JCB

The HOPS tethering complex is required to maintain signaling endosomes identity and TORC1 activity

Jieqiong Gao, Raffaele Nicastro, Marie-Pierre Péli-Gulli, Sophie Grziwa, Zilei Chen, Rainer Kurre, Jacob Piehler, Claudio De Virgilio, Florian Fröhlich, and Christian Ungermann

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November 1, 2021

Re: JCB manuscript #202109084

Prof. Christian Ungermann
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Dear Prof. Ungermann,

Thank you for submitting your manuscript entitled "Biogenesis of signaling endosomes depends on late endosome/MVB maturation and its fusion machinery". Your manuscript has been assessed by expert reviewers, whose comments are appended below. Although the reviewers express potential interest in this work, significant concerns unfortunately preclude publication of the current version of the manuscript in JCB.

We share the enthusiasm of reviewer #1 regarding the importance of characterizing the nature and formation of signalling endosomes as well as connecting the metabolic regulation of cell growth and organelle biogenesis. However, as discussed prior to making a formal decision, we agree with the concerns of reviewer #2 regarding appropriate markers for SEs. After having assessed your plan to address this issue, we appreciate that available markers have multiple locations and therefore find that your proposal to perform an extensive additional colocalization analysis as detailed in points i, ii, and iii should provide valuable information that hopefully addresses this issue. In response to reviewer #2 point 3 we are concerned that adding a transmembrane domain to Ivy1 has a large potential to result in artifacts that cannot be interpreted. Therefore, we recommend focusing on the alternative proposals to test whether Ego1 colocalization with Ivy1 better defines the SE compartment, as well as to utilize a split-YFP of Ego1 and Ivy1, and to test if Ego1 with a C-terminal GFP-nanobody tag traps Mup1-GFP to Ivy1 positive puncta. We do not think you need to experimentally address how Ivy1 gets to signaling endosomes for the current study (reviewer #2 point 2). Regarding point 4 of reviewer #2 please clarify your thoughts on potential models in your revised text. In addition, we hope that you will be able to address all of the remaining reviewer comments in your revised manuscript.

Please note that a substantial amount of additional experimental data likely would be needed to satisfactorily address the concerns of the reviewers. As you may know, the typical timeframe for revisions is three to four months. However, we at JCB realize that the implementation of social distancing measures that limit spread of COVID-19 also pose challenges to scientific researchers. Therefore, JCB has waived the revision time limit. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

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If you choose to resubmit, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

Regardless of how you choose to proceed, 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. You can contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for thinking of JCB as an appropriate place to publish your work.

Sincerely,

Lois Weisman, PhD
Monitoring Editor

Andrea L. Marat, PhD
Senior Scientific Editor

Journal of Cell Biology

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

Jieqiong Gao and co-workers describe in this manuscript that the ‚biogenesis of signaling endosomes depends on late endosome/MVB maturation and its fusion machinery'. The authors have shown in their previous work that signaling endosomes in budding yeast provide a platform for TORC1 signaling that is distinct from vacuolar TORC1 signaling. Moreover, these signaling endosomes (ivy1 positive) are distinct from multivesicular bodies (vps4 positive) (MVBs). Here, the authors set out to define the biogenesis of signaling endosomes using a combination of yeast genetics, advanced (3D lattice light sheet) live cell imaging and (quantitative) proteomics.

The results of this manuscript are important as they map the cellular itinerary of signaling endosomes, establish signaling endosomes as a new endosomal sub-compartment and characterize mechanistically how this compartment is connected with the endocytic pathway. It turns out that the formation of signaling endosomes is - at least in part - dependent on the AP3 pathway, and the MVB pathway. Both, signaling endosomes and MVBs require Rab conversion from Vps21 to Ypt7 for maturation to then undergo HOPS dependent fusion with vacuoles and with each other. A fraction of the endocytic cargo (such as nutrient transporters) can also move through signaling endosomes. In addition, this work highlights the intrinsic connections of TORC1 signaling (and hence the metabolic regulation of cell growth) and organelle biogenesis along the endo-lysosomal pathway.

Major point:

- In ESCRT mutants, the markers for signaling endosomes accumulate on class E compartments (Figure 2). In these mutants, do individual signaling endosomes still exist or are they all 'absorbed' into class E compartments? I think it might be important to address this question, since TORC1 signaling from class E compartments and/or signaling endosomes was not affected, while vacuolar TORC1 signaling was strongly reduced (Figure 4).

To me this result is somewhat paradox: While signaling endosomes perhaps no longer exist (instead you have class E compartments), TORC1 signaling from signaling endosomes is is not affected. Yet, vacuoles still exist, but vacuolar TORC1 signaling is affected. Please clarify this point.

- The figure legends for Figure 4 are incomplete and hence information on the WB experiment (was it performed at 30{degree sign}C?) is missing.

- Fig.8: I do not agree with the conclusion that 'HOPS but not CORVET dependent fusion of signaling endosomes with MVBs allows efficient delivery of plasma membrane-derived cargo to the vacuole'. This conclusion implies sequential sorting events in which all Mup1 cargo must visit signaling endosomes that then fuse with MVBs for efficient MVB sorting of cargo into vacuoles. The data in Figure 8 demonstrates that HOPS and CORVET function at different stages in the endo-lysosomal pathway, but I do not see how conclusions on the role of signaling endosomes in cargo sorting are possible without specifically 'eliminating' signaling endosomes (which at the moment might not be possible). Please rephrase.

Minor points:

- Related to the major point above: I find it interesting that signaling endosomes not just 'simply' mature into MVBs to make ILVs, but instead fuse with MVBs to sort cargo. Any idea how to explain this observation? Are they too small to host the formation of ILVs? Do they ever undergo homotypic fusion events?
- In my version of the paper the overlay microscopy images in the supplementary figures looked scrambled.

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

In this manuscript, Gao et al. investigated the biogenesis of signaling endosome(SE), which harbors TORC1 and EGO complex and was recently described as a novel endosomal population. They first showed Ivy1, a putative SE marker, partially colocalizes with other SE proteins, such as EGO1, Gtr2, and Fab1(~30%), but not Vps4. Then, they showed that the identity of Ivy1-labeled SE is directly linked to MVB biogenesis since Ivy1 dots strongly accumulated in Class E compartments when Vps4 was inactive. Because MVB formation depends on multiple fusion events, they next studied the regulation of SE-vacuole fusion, and found that HOPS, but not CORVET, is required for the proper identity of SE and its fusion with the vacuole. Lastly, endocytic cargoes, including alpha-factor and Mup1, can colocalize with Ivy1, and this colocalization was further enhanced when HOPS function was compromised, suggesting that endocytosed cargos can move through SEs before reaching the vacuole lumen. Overall, the data presented here are of high quality, and the paper is well-written. However, this reviewer is not convinced that Ivy1 dots can represent signaling endosomes. Only~ 30% colocalization between Ego1/Kog1 and Ivy1 was observed. In contrast, nearly 80% colocalization between Ypt7 and Ivy1 was observed. This is consistent with previous publications showing Ivy1 is a Ypt7 effector, but does not justify the use of Ivy1 to label SE. Please see below for more details. The authors need to tone down significantly.
Major concerns:
1. How do we define signaling endosomes in yeast? Are they Ivy1-positive dots? Or are they vacuole-attached compartments that contain both TORC1 and Ego complex? In their previous studies, the authors have shown that TORC1 at the SE can phosphorylate Fab1 and Vps27. So, it is my understanding that SE is an endosomal compartment that contains TORC1 and Ego complexes. As shown in figure 1C, only 30% of Ego1 and Kog1 signal colocalize with Ivy1 dots. In other words, the majority of the TORC1 and Ego complexes do not colocalize with Ivy1. This raises the concern of whether Ivy1 can truly represent SE.

2. Along the line of whether Ivy1 can represent signaling endosomes, previous studies (Lazar T. et al. 2002, Numrich et al. 2015) have shown Ivy1 is an inverted bar-domain protein that interacts with phospholipids, Ypt7, and Vps33 (a HOPS component). All these interactions could contribute to its membrane association. However, none of the interactions can explain why Ivy1 could uniquely label signaling endosomes since phospholipids, Ypt7, and Vps33 are common components of late endosomes. In addition, the fact that Ivy1 interacts with Ypt7 and Vps33 complicates the interpretation of using vps11ts mutants, which is also a HOPS component.

3. Because Ivy1 is not a transmembrane protein, the authors need to address the concern that Ivy1 may dissociate from the membrane and re-attach to other Ypt7-, Vps33-, or phospholipids-containing endosomes. For example, in Figure 8C-D, the authors showed that the vps11-1 mutant has more colocalization between Ivy1 and Mup1 at 37 degree. An alternative explanation could be Ivy1 fell off its original membrane and relocalized to Mup1-labeled late endosomes, instead of the accumulation of SE.

4. In the 2019 Mol. Cell paper, Hatakeyama et al.showed that TORC1 on the signaling endosomes inhibits the ESCRT function by phosphorylating Vps27. In the current study, it is also shown that signaling endosomes contain very little Vps4. Both data consistently suggest that the ESCRT machinery may not function on the signaling endosomes. Then, the authors showed endocytic cargoes like Mup1 and alpha-factor can traffick normally through the signaling endosomes. I found this set of data confusing. If there is no ESCRT function on the signaling endosomes, how do signaling endosomes internalize these cargo proteins and become MVBs?

5. In figure 4A, the effect of vps11-1 on ET function is not very obvious. The authors need to verify with a second cargo, such as Fab1 or Vps27 phosphorylation. In addition, the VT cargo Sch9C-term-GFP-Pho8N-term also uses the AP3 pathway for its trafficking to the vacuole. This complicates the interpretation of VT activity in the vps11-1 mutant. If Sch9C-term-GFP-Pho8N-term cannot be delivered to the vacuole(since HOPS is dysfunctional), it is not surprising that no phosphorylation cannot be detected.

Minor concerns:

6. The mass spectrometry data in figure7 did not answer "which other cargoes may pass through SE." Also, they needed to be verified by other methods such as western blots.

7. In Figure 9, the signaling endosome appears to be a compartment detached from the vacuole. However, most of the live-cell imaging data provided by this manuscript as well as the data shown in the 2019 Mol. Cell paper indicated that signaling endosomes are attached to the vacuole. Please make sure the model is consistent with the data.

We would like to thank both reviewers for their insightful comments, which helped us to improve our manuscript. We are particularly grateful that you gave us the chance to respond to the criticism beforehand.

One main issue of the reviewers has been the use of Ivy1 as a signaling endosome marker. During the revision, we have now thoroughly characterized the localization of Ivy1 relative to several endosomal proteins as well as Tor1 and Ego1 as signaling complex components. We also included Vps4 in this analysis. Based on this, a picture emerges that signaling endosomes are only observed when both the HOPS tethering complex is functional (using a specific *vps11-1* allele), and MVBs can form. If MVB biogenesis is perturbed, SE markers like Ivy1 and Ego move to the Class E endosome (as many other endosomal proteins). If HOPS is perturbed – which is the main focus of this study – Ivy1 positive structures accumulate. These structures now lack Ego1 and Tor1. We speculate that this is due to the rerouting of the EGO complex to the Golgi.

We agree with the reviewers that we need to distinguish Ivy1 from SEs and did so throughout the text now. Our analysis reveals, however, an exciting crosstalk between MVBs and SEs, which is controlled by HOPS and likely retromer. We speculate that SEs are a dynamic structure that undergoes fusion and fission and is maintained during signaling. We have taken this into account and therefore needed to restructure the manuscript, but also removed Figure 8 (relative localization of Ivy1 to the endocytic cargo Mup1) as we cannot make any conclusion on endocytic trafficking due to the changes that occur to Ivy1 during the restrictive temperature of the *vps11-1* mutant.

We have therefore thoroughly reworked the manuscript and feel that this overall strongly improved the study. In particular, we have

- employed three-color imaging to determine how the SE proteins Tor1 and Ego1 colocalize with Ivy1, and observed that a large fraction of this colocalization population overlaps with Vps21 and Ypt7 (new Figure 1).
- included a reverse quantification by analyzing all markers relative to Ivy1 and now show that Ego1, Gtr2, and Kog1 increase in their colocalization with Ivy1 (Figure S1A).
- moved the mass spec analysis of the vps11-1 and vps11-3 mutants to the initial characterization of the *ts* strains (now Figure 4F, G).
- extended the monitoring of Cps1 trafficking by using three-color imaging with the additional markers Tor1 and Ego1. We show that all SE-localized markers are lost at the expense of the expanded class E compartments in the ESCRT mutant (Figure 3E, F).
- provided evidence that retromer is required for Ivy1 and Ego1 localization to dot-like structure (Figure 6E, F).

- provided the evidence that the two vacuolar (VT) and endosomal (ET) TORC1 reporters can arrive successfully at the vacuole and SE in the *vps11-1* mutant at 30°C, the temperature at which we monitored the TORC1 activity (Figure 7A, Figure S4A-E).

