THE EMBO JOURNAL

Manuscript EMBO-2016-96048

Sphingolipid metabolic flow controls phosphoinositide turnover at the trans Golgi network

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Review timeline:

Submission date:	11 November 2016
Editorial Decision:	16 December 2016
Revision received:	14 March 2017
Editorial Decision:	29 March 2017
Revision received:	31 March 2017
Accepted:	04 April 2017

Editor: Andrea Leibfried

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 16 December 2016

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are shown below.

As you will see, the referees appreciate your work. However, they also think that the relevance of and the direct evidence for your findings needs to be better shown (referee #1, point 1 and 2; referee #2, point 1; referee #3, point 1 and 2 of major concerns, point 1, 5, 6). Furthermore, the referees raise several technical issues (referee #1, 'other points'; referee #2, request for control in point 2; referee #3, specific major concerns 2-4).

Given the referees' positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers and especially those mentioned above.

REFEREE REPORTS

Referee #1:

Capasso et al

This is an elegant study in which Capasso et al pursue fundings of the effects of FB1 and myriocin, two inhibitors of sphingolipid (SL) synthesis, on regulation of synthesis of sphingomyelin. This leads them to define a negative feedback circuit in which SM synthesis is 'sensed' through the generated DAG and activation of PKD. While many of the individual components have been studied in previous studies, this work brings together all the key players and makes sense of the specific mechanisms of regulation involved.

1. This study does not address the consequences of perturbing this signaling mechanism on SL homeostasis. What happens to total levels of SM, ceramide, and GlcCer etc if this mechanism is interrupted (e.g. loss of PKD or Sac1)?
2. While the authors have focused on SL homeostasis, a clear connection is presented to cholesterol, yet there are no data on this point? Could the authors measure levels of sterols or synthesis of sterols if this mechanism is interfered with? This could add to the significance of this work. At the very least, the authors should discuss that.
3. There are published data on regulation of CERT by phosphorylation. How can this be incorporated into this mechanism?

Other Points

1. Fig 1B. did Brefeldin also increase the synthesis of GlcCer (did the % of SM increase with BFA)?
2. How come the anti PI4P antibody does not recognize PI4P in the plasma membrane in Figure 3A? Also, how come the FAPP2 does not detect PM PI4P and the ORP5 PH does not appear to detect Golgi PI4P? These results raise issues as to the 'absolute' specificity of these probes. These should be discussed.
3. The results shown in EV11B are somewhat out of place. They interrupt the development of the main sequence of causally related events, as the role of loss of PI4P is in 'later' feedback' mechanism.
4. EV14A is not very clear. What is being proposed about how PKD interacts with OSBP and what is being proposed about PI4P shuttling and regulation of Sac1?

Referee #2:

A major open question in lipid cell biology is how cells maintain lipid gradients along the secretory pathway in the face of extensive membrane trafficking and altering metabolic lipid fluxes. This study describes a novel molecular circuit that links sphingomyelin (SM) metabolic flow to the regulation of PI4P turnover at the TGN. By subjecting cells to controlled perturbations in sphingolipid metabolic input, the authors demonstrate that an acute increase in SM biosynthesis leads to PKD-mediated phosphorylation of OSBP, which, in turn, induces relocation of PI4P to the ER and subsequent dephosphorylation of PI4P by Sac1. Depletion of the PI4P pool at the TGN releases ceramide transfer protein CERT to the cytosol, thus reducing delivery of ER ceramides to the trans-Golgi for conversion to SM. Based on these findings, the authors postulate a critical role of OSBP in a negative feedback loop that serves to keep the rate of SM production constant in the face of acute fluctuations in the levels of sphingolipid precursors.

Overall, I find the study very interesting and well executed. An important aspect of the work is that it extends our appreciation of the cellular function of OSBP from being merely a sterol transporter to a key homeostatic regulator of sphingolipid levels at the TGN. My main concern is that the proposed role of OSBP as homeostatic regulator of subcellular sphingolipid pools is largely based on indirect evidence. As outlined below, the authors should further exploit their elegant method of measuring adaptive metabolic responses to perturbations in sphingolipid flows to challenge central aspects of their model.

