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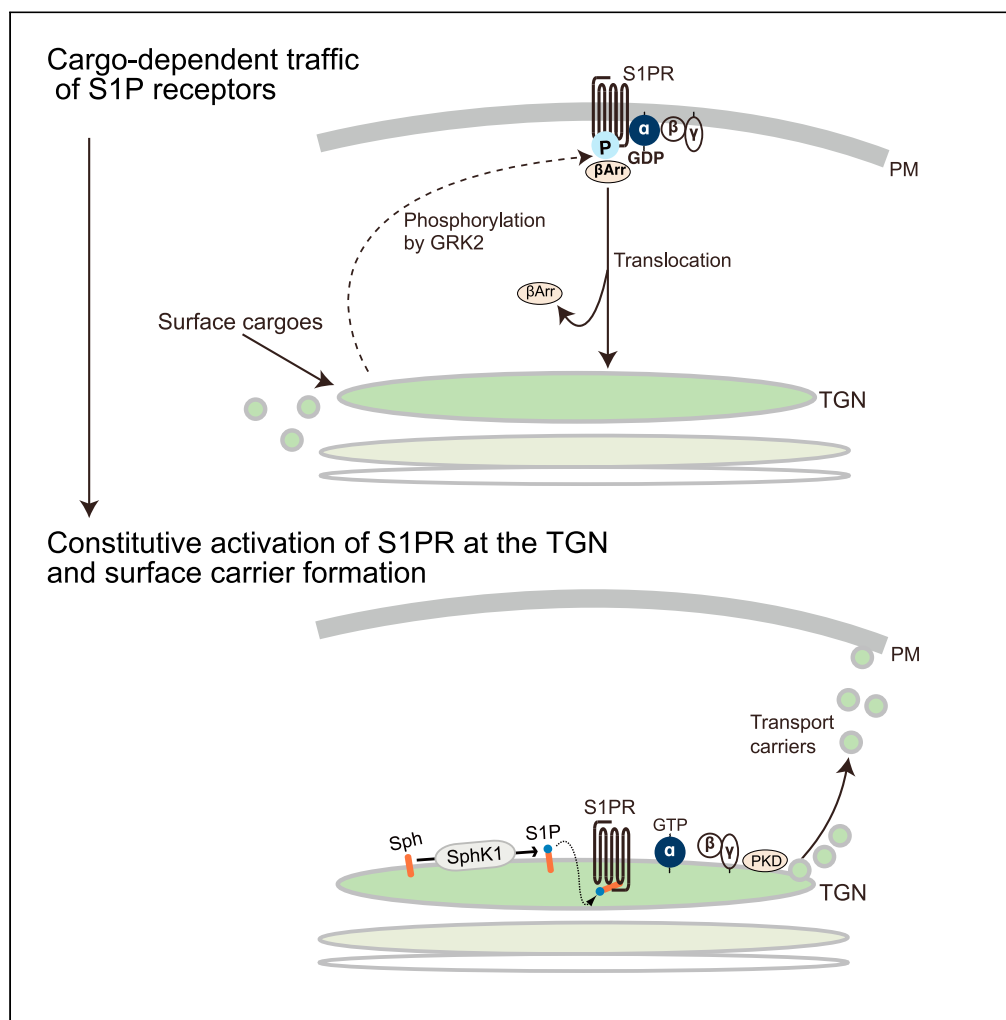
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## Article

# Constitutive activation of S1P receptors at the *trans*-Golgi network is required for surface transport carrier formation



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## Highlights

S1P receptors traffic from the PM to Golgi in a surface cargo-dependent manner

S1PR trafficking follows GRK2-dependent phosphorylation and β-arrestin binding

S1PRs at the Golgi are continuously activated by S1P while sending G-protein signals

S1PR/Gβγ signals at the Golgi are indispensable for surface transport carrier formation

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## Article

Constitutive activation of S1P receptors at the *trans*-Golgi network is required for surface transport carrier formation

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## SUMMARY

**The importance of the G-protein  $\beta\gamma$  subunits in the regulation of cargo transport from the *trans*-Golgi network (TGN) to the plasma membrane (PM) is well accepted; however, the molecular mechanism underlying the G-protein activation at the TGN remains unclear. We show here that sphingosine 1-phosphate (S1P) receptors at the PM were trafficked to the TGN in response to a surface transport cargo, temperature-sensitive vesicular stomatitis virus glycoprotein tagged with green fluorescent protein accumulation in the Golgi. The receptor internalization occurred in an S1P-independent manner but required phosphorylation by G-protein receptor kinase 2 and  $\beta$ -arrestin association before internalization. Continuously activated S1P receptors in a manner dependent on S1P at the TGN kept transmitting G-protein signals including the  $\beta\gamma$  subunits supply necessary for transport carrier formation at the TGN destined for the PM.**

## INTRODUCTION

The Golgi complex is a highly dynamic cellular organelle, carrying out the key functions of processing, sorting, and trafficking of newly synthesized membranes and secretory proteins (Tang and Wang, 2013; Wang and Seemann, 2011). The generation of functional transport carriers from the *trans*-Golgi network (TGN) to the plasma membrane (PM) requires a series of processes including membrane deformation caused by lipids such as diacylglycerol and phosphatidic acid, recruitment of pro-fission proteins such as protein kinase D and CtBP1/BARS, and vesicle formation (Campelo and Malhotra, 2012; Liljedahl et al., 2001; Yeaman et al., 2004). Various attempts were made to identify factors to initiate the fission of PM-destined vesicles from the TGN. A series of studies have implicated the G-protein  $\beta\gamma$  subunits ( $G\beta\gamma$ ) of heterotrimeric G-proteins in the regulation of vesicle formation from the TGN to PM (Díaz Añel and Malhotra, 2005; Irannejad and Wedegaertner, 2010; Jamora et al., 1997). However, the precise mechanism underlying the G-protein activation for this phenomenon remains unclear.

Sphingosine 1-phosphate (S1P) is a phosphorylated product of sphingosine catalyzed by sphingosine kinase (SphK) and has emerged as a potent lipid mediator with diverse effects on multiple biological processes including cell growth, survival, differentiation, motility, and cytoskeletal organization; neurotransmitter release; and endosome maturation (Kajimoto et al., 2007, 2013; Pyne and Pyne, 2000; Spiegel and Milstien, 2002). Most of these processes are mediated by five S1P-specific G-protein-coupled receptors (GPCRs; S1P<sub>1-5</sub>Rs) and show distinct expression in tissues and cells, and also unique G-protein-coupling patterns suggesting distinctive cellular functions (Blaho and Hla, 2014; Bryan and Del Poeta, 2018; Rosen et al., 2009).

We have recently observed in our laboratory the constitutive activation of S1P<sub>1</sub>R and S1P<sub>3</sub>R by S1P on the multivesicular endosomes and that the resultant release of the free  $G\beta\gamma$  regulated the cargo sorting into exosomal intraluminal vesicles within multivesicular endosomes (Kajimoto et al., 2013, 2018). This observation has led to the hypothesis that a similar S1P signaling paradigm may be true for other organelles such as Golgi apparatus. In the present studies, we have uncovered that the receptor-mediated S1P signal in the Golgi apparatus plays an important role in the constant supply of the  $G\beta\gamma$  at the TGN, which is a prerequisite for the surface transport carrier formation. We also show the mechanisms by which S1P receptors (S1PRs) in the PM trafficked to the Golgi in response to a cargo accumulation in the Golgi and started agonist-dependent S1PR stimulation at the TGN.

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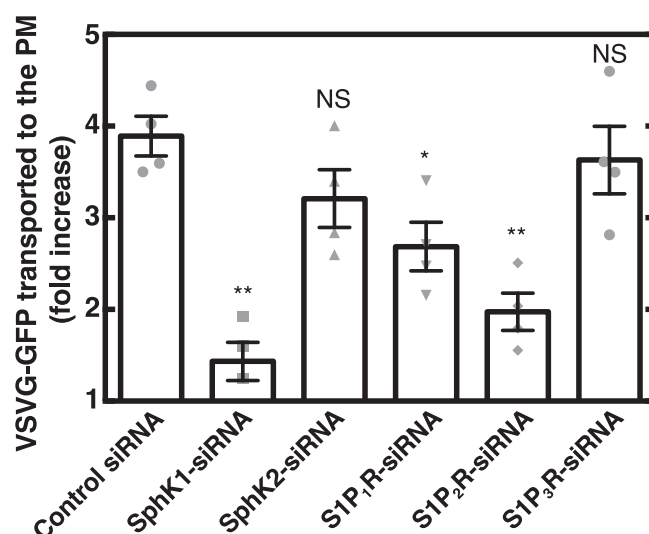
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**Figure 1. Requirement of S1P signal-related molecules for the surface cargo transport from the TGN to PM**

HeLa cells were transiently cotransfected with a vector encoding VSVG-GFP and siRNAs targeted against SphK1, SphK2, S1P<sub>1</sub>R, S1P<sub>2</sub>R, or S1P<sub>3</sub>R. After 1 day of transfection, cells were cultured overnight at 40°C for the synchronized retention of VSVG-GFP in the ER. Cells were then incubated at 20°C for 3 h, followed by incubation at 37°C for 20 min. VSVG-GFP trafficked to the PM was quantitated by biotinylation from outside of the cells, solubilization and pull-down by streptavidin agarose, followed by fluorescence measurement of biotinylated VSVG-GFP. The results are expressed as a fold increase of VSVG-GFP transported to the PM during the incubation at 37°C over that at 20°C (\*\*,  $p < 0.01$ ; \*,  $p < 0.05$ , NS, not significant versus control siRNA; Welch's  $t$  test). Bars represent mean  $\pm$  s.e.m.