- demonstrated that the HOPS mutant affects ET function by using Vps27 modification as a second read-out (Figure 7D).

- deleted the Mup1 trafficking part relative to Ivy1 (previous Figure 8).

- adjusted and expanded the text to focus on the function of HOPS on the identity of SEs as a dynamic structure, which resulted in the adjustment of manuscript title and restructure of the figures and writing.

Please find below a detailed response to all specific comments of the reviewers.

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

Jieqiong Gao and co-workers describe in this manuscript that the ‚biogenesis of signaling endosomes depends on late endosome/MVB maturation and its fusion machinery'. The authors have shown in their previous work that signaling endosomes in budding yeast provide a platform for TORC1 signaling that is distinct from vacuolar TORC1 signaling. Moreover, these signaling endosomes (ivy1 positive) are distinct from multivesicular bodies (vps4 positive) (MVBs). Here, the authors set out to define the biogenesis of signaling endosomes using a combination of yeast genetics, advanced (3D lattice light-sheet) live-cell imaging and (quantitative) proteomics.

The results of this manuscript are important as they map the cellular itinerary of signaling endosomes, establish signaling endosomes as a new endosomal sub-compartment and characterize mechanistically how this compartment is connected with the endocytic pathway. It turns out that the formation of signaling endosomes is - at least in part - dependent on the AP3 pathway, and the MVB pathway. Both, signaling endosomes and MVBs require Rab conversion from Vps21 to Ypt7 for maturation to then undergo HOPS dependent fusion with vacuoles and with each other. A fraction of the endocytic cargo (such as nutrient transporters) can also move through signaling endosomes. In addition, this work highlights the intrinsic connections of TORC1 signaling (and hence the metabolic regulation of cell growth) and organelle biogenesis along the endo-lysosomal pathway.

Thank you for the overall positive evaluation.

Major point:

- In ESCRT mutants, the markers for signaling endosomes accumulate on class E compartments (Figure 2). In these mutants, do individual signaling endosomes still exist or are they all 'absorbed' into class E compartments? I think it might be important

to address this question, since TORC1 signaling from class E compartments and/or signaling endosomes was not affected, while vacuolar TORC1 signaling was strongly reduced (Figure 4).

To me this result is somewhat paradox: While signaling endosomes perhaps no longer exist (instead you have class E compartments), TORC1 signaling from signaling endosomes is not affected. Yet, vacuoles still exist, but vacuolar TORC1 signaling is affected. Please clarify this point.

We thank the reviewer for these important points. To test if the individual signaling endosomes still exist in the *vps4* mutant, we performed three-color imaging of the Cps1 protein as a substrate of ESCRT-III with Ego1 or Tor1 and Ivy1. We did this in particular in *vps4* mutant cells. Under these conditions, Tor1 and Ego1 move together with Ivy1 to the Class E endosome (Figure 3 E, F). We suspect that, under these conditions, endosomal signaling occurs from this aberrant structure. As the vacuole will receive less flux of nutrients, we suspect that this results in the reduction in vacuolar TORC1 activity. We have now extended this part of the text to meet the reviewers' point (see results part on Figure 7G, H).

- The figure legends for Figure 4 are incomplete and hence information on the WB experiment (was it performed at 30{degree sign}C?) is missing.

We have added it now.

- Fig.8: I do not agree with the conclusion that 'HOPS but not CORVET dependent fusion of signaling endosomes with MVBs allows efficient delivery of plasma membrane-derived cargo to the vacuole'. This conclusion implies sequential sorting events in which all Mup1 cargo must visit signaling endosomes that then fuse with MVBs for efficient MVB sorting of cargo into vacuoles. The data in Figure 8 demonstrates that HOPS and CORVET function at different stages in the endo-lysosomal pathway, but I do not see how conclusions on the role of signaling endosomes in cargo sorting are possible without specifically 'eliminating' signaling endosomes (which at the moment might not be possible). Please rephrase.

We agree with the reviewer and therefore removed the figure entirely. As *vps11-1* cells show relocalization of Ego1 to the Golgi at restrictive temperature, we cannot be sure which structure we are following that colocalizes with Mup1.

Minor points:

- Related to the major point above: I find it interesting that signaling endosomes not just 'simply' mature into MVBs to make ILVs, but instead fuse with MVBs to sort cargo. Any idea how to explain this observation? Are they too small to host the formation of ILVs? Do they ever undergo homotypic fusion events?

Indeed, Ivy1-positive structures seem to coalesce into a single structure if HOPS function is reactivated. We interpret this as homotypic fusion events. However, at this stage, we cannot be entirely certain as, so far, we have not visualized this even at high resolution or determined fusion directly. This will be an issue for future studies (see Figure 8A-E).

- In my version of the paper the overlay microscopy images in the supplementary figures looked scrambled.

We are sorry that this occurred and only noticed the issue once we saw the review. Thanks very much for pointing this out, we will recheck the figures after converting them to PDF files before the next submission.

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

In this manuscript, Gao et al. investigated the biogenesis of signaling endosome (SE), which harbors TORC1 and EGO complex and was recently described as a novel endosomal population. They first showed Ivy1, a putative SE marker, partially colocalizes with other SE proteins, such as EGO1, Gtr2, and Fab1(~30%), but not Vps4. Then, they showed that the identity of Ivy1-labeled SE is directly linked to MVB biogenesis since Ivy1 dots strongly accumulated in Class E compartments when Vps4 was inactive. Because MVB formation depends on multiple fusion events, they next studied the regulation of SE-vacuole fusion, and found that HOPS, but not CORVET, is required for the proper identity of SE and its fusion with the vacuole. Lastly, endocytic cargoes, including alpha-factor and Mup1, can colocalize with Ivy1, and this colocalization was further enhanced when HOPS function was compromised, suggesting that endocytosed cargos can move through SEs before reaching the vacuole lumen. Overall, the data presented here are of high quality, and the paper is well-written. However, this reviewer is not convinced that Ivy1 dots can represent signaling endosomes. Only~ 30% colocalization between Ego1/Kog1 and Ivy1 was observed. In contrast, nearly 80% colocalization between Ypt7 and Ivy1 was observed. This is consistent with previous publications showing Ivy1 is a Ypt7 effector, but does not justify the use of Ivy1 to label SE. Please see below for more details. The authors need to tone down significantly.

Major concerns:

1. How do we define signaling endosomes in yeast? Are they Ivy1-positive dots? Or are they vacuole-attached compartments that contain both TORC1 and Ego complex? In their previous studies, the authors have shown that TORC1 at the SE can phosphorylate Fab1 and Vps27. So, it is my understanding that SE is an endosomal compartment that contains TORC1 and Ego complexes. As shown in figure 1C, only 30% of Ego1 and Kog1 signal colocalize with Ivy1 dots. In other words, the majority of the TORC1 and Ego complexes do not colocalize with Ivy1. This raises the concern of whether Ivy1 can truly represent SE.

We thank the reviewer for these important points, which we took as a lead during our revision.

The term signaling endosome was coined based on the distinct localization of the Rag GTPase complex (called EGO complex in yeast) and its interacting TORC1 complex to the vacuole and endosome (Hatakeyama et al., 2019). Both are peripheral membrane protein complexes, and Ego1 (and thus the complex) seems to be primarily (but not exclusively) sorted via the AP-3 pathway (Hatakeyama et al., 2019). One key

observation was that Vps27, an early ESCRT subunit, is a substrate of endosomal TORC1, and thus phosphorylated if endosomal TORC1 is active. The same applies to Fab1, the PI3P-5 kinase (Chen et al., 2021).

During our initial characterization of Ivy1 as a Ypt7-effector, we noticed that Ivy1 and the EGO complex colocalize to dots, which we initially interpreted as vacuolar domains (Numrich et al., 2015), but turned out to be endosomes. Strikingly, Ivy1 did not colocalize with Vps4, the ESCRT-IV subunit, which is needed for the formation of intraluminal vesicles. This suggested that the biogenesis of SEs is linked to endosomal TORC1 activity.

Which marker is now best to trace SEs? All available markers have multiple localizations – there is no exclusive Rab5 as Vps21 and its homologs are found on EGO-positive and Vps4 positive dots/endosomes (our study). To test if we could take Ivy1 as a reference marker for SEs, we initially did a reverse quantification by analyzing all markers relative to Ivy1 and now show that Ego1, Gtr2, and Kog1 have more colocalization with Ivy1 as there is a reduced number of Ego1, Gtr2, and Kog1 dots compared to Ivy1 dots in the cell.

We then analyzed the localization of Ego1 or Tor1 relative to Ivy1 and the Rabs Vps21 or Ypt7 or the MVB marker Vps4 using three-color imaging. We observed that a large fraction of Ego1 or Tor1 colocalized with Ivy1 and this population also colocalized with Vps21 or Ypt7. The picture is, however, a little more complex as a fraction of Tor1 (28 ± 0.5%) and Ego1 (10 ± 2.6%) also colocalizes with Vps4 (Figure 1A-D).

We further colocalized Ivy1 with ET (reporter of endosomal TORC1 activity) (Hatakeyama et al., 2019) and noticed that more than 50% of Ivy1 dots were positive for ET (Figure 1E-G).

We have thus rephrased the entire text to meet these observations. Within the manuscript we now point out that we used Ivy1 as a reference marker, which does not colocalize strongly with MVBs, yet marks a population of endosomes that are ESCRT-III minus. TORC1 phosphorylates Vps27 (Hatakeyama et al., 2019) and Fab1 (Chen et al., 2021), which may explain this. We therefore took Ivy1 dots as a marker of a SE population that we were able to follow. As we also noticed later on, HOPS, ESCRT, and retromer mutants cause a redistribution of Ego1 and Tor1 relative to other markers and therefore did not refer to SEs once HOPS was inactivated.

2. Along the line of whether Ivy1 can represent signaling endosomes, previous studies (Lazar T. et al. 2002, Numrich et al. 2015) have shown Ivy1 is an inverted bar-domain protein that interacts with phospholipids, Ypt7, and Vps33 (a HOPS component). All these interactions could contribute to its membrane association. However, none of the interactions can explain why Ivy1 could uniquely label signaling endosomes since phospholipids, Ypt7, and Vps33 are common components of late endosomes. In addition, the fact that Ivy1 interacts with Ypt7 and Vps33 complicates the interpretation of using vps11ts mutants, which is also a HOPS component.

The reviewer is right. Ivy1 binds Ypt7 and Vps33, based on yeast two-hybrid analyses (Lazar et al., 2002). And as pointed out in the manuscript, it has its limitations as it is also found on vacuoles.

We confirmed before that it is a Ypt7 effector, but did not find any evidence that it binds Vps33 or HOPS (Numrich et al., 2015). Ivy1 also binds PI3P and Fab1 (Numrich et al., 2015; Malia et al., 2018; Chen et al., 2021), though we did not find strong evidence for general phospholipid binding as proposed by Lazar et al. (2002). We also recently discovered that Ivy1 is phosphorylated at its unstructured N- and C- terminal extensions and that this changes its localization (Grziwa et al., in preparation). Therefore, we postulate that Ivy1 may use multiple cues to localize in part to SEs due to its interaction with PI3P and Fab1. Its localization to vacuoles seems to be mainly driven by Ypt7 binding.

We have further clarified these points throughout the text.

3. Because Ivy1 is not a transmembrane protein, the authors need to address the concern that Ivy1 may dissociate from the membrane and re-attach to other Ypt7-, Vps33-, or phospholipids-containing endosomes. For example, in Figure 8C-D, the authors showed that the vps11-1 mutant has more colocalization between Ivy1 and Mup1 at 37 degree. An alternative explanation could be Ivy1 fell off its original membrane and relocalized to Mup1-labeled late endosomes, instead of the accumulation of SE.

We agree completely with the reviewer that we cannot exclude if Ivy1 may relocalize from its original membrane to another membrane during the HOPS inactivation. We have thus removed this figure entirely and thoroughly rephrased the text when we discuss the effects of the HOPS mutant.

4. In the 2019 Mol. Cell paper, Hatakeyama et al.showed that TORC1 on the signaling endosomes inhibits the ESCRT function by phosphorylating Vps27. In the current study, it is also shown that signaling endosomes contain very little Vps4. Both data consistently suggest that the ESCRT machinery may not function on the signaling endosomes. Then, the authors showed endocytic cargoes like Mup1 and alpha-factor can traffick normally through the signaling endosomes. I found this set of data confusing. If there is no ESCRT function on the signaling endosomes, how do signaling endosomes internalize these cargo proteins and become MVBs?

We envision at present two scenarios, though we cannot yet say, which of these apply. One model is that SEs are suppressed in fusion as long as signaling inputs activate endosomal TORC1. Once TORC1 is inactive, they fuse with MVBs, where ESCRTs cause cargo sorting into intraluminal vesicles.