1) According to the model presented in Fig. EV14, removal of OSBP should break the negative feedback loop that serves to keep the rate of SM production constant irrespective of instant changes in the levels of sphingolipid precursors. The authors should experimentally validate this prediction by analyzing the metabolic response to increasing concentrations of D-Sph (or C6-D-Cer) in control

and OSBP-KD cells, as in Fig. 1A.

2) The authors find that overexpression of OSBP reduces the TGN-associated PI4P pool (Fig. 7B). To ascertain that this reduction is indeed due to an OSBP-mediated relocation of PI4P to the ER and its subsequent turnover by Sac1 (as predicted by their model), they should also analyze the impact of overexpressing OSBP with a mutated FFAT-motif on the TGN-associated PI4P pool.

3) Note that panel D in Fig. 7 is missing, even though the main text contains a reference to Fig. 7D (on p. 16).

Referee #3:

In this manuscript the authors tackle the mechanism by which cells exhibit different amounts of sphingolipids (SL) in pre- and post-Golgi compartments. This issue is critical in cell biology since it regulates many cellular processes such as sorting, trafficking and recycling. Indeed the authors already showed that SL required PtdIns4(P) for their transport to the TGN (D'Angelo et al. 2013). Here they provide an exhaustive study allowing them to establish a model where SL flow controls PtdIns4(P) levels in the TGN, and a negative feedback circuit. More precisely by dissecting the different molecular actors involved in this homeostatic circuit, the authors revealed that SL dependent signalling pathways leads to PtdIns(4)P consumption that in turn induce the release of PtdIns4(P) effectors from TGN membranes.
Although the paper is quite complex to read and could possibly be simplified for non-specialists, the findings are very interesting and generally well supported by the data. I have a few suggestions and questions that might improve the clarity and quality of the data.

Major concerns

Although the bulk of data indicate that at the TGN there is a PI4P-based feedback system to regulate the levels of SL two main aspects should be clarified/discussed in the paper:

1) The timing and dynamics of this process. In other words, the big supply of SL precursors should induce, at early time-points, a strong increase of SM, following the strong recruitment of CERT, FAPP2, OSB1 and their activity.
2) What is the effect of SL precursor addition and of the activation of this feedback system on lipid trafficking? Is the composition of post-TGN compartments altered?

Specific major concerns:

1) It is clear that the distribution of CERT is dependent on the levels of sphingolipids. This issue is very important and it should be better analysed:

a) Authors should quantify the amount of CERT in the different subcellular compartments at steady-state and upon the different treatments;
b) Fig. EV4 is confusing: why at steady-state (CTRL in the figure) CERT is present in small dots resembling post-TGN carriers?

2) Upon C6-D-Cer treatment CERT is shifted in the cytosol. By looking at the picture the signal resembles an ER signal, the authors should specify/comment this. Moreover it seems that the overall fluorescent signal is decreased (Fig 1C, EV3). Is this due to the decrease of levels of CERT expression? Does the sphingolipid flow affect the expression of CERT (at protein or at mRNA level)? Does the sphingolipid flow affect ER-GOLGI contact sites?

3) Fig 2A: I am surprised how Sec31 looks like in control cells? Sec31 should be localized at the ER and in ERES.

4) Upon D-C6-Cer addition the distribution of TGN results are slightly different: at least in some cells the TGN46 positive compartments appear enlarged, while upon the same conditions the

localization of giantin, GRASP65 are unaffected. It would be therefore very interesting to understand if exclusively the TGN compartments are subjected to changes in dependence on SL levels. Have the authors checked this point? At least they should comment on it.

5) In Figure 3 the authors show that C6-D-Cer and D-Sph treatment induces the disappearance of PtdIns4P from the Golgi. This is a critical finding for proposing the model where SL flow induces PtdIns4P consumption. However, the authors showed only immunofluorescence data where PtdIns4P seems to disappear from the Golgi and the signal appears to be overall much weaker in the whole cell (EV7 especially). It might be necessary to support the authors's conclusion using alternative methodology in order to monitor dynamically the fate of PtdIns4(P) (biochemistry or live imaging).

Furthermore, as shown in Figure 3, the addition of C6-D-Cer induces a consumption of PI4P, however there are some points, technically and conceptually, that are unclear:
a) Why is the Ab anti-PI4P unable to recognize the plasma membrane pool?
b) The authors claim that only the TGN pool is sensitive to SL flow. I expect to find an effect, at longer times, also on the plasma membrane pool if the source is impoverished.
As mentioned above, what is the impact on lipid/membrane trafficking? Maybe the authors should analyse what happens to ORP5 and ORP8L at longer time points?

