EMBO reports

A secreted factor NimrodB4 promotes the elimination of apoptotic corpses by phagocytes in *Drosophila*

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1st Editorial Decision 5th Feb 2021

Dear Bruno,

Thank you for the submission of your research manuscript to our journal. We have now received the full set of referee reports that is copied below.

As you will see, the referees acknowledge that the findings are interesting and consider the conclusions overall well supported by the data. However, the referees also point out several concerns and have a number of suggestions for how the study should be strengthened, which should be addressed in the revision.

Given these constructive and very positive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as detailed above and in their reports) must be fully addressed and their suggestions taken on board. Please address all referee concerns 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.

We invite you to submit your manuscript within three months of a request for revision. This would be May 5th in your case. However, we are aware of the fact that many laboratories are not fully functional due to COVID-19 related shutdowns and we have therefore extended the revision time for all research manuscripts under our scooping protection to allow for the extra time required to address essential experimental issues. Please contact us to discuss the time needed and the revisions further.

IMPORTANT NOTE: we perform an initial quality control of all revised manuscripts before re-review. Your manuscript will FAIL this control and the handling will be DELAYED if the following APPLIES:

1) A data availability section is missing.
2) Your manuscript contains error bars based on n=2. Please use scatter blots showing the individual datapoints in these cases. The use of statistical tests needs to be justified.

When submitting your revised manuscript, please carefully review the instructions that follow below. Failure to include requested items will delay the evaluation of your revision.

When submitting your revised manuscript, we will require:

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).
Please download our Figure Preparation Guidelines (figure preparation pdf) from our Author Guidelines pages
https://www.embopress.org/page/journal/14693178/authorguide for more info on how to prepare your figures.

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

4) a complete author checklist, which you can download from our author guidelines (). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

5) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript (). Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines
()

6) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here:

- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

7) Please note that a Data Availability section at the end of Materials and Methods is now mandatory. In case you have no data that requires deposition in a public database, please state so instead of refereeing to the database.
See also < https://www.embopress.org/page/journal/14693178/authorguide#dataavailability>).
Please note that the Data Availability Section is restricted to new primary data that are part of this study.

8) We would also encourage you to include the source data for figure panels that show essential data. Numerical data should be provided as individual .xls or .csv files (including a tab describing the data). For blots or microscopy, uncropped images should be submitted (using a zip archive if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available .

9) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at .

10) Regarding data quantification
The following points must be specified in each figure legend:
- the name of the statistical test used to generate error bars and P values,
- the number (n) of independent experiments (please specify technical or biological replicates) underlying each data point,
- the nature of the bars and error bars (s.d., s.e.m.)
Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.
- Please also include scale bars in all microscopy images.

11) As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

You are able to opt out of this by letting the editorial office know (emboreports@embo.org). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

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.

Kind regards,

Martina

Martina Rembold, PhD
Senior Editor
EMBO reports

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Referee #1:

This study from Petrignani and colleagues identifies a novel regulator of efferocytosis in Drosophila - NimrodB4. They show that NimB4 operates as a secreted factor which binds to PS on the surface of apoptotic corpses and is important for phagosome maturation within the phagocyte. This is the first demonstration of a secreted factor that is able to enhance apoptotic uptake in Drosophila expanding parallels with the efferocytic process in vertebrates. The study provides novel and exciting results and the experimental data are in most part very convincing. With the following revisions I think this paper would be entirely appropriate for publication in EMBO reports.

1) The authors claim that NimB4 is a secreted protein enriched in phagocytes yet don't provide real proof of NimB4 secretion from macrophages. The authors could show this more directly using NimB4-GFP or hemo>uas-nimb4-rfp detected extracellularly (i.e in the hemolymph pre/post wound)

2) To conclusively prove their findings in vivo it should be possible for the authors to induce apoptosis in 3rd instar larvae (for example temperature controlled hid expression in few cells [i.e. wing disc] and monitor NimB4-gfp upregulation/secretion and binding to apoptotic cells at the disks?). This would provide more conclusive evidence to support their hypothesis.

3) In Figure 2 the authors show that NimB4 binds PS on the surface of apoptotic cells and it is outcompeted by Annexin V. They should also show no binding to non-apoptotic cells.

4) In Figure 3 it would be great to show unengulfed apoptotic bodies decorated with NimB4-GFP (or -RFP) and not just engulfed corpses. I appreciate that it might be impossible to catch them in vivo before they are internalised but if it were possible then again it would strengthen the model greatly.

5) In the experiment presented in figure 4D the authors should demonstrate that overexpression of NimB4-RFP can rescue phagocytic uptake at 120 minutes. (same approach to that used in fig. S6D where they measure lysotracker fluorescence)

6) In the experiment presented in Figure 6C the authors state "We observed by flow cytometry a reduced fluorescence intensity of pHrodo-labeled S. aureus bioparticles in the NimB4sk2 mutant macrophages compared to wild-type (Figure 6C) consistent with defective acidification of the phagosome." It is surprising that the authors use pHrodo particles having already demonstrated that NimB4 macrophages have defects in apoptotic body processing but not in bacterial uptake "NimB4 is required for the phagocytosis of apoptotic cells but not bacteria.". The use of bacteria is justified by the fact that there is no difference in their uptake (fig4D). However, no phagocytic difference was detected when phagocytes were challenged with apoptotic bodies up to the 60 min timepoint (fig4D) so can the authors look at apoptotic corpse acidification using this time point?