Alternatively, TORC1 on SEs may inhibit ESCRTs due to phosphorylation of Vps27 (Figure 7D). If signaling is lost, a phosphatase may activate Vps27 and the cargo will become a substrate to the ESCRT machinery. Both models are not exclusive as there could be fusion between SEs and MVBs. We also discuss both in the text now.

5. In figure 4A, the effect of vps11-1 on ET function is not very obvious. The authors need to verify with a second cargo, such as Fab1 or Vps27 phosphorylation. In addition, the VT cargo Sch9C-term-GFP-Pho8N-term also uses the AP3 pathway for its trafficking to the vacuole. This complicates the interpretation of VT activity in the vps11-1 mutant. If Sch9C-term-GFP-Pho8N-term cannot be delivered to the vacuole(since HOPS is dysfunctional), it is not surprising that no phosphorylation cannot be detected.

We thank the reviewer for these important points. As the reviewer suggested, we first determined the localization of VT and ET in wt, *vps11-1,* and *vps11-3* cells at 24°C and 30°C. We observed that both VT and ET reporters can arrive successfully at the vacuole and SE, respectively, at both temperatures (Figure 7A, Figure S4A-E).

We then followed phosphorylation of Vps27 as a second TORC1 target at endosomes and detected less overall Sch9 phosphorylation *in vps11-1*, but not in *vps11-3* cells, and an upshift of the Vps27-specific phosphorylation bands (Figure 7D,E) (Hatakeyama et al., 2019). This demonstrates that the impairment of HOPS function affects both endosomal and vacuolar TORC1 activities.

Minor concerns:

6. The mass spectrometry data in figure7 did not answer "which other cargoes may pass through SE." Also, they needed to be verified by other methods such as western blots.

The reviewer is right. The figure shows the change in the relative distribution of cargoes in the mutant using mass spectrometry as an unbiased method, which does not rely on tagged proteins. Trafficking defects in the *vps11-1* mutants have been analyzed by Peterson and Emr (2002). We rephrased the text of this part.

7. In Figure 9, the signaling endosome appears to be a compartment detached from the vacuole. However, most of the live-cell imaging data provided by this manuscript as well as the data shown in the 2019 Mol. Cell paper indicated that signaling endosomes are attached to the vacuole. Please make sure the model is consistent with the data.

The reviewer is right, the model is revised now.

The HOPS tethering complex is required to maintain the identity of signaling endosomes and TORC1 activity

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Keywords: signaling endosome, MVB, endosome, Fab1, TORC1, HOPS, membrane fusion
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Abstract

The endomembrane system of eukaryotic cells is essential for cellular homeostasis during growth and proliferation. Previous work showed that a central regulator of growth, namely the target of rapamycin complex 1 (TORC1), binds both membranes of vacuoles and signaling endosomes (SE) that are distinct from multivesicular bodies (MVB). Interestingly, the endosomal TORC1 pool, which binds membranes in part via the EGO complex, critically defines vacuole integrity. Here, we demonstrate that SEs originate at a branchpoint between the biosynthetic and endocytic pathways toward the vacuole and depend on MVB biogenesis. Importantly, function of the AP-3 HOPS tethering complex is essential to maintain the identity of SEs and proper endosomal and vacuolar TORC1 activities. In HOPS mutants, the EGO complex redistributed to the Golgi, which resulted in a partial mislocalization of TORC1. Our study uncovers that SE function requires a functional HOPS complex and MVBs, suggesting a tight link between trafficking and signaling along the endolysosomal pathway.

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Introduction

The endocytic pathway connects the plasma membrane to the endolysosomal compartment with its early (EE) and late endosome (LE) and the lytic lysosome, where proteins are selectively degraded (Huotari and Helenius, 2011; Langemeyer et al., 2018). This connection allows a constant adjustment of the plasma membrane protein and lipid content in response to environmental cues or metabolic needs. Consequently, proteins are continuously surveyed, and are selectively removed by endocytosis if they are bound to a ligand or cargo (Sardana and Emr, 2021). During endocytosis, internalized proteins are packaged into small vesicles, which are first delivered to the EE. Here, some proteins release their cargo and are sorted via the recycling endosome to the PM, whereas others are transferred from the EE to the LE (Huotari and Helenius, 2011). This process requires both maturation of EE to LE, but also multiple fusion events among EEs and LEs (Zeigerer et al., 2012). To allow membrane protein degradation, ESCRT (endosomal sorting complex required for transport) complexes sort these proteins into intraluminal vesicles (ILVs)(Zhen et al., 2021). Consequently, maturation changes the tubular EE into a spherical structure with multiple intraluminal vesicles. Mature LEs, now also called multivesicular bodies (MVB), finally fuse with the lysosome to allow protein degradation for reuse of nutrients (Sardana and Emr, 2021; Huotari and Helenius, 2011).

Rab GTPases are crucial regulators of membrane trafficking, docking and fusion events (Barr, 2013; Wandinger-Ness and Zerial, 2014; Hutagalung and Novick, 2011; Goody et al., 2017). All Rabs can bind to both GTP and GDP. For activation, a guanine nucleotide exchange factor (GEF) promotes loading of the Rab with GTP as a prerequisite for its ability to bind to effector proteins. Inactivation of the Rab requires a GTPase activating protein (GAP), which allows extraction of the Rab-GDP by the chaperone GDI. In the endolysosomal system, Rab5 functions on early endosomes and interacts, among others, with the effector tethering complex CORVET (class C core vacuole/endosome tethering) to promote early endosome fusion (Balderhaar and Ungermann, 2013; Balderhaar et al., 2013). During endosome maturation, Rab5 recruits and activates the Mon1-Ccz1 GEF complex, which in turn activates Rab7 on late endosomes (Nordmann et al., 2010; Langemeyer et al., 2020). In yeast, the Rab7-homolog Ypt7 then recruits the heterohexameric HOPS complex

(homotypic fusion and vacuole protein sorting)(Wurmser et al., 2000; Bröcker et al., 2012; Seals et al., 2000). HOPS has two binding sites for Ypt7, bridges late endosomes and vacuoles to promote the assembly of SNAREs from both organelles, and thus drive fusion (Wickner and Rizo, 2017; Mima and Wickner, 2009; Baker et al., 2015; Bröcker et al., 2012; Beek et al., 2019). Importantly, HOPS also supports fusion of autophagosomes and AP-3 vesicles with the yeast vacuole (Schoppe et al., 2020; Gao et al., 2018; Beek et al., 2019; Cabrera et al., 2010).

Endosomal maturation is accompanied by changes in the lipid composition, most prominently in phosphoinositides. EEs are marked by phosphatidylinositol-3-phosphate (PI3P), which is generated by the Vps34 PI-3 kinase complex (Schu et al., 1993), whose activity is promoted by Rab5 (Tremel et al., 2021). At LEs, PI3P is further phosphorylated by the only PI3P 5-kinase Fab1 (PIKfyve in metazoans)(Hasegawa et al., 2017; Ho et al., 2012). Both lipid kinases function as part of large complexes and localize to multiple membranes of the endolysosomal system. Proteins can specifically bind to phosphorylated inositol head group, often in coincidence of binding to Rab GTPases or other membrane proteins (Balla, 2013). Consequently, changes in Rab composition and PIPs also result in a change in the general membrane composition of maturing organelles.

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The endolysosomal system of yeast seems to be less complex than the mammalian system (Day et al., 2018). It thus came as a surprise when signaling endosomes (SE) were described as a novel endosomal population distinct from MVBs in yeast (Hatakeyama and Virgilio, 2019b; Hatakeyama et al., 2019; Hatakeyama and Virgilio, 2019a). These endosomes harbor, like vacuoles, the highly conserved TORC1 (target of rapamycin complex 1) kinase and its regulatory EGO (Exit from G0) complex (Chen et al., 2021; Hatakeyama et al., 2019), named Rag-Ragulator complex in metazoans (Kanarek et al., 2020). Interestingly, SEs lack the ESCRT-IV ATPase Vps4 required for ILV formation (Babst et al., 1998), but contain a population of the Fab1 lipid kinase (Chen et al., 2021). We recently showed that Fab1 is a substrate of the TORC1 complex, and that Fab1 phosphorylation promotes its localization to SEs in addition to its localization to MVBs and vacuoles (Chen et al., 2021). This suggests that the activity of TORC1 controls Fab1 and thus the biogenesis of SEs. However, the exact link between MVBs and SEs remains unresolved.

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One additional marker protein found on SEs is the I-BAR protein Ivy1 (Chen et al., 2021). Ivy1 is an effector of Ypt7, binds PI3P, and can inhibit Fab1 (Numrich et al., 2015; Malia et al., 2018; Lazar et al., 2002). It also dynamically relocalizes from puncta to vacuoles and vacuolar microdomains in response to nutrient starvation or cellular stress (Numrich et al., 2015; Zweytick et al., 2014; Varlakhanova et al., 2018a; Ishii et al., 2019). We showed before that a fraction of Ivy1 co-localizes with the EGO complex in endosomal dots, which are distinct from MVBs as they lack Vps4 (Chen et al., 2021; Hatakeyama et al., 2019). Here, we focus on the biogenesis of SEs as a novel endosomal population. Our data reveal that SEs harbor not just a pool of TORC1 and the EGO complex, but also the Rab7-like Ypt7 and the Rab5-like Vps21. Importantly, both ESCRTs and HOPS are important to maintain the identity of SEs. Our data suggest that SEs are dynamic structures, which form at an interface between the endocytic pathway and the Golgi by continuous fission and fusion processes.

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Results

SEs and MVBs are distinct endosomal populations

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We previously showed that Ivy1, Fab1, and TORC1 localize to the vacuole and to endosomal dots proximal to the vacuole, which we coined signaling endosomes (SEs) (Hatakeyama et al., 2019; Chen et al., 2021). To understand the dynamics and function of signaling endosomes in the context of the endosomal pathway, we wondered if we could take Ivy1 as a reference marker of signaling endosomes given that the protein, as the other described and above-mentioned SE marker proteins, localizes dynamically to endosomes and vacuoles (Malia et al., 2018; Varlakhanova et al., 2018a; Numrich et al., 2015; Chen et al., 2021). However, this dynamic localization also applies to all other marker proteins of SEs and MVBs. We thus reasoned that the analysis of the relative localization of these markers to each other should reveal, how signaling endosomes form or maintain their identity.

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As a start, we analyzed the localization of Ivy1, marked C-terminally with a Halo tag, relative to several endosomal markers and signaling proteins using three-color imaging. As a marker of the TORC1 complex we selected the catalytic subunit Tor1, which is preferentially found on endosomes

(and vacuolar membranes) if N-terminally tagged with GFP (Hatakeyama et al., 2019; Chen et al., 2021). As a marker of the EGO complex we selected its myristoylated subunit Ego1. We colocalized these with endosomal and vacuolar markers: mCherry-tagged Vps4 (a subunit of ESCRT complex), Vps21 (a Rab5-like protein at endosomes), or Ypt7 (a Rab7-like protein at late endosomes) (Figure 1A, B). We then scored the level of triple colocalization (dark grey), dual colocalization (red, blue), or no colocalization (light grey) (Figure 1C, D).

Our analysis revealed that Ivy1 colocalized well with Tor1 and Ego1 (between 40-50%, blue fraction and dark grey, columns 1, 4). A large fraction of Ivy1/Tor1 and Ivy1/Ego1 positive structures was also positive for Vps21 and even more so for Ypt7, in the case of Ego1 (dark grey part, columns 2, 3 and 5, 6). Importantly, Ivy1/Tor1 or Ivy1/Ego1 positive structures hardly overlapped with Vps4 (dark grey part, columns 1, 3), suggesting that Ivy1 marks a fraction of endosomes that is distinct from MVBs (Hatakeyama and Virgilio, 2019b; Hatakeyama et al., 2019). We noticed in addition that a fraction of Tor1 (28 ± 0.5%) and Ego1 (10 ± 2.6%) also colocalized with Vps4 (red part, columns 1, 4). For some Tor1 and Ego1 dots (20-30%), no colocalization was found (light grey part of columns). As Vps4 marks ESCRT III-positive late endosomes and endosomal TORC1 phosphorylates Vps27 as an ESCRT-0 subunit (Hatakeyama et al., 2019; Hatakeyama and Virgilio, 2019a; b; Lahiri and Klionsky, 2019), we decided to focus on the Ivy1-positive endosomal population. We reasoned that this is likely an endosomal pool where signaling via TORC1 occurs, which prevents ESCRT function (Hatakeyama et al., 2019).

We previously established reporter constructs to determine endosomal (ET) and vacuolar (VT) TORC1 activities (Hatakeyama et al., 2019). They consist of fusion proteins, which target a truncated form of the TORC1 substrate Sch9 either to endosomes or the vacuole (Hatakeyama et al., 2019). To confirm if we could take Ivy1 as an apparent marker of SEs, we co-localized mScarlet-tagged Ivy1 or mCherry-tagged Kog1 (a TORC1 subunit) with GFP-tagged ET and VT. More than 50% (52.9 ± 2.8%) of Ivy1 and 80% (83.33 ± 1.6%) of Kog1 colocalized with ET (Figure 1E-G). In addition, VT colocalized with both proteins, indicating that Ivy1 has two populations that overlap with TORC1, SEs and the vacuole (Figure 1E-G).