6) Does the Golgi delocalization of CERT upon SL precursors addition occur through PKD phosphorylation? Or is this just a consequence of the decreased levels of PI4P?

Minor concerns

1) Page 8 of Results: authors refer to Fig "2D" while they wrote "Fig. 2C"
2) In my opinion the experiments with the use of C6-LCere and LCL29 do not assess whether the large supply of sphingolipid precursor could be poisoning for the cells. Authors should perform tests of cell viability.
3) Figure 3D, the cells are permeabilized to allow the detection of Golgin97 and in these conditions it is difficult to assess the plasma membrane localization of GFP-tagged, overexpressed proteins. Authors should repeat the experiments in non-permeabilized conditions.
4) In Figure 4C as they only show indirect evidences, would it be possible to confirm these data by using another method/probe to assess DAG production? In addition it would be nice to add another panel at an intermediate time point between 10 and 60minutes, in order to compare it with the biochemical data of panel 4D.
5) Figure 6B the panels are extremely small and this is not so obvious that only PI4KIIIbeta impairs CERT Golgi recruitment.
6) Panel D of the figure 7 is missing

Any additional non-essential suggestions for improving the study

1) All data rely on the use of 2 compounds (FB1 and myriocin); would the results be similar if authors use other inhibitors?
2) Why did the authors inverted fluorophore to reveal CERT and Golgin 97 between panel 1C and EV3? Panel 1C CERT is 488 and Golgin97 546 while in EV3 this is the reverse?
3) The data on ARF overexpression (ARF1-myc) could be corroborated by detection of the endogenous ARF proteins either by IF or by biochemistry.

1st Revision - authors' response 14 March 2017

Response to Referees

We thank the Referees to have read our manuscript in detail and to have suggested new experiments to corroborate our conclusions. We have approached experimentally as many Referees comments as possible and we think that this has improved the quality and the significance of our work. Replies to specific comments are detailed below.

Referee #1:

**1. This study does not address the consequences of perturbing this signaling mechanism on SL homeostasis. What happens to total levels of SM, ceramide, and GlcCer etc if this mechanism is interrupted (e.g. loss of PKD or Sac1)?*

We have now analysed the total levels of SM ceramide and HexCer in Sac1-KD HeLa cells by LC-MS. Our data show that compared to control, Sac1-KD cells have reduced sphingoid bases levels (both Sph and dhSph) and increased levels of ceramide, SM and HexCer. These data indicate that Sac1 (and by extension PtdIns(4)*P*) has a role in the control of SL homeostasis and specifically in the conversion of sphingoid bases in more complex metabolites. Moreover, the overall SL levels are significantly increased in Sac1-KD cells suggesting that PtdIns(4)*P* dephosphorylation is involved in the control of global SL levels (**Fig EV19**).

**2. While the authors have focused on SL homeostasis, a clear connection is presented to cholesterol, yet there are no data on this point? Could the authors measure levels of sterols or synthesis of sterols if this mechanism is interfered with? This could add to the significance of this work. At the very least, the authors should discuss that.*

We have now measured the levels and synthesis of cholesterol and cholesterol esters (by HPTLC and GC/MS) in cells where the SL flow was perturbed. According to the data obtained increased SL flow induces an increase in cholesterol esters production, which we interpret as a redirection of cholesterol flux towards the lipid droplets compartment (**Fig EV16**).

