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Rab22a controls MHC-I intracellular trafficking and antigen cross-presentation by dendritic cells

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 22 April 2016

Thank you for the submission of your research manuscript to EMBO reports. We have now received reports from the three referees that were asked to evaluate your study, which can be found at the end of this email.

As you will see, all three referees acknowledge the potential interest of the findings. However, all three referees have raised a number of concerns and suggestions to improve the manuscript or to strengthen the data. Although EMBO reports focuses on novel functional, rather than detailed mechanistic insight, in this case more data on the molecular role of Rab22a (referee #3) in the described processes and on the fate of MHC-I (all referees - especially point 2 by referee #1 and point 2 by referee #2) is required. Also the use of primary DCs as suggested by referee #2, the concerns of referee #1 regarding the experimental procedure used for the data in Fig. 2E are of major importance. Finally, better and higher resolution IF data (referee #3) might be very helpful.

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that all referee concerns (as detailed in their reports) must be fully addressed in 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.

Supplementary/additional data: The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf labeled Appendix. The Appendix includes a table of content on the first page, all figures and their legends. Please follow the nomenclature Appendix Figure Sx throughout the text and also label the figures according to this nomenclature. For more details please refer to our guide to authors.

Important: All materials and methods should be included in the main manuscript file.

Regarding data quantification and statistics, can you please specify the number "n" for how many experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends? This information must be provided in the figure legends. Please also include scale bars in all microscopy images.

We now strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure or per figure panel.

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.

REFEREE REPORTS

Referee #1:

In this manuscript the authors suggest that Rab22a controls MHC-I trafficking through the endocytic system and impacts antigen cross-presentation. They show that Rab22a localizes to late endosomes and phagosomes, that knockdown of Rab22a in a DC-like cell line results in loss of perinuclear distribution of H2-Kb and impacts cross-presentation in this cell line and in BMDCs. They also claim that loss of Rab22a impairs recruitment of Kb to phagosomes. They demonstrate the impact on cross-presentation using two systems: 1) Soluble OVA or OVA-coated beads; 2) T.gondii expressing OVA.

The manuscript convincingly and elegantly shows that knockdown of Rab22a impacts antigen cross-presentation. However, there are major issues with the interpretation as well as some of the experiments presented to support the interpretation. The authors suggest that the effect on cross-presentation is a result of the loss of H2-Kb from phagosomes. This inference is based on a single experiment where they show by FACS that loss of Rab22a impacts the recruitment (Fig. 2E). The experimental procedure used is not clear, and I suspect there is something wrong with the description under 'FACS Analysis' in the Materials and Methods section. There is no mention there of phagosome purification at the different time points indicated in Fig. 2E: the description implies that cells were incubated in a lysis buffer containing NP40 overnight after which the samples were stained with respective antibodies. I suspect that the authors are referring to purified phagosomes here, but even then treatment with NP40 without fixation will solubilize membranes and remove any associated H2-Kb. If they do mean cells, then were the cells were fixed prior to the incubation with lysis buffer and if so how were the phagosomes then isolated? The established protocol in the field involves lysis of cells in a hypotonic buffer prior to phagosome isolation, as they describe earlier in the Materials and Methods section, followed by fixation prior to treatment with a mild detergent and immunostaining. If Fig. 2E does refer to purified phagosomes this should be clarified, and in

addition the authors should show the raw data for the FACS, including the gating on the bead population, and an isotype control should be included.

The authors suggest that elimination of cross-presentation is a result of loss of H2-Kb in endocytic compartments and elimination of its trafficking through phagosomes. Unlike well-studied Rabs, such as Rab5, Rab7, and Rab11, the functional role of Rab22a remains incompletely understood, especially in the context of immune cells and pathogens. Although it has been suggested to play a role in the trafficking of MHC-I through the recycling compartments, there is little direct evidence supporting this in immune cells. However, it has been shown that Rab22 impacts phagosomal maturation, sharing this property with Rab5: (Higher order Rab programming in phagolysosome biogenesis", JCB Vol 174(7), 2006, 923-929). Therefore, the data can also be interpreted in this context. It could be argued that elimination of Rab22a accelerates phagosomal/endosomal maturation, resulting in faster degradation of phagocytosed/endocytosed OVA as well as any recycling Kb that might be present. This could explain both the loss of phagosomal and perinuclear Kb and decreased cross-presentation. Additional data presented is also consistent with this hypothesis. For example, although the blot presented in Fig 4D is not quantitated, it appears that OVA levels are lower upon Rab22a knockdown, supporting the idea that this accelerates OVA degradation. It is well established that antigen cross-presentation greatly depends on the slow maturation of phagosomes, and the authors might want to test this alternative hypothesis or provide more convincing data refuting it.

Minor comments:

The authors should also test the maturation status of BMDC following knockdown of Rab22a. Mature DCs are known to be impaired in their ability to cross-present.

Also, the quality of the Rab22a blot in Fig. EV1B is unacceptable and it should be repeated.

Referee #2:

Cebrian and colleagues examine the role of the small GTPase Rab22 in cross-presentation by bone marrow-derived dendritic cells. They find that Rab22 knockdown compromises cross-presentation of both soluble and particulate antigens as well of a cytosolic antigen secreted by an intracellular parasite. This is presumably due to the requirement of Rab22 for formation of an intracellular pool of MHC class I molecules. Rab22 is not required for endogenous MHC class I presentation or for normal formation of the TGN and Golgi compartments. The experiments are well done and described and the conclusions are based on sound evidence. Although most results are not surprising given the recent report by Nair-Gupta et al on the role of MHC class I molecules in a Rab11+ compartment in cross-presentation, the present study is important since the roles and localization of Rab11 and Rab22 are non-overlapping. Moreover, Rab22 has been reported to be required for constitution of a juxtanuclear recycling compartment while this is presumably not the role of Rab11. Although extensive mechanistic understanding is not required for this journal, the authors should address two points, one of them to corroborate key findings in primary cells, and a second to address a mechanistic issue.