To further determine the identity of Ivy1 dots, we colocalized C-terminally mGFP or mCherry-tagged Ivy1 with functionally tagged markers of the endosome or vacuole carrying the other fluorophore. In particular, we analyzed Vps4, Vps8 (a subunit of CORVET complex at early endosomes), Vps21, Ypt7, Ego1, Gtr2 (a subunit of the heterodimeric Rag GTPase module that controls TORC1 at SEs), Kog1, and Fab1 (a PI3P 5-kinase). In agreement with our previous studies (Chen et al., 2021; Malia et al., 2018; Numrich et al., 2015), we observed that Ivy1 strongly colocalized with Ypt7, and a fraction of Ivy1 colocalized with Vps8, Vps21, Ego1, Gtr2, Kog1, and Fab1 (Figures 2A, B, and C). As observed before (Figure 1), Ivy1 localized only very weakly with Vps4 (Figures 2A, and 2C). In a reverse quantification, we noticed that Ego1, Gtr2, and Kog1 showed increased colocalization with Ivy1, while Vps21 and Vps8 showed less colocalization. This is because Ego1, Gtr2, and Kog1 form fewer dots than Ivy1, whereas Vps21 dots are more abundant. Thus, we hereafter took Ivy1 as a reference marker protein to study SEs in more detail.

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Ivy1 and Vps4 positive endosomes differ in their mobility relative to the vacuole

To determine the dynamics of Ivy1-positive structures, we took advantage of lattice light-sheet microscopy (LLSM) to trace Ivy1-mGFP, which enabled us to follow the molecular events in living cells with high spatiotemporal resolution and utmost detection efficiency of lowest signals (Chen et al., 2014). Intriguingly, we detected two classes of fluorescent signals of Ivy1-mGFP (Figure 2E): hyper-dynamic Ivy1 signals at the vacuolar membrane (class I) and rather immobile Ivy1 dots next to the vacuole (class II) (Figures 2D-G, and Video 1). Ivy1 signals on the vacuole membrane were dim, but mobile, whereas Ivy1 dots were rather bright (Figure 2F, G). To compare the dynamics of Ivy1 positive endosomal compartments and MVBs in the cells, we monitored Ivy1-mGFP relative to Vps4-mCherry by LLSM and observed that Ivy1 puncta moved much slower than Vps4 positive dots (Figure 2H, Video 2). This suggests that SEs and MVBs differ not only in some key proteins, but also in their relative mobility at the vacuole.

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SE identity depends on MVB biogenesis

Since SEs also carry endosomal proteins such as the CORVET subunit Vps8 and Vps21, but also the Rab7-like Ypt7 (Figures 1, 2), we wondered if impaired MVB biogenesis would affect SE identity. We therefore analyzed the localization of Ivy1-mGFP in wild-type and *vps4Δ* cells. Loss of ESCRT proteins results in the accumulation of multilamellar structures, called Class E compartment, next to the vacuole, where all endosomal proteins accumulate (Raymond et al., 1992; Rieder et al., 1996; Babst et al., 1998; Adell et al., 2017; Russell et al., 2012). When we analyzed Ivy1 in *vps4*Δ cells, the protein strongly accumulated in bright puncta next to the vacuole, and most cells lost the vacuolar localization of Ivy1 (Figures 3A, B and Videos 3 and 4). However, Ivy1 dots were still positive for Vps8, Vps21, Ypt7, Ego1, Fab1 and Kog1 (Figures 3A, B and Figure S1B). The same observation was made upon inactivation of Vps4 in a *vps4* temperature-sensitive (ts) strain (Figure S1C, D)(Babst et al., 1997). At permissive temperature (24°C), mCherry-tagged Ivy1 and mNeon-tagged Fab1, one of the proteins we tested for colocalization with Ivy1 in *vps4*Δ cells, partially co-localized in dots and at the vacuolar membrane. However, when shifted to the non-permissive temperature (37°C), Ivy1 strongly accumulated as in the *vps4*Δ cells in dots (Figure S1C, D), which were partially positive for Fab1 and likely correspond to Class E compartments (Adell et al., 2017)(Figure S1C-E).

To confirm that Ivy1 was indeed present on Class E compartments in *vps4* mutant cells, we analyzed the colocalization of mCherry-tagged Ivy1 with GFP-tagged ESCRT substrate carboxypeptidase S (Cps1) in wild-type and *vps4*Δ cells. Upon deletion of *vps4*, Ivy1 colocalized more strongly with Cps1, which accumulated at Class E compartments (Figure S1F, G). To further test if SEs remain as distinct endosomes in *vps4*Δ cells, we analyzed the colocalization of mGFP-tagged Tor1 or Ego1 with Halo-tagged Ivy1 and mCherry-tagged Cps1. We observed that 35.85 ± 0.9% of the Tor1 dots and 76.27 ± 4.2% of the Ego1 dots colocalized with Ivy1 and Cps1 (Figure 3F, dark grey part). There was almost no colocalization of Tor1 or Ego1 with just Ivy1 (blue part) and very little with just Cps1 (red part), suggesting that SEs were lost at the expense of the expanded class E compartments of the ESCRT mutant (Figure 3E-G). We thus conclude that the maintenance of SEs as an endosomal population is directly linked to the biogenesis of MVBs.

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HOPS function is required to maintain SE number and endolysosomal trafficking

The biogenesis of the late endosome depends on multiple fusion events at early and late endosomes as a prerequisite for MVB formation (Zeigerer et al., 2012). EE fusion requires the CORVET tethering complex, whereas the fusion of MVBs with vacuoles depends on HOPS (Beek et al., 2019; Balderhaar and Ungermann, 2013). We therefore asked whether HOPS or CORVET were required to maintain the identity of SEs. In a previous study, two temperature-sensitive alleles for Vps11 have been identified, which disable HOPS (*vps11-1*) or CORVET (*vps11-3*) function, whereas a *vps18-1* mutant specifically impairs HOPS (Robinson et al., 1991; Peterson and Emr, 2001). All mutants are functional at the permissive temperature of 24°C but show a protein sorting defect toward the vacuole and a partial growth defect at 37°C (Peterson and Emr, 2001). We therefore tagged Ivy1 with mGFP in these strains and analyzed its localization relative to FM4-64-stained vacuoles at permissive (24°C) or restrictive (37°C) temperature. Both, *vps11-1* and *vps18-1* cells strongly accumulated Ivy1-mGFP in 4-fold more dots proximal to the vacuole at the restrictive temperature (Figure 4A, B and E), whereas the *vps11-3* mutant had no effect on Ivy1 localization (Figure 4C, E). This indicates that the inactivation of HOPS, but not of CORVET, affects the number of observed Ivy1-positive structures in the cell. We previously showed that Ivy1 accumulates at SEs in cells expressing a phosphomimetic Fab1^{6D} allele (Chen et al., 2021). We thus wondered if the number of Ivy1 dots would increase in a *vps11-1 fab1^{6D}* double mutant. Indeed, this was observed (Figure 4D and E), suggesting that impairment of HOPS and Fab1 both affect the formation of SE independently.

As the *vps11-1* mutant caused a strong increase in Ivy1 positive dots, we asked if we could see general changes in the endosomal and vacuolar proteome due to HOPS inactivation. We therefore turned to a recently established method of SILAC-based vacuolar proteomics, which allows the identification of all vacuolar proteins in comparative analyses (Eising et al., 2019). We reasoned that mutants impaired in HOPS (*vps11-1*) or CORVET (*vps11-3*) should differ in their vacuolar proteome at the restrictive temperature and thus reveal impaired cargo trafficking. We therefore isolated vacuoles from "light" labeled wild-type cells and compared them either to vacuoles from "heavy" labeled *vps11-1* or to vacuoles from "heavy" labeled *vps11-3* cells (Figure 4F). We plotted the ratios of *vps11-1* over wild-type on the x-axis against the ratios of *vps11-3* over wild-type on the

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y-axis. This analysis revealed that all subunits of HOPS and CORVET were affected in both mutants (light blue dots). The effect of the *vps11-1* mutant appears to be stronger than the *vps11-3* mutant. However, we were able to identify clear differences regarding the vacuolar proteome of both analyzed mutants. As expected, the *vps11-1* mutation affected the abundance of EGO complex subunits (red dots), cargoes of the autophagy-related Cytosol-to-vacuole (CVT) pathway (orange dots), and AP-3 cargo proteins (purple dots). In contrast, the *vps11-3* mutant mostly affected the abundance of proteins following the endo-lysosomal pathway, especially plasma membrane proteins (light green dots) and vacuolar hydrolases such as CPY and Cps1 (dark green dots) (Figure 4G). Together, this indicates that the CORVET-specific *vps11-3* allele impairs endocytosis, while the *vps11-1* allele affects HOPS function, which is required for all fusion events at late endosomes and vacuoles (Wickner and Rizo, 2017; Beek et al., 2019; Peterson and Emr, 2001). Vacuolar proteomics of temperature-sensitive alleles can thus recapitulate the affected trafficking defects (Lin et al., 2008; Cabrera et al., 2013; Markgraf et al., 2009; Peplowska et al., 2007).

HOPS is required to maintain identities of endosomal structures

Vacuolar proteomics can reveal the overall changes in protein abundance on vacuoles and associated compartments, yet cannot resolve how a HOPS mutant affects the relative distribution of endosomal proteins at SEs, MVBs, and vacuoles. We therefore co-localized both Vps4 and Ivy1 with several endosomal and Golgi markers relative to the vacuole in *vps11-1* mutant at the permissive and restrictive temperature. For this, we used tagged constructs with mCherry or mGFP fluorophores that maintain the functionality of the proteins (Numrich et al., 2015; Adell et al., 2017).

We initially focused on Ivy1 as a protein found at SEs. At the permissive and restrictive temperatures (24°C, 37°C, respectively), Ivy1 dots were still strongly positive for Ypt7 and to a large extent (26.8 ± 1.8 %, 32.74 ± 3.7%) also for Fab1, the SNARE Pep12 (40.75 ± 3.2%, 29,67 ± 2.4%), the Ypt7 GEF Ccz1 (33.28 ± 2.5%, 22.66 ± 4%), the HOPS subunits Vps39 (43.7 ± 3.5%, 41.52 ± 2.5%) and Vps41 (41.1 ± 1.2%, 36.32 ± 1.6%) (Figure 5A-C and Figure S2A-C). We also did not detect an increase in Ivy1 colocalization with Vps4 or the AP-3 marker Apl5 (Figure 5A and Figure S2A).

This picture changed when we analyzed early endosomal markers. Both the Rab5-like Vps21 and the CORVET subunit Vps8 colocalized with Ivy1 at the permissive temperature like in wild-type (Figure 5B, C). However, at the restrictive temperature, Vps8 and Vps21 positive dots colocalized significantly less with Ivy1 (Figure 5B, C). This suggests that loss of HOPS function results in a change of surface composition of Ivy1-marked structures, and thus likely in the entire SE pool. Importantly, Ivy1-positive dots were still positive for the endosomal SNARE Pep12, Fab1 and Ypt7, but lost the early endosomal markers Vps21 and Vps8 (Figure 5C).

To determine if HOPS inactivation also changed the late endosomal identity, we traced the colocalization of Vps4 with the same markers. Vps4 largely colocalized with Vps8 (82.7 ± 2.85%), Vps21 (67.75 ± 4.4%) and Pep12 (73.44 ± 5.7%), which decreased by 10-30% upon HOPS inactivation (Figure 5D). Interestingly, Vps4 also colocalized well with the subunit of the Ypt7 GEF-subunit, Ccz1 (75.95 ± 3%), and to a lesser degree with Vps39 (24.95 ± 2%) and Fab1 (25.04 ± 2.7%), while only little Ypt7 (10.7 ± 1.1%) was found at these structures (Figure 5D, Figure S3A-C). At the restrictive temperature, the colocalization of Vps4 with Ccz1, Vps41 or Fab1 decreased strongly, suggesting that also the composition of Vps4-positive late endosomes changes upon HOPS inactivation (Figure S3B-C).

