Cdc42 couples T cell receptor endocytosis to GRAF1-mediated tubular invaginations of the plasma membrane

Point-by-point response to referees' comments

Figure and line numbering refer to the revised version of the manuscript. Changes in the manuscript are highlighted in yellow.

Reviewer 1

The lack of effect of the expression of the dominant negative CDC42 mutant is quite surprising. The explanation of the authors is not convincing as, independently of its cytosolic localization, the mutant has been demonstrated to be a dominant negative mutant (as it is the case for many other small GTPase dominant negative mutants that are mainly cytosolic but are dominant negative probably because they sequester effectors in the cytoplasm and/or are not able to stimulate them to act). I understand that it is not easy to design experiments to establish why this mutant in this pathway does not exert its inhibitory function but the authors should at least try to discuss more deeply the issue. One possible explanation could be the presence of other small GTPases that make up for the function of Cdc42..

We agree with the reviewer, the lack of effect of this mutant is surprising and we have not been able to design experiments to address this specific question.

However, we want to point out that none of the work previously done on the GRAF1-mediated endocytic route has reported an effect of Cdc42-T17N on this pathway. Only Cdc42-Q61L has been shown to stall the formation of GRAF1-positive endocytic vesicles. Francis et al. did not observe any consequence of Cdc42-T17N expression on the formation of tubules or dextran uptake [1]. Similarly, Lundark et al report that only Cdc42-Q61L has an effect on tubule formation [2]. Moreover, we could find only two examples of a negative effect of Cdc42-T17N on the CLIC/GEEC pathway in the literature [3,4]. Of note, reference [4] reports a decrease of dextran endocytosis upon Cdc42-T17N expression, but no effect of Cdc42-Q61L, illustrating that the effect of these mutants can be cell or cargo specific.

We also want to mention that we used Cdc42-Q61L primarily to stall GRAF1-positive endocytic invaginations, in order to investigate if TCR endocytosis would be related to this pathway. Indeed, the contribution of Cdc42 to this pathway seems very complex and remains to be fully elucidated, but this was not the purpose of the submitted manuscript.

It seems that the effect of Cdc42-Q61L on the GRAF1 pathway is less due to the constitutive activity of Cdc42 than to the fact that the Q61L mutation forces GRAF1 to interact with Cdc42 indefinitely [1]. As stated in a detailed study, "*GRAF1-mediated inactivation of Cdc42 is not vital for the formation and budding of endocytic carriers, but for their further maturation*" [1]. In short, because of the Q61L mutation, both GRAF1

and Cdc42 remain bound to the membrane and keep promoting its tubulation inside the cell, when this step is normally very transient and is associated with the detachment of both GRAF1 and Cdc42 from the membrane after initiation of the invagination.

As suggested by the reviewer, it is very likely than another GTPase takes on this short step in cells expressing Cdc42T17N, which would explain why it has never been reported to have any effect on the GRAF1-mediated endocytic route. Following the reviewer's suggestion, we have discussed this point in greater length in the revised version of our manuscript (lines 402-410)

Minor points

In the conclusions the authors talk about the CLIC/GRAF1 pathway: shouldn't it be CLIC/GEEC?

We agree with the reviewer, this sentence was confusing. We wanted to refer to GRAF1 contribution only in the context of the endocytic part of this pathway, the CLathrin-Independent Carrier (CLIC), and not to the intracellular component, the GPI-Enriched Endocytic Compartments (GEEC). We have changed the text to avoid any confusion. It now reads: "…the CLIC endocytic pathway regulated by the small GTPase Cdc42 and the BAR-domain containing protein GRAF1..." (line 489)

There should be a separation between Figure 1 legend and the main text (lines 269-270)

The page layout is not final, and has changed after we submitted the manuscript. Nevertheless, we have added a space to facilitate the reading of the revised manuscript.

"doted" should be "dotted" (line 318).

We thank reviewer for spotting this typo, which we have now corrected (line 327).

References

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3. Gauthier, N.C.; Monzo, P.; Kaddai, V.; Doye, A.; Ricci, V.; Boquet, P. *Helicobacter pylori* VacA Cytotoxin: A Probe for a Clathrin-independent and Cdc42-dependent Pinocytic Pathway Routed to Late Endosomes. *Mol. Biol. Cell* **2005**, *16*, 4852–4866.

4. Sabharanjak, S.; Sharma, P.; Parton, R.G.; Mayor, S. GPI-anchored proteins are delivered to recycling endosomes via a distinct cdc42-regulated, clathrin-independent pinocytic pathway. *Dev. Cell* **2002**, *2*, 411–23.