EMBO reports

Translocation of nutrient transporters to cell membrane via Golgi-bypass in Aspergillus nidulans

George Diallinas, Sofia Dimou, Olga Martzoukou, Mariangela Dionysopoulou, Vangelis Bouris, and Sotiris Amillis
DOI: 10.15252/embr.201949929

Corresponding author(s): George Diallinas (diallina@biol.uoa.gr)

Review Timeline:

Submission Date:	19th Dec 19
Editorial Decision:	23rd Dec 19
Revision Received:	23rd Jan 20
Editorial Decision:	9th Apr 20
Revision Received:	15th Apr 20
Accepted:	24th Apr 20

Editor: Martina Rembold

Transaction Report: This manuscript was transferred to EMBO reports following peer review at The EMBO Journal.

1st Editorial Decision 23rd Dec 2019

Thank you for the transfer of your manuscript and the associated referee reports from The EMBO Journal to EMBO reports.

We note that the referees considered the findings of interest but they also noted that further analyses would be needed to support a more generalized model of non-conventional trafficking of nutrient transporters for potential publication in The EMBO Journal. Yet, given the potential interest of your findings and given EMBO report's focus on single key findings, we would like to give you the opportunity to revise your study for EMBO reports. Please address the remaining concerns from referee 1, in particular the point regarding the early, apical localization of GFP-UpA, and also the remaining concerns from referee 2. Please make sure to present your data in the most accurate way and to avoid any overstatements regarding the generality of the transport pathway.

Please address all referee concerns (as detailed above and in their reports) and take their suggestions taken on board and please provide a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss the revisions further.

IMPORTANT NOTE: we perform an initial quality control of all revised manuscripts before re-review. Your manuscript will FAIL this control and the handling will be DELAYED if the following APPLIES:

1) A data availability section providing access to data deposited in public databases is missing (if relevant).
2) Your manuscript contains error bars based on n=2. Please use scatter blots showing the individual datapoints in these cases. The use of statistical tests needs to be justified.

When submitting your revised manuscript, please carefully review the instructions that follow below. Failure to include requested items will delay the evaluation of your revision.

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).
Please download our Figure Preparation Guidelines (figure preparation pdf) from our Author Guidelines pages
https://www.embopress.org/page/journal/14693178/authorguide for more info on how to prepare your figures.

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point

responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

4) a complete author checklist, which you can download from our author guidelines (). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

5) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript (). Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines
()

6) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here:

- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

7) We would also encourage you to include the source data for figure panels that show essential data. Numerical data should be provided as individual .xls or .csv files (including a tab describing the data). For blots or microscopy, uncropped images should be submitted (using a zip archive if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available .

8) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at .

9) Regarding data quantification:
- Please ensure to specify the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments underlying each data point (not replicate measures of one sample), and the test used to calculate p-values in each figure legend. Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.
IMPORTANT: Please note that error bars and statistical comparisons may only be applied to data

obtained from at least three independent biological replicates. If the data rely on a smaller number of replicates, scatter blots showing individual data points are recommended and any statistical tests must be justified.
- Graphs must include a description of the bars and the error bars (s.d., s.e.m.).
- Please also include scale bars in all microscopy images.

10) As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

You are able to opt out of this by letting the editorial office know (emboreports@embo.org). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

EMBO reports-Answers to points raised by the editor and reviewers

Referee #1:

The subsequent parts of the manuscript largely characterise the GFP-UapA secretion pathway further. They show that secretion depends on ER exit sites (gone in sec24 mutants; yet GFP-UapA does not accumulate in ER - strange, see Fig. 4B), does not involve Rab11 or Rab5 homologues, confirm previous findings that sorting to the plasma membrane occurs independently of post-Golgi transport factor AP-1. Moreover, they show that GFP-UapA secretion to the plasma membrane does not require microtubules, but depends on F-actin, probably via an involvement of F-actin in COPII vesicle formation. Finally, they show that secretion requires the plasma membrane syntaxin SsoA. While these results nicely characterise the GFP-UapA secretion pathway, I consider them of secondary importance for the major conclusion. In fact, some parts slightly distracts the reader from the focus of the study. An important additional result is the confirmation of the key results in 2 other H+ symporters (AzgA and FurA), suggesting that the secretion pathway described in this paper is of general relevance for secretion of nutrient transporters.

In respect to this general comment in the revised version we transferred some of our findings (e.g. the entire Figure 7 and parts of Figure 5) in Supplementary material as we agree with the reviewer that they distract the reader from the focus of the study. On the other hand, we added some data further supporting that the trafficking of the other two transporters (AzgA and FurA) is also COPII- and SsoA-dependent, leaving little doubt that all three transporters studied follow the same pathway for translocation to the PM.

I have assessed the manuscript as a new submission, but had a look at my previous comments and their reply. Indeed, the authors have made a significant effort in addressing my points. Indeed, this manuscript has improved and is now providing more compelling evidence that secretion of GFP-UapA occurs in Golgi mutants. This, in combination with more circumstantial results, strongly support the claim that the transporter is delivered independently of the Golgi apparatus. A major strength of this paper is the direct comparison of UapA localisation with other secretory cargo (namely the v-SNARE SynA and the chitin synthase ChsB). It is fair to say, GFP-UapA is behaving differently from these two well-characterised secretory cargoes.

We are very happy the reviewer is convinced that the transporters studied are delivered independently of the Golgi apparatus via a mechanism distinct from that of polar markers, which is the major conclusion of this work.

However, there are still a lot of open questions. During most of the initial 170 min observation, the GFP-UapA signal is located apical (and at the septum), which does not really support the claim of lateral secretion from ER exit sites. The sudden change in localization (from being at the hyphal tip to concentrating at the lateral regions of the hypha) is suspicious. Could it be that the secretion mechanism changes during hyphal growth? The authors link this to growth speed, but as highlighted below, their reference does not support this conclusion. All tests were focusing on these "late" steady-state distribution (compare 240 min to 170 min in Figure 1), so I am not 100% convinced that it is clear what we are

dealing with. Is a localization at 4h after transcription is initiated really indicative of delivery? I would feel much more content if the study would also investigate the "early" localisation at 170 min (see Fig. 1B, 1D).

It is well established that In *A. nidulans,* mature hyphal tips grow at least 5 times faster than germlings tips (Horio and Oakley, 2005). An analogous situation occurs in other filamentous ascomycetes studied (Berepiki et al., 2011). Fast growth in mature hyphae is shown to be associated with the formation of the Spitzenkörper, an apical vesicular center that is responsible for the final steps in vesicle fusion to the apex (Taheri-Talesh et al., 2008). Germlings, which have relatively slower growth rates, lack a visible Spitzenkörper. In addition, it is also well established, that germlings and mature hyphae have a very distinct distribution of actin (Taheri-Talesh et al., 2008; Schultzhaus et al., 2016). In germlings the apical region is characterized by a dense network of actin cable, whereas in mature hyphae actin cables do not any more form a dense network, but instead are less abundant and mostly associated with the cell periphery (Taheri-Talesh et al., 2008). These well-established observations and our present finding that UapA localization is strictly dependent on actin polymerization very probably justify why we have observed a dramatic change in the apical localization of UapA during transition from germlings to mature hyphae. It seems that UapA localization follows the localization of actin. We did our best to explain this issue in the revised manuscript, both in the relative Results section and in Discussion. Thus we added an extra experiment that follows, in a single hyphae, the apical localization of UapA during transition from slow to fast growth (see Fig.1C, compare 189 to 400 min), and we also added a relative cartoon (Fig. 1E) showing the distinct distribution of actin in germlings and mature hyphae.