HOPS and retromer function maintain SE identity

Given that both Ivy1- and Vps4 positive endosomes seem to require HOPS to maintain their identity, we asked if Ego1 and TORC1 would remain at endosomes if HOPS is inactivated. At the permissive temperature, Ego1 and Kog1 colocalized with Ivy1 as in wild-type (Figure 6A, B, Figure 2C). However, at the restrictive temperature, colocalization between Ego1 or Kog1 dots and Ivy1 was largely lost (Figure 6A, B). As Ego1 reaches the vacuole surface by the AP-3 pathway, we tested if some Ivy1 or Ego1 was found also on the Golgi (marked by Sec7), but did not detect any overlap at the permissive temperature (Figure 6C, D). Surprisingly, at the restrictive temperature, we observed that Ego1 now colocalized with the Golgi marker Sec7 (Figure 6C, D). The number of Sec7 dots stayed, however, the same, indicating that HOPS inactivation did not affect Golgi function per se. For Kog1, we did not detect colocalization with Sec7 and currently do not know the identity of the

remaining dots (Figure 6C, D). These data suggest that loss of HOPS results in an accumulation of the EGO complex at the Golgi, while TORC1 is found elsewhere.

Previous work in mammalian cells showed that retromer plays a critical role in TORC1 signaling by controlling a Rab7 GAP and thus Rab7 levels at lysosomes (Kvainickas et al., 2019). A possible explanation for the Golgi localization of Ego1 is that Ego1 became a substrate of retromer, which is found on SEs (Figure 6A, B). We thus analyzed the localization of Ivy1 relative to Ego1 in *vps35Δ* cells and observed a loss of both Ivy1 and Ego1 dots at the expense of vacuolar localization. This suggests that the localization of the EGO and TOR complexes to SEs requires both retromer and HOPS function (Figure 6E, F).

Since Ego1 reaches the vacuolar surface via the AP-3 pathway, we further tested if other AP-3 cargoes also accumulate at the Golgi if HOPS is inactivated. We therefore monitored AP-3-dependent trafficking of the artificial cargo GNS to the vacuole (Reggiori et al., 2000). This GNS cargo consists of the N-terminally tagged cytosolic part of the vacuolar SNARE Nyv1, a bona fide AP-3 cargo (Wen et al., 2006), linked to the longer transmembrane domain of Snc1. If the AP-3 pathway is defective, GNS is rerouted via the plasma membrane to the vacuole (Reggiori et al., 2000). We therefore followed GNS in the *vps11-1* mutant and observed that it localized to the vacuole at the permissive temperature. At the restrictive temperature, GNS also stained the plasma membrane, indicative of an AP-3 defect (Figure S2B). This indicates that the inactivation of HOPS does not result in a general rerouting of AP-3 cargoes to the Golgi or its retention. It rather suggests that the localization of the EGO and TOR complexes to SEs is determined by dynamic fission and fusion processes that require a functional retromer and HOPS complex.

HOPS function is required for TORC1 signaling

As a complete impairment of HOPS function results in the redistribution of Ego1 from SEs and other locations to the Golgi, we expected an alteration in vacuolar and endosomal TORC1 activities. Using our previously described reporter system to measure vacuolar (VT) and endosomal (ET) TORC1 activities (Hatakeyama et al., 2019), we independently confirmed this expectation. These reporters are found in wild-type cells at SEs and vacuoles (Figure 1E-G). As TORC1 activity is temperature

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sensitive, we here used temperatures between 24°C and 30°C. Under these conditions, the VT reporter arrived successfully at the vacuole, whereas the ET reporter colocalized with Kog1 and Ego1 (Figure 1E-G, Figure 7A, Figure S4A-E).

We then determined VT and ET activities. Cells containing the *vps11-1* allele, but not *vps11-3* cells, exhibited a significant reduction in vacuolar TORC1 (VT) activity at 24°C that became even more pronounced at 30°C, while the ET activities were even slightly, but significantly, increased in *vps11-1*, but not *vps11-3* cells (Figure 7B, C). As additional readouts, we followed the phosphorylation of Sch9 and Vps27, which are substrates of vacuolar and endosomal TORC1, respectively (Hatakeyama et al., 2019). We detected less Sch9 phosphorylation (assayed by immunoblot analyses using phosphospecific antibodies that target the TORC1 residue T737 in Sch9) and higher Vps27 phosphorylation (assayed by a slower electrophoretic migration in Phos-tag gel analyses) in *vps11-1*, but not in *vps11-3* cells (Figure 7D,E)(Hatakeyama et al., 2019). This shows that the impairment of HOPS function, in parallel to affecting the endosomal localization of various proteins, also significantly disturbs the partitioning of TORC1 signaling between endosomes and vacuolar membranes.

Because VT primarily defines rapamycin-sensitive growth through its vacuolar target Sch9 (Urban et al., 2007; Hatakeyama et al., 2019), these data explain why a moderate reduction in HOPS function at semi-permissive temperatures (in *vps11-1* and *vps18-1* cells), but not a reduction in CORVET function (in *vps11-3* cells), resulted in rapamycin-sensitive growth (Figure 7B-E). Notably, in line with its rapamycin-sensitive growth at 30°C (Figure 7F), the *vps4*Δ strain also exhibited significantly lower VT, but not ET activity (Figure 7G, H). As *vps4*Δ strains have no AP-3 sorting defect (Babst et al., 1997), this corroborates our conclusion above that defective MVB biogenesis has a significant impact on TORC1 signaling. Taken together, HOPS is needed to maintain proper TORC1 activities at SEs and vacuoles, which is also in agreement with previous genetic analyses (Kingsbury et al., 2014; Hatakeyama et al., 2019; Zurita-Martinez et al., 2007).

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During maturation of endosomes, Rab5 on EEs is replaced by Rab7 on LEs as a prerequisite of their fusion with the lysosome (Borchers et al., 2021). We assumed that HOPS inactivation may trigger endosomal maturation while preventing fusion. To test this, we initially analyzed MVBs in the *vps11-1* mutant by monitoring colocalization of mCherry or mGFP-tagged CORVET (Vps8), the Rab5-like Vps21, the endosomal SNARE Pep12 and the Rab7-like Ypt7 protein, tagged with the corresponding fluorophore. At the permissive temperature, Vps4 strongly colocalized with Vps8 and Vps21, and to a lesser degree with Pep12 (Figure 5A,B). Colocalization with these endosomal markers was reduced at the restrictive temperature. However, only some dots were positive for Ypt7 at either temperature. This was different for SEs, when we monitored colocalization of mScarlet or mGFP-tagged Ivy1 with the same markers. At the permissive temperature, Ivy1 colocalized partially (some 30%) with Vps8, Vps21 and Pep12, and strongly with Ypt7 (Figure 5C,D). However, at the restrictive temperature Ivy1 lost its colocalization with Vps8 and Vps21, whereas it was still positive for Pep12 and Ypt7 (Figure 5C,D). This indicates that impaired HOPS function promotes maturation of SEs, whereas MVBs retain their endosomal markers. ¶
→As we observed a difference in Ypt7 levels, we asked if SEs and MVBs differ in their amount of Mon1-Ccz1 as the Ypt7 GEF in the *vps11-1* mutant. We observed similar colocalization of Ivy1 and Ccz1 at the permissive and restrictive temperature, in agreement with the Ypt7 localization to SEs (Figure S2A,B and 5C). In contrast, colocalization of Vps4 and Ccz1 clearly dropped at the restrictive temperature (Figure S2C,D). This suggests that the Mon1-Ccz1 may respond differently on MVBs and SEs to HOPS inactivation. ¶
→We finally asked if we could use the *vps11-1* mutant to reveal the topology of HOPS at SEs and MVBs. The two Ypt7-interacting subunits, Vps41 and Vps39, localize to opposite ends of HOPS (Bröcker et al., 2012). Vps11 directly binds to Vps39 and mutations in the *vps11-1* allele lie in the identified binding site (Ostrowicz et al., 2010; Plemel et al., 2011; Peterson and Emr, 2001). We thus reasoned that the *vps11-1* allele should cause a release of Vps39 or even HOPS disassembly (see below), but should not impair binding of Vps39 or Vps41 to Ypt7. When we analyzed Vps41 and Vps39 localization relative to Ivy1, we observed no change when *vps11-1* cells were shifted to the restrictive temperature (Figure S2A,B). However, when we monitored colocalization with the MVB marker Vps4, colocalization with Vps41 was lost at the restrictive temperature while Vps39 was unaffected (Figure S2C,D). We thus suggest that HOPS binds to MVBs via Vps39, whereas HOPS has either no preferred orientation to bind Ypt7 on SEs or even bridges SEs.

HOPS is required for reformation of Ivy1 positive structures next to the vacuoles

As HOPS inactivation causes an accumulation of Ivy1-positive structures due to a possible fusion defect, we wondered if we could observe recovery of Ivy1-positive structures with vacuoles by shifting *vps11-1* mutant cells back to the permissive temperature (Figure 8A). To monitor this, we shifted cells to 37°C to accumulate Ivy1-mGFP dots, and then traced Ivy1 dots by LLSM after cells were exposed to the permissive temperature (Figure 8B). Over the first 30 min, we observed that the number of Ivy1 puncta strongly decreased at the expense of one large dot (Figure 8B). At 32 min, this bright dot suddenly disappeared (Figure 8C) and Ivy1 fluorescence thereafter equally distributed over the entire vacuole surface (Figure 8B, D, Videos 5,6). Following this event, Ivy1 dots then reappeared proximal to the vacuole, suggesting either reformation of SEs or relocalization of Ivy1 (Figure 8E). These data suggest that Ivy1-positive structures may initially undergo homotypic fusion before fusing with the vacuole or MVB, and all fusion or reformation events are HOPS dependent. During this process, they most likely also acquire the EGO and TORC1 signaling complexes, resulting in the reformation of SEs.

Endosomal cargo can pass through Ivy1 positive structures

Previous analyses suggested that SEs have a key function in endosomal TORC1 activity to control protein synthesis, macroautophagy and ESCRT-mediated microautophagy at the vacuole (Hatakeyama et al., 2019; Hatakeyama and Virgilio, 2019b; Lahiri and Klionsky, 2019). However, it has not been resolved how endosomal TORC1 may sense the nutrient status of the cell. We considered the possibility that SEs are part of the endocytic pathway and may thus detect the flux of cargo or possibly receptor proteins. To test if Ivy1-positive SEs are connected to the endocytic pathway, we monitored the trafficking of Cy5-labeled α-factor via its pheromone receptor Ste2 from the plasma membrane through the endocytic pathway to the vacuole (Arlt et al., 2015; Day et al., 2018). For this, α-factor was added to cells expressing mGFP-tagged Ivy1, and both signals were recorded by 3D LLSM over time. Due to the time needed between α-factor addition to cells and their mounting at the LLSM stage, we only observed events at the Ivy1-decorated SEs. We observed α-factor and Ivy1 in the same structure over time, followed by the appearance of α-factor in the vacuole

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lumen (Video 7, Figure 9B and C), suggesting that endosomal cargo can pass through endosomal structures marked by Ivy1.

Discussion

Signaling endosomes are a novel endosomal population in yeast, which harbors endosomal TORC1 (Hatakeyama and Virgilio, 2019b; Lahiri and Klionsky, 2019; Hatakeyama et al., 2019; Chen et al., 2021). Here, we set out to determine the identity of SEs and their link to the endocytic pathway using Ivy1 as a reference marker. We show that these SEs are distinct structures with slower mobility than MVBs, localize close to the vacuole, yet are tightly connected to MVB biogenesis. If the ESCRT-IV protein Vps4 is lacking or impaired, Tor1, Ego1, and Ivy1 as proteins found on SEs shift largely to Class E compartments as shown for many other endosomal proteins (Russell et al., 2012). Using a HOPS inactivating *vps11-1* allele (Peterson and Emr, 2001), we uncover that most of the Ivy1-positive structures remain endosomal, but lose SE-specific signaling markers such as the TORC1 subunit Kog1 and Ego1, which is then found at the Golgi (Figure 6). Once HOPS is reactivated, these Ivy1-positive structures reform into a punctum next to the vacuole, suggesting that they are reformed SEs. In agreement with the signaling function of SEs, HOPS inactivation impairs vacuolar TORC1 activity even at semi-permissive temperatures and slightly enhances endosomal TORC1 activity (Figure 7). This is explained by the depletion of Ego1 (and hence EGOC; (Nicastro et al., 2017)) at vacuolar membranes and its clustering at SEs and the Golgi compartment. Overall, we reveal that SEs form at a branch between endocytosis and MVB biogenesis, thus linking signaling to protein trafficking (Figure 9D).

SEs as an endosomal population have escaped attention in previous studies. One reason for this could be that SEs harbor basically all endosomal markers, such as the Rab5-like Vps21, CORVET, or the SNARE Pep12. Also, the ESCRT-0 subunit Vps27 is present on SEs (Hatakeyama et al., 2019), and Vps27 has been taken as a bona fide marker of endosomes in many studies (Kama et al., 2011; Dobzinski et al., 2015; Kanneganti et al., 2011; MacDonald et al., 2012; Bilodeau et al., 2003; Katzmann et al., 2003; Curwin et al., 2009). However, as shown here and before (Chen et al., 2021), SEs, which we follow using Ivy1 as a reference marker, lack the ESCRT-IV subunit Vps4 and

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Moved up [1]: We plotted the ratios of *vps11-1* over wild-type on the x-axis against the ratios of *vps11-3* over wild-type on the y-axis. This analysis revealed that all subunits of HOPS and CORVET were affected in both mutants (light blue dots). The effect of the *vps11-1* mutant appears to be stronger than the *vps11-3* mutant. However, we were able to identify clear differences regarding the vacuolar proteome of both analyzed mutants.