The key claim of the paper is a critical role of Rab22 in cross-presentation, which is demonstrated unambiguously. Rab22 has previously been shown to be implicated in recycling of class I molecules, however this role had not been studied in DCs, i.e. the cells in which class I recycling is likely to be functionally more relevant than in other cell types studied previously. MHC class I trafficking in dendritic cells and in cross-presentation is a poorly understood issue, so that the results of this paper will be of interest to a wider community of immunologists interested in antigen presentation.

Comments in detail:
- Key experiments studying the intracellular localization of Rab22 and its role in MHC class distribution (Fig. 1 and 2C,D,E) are performed in the Jaw cell line stably transformed by a lentivirus. However in this line Rab22 expression is reduced by only 60%, while an almost complete knockdown is obtained in primary BM-DCs. In order to rule out some oddity of the Jaw line, it is critical to confirm the findings obtained in Jaw, including the surprising co-localization of Rab22

and Lamp1, using primary DCs. The authors might also want to discuss the latter finding.
- In model lines such as HeLa or CHO, Rab22 mainly affects slow recycling of MHC class I molecules, while the fast recycling pathway is not affected. MHC class I molecules can use both fast (Rab35-dependent) and slow recycling pathways The authors should examine recycling of the transferrin receptor to check whether fast recycling is modified by Rab22 knockdown.
- The final paragraph discusses issues and findings that are not relevant in the context of this study, this paragraph can be eliminated.

Referee #3:

This brief manuscript reports the potentially interesting finding that Rab22a plays a significant role in the cross presentation of exogenous antigens on MHCI in dendritic cells (DCs). The authors show evidence that a partial knockdown of Rab22a (only 40-50%) results in the loss of an intracellular pool of MHCI, its resulting failure to be recruited to phagosomes, and a diminution of peptide-MHCI presentation to T cells. What we do not learn is very much about why this all happens, and this absence of any mechanistic insight is the paper's weakness. Even the significant loss of intracellular MHCI goes without much characterization, presumably it is degraded? Such a fate would be consistent with the colocalization studies that, in my opinion, show the best localization of Rab22a to lysosomes rather than other endosomal compartments (the authors' interpretation to the contrary not withstanding). The limited resolution of the IF images shown precludes very many other conclusions, none of which anyway would inform the issue of mechanism.

Apart from the overinterpreted IF, the paper is fine as far as it goes, but unfortunately, it does not go very far to letting the reader know "why" Rab22a is important. Simply showing that it may does not create a paper that will be of wide interest.

1st Revision - authors' response 22 July 2016

We thank you for your letter and we are glad to receive such constructive critics from the referees. We acknowledge the concerns raised up by them, and we have fully addressed these remarks in a point-by-point response, as it is detailed in the other letter. We consider that the experimental advance achieved during this three months of work has significantly strengthen our study by:

i) Providing a deeper insight on the molecular bases of MHC-I intracellular transport in dendritic cells, and particularly in better understanding the role of Rab22a during this process (requested by all referees, especially point 2 by referee #1 and point 2 by referee #2).

ii) Confirming key results from our study in primary BMDCs (point 1 by referee #2).

iii) Clarifying experimental approaches used in our manuscript (point 1 by referee #1) and improving the quality of IF images in the new uploaded high resolution figures (requested by referee #3).

We have also specified in the respective figure legends: the number "n" corresponding to the different experiments performed, the bars and error bars, and the tests used to calculate the p-values. As requested, we have also included scale bars in all the microscopy images shown.

The identification of molecular effectors controlling the transport of MHC-I molecules from the endocytic recycling center to dendritic cell phagosomes to allow cross-presentation is only started to be explored [1]. Moreover, we would like to call to the editor’s attention the fact that the last issue of Immunological Reviews is entirely dedicated to antigen presentation. Some of the articles published there highlight the potential role of recycling compartments in regulating MHC-I intracellular trafficking during antigen cross-presentation by dendritic cells [2,3], illustrating that this topic is of high interest and timely. We consider that our detailed study on the GTPase Rab22a has a lot to add to this field of research. Another asset of our work is to shed light on how cross-presentation is achieved for antigens present in compartments with very little inter-connection with the endocytic pathway, such as the parasitophorous vacuole (PV) of *Toxoplasma gondii*. In this report, we show that Rab22a regulates the intracellular distribution of MHC-I molecules in dendritic

cells. Silencing the expression of this GTPase causes the disappearance of the perinuclear pool of MHC-I, inhibits the recycling capacity of these molecules and profoundly alters the cross-presentation of soluble and particulate antigens. Interestingly, endogenous Rab22a was recruited to the PV upon active infections of *T. gondii* and the knock-down of this GTPase impaired cross-presentation of parasite-associated antigens. It is worth noticing that this is the first report of an endogenous Rab protein associated to the PV of this microorganism.

In summary, cross-presentation is a key event in the cytotoxic immune response to numerous intracellular parasites and tumor cells. Identification of Rab22a as a key factor that controls the intracellular transport of MHC-I molecules and that is necessary selectively for cross-presentation of endocytosed and *T. gondii*-associated antigens will be of interest for a large audience of cell biologists, immunologists and microbiologists.