Finally, many results and conclusions are still given or supported as single images. The provided images give an impression of the signal intensity variation, which in Golgi mutants is directly correlated to secretion efficiencies. I also find the study not focused enough. In my mind, the lack of a role of Rab11 or Rab5 in GFP-UapA is not really adding much to the study.

When single images are shown, these reflect many results from at least 3 individual experiments and observations in at least 15 hyphae. But the reviewer is absolutely right, in the previous version we did not highlight quantification as it should be. In the revised manuscript we show an improved full range of quantification data, for all major figures concerning the fraction of UapA reaching the PM.

The data on Rab5 were transferred to supplementary material, as we agree that since early endosome are known not to be involved in UapA trafficking or recycling, they somehow distract the reader.

We kept the Rap11(RabE) data as this key post-Golgi protein shown to be necessary at multiple points of post-Golgi polar cargo trafficking (i.e. exit form TGN, microtubule-dependent traffic, function of the Spitzenkörper and exocyst, and recycling via endosomes or/and the TGN; see Martzoukou et al., 2018). Thus, its absolute redundancy for transporter trafficking confirms that the sorting route discovered in this work is TGN-independent and distinct from that of polar markers.

Summary: I come to the same conclusion as before- there certainly "is something", but I do not find this study fully convincing; the strongest part is the localisation of GFP-UapA in Golgi-mutants; the absence of co-localization with Golgi markers is not really a supportive result, as GFP-UapA signals maybe too faint (or it is secreted to fast) to be seen; that SynA is locating with Golgi markers could be due to different expression strength (in fact no GFP-UapA signals are seen in the cytoplasm at all); most importantly, a big question around the read-out (the localization), which seems to change fundamentally from 170 min to 240 min (although change in growth rate according to Horio and Oakley, 2005 is not the cause); I think this phenomenon needs to be better understood, or the effect of Golgi-mutations on this early localization needs to be investigated.

We agree that absence of localization in the Golgi, being a negative result, is not *de facto* proof of Golgi-bypass, but under the light of detectable Golgi/TGN localization of polar cargoes and mostly quantified results obtained in Golgi mutants, we believe that transporters *do* bypass the Golgi, something that the reviewer also seems to be convinced of by his earlier general comments. We exclude that we do not see UapA in the Golgi due to low expressions as in the very original version of our manuscript we also used conditions that over-express UapA, and the results/conclusions were practically identical. Overexpression of UapA led to ER-overload (visible as more prominent perinucler fluorescent ER rings) and increased amounts of UapA reaching the PM, but never led to Golgi-like fluorescent signals. In the revised manuscript we decided not to include to UapA overexpression experiments as this is not only a non-physiological case, but it might also distract the reader from the focus of the study.

Some additional comments:

Abstract: What is meant by "a novel genetic system"-why is it "novel"?

We consider our system novel because, to our knowledge, no previous report has developed a system to follow *de novo* made transporter/cargo trafficking for a time window that Golgi functioning is shut off and cells are alive. During manuscript revision, however, we became aware of a new article studying the trafficking of *de novo* made GLUT4 transporter (Camus et al., 2020). To our satisfaction, this work showed that GLUT4 is sorted to the PM via an early secretary compartment, thus bypassing the Golgi. In the revised manuscript we refer to this new work on GLUT4 and adapt our speculative model, shown in Figure 9, to consider an alternative possible step where transporter-specific vesicles fuse to create a hypothetical ER-to-PM intermediate, form where 'new' vesicles are directed to the PM.

Abstract: Explain abbreviation PM-**Point addressed**

Typo: "independent, mechanism,"- **Point addressed**

Page 4: The first part of the results is really an extension of the discussion or introduction. I like the arguments that the authors build here. This part is important, but in my mind it should be part of the Introduction.

Text revised and this part now makes part of the introduction.

Page 5: explain "N source"- **Point addressed.**

Figure 1B, 240 min: Why is there strong subapical but no apical localization when the opposite is holds true for 170 min?

Point addressed. We should note that 240 min were referring only to UapA derepression time. The difference in localization was a matter of the selection of a longer, mature hypha which shows an acceleration in apical extension. For more details see our answer above and revised text and Figure 1

Fig. 1B, page 5: The author's state that UapA-GFP labels a network after 80-120 min, that is has a typical ER appearance. They also state that "importantly" no Golgi was stained. I do not really see this from the data. Golgi would be vesicular which the images at 120 min would support as much as the claimed network localization. I also miss staining of the nuclear envelopes (part of the ER)?

Golgi structures in *A. nidulans* and other fungi appear as distinct, rather immotile, cytoplasmic puncta, as those seen in experiments following SedV or PH markers (see Figures 1C, 2). Within the limits of fluorescent microscopy, de novo made UapA signals never mark similar cytoplasmic structures. Staining of perinuclear ER rings is visible only upon transporter overexpression (see also our relative comment above). Transporters on their way to the PM label mostly a membranous network, characteristic of Aspergillus ER, as defined through several previous studies suing ER-resident markers (see Erpapazoglou et al., 2006; Evangelinos et al., 2016). Passage of transporters from the ER is confirmed in this work via experiments performed in *sec13* and *sec24* transcriptionally repressed strains. Labeling of nuclei (via the use a fluorescent histone H1) has also been performed in some control experiments, but we did not see a reason to include them in the final version of this work, as the positioning of nuclei in both germlings and hyphae is very well established.

Figure 1C: I find it a bit hard to believe that the localization changes so dramatically within 70 minutes- from being apical at 170 min to being absent from the apex to very strong in the subapical region. The authors explain this by different growth speed and cite Taheri-Talesh et al 2008 for this. However, I cannot find any data in this paper that support this claim. In fact, Taheri-Talesh et al 2008 cite Horio and Oakley, 2005. In this paper, the authors show that germlings grow slowly until they are 75 min old, but accelerate when they are 136.3-235.8 min old. Thus, the fast growth rate occurs already in the provided image at 120-170 min; yet the localization is dramatically different in 170 min and 240 min old germlings. I think this needs correction.

Point addressed. See our answer above and revised text and Figure 1. In brief, the change in apical UapA localization is in line with the significant changes underlying transitions from slow-growing germlings to fast-growing mature hyphae, and in particular the reorganization of apical actin cables.

Page 6: The authors say "our findings strongly suggested that neosynthesized UapA"-- did I miss something here? The faint signal at 100 minutes could be Golgi or ER or vesicles, maybe

even misfolded protein aggregates. Which "strong" evidence for an ER localisation do the authors provide?

We removed the word “strongly: and reduced speculation for Golgi bypass at this point. This conclusion becomes stronger later.

Page 6: Why is it surprising that newly synthesised UapA is not found in rapidly moving early endosomes? Endocytosis and membrane recycling is not yet considered at this point in the manuscript.

The reviewer is right, in the way the sentence was written the use of the word “surprising” led to a misconception. We removed it and rephrased the sentence.