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Deleted: As expected, the *vps11-1* mutation affected the abundance of Ego-complex subunits (red dots), CVT cargoes (orange dots) and AP-3 cargo proteins (purple dots). These data agree with the re-localization of Ego1 to the Golgi and the reduced VT activity in the *vps11-1* mutant, when HOPS function is impaired, yet did not uncover possible cargoes (Figure S1E). In contrast, the *vps11-3* mutant mostly affected the abundance of proteins following the endo-lysosomal pathway, especially plasma membrane proteins (light green dots) and vacuolar hydrolases such as CPY and Cps1 (dark green dots)(Figure 7E). Together this indicates that the more CORVET-specific *vps11-3* allele impairs endocytosis, while the *vps11-1* allele affects HOPS function. Overall, this shows that vacuolar ... [1]

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are thus unable to form intraluminal vesicles, yet all SE-specific markers accumulate at class E compartments if *VPS4* is deleted (Figure 3). This shows that the function of the SE as a dynamic endosomal compartment requires functional MVBs. Even the CORVET subunit Vps8 as an effector of the Rab5-like Vps21 may not be the best marker to trace endosomes in general and thus follow their fusion with the vacuole (Day et al., 2018; Casler and Glick, 2020).

We realize that SEs are difficult to trace and used here mainly Ivy1 as a reference marker. Ivy1 is an IBAR protein with a preference for negative curvature, binds both PI3P and Ypt7, can inhibit Fab1 function, and is found in endosomal dots and on the vacuole similar to Tor1 and Ego1 (Lazar et al., 2002; Numrich et al., 2015; Malia et al., 2018). Ivy1 also relocalizes from dots to the vacuole in response to changes in amino acids, and is found in vacuolar domains after long starvation (Sullivan et al., 2019; Varlakhanova et al., 2018b; a; Murley et al., 2017; Toulmay and Prinz, 2012; Numrich et al., 2015). We are aware that Ivy1 as a peripheral membrane protein may relocalize from endosomes to vacuoles without membrane fusion. However, we follow Ivy1 here under normal growth conditions and observe clear colocalization of Tor1 or Ego1 together with Ivy1 and endosomal proteins such as Vps21, but also Ypt7 (Figure 1A-D). Importantly, Ivy1 dots also colocalized well with endosomal TORC1 (ET) (Figure 1F-G). Moreover, Ivy1 structures accumulate if HOPS is impaired, suggesting that they require HOPS to fuse with MVBs and the vacuole. These data indicate that Ivy1 dots correspond largely to SEs, possibly at different stages of their maturation (Figure 1A-D). In addition to the more static Ivy1-positive dots (which we here consider SEs), we observe a rather mobile Ivy1 fraction on vacuoles (Figure 2F, G), which was also observed when we reactivated HOPS and traced the vacuolar pool of Ivy1 on the vacuole over time (Figure 8). We consider it likely that these mobile dots on vacuoles correspond to individual Ivy1 molecules, but not endosomes. In comparison to Ivy1 dots, MVBs (as monitored by Vps4 mobility) are more mobile (Figure 2H), though we do not know the reason for this difference in mobility presently.

Several studies have used ultrastructural analyses to dissect the yeast endocytic pathway by following the endocytic uptake of nanogold particles (Griffith and Reggiori, 2009; Prescianotto-Baschong and Riezman, 2002). Here, tubular intermediates appeared, which were interpreted as early endosomes. As we find colocalization of Ivy1 with α-factor as an endocytic cargo, we speculate

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Deleted: As shown here, endosomes can lose Vps8 and Vps21, yet remain as Ypt7 positive dot-like structures next to the vacuole if HOPS is inactivated (Figure 5). It is thus possible that SEs and MVBs first mature to Ypt7-positive structures, then fuse with themselves and finally with the vacuole (Figure 9). How this possible order of events is coordinated or controlled is an issue for future studies. We speculate that TORC1 activity on SEs can affect this process.

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that the biogenesis of SEs and TORC1 signaling is linked to nutrient transporter shuttling (Figure 9D). How such a link between trafficking and signaling may work is presently unclear. We favor a model, where nutrient transporters themselves either activate TORC1 or bring along signaling molecules. This would allow TORC1 to translate trafficking of nutrient transporters into the metabolic state of the cell. If TORC1 is then active, it may phosphorylate several proteins such as the Fab1 complex (Chen et al., 2021), which may stabilize the SE, affect signaling and thus growth.

Our data uncover a key role of the HOPS complex in maintaining SE identities. HOPS is a tethering complex that binds SNAREs and promotes fusion of Ypt7-positive membranes (Wickner and Rizo, 2017; Zick and Wickner, 2016; Mima and Wickner, 2009; Bröcker et al., 2012; Lürick et al., 2017; Ho and Stroupe, 2015). The two *vps11* alleles clearly affect HOPS and CORVET differently (Peterson and Emr, 2001). The *vps11-1* allele affects primarily HOPS and thus fusion events at the vacuole, but still allows CORVET-dependent endocytosis, whereas the *vps11-3* allele blocks the latter process without interfering with fusion at the vacuole. In agreement, vacuolar proteomics clearly show that the *vps11-3* mutant strongly blocks endocytosis, whereas the *vps11-1* analysis shows that both HOPS and the EGO complex are lost from vacuolar fractions at the restrictive temperature (Figure 4F, G). If SEs then harbor both HOPS and Ypt7, why do not they fuse with the vacuole? We speculate that signaling at the SE may also block the fusion machinery, by phosphorylation of Vps27 or other proteins (Figure 7C, D) (Hatakeyama et al., 2019; Hatakeyama and Virgilio, 2019a). In this case, loss of signaling may revert this process and promote fusion of SEs with MVBs or the vacuole. We are currently testing this hypothesis.

SEs are possibly also connected to the AP-3 pathway (Nagano et al., 2019; Toshima et al., 2014), and thus may exist at a branch between the biosynthetic sorting pathway to the vacuole and the endocytic pathway (Figure 9D). This would explain why the EGO complex, an identified substrate of the AP-3 pathway (Hatakeyama et al., 2019), localizes to SEs and vacuoles. This localization may be far more dynamic than anticipated as Ego1 (and likely the entire EGOC) appears at the Golgi if HOPS has been inactivated. Furthermore, Ego1 and Ivy1 dots are reduced in retromer mutant, suggesting a role of sorting nexins or retromer in retrograde transport at SEs. Localization of EGOC

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Deleted: In turn, less active TORC1 may favor fusion of SEs with MVBs or the vacuole. It is possible that the observed early endosomal membrane recycling pathway, which also requires the EGO complex, is linked to the biogenesis of SEs that we describe here (MacDonald and Piper, 2017).

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and TORC1 to SEs may thus require a balance between the HOPS dependent fusion and a retromer-dependent recycling pathway.

In summary, our data reveal that signaling endosomes are tightly connected to the biogenesis of late endosomes in yeast, are linked to the endocytic pathway, and may thus receive signal input for endosomal TORC1 activity for their stabilization as an endosomal structure. We uncover a key role of the HOPS complex in keeping SE identities, suggesting that fusion regulation may be part of the signaling cascade. Future studies need to dissect how TORC1 or other signaling complexes promote SE formation, sense endocytic trafficking, and thus translate this into metabolic adjustments.

Materials and methods

Yeast strains and molecular biology

Strains used in this study are listed in Table S1. Deletions and tagging of genes in the cells were done by PCR-based homologous recombination with corresponding primers and templates (Janke et al., 2004; Puig et al., 1998). Mutations in Fab1 were generated by a CRISPR-Cas9 approach (Generoso et al., 2016). Vps4-mCherry has an HA-tag as a spacer before the mCherry tag, which maintains protein functionality (Adell et al., 2017). Plasmids are listed in Table S2.

Fluorescence microscopy

Yeast cells were grown in a synthetic complete medium (yeast nitrogen base without amino acids and with ammonium sulfate) containing 2% glucose to log phase at 30°C. Selective temperature-sensitive (*ts)* strains were cultured in a synthetic complete medium at 24°C to log phase and then shifted to 37°C for 1 h. Cells were imaged on a DeltaVision Elite imaging system based on an inverted microscope with 100x NA 1.49 objectives, an sCMOS camera (PCO, Kelheim, Germany), and an Insight SSI (TM) illumination system. Stacks of 6 to 8 images with 0.2-0.35 µm spacing were taken, and images were deconvolved using the SoftWoRx software (Applied Precision, Issaquah, WA). To analyze the localization of ET or VT relative to the vacuole, Ivy1 and Kog1, images were

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captured with an inverted spinning disk confocal microscope (Nikon Ti-E , VisiScope CSU-W1, Puchheim, Germany) that was equipped with a Photometrics pco.edge 4.2 sCMOS camera, and a 100x NA1.3 oil immersion Nikon CFI series objective (Egg, Switzerland).

Real-time 3D lattice light-sheet microscopy (LLSM) and image processing

Wild-type cells expressing Ivy1-mGFP were grown in a synthetic complete medium to log phase, and vacuoles were stained with FM4-64 or CMAC (videos 1, 2, 3, and 4). *vps11-1 ts* cells expressing Ivy1-mGFP were grown in synthetic complete medium at 24°C to a log phase, incubated at 37°C for 1 h, and vacuoles were stained with CMAC. For α-factor uptake, cells expressing Ivy1-mGFP were grown in synthetic complete medium to a log phase, incubated on ice for 15 min, and then 2.5 µM labeled α-factor were added to the cells for additional 15 min incubation on ice.

5 µl of cells were spotted on the top of 5 mm round glass coverslips (Art. No. 11888372, Thermo Scientific) coated with concanavalin A for 5 min at room temperature to make them adhere. They were then mounted on a sample holder specially designed for LLSM, which was an exact home-built clone of the original design by the Eric Betzig group (Chen et al., 2014). The holder was inserted into a sample bath containing synthetic complete medium at room temperature (25°C). A two-channel image stack was acquired in sample scan mode through a fixed light sheet with a step size of 500 nm which is equivalent to a ~271 nm slicing with respect to the z-axis considering the sample scan angle of 32.8°. We used a dithered square lattice pattern generated by multiple Bessel beams using an inner and outer numerical aperture of the excitation objective of 0.48 and 0.55, respectively. Each 3D image stack (512×320×150 voxels) contained 50-100 cells and was imaged at 30-40 frames. For time-lapse movies, we recorded protein dynamics with a full 3D stack every 1 sec for a total time of 1 min (60 time points). For *vps11-1* cells recovery assay, we recorded a full 3D stack every 1 min for a total time of 40 min (40 time points). For α-factor uptake assay, we recorded a full 3D stack every 30 sec for a total time of 15 min (30 time points). The different channels were sequentially excited using a 405 nm laser (LBX-405, Oxxius, Lannion, Franc) for CMAC, a 488 nm laser (2RU-VFL-P-300-488-B1R, MPB Communications Inc., Pointe-Claire, Canada) for GFP (Ivy1 or Vps4), a 560 nm laser (2RU-VFL-P-500-560-B1R, MPB Communications Inc.) for mCherry

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(Ivy1). Fluorescence was detected by a sCMOS camera (ORCAFlash 4.0, Hamamatsu, Japan) using a quadband emission filter (446/523/600/677 HC Quadband, Semrock) and an exposure time of 13.2 ms for monitoring protein dynamics in each channel, and an exposure time of 23.2 ms for the *vps11-1* cells recovery and α-factor uptake assay. The final pixel size in the image is 104.5 nm. The raw data was further processed by using an open-source LLSM post-processing utility called LLSpy v0.4.9 (https://github.com/tlambert03/LLSpy) for deskewing, deconvolution, 3D stack rotation, and rescaling. Deconvolution was performed by using experimental point spread functions and is based on the Richardson-Lucy algorithm using 10 iterations. Finally, image data were analyzed using spot detection and tracking in Imaris 9.5 (Bitplane, Zurich, Switzerland). By using the built-in spot detection routine, single fluorescent signals were classified as diffraction-limited ellipsoids with a diameter of 250 nm in x- and y-, and 600 nm in z direction. These spots were tracked with the built-in autoregressive motion model using a maximum single step displacement of 1.2 µm and a maximum gap size of 0 time points. Only trajectories longer than 2.5 s were considered for further analysis. Mean track intensity and speed box-plots were generated by Imaris built-in Vantage plot tool. Here box-plots show minimum (Q0 percentile) and maximum (Q4 percentile) values, the box defines data points within Q1 and Q3 percentile and the line the median (Q2 percentile).