For all this, we feel that it is scientifically justified to resubmit the manuscript "Rab22a controls the intracellular trafficking of MHC class I molecules and antigen cross-presentation by dendritic cells" to be revised and considered for publication in EMBO Reports as a Scientific Report.

Authors declare not having any conflicting financial interest.

References

1. Nair-Gupta P, Baccarini A, Tung N, Seyffer F, Florey O, Huang Y, Banerjee M, Overholtzer M, Roche PA, Tampe R, et al. (2014) TLR signals induce phagosomal MHC-I delivery from the endosomal recycling compartment to allow cross-presentation. *Cell* **158:** 506-21
2. van Endert P (2016) Intracellular recycling and cross-presentation by MHC class I molecules. *Immunol Rev* **272:** 80-96
3. Blander JM (2016) The comings and goings of MHC class I molecules herald a new dawn in cross-presentation. *Immunol Rev* **272:** 65-79

Referee #1:

In this manuscript the authors suggest that Rab22a controls MHC-I trafficking through the endocytic system and impacts antigen cross-presentation. They show that Rab22a localizes to late endosomes and phagosomes, that knockdown of Rab22a in a DC-like cell line results in loss of perinuclear distribution of H2-Kb and impacts cross-presentation in this cell line and in BMDCs. They also claim that loss of Rab22a impairs recruitment of Kb to phagosomes. They demonstrate the impact on cross-presentation using two systems: 1) Soluble OVA or OVA-coated beads; 2) T. gondii-expressing OVA.

The manuscript convincingly and elegantly shows that knockdown of Rab22a impacts antigen cross-presentation. However, there are major issues with the interpretation as well as some of the experiments presented to support the interpretation. The authors suggest that the effect on cross-presentation is a result of the loss of H2-Kb from phagosomes. This inference is based on a single experiment where they show by FACS that loss of Rab22a impacts the recruitment (Fig. 2E). The experimental procedure used is not clear, and I suspect there is something wrong with the description under 'FACS Analysis' in the Materials and Methods section. There is no mention there of phagosome purification at the different time points indicated in Fig. 2E: the description implies that cells were incubated in a lysis buffer containing NP40 overnight after which the samples were stained with respective antibodies. I suspect that the authors are referring to purified phagosomes here, but even then treatment with NP40 without fixation will solubilize membranes and remove any associated H2-Kb. If they do mean cells, then were the cells were fixed prior to the incubation with lysis buffer and if so how were the phagosomes then isolated? The established protocol in the field involves lysis of cells in a hypotonic buffer prior to phagosome isolation, as they describe earlier in the Materials and Methods section, followed by fixation prior to treatment with a mild detergent and immunostaining. If Fig. 2E does refer to purified phagosomes this should be clarified, and in addition the authors should show the raw data for the FACS, including the gating on the bead population, and an isotype control should be included.

Response: We thank the referee for the very constructive and relevant critics, and also for the nice comments on the manuscript. As he/she points out, there was a mistake in the description of the

experimental procedure in the Materials and Methods section. We apologize for this error and we have corrected it in this new version of the manuscript. We have confused the protocol's description because we have in our lab two different experimental procedures to study phagosome populations by FACS analysis: one protocol to study the acquisition/recruitment of different markers to DC phagosomes, and the other to study phagosomal OVA degradation. The first one involves the use of a hypotonic buffer (PBS 8% sucrose, 3 mM imidazole, 1 mM DTT and 1 X protease inhibitor cocktail) to disrupt the cells with a syringe and a 22G needle, as we do for phagosome purification. Afterwards, samples are fixed with 1% PBS/PFA and labeled ON at 4°C with anti-Rab22a or anti-H-2K^b antibodies. The second protocol does not involve any hypotonic buffer or cellular disruption, but we do use a lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% NP-40, 1 mM DTT and 1 X protease inhibitor cocktail) ON at 4°C. Since in this new version of our manuscript we have also performed phagosomal degradation assays, we have included both protocols. The referee will find a detailed description of them in the Materials and Methods sections (second paragraph of FACS analysis). As the referee requested, we have also included in our manuscript the raw data from the FACS analysis. The gating on a particular FSC/SSC region corresponding to a single beads population (red circle) is shown in Fig EV2E. For the analysis, we acquired more than ten thousands phagosomes of each sample. In Fig EV2F, the fluorescent intensity profiles of H-2K^b staining obtained in Scramble and Rab22a KD DC phagosomes from one representative of the three independent experiments performed are shown. We also aimed to confirm this result in primary transduced BMDCs. Because it is a technically challenging assay to perform (large amounts of cells are needed to obtain enough phagosomes), we only analyzed phagosomes after 3 hours of internalization, since this represent the time point where the biggest differences are observed with JAWS-II DC isolated phagosomes (Fig 2E). Again, ten thousand phagosomes were analyzed from Rab22a KD and Scramble BMDCs of each condition; isotype control and anti H-2K^b antibodies (H-2K^b staining was performed in triplicates). We performed two independent experiments and twice we observed a significant impairment of MHC-I phagosomal recruitment in BMDC phagosomes, as compared to Scramble BMDCs (Fig EV2G). We also tried to further confirm this result by a second experimental approach (phagosome purification and Western blot analysis), but unfortunately none of the anti-MHC-I antibodies available in our lab is suited for Western blot and although we tested them, they didn't work.