7) In figure 5 the authors show that NimB4 mutant macrophages have more lysotracker positive (acidified) vesicles compared to wildtype. However, in figure 6 they show that Lamp1 and lysotracker do not colocalise in the mutant and therefore conclude that a loss of NimB4 impairs the fusion of the phagosomes with lysosomes. If this is the case then the phagosomes should not acidify which then begs the question as to why the mutants have increased lysotracker positive vesicles. How are the phagosomes acidifying if they are not fusing to lysosomes? These two findings seem to contradict one another. Can the authors explain this?

8) The vesicles in the TEM sections of NimB4 mutant macrophages in Figure 5C do not look like they contain apoptotic bodies which are usually seen as dark structures in EM. Instead these vesicles look empty. Perhaps they are lysosomes. The images in Fig 3A are much more convincing of an accumulation of apoptotic corpses in embryonic mutant macrophages as these are clearly Dcp-1 positive. If the authors want to include TEM sections then it would be more informative to capture sections of these embryonic macrophages in NimB4 mutants in vivo.

9) Does NimB4-gfp colocalize with Rab5/7 vesicles?

10) Can NimB4 overexpression in macrophages rescue the processing defect/vacuolation phenotype observed in Draper mutants?

Minor points:

1) The authors show that "clean injury induces NimB4 expression in the hemocytes with an acute phase profile 1 hour following challenge". Do the authors know what signal is responsible for the upregulation? Is NimB4 upregulation triggered just by clean injury or is there any effect after septic injury? Whilst answering these experimentally are probably beyond the scope of the paper it would be nice to at least discuss/speculate on these in the paper.

2) Sup fig 1B staining is not very convincing: immunostaining without permeabilization shows membrane staining while it is not detected after permeabilization. Also, I would expect NimB4 to localize to secretory vesicles not to be diffuse in the cytosol.

3) Fig S6D- the stats bar on left is misplaced (NimB4sk2 ; Lpp> + VS NimB4sk2 ; Lpp>NimB4-RFP).

4) Is there any compensation of other nimrod family members in the NimB4 KO?

5) fig S2a. The western blot shows a 27kDa RFP band in all samples expressing an RFP-tagged construct. Does this mean that all the products are cleaved?
6) Fig 4D Legend is incorrect - it should read: Ex vivo phagocytosis assay using red apoptotic cells stained with CellTrace Red (left), or BioParticles-Texas Red Conjugate from Staphylococcus aureus Wood strain (right).

Referee #2:

The manuscript by Petrignani et al., describes the role of the NimrodB4 protein in the uptake and degradation of apoptotic material in Drosophila phagocytic cells.

The authors show that NimrodB4 (NIMB4) is a secreted member of the Nimrod family of proteins, as opposed to the transmembrane proteins Draper and SIMU. NIMB4 is expressed by macrophages and glial cells. It binds apoptotic corpses via phosphatidyl serines.

The study of NIMB4 mutant (NIMB4sk2) Drosophila highlighted an accumulation of vacuoles and a defective debris clearance during development. Importantly, the initial binding of the corpses was not affected in the mutants. Expression of NIMB4 as an RFP fusion construct was sufficient to complement the defects in the NIMB4 mutant. Therefore the manuscript by Petrignani et al. reveals NIMB4 as a bridging molecule promoting phagocytosis in a paracrine and autocrine manner.

The authors then describe further the role of NIMB4 on phagosome maturation and phagolysosome biogenesis, showing that the NIMB4 mutants fail to acquire the Lamp1 late endosomal / lysosomal marker. Expression of Rab7 and not Rab5 rescues the defective phenotype. Therefore, the blockade is at the level of the transition to Rab7 positive compartments.

Overall the manuscript is of very high scientific standards and it reaches the criteria of significant novel advance in the field. Of course, one would like to know more about the precise role of NIMB4 on the Rab5-Rab7 conversion, a potential link with the exchange factors or the fusion machinery, but the manuscript already presents a lot of data, and I realize that investigating this question would be beyond the scope of the present paper. It could be however discussed more in the

discussion section by adding references to reviews and articles on phagosome maturation in general, in addition to the references to efferocytosis.

Minor comments and typos:
Page 7, line 36 : « Interestingly, we observed a similar increase in the number of LAMP1-mcherry positive structures when wild-type macrophages were pre-treated with chloroquine, a specific inhibitor of phagosome maturation (Supplementary Figure 7A, B) » : could the authors rephrase the sentence to avoid stating that chloroquine is a specific inhibitor of phagosome maturation, but saying that it inhibits endosomal acidification ?
Page 8, line 11 : promoting the phagosome-lysosome fusion is these cells is sufficient to rescue the NimB4 deficiency (Figure 7A-C) : should read in these cells

Referee #3:

Petrignani et al. describe a new component of phagocytosis process in Drosophila, NimrodB4. Interestingly, NimB4 is a secreted molecule, but functions primarily in phagosome maturation rather than facilitating binding to phagocytes, indicating a more complex role for so-called bridging molecules. The study is very strong, providing a thorough analysis of the gene through expression and functional analysis, both ex vivo and in the fly. One addition that could make the paper even stronger is to generate double mutants with draper or simu (or Crq or integrins or Ced-12) to see if NimB4 functions in the same pathways or not. Minor concerns are listed below but several of them should be addressed before publication.