ET/VT assay to determine TORC1 activity

wt, *vps11-1*, and *vps11-3* cells were transformed either with the ET reporter (FYVE-GFP-Sch9$^{C\text{-}term}$) harboring plasmid (p3027) or the VT reporter (Sch9$^{C\text{-}term}$-GFP-Pho8$^{N\text{-}term}$) harboring plasmid p2976. The cells were grown at 24°C or 30°C in a synthetic complete (2 % glucose, YNB, ammonium sulfate, all amino acids) until mid-log phase, and 10 ml of cell culture were mixed with TCA (trichloroacetic acid) at a final concentration of 6%. After centrifugation, the pellet was washed with cold acetone and dried in a speed-vac. The pellet was resuspended in lysis buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 6 M urea, 1% SDS), the amount being proportional to the OD_{600nm} of the original cell culture. Proteins were extracted by agitation in a Precellys machine after the addition of glass beads. After the addition of 2X Laemmli buffer (350 mM Tris-HCl, pH 6.8, 30% glycerol, 600 mM DTT, 10% SDS, and 0.02% bromophenol blue), the mix was boiled at 98°C for 5 minutes. The analysis was carried

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out by SDS-PAGE using phosphospecific anti-Sch9-pThr737, anti-Sch9, and anti-GFP antibodies. For Vps27 phosphorylation state analysis, EDTA-free protein extracts were run on a 6% gel containing 50 μM Mn^{2+}-Phos-tag, and probed with anti-Vps27 antibodies.

Vacuole isolation and proteomics

Wild-type and *ts* mutant cells were grown in 500 ml synthetic medium with 30 mg/l normal lysine or 30 mg/l heavy lysine (L-Lysine $^{13}C_6{}^{15}N_2$; Cambridge Isotope Laboratories) at 23°C and then incubated at 37°C for 1 h to an OD_{600} of ~0.8 to 1.0, respectively. The vacuole isolation assay was performed as described before (Gao et al., 2018). Isolated vacuoles were precipitated with 20% trichloroacetic acid (TCA), incubated on ice for 20 min, and resuspended in ice-cold acetone twice by sonication. The vacuole precipitate was further purified using PreOmics IST kit (Martinsried, Germany) for the final mass spectrometry measurements.

Reversed-phase chromatography was analyzed by a Thermo Ultimate 3000 RSLCnano system connected to a Q Exactive-Plus mass spectrometer (Thermo). Peptides were separated and eluted as described previously (Eising et al., 2018). The MS results were analyzed by MaxQuant (REF) (version 1.6.14.0, www. maxquant.org/) as described before (Fröhlich et al., 2013), and the plots were performed with the R software package (www.r-project.org/).

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Authors' contribution

JG performed all localization and trafficking experiments with support from SG, ZC, and MPPG. RN, MPPG, and CDV analyzed the TORC1 activity. LLSM analysis was done with RK and JP. All mass

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spectrometry analyses were done by FF. CU supervised the study and wrote the manuscript together with JG, and with the support of all authors.

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Figure legends

Figure 1. Ivy1 localizes to signaling endosomes. (A, B) Localization of Tor1 or Ego1 relative to Ivy1 and Vps4, Vps21, or Ypt7. Cells expressing mGFP-tagged Tor1 or Ego1 with Halo-tagged Ivy1 and mCherry-tagged Vps4, Vps21, or Ypt7 were grown in a synthetic complete medium. The cells were incubated with the Janelia fluor 646 Halo-tag ligand for 1 h and washed 8 times before taking images. The cells were analyzed by fluorescence microscopy and shown as individual slices. Scale bar, 5 μm. **(C)** Quantification of Tor1 or Ego1 dots co-localizing with Ivy1 and/or Vps4, Vps21, or Ypt7 puncta (from A, B). Tor1 dots (n ≥ 100), Ego1 dots (n ≥ 100), Ivy1 dots (n ≥ 150), Vps4 dots (n ≥ 500), Vps21 dots (n ≥ 500), Ypt7 dots (n ≥ 100) from three independent experiments were quantified by Image J. **(D)** Schematic representation showing the observed populations of fluorescent mGFP-Tor1 and Ego1-mGFP dots in cells. Dark grey dots indicate the fraction of Tor1 (top panel) or Ego1 (bottom panel) co-localizing with Ivy1, and Vps4 (left), Vps21 (middle), or Ypt7 (right) puncta. Blue spots indicate the fraction of Tor1 or Ego1 co-localizing just with Ivy1. Red dots

indicate the fraction of Tor1 or Ego1 co-localizing with just Vps4 (left), just Vps21 (middle), or just Ypt7 (right). Light grey dots indicate the fraction of Tor1 or Ego1 without any colocalization with the selected markers. **(E, F)** Localization of Kog1 or Ivy1 relative to vacuolar TORC1 (VT) and endosomal TORC1 (ET) reporters. Cells expressing mCherry-tagged Kog1 or mScarlet-tagged Ivy1 were transformed with ET (FYVE-GFP-Sch9$^{C\text{-}term}$) or VT (Sch9$^{C\text{-}term}$-GFP-Pho8$^{N\text{-}term}$) reporters. The cells were grown in a synthetic medium, analyzed by fluorescence microscopy and shown as individual slices. Scale bar, 5 µm. **(G)** Quantification of the number of Kog1 or Ivy1 dots co-localizing with ET-positive dots. Kog1 dots (n ≥ 100), Ivy1 dots (n ≥ 150), ET dots (n ≥ 200) were quantified by Image J. Error bars represent standard deviation (SD) of three independent experiments.

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Figure 2. Ivy1-positive structures mark SEs that are distinct from MVBs. (A, B) Localization of Ivy1-positive dots relative to endosomal proteins. Cells expressing mGFP-tagged Ivy1 and mCherry-tagged Vps4, Vps8, Vps21, Kog1 or mCherry-tagged Ivy1 and GFP-tagged Ypt7, Ego1, Gtr2, mNeon-tagged Fab1 were grown in a synthetic medium. Vacuoles were stained with CMAC. The cells were analyzed by fluorescence microscopy and individual slices are shown. Scale bar, 5 µm. **(C)** Quantification of Ivy1 dots co-localizing with endosomal proteins. Ivy1 dots (n ≥ 150), Vps4 dots (n ≥ 300), Vps8 dots (n ≥ 50), Vps21 dots (n ≥ 200), Ypt7 dots (n ≥ 150), Ego1 dots (n ≥ 50), Gtr2 dots (n ≥ 50), Kog1 dots (n ≥ 50), or Fab1 dots (n ≥ 50) were quantified by Image J. Error bars represent standard deviation (SD) of three independent experiments. **(D)** Ivy1 localization by lattice light-sheet microscopy (LLSM) after 3D deconvolution (video 1). Cells expressing mGFP-tagged Ivy1 were grown in a synthetic medium. Vacuoles were stained with FM4-64 and visualized relative to Ivy1-mGFP by Imaris. Scale bar, 5 µm. 200-500 cells were analyzed in each independent experiment. **(E)** Schematic model showing the location of fluorescent Ivy1-mGFP expressed in yeast cells. The green ring corresponds to Ivy1 localization on the vacuolar membrane (Class I), green spots indicate SEs (Class II), and magenta spots are MVBs. **(F)** Fluorescence intensity distribution of all tracked spots for Ivy1-mGFP from video 1. **(G)** Speed distribution based on trajectory displacements per time point of all tracks for Ivy1-mGFP from video 1. The data were analyzed as

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in Figure 2F. **(H)** Speed distribution for Ivy1-mCherry (SEs) and Vps4-HA-mGFP (MVBs) from video 2. The data were analyzed as in Figure 2F.

Figure 3. SE identity depends on MVB functionality. (A, B) Localization of Ivy1 relative to endosomal proteins in ESCRT mutants. Wild-type or *vps4Δ* cells expressing mGFP-tagged Ivy1 and mCherry-tagged Vps8, Vps21, Ypt7, Kog1 or mCherry-tagged Ivy1 and GFP-tagged Ego1, mNeon-tagged Fab1 were grown in a synthetic medium. Vacuoles were stained with CMAC. The cells were analyzed by fluorescence microscopy and shown as individual slices. Scale bar, 5 µm. **(C)** 3D track of the mean fluorescence intensity of Ivy1-mGFP from videos 3 and 4. Wild-type or *vps4Δ* cells expressing mGFP-tagged Ivy1 were grown in a synthetic medium. Vacuoles were stained with CMAC and the data were analyzed as in Figure 2F. **(D)** Quantification related to panels A and B. Ivy1 dots (n ≥ 200), Vps4 dots (n ≥ 400), Vps21 dots (n ≥ 300), Ypt7 dots (n ≥ 100), Ego1 dots (n ≥ 50), Kog1 dots (n ≥ 100), or Fab1 dots (n ≥ 50) were quantified by Image J. Error bars represent standard deviation (SD) of three independent experiments. n.s, p>0.05 (Student's *t*-test). **(E)** Localization of Tor1 or Ego1 relative to Ivy1 and Cps1 in *vps4Δ* cells. Cells expressing mGFP-tagged Tor1 or Ego1 with HaloTag-Ivy1 and mCherry-Cps1 were grown in a synthetic medium. The cells were incubated with the Janelia fluor 646 HaloTag ligand for 1 h and washed 8 times before imaging. The cells were analyzed by fluorescence microscopy and shown as individual slices. Scale bar, 5 µm. **(F)** Quantification of Tor1 or Ego1 dots co-localizing with Ivy1 and/or Cps1 from panel E. Tor1 dots (n ≥ 100), Ego1 dots (n ≥ 100), Ivy1 dots (n ≥ 200), Cps1 dots (n ≥ 200) from three independent experiments were quantified by Image J. **(G)** Schematic model showing the different populations of mGFP-Tor1 and Ego1-mGFP dots analyzed in panel F. Dark grey spots indicate Tor1 or Ego1 dots co-localizing with both Ivy1 and Cps1. Blue and red spots indicate Tor1 or Ego1 dots co-localizing with just Ivy1 or just Cps1, respectively. Light grey spots indicate Tor1 or Ego1 dots that show colocalization neither with Ivy1 nor with Cps1.

Figure 4. Ivy1-positive structures accumulate in HOPS mutants. (A-C) Localization of Ivy1 relative to the vacuole. Selected temperature-sensitive (*ts*) strains (*vps11-1*, *vps18-1*, *vps11-3*)

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expressing mGFP-tagged Ivy1 were grown at 24°C in a synthetic medium, and then shifted to 24°C or 37°C for 1 h. Vacuoles were stained with FM4-64. The cells were analyzed by fluorescence microscopy and shown as individual slices. Scale bar, 5 µm. **(D)** *vps11-1 fab1*^{6D} cells expressing mGFP-tagged Ivy1 were stained with CMAC and analyzed as before. **(E)** Quantification of Ivy1 dots per cell in the indicated mutant strains grown at 24 and 37°C. Ivy1 dots (n ≥ 200) were analyzed. Error bars represent standard deviation (SD) of three independent experiments. n.s, p>0.05, **, p ≤ 0.01, ***, p ≤ 0.001 (Student's *t*-test). **(F)** Design of the experimental procedure to determine vacuolar proteomics. **(G)** Vacuolar proteomic analysis. Wild-type and *vps11-1* or *vps11-3* cells were grown in light lysine (wt cells) or heavy lysine (mutant cells) containing SILAC medium as described in Methods and incubated at 37°C for 1 h. The vacuoles were isolated and analyzed by mass spectrometry. Intensities of identified proteins are plotted in normalized heavy over light SILAC ratios. Selected vacuolar proteins are marked.

Figure 5. Ivy1-structures lose early endosomes marker proteins upon HOPS inactivation. (A) Quantification of Ivy1 dots co-localizing with Vps4, Apl5, or Fab1 puncta in *vps11-1* mutant cells grown at 24°C or 37C. Ivy1 dots (n ≥ 200), Vps4 dots (n ≥ 400), Apl5 dots (n ≥ 500), or Fab1 dots (n ≥ 50) were quantified by Image J. Error bars represent standard deviation (SD) of three independent experiments. n.s, p>0.05 (Student's *t*-test) (related to Figure S2A). **(B)** Localization of Ivy1 relative to the endosomal Rabs Vps21 and Ypt7, or the SNARE Pep12. *vps11-1 ts* cells expressing mGFP-tagged Ivy1 and mCherry-tagged Vps21, Pep12 or mScarlet-tagged Ivy1 and mGFP-tagged Ypt7 were grown at 24°C in a synthetic medium, and then shifted to 24°C or 37°C for 1 h. Vacuoles were stained with CMAC. The cells were analyzed by fluorescence microscopy, and individual slices are shown. Scale bar, 5 µm. **(C)** Quantification of Ivy1 dots that co-localize with Vps8, Vps21, Ypt7, or Pep12 puncta. Ivy1 dots (n ≥ 150), Vps8 (n ≥ 100), Vps21 dots (n ≥ 300), Ypt7 dots (n ≥ 100), or Pep12 dots (n ≥ 150) were quantified by Image J. Error bars represent standard deviation (SD) of three independent experiments. n.s, p>0.05; **, p ≤ 0.01; ***, p ≤ 0.001 (Student's *t*-test) (related to Figure S2A). **(D)** Quantification of Vps4 dots that colocalize with Vps8, Vps21, Ypt7, or Pep12 puncta. Dots of Vps4 (n ≥ 400), Vps8 (n ≥ 100), Vps21 (n ≥ 300), Ypt7 (n ≥ 100), or Pep12 (n ≥ 150)

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were quantified by Image J. Error bars represent standard deviation (SD) of three independent experiments. *, p ≤ 0.05, **, p ≤ 0.01 (Student's *t*-test) (related to Figure S3A).