The authors suggest that elimination of cross-presentation is a result of loss of H2-Kb in endocytic compartments and elimination of its trafficking through phagosomes. Unlike well-studied Rabs, such as Rab5, Rab7, and Rab11, the functional role of Rab22a remains incompletely understood, especially in the context of immune cells and pathogens. Although it has been suggested to play a role in the trafficking of MHC-I through the recycling compartments, there is little direct evidence supporting this in immune cells. However, it has been shown that Rab22 impacts phagosomal maturation, sharing this property with Rab5: (Higher order Rab programming in phagolysosome biogenesis", JCB Vol 174(7), 2006, 923-929). Therefore, the data can also be interpreted in this context. It could be argued that elimination of Rab22a accelerates phagosomal/endosomal maturation, resulting in faster degradation of phagocytosed/endocytosed OVA as well as any recycling Kb that might be present. This could explain both the loss of phagosomal and perinuclear Kb and decreased cross-presentation. Additional data presented is also consistent with this hypothesis. For example, although the blot presented in Fig 4D is not quantitated, it appears that OVA levels are lower upon Rab22a knockdown, supporting the idea that this accelerates OVA degradation. It is well established that antigen cross-presentation greatly depends on the slow maturation of phagosomes, and the authors might want to test this alternative hypothesis or provide more convincing data refuting it.

Response: This is a very important point and we have decided: i) to investigate the role of Rab22a in the recycling of MHC-I molecules in DCs, and ii) to study the role of Rab22a in phagosomal maturation in DCs. To better understand the role of Rab22a in MHC-I molecules recycling in cells of the immune system, particularly in DCs, we have performed recycling assays by FACS analysis, as explained in the Materials and Methods section. Interestingly, we observed a significant reduction in the ability of MHC-I molecules recycling (Fig 2F and Fig EV2H), but not of TfR recycling (Fig 2G and Fig EV2I), in Rab22a KD DCs, as compared to Scramble DCs. This confirms previous results obtained in different cell types by others [1,2], and suggests a specific role for Rab22a in MHC-I molecules intracellular trafficking. On the other hand, to study phagosomal maturation in Rab22a KD DCs, we decided to assess phagosomal antigen degradation by using OVA-coated beads and FACS analysis. For this, Scramble and Rab22a KD DCs were incubated with 3 μm LB (pulse +

chase), as indicated in the Materials and Methods section, and after phagosomal isolation beads were stained for OVA. The amount of intact and degraded OVA was quantified, and we observed not significant differences between both DC types (Fig 3F and Fig EV3B), suggesting that phagosomal maturation is not altered in Rab22a KD DC phagosomes. As the referee indicates, it has been reported that Rab22a and Rab5 play an important role in regulating phagosomal maturation [3]. But the experiments of this study were conducted with *Mycobacterium tuberculosis*-containing phagosomes in macrophages, a model quite different from LB phagosomes in DCs. As it was also noted by the referee, DCs possess unique endocytic specializations and their phagosomes mature very slow since they need to preserve important antigenic peptides to achieve antigen presentation efficiently. It will be interesting to address in future studies if the activity of Rab22a regulates DC phagosomal maturation after *M. tuberculosis* or *T. gondii* infection, two pathogens that are internalized by different means. *M. tuberculosis* is internalized by receptor-mediated phagocytosis, and *T. gondii* actively invades the host cell and survives inside of a highly specialized parasitophorous vacuole. In any case, both microorganisms induce strong alterations in the maturation of the phagosome/vacuole.

Minor comments:
The authors should also test the maturation status of BMDC following knockdown of Rab22a. Mature DCs are known to be impaired in their ability to cross-present.

Response: Following the reviewer's advice, we decided to study the activation/maturation capacity of BMDCs after the silencing of Rab22a by treating them with 10 µg/ml of LPS during 20 hours. After this treatment, BMDCs were collected and labeled for CD11c, CD80, MHC-II (IA^b) and CD86 on ice. Then, we analyzed by FACS the MFI of CD80, IA^b and CD86 cell surface expression on CD11c positive cells. As it is shown in Fig EV3C, the percentages of CD11c positive cells were similar in Scramble and Rab22a KD BMDCs (left panels), and we confirmed the immature status of our BMDCs since all the maturation markers analyzed increased after LPS treatment. No significant difference of maturation was observed between Scramble and Rab22a KD BMDCs. MFI values are specified beside the legends in the FACS profiles.

Also, the quality of the Rab22a blot in Fig. EV1B is unacceptable and it should be repeated.
Response: As the referee suggested, we changed the Western blot image of Fig EV1B for a different one where the quality of the Rab22a blot is better. Here, we also included the phagosomal maturation marker Lamp1, that although is not a blot of high quality, it is an important control of phagosome purification. The kinetic of early Rab22a recruitment to JAWS-II DC phagosomes was further confirmed by FACS analysis (Fig EV1C).

References

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3. Roberts EA, Chua J, Kyei GB, Deretic V (2006) Higher order Rab programming in phagolysosome biogenesis. *J Cell Biol* **174:** 923-9

Referee #2:

Cebrian and colleagues examine the role of the small GTPase Rab22 in cross-presentation by bone marrow-derived dendritic cells. They find that Rab22 knockdown compromises cross-presentation of both soluble and particulate antigens as well of a cytosolic antigen secreted by an intracellular parasite. This is presumably due to the requirement of Rab22 for formation of an intracellular pool of MHC class I molecules. Rab22 is not required for endogenous MHC class I presentation or for normal formation of the TGN and Golgi compartments. The experiments are well done and described and the conclusions are based on sound evidence. Although most results are not surprising given the recent report by Nair-Gupta et al on the role of MHC class I molecules in a Rab11+ compartment in cross-presentation, the present study is important since the roles and localization of Rab11 and Rab22 are non-overlapping. Moreover, Rab22 has been reported to be required for constitution of a juxtanuclear recycling compartment while this is presumably not the

role of Rab11. Although extensive mechanistic understanding is not required for this journal, the authors should address two points, one of them to corroborate key findings in primary cells, and a second to address a mechanistic issue.