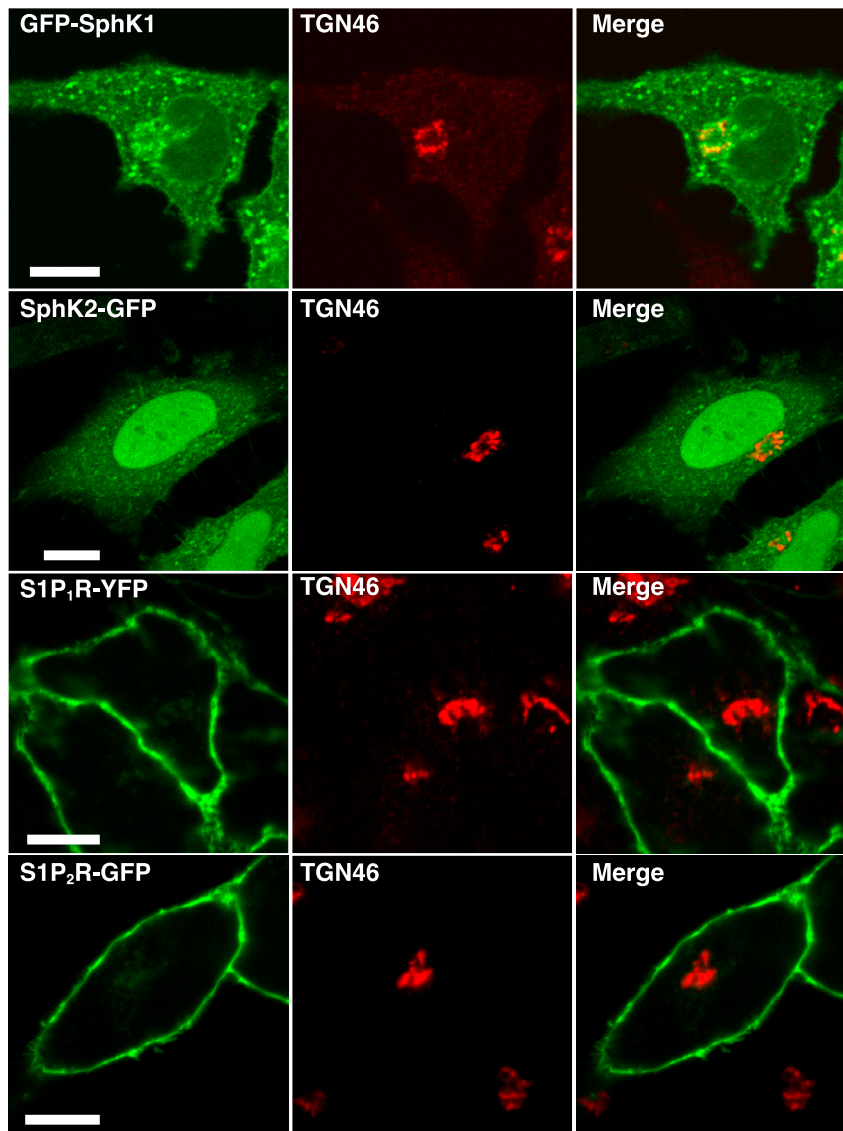
## RESULTS

### Involvement of S1P signal-related proteins in the cargo transport from the TGN to PM

To study the regulation of cargo transport from the TGN to PM, we used temperature-sensitive (ts045) vesicular stomatitis virus glycoprotein (VSVG)-green fluorescent protein (GFP) as a Golgi cargo protein for the surface transport pathway. This glycoprotein is synthesized but does not fold properly at the non-permissive temperature of 40°C and is retained in the endoplasmic reticulum (ER). When transferred to 20°C, VSVG-GFP localizes in the Golgi and, upon being transferred to the permissive temperature of 37°C, it traffics from the Golgi to the PM (Bergmann, 1989). HeLa cells were transfected with a vector encoding VSVG-GFP together with siRNAs targeted against various S1P signal-related proteins. One day after transfection, the cells were cultured at 40°C to retain this protein in the ER and then 20°C to arrest in the Golgi. When transferred to 37°C, a synchronized movement of VSVG-GFP to the PM via the Golgi complex was measured by the cell surface biotinylation of VSVG-GFP, solubilization, pull-down by streptavidin-agarose followed by fluorescence measurement. Upon temperature shift to 37°C, VSVG-GFP was transported efficiently (about a 4-fold increase) to the PM compared with the amount at 20°C in control siRNA-transfected cells (Figure 1). When sphingosine kinase 1 (SphK1), an enzyme to catalyze the formation of S1P, was depleted, temperature shift-induced transport of VSVG-GFP to the PM was significantly ( $P < 0.01$ ) inhibited. However, another subtype SphK2 knockdown showed negligible effects on the transport. As S1P is known to exert its actions mainly through S1PRs (Rosen et al., 2009), the involvement of the most ubiquitously expressed receptors S1P<sub>1</sub>R, S1P<sub>2</sub>R, and S1P<sub>3</sub>R in this phenomenon was assessed. Under the conditions of individual S1PR depletion by the selective siRNAs, S1P<sub>2</sub>R depletion had the strongest inhibition, with a moderate but reproducible suppression in S1P<sub>1</sub>R downregulation, whereas S1P<sub>3</sub>R knockdown showed no apparent effects (Figure 1).

### Cellular distribution of S1P signal-related molecules involved in the regulation of the Golgi to PM transport

To get more insight into the regulation of Golgi surface transport through S1P signal-related molecules, we next studied the cellular distribution of these molecules. Individually fluorophore-tagged molecule-expressing HeLa cells were cultured under standard conditions, and the cellular localization of the molecule was assessed in terms of colocalization with a *trans*-Golgi marker, TGN46. GFP-SphK1 was colocalized well with TGN46, with a poor co-distribution of SphK2-GFP with this Golgi marker (Figure 2). This result matched the observation that downregulation of SphK1 expression by siRNA more strongly impaired the Golgi to PM transport than that in



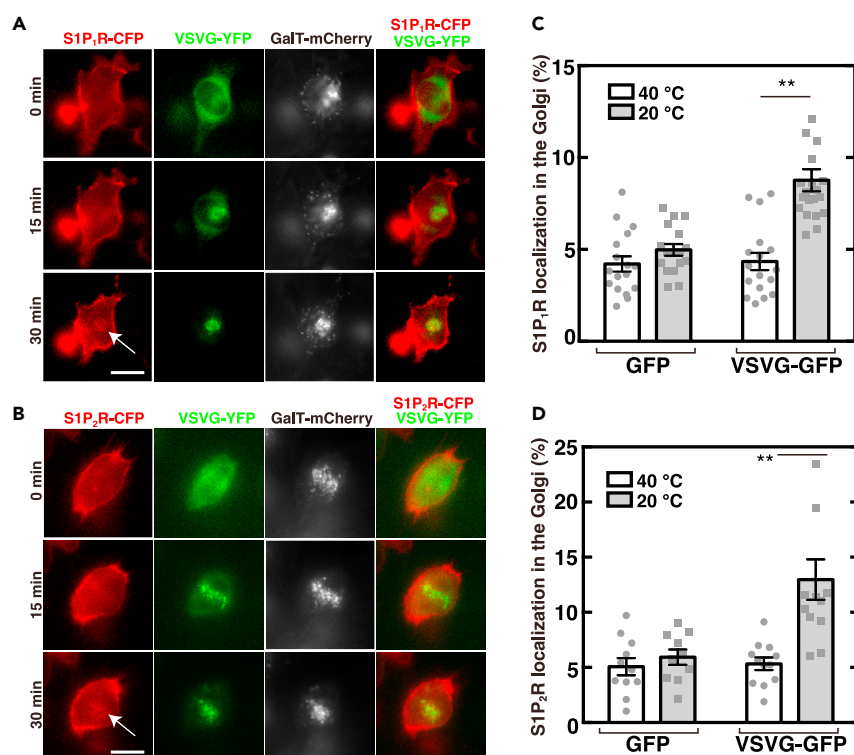
**Figure 2. Cellular distribution of S1P signal-related molecules involved in the Golgi to PM transport**

HeLa cells transiently expressing GFP-SphK1, SphK2-GFP, S1P<sub>1</sub>R-YFP, or S1P<sub>2</sub>R-GFP were cultured overnight at 37°C under standard conditions, fixed, permeabilized, stained with an antibody against TGN46, a TGN resident marker, and analyzed by confocal microscopy. Scale bars, 10  $\mu$ m.

SphK2-depleted cells (Figure 1) and suggests that this isozyme plays a role in the Golgi surface transport. On the other hand, both S1P<sub>1</sub>R and S1P<sub>2</sub>R were poorly colocalized with TGN46 under standard conditions.