3. There are published data on regulation of CERT by phosphorylation. How can this be incorporated into this mechanism?

As correctly reported by this Referee, CERT is subjected to regulation by phosphorylation. Different kinases have been found to phosphorylate CERT at different phospho-sites (Kumagai et al. J Biol Chem. 2014; Tomishige et al. Mol Biol Cell. 2009; Fugmann et al. J Cell Biol. 2007; Kumagai et al. J Biol Chem. 2007). A key phosphorylation in the regulation of CERT 'cycle' is that operated by PKD at Serine 132 (Fugmann et al. J Cell Biol. 2007). When phosphorylated by PKD at S132 CERT shows reduced affinity for PtdIns(4)*P* (Fugmann et al. J Cell Biol. 2007). This is relevant here as in this manuscript we describe a regulatory circuit that 'inactivates' CERT through a PKD-dependent consumption of the Golgi PtdIns(4)*P* pool. It is conceivable to hypothesize that PKD activation induced by SL flow counteracts CERT function by reducing both the levels of PtdIns(4)*P* at the TGN and the ability of CERT to bind it. We have now experimentally evaluated the contribution of PKD phosphorylation on CERT (by the use of the CERT phospho-depleted mutant S132A to its localization at the Golgi under SL challenges. We found that CERT-S132A is sensitive to both SL flow reduction (it is effectively recruited to TGN under myriocin treatment) and increase (it is redistributed to the cytosol following C6-D-Cer treatment). These data suggest that the contribution of direct CERT phosphorylation by PKD is minor when compared with the primary effect on PtdIns(4)*P* levels in the phenomenon we analyse in this paper (**Fig EV16**). Nonetheless CERT phosphorylation at S132 influences its localization (as testified by the increased association with membranes of the CERT-S132A mutant under basal conditions) thus adding a parallel regulatory layer to our circuit.

Other Points

1. Fig 1B. did Brefeldin also increase the synthesis of GlcCer (did the % of SM increase with BFA)?

We have now reported the effect of BFA treatment on GlcCer (**Fig 1B,** insets). As reported in the figure GlcCer production was not influenced by BFA. Also BFA treatment did not significantly change the SL-precursors uptake (not shown), as a consequence the % of precursor converted in SM (at high SL precursor concentrations) is increased in BFA treated cells.

2. How come the anti PI4P antibody does not recognize PI4P in the plasma membrane in Figure 3A? Also, how come the FAPP2 does not detect PM PI4P and the ORP5 PH does not appear to detect Golgi PI4P? These results raise issues as to the 'absolute' specificity of these probes. These should be discussed.

We apologize with this referee for having failed to convey complete information on the tools used in this study. Recent studies have highlighted the existence of multiple pools of PtdIns(4)*P* at the TGN, PM and endo/ lysosomal compartments (Hammond et al. JCB 2014). Nonetheless, with the exception of the P4M-SidM probe developed by Hammond et al. in Tamas Balla laboratory, antibodies and protein domains used as PtdIns(4)*P* probes often display selectivity for one specific subcellular PtdIns(4)*P* pool depending on the probe and on the staining protocol used.

Specifically, the antibody that we have used to detect PtdIns(4)*P* in this study has been extensively characterized (Hammond et al. Biochem J 2009). This antibody effectively and specifically recognizes both the PM and Golgi PtdIns(4)*P* pools, but as stated by the authors *'the conditions that preserve plasma membrane staining are diametrically opposed to those best for Golgi staining'*. In this study we deliberately decided to use the conditions *'best for Golgi staining'* provided that the Golgi PtdIns(4)*P* was found to be specifically sensitive to our treatments as inferred by the use of different PtdIns(4)*P* binding domains.

As for the second question, it has been reported that protein domains having affinity for phosphoinostitides often bind a second factor on membranes that participates in defining their subcellular localization (Carlton and Cullen, Trends Cell. Biol., 2005). This property is referred to as 'coincident detection' alluding to the fact that these co-receptor and phosphoinostitides are bound simultaneously by protein domains. In the case of FAPP2-PH domain (and of PH domains of other Golgi localized PtdIns(4)*P* binding proteins) the co-receptor that is coincidentally detected along

with PtdIns(4)*P* is the Golgi GTPase ARF1 (Godi et al, Nat. Cell Biol., 2004). Both ARF1 and PtdIns(4)*P* binding are necessary (but not sufficient) for FAPP2 localization to the Golgi (Godi et al, Nat. Cell Biol., 2004; D'Angelo et al. Nature, 2013). The PH domains of ORP5 and ORP8L also are supposed to bind a co-receptor at the plasma membrane where its nature is still not defined (Pietro De Camilli personal communication). Nonetheless as for FAPP2-PH also ORP5 and ORP8L PHs require PtdIns(4)*P* for membrane binding (Chung et al., Science, 2015). In all, the current view on PtdIns(4)*P* binding proteins is that PtdIns(4)*P* provides an anchorage to a subset of intracellular membranes where a second factor determines the specificity among the different PtdIns(4)*P* pools.