The key claim of the paper is a critical role of Rab22 in cross-presentation, which is demonstrated unambiguously. Rab22 has previously been shown to be implicated in recycling of class I molecules, however this role had not been studied in DCs, i.e. the cells in which class I recycling is likely to be functionally more relevant than in other cell types studied previously. MHC class I trafficking in dendritic cells and in cross-presentation is a poorly understood issue, so that the results of this paper will be of interest to a wider community of immunologists interested in antigen presentation.
Response: We thank the referee for the comments and critics. Before answering all the comments in detail, I would like to mention that indeed, some preliminary data from our lab not included in this manuscript, indicate that Rab11a and Rab22a play non-overlapping roles and locations in the endocytic pathway of DCs. After 1 hour of phagocytosis of 3 µm LB by DCs, we have observed three different populations of phagosomes by IF staining and confocal microscopy: Rab11a-positive/Rab22a-negative, Rab11a-negative/Rab22a-positive and Rab11a/Rab22a-positive. Although this is just one result, it goes in the same direction as the referee discusses. Moreover, the role of recycling compartments in MHC-I trafficking during antigen cross-presentation is only starting to be explored, but it is gaining a lot of attention lately [1,2]. So, we consider that Rab22a has a key role in regulating this immunological process, as we show in our manuscript, and that this study will add relevant information to the field of study.

Comments in detail:
- Key experiments studying the intracellular localization of Rab22 and its role in MHC class I distribution (Fig. 1 and 2C, D, E) are performed in the Jaw cell line stably transformed by a lentivirus. However in this line Rab22 expression is reduced by only 60%, while an almost complete knockdown is obtained in primary BM-DCs. In order to rule out some oddity of the Jaw line, it is critical to confirm the findings obtained in Jaw, including the surprising co-localization of Rab22 and Lamp1, using primary DCs. The authors might also want to discuss the latter finding.

Response: We understand the concern pointed out by the referee and we have performed all the key experiments requested in primary BMDCs.

Figure 1: Actually, the entire Fig 1 (including Fig EV1A) was already done with BMDCs in the previous version of the manuscript. We started our study describing the endocytic location of Rab22a in primary BMDCs. In this part of the article, only Fig EV1B and EV1C were done with the DC line JAWS-II, just to confirm the early phagosomal acquisition of Rab22a also in these cells. All this information is detailed in the main text, and the DC type used for each experiment is specified in the Figure legends. Regarding the high colocalization obtained for Rab22a and Lamp1 (Pearson correlation coefficient = 0.8412 ± 0.03562), it was very surprising for us too since this was not reported before for other cell types [3]. As we mention in the Discussion section, we reason that in DCs the wider distribution of Rab22a along the endocytic network than in other cell types could help them intercept exogenous antigens in order to guarantee an efficient cross-presentation. It is also interesting to note that, even if Rab22a highly localizes to late endosome/lysosomes, the phagosomal recruitment kinetics of Rab22a and Lamp1 are completely different. This might suggest that only Rab22a from early endosomes and recycling vesicles are present in DC phagosomes. Furthermore, we have addressed in the new version of our manuscript that Rab22a does not control phagosomal maturation, as we determined by measuring OVA phagosomal degradation (Fig 3F and Fig EV3B).

Figure 2C and D: We silenced the expression of Rab22a in BMDCs by using the same lentivirus-delivered shRNA as we did for JAWS-II DC. By previously doing the cross-presentation assays with transduced BMDCs, we knew already that the effect of Rab22a knock-down was much more efficient in this DC type than in JAWS-II DCs (Fig 3G). In this occasion, we also observed a drastic reduction of Rab22a expression in primary transduced BMDCs by performing IF staining and confocal microscopy (Fig EV2B). In the same figure, we noted a marked disruption of the intracellular pool of MHC-I molecules, but the staining of these molecules at the cell surface was normal. As we did previously with JAWS-II DCs, we quantified and confirmed this observation by flow cytometry analysis. Again, we observed no significant difference of MHC-I expression at the cell surface in intact cells, but in saponin-permeabilized cells we did find a clear reduction of the

intracellular pool of MHC-I molecules in Rab22a KD BMDCs, as compared to Scramble BMDCs (Fig EV2C and D).

Figure 2E: This is indeed a key result of our study. Because it is a technically challenging assay to perform with BMDCs (large amounts of cells are needed to obtain enough phagosomes to analyze), we decided to transduce primary BMDCs and to recover phagosomes only after 3 hours of internalization, since this represents the time point where the biggest differences are observed with JAWS-II DCs (Fig 2E). Ten thousand isolated phagosomes were analyzed from Rab22a KD and Scramble BMDCs of each condition; isotype control and anti H-2K^b antibodies (H-2K^b staining was performed in triplicates). We performed two independent experiments and twice we observed a significant impairment of MHC-I phagosomal recruitment in BMDC phagosomes, as compared to Scramble BMDCs (Fig EV2G). The gating on a particular FSC/SSC region corresponding to a single beads population (red circle) is shown in Fig EV2E.