### Cargo-dependent accumulation of S1PRs to the Golgi complex

The discrepancy between functional studies using S1PR-depleted cells (Figure 1) and cellular distribution studies (Figure 2) facilitated us to ask whether the distribution of S1PRs may change dynamically depending on cellular conditions. To test this possibility, we next carried out living cell studies where S1PR distribution was monitored under conditions that affect Golgi transport needs. HeLa cells expressing S1P<sub>1</sub>R-CFP together with both a Golgi cargo marker, VSVG-yellow fluorescent protein (YFP), and a Golgi resident marker, GalT-mCherry, were cultured first at a non-permissive temperature of 40°C and then shifted to a permissive temperature of 37°C and the cellular distribution of fluorescence proteins was analyzed using a time-lapse monitoring system. Surprisingly, upon temperature shift to 37°C, S1P<sub>1</sub>R accumulated to the Golgi as indicated by the Golgi marker, Gal T-mCherry, in a time-dependent manner as the Golgi cargo protein, VSVG-YFP, gathered to the



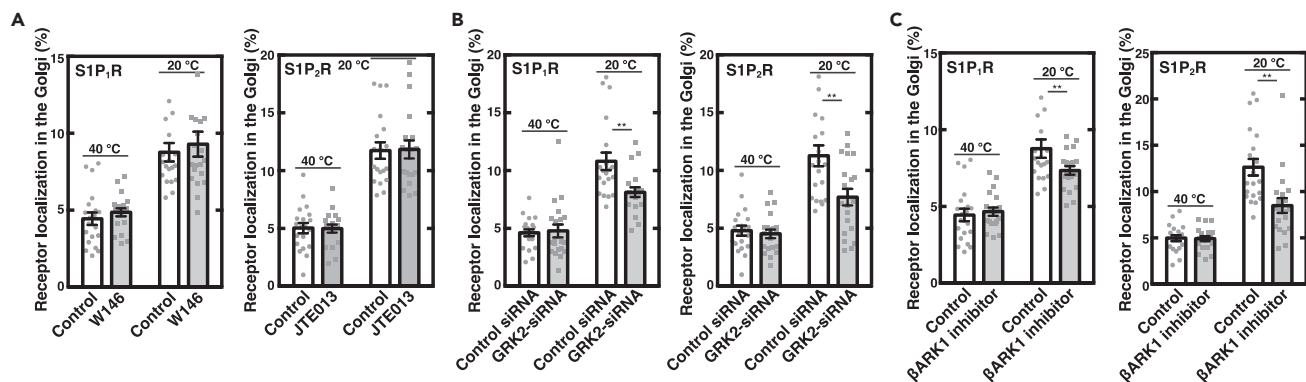
**Figure 3. Cargo accumulation in the Golgi induces S1PRs to traffic from the PM to Golgi**

(A and B) HeLa cells expressing VSVG-YFP, GalT-mCherry, and either S1P<sub>1</sub>R-CFP or S1P<sub>2</sub>R-CFP were cultured overnight at 40°C. Upon temperature shift to 37°C, each fluorescence protein was monitored for live cell imaging using a fluorescence microscopy equipped with a time-lapse recording system. Note that, upon temperature shift in 30 min, the majority of VSVG-YFP accumulated in GalT-mCherry-positive Golgi areas and some of the S1PRs also gathered in these areas. Scale bars, 10  $\mu$ m.

(C and D) HeLa cells expressing VSVG-GFP and either S1P<sub>1</sub>R-mCherry or S1P<sub>2</sub>R-mCherry were cultured overnight at 40°C. In some experiments, GFP was expressed instead of VSVG-GFP as specified in the figures. Aliquots of the cells were further cultured at 20°C for 3 h for the efficient localization of VSVG-GFP in the Golgi. Cells were fixed, stained by using anti-TGN46 antibody and anti-rabbit Alexa 647 antibody, and analyzed for each fluorophore distribution by confocal microscopy. In each temperature condition, S1PR localization in the Golgi was expressed as a percentage of the fluorescence of S1PR-mCherry in the TGN46-positive areas of the total. Results are expressed as the mean on scatter-dot plots (\*\*,  $p < 0.01$ ; Welch's  $t$  test). Bars represent mean  $\pm$  s.e.m.

Golgi (Figure 3A, see arrow). Quantification results with fixed cell studies also confirmed that S1P<sub>1</sub>R-mCherry accumulates to the Golgi when the cargo protein gathered to the Golgi (Figures 3C and S1A), suggesting that S1P<sub>1</sub>R-mCherry trafficked to the Golgi in a cargo-dependent manner. This cargo-dependent S1P<sub>1</sub>R traffic to the Golgi was also observed in human neuroblastoma cell line, SH-SY5Y cells (Figures S2A and S2B), suggesting a more general phenomenon. When non-cargo protein GFP was expressed instead of VSVG-GFP, S1P<sub>1</sub>R-mCherry did not accumulate to the Golgi (Figures 3C and S1A). When S1P<sub>2</sub>R was used, similar results were obtained (Figures 3B, 3D, and S1B), i.e., S1P<sub>2</sub>R also accumulated to the Golgi in a cargo-dependent manner.

To get more insight into the mechanism underlying S1PR trafficked to the Golgi from the PM in response to the Golgi cargo accumulation, the effect of antagonists W146 and JTE013 for S1P<sub>1</sub>R and S1P<sub>2</sub>R, respectively, on the translocation of the receptors to the Golgi complex under the temperature shift from 40°C to 20°C in VSVG-GFP expressing cells was studied. W146 showed no detectable effects on the translocation of S1P<sub>1</sub>R to the Golgi complex during temperature shift-induced VSVG-GFP accumulation to this organelle (Figure 4A). Similarly, the S1P<sub>2</sub>R antagonist, JTE013, had no effects on the receptor accumulation to the Golgi. These results indicate that the mechanism underlying the S1PRs translocation from the PM under the Golgi cargo-dependent situations may not involve agonist (S1P)-induced activation of the receptors, as the exogenous addition of S1P to the HeLa cells expressing S1P<sub>1</sub>R or S1P<sub>2</sub>R caused internalization of the receptor, which was almost completely blocked by W146 or JTE013, respectively (Figure S3). One of



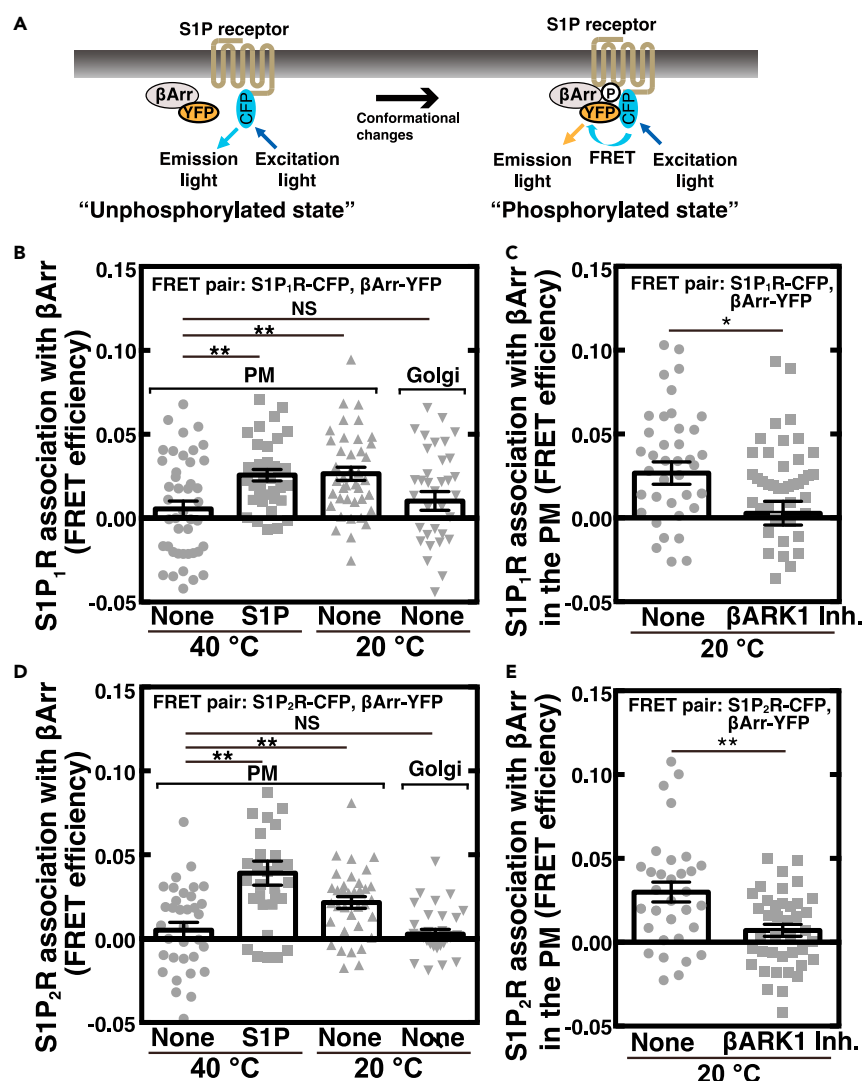
**Figure 4. Golgi cargo-induced S1PR traffic from the PM to Golgi takes place in a ligand-independent but GRK2 activity-required manner**

(A–C) HeLa cells were transiently cotransfected with vectors encoding both VSVG-GFP and either S1P<sub>1</sub>R-CFP or S1P<sub>2</sub>R-CFP (A and C) and cultured for 2 days. In some experiments, cells were cotransfected together with siRNAs targeted against GRK2 (B). Cells were cultured overnight at 40°C, and before 30 min of the temperature shift to 20°C, cells were pretreated with vehicle control, 10  $\mu$ M W146 or JTE013 (A) and with 0.3 mM  $\beta$ ARK1 inhibitor (C). Cells were further incubated for 3 h at 20°C and analyzed for S1PR localization in the Golgi as in Figures 3C and 3D. Results are expressed as the mean on scatter-dot plots (\*\*,  $p < 0.01$ ; Welch's  $t$  test). Bars represent mean  $\pm$  s.e.m.

the possibilities of this receptor trafficking to the Golgi may involve the mechanism of an agonist-independent activation or conformational changes of the receptor and a subsequent signal transduction, which allows the receptor to traffic inside the cells. Most of the GPCRs, including S1PRs, are known to undergo phosphorylation by the selective G-protein-coupled receptor kinase (GRK) after conformational changes of the receptors and subsequent internalization (Benovic et al., 1987; Kajimoto et al., 2007; Krasel et al., 2005). To assess the involvement of GRK2, which is known to catalyze the phosphorylation of S1PRs (Arnon et al., 2011; Penela et al., 2008), the effects of knockdown of GRK2 using siRNA and a pharmacological inhibitor on the S1PR localization in the Golgi were tested. Upon temperature shift from 40°C to 20°C in both individual S1PR and VSVG-GFP-expressing cells, GRK2 knockdown resulted in a significant reduction of the accumulation of S1P<sub>1</sub>R and S1P<sub>2</sub>R in the Golgi compared with the respective control siRNA treatment (Figure 4B). Similarly,  $\beta$ ARK1 inhibitor, a GRK2 inhibitor, caused an inhibition of the accumulation of these receptors in the Golgi (Figure 4C). These results indicate that, under the conditions where the amount of a cargo increases in the Golgi, S1PRs undergo phosphorylation by GRK2 followed by internalization toward the Golgi compartment.