Specific comments:
1. Sup Fig 1 - some definition/explanation of clean injury in the text on p. 4 would be helpful to the reader.
2. Sup Fig 2 B- The expression in nephrocytes is intriguing. A control for Lpp-GAL4 (UAS-mcd8 GFP) expr should be done to confirm that expression is normally limited to the fat body and not also expressed in the nephrocytes. This would be worthwhile for Hml-GAL4 as well. Many GAL4 lines are expressed more broadly than reported.
3. Sup Fig 3A - It appears as if the nimb4sk mutant has an insertion, not a deletion. Perhaps the lines are mislabeled between w1118 and nimb4sk. The sequence alignment is off too.
4. At the top of page 5, it says that embryonic glia are full of corpses, yet NimB4 did not have expression in glia. It is difficult to discern glia in Figure 3A (and the figure legend only refers to macrophages). Perhaps the authors are referring to larval glia in Figure 3B? If the authors wish to mention embryonic glia, additional experiments/images are necessary to demonstrate that embryonic glia have the defect (such as labeling with anti-repo).
5. Fig. 7 - UAS-Rab5 has a pronounced defect on its own so this should be mentioned and perhaps considered in making conclusions. Statistical comparisons should be made between NimB4; UAS-Rab5/7 and NimB4 alone, not w1118.
6. Draper motor defects have been shown to be due to muscle, not brain (Draper et al. Am J Pathol 2014). Have the authors ruled out a muscle defect? The conclusions regarding neurodegeneration should mention this caveat unless brain morphology is characterized. Similarly the effect on survival is not very strong and could be caused by other problems other than neurodegeneration.
7. Fig Sup 6 and 7 - The RNAi shows a cell-autonomous effect, but the rescue indicates a non-autonomous effect. Further discussion of this finding is needed.

Dear editor,

We want to thank the reviewers for carefully examining our work and providing constructive suggestions, which have helped us improve our manuscript. We have performed additional experiments that address the comments of the reviewers and significantly strengthen our initial conclusions. The main changes are:

- We showed that NimB4 exclusively binds to apoptotic cells (**Fig EV2A**)
- We showed that NimB4 binds to apoptotic cell in vivo (**Fig EV2B, C, D**)
- We showed that the genomic fragment containing a wild-type copy of *NimB4* could rescue the phagocytic defect caused by *NimB4*^{sk2} mutation (**Fig 4D**)
- We confirmed the phagosome maturation defect in the *NimB4*^{sk2} mutant using pHrodo-succinimidyl ester (SE) apoptotic cells (**Fig 6C**).
- We analyzed the lysotracker signal in hemocytes double mutants for *NimB4*^{sk2}, *draper,* croquemort, *NimC1*$^{\Delta}$, and *Eater*^{1} (**Fig EV4A**)

The revision has reinforced the conclusion of our paper without challenging the main conclusions, which reveal the role of NimB4 in the phagocytosis of apoptotic cells. You will find below a point-by-point answer to reviewer's comments.

With best regards,

Bianca Petrignani and Bruno Lemaitre

Point by Point answer

(New figures and results are indicated in bold)

Reviewer #1:

This study from Petrignani and colleagues identifies a novel regulator of efferocytosis in *Drosophila* - NimrodB4. They show that NimB4 operates as a secreted factor which binds to PS on the surface of apoptotic corpses and is important for phagosome maturation within the phagocyte. This is the first demonstration of a secreted factor that is able to enhance apoptotic uptake in Drosophila expanding parallels with the efferocytic process in vertebrates. The study provides novel and exciting results and the experimental data are in most part very convincing. With the following revisions I think this paper would be entirely appropriate for publication in EMBO reports.

1) The authors claim that NimB4 is a secreted protein enriched in phagocytes yet don't provide real proof of NimB4 secretion from macrophages. The authors could show this more

directly using NimB4-GFP or Hemo>uas-Nimb4-RFP detected extracellularly (i.e in the hemolymph pre/post wound).

R1. We agree that we could show more directly the secretion of NimB4 in the hemolymph from macrophages. As such, we have analyzed the fate of NimB4-RFP protein when expressed in hemocytes of *Hml>UAS-NimB4-RFP.* We observed RFP signals in nephrocytes known to recycle hemolymphatic proteins consistent with the notion that NimB4 is indeed secreted. **This is now shown in Fig EV1F which includes a picture of *Hml> NimB4-RFP* larvae and *Hml> SP^{VKG}-RFP* (used as a control) larvae.**

2) To conclusively prove their findings in vivo it should be possible for the authors to induce apoptosis in 3rd instar larvae (for example temperature controlled hid expression in few cells [i.e. wing disc] and monitor NimB4-gfp upregulation/secretion and binding to apoptotic cells at the disks?). This would provide more conclusive evidence to support their hypothesis.

R2. We followed the reviewer's suggestion and expressed the pro-apoptotic gene *Reaper* in the imaginal wing disc with the driver *Ap-GAL4*. We observed, that as expected, *NimB4-GFP* binds to the wing disc when *Reaper* is expressed and induces apoptosis. **This is shown in Fig EV2B that includes pictures of the third instar imaginal wing disc with and without the expression of *Reaper*.**

3) In Figure 2 the authors show that NimB4 binds PS on the surface of apoptotic cells and it is outcompeted by Annexin V. They should also show no binding to non-apoptotic cells.

R3. This is an excellent suggestion. For this, we have repeated our analysis using non apoptotic S2 cells (without cycloheximide treatment). **This is illustrated in Fig EV2A that shows that NimB4-RFP is binding exclusively to apoptotic S2 cells after treatment with cycloheximide**.