- *In model lines such as HeLa or CHO, Rab22 mainly affects slow recycling of MHC class I molecules, while the fast recycling pathway is not affected. MHC class I molecules can use both fast (Rab35-dependent) and slow recycling pathways The authors should examine recycling of the transferrin receptor to check whether fast recycling is modified by Rab22 knockdown.*

Response: This represents also a very relevant question in our study and we decided to investigate both MHC-I molecules and transferrin receptor (TfR) recycling capacities after silencing the expression of Rab22a in DCs. For this, we used a flow cytometry-based approach previously described by others [4], that we detail in the Materials and Methods section of our manuscript. The role of Rab22a in the recycling of MHC-I molecules in cells of the immune system, particularly in DCs, has not been addressed so far. We performed the recycling assays with JAWS-II DCs and we observed a significant inhibition in the recycling of MHC-I molecules (Fig 2F and Fig EV2H), but not of TfR (Fig 2G and Fig EV2I), in Rab22a KD DCs, as compared to Scramble DCs. We speculate that the fast recycling pathway, which is not affected by the KD of Rab22a, could be used to compensate other endocytic functions, such as OVA-mediated endocytosis. The result of TfR recycling is also interesting because there are some contradictory data in the bibliography [5,6]. Taken into account the clear impact on the recycling of MHC-I molecules in Rab22a KD DCs, it is still not clear for us why the amount of MHC-I molecules at the cell surface is not affected in these cells. Maybe the presence of these molecules at the plasma membrane is mainly coming from the classical secretory pathway, or the endocytosis of MHC-I molecules is indeed altered and in the recycling experiments, we force their internalization due to the presence of anti MHC-I by inducing clustering of these molecules at the cell surface during binding at 4°C.

- *The final paragraph discusses issues and findings that are not relevant in the context of this study, this paragraph can be eliminated.*
Response: As suggested by the referee, we have eliminated this last paragraph of the discussion about the role of Rab22a during carcinogenesis.

References

1. Blander JM (2016) The comings and goings of MHC class I molecules herald a new dawn in cross-presentation. *Immunol Rev* **272:** 65-79
2. van Endert P (2016) Intracellular recycling and cross-presentation by MHC class I molecules. *Immunol Rev* **272:** 80-96
3. Mesa R, Salomon C, Roggero M, Stahl PD, Mayorga LS (2001) Rab22a affects the morphology and function of the endocytic pathway. *J Cell Sci* **114:** 4041-9
4. Osborne DG, Piotrowski JT, Dick CJ, Zhang JS, Billadeau DD (2015) SNX17 affects T cell activation by regulating TCR and integrin recycling. *J Immunol* **194:** 4555-66
5. Weigert R, Yeung AC, Li J, Donaldson JG (2004) Rab22a regulates the recycling of membrane proteins internalized independently of clathrin. *Mol Biol Cell* **15:** 3758-70
6. Magadan JG, Barbieri MA, Mesa R, Stahl PD, Mayorga LS (2006) Rab22a regulates the sorting of transferrin to recycling endosomes. *Mol Cell Biol* **26:** 2595-614

Referee #3:

This brief manuscript reports the potentially interesting finding that Rab22a plays a significant role in the cross presentation of exogenous antigens on MHCI in dendritic cells (DCs). The authors show evidence that a partial knockdown of Rab22a (only 40-50%) results in the loss of an intracellular pool of MHCI, its resulting failure to be recruited to phagosomes, and a diminution of peptide-MHCI presentation to T cells. What we do not learn is very much about why this all happens, and this absence of any mechanistic insight is the paper's weakness. Even the significant loss of intracellular MHCI goes without much characterization, presumably it is degraded? Such a fate would be consistent with the colocalization studies that, in my opinion, show the best localization of Rab22a to lysosomes rather than other endosomal compartments (the authors' interpretation to the contrary notwithstanding). The limited resolution of the IF images shown precludes very many other conclusions, none of which anyway would inform the issue of mechanism.

Apart from the over interpreted IF, the paper is fine as far as it goes, but unfortunately, it does not go very far to letting the reader know "why" Rab22a is important. Simply showing that it may does not create a paper that will be of wide interest.

Response: We thank the referee for the critics and comments on our study, and we would like to answer his/her concerns as detailed as we can. We acknowledge that the previous version of our manuscript was missing some mechanistic insights to explain the fate of MHC-I molecules when Rab22a expression is silenced in DCs. The role of Rab22a in the recycling of MHC-I molecules in cells of the immune system, particularly in DCs, has not been addressed so far. To better understand the role of this GTPase in the intracellular mechanism of MHC-I trafficking, we decided to address the MHC-I recycling capacity of Rab22a KD DCs. By using a flow cytometry-based approach previously described by others [1], we observed a significant inhibition in the recycling of MHC-I molecules (Fig 2F and Fig EV2H), but not of TfR (Fig 2G and Fig EV2I), in Rab22a KD DCs, as compared to Scramble DCs. We speculate that the fast recycling pathway, which is not affected by the KD of Rab22a, could be used to compensate other endocytic functions, such as OVA-mediated endocytosis. The result of TfR recycling is also interesting because there are some contradictory data in the bibliography [2,3]. Taken into account the clear impact on the recycling of MHC-I molecules in Rab22a KD DCs, it is still not clear for us why the amount of MHC-I molecules at the cell surface is not affected in these cells. Maybe the presence of these molecules at the plasma membrane is mainly coming from the classical secretory pathway, or the endocytosis of MHC-I molecules is indeed altered, and in the recycling experiments we force their internalization due to the presence of anti MHC-I by inducing clustering of these molecules at the cell surface during binding at 4°C. We would also like to mention that we confirmed key results of our study concerning the role of Rab22a in MHC-I intracellular trafficking with primary transduced BMDCs (Fig EV2B, C, D and G), as it was requested by other referee. Mechanistically speaking, we think that the disappearance of the intracellular pool of MHC-I molecules in Rab22a KD DCs is mainly due to a disorganization of the recycling center and to a substantial defect in the capacity of MHC-I recycling.