We have now included this information and discussed it in the new version of our manuscript. In addition and for completeness we have now used the recently developed PtdIns(4)*P* probe (i.e., diP4M-SidM-mCherry) (Hammond et al. JCB 2014) that reveals all the different intracellular PtdIns(4)*P* pools. The use of this new tool confirms that increased SL flux specifically impacts on the Golgi PtdIns(4)*P* pool (**Fig EV11A**, and **B**, and **Movie 6**).

3. The results shown in EV11B are somewhat out of place. They interrupt the development of the main sequence of causally related events, as the role of loss of PI4P is in 'later' feedback' mechanism.

We have now shifted the results presented in this figure later in the text (**Fig EV21A**) so to not interrupt the 'narrative' of the manuscript.

4. EV14A is not very clear. What is being proposed about how PKD interacts with OSBP and what is being proposed about PI4P shuttling and regulation of Sac1?

According to our model PKD phosphorylates OSBP1 [most probably at Ser 240 (Nhek et al., 2010)]. OSBP1 phosphorylation by PKD is reported to inhibit its localization to the TGN possibly modulating its affinity for the ER and TGN binding factors VAPs and PtdIns(4)*P* respectively. Our data suggest that OSBP1 phosphorylation fosters PtdIns(4)*P* relocation to the ER and Sac1-dependent PtdIns(4)*P* dephosphorylation. We have now included this description in the discussion and in the figure legend.

Referee #2:

**1) According to the model presented in Fig. EV14, removal of OSBP should break the negative feedback loop that serves to keep the rate of SM production constant irrespective of instant changes in the levels of sphingolipid precursors. The authors should experimentally validate this prediction*

by analyzing the metabolic response to increasing concentrations of D-Sph (or C6-D-Cer) in control and OSBP-KD cells, as in Fig. 1A.

We have performed the experiment suggested by this Referee. As now reported in **Fig. EV20B** the silencing of OSBP made HeLa cells more resistant to SL challenges in terms of SM synthesis inhibition as predicted by the model.

2) The authors find that overexpression of OSBP reduces the TGN-associated PI4P pool (Fig. 7B). To

ascertain that this reduction is indeed due to an OSBP-mediated relocation of PI4P to the ER and its subsequent turnover by Sac1 (as predicted by their model), they should also analyze the impact of overexpressing OSBP with a mutated FFAT-motif on the TGN-associated PI4P pool.

We have now overexpressed an OSBP1 FF>AA mutant (Mesmin et al, Cell, 2013) and found that it does not induce a reduction of the TGN-associated PtdIns(4)*P* pool at expression levels where wt OSBP1 does (**Fig. EV20A**).

3) Note that panel D in Fig. 7 is missing, even though the main text contains a reference to Fig. 7D (on p. 16).

We apologize for this inconsistence. We have now amended the figure and text accordingly.

Referee #3:

Although the bulk of data indicate that at the TGN there is a PI4P-based feedback system to regulate the levels of SL two main aspects should be clarified/discussed in the paper:

**1) The timing and dynamics of this process. In other words, the big supply of SL precursors should induce, at early time-points, a strong increase of SM, following the strong recruitment of CERT, FAPP2, OSB1 and their activity.*

To address this point we have now measured the conversion of C6-D-Cer in C6-D-GlcCer and C6-D-SM at early time points after C6-D-Cer (10μM) treatment. Our results show that (as anticipated by this Referee) at early times cells display a highly efficient SM production. Following on this early phase, the metabolic flux to SM is slowed down while that to C6-D-GlcCer remained constant (**Fig. EV2**).

**2) What is the effect of SL precursor addition and of the activation of this feedback system on lipid trafficking? Is the composition of post-TGN compartments altered?*

According to our working model the activation of the feedback mechanism described in this manuscript should result in the blockade of Cer transport to the TGN. We have now directly tested this hypothesis and found that following on SL precursors load, C5-BODIPY-Cer is no longer transported to the Golgi (**Fig. EV4D**). Previous reports have shown that following on SL precursors addition, the formation of post-Golgi-carriers is strongly impaired and that this effect requires the conversion of SL precursors to sphingomyelin (Duran et al. EMBO J, 2012). We confirm this evidence in our hands (not shown) and we conclude that under conditions where the described feedback mechanism is activated both lipid trafficking (to the TGN) and membrane trafficking (out of the TGN) are blocked. This combined inhibition results in that acute oscillations in the SL new-synthetic load are not immediately translated into an alteration of the lipid composition of the post-TGN compartments.