Page6: Typo: "apical parker,"- **corrected**

Page 6, Page 1C: The authors look at localization of Synaptobrevin A tagged with GFP (SynA-GFP) and find it in vesicles. They claim that these are Golgi bodies. However, almost all organelles shown a vesicular appearance- how can they exclude that these structures are vacuoles? The authors provide more compelling evidence for their claim later- but at this point, I am not convinced.

In *A. nidulans* vacuoles are very distinct morphologically (even visible in Brightfield) and in size compared to Golgi/ER and moreover can be easily stained with CMAC. In control experiments not shown in the present manuscript, CMAC staining has been used. Standard polar markers, such as SynA, do not co-localize with stained vacuoles, at least under the conditions we used to study transporter trafficking. As the reviewer also noticed, later we do show that SynA-GFP co-localize significantly with a standard late Golgi marker.

Page 6, Fig. 1B: There are now arrows in Figure 1B! The argument that diffusion is excluded is relatively weak (no gradient visible...). Intensity scans are needed here. Clearly most UapA is located at the apical region of the hypha, so diffusion is a possibility.

Arrows are now shown in Figure 1B. We consider diffusion a rather unlikely possibility not only due to lack of a visible gradient, but also because punctuate cortical foci appear on the PM rather randomly and far away from the apical region (indicated now by arrows). Also, in longer hyphae, where no UapA could be detected in the PM of the apical region, we could see the appearance of cortical foci in subapical regions.

Figure 1D: Nice experiment, I am convinced now that endocytosis does not participate in the localization of UapA. However, the apical localization is now even more prominent- again leaving me a little confused about the statement that "localization to the PM takes place by direct short-range lateral sorting from the ER" (middle page 6).

The apparent small difference in the samples shown in figure 1B and 1G is not significant when other images of more germlings are examined.

Page 7 and Figure 2: The GFP-UapA signals are much too weak to provide co-localisation information. As seen in Fig. 1B, distinct signals are rare. Why if a lack of co-localisation is due

to the weak signal intesity? Triple-GFP tags are needed to be sure that GFP-UapA can be detected.

Triple-GFP tags block UapA trafficking, while single GFP tagging has no effect on trafficking and transport kinetics. As explained above, overexpression of UapA leads to the same major conclusion of this work: ER and PM localization, but not Golgi localization of UapA.

Page 10: Would one not expect accumulation of GFP-UapA in the ER in Sec24 mutants? This is visible for ChsB (Figure 4C), but not seen in Figure 4B for UapA! The authors state that UapA stays in the network- but I cannot see this being supported by the image provided.

In the revised manuscript we replaced the image of UapA-GFP in Sec24 repressed background (Fig.4B) and added quantification data form relative experiments. We think that that now the pictures shown for both UapA and ChsB (also quantified) are very similar, both showing accumulation of cytoplasmic structures rather than PM localization. A possibly interesting point to compare is the relative UapA localization in the absence of Sec24 (Fig.4B, lower panel) compared to that in the absence Sec13 (Fig.4E, lower panel). In the latter case UapA labels more well-defined punctuate structures. This might suggest that in the absence of Sec13, which necessary for the final maturation of COPII in ERes, pre-budding cargo-specific ER microdomains are formed, but these domains cannot be formed when Sec24, the cargo recognition protein, is missing.

Referee #2:

The authors have significantly improved the manuscript in response to the reviewers' comments. The results are now more compelling, and I believe they make a much stronger case for the unconventional secretion of nutrient transporters in Aspergillus. There is also improved data on molecular machinery, and the authors now propose a model for the trafficking route, which I think is a good addition. There are a few outstanding issues though, as expanded upon below.

A major point in the original review was the lack of quantitation of trafficking. Unfortunately, this issue persists. The data would be more complete and robust if the amount of trafficking to the plasma membrane was quantified. Currently we have to rely on single images, which, although they may be representative, represent an incomplete dataset.

In the revised manuscript we present (in all major figures) a full range of quantification results, for both UapA and apical markers. The reviewer was absolutely right that these data are necessary to make our work convincing.

Minor points:

Figure 1 panel labeling is incorrectly referred to in the main text.-**corrected**

In Figure 2, there appears to be a low amount of co-localization of UapA with the trans-Golgi marker. Can the authors account for this? In Fig 2B, the green signal is also weaker in the merge than it is in the single channel for UapA, which gives a false impression of no/low co-localization. The levels should be adjusted so they are consistent between panels.

This low apparent colocalization (close to 0.2) is due to the very close proximity of ER and Golgi structures. A similar level of apparent colocalization is estimated when well-established markers of ER- and Golgi-resident markers are followed. We also did our best to adjust to green signal in Fig.2B.

In Fig 5C, the images are not the best quality.

We replaced Fig.5c with a better quality image.

The text in the introduction needs modifying. It is stated that "genetic knock-out of proteins involved in TGN-dependent membrane cargo sorting (e.g. Arfrp1, golgin-160 or AP-1)" does not affect GLUT4 traffic. The implication here is that GLUT-4 is transported in a Golgi-independent way. However, there are many routes out of the TGN, and in many cases the machinery is poorly defined, so stating that loss of any or even all of these three proteins has no effect on secretion is not actually good evidence that transport is Golgi-independent. The text should be modified here.

This paragraph is revised accordingly and a new reference included (Camus et al., 2020)

In the Results, the first sentence states that AP1 is a key effector of conventional secretion. Presumably the authors are referring specifically to Aspergillus here, because in mammalian cells it is not true for all cargoes. This needs to be made clear.

Text revised accordingly.

Referee #3:

Despite the improved quantitation of morphological data, the paper simply does not establish what the authors claim, due to their lack of establishing ANY basolateral cargo that follows a different sorting route, nor establishing whether basolateral sorting is the default route. Their phenomenon appear to have nothing to do with nutrient transporters. Instead, it appears that in the A. nidulans secretory pathway their is an apical delivery pathway involving the Golgi and a basolateral delivery pathway that does not involve the Golgi, or at least involves it much less. But without a systematic analysis of cargoes, including heterologous cargoes with known sorting routes, even these conlcusions are not sufficiently established for EMBO Journal. Furthermore, one pathway is likely to be a default or "bulk flow" pathway and the other one involving specific sorting events. This is not addressed at any level.

To our opinion, the two issues raised are not justified. First, the major point of this work is not whether there are distinct trafficking routes for basolateral and apical cargoes in general, although this is an interesting point that our findings do support. The major novel point of our work is that several transporters, used as model non-polar cargoes, reveal that there is a Golgi-independent trafficking route, at least for specific nutrient transporters. Her/his proposal to use heterologous known basolateral cargoes (from mammals?) find us in total disagreement, as membrane cargoes are co-translationally

translocated and folded in the ER, and then follow trafficking routes, processes which are all highly dependent on specific interaction with specific lipids. Our long standing experience has shown that metazoan membrane proteins are not property secreted in fungi (blocked in the ER), so using them for studying trafficking in fungi is of no value. We definitely want to continue our studies using other endogenous specific cargoes that are localized basolaterally, or in distinct PM domains, but his is beyond the scope of the present work.