4) In Figure 3 it would be great to show unengulfed apoptotic bodies decorated with NimB4-GFP (or -RFP) and not just engulfed corpses. I appreciate that it might be impossible to catch them in vivo before they are internalized but if it were possible then again it would strengthen the model greatly.

R4. Following the reviewer's suggestion, we collected hemolymph of NimB4-GFP larvae 1h after clean injury, which was stained with DAPI to mark apoptotic cells. By this approach, we could observe 'corpses' with the shape and the dimensions of apoptotic cells decorated with NimB4-GFP. **This is shown in Fig EV2C, of the revised version**.

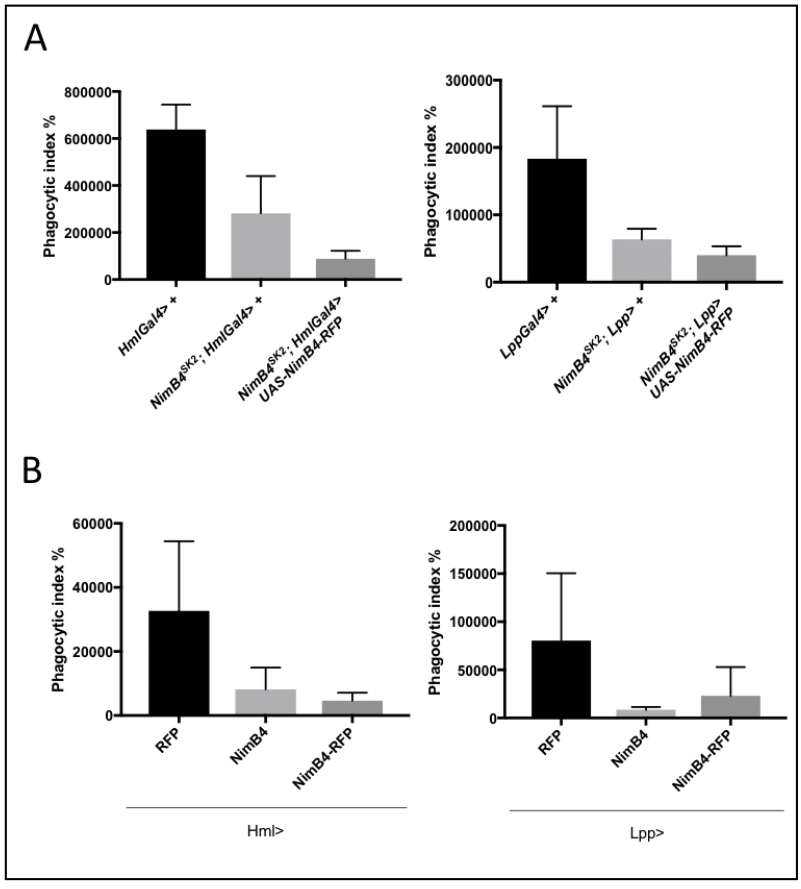
5) In the experiment presented in figure 4D the authors should demonstrate that overexpression of NimB4-RFP can rescue phagocytic uptake at 120 minutes. (same approach to that used in fig. S6D where they measure lysotracker fluorescence)

R5. To show the rescue of phagocytosis at 120 minutes, we expressed NimB4-RFP using the hemocyte driver (*HmlGal4*) and the fat body driver (*LppGal4*). Surprisingly we observed that over-expression of NimB4-RFP acts as a dominant negative, blocking phagocytosis of

apoptotic cells. The phagocytic index of Gal4> NimB4-RFP was even lower than the mutant *NimB4*^{sk2}. This result could be explained by the presence of the tag in NimB4-RFP or by the over-load of NimB4 that blocks the phagocytosis process (see Figure R1A below). To distinguish between these two explanations, we repeated the same experiment by expressing *UAS-NimB4* without the RFP tag. The overexpression of NimB4 from the fat body and the hemocytes also negatively impacted the phagocytosis of apoptotic cells (see Figure R1B below). This result lets us think that excessive presence of NimB4 inhibits the apoptosis, by saturating binding sites. Although it reinforces our conclusion that NimB4 impacts efferocytosis, we did not include those data as they are based on over-expression. One consequence of those new findings is that the rescue observed in Fig EV6C, D is incorrect. Most probably, the decrease of the lysotracker staining corresponds to the decrease of phagocytosis. Hence, we decided to remove Fig EV6C, D from the manuscript.

To definitively confirm the role of NimB4 in efferocytosis, we did a rescue of *NimB4*^{sk2} using a genomic fragment containing a wild-type copy of NimB4 (genotype: *NimB4*^{sk2}, [*NimB4+*]). The addition of the [NimB4+] element fully rescues the phagocytic defect caused by *NimB4*^{sk2}at 120 min (See Figure revised 4D).

Figure R1. Phagocytic index of Gal4>NimB4-RFP and Gal4>NimB4.



6) In the experiment presented in Figure 6C the authors state "We observed by flow cytometry a reduced fluorescence intensity of pHrodo-labeled S. aureus bioparticles in the *NimB4*^{sk2} mutant macrophages compared to wild-type (Figure 6C) consistent with defective

acidification of the phagosome." It is surprising that the authors use pHrodo particles having already demonstrated that NimB4 macrophages have defects in apoptotic body processing but not in bacterial uptake "NimB4 is required for the phagocytosis of apoptotic cells but not bacteria.". The use of bacteria is justified by the fact that there is no difference in their uptake (fig4D). However, no phagocytic difference was detected when phagocytes were challenged with apoptotic bodies up to the 60 min timepoint (fig4D) so can the authors look at apoptotic corpse acidification using this time point?