Specific major concerns:

**1) It is clear that the distribution of CERT is dependent on the levels of sphingolipids. This issue is very important and it should be better analysed:*

a) Authors should quantify the amount of CERT in the different subcellular compartments at steady-state and upon the different treatments;

We have now evaluated the distribution of CERT upon SL challenges by new experiments. First we have visualized this phenomenon by live imaging (**Fig. EV5A** and **Movie 1**). Then, by immunofluorescence (**Fig. EV5B**) using different organelle markers, we have analysed the amount of CERT in the different subcellular compartments at steady state and upon the different treatments.

b) Fig. EV4 is confusing: why at steady-state (CTRL in the figure) CERT is present in small dots resembling post-TGN carriers?

As already reported by Fugman et al., (JCB 2007) overexpressed CERT (and specifically the PKD phospho-depleted mutant S132A) decorates a population of cytosolic vesicles. They also show that '*Some of the vesicular structures were found to contain the cargo protein ssHRP, providing evidence that these structures represent Golgi-derived transport carriers'*. Consistently, we do find overexpressed CERT (both wt and S132A) at cytosolic vesicles, which do not overlap with endosomal/lysosomal markers (EEA1, M6PR, LAMP1, Tfn) and that partially co-localize with CD8alpha positive post-Golgi carriers (**Fig EV5B** and **C**, and **Movie for Referees 1**).

2) Upon C6-D-Cer treatment CERT is shifted in the cytosol. By looking at the picture the signal resembles an ER signal, the authors should specify/comment this. Moreover it seems that the overall fluorescent signal is decreased (Fig 1C, EV3). Is this due to the decrease of levels of CERT expression? Does the sphingolipid flow affect the expression of CERT (at protein or at mRNA level)? Does the sphingolipid flow affect ER-GOLGI contact sites?

We have now evaluated the distribution of CERT following C6-D-Cer treatment and found that it induces a redistribution of CERT from the Golgi to a diffused compartment that does not overlap with the ER (**Fig EV5B**). Moreover we have tested whether C6-D-Cer treatment impacts on CERT mRNA or protein levels and found that it doesn't (**Fig EV4B**, and **C**)

As pointed out by this Referee according to our model, sphingolipid flow should impact on ER-TGN membrane contact sites as it controls PtdIns(4)*P* (see Mesmin at al. Cell, 2013). This is a very interesting aspect and is matter of intense investigation in the lab at the moment. Our new data show that SL flow specifically perturbs TGN structure (**Fig EV12, EV14** and **Movies 7-10**) that could possibly interfere with the ER/TGN MCSs, nonetheless we think that the dissection of this specific issue goes beyond the scope of this paper and deserves a dedicated study.

3) Fig 2A: I am surprised how Sec31 looks like in control cells? Sec31 should be localized at the ER and in ERES.

As correctly stated by this Referee the mammalian Sec31 decorates ER exit sites (ERES) as accurately demonstrated by Tang et al. (JBC 2000) who first cloned it. Our immunolocalization of Sec31 is superimposable to that shown by Tang et al. We, like them find that *'Sec31A is associated with vesicular structures that are concentrated at the perinuclear region but are also found scattered at the cell periphery'*. Thus we are confident that our staining represents the genuine Sec31 localization at ERES.

4) Upon D-C6-Cer addition the distribution of TGN results are slightly different: at least in some cells the TGN46 positive compartments appear enlarged, while upon the same conditions the localization of giantin, GRASP65 are unaffected. It would be therefore very interesting to understand if exclusively the TGN compartments are subjected to changes in dependence on SL levels. Have the authors checked this point? At least they should comment on it.

We have now evaluated the effect of D-C6-Cer treatment on Golgi structure by immunoflurescnce, electron microscopy and electron tomography experiments (**Fig EV12, Movies 7-10**). According to our results D-C6-Cer treatment induces a morphological alteration of the Trans Golgi/ TGN characterized by the 'curling' of late cisternae and by formation of inward budded vesicles at the

trans Golgi. Interestingly these effects were counteracted by SMS1-KD (**Fig EV14**) suggesting that the activation of the SMS1 dependent feedback and the loss of PtdIns(4)*P* cause TGN alterations.