2nd Editorial Decision 9th Apr 2020

Thank you for the submission of your revised manuscript to EMBO reports. I apologize once more for the unusual delay in handling your manuscript but we received the delayed report from referee 1 only very recently. As we communicated previously, referee 2 now supports publication of the current dataset while referee 1 remains concerned about the conclusiveness of the cell biological and imaging data. As outlined in our earlier communication, we have assessed this report carefully and we focused solely on substantial experimental issues that are directly relevant to the key claims of the paper. We also ensure that the issues we considered were objective, constructive, addressable and consistent with the preceding per review process - that is, we explicitly discounted any new, peripheral or in our view excessive issues. We note that both referees consider the genetic data on Golgi-independent trafficking of the nutrient transporters convincing. However, we also had to note that referee 1 remained concerned about the conclusiveness of the localization data. Since the localization data is critical in supporting the genetic data, we decided to consult further on the issues we had identified as requiring resolution with referee 2 as well as with an additional independent expert on fungal biology (the expertise of referee 1). As we outline before, we asked for specific input on two issues during arbitrating: (1) the conclusiveness of ER localization of UapA and its absence from the Golgi and (2) the conclusiveness of the co-localization data with Golgi markers and how these aspects and the key claims can be most appropriately presented.

As you will see in the appended report below, referee 2 noted that s/he considers the ER localization overall convincing but also points out that a co-localization of UapA with the Golgi cannot be rigorously excluded based on the images provided. The additional advisor supported these comments. Taking the advice of both experts into account alongside referee 1's report, we have therefore decided to proceed with publication in EMBO reports, conditional on addressing the following points:

1) We note that the imaging data is in agreement with the genetic data but it cannot conclusively exclude that a subpool of the transporter localizes to the Golgi. Please acknowledge this limitation clearly in the text and discussion.

2) Please provide merged images for Figure 2B with a brighter green signal, to allow for a better subjective assessment of the level of co-localization. Please comment on potential co-localization seen in the quantification.

3) Please provide source data for all confocal imaging data as well as all other key data for publication alongside the figures in line of general journal policy.

4) If you have co-localization data for newly synthesized UapA-GFP and ER markers for Figure 1, please add these. Otherwise, please rephrase the conclusion "Subsequently UapA-GFP; labels a membranous cytoplasmic network (80-120 min) very typical of A. nidulans ER" to better reflect the weak staining and absence of perinuclear staining.

5) Please add a careful description of the strains and promoters used to express SynA, GFP-UapA and of all other strains. Please add information on the expression level that is expected and whether it is similar to endogenous expression. Along these lines, please move Table S1 (strains used) to the main methods section since it provides information on these issues.

6) Please acknowledge the vesicular structures observed for UapA in Fig. S1 and Fig 2B or upon Latrunculin treatment. Please discuss their potential nature and whether these might be Golgi-derived vesicles.

7) Please address all other concerns from referee 1 in a point-by-point response to the editors and the text and/or figure legends, as appropriate.

From the editorial side, there are also a few things that must be addressed before we can proceed with the publication of your study.

1) I attach to this email a copy of your manuscript file with comments by our data editors. Please address all comments and upload a revised file with tracked changes with your final manuscript submission.

2) Please abbreviate the names in the Author Acknowledgement section using initials, e.g., SD for S. Dimou

3) Please update the references to the numbered format of EMBO reports. The abbreviation 'et al' should be used if more than 10 authors. You can download the respective EndNote file from our Guide to Authors
https://drive.google.com/file/d/0BxFM9n2lEE5oOHM4d2xEbmpxN2c/view

4) Please add callouts to the panels of Figure 8, where appropriate.

5) Supplementary information:
- Please update the title to "Appendix" and add a table of content to the first page including page numbers.
- The nomenclature for Supplementary figures is "Appendix Figure Sx". Supplementary tables are called "Appendix table Sx". Please update the legends and all callouts in the text accordingly.

- Movie: Please note the nomenclature Movie EV1. Please remove the legend from the Appendix/Supplement and provide it as a simple README.txt file. Then zip the movie together with its legend and upload the zip file.

6) Appendix figure legends:
- Fig. S1A: please specify the meaning of the dotted box and provide scale bars for the magnified images.
- Fig. S1B: please specify the bars and error bars, the statistical test used and the exact meaning of ** in the legend. Please specify the nature of the replicates 'n' (biological, technical)
- Fig. S2: Please define the error bars, the number of experiments (biological, technical) and the meaning of *, **, ***, ****. Please also define the statistical test used.
- Fig. S3: please provide scale bars for the magnified boxes. Please provide a separate legend for panel C. Please show the quantification as scatter blots instead of the bar blot, since it is based on only two biological replicates.
- Table S1: please add the references listed in the table to the Appendix reference list.

7) Main figure legends: Please add scale bars to the magnified boxes in Fig. 1,2,3,5,6.

8) Methods: please specify the catalog number for all antibodies.

9) Data availability section: This section is mandatory but should only report on primary datasets deposited in a public database. In your case, please add a statement that no data were deposited in a public database and please remove the current statement.

10) Finally, EMBO reports papers are accompanied online by a synopsis that consist of A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is 550x200-400 pixels large (width x height) in .png format. You can either show a model or key data in the synopsis image. Please note that the size is rather small and that text needs to be readable at the final size. Please send us this information along with the revised manuscript.

We look forward to seeing a final version of your manuscript as soon as possible.

****

Referee #1:

I had an extensive read of the manuscript by Dimou et al. The authors use live cell imaging techniques to visualise sorting of de novo synthesized UpaA to the plasma membrane in the fungus Aspergillus nidulans. In a first set of experiments, they do not find any indication that fluorescent UpaA is passing through vesicular structures that could resemble the Golgi apparatus. Green-fluorescent UpaA does not co-localise with two red-fluorescent Golgi markers, and sorting into the plasma membrane is not abolished when main Golgi-dependent effectors are down-regulated. However, UpaA sorting requires COPII vesicle formation at ER exit sites, clathrin heavy chain, the plasma membrane t-SNARE SsoA and F-actin, suggesting that UpaA-carrying transport vesicles bypass the Golgi and fuse with the lateral plasma membrane. Such secretion pathway would be new and its discovery a significant step towards understanding of membrane trafficking pathways in fungi.
However, I have several concerns about this work. This is a cell biological study, which has localisation data as its main, often exclusive read-out. Such live cell imaging approach is powerful, but requires additional care, as proteins can be mislocated due to overexpression or the presence of unlabelled protein, transcribed from the endogenous gene. In the end, a picture shows a localisation, so care has to be taken that the conditions to come to this picture are right and carefully chosen.
The current study falls short here. Firstly, the experimental setup is not well characterised. While the authors compare revealingly UpaA sorting with secretion of other cargo (e,g, SynA or ChsB), they do not provide information on the expression strength of the promoters used (which promoter was used to induce SynA? How much SynA is produced? Is the endogenous copy still expressed?).

