THE EMBO JOURNAL

Endosome maturation factors Rabenosyn-5/VPS45 and Caveolin-1 regulate ciliary membrane and Polycystin-2 homeostasis

Noémie Scheidel, Julie Kennedy, Oliver E. Blacque

Review timeline:

Submission date:	15 September 2017
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Revision received:	14 December 2017
Editorial Decision:	15 January 2018
Revision received:	8 February 2018
Accepted:	16 February 2018

Editor: Ieva Gailite

Transaction Report:

(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 25 October 2017

Thank you for submitting your manuscript for consideration by the EMBO Journal. First of all, I would like to sincerely apologise for the delay in communicating the editorial decision, caused by delayed submission of reviewers' reports. We have now received two referee reports on your manuscript, which I have copied below.

As you can see, both referees express interest in the presented role of endosomal proteins in regulation of ciliary homeostasis. However, they also raise several concerns that need to be addressed before they can support publication here. Therefore I would like to invite you to submit a revised version of the manuscript, addressing the comments of both referees. If you have the data, you are welcome to add the analysis of biochemical interactions of endosomal components and PKD-2, but this will not be required for a re-consideration of the revised version.

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, please contact me as soon as possible upon publication of any related work in order to discuss how to proceed. If you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension. I should add that it is EMBO Journal policy to allow only a single major round of revision.

Please feel free to contact me if you have any further questions regarding the revision. Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Referee #1:

Scheidel et al. screened for genes affecting the ciliary membrane and PKD-2 homeostasis of the amphid and phasmid neurons in C. elegans and found that vps-45, rabs-5 and cav-1 are required. The authors show beautiful pictures with fluorescent and electron microscopy to show the genetic interaction among the genes involved. These genes in ciliary membrane regulation are important and the descriptions are new. The manuscript is quite interesting. However, as described below I think that the manuscript is not suitable at the present form in the EMBO Journal.

Major points:
(1) The mutant alleles of vps-45 and rabs-5 show a temperature-sensitive larval arrest phenotype. The authors must recognize this fact because they cultured these mutants at 15˚C (described in the Materials and Methods). It should be noted that these mutants can survive at 15˚C for many generations but die at higher temperature such as 25˚C, although mutants appear deletion-based null but not point mutations. This fact implicates that the phenomenon involving these proteins is temperature-sensitive, although the exact molecular mechanisms are not clear. This makes me suspect that functions of some molecules interacting with VPS-45 and RABS-5 may be modulated by temperature. I could not find the description in the manuscript whether the phenotypes in this manuscript were examined in 15˚C or 25˚C. Because these phenotypes are described for the first time, the temperature sensitivity should be important. More specifically, which temperature were the phenotypes found in this manuscript detected at: 15˚C or 25˚C? If the descriptions in Materials and Methods (cultured in 15˚C) are used throughout the manuscript, is it true to say negative phenotypes in relation to rab-5 etc? Are Is there any possibility that the same phenotypes are found at 25˚C. Instead, if the authors actually examined the phenotypes at 25˚C, the results may be different. Please clarify these possibilities by comparing the phenotypes at both permissive and restrictive temperature. Also it is important how the phenotypes described in the manuscript are affected, for example, by shift up experiments.

(2) The genes vps-45 and rabs-5 are spatially and temporally expressed in a ubiquitous manner. The authors described the phenotypes in ciliary membranes, but it is important that the observed phenotypes are related to whether development, maintenance or both of ciliary structure and function? In this sense, some experiments for temporal and spatial contribution to answer these questions are important by using some of the many such techniques.

(3) The authors mostly show genetic interactions and morphological phenotypes. The molecular interactions among important players are not well-described. They should show biochemical interactions among VPS-45, RABS-5, CAV-1 and PKD-2 etc.

Mionor points:
Descriptions on references are not complete: I could not find the review article pointed out in P21 of the text in the reference list. Also many papers are listed multiple times in the Reference section.