**5) In Figure 3 the authors show that C6-D-Cer and D-Sph treatment induces the disappearance of PtdIns4P from the Golgi. This is a critical finding for proposing the model where SL flow induces PtdIns4P consumption. However, the authors showed only immunofluorescence data where PtdIns4P seems to disappear from the Golgi and the signal appears to be overall much weaker in the whole cell (EV7 especially). It might be necessary to support the authors's conclusion using alternative methodology in order to monitor dynamically the fate of PtdIns4(P) (biochemistry or live imaging).*

We have now monitored dynamically PtdIns(4)*P* by the use of diP4M-SidM-mCherry (see **Fig EV11A**, and **B** and **Movie 6**) by live imaging. Our data support that the Golgi PtdIns(4)*P* pool is specifically sensitive to increased SL flow.

Furthermore, as shown in Figure 3, the addition of C6-D-Cer induces a consumption of PI4P, however there are some points, technically and conceptually, that are unclear:

a) Why is the Ab anti-PI4P unable to recognize the plasma membrane pool?

Please see reply to Referee1 'other points' 2.

b) The authors claim that only the TGN pool is sensitive to SL flow. I expect to find an effect, at longer times, also on the plasma membrane pool if the source is impoverished.
As mentioned above, what is the impact on lipid/membrane trafficking? Maybe the authors should

analyse what happens to ORP5 and ORP8L at longer time points?

We have now approached this issue by performing the experiment suggested by this Referee. We found that even after 16 hours of treatment with D-C6-Cer the localization of the PM PtdIns(4)*P* probes ORP5 and ORP8L PH domains is not affected. Notably also probes for other phosphoinositides (i.e., PLCδ-PH, or FYVE) were not influenced in their localization after long D-C6-Cer treatments (**Fig EV11C**). These data suggest that the Golgi PtdIns(4)*P* pool is metabolically isolated from that at the PM and from other phosphoinositides. This evidence was unexpected and invites new investigation on the specific issue that we intend to explore in the future.

**6) Does the Golgi delocalization of CERT upon SL precursors addition occur through PKD phosphorylation? Or is this just a consequence of the decreased levels of PI4P?*

We have now experimentally evaluated the contribution of PKD phosphorylation on CERT (by the use of the CERT phospho-depleted mutant S132A to its localization at the Golgi under SL challenges and found it to be minor when compared with the primary effect on PtdIns(4)*P* levels (**Fig EV16**). Nonetheless CERT phosphorylation at S132 influences its localization thus adding a parallel regulatory layer to our circuit.

Minor concerns

1) Page 8 of Results: authors refer to Fig "2D" while they wrote "Fig. 2C"

We apologize for this inconsistence. We have now amended the figure and text accordingly.

2) In my opinion the experiments with the use of C6-LCere and LCL29 do not assess whether the large supply of sphingolipid precursor could be poisoning for the cells. Authors should perform tests of cell viability.

LCL29 and C6-D-Cer (not C6-L-Cer) are supposed to induce apoptosis. We have now tested this experimentally (**Fig EV10**) and found that LCL29 is the most potent inducer of apoptosis with significant PARP1 cleavage being visible after 16 hours of treatment (10μM). C6-D-Cer was found to be much less effective in inducing PARP cleavage while, as expected, C6-L-Cer was completely

non-effective. Notably, at 2 hours treatment (i.e., the time at which PtdIns4P was evaluated), none of these compounds induces significant apoptosis.

3) Figure 3D, the cells are permeabilized to allow the detection of Golgin97 and in these conditions it is difficult to assess the plasma membrane localization of GFP-tagged, overexpressed proteins. Authors should repeat the experiments in non-permeabilized conditions.

We have now done this and results in **Fig EV11C** have been obtained by imaging non-permeabilized cells.

4) In Figure 4C as they only show indirect evidences, would it be possible to confirm these data by using another method/probe to assess DAG production? In addition it would be nice to add another panel at an intermediate time point between 10 and 60minutes, in order to compare it with the biochemical data of panel 4D.