R6. Following the reviewer's suggestion, we use a pHrodo-succinimidyl ester (SE), a pH-sensitive fluorescent dye that labels the apoptotic cells for monitoring the phagosome maturation. **The new panel Fig 6C shows as expected a decrease of fluorescence in the *NimB4*^{sk2} and the *Draper* mutant when apoptotic cells are labeled with pHrodo-succinimidyl ester 1h after incubation**.

7) In figure 5 the authors show that NimB4 mutant macrophages have more lysotracker positive (acidified) vesicles compared to wildtype. However, in figure 6 they show that Lamp1 and lysotracker do not colocalize in the mutant and therefore conclude that a loss of NimB4 impairs the fusion of the phagosomes with lysosomes. If this is the case then the phagosomes should not acidify which then begs the question as to why the mutants have increased lysotracker positive vesicles. How are the phagosomes acidifying if they are not fusing to lysosomes? These two findings seem to contradict one another. Can the authors explain this?

R7. Following the internalization of apoptotic cells, the phagosome sequentially forms early phagosomes, late phagosomes and, phagolysosomes. During phagosome maturation, various intracellular organelles are sequentially incorporated into phagosomes (early endosomes, late endosomes and lysosomes). These vesicles provide to the phagosome a variety of protein and lipid materials. Phagosome luminal pH starts to decrease immediately after the completion of engulfment and reaches the lowest level after the fusion with the lysosome (Zhen Zhou and Xiaomeng Yu, 2008). Lysotracker can detect any acidic organelles such as late endosome, late phagosome, phagolysosome and lysosome. The use of Lamp1 and RAB7 reporters strongly suggest that phagosomes of NimB4 hemocytes do not fuse to the lysosome. As such, we attribute the accumulation of acidic vesicles that we observe in the *NimB4*^{sk2} mutant to an accumulation of late phagosomes, which are acidic (hence Lysotracker positive) although not yet fused with lysosomes.

8) The vesicles in the TEM sections of *NimB*4 mutant macrophages in Figure 5C do not look like they contain apoptotic bodies which are usually seen as dark structures in EM. Instead these vesicles look empty. Perhaps they are lysosomes. The images in Fig 3A are much more convincing of an accumulation of apoptotic corpses in embryonic mutant macrophages as these are clearly Dcp-1 positive. If the authors want to include TEM sections then it would be more informative to capture sections of these embryonic macrophages in NimB4 mutants in vivo.

R8. We agree with the reviewer that it is surprising to observe no apoptotic bodies in the enlarged vesicles of macrophages. However, we can exclude that vesicles are enlarged

lysosomes as lysosomes are usually dark structures in EM section. Also, the fluorescent microscopy image in Figure 5E shows enlarged Rab7 positive vesicles. As such, we suspect that the enlarged vesicles observed in the TEM section are empty late phagosomes.

9) Does NimB4-GFP colocalize with Rab5/7 vesicles?

R9. As NimB4 is a secreted protein, it cannot bind directly to Rab5/7 or to other proteins present on the intracellular part of the phagosome. The most likely scenario is that NimB4 binds to a receptor present at the first steps of phagosome formation. However, with our tools we cannot exclude the binding of NimB4 to Rab5/7. As both endogenous reporters of NimB4 and Rabs present a GFP tag, we could not test this hypothesis during the revision period.

10) Can NimB4 overexpression in macrophages rescue the processing defect/vacuolation phenotype observed in Draper mutants?

R10. Unfortunately, because of the dominant negative effect of the over-expression of NimB4 (see R5) we did not consider to rescue *draper* mutant with our over-expression line UAS-NimB4.

Minor points:

The authors show that "clean injury induces NimB4 expression in the hemocytes with an acute phase profile 1 hour following challenge". Do the authors know what signal is responsible for the upregulation? Is NimB4 upregulation triggered just by clean injury or is there any effect after septic injury? Whilst answering these experimentally are probably beyond the scope of the paper it would be nice to at least discuss/speculate on these in the paper.

We agree with the reviewer that this is an interesting question but we felt that it was beyond the scoop of the article that focus on NimB4 function. **In the revised version, we added a few sentences about this topic in the discussion.**

Sup fig 1B staining is not very convincing: immunostaining without permeabilization shows membrane staining while it is not detected after permeabilization. Also, I would expect NimB4 to localize to secretory vesicles not to be diffuse in the cytosol.

We agree with the review that the staining experiment with and without triton is not very convincing. For this reason, we decided to remove the immunostaining done without triton. Instead, we looked in live confocal the localization NimB4-GFP in the cell. **In the revised version, we have added in Figure 1 panel C, a confocal live image of *NimB4-GFP* hemocytes. Interestingly, we observed that without fixation NimB4-sGFP localize in small spots throughout the cytoplasm, likely associated with the endosomal compartments.**

Fig S6D- the stats bar on left is misplaced (*NimB4^{sk2}; Lpp> + VS NimB4^{sk2}; Lpp>NimB4-RFP*).

As discussed in R5, S6D has been removed.

Is there any compensation of other nimrod family members in the *NimB4*^{sk2}?

