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 2

This reviewer agree with most part of this manuscript but still have some concerns. Several concerns.

Spelling. I think "internalization" would be suitable instead of "internalization" used in this manuscript. Am I wrong?

"Internalisation", like "organisation", or "signalling" is UK English spelling, which we prefer to use over the US spelling ("Internalization", "organization", or "signaling")

Line 241

"Expressing a mutant of GRAF1 containing the membrane binding BAR and PH domains but lacking the SH3 and GAP domains". Is this mutant of GRAF1 is a constitutively active form or dominant negative form or non-functional form? Please explain.

This mutant, as described in the text, lacks the SH3 and GAP domains and consists essentially of the membrane binding BAR and PH domains [1]. It has been shown to promote the formation of tubules [1], and we used it in this context. It has been hypothesised that this mutant could potentially act as a dominant negative GRAF1, but never formally demonstrated, this is why we do not mention it as such.

Line 246

What does mean "GRAF1-BAR-PH"? There are no explanation. I assume that GRAF1 containing the membrane binding BAR and PH domains but lacking the SH3 and GAP domains (GRAF1-BAR-PH).

The reviewer is twice correct: 1) we mean the mutant of GRAF1 lacking the SH3 and GAP domains described above. And 2), we have indeed forgotten to define this abbreviation. We have revised the text accordingly (line 247).

In Figure 1 D What do mean yellow arrow and endogenous GRAF1-488?. GRFA1 which contains aa1-488, or endogenous GRAF1 was detected using Alexa 488?

We indeed mean endogenous GRAF1 detected with a primary antibody against GRAF1 and a secondary antibody labelled with Alexa-488. We agree with the reviewer, this labelling was not very clear. We have removed the reference to 488 to avoid any confusion. The arrows indicate tubules and we had indeed forgotten to describe them in the figure legend. This has now been corrected (line 274).

Line 298. They found that only 3% ± 7% of GRAF1-positive tubules to be positive for Alexa546-Tf and only 11% ± 14% (activated cells) and 0% (resting cells) of the GRAF1-tubules were positive for the T cell-specific adaptor protein Lat in cells expressing Cdc42-Q61L and GFP-GRAF1.

How do authors conclude that "this result suggests that Lat endocytosis is not mediated by Cdc42 and therefore is distinct from that of TCR"? I cannot well catch their logic how authors conclude "not mediated by Cdc42".

The hypothesis that we want to express in the submitted manuscript is that Cdc42 and GRAF1 contribute to the endocytosis of TCR. Accordingly, we show TCR is in tubules induced by Cdc42-Q61L. We also show that transferrin, whose internalisation pathway is distinct from TCR and not mediated by Cdc42, is not in these tubules. Finally, we show that Lat, whose internalisation pathway remains unknown, is not in the Cdc42-Q61L-induced tubules, like transferrin. For us, this suggests that Lat, like transferrin, does not use a Cdc42-mediated endocytic route, unlike TCR. We have amended the main text to make this point easier to understand (lines 304-308)

Lien 388. They said that Cdc42-T17N is a dominant negative mutant, however, did not influence TCR 388 endocytosis when compared to controls. I think it is necessary to check loss of CdC42. I wonder what happens if authors use knock-down instead of Dominant Negative form?

We used siRNAs against Cdc42 to silence its expression in Jurkat T cells. Unfortunately, we could never get a higher downregulation than 62% (see Figure R1 in this document). T cells express very high levels of Cdc42 and consequently the 38% remaining still represented a high amount of available Cdc42. Accordingly, we could not measure any significant difference in TCR uptake between control cells and cells in which Cdc42 had been silenced (see Figure R1).

We have nevertheless discussed this point in greater length in the revised version of the manuscript (lines 402-410).

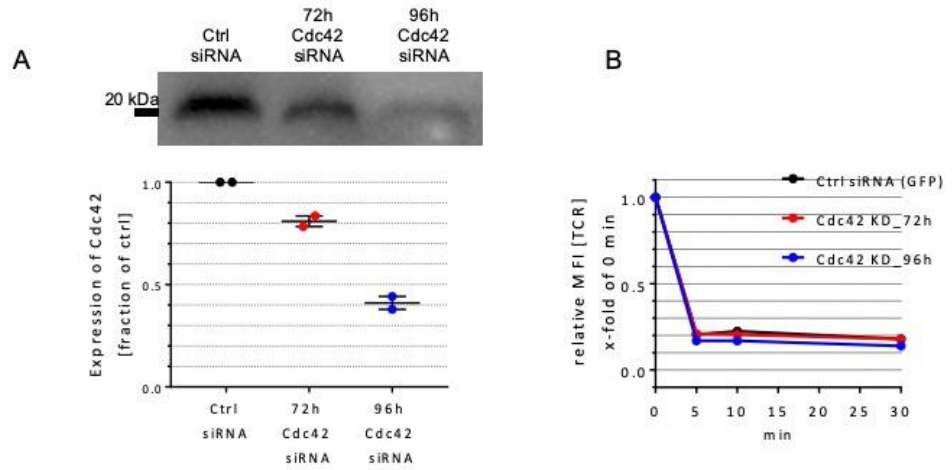


Figure R1. A 40% downregulation of Cdc42 has no impact on TCR uptake in activated Jurkat T cells. **A.** Representative image and quantification of western blots (n = 2) of cell lysates made from Jurkat

T cells electroporated with siRNA against GFP (control) or against Cdc42 after 72h or 96h. **B**. Flow cytometry-based uptake measurements of the Jurkat T cells treated as in A.

In Figure 5. A. Left panel is probably remaining TCR-CD3 detected by an antibody against TCRβ454 at the cell surface at the indicated time points. How about right panel? Is total surface TCR-CD3 assumed from Fig.4 or not? Please explain and label the panel.

We thank the reviewer for having spotted that the figure legend of Figure 5 was incomplete. We have re-labelled the figure panels, completed the figure legend and added a line in the main text to refer better to this panel (lines 446-447).

In supplemental Figure S1. What does mean GRAF1-647?

Same as previously, it means endogenous GRAF1 detected with a primary antibody against GRAF1 and a secondary antibody labelled with Alexa-647. We have removed the reference to 647 to avoid any confusion

Minor points. Line 76. "CD44, two should be "CD44, two".

The reviewer is correct; there was a mistake with the font size, which we have now corrected (line 76).

Line 269 and 270 and line 420 and 421. Between these two lines, please make one space.

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

Reference

1. Lundmark, R.; Doherty, G.J.; Howes, M.T.; Cortese, K.; Vallis, Y.; Parton, R.G.; McMahon, H.T. The GTPase-activating protein GRAF1 regulates the CLIC/GEEC endocytic pathway. *Curr. Biol.* **2008**, *18*, 1802–8.