It is not clear if the GFP-UpaA fusion protein represents an additional copy, or if the endogenous copy is tagged. Also, some conclusions are contradixctory (e.g. Fig. 1 states that UpaA locates in the ER - where is the evidence for that?- but not in vesicular structcures that could be Golgi, while other Figures clearly show vesicular structures (Supp Fif 1, Fig. 2B and others).
Moreover, the absence of a signal could be due to low protein numbers and single GFP tagging. If a protein travels fast through the secretion pathway, one will not see it in the Golgi or the ER. In the end, it is the residential time that determine if a protein can be seen at a given point of its secretory pathway. The authors are aware of this and test sorting of UpaA in several mutants. Indeed, I do think they have a point when arguing that nutrient transporter s most likely bypass the Golgi. However, it is the collective information that brings me to this conclusion. Looking at the individual results, I find a lot of questionable statements and not fully supported conclusion.
My conclusion is, that the paper has a good point and I do "believe" the main message- but I am not really convinced by what I read. I therefore cannot support publication in EMBO Reports.
Specific points:
The Introduction would benefit from shortening. Right now it reads like a review, it is not really bringing across why the authors have done their study. In fact, at the end of the discussion, the reader gets the feeling that all is clear and not many questions are open. This does not help the "novelty" aspect of this research.
Page 4: The system they developed needs to be explained and better characterized. Which promoter was used? In the figure legend the authors say that the native uapA promoter was derepressed- is this correct? And is this system really novel? UapA-GFP expression from its endogenous promoter was already established in their paper Gournas et al 2010, Mol Mivcrobiol.?
On page 5, the authors say: "Subsequently UapA-GFP; labels a membranous cytoplasmic network (80-120 min) very typical of A. nidulans ER" this conclusion is not justified by the images. For "membranes" can be identified, the fluorescence is at best faint. That the fluorescent is representing "very typical of Aspergillus ER " is simply not true (e.g. no nuclear envelopes are seen, no ER at the periphery of the cell). Later the authors state further "Thus, in long hyphae, UapA is imaged to clearly label an ER-like membranous network"- this is not supported by the data shown in Figure 1.

End of page 5: "These observations will become more apparent later, when actin polymerization is shown to be essential for UapA trafficking." This is an odd way of highlighting a potential result, but delaying the presentation of the evidence.

End of page 5: "Overall, results highlighted in Figure 1A-E, suggested that neosynthesized UapA labels the ER...", two flaws: (i) no evidence for ER localisation is given in Figure 1, (ii) Figure 1E show no results, but a graph

Beginning Page 6: Why is the analysis of the dynamic localization of Synaptobrevin A "Most important"? Information on the experimental setup needs to be provided here; how can the GFP-vSNARE appear in endosomes before it is shown in the plasma membrane? And are these structures endopsomes and Golgi vesicles?

Beginning Page 6: It is obvious that Synaptobrevin-GFP appearance differs from UapA (Figure 1F compared to 1B). However, no information on the inducible promoter is given in the text. In the figure legend, the authors refer to Material and Methods, but again I was not successful to get an idea of which promoter was used to induce SynA-GFP. Maybe the observed localisation in vesicles is an overexpression artefact?

Page 6, middle: The authors state that they do not see a gradient of GFP-UpaA at the tip and thus

lateral diffusion down the length of the hypha is unlikely. They also state that diffusion of transmembrane proteins in the plasma membrane is slow. From this they conclude "This shows that UapA localization to the PM takes place by direct short-range lateral sorting from the ER network". Again, much too farfetched in my opinion: (i) Figure 1B shows a gradient at 140 min- which is the time the lateral signals appear (arrowhead), (ii) lateral diffusion of transmembrane proteins can, indeed be fast (minutes); (iii) even if these two points are dismissed, the authors have NOT shown " direct short-range lateral sorting from the ER network" as they state.

Page 6, second paragraph: The authors stare that Gournas et al (2010) and Karachaliou et al. (2013) "have strongly supported that internalized UapA is not recycled back to the PM after endocytosis", from which they conclude that " Thus, the subcellular localization of UapA shown in Figures 1B, 1C and 1D reflects strictly secretion of neosynthesized UapA". This is an important argument. I therefore looked into both papers, but could not find any of the cited evidence. In fact, the word recycling/recycle is only mentioned in the introduction. Did I overlook this?

Page 6, Fig. 1G: The authors state that the localisation results are "The result practically identical to the one obtained in the wild-type strain". I disagree here- we clearly see a septum stained in 1G (160 min), which does not show up in Fig. 1Band 1C (even after 400 minutes!). Thus, there is something else going on.

Fig. S1: Co-localisation with FM4-64 shown in Fig. S1 is not convincing- resolution too low! Also, these images show intracellular UapA dots- which were not shown and mentioned in Figure 1! Could they be Golgi vesicles?

The localisation experiments shown in Figure 2 are good and important. The way they are shown in Figure 2 A, 2B is not very convincing, though. The green channel need to be exposed more to judge the overlay. I have redone this with the images provided. Indeed, I do not see significant correlation of red and green in the left panels (Fig. 2A, SedV; see left inserted figure), but the right panels (Fig. 2B, PHOSBP) are more concerning. Firstly, GFP-UpaA suddenly localises in vesicular structures (which was categorically excluded in the previous part of the paper). Secondly, I do see some co-localisation after bringing up the green channel (see arrowheads inserted Figure in this document). I am a bit puzzled now.

The fluorescent SynA/Golgi marker data seem convincing. However, I would still need to know the expression strength of the promoter used to induce SynA- see point above.

Page 9: The author show appearance of UapA in the plasma membrane in mutants defective in Golgi-dependent secretion steps. The data are convincing, but I wonder why the investigation was done after 6-8 h of derepression? All previous results were presented in a 1-3h time window? Could it be that the promoter is not 100 tight, allowing some Golgi-based secretion over time? If Golgi is not involved, 140-170 minutes (as shown in Fig. 1B, Fig 2A,B) should reveal the effect.

Page 9: What was the time window for the test of conventional polar cargoes, such as SynA (synaptobrevin A) or ChsB (chitin synthase) in the absence of either SedV or HypB? Also 6-8h? This information is missing from the text and the figure legend.

Page 10: The authors state that UpaA remains in an ER network in sec24 mutants. Figure 4B does NOT suggest that the fluorescent protein accumulates in the ER (no nuclear envelopes labelled- but they are labelled in Fig. 4C (ChsB-GFP). Thus, the conclusion is not justified by the data. On the

other hand, a role of Sec24 in UpaA secretion is clearly shown, which suggests that UpaA leaves the ER in COPII vesicles (which, as the authors state clearly, normally travel to the cis-Golgi.

Page 11: The authors refer to Martzoukou et al., 2018, (Genetics 209: 1121) for evidence that UapA sorting to the plasma membrane depends on the clathrin heavy chain ClaH, but not on the clathrin light chain ClaL. As both chains cooperate to make the clathrin coat, I was surprised and looked into the paper for the evidence that one but not the other determines UapA sorting. I could not find any supporting evidence.

Page 11: The authors find that UapA sorting to the plasma membrane is impaired in clathrin heavy chain mutants, but not in Rab11 or AP-1 mutants. They conclude that "clathrin heavy chain is critical for the trafficking of de novo made UapA to the PM, but two key TGN-localized upstream effectors of ClaH function, AP-1 and RabE are not". From the absence of co-localization, they conclude a vesicle-independent role of clathrin in UpaA sorting to the plasmas membrane. Looking at the figure 5C, left panel, I have to say that I am not at all convinced that the data shown merit this conclusion.

