Coupled Sterol Synthesis and Transport Machineries at ER-Endocytic Contact Sites

Javier Encinar del Dedo, Isabel María Fernández-Golbano, Laura Pastor, Paula Meler, Cristina Ferrer-Orta, Elena Rebollo, and Maria Isabel Geli

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Dear Dr. Geli,

Thank you for submitting your manuscript entitled "Coupled Sterol Synthesis and Transport at ER-Endocytic Contact Sites". The manuscript was assessed by expert reviewers, whose comments are appended to this letter. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

You will see that all three reviewers thought your study was a potentially significant advance, but also raised a large number of concerns about almost every aspect of your study. They have all written thoughtful, constructive critiques. Rev 2 asks for better evidence that sterol synthesis and transport are coupled. Addressing these concerns would substantially improve the study. The other two reviewers have a number of good suggestions for obtaining more substantial insight into the role of osh2 and other ORPs in sterol and PIP trafficking. They also raise important technical concerns about the specificity of OSW-1 (rev 3, pt 1) and the D4H sensor (rev 3, pt 4) that need to be addressed. An investigation of how and why sterols accumulate in the ER of cells lacking Osh2 or Osh4 (rev 1, pt 3) would be a welcome addition, but is not required. Please let us know if you would like to discuss potential revision plans, especially if you would like to refocus your study in light of the reviewers' comments.

While you are revising your manuscript, please also attend to the following editorial points to help expedite the publication of your manuscript. Please direct any editorial questions to the journal office.

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Text limits: Character count for a Report is < 20,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

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November 2, 2020

Re: JCB manuscript #202010016

Dr. Maria Isabel Geli
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Dear Dr. Geli,

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***IMPORTANT: It is JCB policy that if requested, original data images must be made available. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original microscopy and blot data images before submitting your revision.***
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When submitting the revision, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

We hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised in this letter.

Thank you for this interesting contribution to Journal of Cell Biology. You can contact us at the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Sincerely,

William Prinz, PhD
Monitoring Editor

Andrea L. Marat, PhD
Senior Scientific Editor

Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

In this work, the authors show that Osh2 is required to extract sterols from ERSSS (ER sterol synthesis and exit sites), and that Osh2 may form a complex with sterol synthesizing enzymes and proteins involved in endocytosis. Moreover, the sterol dynamics are very different between mother and daughter cells. Overall, there are some interesting findings but major concerns remain.

1. Sterol delivery from sites of synthesis to the PM remains a key question in cell biology. The findings that support sterol extraction by Osh2 was presented in figure 2. D4H positive dots were increased at cortical ER in osh2 null cells. This is the key observation of this work, and needs to be further strengthened. For instance, is the FFAT motif important for this function? Is PIP binding (e.g. HH mutants) required? Can a sterol binding mutant of Osh2 complement this?

2. In this regard, some relevance to mammalian cells would strengthen the study. Mammalian ORP2, and to some extent ORP1S can deliver cholesterol to the PM (PMID: 30581148). Can ORP2 or ORP1S rescue the D4H phenotype in figure 2?
3. In osh2 or osh4 null cells, more D4H spots were detected in the ER (Fig. 2 & S2). At least in mammals, sterols in the ER are very low and are hardly detectable by D4H. It is likely that there are specialized, but non-overlapping regions in the ER for osh2/4 function. Does Osh4p also associate with some the same ERG mutants? This would be a good control. What happens to ER D4H in cells null for both osh2 and osh4? Why is not increased ER sterol (D4H positive spots) converted to sterol esters? Where is Are1 and Are2 in osh2 null or osh2osh4 null cells?

4. There is also a general concern about whether D4H indeed reflects sterol levels. At least some of the D4H imaging should be verified with another probe, such as purified AloD4, PFO etc.

5. Figure 3. These physical interactions are not very meaningful without functional validation. Are any of the interactions important for Osh2 to extract sterol from the ER?

6. The data used to argue against counter transport by Osh2 is not convincing. ORPs can also use PIP2 as a counter-current so reducing PI4P alone may not impact Osh2 function. PIP2 is more abundant than PI4P at the PM. Osh2 can associate with PIP2 (see T. Schulz and W. Prinz, BBA, 2007). Is there any change in PM PIP2 in osh2 null cells or osh2,3 null cells or in cells overexpressing OSH2? This PIP2 angle is important as PIP2 plays a key role in actin dynamics and endocytosis. Perhaps some of the effects associated with osh2 are due to PIP2.

7. The quality of figure 1E appears very poor, so is Figure 2B.

Reviewer #2 (Comments to the Authors (Required)):

In this paper, the authors use a recently developed sterol probe (D4H-GFP) which labels accessible sterols or high concentration sterol regions and identify a differential labeling in mother (cER rim and vesicles) and daughter cells (even distribution in the PM). By using a combination of co-localization, co-IPs, mutants and drugs experiments, they show that the D4H labelled cortical punctae correspond to ER-endocytes contact sites where sterol synthesizing enzymes, the sterol transporter Osh2 and the endocytic machinery interact to facilitate endocyte formation by coupling sterol synthesis and transport. They named this new domain ERSSSES for ER Sterol Synthesis and Exit Sites. In parallel, they show that these domains are involved in endocyte formation in mother but not in daughter cells where endocytosis is coupled to sterol loading from PM and secretion. Previously, the authors have shown that the sterol transport activity of Osh2 and interaction with Myo5 at ER-endocytes MCSs was required to promote actin polymerization and membrane invagination at endocytic sites. The novelty of this work is the identification of a subset of sterol synthesizing enzymes as Osh2 and Myo5 interactors at ER-endocyte MCSs, the putative coupling of sterol synthesis and sterol transport in the formation of the endocytes and a differential mechanisms of sterol loading to endocyte in mother and daughter cells. The formation of a complex between some ERG subunits, Osh2 and Myo5 is very interesting and convincing but the functional role of this association and the use of the term "on site coupling of sterol synthesis and transfer" required more investigation. The experiments showing that endocytosis in daughter cells might relies on a different mechanism of sterol uptake are also convincing but the authors have to clarify some points regarding the presence of ERSSSES in daughter cells.

Major points
- Coupled synthesis and transfer. In the paper, the authors clearly show that Osh2 is required to extract sterols from ERSSSES and that global sterol synthesis is required, which seems logical to provide substrate for transfer. However, they do not provide clear evidence that local sterol synthesis at ERSSSES is required. In Fig2C, the fact that D4H intensity decreases in WT after sterol synthesis inhibition suggests that Osh2 can transport lipids after synthesis. To show a coupling between synthesis and transport, the author might, as examples, express Osh2 under an inducible promotor in osh2Δ background after incubation with FPM to show if they observe a decrease of
D4H signal at cER rims after synthesis or try to relocate one of this enzymes involved in sterol synthesis in another part of the ER to prove that local synthesis at ER-SSS is required for transfer by Osh2 (as it has been performed recently for phagophore formation (https://doi.org/10.1016/j.cell.2019.12.005)). In addition, only a few enzymes involved in ergosterol synthesis, not involved in the final step of synthesis, are located at ER-SSS and also at lipid droplet surface. Thus, the authors have to provide more evidences of coupled synthesis and transfer or to change the title and writing of the manuscript.

- The authors have to clarify if ER-SSS are also present in daughter cells and in which cells (mother or daughter) they performed their quantification in the first part of the article (Fig 1 to 3). Indeed, D4H labeling pattern in daughter cells is sometimes unclear. In Fig.1A or S1, the signal looks evenly distributed around the PM whereas in other cells (Fig.1B or Fig2), we can clearly see some dots in the cortical region of daughter cells that in some pictures co-localized with Osh2, ERG6 or Sec61 (Fig 3A, B, Fig S2B). In the same way, the ERG6-27 proteins also form dots co-localizing in daughter cells (Fig S3A) and Osh2 and Osh6 dots are also present in daughter cells (Fig3H). Thus, the authors should clarify 1) in how many daughter cells (or depending on the daughter cell size) they observed cortical D4H dots and/or evenly distributed D4H signal and 2) is ER-SSS sites also exist in daughter cells.

Minor points
- As PM sterols in mother cells is supposed to be less accessible (i.e. not labelled by D4H), is sterol transport from ER at ER-endocytes MCSs required only to promote actin polymerization or also to provide lipids to sustain endocytes growth? This can make a difference in term of the quantity of lipid transported. Coupling between lipid synthesis and phagophore growth have been recently described in yeast (https://doi.org/10.1016/j.cell.2019.12.005). Can the authors discuss this point in the discussion?
- It is strange that no PM labeling with D4H probe is observed at all in the mother cells whereas it has been observed in all the organisms studied until now with this probe, including mammals where PM subdomains enriched in sterols also exists. In addition, this probe seems to be sensitive to both sterol accessibility and concentration. Concerning the labeling at cER rims, how the authors can differentiate between a local enrichment of sterol and/or a higher accessibility.
- Could the authors provide a quantitative analysis of the immune-EM labeling performed in Fig. 1E and 2B as it has been performed in Encinar del Dedo 2017.

