molecular systems biology

Bicoid gradient formation mechanism and dynamics revealed by protein lifetime analysis

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Review timeline:

Submission date:	3rd April 2018
Editorial Decision:	22nd May 2018
Revision received:	6th July 2018
Editorial Decision:	25th July 2018
Revision received:	6th August 2018
Accepted:	7th August 2018

Editor: Maria Polychronidou.

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 22nd May 2018

Thank you again for submitting your work to Molecular Systems Biology. We have now heard back from two of the three referees who agreed to evaluate your study. Unfortunately, after several reminders, we have not managed to obtain a report from reviewer #1. In the interest of time, we decided to proceed with making a decision based on the two available reports. As you will see below, the reviewers think that the application of the timer protein to a morphogen gradient system is an elegant approach and they appreciate the carefully performed quantitative analyses. They raise however a series of concerns, which we would ask you to address in a revision.

The reviewers' recommendations are rather clear therefore I think that there is no need to repeat the points listed below. Please let me know in case you would like to discuss further any of the reviewers' comments.

REFEREE REPORTS.

Reviewer #2:

This manuscript by Durrieu et al addresses the fundamental question of the mechanism of Bicoid gradient formation in the early Drosophila embryo. The question has received a lot of attention and has lead to several proposed models. In this manuscript the authors make use of a new tool - Bicoid fused to an engineered fluorescent timer, which allows them to determine the age of Bicoid protein in space and over time. The authors show that this parameter can be used to distinguish between four possible previously published models of Bcd gradient formation: SDD, nuclear shuttling, RNA gradient and RNA diffusion. Their main conclusion is that the SDD model is the one that most closely recapitulates the data at nuclear cycle 14. In addition, they also find that to explain the

observed temporal dynamics of the Bicoid profile and protein age, the synthesis and degradation rates of Bicoid have to change over time.

Altogether, the novel observations in the manuscript based on the tFT-Bcd and high quality imaging, combined with rigorous quantitative analysis, form an important contribution to the Bicoid field, and will also be of interest to biologists studying morphogen gradient formation in other systems. However, some aspects of the analysis that I outline below are still missing or need better explanation. Overall, I find the manuscript poorly written - many references to figures or precise pointers to Supplementary information are missing, there are multiple imprecise or curtailed explanations. Some key points are noted below, but the writing could be improved beyond these.

Main points:

- The finding that synthesis and degradation rates change over time is a key prediction of the study, however, there is no independent validation of this result. It would significantly strengthen the conclusion if the finding can be corroborated with a FRAP, photoconversion or alternative experiment at different developmental time points. Furthermore, the authors should provide an explanation of the potential causes of the time-dependence, at least at the level of discussion.

- It is not clear whether the consideration of time-dependence in the synthesis and degradation rates could affect the fit to the shuttling, RNA gradient and RNA diffusion models. The authors should comment on and address this point.

-As a key result, Fig. 3a should be extended or amended to demonstrate the sensitivity of the result on the model parameters. The comparison to Fig. 1e in the main text is obscure (for example Fig. 1e and 3a are in different units; Fig. 3a does not contain information about parameter ranges.)

- It is unclear how the chosen maturation rates of mCherry and sfGFP affect the simulated mCherry/sfGFP ratio in the different models. Fig 3a, which captures the main result of the study, shows a minor difference in the ratio between the SDD and shuttling models. However, it is unclear whether the better fit of the experimental data to the SDD model depends on the choice of maturation rates. The authors should further provide a systematic explanation of how they connect the two step mCherry rate to an effective one of 50 min in the main text. And why do they use 20 min for sfGFP in the main text and report 27 min in the Supplementary information?

- The authors should explain how the brightness (quantum yield, extinction coefficient) of the fluorophores are taken into account in the model fitting.

- Fig. S5 does not contain data on the fmCherry maturation rate, as stated in line 171 of the main text. The rate should be measured in a comparable way to mCherry and reported.

Additional points:

- Fig. 1e - The authors should explain what parameter ranges are considered and how the standard deviations are derived. They should also comment on the nearly absent sd of the shuttling model. fig1d and e - explanation of what is plotted is needed.

- Line 180 - I guess fig. S4m,n is meant. A more detailed explanation of the effects of normalization should be provided within the main text, in particular because Fig 2 ii contains a normalized graph but the rationale behind this is not made clear.

- Fig. 4 - it would be useful to add plots of the intensities and ratio over time for several positions.

- Fig 5a - which orange line?