Referee #2:

The manuscript by Scheidel, Kennedy, and Blacque identifies novel roles for endosomal proteins RABS-5 and VPS-45 in ciliary morphology, and function. Their work adds to the list of the proteins that localize to the PCMC and is expected to better our understanding of how events at the ciliary base contribute to ciliary membrane protein localizations, and ciliary length regulation in different types of cilia in living animals. The differential localization of RABS-5 between different types of ciliated neurons is particularly exciting. One of the most significant findings is the localization of RABS-5 at the PCMC in the male mating neurons, and the suggestion that this could be because these male neurons have different PCMC endocytosis requirements due to their ability to release ciliary extracellular vesicles. This finding opens up a thought that there are different means to establishing ciliary diversity, and that ciliary functional diversity can be achieved by regulating not just axonemal ultrastructure but, possibly also membrane events at the ciliary base/PCMC. (Authors need to emphasize the significance of this finding, which is currently buried deep in the manuscript.) This manuscript will appeal any reader interested in cell biology and is well suited for EMBO.
rabs-5 mutants are Dyf (but not completely dye filling defective) and roaming defective but not osmotic avoidance defective. rabs-5 appears to be expressed in some but not all ciliated sensory neurons. Double labeling with rabs-5p::gfp and a red dye shows overlap in some but not all dye

filling neurons in the head and tail (what are these amphid and phasmid cells? rabs-5 expression is noticeably brighter in the posterior pair of phasmids). Consistent with cell-specific functions, authors show that RABS-5, VPS-45, and CAV-1 play unique functions in male-specific ciliated sensory neurons that shed and release PKD-2::GFP labeled EVs. Combined, these data indicate that rabs-5 is not a general ciliogenic factor, but rather may perform more cell-specific functions. Authors did not drive this point home, and I think they should.
Authors present convincing data showing the RABS-5 and VPS-5 act in the PCMC of ciliated sensory neurons - the staging area of inbound and outbound ciliary proteins. They use fluorescently-tagged ciliary and endocytic compartmental markers to visualize neuronal transport and ciliary structure in living animals and TEM to visualize ultrastructure in fixed animals. This data supporting a role for these proteins in endocytosis is rock solid. However, authors may have overlooked two important phenotypes revealed by electron microscopy.
(In general, for TEM analysis, it is difficult to determine if sections were taken in the same region for each genotype. This reviewer would like to see the TEMs show similar levels of the axoneme. For e.g., in Fig EV5B, the wild type IL2 cilia are being shown at a more anterior level than the mutants. In Fig EV5A, the vps-45 mutant CEM axoneme is at a different level compared to the other two genotypes)
Figure EV4 middle segments and TZ regions do not fit wild-type in rabs-5 or vps-45. In the middle segment, there are singlets and unwound B-tubules, which are unexpected in this area. In the transition zone region, not all cilia display Y links. Also, the top inset of rabs-5 TZ was done with a different fixation method (tannic acid, I assume), because the protofilaments are beautifully visible and countable - and in this particularly, there are many more inner singlets than are expected.
Hence, rabs-5 and vps-45, while not essential for ciliogenesis, do impact axonemal ultrastructure in amphid channel cilia. This suggests that RABS-5 and VPS-45 may not only act to control ciliary membrane homeostasis, but may impact ciliary components that rely on the PCMC for ciliary entrance and/or exit.
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Hence rabs-5 and vps-45 mutants have phenotypes that extend beyond the PCMC and extend to the CEM ciliary axoneme and EV distribution profile.
Authors present a very nice summary of PCMC phenotypes in Figure 4 - I suggest they do the same for axonemal and EV phenotypes in EV4 and EV5.

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Fig 2E. why are there 3 cells instead of 2 in GFP-tagged RABS-5ΔRBD animals?
Fig 3A. capitalize I in PI3
Fig 3C. Dyf not DyF
EV5 label CEM distal not distral
Reference list is a mess

I apologize to the authors for the delay in review.