To answer this point, we performed qPCR analysis of all the *NimB* genes in the *NimB4*^{sk2} mutant. We used three different samples: hemocyte, fat-body, and whole larvae. The results shown below did not reveal any compensatory expression of the other NimB in NimB4. We did not include this information to no overload the manuscript

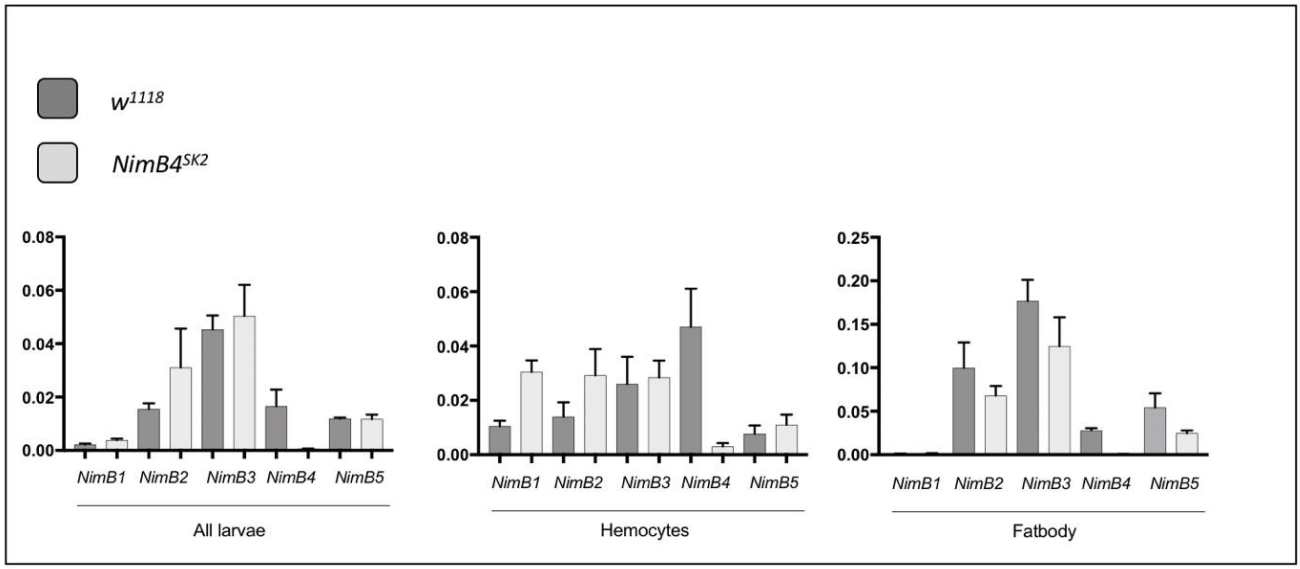


fig S2a. The western blot shows a 27kDa RFP band in all samples expressing an RFP-tagged construct. Does this mean that all the products are cleaved?

In figure 1, we consistently observed in the western blot a 27KD band that we attribute to the cleavage of RFP. This indeed suggests that all the products are cleaved.

Fig 4D Legend is incorrect - it should read: Ex vivo phagocytosis assay using red apoptotic cells stained with CellTrace Red (left), or BioParticles-Texas Red Conjugate from Staphylococcus aureus Wood strain (right).

We thank the reviewer for this comment and corrected the legend of figure 4D.

Reviewer #2:

The manuscript by Petrignani et al., describes the role of the NimrodB4 protein in the uptake and degradation of apoptotic material in Drosophila phagocytic cells. The authors show that NimrodB4 (NIMB4) is a secreted member of the Nimrod family of

proteins, as opposed to the transmembrane proteins Draper and SIMU. NIMB4 is expressed by macrophages and glial cells. It binds apoptotic corpses via phosphatidyl serines. The study of NIMB4 mutant (*NIMB4*^{sk2}) Drosophila highlighted an accumulation of vacuoles and a defective debris clearance during development. Importantly, the initial binding of the corpses was not affected in the mutants. Expression of NIMB4 as an RFP fusion construct was sufficient to complement the defects in the NIMB4 mutant. Therefore, the manuscript by Petrignani et al. reveals NIMB4 as a bridging molecule promoting phagocytosis in a paracrine and autocrine manner.
The authors then describe further the role of NIMB4 on phagosome maturation and phagolysosome biogenesis, showing that the NIMB4 mutants fail to acquire the Lamp1 late endosomal / lysosomal marker. Expression of Rab7 and not Rab5 rescues the defective phenotype. Therefore, the blockade is at the level of the transition to Rab7 positive compartments.
Overall the manuscript is of very high scientific standards and it reaches the criteria of significant novel advance in the field. Of course, one would like to know more about the precise role of NIMB4 on the Rab5-Rab7 conversion, a potential link with the exchange factors or the fusion machinery, but the manuscript already presents a lot of data, and I realize that investigating this question would be beyond the scope of the present paper. It could be however discussed more in the discussion section by adding references to reviews and articles on phagosome maturation in general, in addition to the references to efferocytosis.

11) Of course, one would like to know more about the precise role of NIMB4 on the Rab5-Rab7 conversion, a potential link with the exchange factors or the fusion machinery, but the manuscript already presents a lot of data, and I realize that investigating this question would be beyond the scope of the present paper. **It could be however discussed more in the discussion section by adding references to reviews and articles on phagosome maturation in general, in addition to the references to efferocytosis.**

R11. As suggested by the reviewer, we have further developed this point in the discussion and added new references.

Minor comments and typos:

Page 7, line 36: « Interestingly, we observed a similar increase in the number of LAMP1-mcherry positive structures when wild-type macrophages were pre-treated with chloroquine, a specific inhibitor of phagosome maturation (Supplementary Figure 7A, B) » : could the authors rephrase the sentence to avoid stating that chloroquine is a specific inhibitor of phagosome maturation, but saying that it inhibits endosomal acidification ?