Page 12: The authors find that F-actin is required for delivery of UpaA. In the presence of the actin-drug Latrunculin, UpaA is located in vesicular structures (*Figure 6A). It would be most interesting to see if these vesicular structures are co-localising with red-fluorescent Golgi markers! There is a real possibility that post-Golgi vesicles are not made (as myosin II is involved in their formation) and that UpaA is now accumulating in Golgi vesicles.

Page 13, first paragraph: The experimental setup for visualisation of de novo made SsoA needs to be explained (which promoter, how strong compared to the SsoA promoter? Also, the authors say that SsoA behaviour "somehow resembles the picture obtained with UapA". This needs explanation

Referee #2:

The authors have addressed the points I raised previously when the paper was under consideration at EMBO Journal. I think it is an interesting and well executed paper that is suitable for publication in EMBOR.

****

Additional feedback from Referee #2:

"1.) ER localisation. The images in Fig 1D, but more convincingly in Figs 2A and B, are consistent with ER localisation. The staining looks like one would expect for ER, being mainly reticular. To absolutely nail the point the authors could do a double labelling with an ER marker, but I understand they have shown ER colocalization in previous work. It could nevertheless be requested. However, considering the proteins being made are transmembrane proteins, they would have to be made in the ER. So alternatively the authors could make these points clearer in the discussion and refer to their previous work.

2.) Regarding the low expression-in Fig 2A the staining is pretty bright and clear, and does not show

colocalisation with Golgi. So, consistent with a non-Golgi route of transport. In Fig 2B, I would have liked to have seen a stronger green signal in the merge. I have the impression that there may be some colocalisation with the Golgi marker in the merge. Better images could make this clearer. The quantitation would seem to indicate a low degree of colocalisation, but it must be done on images with bright enough green and red signals. This could also be requested.

The functional data are strong though- clearly blocking Golgi function doesn't affect the amount of UapA at the PM, but does for other secretory cargoes. This strongly supports the model for unconventional (non-Golgi secretion).

Regarding the expression levels of the proteins and promoters used, my understanding is that they tagged the endogenous versions of the proteins, and therefore that they would be expressed at or close the normal levels. This could easily be clarified in the text. I don't think expression levels can account for the experimental differences between UapA and the other cargoes."

Answers to Referee #1

Major concerns

While the authors compare revealingly UapA sorting with secretion of other cargo (e.g., SynA or ChsB), they do not provide information on the expression strength of the promoters used (which promoter was used to induce SynA? How much SynA is produced? Is the endogenous copy still expressed?). It is not clear if the GFP-UapA fusion protein represents an additional copy, or if the endogenous copy is tagged. Also, some conclusions are contradictory (e.g. Fig. 1 states that UapA locates in the ER - where is the evidence for that?- but not in vesicular structures that could be Golgi, while other Figures clearly show vesicular structures (Supp Fif 1, Fig. 2B and others). Moreover, the absence of a signal could be due to low protein numbers and single GFP tagging. If a protein travels fast through the secretion pathway, one will not see it in the Golgi or the ER.

We understand that in the previous versions of the manuscript not much information was given for the on the expression strength of the promoters used. We thus added a very detailed description for that in Material and methods. What follows here is a summary of this new information.

- In all cases, transporters and other cargoes or markers tagged with GFP or mRFP, are expressed in the total absence of the endogenous untagged copy. This is apparent from the strain list in Table 1, but was also already stated at page 4 in the the begging of the Resits section ("*strains containing an in-locus targeted uapA-gfp allele"*). In brief, replacement of the endogenous copy was achieved, in each case, by a standard reverse genetic methodology, using liner DNA cassettes, via targeted homologous recombination. The desired recombination events were confirmed by PCR.
- In all cases we avoided overexpression of a cargoes and other markers, except for a case where overexpression of UapA was used as a tool to mark better the ER (see below).
- For UapA, we used either its *native* promoter or the regulatable $alcA_p$ promoter (Waring et al., 1989), which can be controlled to drive expression of UapA at levels similar to those obtained with the native promoter. Both promoters can be tightly repressed, either by ammonium ions as N source (the *uapA* native promoter), or by glucose as C source (the $alcA_p$ promoter), and rapidly derepressed with similar kinetics, by shift to media with nitrate as N source or fructose as C source, respectively. The similarity in expression levels by these two promoters can be seen in Figure 1 of a preprint by Bouris et al 2019 (Nutrient transporter translocation to the plasma membrane via a Golgi-independent unconventional route, Vangelis Bouris, Olga Martzoukou, Sotiris Amillis, George Diallinas, bioRxiv 540203; doi:

https://doi.org/10.1101/540203). For studying the *de novo* localization of UapA, we incubated conidiospores overnight under repression conditions to obtain germlings or young hyphae, and next day shifted the cultures in derepressing conditions. The same approach was used to study the other two transporters (AzgA and FurA) expressed from $alcA_p$.

- In a panel added in the current version (Fig.1F), we *also* over-expressed UapA-GFP via the *alcAp* promoter by shifting the culture to depressed plus *ethanol-induction* conditions. Notice that $alcA_p$ is i) tightly repressed in the presence of glucose, ii) derepressed and expressed at levels similar to those detected from the *uapA* native promoter, when glucose is replaced by fructose, and iii) highly induced leading to significant overexpression (>10-fold) under derepression *plus* ethanol-induction conditions. We used this system to also over-express UapA-GFP in Fig.1F for two main reasons. First to show clearly that *de novo* made UapA can also label the characteristic perinuclear ER rings, and second, to show that even after overexpression we hardly detect any significant fraction of UapA-GFP localizing in punctuate Golgi-like structures. Thus, we could dismiss the possibility that UapA does not label Golgi-like structures because of low expression when transcribed from its native promoter.
- For following the trafficking of other control cargoes, we used again either their native promoter or the $alcA_p$ promoter. In the former case, we could only follow the trafficking of constitutively expressed cargo, as these promoters are not regulatable. In cases, where we used the regulatable *alcA* promoter, we could follow the trafficking of *de novo* made cargo after a shift to derepressing conditions, as we did with transporters. All relevant strains used were the product of in-locus gene replacement of the endogenous genes with the GFP-tagged gene versions of the cargoes.
- For most other markers tagged with fluorescent epitopes (e.g. Sec24, SedV, AP-1, RabE, ClaH, RabB, SsoA), except TubA and PH^{ospb}, we used native promoters for expression. For PH^{ospb}, which is an artificial marker of the TGN (Pantazopoulou et al.,
- Mol Biol Cell. 2009, 20:4335-47), we used the strong constitutive promoter $gpdA_p$ (Punt et al. Gene. 1990, 93:101-9), whereas for TubA we used the *alcA* promoter expressed at moderate levels (derepressed, non-induced).
- In summary, we a) did not use over-expression of cargoes/proteins, unless needed as an extra tool for specific reasoning, b) used native promoters or the *alcAp* regulatable promoter under conditions that ‘mimic’ expression levels and dynamics of native promoters and thus leads to physiological levels of cargo expression, c) always replaced the endogenous copy of a gene by an in-locus integrated copy tagged with GFP, mRFP or other epitopes, d) used over-expression of UapA only to show that the transporter labels perinuclear ER, but not punctuate Golgi-like structures.

Specific points

The Introduction would benefit from shortening.