-It is unclear how and when the production changes in Fig. 5d - the text, figures and figure legends seem contradictory. In the text, it says that the production and degradation decrease (line 295), in the fig. legend to fig. 5d it says that the production increases gradually. Having the relevant plot of the

rates that were used next to Fig. 5d would be useful to clarify this.

- Parameter units should be provided in supplementary tables.

Reviewer #3:

This manuscript presents a quantitative study of the formation of the Bicoid morphogen gradient in the early fly embryo. It's a collaboration between a quantitative imaging lab and the lab that pioneered the tFT reporter's use as a protein age tracer. Consequently, the study is technically impeccable, on par with the state-of-the-art of the Bicoid system. It's the first time this tracer has been used in the fly embryo and a beautiful advertisement for its usage as a quantitative tool. The study confirms many of the known features of the system (1, 2, 3, and 5 in the discussion) and adds the finding of proteasome mediated Bicoid degradation (4). It is a valuable contribution and addition to the field that is interested in the quantitative aspects of Bicoid gradient formation, and it certainly sets a new standard for that field. That being said, however, since its first usage in the early 2000's, the SDD model has in a way always been the golden standard in this field and never been seriously challenged. As such, the novelty and biological relevance of the current findings are somewhat limited. Beyond that, I am uncertain about its broader reach for a general audience.

I have three technical comments:
1. The authors might consider adding to the discussion a paragraph that treats the issue of steady-state in greater depth and what their findings might add to that aspect of the dynamics. The introduction mentions briefly that there is an ongoing debate, and the SDD model can incorporate time dependent parameters, but what have we learned from the current study about the fact that the gradient is never at steady-state and its implications?
2. It was also not entirely clear from reading the manuscript how the authors thought about the degradation properties of Bicoid versus the fusion protein versus the tFT alone. Ultimately, we're interested in Bicoid's properties alone. But how close did we get to that in the current study? Is there still a correction necessary from what was measured with the fusion protein versus the Bicoid protein alone?
3. Shells of embryos is not a nomenclature used in the field. If you need to use it, it should be defined.

Reviewer #2:

However, some aspects of the analysis that I outline below are still missing or need better explanation. Overall, I find the manuscript poorly written - many references to figures or precise pointers to Supplementary information are missing, there are multiple imprecise or curtailed explanations. Some key points are noted below, but the writing could be improved beyond these.

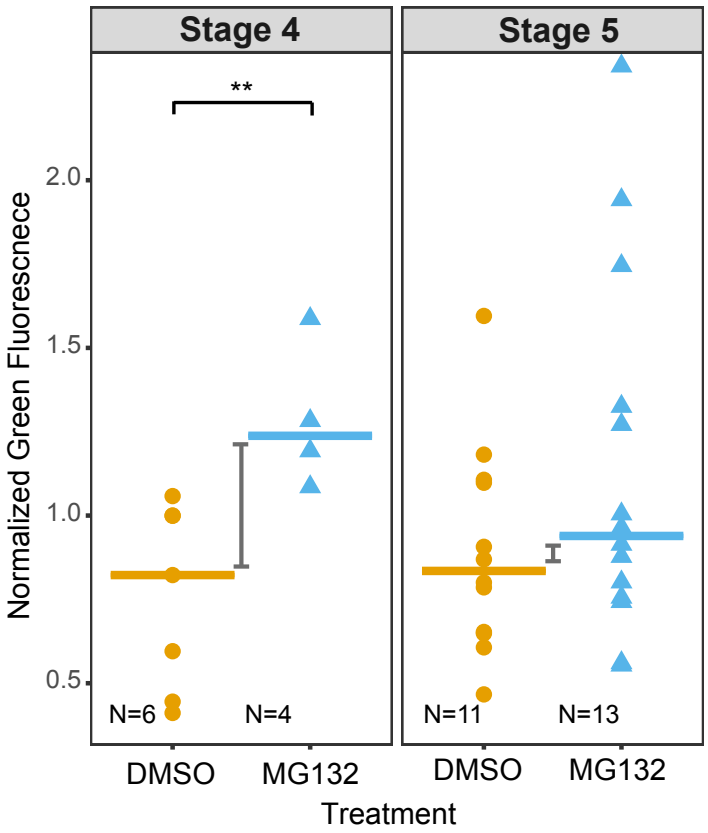
We have improved the writing in the revised version.