1st Revision - authors' response 14 December 2017

Response to reviewers

Referee #1:

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The reviewer is right to point out that the *rabs-5* and *vps-45* deletion alleles (*ok1513*, *tm0246*) appear temperature sensitive, at least in terms of larval lethality when grown at 25^{o}C. We have now clarified the temperature sensitivity of these alleles in the materials and methods section and in the relevant results sections (lines 195-197; 559-561).

In our initial submission, we were satisfied to present phenotypes at 15^{o}C because the nature of the deletions predict strong loss-of-function or null effects, as the reviewer acknowledges. *ok1513* removes 80% of coding sequence, including the FYVE domain essential for RABS-5 ciliary and non-ciliary functions (Fig 2A). *tm0246* removes 84 bp of upstream promoter sequence, together with 30% of coding sequence that includes the start codon, all of exons 1-3 and most of exon 4 (note that we have added a new schematic showing the *tm0246* deletion in Fig EV3F).

Nonetheless, we agree with the reviewer that it would be interesting to assess ciliary phenotypes of mutant worms grown at higher temperatures. Since o*k1513* and *tm0246* homozygote embryos cultured at 25^{o}C arrest at early larval stages, we first examined adult worms cultured for their lifetime at 20^{o}C. We found no difference in the dye filling or cilium structure (axoneme length; PCMC size) phenotypes of these worms compared to those cultured at 15^{o}C. To examine if a tolerated short period of growth at 25^{o}C enhances the ciliary phenotypes, we subjected embryos and L4 larvae (at 15^{o}C) to a further 24 hours of growth at 25^{o}C. Compared to worms at 15^{o}C (and 20^{o}C), the temperature up-shift to 25^{o}C enhances the dye filling, phasmid length and PCMC area phenotypes. However, it should be noted that the severity of most of these phenotypes is only modestly enhanced by the temperature upshift. From these new data, we conclude that the cilium structure defects of *ok1513* and *tm0246* worms can be modestly enhanced by higher growth temperature. These new results are now described in the results section (Figs EV1A, EV1C, EV3C and EV3E) of the revised manuscript (lines 194-202; 302-303).

Our conclusion that the cilia-related RABS-5/VPS-45 pathway may not require RAB-5 is derived from what we described in the ciliary context for mutant alleles of RAB-5 GEFs and worms overexpressing dominant inactive RAB-5(S33N). Thus, our conclusions do not rely on data from the temperature sensitive *rabs-5* and *vps-45* mutants.

(2) The genes vps-45 and rabs-5 are spatially and temporally expressed in a ubiquitous manner. The authors described the phenotypes in ciliary membranes, but it is important that the observed phenotypes are related to whether development, maintenance or both of ciliary structure and function? In this sense, some experiments for temporal and spatial contribution to answer these questions are important by using some of the many such techniques.

In terms of spatial contribution, our manuscript shows that the *rabs-5* and *vps-45* mutant phenotypes are rescued by expressing the corresponding wild type sequences under the control of an *arl-13* promoter, which is active only in ciliated cells (Figs 1B and C, and 3C). These findings indicate that RABS-5 and VPS-45 influence ciliary phenotypes by functioning in ciliated cells. Consistent with this conclusion, the a *rabs-5*p*::gfp* reporter containing 1566-bp of upstream sequence is expressed almost exclusively in ciliated neurons (Figs 1G and EV1E). Please note that we now state in our manuscript that *rabs-5* expression could be under additional cis-regulatory control as part of an operon with the upstream gene, thereby accounting for RABS-5 functions in additional non-ciliated cell types (lines 216-219).