Reviewer #3 (Comments to the Authors (Required)):

In this manuscript Encinar del Dedo and co-workers provide new insights into the distribution of sterol inside the budding yeast, unveiling in particular a close interplay between the sterol transporter Osh2, localized at ER-PM contact sites, a subset of ER-resident ergosterol-synthesizing enzymes (Erg) and actin polymerization processes, ensuring the invagination of the PM during endocytosis, which relies on sterol accumulation in the PM. In the first part of the manuscript, the authors use a genetically-encoded fluorescent sterol sensor(GFP-D4H) to visualize how sterol is distributed inside the cell. They report that sterol distribution is highly polarized, observing a large accumulation of sterol in the PM of the daughter cell, when budding from the mother cell, comparatively to the mother cell. Sterol-rich secretory vesicles moving toward the bud seem to responsible for supplying the PM with sterol. In the mother cell, sterol is mostly seen enriched in punctuate structures corresponding to rims of the cortical ER, i.e. a part of the ER involved in contact sites with the PM. From this result it is suggested that sterol is particularly
available in those sites (referred to as ER Sterol Synthesis and Exit site ERSSES) and then transferred by Osh2 to endocytic sites. In the second part of the manuscript, co-localisation experiments are performed that support this idea. Moreover, GST pull-down and double-hybrid assays reveal that Osh2, in addition to interacting with Myo5 (as shown in Javier Encinar del Dedo et al. 2017, Dev Cell) and VAP Scs2p protein (Levine’s paper), also physically interacts with Erg6 via its sterol-binding domain (ORD). In addition, Myo5 binds to Erg6 and Erg27. The third part of the study aims at demonstrating, by using various compounds to either sequester sterol, inhibit sterol synthesis or possibly inhibit Osh2 transfer activity that (i) the delivery of sterol by Osh2 is critical for endocytic processes in the mother cell but not in the daughter cell (ii) that sterol conveyed by the vesicular secretory pathway is critical to maintain the endocytic capabilities of the daughter cell (iii) that Erg6 and Erg27 as well as their interaction with Myo5 are critical for endocytosis in the daughter cell.

With this study, Gelli’s lab pursues a previous work it published in Dev Cell in 2017, which described a new sterol transfer route, from the ER to the PM, ensured by the multidomain OSBP-related Osh2 protein, attached to VAP at the ER and PIPs/Myo5 at the PM. Here, the study establishes rather convincingly that Osh2 with VAP and Myo5 are part of a complex that also integrates Erg proteins to coordinate lipid metabolism, lipid transfer and vesicular transport. Along with analyzing this, the authors, as they use the D4H probe, maybe for the first time in yeast, offer a more precise and clearer view, quite fascinating in my view, on how ergosterol is distributed between compartments in the yeast. For these two reasons, I would like to recommend this manuscript for publication in JCB as it should interest a large audience in the field. However, while many aspects of the study rely on quite robust microscopy data (notably analysis of time-lapse movies of endocytic events at the end of the manuscript), others are less convincing and should be improved. Also, a major issue to address is whether OSW1 is a faithful pharmacological tool to selectively block Osh2. Moreover, I think that the authors miss the opportunity, although they have seemingly powerful tools to do so, to further examine what the role of Osh4 is in the delivery of sterol in the daughter cell and to provide a more complete analysis of the sterol transfer mechanism of Osh2. I think this is needed to strengthen the main overall aim of the manuscript that is to show the existence of two key sterol routes toward the PM, ensured by different Oshs, that support endocytic processes in the mother and daughter cell.

Major points

- **OSW-1** is a compound that has been found to specifically inhibit OSBP and ORP4 but no other ORPs. To my knowledge it is quite unknown from a biochemical standpoint whether this molecule is capable of blocking (i) the sterol transfer activity of Osh2 and whether it selectively inhibits Osh2 and not Osh4. Referring to sequence analyses that indicate that the ORDs of Osh1 and Osh2 are the most closely related to the ORD of OSBP is far from being sufficient to prove that this molecule does inhibit Osh2. Furthermore, the molecule is used at 8 μM i.e. at a concentration 100 fold-higher than the ones typically used to arrest OSBP’s activity in mammal cell lines (in the nanomolar range, see Mesmin’s papers in Cell and EMBO J). Thus one can seriously question whether the effects seen in multiple experiments (Fig2C, Fig4) are caused by a selective inhibition of Osh2’s activity or rather by a more general, deleterious effect imposed by OSW-1 (notably after a 2 h-treatment). Moreover, a picture of osh2Δ cells with internal patches stained by GFP-D4H in Figure S2B suggests that OSW-1 has an impact on sterol distribution independently from Osh2. I think mandatory that the authors ascertain through biochemical/functional assays whether and to which extent OSW-1 is able to inhibit Osh2 and Osh4’s sterol transfer activity.

- By many aspects, fluorescent microscopy provides convincing data that yet could be bolstered by more quantitative analyses of EM images (as the authors did in their previous study) that are currently lacking in the manuscript. Notably the images shown in panel 1E should be definitively be backed to a quantitation of D4H labelling to fully support the idea that sterol accumulates in cER
rims and ER/endocytic contacts sites
- Explanations for pictures shown in Figure 2B are limited thus it is quite difficult as a reader to understand what one should see (at least the authors should indicate the boundaries of an engrossed cER rims). Besides, the authors should provide a more quantitative analysis of such pictures (more D4H in Osh2Δ cells ?) to strengthen the results reported in Figure 2A.

- Knowing the links between ORPs/Osh proteins and PIP metabolism, the authors should try in this second study following the Dev Cell paper to eventually examine by experimental approaches the link between Osh2’s activity and PI4P at the PM, given that PI4P is very likely a ligand recognized by Osh2. Authors simply refer to a study indicating that PI4P depletion does not impact actin polymerization (Yamanoto), maybe to make the point that Osh2 conveys sterol from a curved and sterol-rich area of the ER to the PM in the Discussion. However, Osh2 ORD can be substituted by the ORD of Osh4 whose transfer function strongly relies on PI4P. Moreover a lack of Osh2 impacts yeast PI4P level (Stefan et al, 2011) Thus how to reconcile these data. The authors should examine how an Osh2 mutant, unable to extract PI4P, behaves in a cellular context, by analyzing whether it moves sterol to the PM and impacts endocytosis.

- The authors show, using sec mutants, that the build-up of sterol in the PM of the daughter cell and therefore its polarized distribution, depend on secretory vesicles moving toward the bud. The absence of Osh4 leads to a similar accumulation of sterol-rich secretory vesicles (Fig. S2A, PI4P is not fully removed so the fusion of vesicles with the PM is blocked) at the expense of the PM of daughter cells. In the Introduction, the authors indicate that "Osh4 instead, is found on Golgi membranes, where its retrieves PI4P in exchange for sterol and contributes to polarized secretion". Why not defining more the link between sterol/PI4P counter-exchange and sterol accumulation as well as endocytic events in the PM of daughter cell? As for Osh2, this should be done by examining a HH/AA mutant of Osh4 unable to extract and transport PI4P.

- It might also be interesting to examine the role of Osh1 as well because it has been recently shown to be involved in post-Golgi secretory processes (10.1016/j.devcel.2019.12.010)

Other comments
P2, line 19 I would be quite precise on the nomenclature of ORPs/Osh proteins. Yeast encodes Osh proteins and not ORPs. This distinction has been made since the sequencing of the seven Osh protein and human ORPs in late 90/early 2000 and should be kept as it is.

P3, line 3 - Most ORPs and Osh proteins localize
P3, line 3 - Osh1 also localizes at the Golgi, maybe at ER-Golgi contacts.
P3, line 17 - I would mention Moser von Filske et al., Science 2015 instead of or in addition to Maeda et al, Nature, 2013 when describing Osh6 as a protein that counter-transfers PS and PI4P
P2, line 22-24 - One can hardly state today that the counter-transport capacity of ORPs/Osh proteins has been mostly shown through the study of Osh4. A large series of studies, focusing on Osh6/Osh7, ORP5/8 and OSBP for instance, has been reported since, showing that this mechanism is performed by many other ORP/Osh proteins to ensure the transfer of sterol and phosphatidylserine between cell compartments. Moreover, line 24, I would not mention there Im et al, 2005 paper which is of course a seminal paper that established from a structural standpoint that ORPs/Osh could be considered as serious candidates for sterol transport but did not report that PI4P is an extractable lipid ligand of Osh4.
P4, last line. In one extra sentence, the authors should more explicitly explain why they conclude that endocytosis is not altered.
P6, Line 16. The authors should better explain why the number of cortical GFP-D4H patches increases along with the brightness of these spots. That is not obvious, unless one considers that sterol level in some cER rims goes above a certain threshold and that these rims are therefore stained by the GFP-D4H probe.
The authors should quantify the increase in the number of non-cortical GFP-D4H patches observed when Osh4 is missing, as this suggests that sterol is extracted by Osh4 from other ER regions (not engaged in contact sites?) to feed up another sterol transfer route(s).

The interaction between Myo5 and Erg27 seems to be particularly weak. Do the authors have particular comments on that?

P10, line 16, the sentence "depletion of Osh2, a major sterol transporter at ERSSSES" is far too much affirmative in this part of the text.

Figure 2A. When compared with the pictures shown in the Figure 1B it is difficult to be convinced that the ER has a normal morphology when looking at the Sec61 labelling (very bright and expanded staining) even in WT strain.

Figure 2C. Why does the RFI value start at 1.4 instead of 1 in osh2Δ cell.

P6, line 20. The author should cite Tong et al, 2013 (10.1016/j.str.2013.05.007), showing why Osh3 is unable to trap sterol.