As it is generally accepted that upon ligand activation of GPCRs phosphorylation of the receptors by GRK results in their association with  $\beta$ -arrestin, leading to their internalization (Laporte et al., 2000), we hypothesized that Golgi cargo accumulation-induced but ligand-independent phosphorylation of S1PRs by GRK2 follows the same scenario, i.e.,  $\beta$ -arrestin binding followed by internalization of the receptor. To address this issue, we undertook fluorescence resonance energy transfer (FRET) studies to visualize the association of the two molecules directly in the cells. HeLa cells expressing  $\beta$ -arrestin 2-YFP, VSVG-mCherry, and either S1P<sub>1</sub>R-CFP or S1P<sub>2</sub>R-CFP were cultured at the non-permissive temperature of 40°C and then at 20°C for an efficient retention of VSVG-mCherry in the Golgi and analyzed for S1PRs and  $\beta$ -arrestin 2 association by FRET analysis (Figure 5A). Under the non-permissive temperature of 40°C, the FRET efficiency was low in the PM but was elevated by S1P (Figures 5B and 5D), confirming the validity of the system that S1P-induced conformationally changed S1P<sub>1</sub>R and S1P<sub>2</sub>R become successfully associated with  $\beta$ -arrestin 2 in the PM. Under these conditions (temperature at 40°C), the amount of S1P<sub>1</sub>R and S1P<sub>2</sub>R in the Golgi compartment was too low for FRET analysis (Figures 3A and 3B and data not shown). Importantly, under the temperature shift to 20°C in the absence of S1P, the FRET efficiency in the PM was elevated to an extent comparable with S1P stimulation in the PM under the non-permissive conditions. Expectedly,  $\beta$ ARK1 inhibitor treatment of the cells resulted in the lowered FRET efficiency between the S1PRs and  $\beta$ -arrestin 2 (Figures 5C and 5E), confirming the importance of phosphorylation of S1PRs by GRK2 necessary for  $\beta$ -arrestin 2 binding. It is noteworthy that, during the incubation at 20°C, FRET efficiency in the Golgi compartment was low (Figures 5B and 5D, Golgi [None] at 20°C). These results imply that, under the conditions when the Golgi cargoes such as VSVG accumulate in the Golgi compartment, phosphorylated S1P<sub>1</sub>R and S1P<sub>2</sub>R catalyzed by GRK2 become associated with  $\beta$ -arrestin 2 followed by internalization and recruitment to the Golgi in a ligand-independent manner, while releasing  $\beta$ -arrestin 2 in the Golgi.





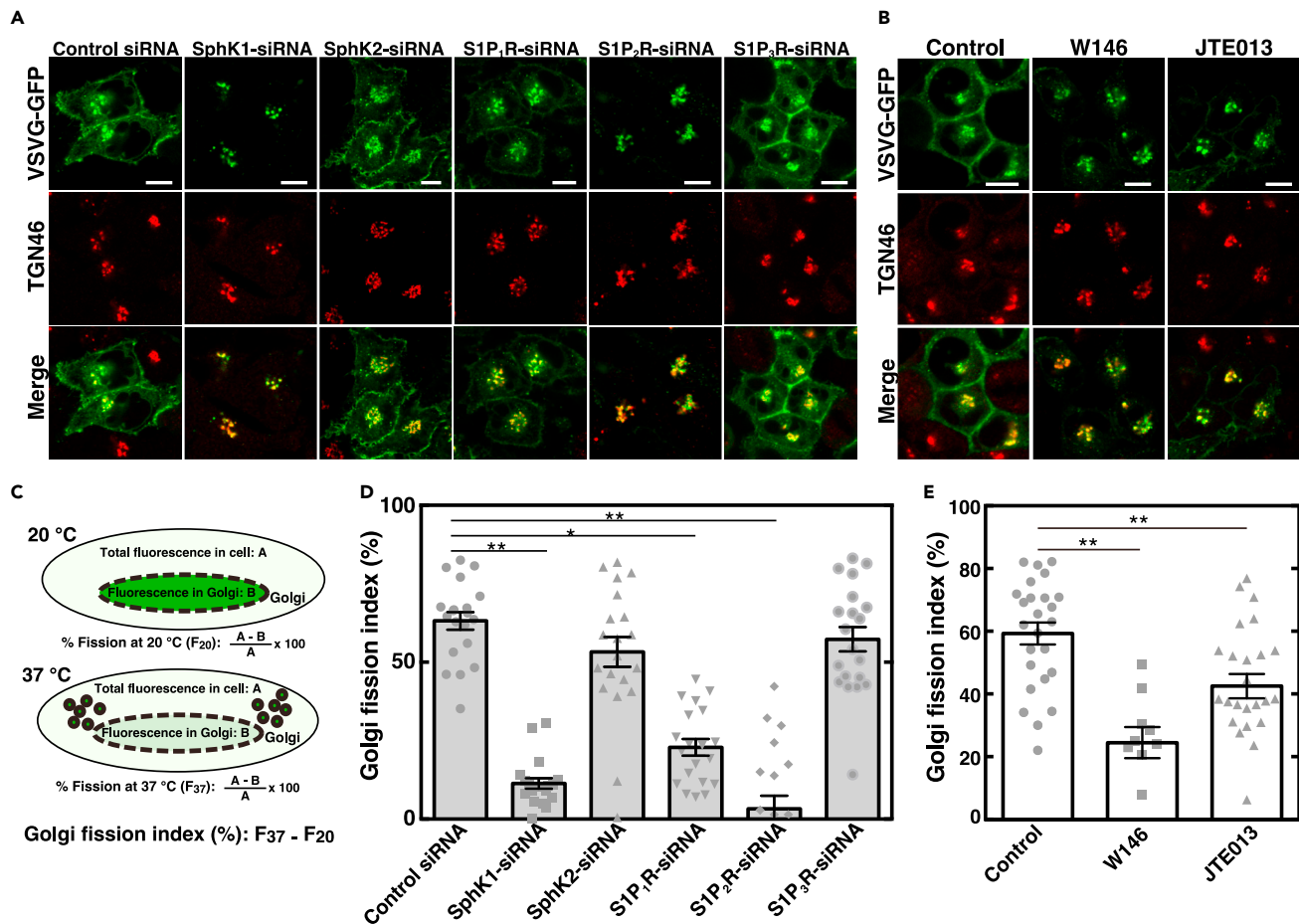
**Figure 5. Cargo accumulation in the Golgi induces S1PR association with β-arrestin 2 in the PM**

(A) Strategy to detect S1PR interaction with β-arrestin (βArr) using FRET is depicted. (B–E) HeLa cells expressing VSVG-mCherry, β-arrestin-YFP, and either S1P<sub>1</sub>R-CFP (B and C) or S1P<sub>2</sub>R-CFP (D and E) were cultured overnight at 40°C. Cells were then incubated for 3 h at 20°C for an efficient retention of VSVG-mCherry in the Golgi. In some experiments, cells were stimulated with 1 μM S1P for 5 min before temperature shift to 20°C (B and D) or pretreated for 30 min with 0.3 mM βARK1 inhibitor (βARK1 Inh.) before temperature shift to 20°C (C and E). Cells were fixed and analyzed for S1PR/β-arrestin association by measuring FRET efficiency using the acceptor photobleaching methods. Results are expressed as the mean on scatter-dot plots (\*\*, p < 0.01; \*, p < 0.05; NS, not significant; Welch's t test). Bars represent mean ± s.e.m.

### Requirement of S1P-dependent S1PR activation at the Golgi for subsequent surface transport carrier formation from the TGN destined to the PM

Next, experiments were performed to ask about the physiological significance of the translocation of S1PRs to the Golgi complex in response to the increase in the amount of the Golgi cargoes. HeLa cells expressing VSVG-GFP were transfected with siRNA against each S1P signal-related molecule. One day after transfection, the cells were cultured at 40°C overnight and then at 20°C for 3 h to synchronize the accumulation of VSVG-GFP in the Golgi. Upon temperature shift from 20°C to 37°C, the ability of transport carrier formation destined for the PM was measured. Importantly, knockdown of the Golgi-localizing enzyme, SphK1, resulted in a robust inhibition of VSVG transport (81%) from the TGN and the majority of the cargo remained in the Golgi (Figures 6A and 6D) in contrast to the majority of the cargo being trafficked out of the Golgi in