Figure 6. HOPS and retromer mutants differentially affect Ego1 localization away from SEs. **(A)** Ivy1 structures lose signaling complexes upon HOPS inactivation. *vps11-1* cells expressing mScarlet-tagged Ivy1 and mGFP-tagged Vps35, Ego1 or mGFP-tagged Ivy1 and mCherry-tagged Kog1 were grown at 24°C in a synthetic medium and then shifted to 24°C or 37°C for 1 h. Vacuoles were stained with CMAC. The cells were analyzed by fluorescence microscopy and individual slices are shown. **(B)** Quantification of Ivy1 dots co-localizing with Vps35, Ego1, or Kog1 puncta. Ivy1 dots (n ≥ 200), Vps35 dots (n ≥ 150), Ego1 dots (n ≥ 150), or Kog1 dots (n ≥ 100) were quantified by Image J. Error bars represent standard deviation (SD) of three independent experiments. *, p ≤ 0.05, **, p ≤ 0.01, ***, p ≤ 0.001 (Student's *t*-test). **(C)** Localization of Sec7 relative to Ivy1, Ego1, or Kog1. *vps11-1* cells expressing mScarlet-tagged Sec7 and mGFP-tagged Ivy1, Ego1 or mGFP-tagged Sec7 and mCherry-tagged Kog1 were grown and analyzed as in (A). **(D)** Percentage of Sec7 dots co-localizing with Ivy1, Ego1, or Kog1 puncta. Ivy1 (n ≥ 200), Sec7 (n ≥ 350), Ego1 (n ≥ 150), or Kog1 dots (n ≥ 100) were quantified by Image J. Error bars represent standard deviation (SD) of three independent experiments. ***, p ≤ 0.001 (Student's *t*-test). **(E)** Localization of Ivy1 relative to Ego1 in wild-type and retromer mutant. Wild-type or *vps35Δ* mutant expressing mCherry-tagged Ivy1 and mGFP-tagged Ego1 were grown in a synthetic medium. Vacuoles were stained with CMAC. The cells were analyzed by fluorescence microscopy and individual slices are shown. Scale bar, 5 µm. **(F)** Quantification of Ivy1 and Ego1 dots per wt or *vps35Δ* cell. Ivy1 dots (n ≥ 200) and Ego1 dots (n ≥ 200) were quantified. Error bars represent standard deviation (SD) of three independent experiments. *, p ≤ 0.05; ***, p ≤ 0.001 (Student's *t*-test).

Figure 7. HOPS mutants affect TORC1 activity. (A) Localization of VT relative to the vacuole. Wt, *vps11-1*, and *vps11-3* cells were transformed with the VT (Sch9$^{C\text{-}term}$-GFP-Pho8$^{N\text{-}term}$) reporter and grown in a synthetic medium at 24°C or 30°C. Vacuoles were stained with FM4-64. The cells were analyzed by fluorescence microscopy and showed as individual slices. Scale bar, 5 µm. **(B)** The

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vps11-1 allele causes changes in both vacuolar and endosomal TORC1 activities. Strains with the indicated genotypes were transformed with ET (FYVE-GFP-Sch9$^{C\text{-}term}$) or VT (Sch9$^{C\text{-}term}$-GFP-Pho8$^{N\text{-}term}$) reporters and grown exponentially at 24°C or 30°C on SDC+all medium. To measure ET/VT activities (PMID: 30527664), proteins were extracted, run on SDS-PAGE, and the phosphorylation levels of the ET/VT reporters were detected by immunoblotting using phospho-specific anti-Sch9-pThr^{737} antibodies. ET/VT input levels were detected with anti-GFP antibodies. Different exposures are shown to better visualize the effects on ET and VT. **(C)** Quantifications of the ET/VT assays in (A). Significance was determined with a two-tailed Student's t-test (**p < 0.005; *p < 0.05). **(D)** Phosphorylation states of vacuolar Sch9 and endosomal Vps27. Wt, *vps11-1*, and *vps11-3* were grown in a synthetic complete medium. Corresponding cells extracts were run on 7.5% and 9% SDS-PAGE and probed with phosphospecific Thr^{737} Sch9 and anti-Sch9 antibodies or run on a 6% gel containing 50 µM Mn^{2+}-Phos-tag and probed with anti-Vps27 antibodies. Quantifications of the Sch9 Thr^{737} phosphorylation assayed in (D). Error bars represent standard deviation (SD) of three independent experiments. **, p ≤ 0.01 (Student's *t*-test). **(F)** Growth of wild-type, *vps4Δ*, *vps11-1*, *vps18-1*, and *vps11-3* on rapamycin-containing plates. The cells were grown in synthetic medium and spotted onto plates containing SDC+all with or without 2 ng/ml rapamycin and grown at either 24°C or 30°C for 2–5 days. **G)** *VPS4* deletion affects vacuolar but not endosomal TORC1 activity. Wild-type and *vps4Δ* cells were transformed with ET (FYVE-GFP-Sch9$^{C\text{-}term}$) or VT (Sch9$^{C\text{-}term}$-GFP-Pho8$^{N\text{-}term}$) reporters and grown exponentially at 30°C in a synthetic medium. ET/VT activities were assessed as in panel B. **(H)** Quantifications of the ET/VT assay in (G). Significance was determined with a two-tailed Student's t test (*, p ≤ 0.05).

Figure 8. HOPS function is required for SE recovery. (A) Schematic diagram of the method for monitoring signaling endosomes fusion with vacuoles. **(B, C)** Tracing of SEs by LLSM during recovery of *vps11-1* mutant cells (from video 5). *vps11-1* cells expressing mGFP-tagged Ivy1 were grown at 24°C in a synthetic medium, incubated at 37°C for 1 h, and then tracked and analyzed by LLSM at 24°C. Vacuoles were stained with CMAC. The 3D stacks were cropped by Imaris after deconvolution, and the different channels were split by ImageJ. Scale bar, 2 µm. *Imaris-defined* XY,

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XZ and YZ planes of the 32 min time point are shown. The analyzed dot is indicated in (B) by white arrows and Ivy1 fluorescent intensity was analyzed by vantage time plots in Imaris, and the plot statistic values were measured by surpass objects with spots (C). **(D)** 3D view of Ivy1 following dots dispersion. The image was extracted from video 5, and all views are shown. **(E)** Quantification of Ivy1 dots in video 6. The numbers of Ivy1 dots were counted manually and analyzed by ImageJ. Error bars represent standard deviation (SD) of three independent experiments.

Figure 9. Plasma membrane-derived cargo can pass through the signaling endosomes. (A) Schematic model of α-factor uptake by yeast cells. Green dots refer to SE, green ring to vacuoles, magenta dots to MVB. **(B)** Trafficking of α-factor relative to SEs. Selected time points from LLSM image (video 7) after 3D deconvolution are shown. Cells expressing mGFP-tagged Ivy1 were grown in a synthetic medium, cooled to 4°C to block endocytosis, and treated with fluorescent α-factor for 15 min at 4°C. After mounting, α-factor was tracked by LLSM at 23°C. Indicated time points refer to the time interval after 5 min when cells were shifted to 23°C. **(C)** 3D track mean fluorescence intensities of Ivy1-mGFP and α-factor from video 7. Respective Ivy1 and α-factor fluorescence intensities were analyzed by vantage time plots in Imaris, the plots statistics values measured by surpass objects with spots. The analyzed dots were indicated in (B) by white arrows. The experiment was done 3 times with similar observations. **(D)** Working model of the signaling endosomes function in endolysosomal trafficking. Endocytic transport of a plasma membrane protein (red) bound to cargo (blue) occur via the early endosome (EE) and multivesicular body (MVB) toward the vacuole. Signaling endosomes (SE) are shown at the interface between EE and the Golgi. Rab5 (5, green) and Rab7 (7, black) indicate membrane identity of each compartment. Vps4 (4, pink) is present on MVBs, Ivy1 on SEs and the vacuole, where also the two pools of EGOC, a substrate of the AP-3 pathway, and TORC1 are observed. A fraction of EGOC and TORC1 also resides on MVBs. HOPS promotes fusion between these compartments. For details see text.

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February 14, 2022

RE: JCB Manuscript #202109084R

Prof. Christian Ungermann
Osnabrück University
Biology/Chemistry
Barbarastrasse 13
Osnabrück 49076
Germany

Dear Prof. Ungermann:

Thank you for submitting your revised manuscript entitled "The HOPS tethering complex is required to maintain the identity of signaling endosomes and TORC1 activity". As you will see, the reviewers are now supportive of publication in JCB. However, we agree with the comments of reviewer #2 that you should slightly extend your analysis of the nature of signaling endosomes to ensure they are as well defined as possible for the field. Therefore in your final revision please attempt the requested quantification using your current data and make the indicated text clarifications. After these minor additional points are addressed we would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

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2) Figures limits: Articles may have up to 10 main text figures.

3) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. * Molecular weight or nucleic acid size markers must be included on all gel electrophoresis.*

4) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends. Please also be sure to indicate the statistical tests used in each of your experiments (either in the figure legend itself or in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). Also, if you used parametric tests, please indicate if the data distribution was tested for normality (and if so, how). If not, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

5) Abstract and title: The abstract should be no longer than 160 words and should communicate the significance of the paper for a general audience. The title should be less than 100 characters including spaces. Make the title concise but accessible to a general readership.

* To shorten your title slightly the following version is suggested:
The HOPS tethering complex is required to maintain signaling endosome identity and TORC1 activity

6) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. * Please provide full descriptions in the text for readers who may not have access to referenced manuscripts. *

7) * Please be sure to provide the sequences for all of your primers/oligos and RNAi constructs in the materials and methods. ** You must also indicate in the methods the source, species, and catalog numbers (where appropriate) for all of your antibodies. * Please also indicate the acquisition and quantification methods for immunoblotting/western blots. **

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a. Make and model of microscope
b. Type, magnification, and numerical aperture of the objective lenses
c. Temperature
d. Imaging medium
e. Fluorochromes
f. Camera make and model
g. Acquisition software
h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

9) References: There is no limit to the number of references cited in a manuscript. References should be cited parenthetically in the text by author and year of publication. Abbreviate the names of journals according to PubMed.

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11) eTOC summary: A ~40-50-word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person.

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Sincerely,

Lois Weisman, PhD
Monitoring Editor

Andrea L. Marat, PhD
Senior Scientific Editor

Journal of Cell Biology

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

The authors have address all my concerns. The manuscript is now much stronger and provides exciting new ideas for signaling endosomes and TORC1 signaling.

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

In this revision, the authors have addressed many of my concerns. Overall, the paper quality has been improved. Signaling endosome is a new concept coined by the authors, and it has generated significant interest in the field. Thus, it is appropriate to publish this paper in JCB.
However, I am still not entirely convinced that Ivy1 can represent signaling endosomes. As the authors stated in this revision, cells have significantly more Ivy1 dots than Tor1 or Ego1 dots, which means a lot of Ivy1 dots cannot be interpreted as signaling endosomes. In addition, the colocalization between Ivy1 and signaling endosome makers(Ego1, Tor1, and Kog1) ranged from 40 to 50%, which indicates nearly half (or more) of the Ivy1 dots do not colocalize with signaling endosomes. I understand that the endosome population is heterogeneous, and it is impossible to find two endosomal proteins to be perfectly colocalizing. Still, future readers of this paper need to be cautioned with whether Ivy1 is the appropriate maker for signaling endosomes.
Thus, I suggest the authors do the following without more experiments:
1. Provide a quantification of average dot numbers per cell for Ivy1, Tor1, Ego1, and Vps4.
2. Indicate how many cells have been counted for each quantification.
3. Provide a reverse quantification for Vps4 dots. In other words, what percentage of Vps4 dots are Ivy1 positive?
4. Include a discussion of the heterogeneity of endosomes and make a clear statement that not all Ivy1 dots can represent signaling endosomes.

Other minor points:
1. Video 2. It appears that the time label is covered by a thumbnail image at the lower right corner.
2. Again, in video 2, why does Ivy1-GFP appear to have many more dots than other IVY1 images/videos? Is this an artifact caused by Vps4 tagging?