Figure 3D. Is there any explanation for the double bands seen on gel for ProtA-Myo5.

Figure 5. Authors should explain why Sla2-GFP is used instead of Sla1-GFP to visualize endocytic events.

Discussion. The authors should compare their results with those published by Stefan and co-workers (10.1186/s12915-020-0758-x), showing that Osh3 maintains a polarized distribution of PI4P in the budding yeast (intriguingly like the sterol distribution with more PI4P in the bud), notably as Osh2 and Osh3 seem to team up or to complement each other at ER-PM contact sites, at least in the context of endocytosis.

Discussion p11, line 23. The sentence is somehow misleading or at least imprecise because unsaturated PS species are predominant in the PM but at a lesser extent than in the ER, and this could modify how sterol is sequestered and embedded in the inner leaflet of the PM and how it is detected by GFP-D4H comparatively to the ER.

Discussion p12. Line2-5. It should be interesting to discuss the paper published by Klemms et al. in JCB, 2009 (10.1083/jcb.200901145) that showed that sterol is more abundant in secretory vesicles. Also it is surprising that the authors do not comment more their results on Osh4.

Discussion. How do the authors unambiguously link the ERSSSES identified in their manuscript with curved structures with a diameter of 30 nm visualized by Voeltz et al.?

Minor comments
There are some typos and grammatical errors throughout the manuscript. I noted some of them.

Abstract (line 10);
P 2 : line 7, 11, 20 (typos)
P6, line 18 (typos)
P7, line 4 typo
P7, line 11 - It is not indicated to what refers the citation number (12)
P11, line 12

Figure 2C : Y-axis - Decimals are separated from the number by a comma and not a dot
Please, find below the point by point answers we offer to all reviewer’s concerns.

Reviewer #1 (Comments to the Authors (Required)):

In this work, the authors show that Osh2 is required to extract sterols from ERSES (ER sterol synthesis and exit sites), and that Osh2 may form a complex with sterol synthesizing enzymes and proteins involved in endocytosis. Moreover, the sterol dynamics are very different between mother and daughter cells. Overall, there are some interesting findings but major concerns remain.

We thank the reviewer for the positive comments and the constructive criticisms.

1. Sterol delivery from sites of synthesis to the PM remains a key question in cell biology. The findings that support sterol extraction by Osh2 was presented in figure 2. D4H positive dots were increased at cortical ER in osh2 null cells. This is the key observation of this work, and needs to be further strengthened. For instance, is the FFAT motif important for this function? Is PIP binding (e.g. HH mutants) required? Can a sterol binding mutant of Osh2 complement this?

As suggested by the reviewer, we have now analyzed a number of informative Osh2 mutants for their capacity to extract sterols from ERSES. The Osh2 mutants included in the study are:

1. A mutant lacking the lipid transfer domain (osh2-ORDΔ).
2. Mutants where the lipid transfer domain of Osh2 has been substituted by the one of Osh4, which transfers sterols and PI4P (osh2-ORD4), or Osh6, which transfer PS and PI4P (osh2-ORD6).
3. A mutant bearing point mutations that impair Osh2 binding to the yeast VAP proteins (Scs2 and Scs22) (osh2-FFAT*) (Encinar Del Dedo et al., 2017).
4. A mutant bearing mutations in the Osh2 H1000, H1001 and R1230, predicted to impair phosphoinositide counter-transport (osh2-HHR*) (de Saint-Jean et al., 2011; Wang et al., 2019).
5. A mutant bearing mutations in the Osh2 K1114, K1116, K1118 (surface patch III), predicted to impair PI(4,5)P₂ counter-transport (osh2-KKK*) (Wang et al., 2019).

The results are shown in figure 2E of the present manuscript. They indicate that sterol binding and phosphoinositide counter-transport, as well as the interaction of Osh2 with the yeast VAPs, are required to sustain the function of Osh2 extracting sterols from ERSES. The lysines in surface patch III are instead dispensable, suggesting that Osh2 is probably not functionally analogous to ORP2 (Wang et al., 2019). Together with the analysis of the PI4P and PI(4,5)P₂ levels in osh2 and osh3 mutants (Fig. S2I of the present manuscript), and the observation that Osh4
can complement the osh2Δ phenotype, when targeted to ERSES (Please see response to point 5 for a more detailed description) (Fig. 2E and 3I), the data strongly suggest that Osh2 uses the PI4P counter transport to sustain sterol extraction from ERSES in vivo.

2. In this regard, some relevance to mammalian cells would strengthen the study. Mammalian ORP2, and to some extent ORP1S can deliver cholesterol to the PM (PMID: 30581148). Can ORP2 or ORP1S rescue the D4H phenotype in figure 2?

While the issue of the possible functional conservation of the ERSES and the localized sterol transport to endocytic sites is certainly exciting, we finally decided not to perform these experiments because, based on sequence comparison and the new results described above, Osh2 seems to rather work as a PI4P/sterol transfer protein, more similar to Osh4 and OSBP. We respectfully think that the analysis of the evolutionary conservation of the mechanisms described in the manuscript will require the localization of the enzymes in the cholesterol biosynthetic pathway in mammalian cells, as well as the identification of lipid transfer proteins, possibly involved in clathrin dependent or independent endocytosis (Caldieri et al., 2017; Fox et al., 2013), which I think is out of the scope of the report.

3. In osh2 or osh4 null cells, more D4H spots were detected in the ER (Fig. 2 & S2). At least in mammals, sterols in the ER are very low and are hardly detectable by D4H. It is likely that there are specialized, but non-overlapping regions in the ER for osh2/4 function. Does Osh4p also associate with some the same ERG mutants? This would be a good control. What happens to ER D4H in cells null for both osh2 and osh4?

This is indeed an open and very interesting issue. Since Osh4 does not significantly reside in the cortex (Ling et al., 2014), we discarded a direct role of Osh4 in sterol extraction from cortical ERSES in mother cells. However, we envisioned that a specialized kind of ERSES might operate for Osh4, maybe at the mother-bud neck where ER rims and Osh4 staining are visible (Ling et al., 2014). We did not find though evidence for an interaction of Osh4 with Erg6 in the two hybrid assay (Fig. 3G of the present manuscript) nor have we been able to detect an interaction between Osh4 and any of the cortical ERSES components (Erg2, Erg6, Erg7 or Erg27) in the immunoprecipitation assays suggested by the reviewer (now included in Fig. S3E). Nonetheless, Osh4 has been shown to interact with Erg11 (Tarassov et al., 2008), and therefore, other type of specialized ER subdomains might serve accessible sterols for Osh4 extraction. In this context, we quantified the number and intensity of ER-associated GFP-D4H patches in the osh4Δ mutant, but the data is very difficult to interpret because, as shown in figure S2B of the present manuscript, the ER collapses in the osh4Δ mutant and therefore, it is impossible to decide whether the accumulated GFP-D4H patches are associated with the ER or correspond to other structures (secretory vesicles, endosomes, Golgi…). Proper analysis of this matter will require the design of an
osh4 conditional mutant to avoid pleiotropic effects, as well as double labeling fluorescence microscopy experiments with markers of the secretory and endocytic pathway.

Why is not increased ER sterol (D4H positive spots) converted to sterol esters? Where is Are1 and Are2 in osh2 null or osh2osh4 null cells?

We think that they might indeed be converted to sterol esters and packed into LDs. Previous published data showed that the osh multiple mutant accumulates lipid droplets, and that such accumulation can be reverted by re-expression of Osh2 (Beh and Rine, 2004). This result actually reinforces the view that Osh2 can extract sterols from the ER. We have not detected though a significant increase in the size or number of LDs in the osh2Δ single mutant as compared to the wild type in our electron microscopy images (Fig. R1), probably because sterols can still be extracted from the ER by other means and/or because the fraction of sterols exported by Osh2 in a wild type is functionally important for endocytosis in mother cells but quantitatively minor (as compared for example to the fraction transported to the daughters). In any case, the observation that GFP-D4H cortical patches only increase their intensity up to a certain threshold upon Osh2 deletion or OSW-1 treatment, suggests the existence of compensatory mechanisms to prevent accumulation of accessible sterols at the ER, which might well be their conversion to sterol esters. It might be worth mentioning here that the GFP-D4H probe does not recognize esterified sterols (Savinov and Heuck, 2017). Attempts to define the subcellular localization of Are1 and Are2 have rendered conflicting results, probably because of their complex transmembrane topology, but the data suggest that they are all over the ER (Yeast RGB data base). Therefore, they will be in contact with excess sterols being accumulated at or overflowing from ERSES in the osh2 mutant.

4. There is also a general concern about whether D4H indeed reflects sterol levels. At least some of the D4H imaging should be verified with another probe, such as purified AloD4, PFO etc.

We were probably not clear enough on this point and therefore, we apologize for the mis-understanding. We actually tried to be very careful not to state that the GFP-D4H labeling reflects the overall sterol concentration. That is why we always talk about cytosolic- accessible sterols rather than sterols. The accessibility of the sterol hydroxyl group is modulated by the membrane composition, as well as by the membrane curvature, so in terms of defining pools that can be extracted by Oshes, it is more informative to define the localization of sterols with cytosolically exposed hydroxyl groups than the actual sterol concentration.