We have revised the sentence according to the reviewer suggestion.

Page 8, line 11: promoting the phagosome-lysosome fusion is these cells is sufficient to rescue the NimB4 deficiency (Figure 7A-C): should read in these cells

We have revised the sentence according to the reviewer suggestion.

Reviewer #3:

Petrignani et al. describe a new component of phagocytosis process in Drosophila, NimrodB4. Interestingly, NimB4 is a secreted molecule, but functions primarily in phagosome maturation rather than facilitating binding to phagocytes, indicating a more complex role for so-called bridging molecules. The study is very strong, providing a thorough analysis of the gene through expression and functional analysis, both ex vivo and in the fly. One addition that could make the paper even stronger is to generate double mutants with draper or simu (or Crq or integrins or Ced-12) to see if NimB4 functions in the same pathways or not. Minor concerns are listed below but several of them should be addressed before publication.

Major points:

12) One addition that could make the paper even stronger is to generate double mutants with draper or simu (or Crq or integrins or Ced-12) to see if NimB4 functions in the same pathways or not. Minor concerns are listed below but several of them should be addressed before publication.

R12. We observed in figure 5A that draper and croquemort mutants present an increased lysotracker signal as the *NimB4*^{Sk2} mutant. This leds to the hypothesis that Draper and Croquemort and NimB4 function in the same pathway and that NimB4 could bind those receptors. As suggested by the reviewer, we analyzed the lysotracker signal in double mutants between NimB4 mutation and mutation in *draper, croquemort, eater*^{1} and *NimC1*$^{\Delta}$ (we omitted SIMU because it is not expressed during the larval stage of development). We observed an increased lysotracker signal in the *NimB4*^{sk2}*; draper* double mutant suggesting an additive effect between *draper* and *NimB4*^{sk2}. Interestingly, we observed no increased signal in the *NimB4*^{sk}*, croquemort* double mutant. This result led us think that croquemort and NimB4 could work in a similar pathway although other interpretations are possible (Expanded figure 4A**). This is now shown in panel A of Fig EV4.**

Specific comments:

Sup Fig 1 - some definition/explanation of clean injury in the text on p. 4 would be helpful to the reader.

In the revised version, we now included a clearer explanation of how we performed clean injury in third instar larvae.

Sup Fig 2 B- The expression in nephrocytes is intriguing. A control for Lpp-GAL4 (UAS-mcd8 GFP) expr should be done to confirm that expression is normally limited to the fat body and not also expressed in the nephrocytes. This would be worthwhile for Hml-GAL4 as well. Many GAL4 lines are expressed more broadly than reported.

We agree that the *Lpp-Gal4* and *Hml-Gal4* could be express more broadly than reported. To address this point, we expressed the *UAS-mcd8-GFP* line with both drivers and look at the fluorescence in nephrocytes. We observed no fluorescence in the nephrocytes of the larvae when *UAS-mcd8-GFP was expressed* from the fat body or the hemocyte. **Those controls have been added in Figure EV1 (panels E and F) the picture of *Lpp-Gal4>UAS-mcd8* and *Hml-Gal4>UAS-mcd8* showing no fluorescence in the nephrocyte.**

Sup Fig 3A - It appears as if the $Nimb4^{sk2}$ mutant has an insertion, not a deletion. Perhaps the lines are mislabeled between w^{1118} and $NimB4^{sk2}$. The sequence alignment is off too.

We are sorry for this confusion and thank the reviewer as we inverted the wild-type and the mutants. **We corrected figure 3A.**

At the top of page 5, it says that embryonic glia is full of corpses, yet NimB4 did not have expression in glia. It is difficult to discern glia in Figure 3A (and the figure legend only refers to macrophages). Perhaps the authors are referring to larval glia in Figure 3B? If the authors wish to mention embryonic glia, additional experiments/images are necessary to demonstrate that embryonic glia have the defect (such as labeling with anti-repo).

We are sorry for this confusion, we were referring exclusively to embryonic hemocytes in the text. **We have revised the sentence according to the reviewer suggestion.**

Fig. 7 - UAS-Rab5 has a pronounced defect on its own so this should be mentioned and perhaps considered in making conclusions. Statistical comparisons should be made between NimB4; UAS-Rab5/7 and NimB4 alone, not w1118.

We agree that the over-expression of UAS-RAB5 has a pronounced defect on its own. **We have revised our conclusion according to the reviewer suggestion and did the statistical analysis**

Draper motor defects have been shown to be due to muscle, not brain (Draper et al. Am J Pathol 2014). Have the authors ruled out a muscle defect? The conclusions regarding neurodegeneration should mention this caveat unless brain morphology is characterized. Similarly, the effect on survival is not very strong and could be caused by other problems other than neurodegeneration.

We agree with the reviewer that Draper motor defect have been shown to be due to muscle not brain. However we didn't investigate the muscle defect in the $NimB4^{sk2}$ mutant. **Following the suggestion, we removed the text regarding NimB4 and neurodegeneration.**

Fig Sup 6 and 7 - The RNAi shows a cell-autonomous effect, but the rescue indicates a non-autonomous effect. Further discussion of this finding is needed.

