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Dear Editor,

We are submitting a revised version of our manuscript entitled " **Overexpression of *YPT6* restores invasive filamentous growth and secretory vesicle clustering in a *Candida albicans arl1* mutant**" for publication in Small GTPases. Please find below a point-by-point response to the reviewers' comments.

We look forward to hearing from you.

Sincerely,

Martine Bassilana

Responses to the reviewers' comments:

Thank you for your helpful comments.

Reviewer #1 (Remarks to the Author):

This concise MS reports interesting observations regarding the interplay between ARL1 and RAB6 during invasive growth of the fungal pathogen Candida albicans. That overexpression of RAB6 suppresses arl1Δ suggests that one or more effectors that are cooperatively recruited by ARL1 and RAB6 limit(s) hyphal growth or that ARL1 and RAB6 act in parallel and non-mutually compensatory pathways to sustain hyphal growth. It also shows that RAB6 affects the clustering of SEC4 secretory vesicles at the apex, which, as the authors discuss, is interesting given that RAB6 and SEC4 are present in this apical accumulation in the hyphal ascomycete Aspergillus nidulans, in which SEC4 and RAB6 ablation results in severe defects in hyphal growth (SEC4 localization and deletion in A. nidulans has been reported in Mol Biol Cell 25: 2428-2443; it might be worth adding this reference

The reference Pantazopoulou *et al.,* 2014 has been added line 123.

as well as extending the discussion on the implications of the authors' observation a little.

We have now added, lines 148-157, an alternative scenario that might explain the Arl1 and Ypt6 genetic interaction during hyphal growth and effect on secretory vesicle distribution. It is possible that alteration of secretory vesicle distribution in the *arl1* mutant is an indirect result of reduced hyphal extension rate, perhaps due to a misregulation of the GARP (Golgi-associated retrograde protein) complex. It reads now: "Alternatively, as the *arl1* deletion mutant has a reduced hyphal extension rate, it is also possible that the alteration of Sec4 distribution results indirectly from reduced growth rather than from Arl1 regulation. Hence, over-expression of Ypt6 could rescue such a defect as a result of increasing the hyphal extension rate. In such a scenario, we can imagine that the Arl1 and Ypt6 genetic interaction during retrograde vesicular transport *via* the GARP (Golgi-associated retrograde protein) complex, observed in *S. cerevisiae* [33-35], is critical specifically for hyphal growth, perhaps for lipid homeostasis [36]. Further characterization of the *arl1* and ypt6 mutants will be necessary to define the specific roles of these small GTPases during hyphal growth".

I have a couple of minor points:

YPT6 gain-of-function mutant (presumably by impaired GTP hydrolysis). To me it is not unexpected that Q71L is a less efficient suppressor than the wt. Langemeyer et al (2014) eLife 3: e01623 discuss that mutations that interfere with GTP hydrolysis in RAS do not translate directly into the same phenotype in RABs.

We agree with this comment that it is not unexpected that the Ypt6[Q71L] mutant is less efficient than Ypt6 in rescuing the *arl1* mutant defect. In addition to the suggestion that GTP-GDP cycling of Ypt6 is critical for the Rab GTPase function, we have now added on lines 72-74: "Alternatively, it is possible that the reduced efficiency of Ypt6[Q71L], compared to Ypt6, to restore invasive growth is due to sub-optimal activation by a GEF [26]."

Statistical analysis in Fig 1E. What test was used to calculate P values?

This was done using a Student *t* test and it is now indicated in the legend of Figure 1, line 224.

Were authors using corrections for multiple comparisons? Are all data from the 3 experiments pooled to calculate means and SD? (if bars are constructed with only three points a scatter plot rather than a bar diagram is appropriate).

Yes, all data were pooled, *i.e.* these data represent the average hyphal length from a total of 200-400 cells each strain, from 3 experiments. This is now clarified in the legend of Figure 1, lines 223-224.

Statistical analysis in Fig 2F: as for 1E (in this case P values are not given, please add them).

Thank you for pointing out this omission. This is now indicated in the legend of Figure 2, lines 246-249. P values are: *arl1 vs* WT: 0.0004*, arl1 + ARL1 vs arl1*: 0.0028 and *arl1 + YPT6 vs arl1*: 0.0042; no statistically significant difference was observed between the values for WT, *arl1 + ARL1* and *arl1 + YPT6*."

It will be interesting to colocalize RAB6 with an early Golgi marker.

We agree with the referee's comment that it would be interesting to colocalize Ypt6 with an early Golgi marker, such as Vrg4. However, we feel that such analyses are beyond the scope of this brief report, which focuses mainly on Arl1 and its genetic interactions with Ypt6. In-depth characterization of Ypt6 in *C. albicans* is currently underway in the laboratory and is part of a work in preparation.

Title: it seems to me that secretory vesicles clustering should be secretory vesicle clustering (vesicle in singular, as an adjective)

Yes, this is absolutely right, this mistake is now corrected. Thank you.

Reviewer #2 (Remarks to the Author):

The manuscript by Wakade et al. is an excellent study addressing the mechanisms by which small G protein regulators of membrane traffic control hyphal growth in the pathogenic yeast Candida albicans. This laboratory has recently demonstrated that the Arf-like protein Arl1 is involved in hyphal growth and invasiveness of C. elegans. Wadade et al. in this manuscript the show that the invasive growth defect of an arl/arl mutant of C. albicans is rescued by overexpression of Ypt6. They also show that Ypt6 is itself important for invasive growth, as a ypt6/ypt6 mutant showed defects in invasive growth. However, Arf1 overexpression did not rescue this growth defect. The reduced length of hyphae in the arl/arl mutant was also rescued by overexpression of Ypt6, but only partially.

Localization of the secretory vesicle Rab protein Sec4 was strikingly altered in an arl/arl mutant, with a less tight localization to the tip, the site of polarized growth. Quantification revealed that the arl/arl mutant had a significantly higher level of non-clustered secretory vesicles compared to wild type, a phenotype rescued by overexpression of Ypt6. These results support the conclusion that Ypt6, the C. elegans orthologue of Rab6, works with Arl1 to mediate polarized delivery of secretory vesicles to the growing tip of hyphae in the pathogenic yeast C. elegans. I have only a few minor comments and questions.

Detailed comments:

1. Does ypt6/ypt6 also have a hyphal growth defect?

Yes, deletion of *YPT6* results in a mutant that has a reduced filament extension rate, as stated lines 137-139 (in preparation). Indeed, we observed that the *ypt6*/*ypt6* mutant had significantly shorter hyphae (average hyphal length of 15 ± 3.5 µm, compared to 22 ± 3 µm for the complemented strain, *n* = 100 cells). This is part of a work in preparation.

2. When it is first used, could the authors define "yemCherry" (is it "yeast enhanced monomeric Cherry?).

Yes, yemCherry is for yeast enhanced monomeric Cherry, and this is now stated in the Materials and Methods section, lines 183-184.