With respect to the question of the specificity of the probe at recognizing sterols, I have to say that GFP-D4H has been used in a wide variety of cell types to follow the dynamics of sterols in vivo, including a recently published article in the Journal of Cell Biology, where GFP-D4H is used to follow sterols in S. pombe (Marek et al J Cell Biol 2020). Similar to our work, the article from S. Martí demonstrates that
the probe recognizes sterols using mutants in the ergosterol biosynthetic pathway
and drugs that inhibit sterol synthesis, which is, to our understanding, the best way
to demonstrate its specificity. We respectfully think that following sterols with AloD4
will not add much since it is structurally very similar to the D4 domain of PFO,
which is what we are using (Gay et al Biophys, J. 2015, Fig. R2). On the other
hand, fixing the cells will alter the lipid organization and will certainly not allow us to
follow the sterol dynamics. Instead, we have worked out experimental conditions
and acquisition fluorescence microscopy settings to visualize PM sterols with very
low concentrations of filipin in vivo, not to disturb growth or endocytosis. Under
these conditions, filipin staining also appears polarized (Fig. R3A), co-localizing
with the GFP-D4H staining in daughter cells (Fig. R3B). The gradient towards
daughter cells is not as sharp as the cytosolic GFP-D4H staining, but one needs to
keep in mind that filipin does not discern between the inner and outer leaflet nor
does it require the hydroxyl group to be accessible for binding (Maekawa, 2017).
We also know now that an extracellular GFP-D4H probe shows a less pronounced
polarized pattern, similar to filipin (Fig. R3C). We have not added all these data in
the manuscript because of space constrains, but we could try to do so if the editors
and the reviewers consider it essential.

Under these experimental conditions, we do not see the sterols at ERSES, either
because filipin does not penetrate into the cell, or because the actual sterol
concentration at ERSES is not that high, as compared to the plasma membrane.
The sterol hydroxyl group at ERSES might be particularly accessible because of
the membrane curvature at the ER rims (Fig. 1E of the present manuscript). Higher
filipin concentrations kill the cells and block endocytosis (Encinar Del Dedo et al.,
2017). Thus, to further demonstrate that the GFP-D4H probe recognizes sterols at
ERSES, we have now added in figure S1D and E a fluorescence micrograph of
yeast expressing GFP-D4H upon treatment with Terbinafine (TBF), which inhibits
Erg1 (Bhattacharya et al., 2018), and a micrograph of the GFP-D4H staining in an
erg11 mutant. In both cases, the polarized PM staining and the cortical GFP-D4H
patches disappear. We have also performed an assay to follow the kinetics of the
GFP-D4H cortical patch staining at ERSES upon addition of TBF. There, we
observe immediate intensity decay upon drug imposition (Fig. 2C). Together with
the observation that the cortical GFP-D4H staining at ERSES co-localizes with the
sterol biosynthetic machinery and the sterol transporter Osh2, we believe that
these data now convincingly demonstrates the specificity of the GFP-D4H staining
at ERSES too.

5. Figure 3. These physical interactions are not very meaningful without functional
validation. Are any of the interactions important for Osh2 to extract sterol from the
ER?

We completely agree with the reviewer that demonstrating the relevance of the
Osh2/Erg interaction for the Osh2-dependent sterol extraction from ERSES was
indeed a very important issue in this work, but difficult to address because of the
redundancy in the system (Fig. R4). To do so, we have generated an osh2-ORD4-
PPPVP* mutant, bearing the Osh4 ORD, which cannot efficiently interact with
Erg6, and the mutation of the PPPVP motif that mediates the Osh2/Myo5 interaction (Encinar Del Dedo et al., 2017). This mutant cannot directly or indirectly interact with the Erg machinery. We now show in figure 3I that the osh2-ORD4-PPPVP* mutant is unable to complement the sterol extraction defect in the osh2∆ mutant (Fig. R4). Reciprocally, we show that whereas expression of Osh4 does not complement the osh2∆ sterol extraction defect, a chimera of Erg6 and Osh4 does (Fig. 3I of the present manuscript). Finally, and consistent with a scaffolding role of Erg6 at ERSES supporting sterol extraction by Osh2, we observed that deletion of this enzyme installs a defect similar to deletion of Osh2, and that such defect can be complemented by a catalytically inactive Erg6 (Erg6-D152L) (Fig. 3K).

6. The data used to argue against counter transport by Osh2 is not convincing. ORPs can also use PIP2 as a counter-current so reducing PI4P alone may not impact Osh2 function. PIP2 is more abundant than PI4P at the PM. Osh2 can associate with PIP2 (see T. Schulz and W. Prinz, BBA, 2007). Is there any change in PM PIP2 in osh2 null cells or osh2,3 null cells or in cells overexpressing OSH2? This PIP2 angle is important as PIP2 plays a key role in actin dynamics and endocytosis. Perhaps some of the effects associated with osh2 are due to PIP2.

In the previous version of the manuscript, we were indeed careful not to discard the counter transport hypothesis. In any case, as discussed above, we now show that the osh2-HHR* (but not the osh2-KKK*) mutant has a defect extracting sterols from ERSES (Fig. 2E), indicating that phosphoinositide counter-transport indeed fuels sterol extraction by Osh2. On the other hand, Stefan and coworkers previously showed that depletion of Osh2 and Osh3 causes an increase in the PI4P plasma membrane levels, which is most apparent in mother cells (Stefan et al., 2011). We have reproduced this result and show that expression of Osh2, but not the Osh2-HHR* mutant, partially rescues de PI4P transport defect in the osh2∆ osh3∆ double knock out (Fig. S2I of the present manuscript). On the contrary, depletion of Osh2 and Osh3 does not have a major impact on the plasma membrane PI(4,5)P2 levels (if at all, we observe a slight decrease caused by Osh3 depletion) (Fig. S2I of the present manuscript). Together with the observations that: 1. Osh4 can restore sterol extraction from ERSES, when either hooked to Erg6 (Fig. 3I of the present manuscript) or to the N-terminus of Osh2 (Fig. 2E of the present manuscript) and that; 2. The capacity of the Osh2 mutants and chimeras to extract sterols from ERSES parallels their capacity to sustain endocytosis in mother cells (Fig. 2E, 3I and S3H and (Encinar Del Dedo et al., 2017)), the data strongly indicate that Osh2 uses PI4P counter-transport in vivo to sustain steady sterol transport from the ER to endocytic sites. We have modified the discussion accordingly.

7. The quality of figure 1E appears very poor, so is Figure 2B

We have now collected more than 100 immuno-electron microscopy images of ER rims and endocytic invaginations decorated with one immuno-gold particle that labels the position of the GFP-D4H probe. Using a number of parameters to define the position of the immuno-golds, as well as the size and shape of the associated
invaginations (Fig. S2A), we have applied statistics to demarcate the position of sterols along endocytic invaginations with a resolution of less than 20 nm (Fig. 1E and F, and S2A), as previously done for other endocytic proteins (Idrissi et al., 2012; Idrissi et al., 2008). Using this approach, we show that the GFP-D4H probe coincides with the endocytic actin nucleating promoting factor Myo5, close to the donor plasma membrane, and to a lesser extent, with the amphiphysin-like protein Rvs167 and the N-WASP homolog Las17, at the invagination neck. Interestingly, an article from A. Menon’s laboratory identified Myo5 and Rvs167 as sterol binding proteins (Chauhan et al., 2020), suggesting that the localized transfer of sterols at endocytic sites might directly contribute to the recruitment and/or activation of these proteins to initiate actin polymerization or effect vesicle scission, respectively. Consistently, defects in actin polymerization and vesicles scission are the two main phenotypes installed upon depletion of Osh2 (Encinar Del Dedo et al., 2017). Further, quantitative immunoelectron microscopy provides evidence showing that engrossed ER rims labeled with GFP-D4H accumulate in the osh2Δ mutant (Fig. 2B).

Reviewer #2 (Comments to the Authors (Required))

In this paper, the authors use a recently developed sterol probe (D4H-GFP) which labels accessible sterols or high concentration sterol regions and identify a differential labeling in mother (cER rim and vesicles) and daughter cells (even distribution in the PM). By using a combination of co-localization, co-IPs, mutants and drugs experiments, they show that the D4H labelled cortical punctae correspond to ER-endocytes contact sites where sterol synthesizing enzymes, the sterol transporter Osh2 and the endocytic machinery interact to facilitate endocyte formation by coupling sterol synthesis and transport. They named this new domain ERSES for ER Sterol Synthesis and Exit Sites. In parallel, they show that these domains are involved in endocyte formation in mother but not in daughter cells where endocytosis is coupled to sterol loading from PM and secretion. Previously, the authors have shown that the sterol transport activity of Osh2 and interaction with Myo5 at ER-endocytes MCSs was required to promote actin polymerization and membrane invagination at endocytic sites. The novelty of this work is the identification of a subset of sterol synthesizing enzymes as Osh2 and Myo5 interactors at ER-endocyte MCSs, the putative coupling of sterol synthesis and sterol transport in the formation of the endocytes and a differential mechanisms of sterol loading to endocyte in mother and daughter cells. The formation of a complex between some ERG subunits, Osh2 and Myo5 is very interesting and convincing but the functional role of this association and the use of the term "on site coupling of sterol synthesis and transfer" required more investigation. The experiments showing that endocytosis in daughter cells might relies on a different mechanism of sterol uptake are also convincing but the authors have to clarify some points regarding the presence of ERSES in daughter cells.