Main points:

- The finding that synthesis and degradation rates change over time is a key prediction of the study, however, there is no independent validation of this result. It would significantly strengthen the conclusion if the finding can be corroborated with a FRAP, photoconversion or alternative experiment at different developmental time points.

We agree that further work would be needed to validate whether there are temporal changes in production or degradation during development. Within the framework of this project, it is not feasible to experimentally address these points fully – as this would require significant new work that would represent a paper in itself. Therefore, we have re-focused the results section onto aspects of our results that are clearly supported by our data (pages 20 and 21, lines 460-473), and we have moved the more hypothetical aspects to discussion (page 23, lines 503-514). In addition, we have performed a proteasome inhibitor experiment to investigate the effects of stopped degradation at different stages of development. While we see a clear impact of the inhibitor at stage 4, we do not see a strong effect when injected during cellularization (see Figure below). This result is consistent with the idea of halted/ slowed degradation at this stage. However, we are uncertain about the extent to which cellularization has been completed in these embryos and whether this hinders access of the inhibitor to the cells. Given the situation that the literature is very divergent on the degradation rates and their changes during cycle 14**, we decided to remove any conclusions about degradation rates at this very specific time point, and to restrict our interpretation to the clearer results.

**–Liu *et al.*[1] determined the strength of the degradation by incubating purified Bcd protein with extracts from 0-1, 1-2 or 2-3 hrs old embryos that had been treated with CHX to inhibit translation. Bcd levels were then assessed in Western Blots. They find that degradation is similar in 0-1 and 1-2 hr embryo extracts, and then slows down significantly in 2-3 hr old embryos (this period corresponds to the cellularization of the blastoderm, during cycle 14). Drocco *et al.*[2], measured the Bcd degradation rate by a method based on photoconversion of Dronpa-Bcd on live embryos, and found it increases drastically at the beginning of cycle 14. Therefore, both of these approaches predict time-varying degradation rates, but with opposing reports for the behaviour of the Bcd lifetime in cycle 14.



Reviewer Response Figure 1. Bcd proteasomal degradation decreases in Stage 5 embryos. Stage 4 or Stage 5 embryos expressing the tFT-Bcd construct were injected with MG132 (orange) or DMSO (blue), and imaged on a confocal microscope 30 min later. Average GFP levels in the anterior third of the embryo were quantified from single, equatorial z-slices. The symbols indicate individual embryo measurements, and the lines signal the median of the distribution. The gray brackets illustrate the difference in GFP fluorescence at each embryonic stage. Statistical significance was assessed by a two-sample Wilcoxon test (Stage 4 p-value = 0.01, Stage 5 p-value = 0.30).

Furthermore, the authors should provide an explanation of the potential causes of the time-dependence, at least at the level of discussion.

We now introduce explanations for the temporal trajectory of the Bcd age measurements in the Results (page 19, lines 440-442), and Discussion (page 23, lines 528-540).

- It is not clear whether the consideration of time-dependence in the synthesis and degradation rates could affect the fit to the shuttling, RNA gradient and RNA diffusion models. The authors should comment on and address this point.

The fittings for model selection in Figure 1 were performed on data from embryos in

early nuclear cycle 14, assuming that the gradient was in steady state. This assumption seems to be challenged by our later results in Figure 6, where we observe that the production and degradation rates change during this stage of development. However, we do find a short period during nuclear cycle 14 where the system is roughly in steady-state – identified by the average Bcd concentration and age remaining relatively constant- and the fittings were done in this regime. We have added a panel, Figure 6C, where we highlight this observation, and a paragraph discussing it on the main text (pages 20-21, lines 466-473). To illustrate this, we have plotted the data from Figure 6C and D in a new format, that shows that the ratio is relatively constant for a period of time between nuclear cycle 13 and 14, presumably because the system is in a quasi-equilibrium. Of course, this steady-state is much shorter than previously thought, which could have consequences in the gradient interpretation, and as well on our fittings. See lines 542-551 (pages 23 and 24) for discussion in the manuscript.

We agree that introducing time dependence in these models would alter the fit quality. However, for the RNA gradient and diffusion models, it would still not be possible to fit the protein age using reasonable parameters – as these models predict curve shapes that are fundamentally different from what we measured. In the shuttling model degradation is not present, so a time-varying degradation rate cannot be included. The fact that we find that there is significant degradation and that this impacts the gradient is evidence against the idea that nuclear shuttling is sufficient alone for gradient formation. Of course, there is also time-varying Bcd production. However, for relatively slow changes in production this is unlikely to have a significant effect on protein age, as changes in protein production generally lead to only transient shifts in the tFT reporter, as shown in Theory Figure 1 in the Appendix, and the more radical stop of the productions happens in mid-late cycle 14, while the fittings of the models were performed in early cycle 14.