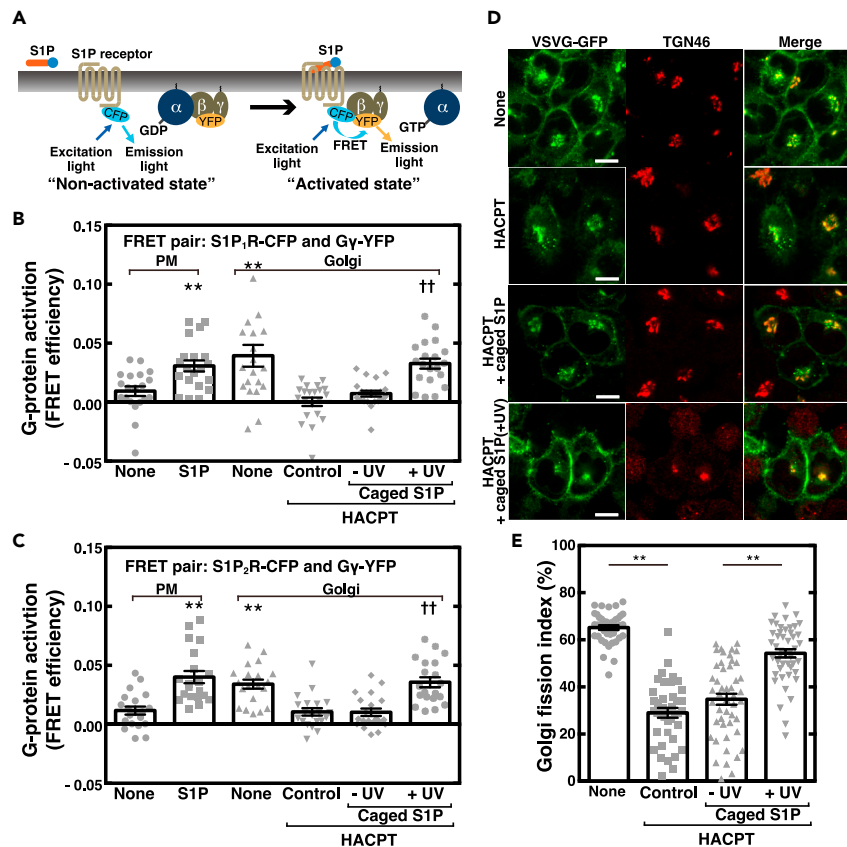
**Figure 6. Requirement of S1P/S1PR axis for the formation of transport carriers from the Golgi destined for the PM**

HeLa cells were transiently cotransfected with a vector encoding VSVG-GFP and siRNAs targeted against SphK1, SphK2, S1P<sub>1</sub>R, S1P<sub>2</sub>R, or S1P<sub>3</sub>R (A and D). After 1 day of transfection, VSVG-GFP-expressing cells were cultured overnight at 40°C and then for 3 h at 20°C for the efficient retention of VSVG-GFP in the Golgi. Cells were further incubated for 20 min at 37°C to allow transport carrier formation, fixed, permeabilized, immunostained with anti-TGN46 antibody, analyzed by confocal microscopy (A), and further evaluated for Golgi fission (D) according to the definition as illustrated in (C). Results are expressed as the mean on scatter-dot plots (\*\*,  $p < 0.01$ ; \*,  $p < 0.05$  versus control siRNA; Welch's t test). In some experiments, the cells expressing VSVG-GFP (B and E) were cultured overnight at 40°C followed by 3-h incubation at 20°C as described earlier. Twenty minutes before shifting to a permissive temperature of 37°C, cells were preincubated with 1  $\mu$ M W146 or 1  $\mu$ M JTE013. After incubation for 20 min at 37°C, cells were fixed, permeabilized, immunostained with anti-TGN46 antibody, and subjected to morphological (B) or functional analysis (E). Scale bars, 10  $\mu$ m. Results are expressed as the mean on scatter-dot plots (\*\*,  $p < 0.01$ ; Welch's t test). Bars represent mean  $\pm$  s.e.m.

the control, as judged by a criterion indicated by Golgi fission index (Figure 6C). In the case of S1PR knock-down, S1P<sub>2</sub>R depletion had the strongest effect (95% inhibition of transport) with S1P<sub>1</sub>R reduction being a moderate (64%) but significant suppression, whereas S1P<sub>3</sub>R knockdown showed no detectable effects on the cargo transport from the Golgi, as cells with morphologically abnormal nuclei were excluded from the analysis in S1P<sub>3</sub>R-depleted cells. Supporting the results of receptor depletion experiments, pharmacological antagonists W146 and JTE013 for S1P<sub>1</sub>R and S1P<sub>2</sub>R, respectively, suppressed the transport of VSVG-GFP (Figures 6B and 6E). These results imply that a receptor-mediated S1P signal at the Golgi compartment may play a role in the transport carrier formation destined to the PM.

### Importance of S1P-dependent S1PR-coupled G-protein activation for the G $\beta\gamma$ supply necessary for the transport carrier formation at the TGN destined for the PM

To identify downstream signaling events after S1PR activation, we have dissected the signaling pathways necessary for surface transport carrier formation. S1PRs belong to GPCRs and S1P<sub>1</sub>R is known to be coupled exclusively with Gi/o, whereas S1P<sub>2</sub>R is coupled with various G-proteins such as Gi/o, Gq, or G12/13, depending on cell types (Rosen et al., 2009). As the G $\beta\gamma$  subunits are common among these



**Figure 7. Constitutive activation of S1PRs in the Golgi in a ligand-dependent manner with a concomitant G-protein subunit dissociation**

(A) The strategy to detect S1PR-mediated G-protein activation using a FRET pair of S1PR-CFP and the Gy-YFP is depicted. (B and C) HeLa cells transiently expressing VSVG-mCherry, G $\beta$ , and Gy-YFP or either S1P<sub>1</sub>R-CFP (B) or S1P<sub>2</sub>R-CFP (C) were cultured overnight at 40°C in the presence of DMSO vehicle or 5  $\mu$ M HACPT. Aliquots of the cells were stimulated for 5 min by 1  $\mu$ M S1P before the end of the incubation at the non-permissive temperature and fixed for FRET analysis. After the incubation at 40°C, cells were further incubated for 3 h at 20°C for the efficient retention of VSVG-GFP in the Golgi. In some experiments, 1  $\mu$ M caged S1P was added 30 min before the end of 20°C incubation and then the preload cells were treated by UV irradiation for 20 s to activate caged S1P by photolysis where indicated. Cells were fixed, permeabilized, immunostained with anti-TGN46 antibody to identify the Golgi areas, and analyzed by confocal microscopy for FRET efficiencies using an acceptor photobleaching method. Results are expressed as the mean on scatter-dot plots (\*\*, p < 0.01 versus None in PM; ††, p < 0.01 versus -UV in Golgi; Welch's t test).

(D and E) HeLa cells expressing VSVG-GFP were cultured overnight at 40°C in the presence of DMSO vehicle or 5  $\mu$ M HACPT and then for 3 h at 20°C. Cells were further incubated for 20 min at 37°C to allow transport carrier formation. In some experiments, 1  $\mu$ M caged S1P was added 30 min before the cell incubation at 37°C. After preload, cells were rinsed and flashed with UV for 20 s for photolysis where indicated and then incubated for 20 min at 37°C. After incubation at 37°C, cells were fixed, permeabilized, immunostained with anti-TGN46 antibody, and analyzed by confocal microscopy for a morphological (D) or a functional study as in Figure 6C (E). Scale bars, 10  $\mu$ m. Results are expressed as the mean on scatter-dot plots. Bars represent mean  $\pm$  s.e.m. (\*\*, p < 0.01; Welch's t test).

G-proteins and ligand-dependent activation of S1PRs follows subsequent dissociation of the G-protein subunits, which results in the association of the G $\beta\gamma$  with the receptors (Kajimoto et al., 2013), we sought to detect S1PR-mediated G-protein activation by a FRET technique using S1PR-CFP and Gy-YFP as a FRET pair in VSVG-mCherry co-expressing cells (Figure 7A). This FRET system was verified by the observation that activation of both S1P<sub>1</sub>R and S1P<sub>2</sub>R in the PM by extracellular S1P resulted in an increase in FRET efficiency, showing a ligand-induced receptor activation caused the G-protein subunit dissociation (Figures 7B and 7C). Unexpectedly, both S1P<sub>1</sub>R and S1P<sub>2</sub>R in the Golgi were constitutively activated during VSVG-mCherry accumulation in the Golgi to an extent similar to exogenous S1P activation of the receptors in the PM (Figures 7B and 7C, compare PM/S1P with Golgi/None). Treatment of the cells with an

SphK inhibitor, HACPT, caused the reset of the G-protein dissociation (Figures 7B and 7C, see Golgi/HACPT/Control), suggesting continuous S1P supply catalyzed by SphK1 is necessary for constant activation of the receptors in the Golgi compartment. This notion was further confirmed by a rescue experiment, i.e., HACPT-induced “reset” was canceled by photolysis-activated caged S1P but not by an inactive one, suggesting a constant S1PR activation in the Golgi by SphK1-catalyzed S1P (Figures 7B and 7C). The importance of S1P-induced activation of the receptors in the Golgi in the transport vesicle formation at the TGN was further demonstrated by morphological studies. HACPT-caused inhibition of transport vesicle formation at the TGN was rescued by a photolysis-activated caged S1P (Figures 7D and 7E). The importance of ligand-dependent S1PR activation at the TGN in surface transport carrier formation was also demonstrated in SH-SY5Y cells by showing the sensitivity of HACPT to the transport carrier formation (Figures S2C and S2D).