We thank the reviewer for the positive comments and the constructive criticisms.

Major points
Coupled synthesis and transfer. In the paper, the authors clearly show that Osh2 is required to extract sterols from ERSES and that global sterol synthesis is required, which seems logical to provide substrate for transfer. However, they do not provide clear evidence that local sterol synthesis at ERSES is required. In Fig2C, the fact that D4H intensity decreases in WT after sterol synthesis inhibition suggests that Osh2 can transport lipids after synthesis. To show a coupling between synthesis and transport, the author might, as examples, express Osh2 under an inducible promoter in osh2Δ background after incubation with FPM to show if they observe a decrease of D4H signal at cER rims after synthesis or try to relocate one of these enzymes involved in sterol synthesis in another part of the ER to prove that local synthesis at ERSES is required for transfer by Osh2 (as it has been performed recently for phagophore formation [https://doi.org/10.1016/j.cell.2019.12.005]). In addition, only a few enzymes involved in ergosterol synthesis, not involved in the final step of synthesis, are located at ERSES and also at lipid droplet surface. Thus, the authors have to provide more evidences of coupled synthesis and transfer or to change the title and writing of the manuscript.

We completely agree with the reviewer that we cannot formally state that synthesis and transport at ERSES is coupled. Despite a subset of sterol biosynthetic enzymes and the sterol transporter Osh2 are located at ERSES, the enzymatic reactions driven by Erg2, Erg7, Erg27 and Erg11 could also occur in the vicinity of the lipid droplets, from where sterols could diffuse to the ERSES to be extracted by Osh2. Therefore, we have rephrased the title and the text so that we talk about coupling of the sterol biosynthetic and transport machineries at ERSES, rather than coupling of sterol synthesis and transport. Also, we have renamed ERSES (ER Sterol Synthesis Exit sites) to ERSES (ER Sterol Exit Sites) to be more accurate. In fact, we never wanted to state that synthesis in the vicinity of the transporter is required for Osh2 to extract sterols, but rather, that the coincidence of the two machineries might generate a local sterol concentration that will favor extraction, while preventing an increase in the overall ER sterol content. We apologize for the misunderstanding. To reinforce the evidence for the functional relevance of the Osh2/Erg interaction though, we have now added the two experiments described above where we show that: 1. disruption of the Erg/Osh2 interaction causes a defect in the sterol extraction from ERSES and, 2. Osh4 can complement the osh2Δ defect when covalently hooked to Erg6 (please refer to the response to the point 5 for reviewer 1 and figure 3I of the present manuscript).

We attempted the Osh2 re-expression experiment using the Rapamycin-induced sequestration system described in the response to reviewer 3 (Fig. R7A) to test the referee’s interesting idea that coupled synthesis might be required for export, but by the time we had physiological levels of Osh2, the treatment of yeast with sterol biosynthesis inhibitors altered the GFP-D4H distribution so much, that it was hard to drive any conclusion. As mentioned before though, we are now careful not to state that Osh2-dependent transport is coupled to sterol synthesis.
- The authors have to clarify if ERSES are also present in daughter cells and in which cells (mother or daughter) they performed their quantification in the first part of the article (Fig 1 to 3). Indeed, D4H labeling pattern in daughter cells is sometimes unclear. In Fig.1A or S1, the signal looks evenly distributed around the PM whereas in other cells (Fig.1B or Fig2), we can clearly see some dots in the cortical region of daughter cells that in some pictures co-localized with Osh2, ERG6 or Sec61 (Fig 3A, B, Fig S2B). In the same way, the ERG6-27 proteins also form dots co-localizing in daughter cells (FigS3A) and Osh2 and Osh6 dots are also present in daughter cells (Fig3H). Thus, the authors should clarify 1) in how many daughter cells (or depending on the daughter cell size) they observed cortical D4H dots and/or evenly distributed D4H signal and 2) is ERSES sites also exist in daughter cells.

We apologize for the lack of information. We realize that we were not sufficiently clear on this point because of space constrains. We talk about daughter cells, but we really meant daughter cells grown up to a diameter of 2 µm. In these cells, the number of cortical ERSES is still very low (Fig. R5, white bars). In larger daughter cells, we observe a steady decrease in the PM levels of accessible sterols and a progressive increase of cortical ERSES (Fig. R5). We have analyzed this by following Erg6-mCherry patches in a strain expressing Sec61-GFP. We cannot use the GFP-D4H probe because the PM staining is too strong. The endocytic requirements in daughter cells are evaluated in cells smaller than 2 µm in diameter, whenever individual endocytic patches can be discern. We have now described better these considerations in the text.

Minor points

- As PM sterols in mother cells is supposed to be less accessible (i.e. not labelled by D4H), is sterol transport from ER at ER-endocytes MCSs required only to promote actin polymerization or also to provide lipids to sustain endocytes growth? This can make a difference in term of the quantity of lipid transported. Coupling between lipid synthesis and phagophore growth have been recently described in yeast (https://doi.org/10.1016/j.cell.2019.12.005). Can the authors discuss this point in the discussion?

This is indeed an exciting question that we are actually planning to address in the future because Erg7, Erg6 and Erg27 all interact with the very long fatty acyl-CoA synthetase Fat1 (Athenstaedt et al., 1999), which preferably activates fatty acids with 20 to 26 carbons. Those very long fatty acids are incorporated into ceramides and a small fraction of phosphatidylinositol, and they are thought to play a role stabilizing highly curved membranes (Schneiter et al., 2004). Unfortunately though, because of the extra amount of data included in the new version of the manuscript, we had to eliminate the discussion section to maintain the report format, and consequently, leave out all arguments not strictly related to the results. We would be happy to include them though, but we would need to reformat the report to an article.
- It is strange that no PM labeling with D4H probe is observed at all in the mother cells whereas it has been observed in all the organisms studied until now with this probe, including mammals where PM subdomains enriched in sterols also exists. In addition, this probe seems to be sensitive to both sterol accessibility and concentration. Concerning the labeling at cER rims, how the authors can differentiate between a local enrichment of sterol and/or a higher accessibility.

With the current tools, it is actually difficult to discern between sterol concentration and accessibility in vivo. Filipin staining would more accurately represent the sterol concentration but its use in vivo is tricky because it is toxic. We could use fluorescent sterol analogs to follow their dynamics independently of its accessibility, but they are exogenously added and then, we would miss the effect of the spatio-temporal organization of the sterol synthesis and transport machineries. In any case, what we wanted to detect in this work was precisely the fraction of sterols with cytosolic-accessible hydroxyl groups (and therefore, more accessible to Oshes). We have added now extra evidence demonstrating that GFP-D4H recognizes sterols at ERSES (Please refer to the response to point 4 of reviewer 1 for further details). The reason why we do not see labeling in the cytosolic leaflet of the PM of mother cells, as compared to mammalian cells, might simply be due to the lower affinity the probe has for ergosterol, as compared to cholesterol (Savinov and Heuck, 2017) and the fact that we use the minimum expression levels possible to avoid disturbing endocytosis and cell growth. The composition of the yeast plasma membrane with ceramides bearing very long saturated acyl chains might also influence the accessibility of the PM sterols.

- Could the authors provide a quantitative analysis of the immune-EM labeling performed in Fig. 1E and 2B as it has been performed in Encinar del Dedo 2017.

We have now included this kind of analysis in figures 1E and F, 2B and S2A of the present manuscript. The data defines the position of the GFP-D4H probe along the endocytic invaginations with unprecedented resolution, showing that it coincides with the endocytic actin nucleating promoting factor Myo5, close to the donor PM, and to a lesser extent, with the amphiphysin-like protein Rvs167 and the N-WASP homolog Las17, at the invagination neck. As discussed before, this is an interesting piece of data because an article from A. Menon’s laboratory identified Myo5 and Rvs167 as sterol binding proteins (Chauhan et al., 2020), suggesting that the localized transfer of sterols at endocytic sites might directly contribute to the recruitment and/or activation of these proteins. Further, the analysis of the localization of the GFP-D4H probe in the osh2Δ mutant rendered additional evidence for its role extracting sterols from the ER, as we quantitatively show that the mutant accumulates GFP-D4H-labeled engrossed ER rims (Fig. 2B).

Reviewer #3 (Comments to the Authors (Required)):

In this manuscript Encinar del Dedo and co-workers provide new insights into the distribution of sterol inside the budding yeast, unveiling in particular a close
interplay between the sterol transporter Osh2, localized at ER-PM contact sites, a subset of ER-resident ergosterol-synthesizing enzymes (Erg) and actin polymerization processes, ensuring the invagination of the PM during endocytosis, which relies on sterol accumulation in the PM. In the first part of the manuscript, the authors use a genetically-encoded fluorescent sterol sensor (GFP-D4H) to visualize how sterol is distributed inside the cell. They report that sterol distribution is highly polarized, observing a large accumulation of sterol in the PM of the daughter cell, when budding from the mother cell, comparatively to the mother cell. Sterol-rich secretory vesicles moving toward the bud seem to responsible for supplying the PM with sterol. In the mother cell, sterol is mostly seen enriched in punctuate structures corresponding to rims of the cortical ER, i.e. a part of the ER involved in contact sites with the PM. From this result it is suggested that sterol is particularly available in those sites (referred to as ER Sterol Synthesis and Exit site ERSES) and then transferred by Osh2 to endocytic sites. In the second part of the manuscript, co-localisation experiments are performed that support this idea. Moreover, GST pull-down and double-hybrid assays reveal that Osh2, in addition to interacting with Myo5 (as shown in Javier Encinar del Dedo et al. 2017, Dev Cell) and VAP Scs2p protein (Levine’s paper), also physically interacts with Erg6 via its sterol-binding domain (ORD). In addition, Myo5 binds to Erg6 and Erg27. The third part of the study aims at demonstrating, by using various compounds to either sequester sterol, inhibit sterol synthesis or possibly inhibit Osh2 transfer activity that (i) the delivery of sterol by Osh2 is critical for endocytic processes in the mother cell but not in the daughter cell (ii) that sterol conveyed by the vesicular secretory pathway is critical to maintain the endocytic capabilities of the daughter cell (iii) that Erg6 and Erg27 as well as their interaction with Myo5 are critical for endocytosis in the daughter cell.