We did not shorten the introduction as other referee(s) suggested to move the part of the rationale of our approach, originally present in the Results section, in the Introduction. We think the introduction need to stay as it is to include the logic of our research.

Page 4: The system they developed needs to be explained and better characterized. Which promoter was used? In the figure legend the authors say that the native uapA promoter was derepressed- is this correct? And is this system really novel? UapA-GFP expression from its

endogenous promoter was already established in their paper Gournas et al 2010, Mol Microbial.?

The system was explained above, as an answer to the major point of referee 1. It is also explained in the cartoons of Figure 1A and Figure 3A. In the current manuscript version, we did our best to provide a better explanation of the system also in the text, found in the revised. Materials and methods section. What is novel is not the idea of repression-derepression via transporter native promoters or from *alcA*$_p$, as this is known for 30 years. What is novel is to follow the trafficking ***de novo*** **made cargoes** in a time window that **Golgi functioning is blocked**.

On page 5, the authors say: "Subsequently UapA-GFP; labels a membranous cytoplasmic network (80-120 min) very typical of A. nidulans ER" this conclusion is not justified by the images. For "membranes" can be identified, the fluorescence is at best faint. That the fluorescent is representing "very typical of Aspergillus ER " is simply not true (e.g. no nuclear envelopes are seen, no ER at the periphery of the cell). Later the authors state further "Thus, in long hyphae, UapA is imaged to clearly label an ER-like membranous network"- this is not supported by the data shown in Figure 1.

By adding a new panel in Figure 1, showing the dynamic localization of *de novo* made over-expressed UapA-GFP we hope to have convinced referee 1 that what we detected is typical perinuclear ER rings, in addition to the cortical ER network, seen most commonly in fungi when a cargo is overexpressed. And as referee 2 also stresses, considering transporters are polytopic transmembrane proteins, they would have to be made in the ER.

End of page 5: "These observations will become more apparent later, when actin polymerization is shown to be essential for UapA trafficking." This is an odd way of highlighting a potential result, but delaying the presentation of the evidence.

We do not agree with this comment.

End of page 5: "Overall, results highlighted in Figure 1A-E, suggested that neosynthesized UapA labels the ER...", two flaws: (i) no evidence for ER localisation is given in Figure 1, (ii) Figure 1E show no results, but a graph

(i) See our comment above, after adding the image with overexpressed UapA. Text revised accordingly. (ii) We rephrased the sentence into “Results 1A-D.

Beginning Page 6: Why is the analysis of the dynamic localization of Synaptobrevin A "Most important"? Information on the experimental setup needs to be provided here; how can the GFP-vSNARE appear in endosomes before it is shown in the plasma membrane? And are these structures endosomes and Golgi vesicles?

Beginning Page 6: It is obvious that Synaptobrevin-GFP appearance differs from UapA (Figure 1F compared to 1B). However, no information on the inducible promoter is given in the text. In the figure legend, the authors refer to Material and Methods, but again I was not

successful to get an idea of which promoter was used to induce SynA-GFP. Maybe the observed localisation in vesicles is an overexpression artefact?

We removed “most important” at this point. It is «important» however, because our analysis shows clearly a very distinct picture of localization of SynA compared to UapA. In this experiment SynA is expressed *de novo* in germlings, via the *alcA*$_p$ promoter, as we did for UapA. At 60 min of expression it is apparent that SynA has not yet reached its final normal destination, the apical tip. The punctuate cytoplasmic structures observed are very probably Golgi-like compartments, but some of them might also be ERes. At later times (80-100 min), when SynA is apically localized and undergoes continuous local recycling at the collar behind the tip, some of these structures might also be endosomes, as they show a degree of motility (see Abenza et al., 2009). As all referees accept, there is a very clear difference in the structures labeled by the two cargoes; UapA labels ER-like membranes (both ER rings, when overexpressed and cortical ER), while SynA labels mostly vesicular-tubular puncta compatible with Golgi or ERes, and later some apical endosomes. The text was revised accordingly.

Page 6, middle: The authors state that they do not see a gradient of GFP-UpaA at the tip and thus lateral diffusion down the length of the hypha is unlikely. They also state that diffusion of transmembrane proteins in the plasma membrane is slow. From this they conclude "This shows that UapA localization to the PM takes place by direct short-range lateral sorting from the ER network". Again, much too farfetched in my opinion: (i) Figure 1B shows a gradient at 140 min- which is the time the lateral signals appear (arrowhead), (ii) lateral diffusion of transmembrane proteins can, indeed be fast (minutes); (iii) even if these two points are dismissed, the authors have NOT shown " direct short-range lateral sorting from the ER network" as they state.

We do not agree with comments i, ii as both are scientifically unjustified. We have given references in the text showing that transmembrane proteins diffuse slowly, not rapidly (Valdez-Taubas and Pelham, 2003; Bianchi et al, 2018). In addition, Referee 1 should consider that UapA is large dimeric protein with at least 28 transmembrane domains. Such proteins do not diffuse long distances (see especially Bianchi et al., 2018,). Some membrane proteins that are not transmembrane can diffuse faster, as t-SNARES (e.g. SsoA). In respect to seeing a gradient this also unjustified. There is no clear gradient but appearance of random cortical puncta. This is even more visible in panel 1D, where we now also added highlighting arrows. In respect to point iii, we agree that we do not have *formal* proof for direct short-range lateral sorting from the ER to the PM, but considering all data presented in this work, this is the most probable scenario. In addition, when we say “direct”, we do not exclude the involvement of an intermediate compartment, as depicted also in our model in Figure 9. We adapted the text accordingly by removing the word direct.

Page 6, second paragraph: The authors stare that Gournas et al (2010) and Karachaliou et al. (2013) "have strongly supported that internalized UapA is not recycled back to the PM after endocytosis", from which they conclude that " Thus, the subcellular localization of UapA shown in Figures 1B, 1C and 1D reflects strictly secretion of neosynthesized UapA". This is an important argument. I therefore looked into both papers, but could not find any of the cited evidence. In fact, the word recycling/recycle is only mentioned in the introduction. Did I overlook this?

In Karachaliou et al, we have shown that blocking endocytosis (Δ*end3* mutant background) or UapA ubiquitination (by knocking-out the major Ub ligase HulA/Rsp5 or the specific arrestin necessary for UapA internalization) does not influence the localization of UapA in the PM. Recycling without endocytosis cannot operate. Most importantly, in the current work, we also showed that blocking Rab5 endosomes, known to be essential for recycling of apical cargoes, did not affect UapA localization in the PM (Appendix Fig. S2).

Page 6, Fig. 1G: The authors state that the localisation results are "The result practically identical to the one obtained in the wild-type strain". I disagree here- we clearly see a septum stained in 1G (160 min), which does not show up in Fig. 1Band 1C (even after 400 minutes!). Thus, there is something else going on.

The first septum in germlings is not always formed at the same time period of germination. This is well known to people working with *A. nidulans.*

Fig. S1: Co-localisation with FM4-64 shown in Fig. S1 is not convincing- resolution too low! Also, these images show intracellular UapA dots- which were not shown and mentioned in Figure 1! Could they be Golgi vesicles?

We decided to remove this supplementary Figure as it adds nothing important to our conclusion and because we agree the resolution is low. The text of the manuscript is revised accordingly.