For the shuttling model, it is plausible that the diffusion constant changes over time, due to, for example, a change in the density of nuclei due to their doubling with each cycle. We have re-run the simulations considering decreasing time-dependent diffusion. We consider a simple phenomenological form for this time dependence to minimize additional parameters. We find that such time dependence does increase the fit quality, but the model is still not as good at explaining our observed data as the SDD model. We now include this analysis in Appendix Figure S12 and lines 516-526 (pages 22-23).

-As a key result, Fig. 3a should be extended or amended to demonstrate the sensitivity of the result on the model parameters. The comparison to Fig. 1e in the main text is obscure (for example Fig. 1e and 3a are in different units; Fig. 3a does not contain information about parameter ranges.)

We agree that Figure 3A is a critical result of the paper and requires further details. We have now split Figure 3 into two, with the fitting to the key data in Figure 3 and the perturbations shown in Figure 4. The revised Figure 3 now includes a panel showing the conversion of protein age into the tandem ratio (panel **B**) and a comparison of the fit quality of the different models (panel **C**). We have also extended the model fitting to test the tandem ratio from the fmCherry-sfGFP Bcd-tFT reporter (panel **A** inset). In this case, we used the fitted parameters to the mCherry-

sfGFP-Bcd-tFT reporter and only allowed the red fluorophore maturation rate and relative fluorescence levels to alter. The resulting fit of the SDD model is very good, though we note that the nuclear shuttling model is also a good fit within the experimental error. We have extended the discussion of these results (page 14, lines 305-310) and given further details of the simulation details in the Methods (pages 29-30, lines 693-712). See also response to below comment.

- It is unclear how the chosen maturation rates of mCherry and sfGFP affect the simulated mCherry/sfGFP ratio in the different models. Figure 3a, which captures the main result of the study, shows a minor difference in the ratio between the SDD and shuttling models. However, it is unclear whether the better fit of the experimental data to the SDD model depends on the choice of maturation rates. The authors should further provide a systematic explanation of how they connect the two step mCherry rate to an effective one of 50 min in the main text. And why do they use 20 min for sfGFP in the main text and report 27 min in the Supplementary information?

The maturation rates are important parameters in the data fitting. We now more rigorously incorporate uncertainty in the maturation rates into our model fitting. In Figure 3**B**, we simulate the effect of varying the maturation rates on the subsequent tandem reporter ratio. For the range 20-50 minutes – which is close to the apparent Bcd lifetime – the variability is largest. As we now show more clearly, this does not alter our key conclusion that the SDD model is the best fit to the data, since the predictions of the alternative models are qualitatively distinct. Importantly, the maturation times of sfGFP, mCherry, and fmCherry are all quite distinct so the tandem reporter works well. However, the uncertainty on the estimated maturation rates does increase the variability on our predictions for the Bcd (effective) diffusion coefficient and lifetime. In Figure 3**C**, we now show the fit quality for a range of fluorophore maturation rates.

We estimated a maturation time for sfGFP of 27 ± 2 min. The error in the estimated rates was assessed by likelihood profile analysis[4] and is now shown in Appendix Figure S6D-F. So long as for the three fluorophores used the maturation times are $\tau_{fmCherry} < \tau_{sfGFP} < \tau_{mCherry}$, our key result that the SDD model is the best fit to the data likely remains correct.

In all the fittings of the tFT-Bcd gradient, we used a two-step model for mCherry maturation (T_1=40 min, T_2 = 9 min). We have clarified the text in lines 281-284, as it was incorrect to infer this implies a maturation time of ~50 min, but we note that this did not affect our simulations as the chemical kinetics were correctly implemented in the fitting algorithm. The global maturation rate of mCherry is expected to be close to the limiting step of 40 min. We thank the reviewer for pointing out this mistake in the text.

- The authors should explain how the brightness (quantum yield, extinction coefficient) of the fluorophores are taken into account in the model fitting.