With this study, Geli’s lab pursues a previous work it published in Dev Cell in 2017, which described a new sterol transfer route, from the ER to the PM, ensured by the multidomain OSBP-related Osh2 protein, attached to VAP at the ER and PIPs/Myo5 at the PM. Here, the study establishes rather convincingly that Osh2 with VAP and Myo5 are part of a complex that also integrates Erg proteins to coordinate lipid metabolism, lipid transfer and vesicular transport. Along with analyzing this, the authors, as they use the D4H probe, maybe for the first time in yeast, offer a more precise and clearer view, quite fascinating in my view, on how ergosterol is distributed between compartments in the yeast. For these two reasons, I would like to recommend this manuscript for publication in JCB as it should interest a large audience in the field. However, while many aspects of the study rely on quite robust microscopy data (notably analysis of time-lapse movies of endocytic events at the end of the manuscript), others are less convincing and should be improved. Also, a major issue to address is whether OSW1 is a faithful pharmacological tool to selectively block Osh2. Moreover, I think that the authors miss the opportunity, although they have seemingly powerful tools to do so, to further examine what the role of Osh4 is in the delivery of sterol in the daughter cell and to provide a more complete analysis of the sterol transfer mechanism of Osh2. I think this is needed to strengthen the main overall aim of the manuscript that is to
show the existence of two key sterol routes toward the PM, ensured by different Oshs, that support endocytic processes in the mother and daughter cell.

We are really grateful for the positive comments, as well as for the constructive criticisms.

Major points

- OSW-1 is a compound that has been found to specifically inhibit OSBP and ORP4 but no other ORPs. To my knowledge it is quite unknown from a biochemical standpoint whether this molecule is capable of blocking (i) the sterol transfer activity of Osh2 and whether it selectively inhibits Osh2 and not Osh4. Referring to sequence analyses that indicate that the ORDs of Osh1 and Osh2 are the most closely related to the ORD of OSBP is far from being sufficient to prove that this molecule does inhibit Osh2. Furthermore, the molecule is used at 8 μM i.e. at a concentration 100 fold-higher than the ones typically used to arrest OSBP's activity in mammal cell lines (in the nanomolar range, see Mesmin's papers in Cell and EMBO J). Thus one can seriously question whether the effects seen in multiple experiments (Fig2C, Fig4) are caused by a selective inhibition of Osh2's activity or rather by a more general, deleterious effect imposed by OSW-1 (notably after a 2 h-treatment). Moreover, a picture of osh2Δ cells with internal patches stained by GFP-D4H in Figure S2B suggests that OSW-1 has an impact on sterol distribution independently from Osh2. I think mandatory that the authors ascertain through biochemical/functional assays whether and to which extent OSW-1 is able to inhibit Osh2 and Osh4’s sterol transfer activity.

We completely agree with the reviewer that there is no evidence indicating that OSW-1 specifically inhibits Osh2. We actually never meant to use the drug as a proof of the specific role of Osh2 in the process, but rather, as a tool to quickly inhibit sterol extraction from ERSES, which we directly show experimentally (Fig. 2C). We were actually quite surprised that it worked so well in that respect. The evidence that shows that Osh2 provides the main lipid transfer activity extracting sterols from ERSES rather comes from the observations that deletion of Osh2, but not other Oshes, increases the number and intensity of cortical GFP-D4H patches (Fig. 2A of the present manuscript), and from the experiment showing that upon sterol synthesis inhibition (with TBF or FPM), sterols are extracted from ERSES in the presence, but not in the absence of Osh2 (Fig. 2C and S2D and E). In any case, we have now added, as suggested by the reviewer, additional functional evidence showing that OSW-1 inhibition of sterol extraction from ERSES specifically requires Osh2 in vivo (Fig. S2F), as well as a control showing that a short OSW-1 treatment does not grossly affect the sterol homeostasis (Fig. S2G). The data shows that upon 1 hour treatment with OSW-1, the cortical GFP-D4H patch intensity increases in wild type cells as well as in Osh1 and Osh3 depleted cells, but it is not altered in an osh2Δ mutant (Fig. S2F). Unfortunately, as discussed in the point 3 for reviewer 1, we could not assay the osh4Δ strain because the ER collapses, making it impossible to decide what is an ERSES. Further, we show that under these conditions the polarized PM GFP-D4H staining
is not significantly altered (Fig. S2G). These data does not discard that OSW-1 inhibits other Oshes or sterol transporters such as Osh4 to a certain extent because, as pointed out by the reviewer, a 2 hour treatment with the drug finally disrupts the polarized GFP-D4H staining. We only used the 2 hour treatment in figure 4A as a control to show that OSW-1 can affect endocytosis in daughter cells when the sterol homeostasis is grossly altered, but not upon acute inhibition of sterol extraction.

With regard to the OSW-1 concentration, we have tried lower concentrations of the drug (Fig. R6) but they are less effective, maybe due to differences in the permeability of the yeast PM, or the presence of many ABC transporters that often extrude the drugs. These factors are indeed known to often complicate the use of pharmacological inhibition in yeast (Bibi et al., 2021; Holmes et al., 2016).

We should mention that we tried to engineer another method to specifically sequester Osh2 in the nucleus, using a Rapamycin inducible system, to back up the OSW-1 results. Overall, sequestration of Osh2 resulted in a 2 fold increase in the number of cortical ERSES and a 1.4 fold increase in their intensity, very similar to what we observe in the osh2Δ strain and in cells treated with OSW-1 for one hour. Unfortunately, the overall sterol staining was grossly altered in this strain, with less visible cortical patches and weak polarized staining, most likely due to the presence of the tor1-1 mutation used to confer Rapamycin resistance (Fig. R7A). We also used another OSBP inhibitor (Itraconazole) with analogous results, but unfortunately again the vehicle (methanol) was also altering the cortical patches to a certain extent. In this context, we have decided to include the above described controls and keep the OSW-1 data, only as the best tool we have in hand to demonstrate that acute inhibition of sterol extraction at ERSES immediately impacts on endocytosis in mother but not in daughter cells. We are careful not to conclude that OSW-1 specifically inhibits Osh2.

Concerning the in vitro sterol extraction assay, we faced several problems. We used 3 constructs to express the Osh2 ORD in E. coli, but the final concentration (in the order of 1µM) and quality of the purified protein was poor in our hands and it was not sufficient to perform DHE extraction assays. On the other hand, the DHE assays are not trivial and they are not established in our laboratory. We planned to make a visit to one of the groups that master this kind of assays but this has not been possible because of the pandemia. Unfortunately also, none of the groups we contacted was able to help with the assay at this point. Since we do not claim the use of OSW-1 as an specific inhibitor of Osh2, but rather, as a tool to inhibit sterol extraction from ERSES in vivo (which we show experimentally), we consider that the data is valid, given that we now provide in vivo evidence showing that OSW-1 does not grossly affect the sterol homeostasis upon acute treatment, and that its inhibitory activity on the sterol extraction from the cortical ERSES requires the presence of Osh2, but not Osh1 or Osh3.

- By many aspects, fluorescent microscopy provides convincing data that yet could be bolstered by more quantitative analyses of EM images (as the authors did in
their previous study) that are currently lacking in the manuscript. Notably the images shown in panel 1E should be definitively be backed to a quantitation of D4H labelling to fully support the idea that sterol accumulates in cER rims and ER/endocytic contacts sites.

As mentioned in the response to reviewers 1 a 2, we have now included this kind of analysis, which is presented in figures 1E and F, 2B and S2A. The data defines the position of the GFP-D4H probe along the endocytic invagination with unprecedented resolution, showing that it coincides with the endocytic actin nucleating promoting factor Myo5, close to the donor plasma membrane, and to a lesser extent, with the amphiphsin-like protein Rvs167 and the N-WASP homolog Las17, at the invagination neck (Fig. 1E and F). As discussed before, this is an interesting piece of data because an article from A. Menon’s laboratory identified Myo5 and Rvs167 as sterol binding proteins (Chauhan et al., 2020), suggesting that the localized transfer of sterols at endocytic sites might directly contribute to the recruitment and/or activation of these proteins to initiate actin polymerization or effect vesicle scission, respectively. Consistently, a delay in the initiation of actin polymerization at endocytic sites and a defect in vesicle scission are the two main phenotypes installed upon depletion of OSH2 (Encinar Del Dedo et al., 2017).