It is possible that an alteration in the recycling route for MHC-I molecules results in an enhancement of the degradative pathway for these molecules, especially when the high colocalization obtained for Rab22a and Lamp1 (Pearson correlation coefficient = 0.8412 ± 0.03562) is taken into consideration. This result was very surprising for us too since it was not reported before in other cell types [4], as we clearly mention it in the Results section. Also, in the Discussion section we hypothesize that in DCs, the wider distribution of Rab22a along the endocytic network than in other cell types could help them intercept exogenous antigens in order to guarantee an efficient cross-presentation. It is also interesting to note that, even if Rab22a highly localizes to late endosome/lysosomes, the phagosomal recruitment kinetics of Rab22a and Lamp1 are completely different. This might suggest that only Rab22a from early endosomes and recycling vesicles are present in DC phagosomes. Furthermore, in the new version of our manuscript we addressed that Rab22a does not control phagosomal maturation, as we determined by measuring OVA phagosomal degradation (Fig 3F and Fig EV3B).

We also acknowledge that IF studies sometimes could guide to over interpretation or to misleading of the results. This is why we tried to confirm the IF observations with other techniques and functional approaches. The IF of Fig 1 are mainly descriptive, but we have statistically determined

the Pearson correlation coefficient for all the three markers of the endocytic network used in the colocalization studies with Rab22a. We have also confirmed the recruitment of Rab22a to DC phagosomes biochemically by purifying phagosomes and Western blot analysis. For JAWS-II DC phagosomes, we have even confirmed this result by FACS analysis. In Fig 2 there is only one IF result (the disruption of the intracellular pool of MHC-I molecules), and we have clearly confirmed this observation by FACS analysis in JAWS-II DCs and BMDCs. The next IF result corresponds to Fig EV4, where we studied the shape of the *cis* and *trans*-Golgi networks in Scramble and Rab22a KD DCs and any evident alteration was observed. Anyways, the correct functionality of the classical secretory pathway was confirmed by performing endogenous MHC-I antigen presentation experiments (Fig 3J). Finally, Fig 4 and Fig EV5 have many IF images. Here we show that Rab22a is recruited to the parasitophorous vacuole (PV) of *Toxoplasma gondii*. Interestingly, this is the first report of an endogenous Rab protein associated to the PV of this microorganism. Furthermore, we show that Rab22a recruitment to the PV is critical to ensure an efficient parasite-associated antigen presentation to CD8+ T lymphocytes (Fig 4B).

References

1. Osborne DG, Piotrowski JT, Dick CJ, Zhang JS, Billadeau DD (2015) SNX17 affects T cell activation by regulating TCR and integrin recycling. *J Immunol* **194:** 4555-66
2. Weigert R, Yeung AC, Li J, Donaldson JG (2004) Rab22a regulates the recycling of membrane proteins internalized independently of clathrin. *Mol Biol Cell* **15:** 3758-70
3. Magadan JG, Barbieri MA, Mesa R, Stahl PD, Mayorga LS (2006) Rab22a regulates the sorting of transferrin to recycling endosomes. *Mol Cell Biol* **26:** 2595-614
4. Mesa R, Salomon C, Roggero M, Stahl PD, Mayorga LS (2001) Rab22a affects the morphology and function of the endocytic pathway. *J Cell Sci* **114:** 4041-9

2nd Editorial Decision 24 August 2016

Thank you for the submission of your revised manuscript to our editorial offices. I apologize getting back to you with delay, but due to the holiday season it took more time than expected to receive the referee reports, which you will find enclosed below. Referee #1 could not look at the manuscript again. Therefore we asked referee #2 also to assess if the concerns of referee #1 have been sufficiently addressed. As you will see, both referees find the manuscript suitable for publication in EMBO reports, but referee #3 mentions more concerns and asks for further mechanistic insight. However, as referee #2 states that his concerns and those of referee #1 have been adequately addressed, and as also referee #3 agrees that the re-submission addressed many of his points, and as EMBO reports emphasizes novel functional over detailed mechanistic insight, we think that the manuscript is now suitable for publication.

Before we can proceed with the formal acceptance of your manuscript, I would like to ask you for some further minor revisions, though.

We noted that in the figure panels 2A and 4D the same blots are shown. This is fine, as they show the same data, however please state this fact in the manuscript text and the figure legend. Further, it seems that the contrast and/or intensity of the Rab22a panel in 2A was more enhanced as in panel 4D. If you show the same part of the blot, please keep also the same color/contrast settings. Or replace 2A with a similar blot, if possible.

As also referee #2 mentions, further language editing by a native speaker would be welcome. For a short report in EMBO reports, we require that the results and discussion sections are combined in a single section called "Results & Discussion". Please do that. Finally, please provide the final manuscript file as .doc/.docx file.

We also strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure or per figure

panel.

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.

REFEREE REPORTS

Referee #2:

Although the mechanism underlying the effect of Rab22 on cross-presentation and MHC class I recycling in dendritic cells remains to be established fully, the revised version of the manuscript addresses all concerns expressed by myself and reviewer 1. The addition of the recycling assay for MHC-I and transferrin receptor adds some mechanistic understanding. Some editing for English wording will be required.