The brightness of the fluorophores is different between sfGFP and mCherry. However, precisely measuring this is challenging. When fitting the data, we have a scaling parameter, which effectively accounts for differences between the brightness

in the fluorophores. In our original submission we used the intensities of the sfGFP and mCherry profiles in the posterior (where we expect them to be similar) to estimate this parameter. However, this approach introduced bias, and so now the scaling parameter is kept as a fully free fitting parameter within the model. As with the uncertainty in the fluorophore folding rates, including such a fitting parameter does not alter our key conclusions, but it does increase the uncertainty in our estimations of the dynamic parameters. We have clarified this issue in the manuscript Results section (page 14, lines 305-310 and 319-320) and Methods (pages 29-30, lines 693-712)

- Fig. S5 does not contain data on the fmCherry maturation rate, as stated in line 171 of the main text. The rate should be measured in a comparable way to mCherry and reported.

We have now included the fmCherry maturation rates estimation in the Appendix Figure S6 (C and F-G). We have slightly expanded the description of fmCherry in the text (page 9 lines 185-187, page 12 255-257 and 272-275, page 13 291-294), and we have separated and expanded the figure on the maturation rates determination (Appendix Figure S6), showing now the likelihood profile analysis to assess the quality of the parameters estimation. Additionally, we have also included fitting of the alternative models for Bcd gradient formation to the tFT fmCherry-sfGFP-Bcd reporter data (Figure 3**A** inset).

Additional points:

- Fig. 1e - The authors should explain what parameter ranges are considered and how the standard deviations are derived. They should also comment on the nearly absent sd of the shuttling model. fig1d and e - explanation of what is plotted is needed.

We have extended the description of this figure, including discussion of errors. Lines 132-161, on pages 7-8.

- Line 180 - I guess fig. S4m,n is meant. A more detailed explanation of the effects of normalization should be provided within the main text, in particular because Figure 2 ii contains a normalized graph but the rationale behind this is not made clear.

As detailed above we have significantly extended our parameter fitting discussion (page 14 lines 305-310 and 319-320, pages 29-30 lines 693-712). We have also streamlined the Appendix Figures to make sure they are clearer in substantiating the results presented in the paper.

- Fig. 4 - it would be useful to add plots of the intensities and ratio over time for several positions.

We have included the data as suggested (Figure 5**C**) and updated the text (pages 17-18, lines 419-424).

- Fig 5a - which orange line?

Fixed (page 41, line 1002).

-It is unclear how and when the production changes in Fig. 5d - the text, figures and figure legends seem contradictory. In the text, it says that the production and degradation decrease (line 295), in the fig. legend to fig. 5d it says that the production increases gradually. Having the relevant plot of the rates that were used next to Fig. 5d would be useful to clarify this.

Thank you for the suggestion. Now the panels relating to the parameter changes are together with the former Fig 5d in the Appendix Figure S11(see our response to the first point). We have also corrected the mistake in the figure legend.

- Parameter units should be provided in supplementary tables.

Done as requested.

Reviewer #3:

I have three technical comments:
1. The authors might consider adding to the discussion a paragraph that treats the issue of steady-state in greater depth and what their findings might add to that aspect of the dynamics. The introduction mentions briefly that there is an ongoing debate, and the SDD model can incorporate time dependent parameters, but what have we learned from the current study about the fact that the gradient is never at steady-state and its implications?

We thank the Reviewer for this suggestion. We have now included a new figure panel that focus more on the issue of the steady-state (Figure 6**C**), and an explanation in the text (pages 20-21, lines: 466-473). Further, we have included a more detailed analysis in the Discussion, and linked our results with the broader debate about the dynamic state of biological systems, lines 542-551 (pages 23-24).

2. It was also not entirely clear from reading the manuscript how the authors thought about the degradation properties of Bicoid versus the fusion protein versus the tFT alone. Ultimately, we're interested in Bicoid's properties alone. But how close did we get to that in the current study? Is there still a correction necessary from what was measured with the fusion protein versus the Bicoid protein alone?

This is an important point and we agree with the reviewer that in the original submission these points were not sufficiently clear. Indeed, all our conclusions relate to tFT-Bcd. However, since the fusion protein is fully functional and embryos develop normally, it is reasonable to assume that we are studying an intact system and that differences between the tagged and the untagged Bcd are likely secondary effects.

Specifically:

1) The construct rescues the null phenotype, so it is fully functional, which for Bcd also means a “normal” gradient (Appendix Figure S3).
2) The measured gradients obtained from our constructs are comparable to previously published quantifications of the Bcd morphogen gradient.

3) The timing of maximum Bcd-sfGFP intensity is similar to previous reports[3]. Therefore, we are confident that the dynamics of our construct are close to wildtype conditions.
4) The control line with the tandem fluorescent timer without the Bcd protein has a completely different behavior, with very low degradation (Figure 2**E**).