- Explanations for pictures shown in Figure 2B are limited thus it is quite difficult as a reader to understand what one should see (at least the authors should indicate the boundaries of an engrossed cER rims). Besides, the authors should provide a more quantitative analysis of such pictures (more D4H in Osh2Δ cells?) to strengthen the results reported in Figure 2A.

We have now slightly colored the cortical ER and point the endocytic invaginations for clarity in figures 1E and 2B. The analysis of the localization of the GFP-D4H probe in the osh2Δ mutant rendered additional evidence for its role extracting sterols from the ER, since we now quantitatively show that GFP-D4H-labeled engrossed ER rims accumulate in the osh2Δ mutant (Fig. 2B).

- Knowing the links between ORPs/Osh proteins and PIP metabolism, the authors should try in this second study following the Dev Cell paper to eventually examine by experimental approaches the link between Osh2’s activity and PI4P at the PM, given that PI4P is very likely a ligand recognized by Osh2. Authors simply refer to a study indicating that PI4P depletion does not impact actin polymerization (Yamanoto), maybe to make the point that Osh2 conveys sterol from a curved and sterol-rich area of the ER to the PM in the Discussion. However, Osh2 ORD can be substituted by the ORD of Osh4 whose transfer function strongly relies on PI4P. Moreover a lack of Osh2 impacts yeast PI4P level (Stefan et al, 2011) Thus how to reconcile these data. The authors should examine how an Osh2 mutant, unable to extract PI4P, behaves in a cellular context, by analyzing whether it moves sterol to the PM and impacts endocytosis.

We thank the reviewer for pointing out these results. Because of them, we actually tried to be careful not to discard the counter transport hypothesis for Osh2 in the
previous version of the manuscript. As mentioned in our response to reviewer 1, we have now analyzed this question in detail. Our results show that the osh2-HHR* (but not the osh2-KKK*) mutant has a defect extracting sterols from ERSES (Fig. 2E), indicating that phosphoinositide counter-transport fuels sterol extraction by Osh2. On the other hand, we have reproduced the data from Stefan and co-workers and further show that expression of Osh2, but not of the Osh2-HHR* mutant, partially rescues de PI4P transport defect in the osh2Δ osh3Δ double knock out (Fig. S2I). On the contrary, depletion of Osh2 and Osh3 does not have a major impact on the PM PI(4,5)P₂ levels (if at all, we observe a slight decrease caused by Osh3 depletion) (Fig. S2I). Together with the observations that: 1. Osh4 can restore sterol extraction from ERSES, when either hooked to Erg6 (Fig. 3I of the present manuscript) or to the N-terminus of Osh2 (Fig. 2E of the present manuscript) and that; 2. The capacity of the Osh2 mutants and chimeras to extract sterols from ERSES parallels their capacity to sustain endocytosis in mother cells (Fig. 2E, 3I and S3H and (Encinar Del Dedo et al., 2017)), the data strongly indicate that Osh2 uses PI4P counter-transport in vivo to sustain steady sterol transport from the ER to endocytic sites. We have modified the discussion accordingly.

- The authors show, using sec mutants, that the build-up of sterol in the PM of the daughter cell and therefore its polarized distribution, depend on secretory vesicles moving toward the bud. The absence of Osh4 leads to a similar accumulation of sterol-rich secretory vesicles (Fig. S2A, PI4P is not fully removed so the fusion of vesicles with the PM is blocked) at the expense of the PM of daughter cells. In the Introduction, the authors indicate that “Osh4 instead, is found on Golgi membranes, where its retrieves PI4P in exchange for sterol and contributes to polarized secretion”. Why not defining more the link between sterol/PI4P counter-exchange and sterol accumulation as well as endocytic events in the PM of daughter cell? As for Osh2, this should be done by examining a HH/AA mutant of Osh4 unable to extract and transport PI4P.

- It might also be interesting to examine the role of Osh1 as well because it has been recently shown to be involved in post-Golgi secretory processes (10.1016/j.devcel.2019.12.010)

We attempted the analysis of the counter transport hypothesis for Osh4 by inspecting the complementation of the osh4∆ mutant with centromeric plasmids expressing wild type OSH4, a PI4P binding-defective osh4-(H143A, H144A) mutant and a sterol binding-defective osh4-Y97F mutant, which we got from Dr. K. G. Kozminski. Very unexpectedly, we found that the mutant osh4 alleles complemented to a similar extent than the WT (Fig. R8). We were so surprised that we even sequenced the plasmids and retransformed the yeast with exactly the same result. We do not really understand this result in the context that the Kozminski’s lab showed that Osh4 can complement the secretion defect of a multiple osh mutant in a sterol and PI4P binding-dependent manner (Smindak et al., 2017). It might be that Osh4 has a role in polarized sterol secretion which is independent of its lipid transfer activity (maybe related to the collapse of the ER we
observe in the mutant). Another more plausible explanation is that Osh4 works together in complex with another Osh with redundant biochemical properties (Osh1, Osh2, Osh5?), which is unstable in its absence, but stabilized upon expression of the wild type or mutant Osh4. Even though very interesting, the issue is not trivial and it will required an extended analysis, which we think is out of the scope of the report. For simplicity, we have left these data out of the manuscript.

Regarding the role of Osh1 in the transport of sterols to daughter cells, we found that its depletion does not have a major effect on the polarized distribution of GFP-D4H. Slightly stronger effects are observed upon depletion of Osh2 and Osh5, even though not comparable to depletion of Osh4 (the data is now included in figure S1G). We have also investigated the role of Osh1 at extracting sterols from ERSES, since it is most related to Osh2, but we did not find any effect in the number or intensity of the cortical GFP-D4H patches (the data is now included in figure S2A).

Other comments

We are really grateful to the reviewer for the careful reading of our manuscript and the constructive criticisms that we have all addressed. We have corrected the errors and include the references suggested. Following, please find some additional explanations to the reviewer questions:

P6, Line 16. The authors should better explain why the number of cortical GFP-D4H patches increases along with the brightness of these spots. That is not obvious, unless one considers that sterol level in some cER rims goes above a certain threshold and that these rims are therefore stained by the GFP-D4H probe.

This is actually what we think. We used a very low expression of the GFP-D4H probe not to disturb growth or endocytosis. Under this circumstance, we are probably not detecting all ERSES with the GFP-D4H probe because the sterols might be occupied by transporters or other sterol binding proteins. We cannot go higher with the expression levels because endocytosis is disturbed and eventually, cell growth. Erg6-GFP or Erg27-GFP are probably more reliable markers to visualize all ERSES.

P6. The authors should quantify the increase in the number of non-cortical GFP-D4H patches observed when Osh4 is missing, as this suggests that sterol is extracted by Osh4 from other ER regions (not engaged in contact sites?) to fed up another sterol transfer route(s).

As mentioned for reviewer 1, we have quantified the number and intensity of non-cortical dots in the osh4Δ strain, showing that both parameters increase in the mutant (Fig. S2B of the current manuscript). The data is not very informative though, because the ER collapses around the nucleus in the osh4Δ strain (Fig. S2B of the current manuscript), making it impossible to distinguish between plausible ER sterol exit sites for Osh4 and other cellular structures (Golgi,
secretory vesicles, endosomes...). We think that some of the GFP-D4H dots accumulating in the \textit{osh4}\(\Delta\) mutant might indeed represent specialized ERSES because, even though we did not detect an interaction of Osh4 with any of the cortical ERSES components, Osh4 has been shown to interact with Erg11 (Tarassov et al., 2008). To properly address the identification of putative Osh4 ERSES we will need to design a conditional \textit{osh4} allele to avoid pleiotropic effects and identify the nature of the other structures in double labeling experiments. In light of our new results, we think that addressing the issue of the complex role of Osh4 in secretion and in the transport of sterols deserves another project on its own and it is therefore out of the scope of the report.

\textit{P7. The interaction between Myo5 and Erg27 seems to be particularly weak. Do the authors have particular comments on that?}

Erg6 is a tetramer (Nes et al., 2004) and it is expressed at higher levels than other Erg proteins (our own observation). This might be important for its scaffolding role at ERSES. The immunoprecipitations assay might simply reflect the stoichiometry. We do not see a stronger interaction of Erg6 with Myo5, as compared to Erg27, in the two hybrid experiments.

\textit{Figure 2A. When compared with the pictures shown in the Figure 1B it is difficult to be convinced that the ER has a normal morphology when looking at the Sec61 labelling (very bright and expanded staining) even in WT strain.}

The images in figures 2A and 1B were taken with different microscopes. We now have substituted the images in figure 2A by images with less enhanced brightness and contrast.

\textit{Figure 2C. Why does the RFI value start at 1.4 instead of 1 in \textit{osh2}\(\Delta\) cell.}

This is because the RFI values of the \textit{osh2} mutant were normalized to the WT, in order to evidence that the intensity of the GFP-D4H patches associated with the ER tips is higher in the \textit{osh2} mutant. For simplicity though, we have now normalized the data in Fig. 2C to the value of each GFP-D4H cortical dot RFI at time 0, so all graphs depart from 1.