In terms of temporal contribution, our revised manuscript now compares the ciliary phenotype of mutants at larval and adult stages (all grown at 15^{o}C). Our new data (Figs EV1A and EV1C, and EV3C and EV3E) shows that *rabs-5* and *vps-45* mutant larvae and adults possess a similar level of phasmid dye-filling and cilium length phenotype when compared to WT controls (lines 203-209; 301). We also see evidence that the mutant PCMCs are already expanded at L1 stage, but for technical reasons, it is hard to image individually separated PCMCs in these very young worms; thus, we don't include the PCMC data in the revised manuscript. Thus, the defective phasmid ciliary phenotypes do not worsen as the mutant worm develop post-embryonically, indicating that RABS-5 and VPS-45 function during early stages of ciliary axoneme development and/or maintenance in the embryo. However, as outlined above in response to the reviewer's first point, a 24 hour growth temperature up-shift (15^{o}C to 25^{o}C) can enhance the ciliary phenotypes of early and late larval *rabs-5* and *vps-45* mutants, indicating that these endosomal proteins must also serve roles in maintaining ciliary structures at later developmental time points. Consistent with the latter, we do not see any obvious change in the expression pattern of a *rabs-5*p*::gfp* reporter between L1 and adult worms, which we present in Fig EV1E (and lines 215-216). We now present the above data and outline the associated conclusions in the results section of the revised manuscript.

(3) The authors mostly show genetic interactions and morphological phenotypes. The molecular interactions among important players are not well-described. They should show biochemical interactions among VPS-45, RABS-5, CAV-1 and PKD-2 etc.

The genetic interactions presented in our study don't necessarily require biochemical interactions between the various components. Indeed, there is a good chance that the endocytic membrane trafficking and vesicle sorting regulators we have studied will not directly interact with each other (exception is the VPS-45 and RABS-5 interaction that is already described in Gengyo-Ando *et al.* (2007), or the interactions will be transient and hard to detect. Also, it must be considered that *C. elegans* is a notoriously challenging model for biochemical experiments. Therefore, although we agree that biochemical studies are warranted, the suggested work is best conducted in another model system, and for this reason, we feel the request is beyond the scope of the current work.

Mionor points:
Descriptions on references are not complete: I could not find the review article pointed out in P21 of the text in the reference list. Also many papers are listed multiple times in the Reference section.

We apologize for the reference list problems. We have now ensured that the references are correct.

Referee #2:
The manuscript by Scheidel, Kennedy, and Blacque identifies novel roles for endosomal proteins RABS-5 and VPS-45 in ciliary morphology, and function. Their work adds to the list of the proteins that localize to the PCMC and is expected to better our understanding of how events at the ciliary base contribute to ciliary membrane protein localizations, and ciliary length regulation in different types of cilia in living animals. The differential localization of RABS-5 between different types of ciliated neurons is particularly exciting. One of the most significant findings is the localization of RABS-5 at the PCMC in the male mating neurons, and the suggestion that this could be because these male neurons have different PCMC endocytosis requirements due to their ability to release

ciliary extracellular vesicles. This finding opens up a thought that there are different means to establishing ciliary diversity, and that ciliary functional diversity can be achieved by regulating not just axonemal ultrastructure but, possibly also membrane events at the ciliary base/PCMC. (Authors need to emphasize the significance of this finding, which is currently buried deep in the manuscript.)

We thank the reviewer for pointing out the importance of our findings with regard to ciliary subtype diversity mechanisms, and we agree this aspect of the work should be more strongly emphasised in the manuscript. We have therefore added an additional line of text in the discussion (lines 476-478).

This manuscript will appeal any reader interested in cell biology and is well suited for EMBO.
rabs-5 mutants are Dyf (but not completely dye filling defective) and roaming defective but not osmotic avoidance defective. rabs-5 appears to be expressed in some but not all ciliated sensory neurons. Double labeling with rabs-5p::gfp and a red dye shows overlap in some but not all dye filling neurons in the head and tail (what are these amphid and phasmid cells? rabs-5 expression is noticeably brighter in the posterior pair of phasmids).

GFP is expressed in all dye-filling amphid neurons and especially strong in AWB. Expression is also found in all 4 phasmid neurons, together with a 5th tail neuron that is likely to be PQR. We make this clear in the revised manuscript by saying that GFP is expressed in all dye filling amphid and phasmid neurons, as well as PQR (although more weakly) (line 214). We also addressed the reviewer's valid point that expression appeared stronger in images we presented for the posterior phasmids. However, upon reanalysis, we don't believe there is evidence of stronger expression in these cells. We have therefore replaced the original suboptimal Fig 1G images with confocal Z-projections, which show the expression pattern more clearly. In addition, we have added new images in Fig EV1E showing that the expression of *rabs-5p::gfp* reporter in young larvae is basically identical to that of adult worms (data also referred to in the results text; lines 215-216).