We have now extended our discussion of the controls regarding the functionality of our tFT-Bcd reporter, lines 189-190, 198-204, 215-217, and a summary in lines 219-225 (pages 9-10).

3. Shells of embryos is not a nomenclature used in the field. If you need to use it, it should be defined.

Yes, this is a new nomenclature. We have now added a clearer definition in the figure legend (page 38, lines 933-936) and a more complete description on how they are produced on the Methods (page 28, lines 651-662). The "shells" are a way of processing 3D images for display purposes. They consist on 3D reconstructions of embryos where the external layer - the cortex - is kept, but the interior - the yolk - is digitally removed. We do this because all the events we study happen in the cortical region, and inclusion of the yolk adds noise and increases the image size.

Reviewer Response References

1. Liu, J., He, F. & Ma, J. Morphogen gradient formation and action. *Fly* **5,** 242–246 (2011).
2. Drocco, J. A., Grimm, O., Tank, D. W. & Wieschaus, E. Measurement and perturbation of morphogen lifetime: effects on gradient shape. *Biophys. J.* **101,** 1807–1815 (2011).
3. Little, S. C., Tkačik, G., Kneeland, T. B., Wieschaus, E. F. & Gregor, T. The Formation of the Bicoid Morphogen Gradient Requires Protein Movement from Anteriorly Localized mRNA. *PLoS Biol* **9,** e1000596 (2011).
4. Raue, A. *et al.* Data2Dynamics: a modeling environment tailored to parameter estimation in dynamical systems. *Bioinformatics* **31,** 3558–3560 (2015).
5. Kavousanakis, M. E., Kanodia, J. S., Kim, Y., Kevrekidis, I. G. & Shvartsman, S. Y. A compartmental model for the bicoid gradient. *Dev. Biol.* **345,** 12–17 (2010).
6. Spirov, A. *et al.* Formation of the bicoid morphogen gradient: an mRNA gradient dictates the protein gradient. *Development* **136,** 605–614 (2009).
7. Dilão, R. & Muraro, D. mRNA diffusion explains protein gradients in Drosophila early development. *J. Theor. Biol.* **264,** 847–853 (2010).

2nd Editorial Decision 25th July 2018

Thank you for sending us your revised manuscript. We have now heard back from reviewer #2 who was asked to evaluate your study. As you will see below, this reviewer is satisfied with the modifications made and thinks that the study is now suitable for publication.

Before we formally accept the study for publication, we would ask you to address the following remaining editorial issues:

REFEREE REPORTS.

Reviewer #2:

The authors have improved the manuscript and addressed my concerns.

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: Prof. Michael Knop
Journal Submitted to: The EMBO Journal
Manuscript Number: MSB-18-8355

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 justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

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

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

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?	For imaging experiments of wt embryos, we estimated that aproximately 5 embryos were enough to get robust results considering the low embryo-embryo variation. In experiments such as embryo injections or western blots, 3 replicates were performed.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	5-15 Drosophila melanogaster embryos were used per treatment
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	We've excluded embryos only based on their "health". On imaging experiments, we excluded embryos that failed starting gastrulation. On injection experiments, we discarded the embyos that were obviously damaged. These criterias were pre-established.
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.	Since all the embryos used were indistiguishable, ensuring no- subjective bias requiered no extra steps.
For animal studies, include a statement about randomization even if no randomization was used.	The allocation of embryos to the different treatments was random.
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.	Group allocation was random. When different treatments were assesed side-by-side, the investigator was blinded. However, the same person performed all steps, so might have remembered some information.
4.b. For animal studies, include a statement about blinding even if no blinding was done	When applicable, the experiments were performed trying to follow a blind protocol, but by the same person.
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.	The performed tests are non-parametric.
Is there an estimate of variation within each group of data?	We did not estimate within group variation.
Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
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/

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).	anti-GFP: rabbit, polyclonal, Abcam, catalog number: Ab6556.
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.	Drosophila melanogaster flys of the y/w background were used for all experiments. Embryos were 2-4 hs old, details on embryo collection can be found in the methods section.Embryos gender was not asessed. The genetic modifications are described in the text and methods. Stocks were kept at 18 deg, crosses and expansion of fly lines were performed at 25 deg, embryos lays were done at RT.
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.	Confirmed

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.	The models equations and parameters are described in the appendix section.

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.	No