\textit{Figure 3D. Is there any explanation for the double bands seen on gel for ProtA-Myo5.}

We always find this double band in native protein extracts for Myo5. It is caused by a proteolytic cut at the hinge between the TH1 domain and the C-terminal extension bearing the TH2 and the SH3 domains. We know that the TH1 domain folds over the C-terminal extension and auto-inhibits its function as an actin nucleating promoting factor (Grotsch et al., 2010). Calcium or PI(4,5)P\(_2\) can release autoinhibition (Fernandez-Golbano et al., 2014; Grotsch et al., 2010), but proteolysis at this hinge might also be physiologically relevant in this context. However, we do not know at this point if this is just an artifact since the myosin
appears as a single band, corresponding to the upper one, in TCA extracts. So, proteolysis might occur during native extraction.

Figure 5. Authors should explain why Sla2-GFP is used instead of Sla1-GFP to visualize endocytic events.

We indistinctly use Sla2-GFP or Sla1-GFP to investigate endocytic defects, the choice depending on the mutant to be analyzed and/or the marker available. In this case, we used Sla2-GFP instead of Sla1-GFP because, in our hands, it is almost impossible to edit the erg mutants genome. When we encounter such a reluctant mutant, we use the endocytic markers expressed from centromeric plasmids. In this context, we find that Sla1-GFP expressed from a plasmid gives more cytosolic background that Sla2-GFP, making it difficult to properly analyze the kymographs. That is why Sla2-GFP is used instead. An explanation is included in material and methods.

Discussion. The authors should compare their results with those published by Stefan and co-workers (10.1186/s12915-020-0758-x), showing that Osh3 maintains a polarized distribution of PI4P in the budding yeast (intriguingly like the sterol distribution with more PI4P in the bud), notably as Osh2 and Osh3 seem to team up or to complement each other at ER-PM contact sites, at least in the context of endocytosis.

As discussed, we have now reproduced the results from Stefan and co-workers showing that PI4P levels are indeed higher in the osh2Δ osh3Δ mutant and also showing that expression of OSH2 but not the osh2-HHR* mutant can partially complement this effect in the osh2Δ osh3Δ background (Fig. S2I). We do not see much of an effect on the PI(4,5)P₂ levels in this mutant (Fig. S2I). Together with the analysis of the osh2-HHR* mutant in the context of the sterol extraction from ERSES (Fig. 2E), the results clearly indicate that Osh2 uses PI4P counter transport to sustain sterol extraction from ERSES.

Discussion p12. Line2-5. It should be interesting to discuss the paper published by Klemms et al. in JCB, 2009 (10.1083/jcb.200901145) that showed that sterol is more abundant in secretory vesicles. Also it is surprising that the authors do not comment more their results on Osh4.

We thank the reviewer for pointing out this reference that we have now included in the manuscript. Because of space constrains we really wanted to focus the report on the description of the ERSES, the functional characterization of the Erg-Osh2 interaction, and its specific function in mother cells. We think that a complete characterization of the Osh4-dependent sterol pathway to the PM of daughter cells requires much more work, possibly describing a different kind of ER sterol exit sites, the characterization of the mechanism maintaining the sterol polarity and possibly, the identification of Osh complexes involved. We really think that all these experiments fall out of the scope of the current report.
Discussion. How do the authors unambiguously link the ERSES identified in their manuscript with curved structures with a diameter of 30 nm visualized by Voeltz et al.?

We cannot really. The curvature of the ER rims can be inferred from the images of our previous paper (Encinar Del Dedo et al., 2017). We only wanted to mention Voeltz et al. because this paper makes a careful description of the ER and mentions the curvature of the rims of the cortical ER. We have referred to our previous paper in the present manuscript.

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Figure R1. The number and size of lipid droplets are not altered by depletion of Osh2. A. Transmission electron micrographs of WT and osh2Δ yeast ultrathin sections showing lipid droplets in the vicinity of the nuclear envelope (Arrows). B. Graphs showing the average ± SEM of the number of lipid droplets per cell section (left) and their size (right) in the indicated strain. Student’s t-test p values indicate the statistical significance of the differences. Bar = 1µm.
Figure R2. Structures from the Alo and PFO D4 domains.
Reproduced from Gay et al Biophys J 2015.
Figure R3. Polarized sterol labeling with intracellular and extracellular GFP-D4H probes and filipin in vivo. Fluorescence micrograph of live yeast stained with 2.5 µg/ml filipin, expressing either a cytosolic GFP-D4H probe (B), an extracellular GFP-D4H probe (PS-GFP-D4H) (C), or none of them (A). The filipin and GFP individual fluorescence channels and the indicated merged fluorescence or bright field (BF) images are shown. Scale bar = 1 µm.
Figure R4. Redundant interacting network at ER-endocytic contact sites. The interaction between the Myo5 SH3 domain and the polyP domain of Osh2 was shown in (Encinar Del Dedo et al., 2017). The interactions of Myo5 and Osh2 and the Ergs were shown in the present manuscript.
Figure R5. ERSSES are barely present in daughter cells smaller than 2 µm of diameter. Graph representing the total average ± SEM number of Erg6-mCherry patches (total = ERSSES + lipid droplets) or patches associated with the rims of cortical ER (cDots) (ERSSES), in daughter cells of the indicated diameters.
Figure R6. The OSBP inhibitor OSW-1 impairs sterol extraction from ERSSES at 8µM. Graph representing the average ± SEM intensity of cortical GFP-D4H patches in yeast expressing Sec61-mCherry, treated with the indicated concentrations of OSW-1 or mock–treated with DMSO. A minimum of 100 patches were recorded in 3 independent experiments. Student’s t-test p values indicate the statistical significance of the differences.
Figure R7. Genetic and pharmacological inhibition of sterol extraction from ERSSes. A. Fluorescence images of yeast expressing the nuclear pore protein Nic96 fused to FKBP (FK506 Binding Protein) and mCherry (Nic96-FKBP-mCherry), together or not with the sterol mCherry-D4H probe (as indicated) in cells expressing either Osh2 or Osh2 fused to FBP (FKBP12-Rapamycin Binding Protein) and GFP (Osh2-FBP-GFP), incubated either in the presence of 10 µM Rapamycin or mock treated with DMSO. Notice that upon Rapamycin treatment, Osh2-FBP-GFP is sequestered on the nuclear envelop through the Rapamycin inducible interaction of FBP and FKBP. Osh2-FBP-GFP sequestration causes an increase of mCherry cortical patches, which are present only when the mCHerry-D4H probe is expressed. Rapamycin had no effect in cells expressing untagged Osh2. The graphs on the right indicate the average ± SEM of the number per cell and the intensity of the cortical mCherry-D4H dots. Student’s t-test p values indicating the statistical significance of the differences are indicated. B. Average ± SEM of the number per cell and the intensity of cortical GFP-D4H dots in yeast treated with 25 µM of Itraconazol or mock treated with methanol. Student’s t-test p values indicate the statistical significance of the differences.
Figure R8. The osh4-(H143A, H144A) and osh4-Y97F mutants complement the GFP-D4H polarity defect of an osh4Δ strain, similar to the wild type. Epifluorescence micrographs of osh4Δ strains expressing the indicated OSH4 alleles, expressed from centromeric plasmids, or the empty vector (p), and GFP-D4H. Graph of average ± SEM frequencies of budding yeast exhibiting polarized PM staining. A minimum of 100 cells were recorded.
June 21, 2021

RE: JCB Manuscript #202010016R

Dr. Maria Isabel Geli
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Dear Dr. Geli:

Thank you for submitting your revised manuscript entitled “Coupled Sterol Synthesis and Transport Machineries at ER-Endocytic Contact Sites”. We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below). In your final revision, please be sure to address the reviewers' final minor concerns. While filipin data as suggested by reviewer #3 would be welcomed, it is not required.

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Reviewer #1 (Comments to the Authors (Required)):

The revised manuscript has much improved clarity on the role of Osh2. One minor comment: Page 4, line 2, ER sterol exit sites.

Reviewer #2 (Comments to the Authors (Required)):

The authors addressed all my concerns and the revisions they performed greatly improved the quality of the manuscript.

Reviewer #3 (Comments to the Authors (Required)):

I consider that the authors responded to most of my questions and concerns, notably by fully addressing whether Osh2 functions as a sterol/PI4P exchanger between the cER and the PM, by clarifying that OSW1 cannot be considered for now as inhibiting Osh2 (yet I consider that not knowing how it works is still a problem) and by providing more quantifications of microscopy data. Also I understand that a deeper analysis of the role of Osh4 in the build-up of sterol in the PM of the bud/daughter cell is out of the aim of this study. Thus given the novel insights that are provided on the links between sterol metabolism, its transport and endocytosis, and now the higher quality of the revised manuscript, I recommend the work of Geli and co-workers for publication.

I have some minor comments.
Introduction "How sterol heterogeneity is generated, or how it contributes to particular cellular functions is fairly unknown" Why not saying "and" instead of "or"
« This probe binds sterols only when its C3 hydroxyl group is exposed" Maybe this sentence should be rephrased
Fig.1B - I would write GFP instead of "---" as a label of the Y-axis
Fig. 1E - To help the reader, maybe also label the Figure with HA-D4H
Fig.1 F - What means "IT"?
Fig. S2A - The font for title and numbering of axes is too small
Fig. S2B - There is a misalignment in the first plot.
Fig. S2F I would directly mention the name of the drug in the Figure in addition to the length of the drug treatment, again to help the reader to navigate between figures and Sup figures

I think that yes it would be interesting to show the microscopy data with filipin, to better compare the present data with early observations showing the links between Osh proteins and sterol distribution (I think about papers published by Beh and Rine notably).