The localisation experiments shown in Figure 2 are good and important. The way they are shown in Figure 2 A, 2B is not very convincing, though. The green channel need to be exposed more to judge the overlay. I have redone this with the images provided. Indeed, I do not see significant correlation of red and green in the left panels (Fig. 2A, SedV; see left inserted figure), but the right panels (Fig. 2B, PHOSBP) are more concerning. Firstly, GFP-UapA suddenly localises in vesicular structures (which was categorically excluded in the previous part of the paper). Secondly, I do see some co-localisation after bringing up the green channel (see arrowheads inserted Figure in this document). I am a bit puzzled now.

We did our best to adapt the green channel for better judging the image. As far as the low co-localization of UapA-GFP with Golgi markers, as already said in the text, this is the usual 'noise' we get even we study the localization of *resident* markers of the ER and the Golgi. This is apparently due to very close proximity, in some parts of the cytoplasm, of ER and Golgi compartments.

The fluorescent SynA/Golgi marker data seem convincing. However, I would still need to know the expression strength of the promoter used to induce SynA- see point above.

See above. SynA is expressed from $alcA_p$ promoter under conditions leading to moderate expression. Notice also that the strain expressing $alcA_p$-SynA shows a growth phenotype identical to an otherwise isogenic wild-type strain.

Page 9: The author show appearance of UapA in the plasma membrane in mutants defective in Golgi-dependent secretion steps. The data are convincing, but I wonder why the investigation was done after 6-8 h of derepression? All previous results were presented in a 1-3h time window? Could it be that the promoter is not 100 tight, allowing some Golgi-based

secretion over time? If Golgi is not involved, 140-170 minutes (as shown in Fig. 1B, Fig 2A,B) should reveal the effect.

This is a valid point. In a wt background UapA is localized in the PM in less than 3 h. However, in Golgi mutants there is a delay in secretion and growth and general, which eventually will lead to unviability. This is in line with the observed more pronounced reduction of growth and swelling of the tip when SedV or GeaA are prepressed, compared to repression of HypB (see Figures 3C and 3D). However, this is already explained in the text. The critical point is that despite the severe cellular defect of cells that do not have Golgi functioning, UapA still finds its way to the PM.

Page 9: What was the time window for the test of conventional polar cargoes, such as SynA (synaptobrevin A) or ChsB (chitin synthase) in the absence of either SedV or HypB? Also 6-8h? This information is missing from the text and the figure legend.

For SynA the conditions and time periods are identical to those UapA. With ChsB, we could only follow localization of continuously expressed cargo, as we used its native constitutive promoter. In both cases, unlike the situation with UapA, Golgi functioning proved essential for localization.

Page 10: The authors state that UapA remains in an ER network in sec24 mutants. Figure 4B does NOT suggest that the fluorescent protein accumulates in the ER (no nuclear envelopes labelled- but they are labelled in Fig. 4C (ChsB-GFP). Thus, the conclusion is not justified by the data. On the other hand, a role of Sec24 in UapA secretion is clearly shown, which suggests that UapA leaves the ER in COPII vesicles (which, as the authors state clearly, normally travel to the cis-Golgi)

In this case we detect UapA mostly into punctate cytoplasmic structures that are compatible with defective ER/ERes and aggregates of UapA in the ER. When Sec24 is absent secretion stops and eventually cells die. This is also obvious from the deformation of germlings shape. We thus do not expect to see a normal appearance of the ER. Why we see a small difference in respect of the appearance of ER rings with ChsB-GFP, but not with UapA-GFP, is not clear to us, but is also an additional indication that these two cargos seem to occupy different ERes leading to different trafficking routes.

Page 11: The authors refer to Martzoukou et al., 2018, (Genetics 209: 1121) for evidence that UapA sorting to the plasma membrane depends on the clathrin heavy chain ClaH, but not on the clathrin light chain ClaL. As both chains cooperate to make the clathrin coat, I was surprised and looked into the paper for the evidence that one but not the other determines UapA sorting. I could not find any supporting evidence.

The referee is correct, the reference given is wrong. The correct one is Martzoukou et al, 2017 in elIFE. Please refer to figure 4 in this article. For the information of referee 1, many surprises have come up in respect transporter trafficking and endocytosis in Aspergillus, such as the non-involvement of ClaL in localization on the PM but its importance in endocytosis, or the non-involvement of AP-2 in clathrin mediated endocytosis of transporters, or the non-involvement of clathrin to the AP-2 dependent endocytosis of apical markers (please see our article in elIFE)

Page 11: The authors find that UapA sorting to the plasma membrane is impaired in clathrin heavy chain mutants, but not in Rab11 or AP-1 mutants. They conclude that "clathrin heavy chain is critical for the trafficking of de novo made UapA to the PM, but two key TGN-localized upstream effectors of ClaH function, AP-1 and RabE are not". From the absence of co-localization, they conclude a vesicle-independent role of clathrin in UapA sorting to the plasmas membrane. Looking at the figure 5C, left panel, I have to say that I am not at all convinced that the data shown merit this conclusion.

There is very clear non-co-localization with ClaH, and only very low co-localization with AP-1/RabE. We do understand that co-localization results suffer from a degree of noise, but under the context of results obtained in the corresponding knockout mutants, we consider them as additional evidence for our basic conclusion, Golgi bypass of the transporters.

Page 12: The authors find that F-actin is required for delivery of UapA. In the presence of the actin-drug Latrunculin, UapA is located in vesicular structures (*Figure 6A). It would be most interesting to see if these vesicular structures are co-localising with red-fluorescent Golgi markers! There is a real possibility that post-Golgi vesicles are not made (as myosin II is involved in their formation) and that UapA is now accumulating in Golgi vesicles.

There is an answer to that, coming from Fig. 6D. Lat B demolishes ERes/COPII formation. Thus, the large punctuate (and not vesicular structures; see their size at 30-50 min of LatB) of UapA, are most probably UapA aggregates.

Page 13, first paragraph: The experimental setup for visualisation of de novo made SsoA needs to be explained (which promoter, how strong compared to the SsoA promoter? Also, the authors say that SsoA behaviour "somehow resembles the picture obtained with UapA". This needs explanation

The setup for visualisation of *de novo* made SsoA is analogous to that for UapA. SsoA is repressed o/n, and derepressed to moderate levels in the next day, via expression from the regulatable promoter $alcA_p$. The $alcA_p$-SsoA strain behaves like its isogenic wt control in growth tests. When we say “resembles the picture obtained with UapA”, it means that we do not detect convincing labelling of Golgi-like structures in the course of SsoA transfer to the PM. Of course, further investigation will be needed to dissect the details of SsoA trafficking, which is beyond the scope of the present study. An explanatory note on that was added in the text.

Accepted **24th Apr 2020**

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: George Diallinas
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2019-49929

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

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

2. Captions

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

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

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

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

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	NA
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	NA
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	YES
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	YES ANOVA/ T-TEST
Is there an estimate of variation within each group of data?	YES

USEFUL LINKS FOR COMPLETING THIS FORM

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

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

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

http://datadryad.org

http://figshare.com

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

http://www.ebi.ac.uk/ega

http://biomodels.net/

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

Is the variance similar between the groups that are being statistically compared?	YES

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	NA
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

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

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NO