We have now used a different DAG probe (i.e., YFP-DBD) for live imaging experiments covering the first 25 min of treatment with C6-D-Cer (**Movie 11**)

5) Figure 6B the panels are extremely small and this is not so obvious that only PI4KIIIbeta impairs CERT Golgi recruitment.

We have now split **Fig 6** in two figures (**Fig 6** and **Fig EV17**) to make the panels more easily readable.

6) Panel D of the figure 7 is missing

We apologize for this inconsistence. We have now amended text accordingly.

Movie for Referees 1: *GFP-CERT decorates a sub-population of Post-Golgi carriers.*

HeLa cells expressing GFP-CERT (green) and a synchronizable and mCherry tagged version of the cargo CD8alpha (red) were imaged for 10 min during cargo release (32 C) after cargo accumulation at the TGN (by keeping cells for 2 hours at 20 C). Note that a subpopulation of mCherry-CD8alpha positive carriers is decorated by GFP-CERT.

2nd Editorial Decision 29 March 2017

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by the three original referees whose comments are enclosed. As you will see, all referees now support publication.

REFEREE REPORTS
Referee #1:

The authors have responded to all my comments satisfactorily.

The study makes important points about the homeostasis of lipid metabolism in the Golgi.

Referee #2:

The authors submitted a significantly improved manuscript in which they adequately addressed all my original concerns. I find their study of great interest and very well executed.

Referee #3:

This study describes a novel molecular circuit that links sphingomyelin (SM) metabolic flow to the regulation of PI4P turnover at the TGN. The topic is very interesting; the experiments are well executed and support the author's conclusions. The authors have satisfactorly addressed all my comments and clarified my doubts. I find that the new version of the manuscript is much improved, and I am happy with it.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Giovanni D'Angelo
Journal Submitted to: the EMBO journal
Manuscript Number: EMBOJ-2016-96048R

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Most experiments were performed at least in quintuplicate and data are reported either as mean values or as representative images. GC-MS based sterol measurements were performed in duplicate and data are reposrted as singole replicates.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	No animal studies are reported in this manuscript
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	All the experimentally produced samples were included in our analysis
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	For fluorescence image analysis, random fields were chosen by observing samples on channels not relevant for the analysis (i.e., DAPI). Images were then acquired in all the channels and were applicable software based automated analysis was performed. For electron microscopy analysis field were selected based on the presence of a morphologically recognizable Golgi complex. For electron tomography field were chosen that contained the structures characterized by transmission electron microscopy.
For animal studies, include a statement about randomization even if no randomization was used.	No animal studies are reported in this manuscript
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	When visual counting of phenotypes was performed, evaluation was done in 'single blind' where the experimentator was unaware of the exact identity of the samples he/ she was observing.
4.b. For animal studies, include a statement about blinding even if no blinding was done	No animal studies are reported in this manuscript.
5. For every figure, are statistical tests justified as appropriate?	Two-tailed Student's T-Test was used as a statistical test.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	When possible (for single cell analysis were the number of obsevation was >100) the values distribution of our measurements was evaluated and found to approximate to normal distribution.
Is there an estimate of variation within each group of data?	The statistical test used assumes unequal variance between groups so to keep the significant assessment more stringent
Is the variance similar between the groups that are being statistically compared?	The statistical test used assumes unequal variance between groups so to keep the significant assessment more stringent

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

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http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

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http://www.ncbi.nlm.nih.gov/gap

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http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	All the antibodies used in this study were previously used and profiled for the assay and species used in this study. References and sources (either commercial anc academic are reported in Table1).
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	In this study we used HeLa cells obtained from American Tissue Type Collection (ATTC, USA) (https://www.lgcstandards-atcc.org/products/all/CCL-2.aspx?geo_country=it).

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	No animal model was used in this study.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	No animal model was used in this study.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	No animal model was used in this study.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	No human subject was involved in this study.
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	No human subject was involved in this study.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	No human subject was involved in this study.
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	No human subject was involved in this study.
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	No human subject was involved in this study.
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	No human subject was involved in this study.
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	No human subject was involved in this study.

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	No data of the kind refrred in this point are reported in this study.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	We commit to provide all numerical row data central to this study once and if it is accepted by the journal.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	No such data are reported in this study
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	No such data have been used in this study
22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	No computational models are reposted in this study

G- Dual use research of concern

23. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	We do not see roon for dual use in our research.