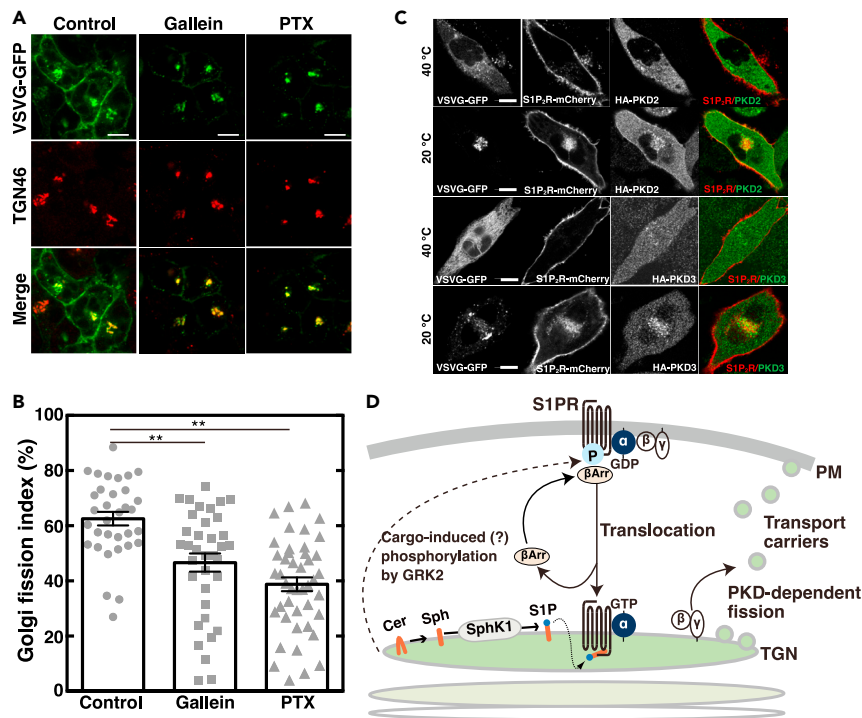
### Importance of S1P/S1PR/G-protein axis-derived $G\beta\gamma$ in the transport carrier formation at the TGN destined for the PM

To identify a molecule after S1P/S1PR/G-protein axis necessary for the transport carrier formation at the TGN, involvement of the  $G\beta\gamma$  was studied for the effects caused by using pharmacological reagents. A small-molecule  $G\beta\gamma$  inhibitor, gallein, caused an inhibition of transport vesicle formation at the TGN (Figures 8A and 8B). Similarly, treatment of cells with pertussis toxin, which is known to ADP-ribosylate the  $G_{i\alpha}$  subunit and to cause GPCR uncoupled from  $G_i$  (Haga et al., 1985), resulted in a suppression of transport vesicle formation (Figures 8A and 8B). Next, the behavior of a well-known pro-fission protein at the TGN, PKD, during surface cargo accumulation in the Golgi was studied. Both PKD2 and PKD3, which were known to play a role in ilimaquinone-induced Golgi fragmentation in HeLa cells (Sonoda et al., 2007), became accumulated in the TGN as S1P<sub>2</sub>R trafficked to the same area in a cargo molecule-induced manner (Figure 8C). These results suggest that S1P/S1PR/G-protein axis derived  $G\beta\gamma$  are important for the transport carrier formation at the TGN to the PM in concert with PKD (Figure 8D).

## DISCUSSION

Originally, heterotrimeric G-proteins are activated in couple with a cognate GPCR activation at the PM. In addition to this canonical G-protein signaling, recent lines of evidence indicate that heterotrimeric G-proteins are also activated independent of GPCRs at different intracellular organelles such as the Golgi complex (Cismowski et al., 1999; Hewavitharana and Wedegaertner, 2012; Takesono et al., 1999). In this context,  $G_i$  is activated in the Golgi by GIV/Girdin, a non-receptor guanine-nucleotide exchange factor (GEF) associated with coat protein complex I (COPI)-coated transport vesicles regulating vesicle trafficking within the Golgi and/or between the ER and the Golgi (Le-Niculescu et al., 2005).

We have revealed in the present studies that receptor-mediated S1P signal at the TGN plays a key role in the regulation of surface transport carrier formation. S1P<sub>1</sub>R and S1P<sub>2</sub>R trafficked to the Golgi complex in a ligand (S1P)-independent but cargo molecule-induced manner (Figure 3) in response to the Golgi cargo molecule VSVG accumulation in the Golgi from the ER. The Golgi cargo accumulation-induced S1PR internalization may be initiated by the receptor phosphorylation catalyzed by GRK2 at the PM because siRNA-induced knockdown of GRK2 as well as a pharmacological inhibition of GRK2 activity resulted in the suppression of both S1P<sub>1</sub>R and S1P<sub>2</sub>R trafficking to the Golgi (Figures 4B and 4C). S1PR internalization followed the association with  $\beta$ -arrestin 2 (Figures 5B and 5D), a protein known to bind to phosphorylated GPCRs by GRK and elicit internalization of the activated receptors for “switch-off” signal (Benovic et al., 1987) or transmitting further signals such as mitogen-activated protein kinases signal into the cells (Gurevich and Gurevich, 2006; Peterson and Luttrell, 2017). It is important to note that the S1PRs trafficked to the Golgi upon the temperature shift (from 40°C to 20°C) became less associated with  $\beta$ -arrestin to a similar level of non-permissive conditions at the PM (Figures 5B and 5D; compare PM [None] at 40°C and Golgi [None] at 20°C) in a striking contrast to that of S1PRs in the Golgi, which underwent continuous S1P-dependent stimulation with a continuous G-protein signal transmission (the  $G\beta\gamma$  supply) in a manner sensitive to the SphK inhibitor, HACPT (Figures 7B and 7C). The absence of  $\beta$ -arrestin, a “switch-off” protein in the Golgi, may account for the continuous S1PR activation in the organelles, which enables a constitutive supply of the  $G\beta\gamma$  necessary for the transport carrier formation at the TGN destined to the PM. In addition to the  $G\beta\gamma$ , S1P<sub>2</sub>R-coupled Gq may participate in phospholipase C-catalyzed generation of diacylglycerol for the recruitment of PKD2 and PKD3 (Figures 8C and 8D). Regarding the origin of the  $G\beta\gamma$  subunits, a previous report suggests that carbachol treatment of lung epithelial A549 cells transfected with M3,  $G_{\alpha q}$ , and YFP- $\beta 1$  caused the  $G\beta\gamma$  subunits translocation from the PM to the Golgi,



**Figure 8. A role of S1PR-activated G-protein-derived Gβγ subunits for the regulation of constitutive transport carrier formation at the TGN destined for the PM**

(A and B) HeLa cells transiently expressing VSVG-GFP were cultured overnight at 40°C in the presence or absence of 100 ng/mL pertussis toxin, followed by 3 h incubation at 20°C for the efficient retention of VSVG-GFP at the Golgi. Thirty minutes before shifting to a permissive temperature of 37°C, cells were preincubated with 10 μM gallein as specified in the figure. After incubation for 20 min at 37°C, cells were fixed, permeabilized, immunostained with anti-TGN46 antibody to mark the Golgi areas, and subjected to morphological (A) or functional analysis as in Figure 6C (B). Scale bars, 10 μm. PTX, pertussis toxin. Results are expressed as the mean on scatter-dot plots (\*\*,  $p < 0.01$ ; Welch's  $t$  test).

(C) HeLa cells transiently expressing VSVG-GFP, S1P<sub>2</sub>R-mCherry, and either HA-PKD2 or HA-PKD3 were cultured at 40°C for 18 h. Some of the cells were then temperature shifted to 20°C and incubated further for 3 h. Cells were fixed, permeabilized, immunostained with anti-TGN46 antibody and anti-HA antibody, and analyzed for each fluorophore distribution by confocal microscopy. Scale bars, 10 μm.

(D) Schematic model for the regulation of transport carrier formation at the TGN destined for the PM through S1PR-mediated βγ subunits. The S1PRs at the PM undergo GRK2-mediated phosphorylation and then β-arrestin association followed by internalization toward the Golgi in response to the Golgi cargo (VSVG, etc.) accumulation. The mechanism underlying the Golgi cargo accumulation-induced GRK activation is currently unclear. S1PRs at the Golgi continue transmitting G-protein signal in a manner activated by SphK1-catalyzed formation of S1P, thereby providing the βγ subunits necessary for PKD-mediated transport carrier formation at the TGN destined for the PM. Cer, ceramide; Sph, sphingosine; β-Arr, β-arrestin.

resulting in Golgi fragmentation and secretion (Saini et al., 2010). Regulation of Golgi transport may differ under basal and agonist-stimulated conditions. Further studies are necessary to establish the involvement of S1P/S1PR/G-protein axis in various cell situations.

Some of the GPCRs are stimulated in a ligand-independent manner, e.g., angiotensin II type 1 receptor (Zou et al., 2004), endothelin type-1a (ET<sub>1A</sub>) receptors (Mederos et al., 2008), and S1P<sub>1</sub>R (Jung et al., 2012) were shown to be activated by mechanical stress signals. Structural changes in the GPCR induced by mechanical stress may initiate subsequent signaling events such as G-protein and β-arrestin signals. However, the manner in which Golgi cargo accumulation is sensed in the Golgi and its signal is converted into GRK2 activation in the PM remains unclear.

We have recently discovered that continuous SphK2/S1P/S1PR/Gβγ signal on the multivesicular endosomes in an intracrine manner regulates F-actin formation on the organelles, which plays a role in cargo sorting into exosomal multivesicular endosomes (Kajimoto et al., 2013, 2018). Continuous activation of

S1P receptors may be true for the G $\beta\gamma$  supply at the TGN for the constitutive generation of transport carriers to the PM. Important questions remain as to how SphK1 is assigned to the regulation of the Golgi function, whereas SphK2 is closely related to the late endosomal functions (Kajimoto et al., 2013). SphKs are shown to be recruited to endosomes via a targeting protein RPK118, which possesses a PX domain with a capacity of binding with several phosphoinositides including phosphatidylinositol-3 phosphate (Hayashi et al., 2002). Further studies are necessary to elucidate the molecular mechanism underlying each SphK targeting to specific organelles to understand vital cell functions of S1P signaling.