Consistent with cell-specific functions, authors show that RABS-5, VPS-45, and CAV-1 play unique functions in male-specific ciliated sensory neurons that shed and release PKD-2::GFP labeled EVs. Combined, these data indicate that rabs-5 is not a general ciliogenic factor, but rather may perform more cell-specific functions. Authors did not drive this point home, and I think they should.

We agree with this assessment, and have therefore added additional text in this regard in the discussion (lines 445-447).

Authors present convincing data showing the RABS-5 and VPS-5 act in the PCMC of ciliated sensory neurons - the staging area of inbound and outbound ciliary proteins. They use fluorescently-tagged ciliary and endocytic compartment markers to visualize neuronal transport and ciliary structure in living animals and TEM to visualize ultrastructure in fixed animals. This data supporting a role for these proteins in endocytosis is rock solid.

However, authors may have overlooked two important phenotypes revealed by electron microscopy. (In general, for TEM analysis, it is difficult to determine if sections were taken in the same region for each genotype. This reviewer would like to see the TEMs show similar levels of the axoneme. For e.g., in Fig EV5B, the wild type IL2 cilia are being shown at a more anterior level than the mutants. In Fig EV5A, the vps-45 mutant CEM axoneme is at a different level compared to the other two genotypes)

For Fig EV5B, we added new WT images of the IL2 pore.

For Fig EV5A, it is difficult to achieve sections where CEM, CEP and OLQ are all at the very same level for each mutant. This is because of: (1) differences in plane of sectioning, and (2) many serial sections lie on grid lines, thus restricting the number of usable sections we can image. Nonetheless, we contend that the cilia being shown in Fig EV5A are at comparable levels. We have added another set of images from an intermediary longitudinal position in the pore; thus, Fig EV5A now shows images from 5 serial sections, spanning the entire cephalic pore to better represent the CEM ultrastructure described in Silva et al. (2017) (the schematic in EV5 was also modified accordingly). We also now show higher magnification images of the

proximal and middle axonemal regions of the CEM cilium, which looks mostly normal in the mutants compared to WT controls. We agree that the distal-most portion of CEM looks abnormal in the images we initially showed for *vps-45*; however, this impression arises because we found it difficult to capture this region of the CEM axoneme due to it being tilted relative to the plane of sectioning. Unfortunately, we don't have sufficiently good images of the distal portion of the CEM cilium in order to make conclusions about its ultrastructure (we now state this in the revised Fig EV5A legend).

Figure EV4 middle segments and TZ regions do not fit wild-type in rabs-5 or vps-45.
In the middle segment, there are singlets and unwound B-tubules, which are unexpected in this area.
In the transition zone region, not all cilia display Y links. Also, the top inset of rabs-5 TZ was done with a different fixation method (tannic acid, I assume), because the protofilaments are beautifully visible and countable - and in this particularly, there are many more inner singlets than are expected.

All of the TEM data in the manuscript is from HPF-fixed samples, which can show individual protofilaments such as those pointed to by the reviewer in Fig EV4.

We thank the reviewer for pointing out possible defects in amphid channel ciliary ultrastructure. With regard to the unwound B-tubule phenotype, we know that 3 of the 10 axonemes in WT worms (both axonemes of ADL, and ASI) display B-tubule seam breaks in both chemical (Jauregui et al. 2008; and our previous work) and HPF (our work) fixed samples. Re-analysis of TEM sections from the middle segment regions of *rabs-5* and *vps-45* mutants reveals no obvious difference in the number of mutant axonemes with unwound middle segment B-tubules compared to controls. Re-analysis of TZ images confirms that a small number (perhaps 10%) of mutant TZs display 10-12 MT singlets, although most TZs display 4-9 singlets (similar to WT controls). Thus, there is some evidence of a defect in the ultrastructure of *rabs-5* and *vps-45* mutant TZs in a small number of axonemes. We now describe this phenotype in the results section of the revised manuscript (lines 329-331).