Referee #3:

The resubmission does a nice job of investigating further the fate of MHCI molecules in Rab22a KD DCs and addressing some of the concerns expressed in the initial review. In addition, the experiments in primary transduced BMDCs were an excellent added control. Unfortunately, the main punchline of this manuscript is predicated on the idea that Rab22a KD DCs have a defect in cross presentation because less MHCI encounters antigen in the phagosome and there is still simply very little data to support this. The authors have made the observations that Rab22a KD DCs have mislocalized MHCI and reduced cross presentation, and then inferred the rest. These are interesting observations, but without at least some hint of a mechanism linking the two, they are just observations and not a complete manuscript.

Reviewers can often be unfair by demanding that manuscripts show direct evidence of a mechanism for every step along a given pathway. In some cases, it is possible to infer some of the steps in the pathway and/or cite previous studies. However, in this case, since the vacuolar pathway is likely not the dominant pathway for antigen processing, it is not suitable to infer this step based on the data presented. Can the authors provide any evidence that antigen loading in the phagosome matters or is happening at all?? It is frustrating to see another manuscript that relies heavily on IF to describe correlations and continue to promote the idea that the vacuolar pathway is important in cross presentation without any data to support such a claim.

An alternative approach is to heed the advice of Reviewer #1 and perform more experiments that address the hypothesis that Rab22a KD is accelerating phagosomal maturation/lysosomal fusion/antigen degradation. This appears to be the case with Rab34 inhibition (Alloatti et al., Immunity, 2015) and Sec22b KD. The half hearted efforts looking at OVA degradation in this manuscript are not enough. It is clearly within the authors' skill set to perform more thorough analysis of phagosome maturation and lysosomal fusion. Also, the CCF4 escape assay pioneered by the first author would be an interesting experiment in Rab22a KD vs. WT.

2nd Revision - authors' response 07 September 2016

Thank you for your letter, we are extremely pleased with this publication in EMBO reports. We have now submitted the last revised version of our manuscript, which contains all the modifications that you have requested us. We really wanted to submit the manuscript before, but we sent it to a professional English corrector for language editing (as requested by referee #2) and it took us a whole week to receive the corrections.

We have replaced Fig 2A with a similar blot to avoid any misunderstanding, and we have included the original source data of this new blot as well in the corresponding power point file. You will also find in this last version of the manuscript that we have changed the results and discussion sections for a single combined section called “Results and Discussion”, as requested for a short report type.

3rd Editorial Decision 13 September 2016

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.

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PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Ignacio Cebrian and Luis S. Mayorga
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2016-42358

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

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 justified
- 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 $\chi 2$ 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.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

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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?	The major conclutions were based on experiments repeated independently three times and, when possible, using two different cell models, the DC line JAWS-II and primary BMDCs. Some control experiments to confirm the asumptions of the main experimet were repeated twice. The N for each experiment is clearly specified in the legend to the figures.
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, in the legend to the figures.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	The Pearson correlation coefficient was estimated as detailed in the manuscript by using the ImageJ software. Two-tailed Student's unpaired t test, and two-way ANOVA test and Bonferroni post-test were performed for the rest of the experiemnts. The GraphPad Prism 5 software was used for all analysis; this software indicates when the data do not meet the normal distribution assumption. All p-values, "n" size and statistical tests are specified in the Figure legends (pages 29-35 of the manuscript).
Is there an estimate of variation within each group of data?	Yes
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).	A complete list of all antibodies used in our study can be found the Materials and Methods section (page 17 and 18 of the manuscript). The catalogs numbers are the following: From Sigma-Aldrich; purified rabbit polyclonal anti-OVA (C6534) , mouse monoclonal anti-α-tubulin (T8203), mouse monoclonal anti-β-actin (A5316). From Santa Cruz; mouse monoclonal anti-Rab22a (390726). From Abcam; mouse monoclonal anti-TGN46 (ab2809). From BD Pharmingen; purified mouse anti-GM130 (610822), purified rat anti-Lamp1 (553792), FITC mouse anti-H-2Kb (553569), FITC hamster anti-CD80 (553768), PE mouse anti I-Ab (553552), PE-Cy7 hamster anti-CD11c (561022)and APC rat anti-CD86 (561964). Mouse monoclonal anti-TfR H68.4 (ATCC). From Aviva Systems Biology; rabbit polyclonal anti-Rab22a (ARP57434), anti-Rab11a (ARP63742) and anti-EEA1 (ARP30074). And purified rabbit anti-HY10, GRA6 (custom made, Biotem).
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	All the cell lines used in this study (JAWS-II DCs, B3Z, HFF, J558 and HEK 293T cells) were imported from Dr. Sebastian Amigorena's lab in France and were routinely tested for Mycoplasma contamination, the all five were negative.

* 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.	C57BL/6 mice from 6 to 10 weeks of age were used to obtain bone marrow stem cells from the femur and tibia. The mice were maintained in specific pathogen-free conditions (SPF), housed in temperature-controlled rooms (22–25°C) and received water and food ad libitum. All animal procedures were performed according to the bioethics rules of the 'Comité Institucional para el Cuidado y Uso de Animales de Laboratorio (CICUAL), Facultad de Ciencias Médicas, Universidad Nacional de Cuyo'. This is detailed in page 17 of the manuscript.
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 accession codes for deposited data. See author guidelines, under '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	The commercial (Sigma-Aldrich) shRNAs sequences were used in our study: Rab22a #1 (target sequence GTACCGGGACGCCACCTCATGCTCTTTACTCGAGTAAAGAGCATGAGGTGGCGTCTTTTTTG) TRC N° 0000380076 and Rab22a #2 (target sequence GTACCGGTGTCAGAGTCGTATCAGTAAGCTCGAGCTTACTGATACGACTCTGACATTTTTTG) TRC N° 0000381994. This information is available on page 18 of the manuscript.
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. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	NA
22. 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

23. 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.	NA