As surface cargo molecules accumulate in the Golgi complex, S1P<sub>1</sub>R and S1P<sub>2</sub>R in the PM internalize and traffic to the Golgi complex where SphK1 constitutively localizes. This trafficking does not require S1P stimulation but involves GRK2-mediated phosphorylation followed by association with  $\beta$ -arrestin for internalization. S1PRs at the Golgi complex, which are free from  $\beta$ -arrestin and undergo continuous activation by locally produced S1P catalyzed by SphK1, keep transmitting G-protein signal, i.e, constant supply of the G $\beta\gamma$ . The G $\beta\gamma$  may utilize the phospholipase C/protein kinase C $\eta$ /protein kinase D pathway (Díaz Añel, 2007) necessary for transport carrier formation at the TGN destined for the PM as illustrated in Figure 8C.

### Limitations of the study

In this study, we have demonstrated that continuous activation of S1P receptors at the TGN in a ligand-dependent manner keeps transmitting G-protein signals including the  $\beta\gamma$  subunits supply necessary for transport carrier formation at the TGN destined for the PM. We have not addressed, however, that the "S1P receptor/G $\beta\gamma$ " scenario at the TGN may be general or VSVG-specific phenomena, because of the complexity of transport carrier systems from the TGN to PM as reviewed recently (Stalder and Gershlick, 2020). Further studies on vesicular trafficking from the TGN to PM using membrane proteins and secretory proteins other than VSVG may shed further light on the understanding of the mechanism underlying surface carrier transport from the TGN.

### STAR★METHODS

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### SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.isci.2021.103351>.

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## AUTHOR CONTRIBUTIONS

T.O. and S.-I.N. designed the study. T.O., S.N., L.Z., N.N.I.M., T.W., T.I., and T.K. performed biochemical and immunocytochemical analysis. S.-I.N. wrote the manuscript together with contributions from T.O.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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## STAR★METHODS

### KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Antibodies</b>		
TGN46	Novus Biologicals, LLC	Novus Cat# NBP1-49643SS, RRID:AB_10011761
<b>Bacterial and virus strains</b>		
DH5alpha	Takara Bio	Cat # 9057
<b>Chemicals, peptides, and recombinant proteins</b>		
S1P	Cayman Chemical Company	Cat # 62570
Caged S1P	Santa Cruz Biotechnology	Cat # sc-207390
W146	Cayman Chemical Company	Cat # 10009109
JTE013	Cayman Chemical Company	Cat # 10009458
βARK1 inhibitor	Cayman Chemical Company	Cat # 21751
HACPT (2-(p-hydroxyanilino)-4-(p-chlorophenyl)thiazole)	Calbiochem	Cat # 567731
Pertussis toxin	Fujifilm Wako Pure Chemical	Cat # 168-22471
Gallein	Tokyo Chemical Industry	Cat # A0601
4% paraformaldehyde	Fujifilm Wako Pure Chemical	Cat # 163-20145
Triton X-100	Nacalai Tesque	Cat # 28229-25
Fugene HD	Promega	Cat # E2311
G418	Nacalai Tesque	Cat # 09380-86
Lipofectamine 2000	Thermo Fisher Scientific	Cat # 11668027
Lipofectamine RNAiMAX	Thermo Fisher Scientific	Cat # 13778100
Cycloheximide	Nakarai Tesque	Cat # 06741-91
Biotin-SS-sulfo-OSu	Dojindo	Cat # 348-09091
Streptavidin agarose	Solulink	Cat # N-1000-002
Protease inhibitor cocktail	nakarai Tesque	Cat # 03969-21
<b>Experimental models: Cell lines</b>		
HeLa cells stably expressing VSVG-GFP	This manuscript	NA
HeLa cells	Riken BioResource Research Center	RCB0007
SH-SY5Y cells	ATCC	CRL-2266
<b>Oligonucleotides</b>		
SPHK1 siRNA 5'- GGGCAAGGCCUUGCAGCUC-3'	Japan Bio Services	NA
SPHK2 siRNA 5'- GCUGGGCUGUCCUUAACCU-3'	Japan Bio Services	NA
S1P1R siRNA 5'- GGAGAACAGCAUUAACUG-3'	Japan Bio Services	NA
S1P2R siRNA 5'- UACCUUGCUCUCUGGCUCU-3'	Japan Bio Services	NA
S1P3R siRNA 5'- GGUCAACAUUCUGAUGUCU-3'	Japan Bio Services	NA
GRK2 siRNA 5'- GCAAGAAAGCCAAGAACAA-3'	Japan Bio Services	NA

(Continued on next page)

### Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Recombinant DNA</b>		
VSVG-mCherry	This manuscript	NA
GFP-SPHK1	<a href="#">Hayashi et al. (1996)</a>	<a href="#">Hayashi et al. (1996)</a> J. Biol. Chem., 277, 33,319–33,324.
SPHK2-GFP	<a href="#">Mohamed et al. (2018)</a>	<a href="#">Mohamed et al. (2018)</a> Kobe J. Med. Sci., 63, E123-E129.
S1P1-CFP	<a href="#">Kajimoto et al. (2013)</a>	<a href="#">Kajimoto et al. (2013)</a> Nat. Commun., 4, 2712.
S1P1-mCherry	This manuscript	NA
S1P2-CFP	<a href="#">Kajimoto et al. (2013)</a>	<a href="#">Kajimoto et al. (2013)</a> Nat. Commun., 4, 2712.
S1P2-mCherry	This manuscript	NA
$\beta$ -Arrestin-YFP	<a href="#">Kajimoto et al. (2013)</a>	<a href="#">Kajimoto et al. (2013)</a> Nat. Commun., 4, 2712.
G $\beta$	<a href="#">Kajimoto et al. (2013)</a>	<a href="#">Kajimoto et al. (2013)</a> Nat. Commun., 4, 2712.
G $\gamma$ -YFP	<a href="#">Kajimoto et al. (2013)</a>	<a href="#">Kajimoto et al. (2013)</a> Nat. Commun., 4, 2712.
GalT-mCherry	This manuscript	NA
VSVG-GFP	<a href="#">Presley et al. (1997)</a>	<a href="#">Presley et al. (1997)</a> Nature, 389, 81–85.
VSVG-YFP	This manuscript	NA
HA-PKD2	<a href="#">Ding et al. (2007)</a>	<a href="#">Ding et al. (2007)</a> J. Biol. Chem., 282, 27,493–27,502.
HA-PKD3	<a href="#">Ding et al. (2007)</a>	<a href="#">Ding et al. (2007)</a> J. Biol. Chem., 282, 27,493–27,502.
<b>Software and algorithms</b>		
Prism 6 for mac OS X	GraphPad Software	NA
Fiji(ImageJ)	<a href="https://imagej.net/Fiji">https://imagej.net/Fiji</a>	NA
MetaFluor	Molecular devices	NA

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for DNA constructs and reagents should be directed to and will be fulfilled by the lead contact, Shun-ichi Nakamura ([snakamur@kobe-u.ac.jp](mailto:snakamur@kobe-u.ac.jp)).

### Materials availability

All plasmids generated in this study are available from the lead contact.

### Data and code availability

The data reported in this paper will be shared by the lead contact upon request. This paper does not report original code.

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

## EXPERIMENTAL MODEL AND SUBJECT DETAILS

### Cell lines

The following cell lines were used in this study.

**HeLa cells.** Origin: Human cervical cancer cell line obtained from a 31-year-old female.

Culture media and conditions: HeLa cells were obtained from Riken BioResource Research Center (RCB0007) and were cultured in DMEM high glucose media (Fujifilm Wako Pure Chemical) and

supplemented with 10% (v/v) inactivated fetal bovine serum (FBS) and penicillin-streptomycin. Cells were maintained in a 37°C incubator under 5% CO<sub>2</sub>. Cells were seeded on glass bottom dish (Mattek, Cat #P35G-0-14-C) for microscope study or 24 well plate (Corning) for biotinylation assay.

**Stable HeLa cells expressing VSVG-GFP.** HeLa cells were transfected with VSVG-GFP in pEGFP vector by using Eugene HD (Promega) and cultured in DMEM high glucose media containing 0.6 mg/mL G418 (Nakarai Tesque) for 2 weeks. The cells expressing VSVG-GFP were sorted by using FACSMelody (BD, NJ). Cells were seeded on glass bottom dish (Mattek, Cat #P35G-0-14-C) for microscope study.

**SH-SY5Y cells.** Origin: Human neuroblastoma cell line obtained from 4-years-old female.

Culture media and conditions: SH-SY5Y cells were obtained from ATCC (CRL-2266) and were cultured in DMEM/F-12 media (Fujifilm Wako Pure Chemical) supplemented with 10% (v/v) inactivated fetal bovine serum (FBS) and penicillin-streptomycin. Cells were maintained in a 37°C incubator under 5% CO<sub>2</sub>. Cells were seeded on glass bottom dish (Mattek, Cat #P35G-0-14-C) for microscope study.

## METHOD DETAILS

### Transfection

Transient transfection of plasmid DNA was carried out using FuGENE HD (Promega) according to the manufacturer's instructions. Cotransfection of plasmid DNA and siRNA was done by Lipofectamine 2000 (Thermo Fisher Scientific) and siRNA was transfected by using Lipofectamine RNAiMAX (Thermo Fisher Scientific) according to the manufacturer's instructions. The following siRNAs were used in this study: sense 5'-GGGCAAGGCCUUG CAGCUCdTdT-3' and antisense 5'-GAGCUGCAAGGCCUUGCCCDdTdT-3' for SphK1, sense 5'-GCUGGGCU GUCCUUAACCUdTdT-3' and antisense 5'-AGGUUGAAGGACAGCCAGCdTdT-3' for SphK2, sense 5'-GG AGAACAGCAUUAACUGdAdC-3' and antisense 5'-CAGUUUAUGCUGUUCUCCdTdT-3' for S1P1R, sense 5'-UACCUUGCUCUCUGGCUCdGdT-3' and antisense 5'-AGAGCCAGAGAGCAAGGUAdTdT-3' for S1P2R and sense 5'-GGUCAACAUCUGAUGUCdGdG-3' and antisense 5'-AGACAUCAGAAUGUUGACCDdTdT-3' for S1P3R.