Hence, rabs-5 and vps-45, while not essential for ciliogenesis, do impact axonemal ultrastructure in amphid channel cilia. This suggests that RABS-5 and VPS-45 may not only act to control ciliary membrane homeostasis, but may impact ciliary components that rely on the PCMC for ciliary entrance and/or exit.

From the re-analysis of our TEM data (see also below for our comment on the CEM cilium), the only axonemal (non-membrane) phenotype we can be confident of in our mutants is the increased MT singlet number in the TZ. However, given that we see this phenotype relatively infrequently, we feel it premature to draw general conclusions at this point regarding roles for RABS-5 and VPS-45 in the regulation of ciliary axoneme ultrastructure.

Figure EV5 presents the same challenge. The CEM cilium has distinct regions (Proximal with 9 doubles, middle with 18 singlets and distal with 9 doublets then singlets. Refer to Silva et al 2017).
Either the sections are taken at different levels or rabs-5 and vps-45 have a profound impact on CEM ciliary ultrastructure. In rabs-5 EVs are present through out the entire cephalic lumen, including the very distal most regions - this is not observed in WT.
Hence rabs-5 and vps-45 mutants have phenotypes that extend beyond the PCMC and extend to the CEM ciliary axoneme and EV distribution profile.

As outlined above, the distal portion of the CEM cilium is not so clear from our images on account of CEM being tilted relative to the sectioning plane. However, we have some good images of the proximal and middle axonemal regions of mutant CEM cilia (now shown at high magnification in Fig EV5), which appear mostly normal. We agree with the reviewer that EVs are present throughout the entire cephalic lumen and we have amended the text and Fig EV5 to reflect this (line 1369).

Authors present a very nice summary of PCMC phenotypes in Figure 4 - I suggest they do the same for axonemal and EV phenotypes in EV4 and EV5.

We have added additional summary text to the schematics in EV4 and EV5

Minor points:

Fig 2E. why are there 3 cells instead of 2 in GFP-tagged RABS-5ΔRBD animals?

The 3rd cell in ΔRBD (and ΔFYVE) expressing worms is PQR; for those images, the right hand side of the tail is shown. We make this clear in the revised legend (lines 1124-1125).

Fig 3A. capitalize I in PI3
Now corrected

Fig 3C. Dyf not DyF
Now corrected

EV5 label CEM distal not distral
Now corrected

Reference list is a mess
We apologize about this issue. Now corrected.

2nd Editorial Decision 15 January 2018

Thank you for submitting a revised version of your manuscript. The manuscript has now been seen by both original referees, who find that their main concerns have been addressed. There remain only a few minor editorial issues that have to be resolved before formal acceptance of the manuscript.

Referee #1:

I think that the manuscript has been appropriately improved by the revision.

Referee #2:

Authors have done a thorough and convincing job of addressing all of my concerns, either with additional data or text clarification. I support publication in EMBO.

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:	Oliver Blacque and Noemi Scheidel
Journal Submitted to:	Embo
Manuscript Number:	EMBOJ-2017-98248R

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:

- è 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.
- è figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- è graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- è if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- è 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:

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

- è the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- è 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.).
- è a statement of how many times the experiment shown was independently replicated in the laboratory.
- è 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?	Experimental sample size was chosen to ensure a power higher than 0.8 based on data from pilot expriments and estimations of the smallest meaningful effect size
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.	Yes, investigators blindly performed experimentation and assessed results on anonymized groups/samples.
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. Student T test was used on data samples following a normal distribution (tested by Shapiro-Wilk normality test) and homoscedasticity (i.e., equal variances tested using Fisher test comparing variances). Unparametric Mann–Whitney U test were used to compare data samples that were not following normal distribution.
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, equality of variance was tested before comparing data samples using Student t-test.

USEFUL LINKS FOR COMPLETING THIS FORM

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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-tun

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/

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.	na