We agree that the RNAi shows a cell-autonomous effect, but the rescue indicates a non-autonomous effect. As mention above in R5; the over-expression of NimB4 used initially to rescue NimB4 as it shows a dominant negative effect blocking the phagocytosis process. **As mentioned in R5 (reviewer 1) we removed the supplementary figure 6 C, D.**

1st Revision - Editorial Decision 17th Jun 2021

Dear Prof. Lemaitre

Thank you for the submission of your revised manuscript to EMBO reports. We have now received the reports from the referees that were asked to assess it (copied below).

As you will see, both referees are very positive about the study and request only a minor changes to clarify text.

Browsing through the manuscript myself, I noticed a few editorial things that we need before we can proceed with the official acceptance of your study.

- Please reduce the number of keywords to 5.

- Please note that a Data Availability section at the end of Materials and Methods is now mandatory. In case you have no data that requires deposition in a public database, please state so instead of refereeing to the database. See also <
https://www.embopress.org/page/journal/14693178/authorguide#dataavailability>). Please note that the Data Availability Section is restricted to new primary data that are part of this study.

- Please update the references to the alphabetical Harvard style. The abbreviation 'et al' should be used if more than 10 authors. You can download the respective EndNote file from our Guide to Authors
https://endnote.com/style_download/embo-reports/

- References to bioRxiv articles (ref 24, Lancaster et al) should be cited the following way:
• Author NAME1, Author NAME2, (YEAR) article title. bioRxiv doi: 1234/002.dfj123 [PREPRINT]

- Please complete the information on funding in our online submission system.

- Figure panels EV3A (NimB4-RFP) and EV3C (NimB4-RFP) still have black boxes at the right bottom corner. I assume these hide an embedded scale bar. If it is not possible to remove this other than using a black box, please state so in the figure legend (e.g., the black box in panel X covers a scale bar...)

- Please provide the tables reporting on Drosophila stocks, plasmids, and primer sequences either as EV tables (Table EVx) or in the form of an Appendix .pdf (Appendix table Sx). Alternatively, you could use the "Reagents and tools table", which will be placed before the methods section. You can find information on this table in the section on Structured methods in our guide to authors:
https://www.embopress.org/page/journal/14693178/authorguide#textformat

- Finally, EMBO reports papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is 550x200-600 pixels large (width x height) in .png format. You can either show a model or key data in the synopsis image. Please note that the size is rather small and that text needs to be readable at the final size. Please send us this information along with the revised manuscript.

We look forward to seeing a final version of your manuscript as soon as possible.

With kind regards,

Martina

Martina Rembold, PhD
Senior Editor
EMBO reports

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Referee #1:

The authors have done an excellent job on revisions and have addressed all my points very clearly. The paper should now be accepted for publications

Referee #3:

The authors have done an excellent job in responding to the previous critiques.

Minor issue: typo in figure 1C - it should read "live confocal"

The authors have addressed all minor editorial requests.

2nd Revision - Editorial Decision 30th Jun 2021

Prof. Bruno Lemaitre
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Station 19
SV 3838
Lausanne, Vaud 1015
Switzerland

Dear Bruno,

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.

At the end of this email I include important information about how to proceed. Please ensure that you take the time to read the information and complete and return the necessary forms to allow us to publish your manuscript as quickly as possible.

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If you do NOT want this File to be published, please inform the editorial office within 2 days, if you have not done so already, otherwise the File will be published by default [contact: emboreports@embo.org]. If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

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Thank you again for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

Kind regards,

Martina

Martina Rembold, PhD
Senior Editor
EMBO reports

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Corresponding Author Name: Bruno Lemaitre, Bianca Petrignani
Journal Submitted to: EMBO reports
Manuscript Number: EMBOR-2020-52262-T

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:

- ➔ 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 iustified
- ➔ 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.

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.

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http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tumo

http://datadryad.org

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http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

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?	Each experiment was repeated independently a minimum of three times (unless otherwise indicated), error bars represent the standard error of the mean of replicate experiments. Data were analyzed using appropriate statistical tests as indicated in figure legends using the GraphPad Prism software.
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?	Significance tests were performed using the Mann-Whitney test. For experiments with more than 2 conditions, significance was tested using ANOVA test followed by post hoc Dunnett's multiple comparison tests (P values of < 0.05 = *, < 0.01 = **, and < 0.001 = ***).
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	To performed the One-Way ANOVA test we verified three assumptions: 1. Assumption of independence (Our experimental plan includes the independence criteria)-2. Assumption of homogeneity (Bartlett's and Brown-forsythe tests)-3.Assumption of normality (Shapiro -Wilk test). For cases where data did not meet these requirements, we used non parametrical statistics (Mann-Whitney tests).
Is there an estimate of variation within each group of data?	One way ANOVA tests (performed with GraphPad Prism) includes the ANOVA table (SS, DF, MS, F).

Is the variance similar between the groups that are being statistically compared?	One way ANOVA tests (performed with GraphPad Prism) includes the Brown-Forsythe test and Bartlett's test.

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).	RFP antibody (Cat ABIN1043867). HRP antibody (Cat #MA1-10371). GFP antibody (cat #EPR14104). Draper antibody (Kurant et al.,2009). Simu antibody (Kurant et al.,2009). Mcherry antibody (Cat #M11217). Dcp-1 antibody (# 9578S).
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	We included in our study experiment with S2 cells. We did not test recently for mycoplasma contamination however we always worked in sterile condition and the growth medium contain antibiotics.

* 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.	Drosophila melanogaster. No gender distinction for larvae experiment. Adult survival are performed with males.
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.	Each website was consulted to confirm compliance with the inclusion criteria.

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
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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
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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