### Construction of plasmids

The expression plasmid for temperature-sensitive (ts045) VSVG-GFP was kindly donated by Dr. J. Lippincott-Schwartz (National Institutes of Health) (Presley et al., 1997). VSVG-YFP and VSVG-mCherry were constructed by inserting VSVG into pEYFP-N1 and pmCherry-N1, respectively. mCherry-mSphK1 was constructed from GFP-mSphK1(WT) (Hayashi et al., 2002), by inserting into pmCherry-C1. hSphK2-mCherry was constructed from hSphK2-GFP (Mohamed et al., 2018) by inserting into pmCherry-N1. Murine S1P1 (mS1P1) cDNA was amplified from mouse brain cDNA, which had been reverse transcribed from fetal mouse brain mRNA (Invitrogen) by PCR (sense primer, 5'-CGGAATTCGCCACCATGGTGCTCCACTAGCATCC-3'; antisense primer, 5'-CGGAA TTCGGGAAGAAGAAATTGACGTTTCCAG-3') and inserted into pEYFP-N1, pECFP-N1 or pmCherry-N1 to make S1P1R-YFP, S1P1R-CFP or S1P1R-mCherry, respectively. Murine S1P2 (mS1P2) cDNA was amplified as described above (sense primer, 5'-CGGAATTCGCCACCATGGGCGGCTTATACTCAGAG-3'; antisense primer, 5'-CGGAATTCGGACCACTGTGTTACCCTCCAG-3') and inserted into pEGFP-N1, pECFP-N1 or pmCherry-N1 to make S1P1R-GFP, S1P1R-CFP or S1P1R-mCherry, respectively. GalT-mCherry: The DNA fragment coding sequence of amino acids 1–82 of  $\beta$ 1,4-galactosyltransferase (GalT) was amplified by using primer sets 5'-AAAGG-TACCATGAGGCTTCGGGAGCCGCTC-5' and 5'-TATGGATCCTTGGCCCCCTCCGGTCCGGAGC-3' and inserted into pmCherry-N1. Mouse  $\beta$ -arrestin 2 cDNA was amplified from mouse brain cDNA by PCR (sense primer, 5'-GCCGGTACCCCGCGGGTCGAGGTTATGG-3'; antisense primer 5'-GCCGGTACCTCATAAGGGCTCTTC TGGC-3') to make an N-terminally yellow fluorescent protein (YFP)-fused construct in pEYFP-C1. Human PKD2 (hPKD2) and human PKD3 (hPKD3) cDNAs were amplified from a human brain cDNA (hPKD2) or human ovary cDNA (hPKD3), by PCR (sense primer, 5'-CCTACGCGTATGGCCACCGCCCCCTTATCCC-3', and antisense primer, 5'-CACATCGATTGAGAGAACTGATGCGCTCCGC-3', for hPKD2; sense primer, 5'-GAAACGCG-TATGTCTGCAAATAATCCCCTCCA-3', and antisense primer, 5'-GCTATCGATTTAAGGATCTTCTCCATAT-CATC-3', for hPKD3) to make N-terminally HA-tagged constructs in pCMV5 (Ding et al., 2007).

### VSVG transport assay by biotinylation

HeLa cells were grown on a 12-well plate and cotransfected with VSVG-GFP plasmid DNA and siRNA and incubated for 24 h at 37°C. Cells were then incubated at 40°C for 18 h with the last 1 h in the presence of

10  $\mu$ g/mL cycloheximide to stop further de novo protein synthesis, and transferred to 20°C for 3 h. Cells were then transferred to 37°C for 20 min, followed by incubation with 1 mg/mL biotin-SS-Sulfo-OSu (Dojindo, Japan) in Hanks' balanced salt solution (HBSS) for 30 min at 4°C for cell surface biotinylation. Cells were then incubated with 100 mM glycine in HBSS for 30 min at 4°C and washed with HBSS, followed by solubilization using lysis buffer (20 mM Tris-HCl (pH7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, protease inhibitor cocktail). Biotinylated proteins were precipitated by streptavidin agarose (Solulink) and GFP signal on the beads was quantified using EnSpire multimode plate reader (PerkinElmer), normalized by GFP signal in the cell lysate.

### VSVG transport assay by confocal microscope

HeLa cells stably expressing VSVG-GFP were seeded on glass bottom dish and transfected with siRNA in some experiments. SH-5Y cells were seeded on glass bottom dish and cotransfected with VSVG-GFP and siRNA. After 24 h cells were incubated at 40°C for 18 h with the last 1 h in the presence of 10  $\mu$ g/mL cycloheximide to stop further de novo protein synthesis, and transferred to 20°C for 3 h. In some experiments inhibitors were added when the incubation at 40°C initiated. Cells were then transferred to 37°C for 20 min, fixed by 4% paraformaldehyde/PBS solution for 20 min and permeabilized by 0.2% Triton X-100-containing PBS for 10 min. Cells were blocked by 1% BSA/PBS for 10 min and then stained by anti-TGN46 antibody. The fluorescent images were obtained using a confocal laser scanning microscope (LSM700, Carl Zeiss) using an  $\times 63$  oil plan-apochromat objective (numerical aperture, 1.4; Carl Zeiss). Golgi fission index was calculated according to the definition as illustrated in Figure 6C by using ImageJ software.

### S1P receptor localization assay

Cells were seeded on glass-bottom dishes and transfected with expression vectors for VSVG-GFP or GFP and S1P<sub>1</sub>R-mCherry or S1P<sub>2</sub>R-mCherry. After 24 h, cells were incubated at 40°C for 18 h with the last 1 h in the presence of 10  $\mu$ g/mL cycloheximide. Cells were incubated at 20°C for 3 h, followed by fixation in 4% paraformaldehyde/PBS solution for 20 min and permeabilization by 0.2% Triton X-100-containing PBS for 10 min. Cells were blocked by 1% BSA/PBS for 10 min and then stained by anti-TGN46 antibody using anti-rabbit IgG Alexa 647 as a secondary antibody. The fluorescent images were obtained using a confocal laser scanning microscope (LSM700, Carl Zeiss) using an  $\times 63$  oil plan-apochromat objective (numerical aperture, 1.4; Carl Zeiss). The percentage of S1P receptor signals localized in Golgi is calculated by using ImageJ software.

### Live cell imaging

HeLa cells were seeded on glass-bottom dishes and transfected with expression vectors for VSVG-GFP, GalT-mCherry and S1P<sub>1</sub>R-CFP or S1P<sub>2</sub>R-CFP. After 24 h, cells were incubated at 40°C for 18 h with the last 1 h in the presence of 10  $\mu$ g/mL cycloheximide. The cells were transferred to 37°C and imaged on an Olympus IX71 microscope (Olympus Optical Co.) equipped with a cooled CCD camera, Cascade II 512 (Photometrics), and the imaging system was controlled by MetaFluor software (Molecular Devices).

### FRET analysis

HeLa cells seeded on glass bottom dish were transiently cotransfected with VSVG-mCherry, S1P<sub>1</sub>R-CFP or S1P<sub>2</sub>R-CFP (donor), G $\beta$  and G $\gamma$ -YFP or  $\beta$ -arrestin 2-YFP (acceptor). After 24 h, cells were incubated at 40°C for 18 h with the last 1 h in the presence of 10  $\mu$ g/mL cycloheximide. Cells were incubated at 20°C for 3 h, followed by fixation in 4% paraformaldehyde/PBS solution for 20 min. Each area of interest was subjected to FRET analysis with acceptor photobleaching method using an LSM 510 META with an  $\times 63$  oil plan-apochromat objective. Following excitation at 458 or 514 nm, CFP emission with a 475–525-nm band-pass barrier filter or YFP emission with 530–600-nm band-pass barrier filter, respectively, was collected. An area of interest was selected for photobleaching of YFP. A protocol was then used, which recorded pre- and post-bleaching images using 458 nm excitation at 10% laser power to limit photobleaching, with a bleaching of the selected area with 100%, 514 nm laser power for 100 of iteration (acceptor photobleaching). FRET was resolved as an increase in the CFP (donor) signal after photobleaching of YFP (acceptor). FRET efficiency (E) can be determined from the relative fluorescence intensity of the energy donor (CFP) before ( $I_{pre}$ ) and after ( $I_{post}$ ) photobleaching of the energy acceptor (YFP):  $E = 1 - (I_{pre}/I_{post})$ .

### **Caged-S1P loading**

Stable HeLa cells expressing VSVG-GFP grown on glass-bottom culture dishes were incubated at 40°C for 18 h with or without 5  $\mu$ M HACPT, then loaded with 1  $\mu$ M caged S1P for 30 min. After washing out the media, dishes were then flashed with UV light (254 nm) for 20 s for photolysis of caged S1P. Next, cells were incubated in culture condition at 37°C for 20 min for VSVG transport assay.

### **QUANTIFICATION AND STATISTICAL ANALYSIS**

Results are expressed as means  $\pm$  s.e.m. Data were analyzed by Welch's t test using Prism 6 for Mac OS X (GraphPad Software). p values < 0.05 were considered significant. Statistical significance is shown as follows: \*p < 0.05, \*\*p < 0.01. p > 0.05 was considered not significant (NS). Error bars indicate s.e.m.