

***In vivo* expansion of functionally integrated GABAergic interneurons by targeted increase in neural progenitors**

Rachel. E. Shaw, Benjamin Kottler, Zoe N. Ludlow, Edgar Buhl, Dongwook Kim, Sara Morais da Silva, Alina Miedzik, Antoine Coum, James J. L. Hodge, Frank Hirth and Rita Sousa-Nunes

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

1st Editorial Decision

2nd October 2017

Thank you for submitting your manuscript to The EMBO Journal. Your study has now been seen by two referees and their comments are provided below.

As you can see from the comments, both referees find the analysis interesting and suitable for publication here. They raise a number of different issues that I am presume you should be able to sort out in a good way. Given the referees' positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing the comments raised in full. I should add that it is EMBO Journal policy to allow only a single major round of revision and that it is therefore important to address the raised concerns at this stage.

REFeree REPORTS

Referee #1:

In this work Shaw and colleagues report the surprising and very interesting observation that the levels of the transcriptional modulator Prospero dictate neural stem cell behavior in *Drosophila*. They find that attenuating Pros levels with a specific RNAi-mediated manipulation can produce supernumerary neural stem cells, as expected, which however proceed to differentiate in a lineage appropriate fashion to give rise to supernumerary neurons. These neurons appear to integrate and function within the circuit indistinguishably from their wild type sisters. These findings are of great interest to the field as they suggest that different levels of Prospero regulate different aspects of neuronal lineage progression. They also make available a tool to potentially easily manipulate neuronal lineage size independently of neuronal subtype and fate acquisition. Finally, these data suggest one way by which expansion and reduction in neuronal lineages might occur during development and evolution.

Overall, the conclusions are of broad interest to the field and are appropriate for publication in the EMBO Journal. There are however a number of technical and textual concerns that need to be addressed to strengthen the major conclusions.

Major concerns:

The most significant concern is the almost sole reliance on a single RNAi line for most of the findings (Pros-RNAiLH). This line is said to reduce levels of Pros less than the other RNAi line used at the beginning of the study (Pros-RNAiKK). However the images of figure EV2 suggest - if anything - the opposite. More convincing data for the difference in down regulation need to be presented. Alternatively, the data should be supported by showing "LH" behaves more like "KK" or a classic Pros mutant in a Pros heterozygous background. In other words, the authors need to nail down the argument that this really is due to changes in Pros levels, especially as they cannot make direct correlations between Pros levels and single stem cell clone size. Perhaps also showing that Pros KK behaves more like Pros LH at 18 degrees would further support their argument. I may have missed this experiment, but I could not find such data in the manuscript. While I am inclined to agree with the interpretation of the authors, it is best to err on the side of caution and be as sure as possible, since this is the major finding of this work.

Minor concerns:

- Figure 3D: the authors state that Chinmo is expressed with the proper temporal order in "LH" knock-down cells. However the image shows persistence of Chinmo at 12h APF in LH conditions, but not wild type conditions.
- Figures 4D and 5: while the measurements show that the additional neurons have calcium transients similar to wild type cells and - interestingly enough - do not hamper locomotor activity, these are fairly crude measures of neuronal function and the statements about that in the results and discussion section need to be toned down accordingly.
- The fact that extra GABA-ergic neurons have no dramatic effects on circuit function has important implications for neuronal circuit wiring: it strongly suggests a degree of developmental plasticity that can tolerate significant changes in neuronal number with minor effects on circuit wiring. This means wiring specificity in neuronal circuits cannot possibly be solely mediated by deterministic recognition cues. The authors may wish to discuss these broader implications of their findings.

Referee #2:

Shaw et al. studied how Pros regulates certain lineages in the Drosophila brain and suggest that this process might cause differences in GABAergic neurogenesis in different species. By manipulating Pros levels with different RNAi lines and temperatures, the authors found that reducing Pros levels led to expansion of both progenitors and neurons in the lineages. They further suggest that the non-tumorigenic neurons resulted from the supernumerary NSCs were physiologically normal and did not affect sensory-motor integration and motor action selection, indicating that these neurons were functionally integrate into neural circuits.

While the overall concept is interesting, there are several missing links in the story.

First, there is basically no evidence that corroborates the authors' claim that the two prosRNAi lines (KK and LH) reduce Pro to different levels. Since this assumption is prerequisite for a major point of the paper, the authors should show -- either in vivo or in vitro -- Pros knocking down efficiency of the different RNAi lines.

Second, the authors show in figure 5 that supernumerary R neurons did not affect motor behavior, thus claiming that expanded GABAergic neuronal progeny is functional. To validate the behavior quantification, the authors should add a control experiment that demonstrates their experimental system is able to detect changes in these behaviors. For example, they may use flies with dysfunctional R neurons or apply optogenetics.

Third, while the authors speculate that Pros-regulated expansion of neurogenesis might be a mechanism for expanding neural circuits in evolution, alternative possibilities should be discussed.

Although the expanded number of GABAergic neurons may be functional as an ensemble, some of these neurons might not be functional. It is unclear to this reviewer how many neurons were quantified and how they were selected. It seems that there are only 5 samples in Figures 4D and 4E. The quantification and statistical analysis for data shown in these figures need to be strengthened.

"In vivo expansion of functionally integrated GABAergic interneurons by targeted increase of neural progenitors"

[Paper #EMBO]-2017-98163]

We thank both referees for their comments and helpful suggestions how to improve our manuscript. We have addressed all of the points raised and outline our response in detail below.

Referee #1:

In this work Shaw and colleagues report the surprising and very interesting observation that the levels of the transcriptional modulator Prospero dictate neural stem cell behavior in Drosophila. They find that attenuating Pros levels with a specific RNAi-mediated manipulation can produce supernumerary neural stem cells, as expected, which however proceed to differentiate in a lineage appropriate fashion to give rise to supernumerary neurons. These neurons appear to integrate and function within the circuit indistinguishably from their wild type sisters. These findings are of great interest to the field as they suggest that different levels of Prospero regulate different aspects of neuronal lineage progression. They also make available a tool to potentially easily manipulate neuronal lineage size independently of neuronal subtype and fate acquisition. Finally, these data suggest one way by which expansion and reduction in neuronal lineages might occur during development and evolution.

Overall, the conclusions are of broad interest to the field and are appropriate for publication in the EMBO Journal. There are however a number of technical and textual concerns that need to be addressed to strengthen the major conclusions.

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Reply: We appreciate the reviewer's point that RNAi always raises concerns of specificity and requires validation. The two *UAS-RNAi* lines utilized in this work are well validated in that they a) abolish detectable Pros antigen when induced at temperatures optimal for GAL4 activity; b) phenocopy *pros* loss-of-function mutations. They are thus widely accepted in the field and were also employed in the cited Narbonne-Reveau *et al.* 2016 study. Concerning, the differential strength of the 2 lines, we observed differential Pros levels within the targeted lineages (regions outlined by dotted line in EV2B) between *prosRNAi,LH* and *prosRNAi,KK* if animals were reared at lower temperatures (≤ 22 °C) such that anti-Pros staining was detectable in RNAi animals. Stainings were of course carried out in parallel for this comparison and imaged under the same conditions. The dark patches devoid of signal in the LH panel are where NSCs (Dpn⁺ cells) reside in the section; note that **in the Dpn⁻ cells**, anti-pros signal is generally stronger in the LH panel than in the KK. This observation is in good agreement with our functional data to which we have now added the suggested *in vivo*

experiment: showing that *prosRNAi, KK* at lower temperatures behaves more like *prosRNAi, LH* at higher temperatures: whereas *en>Dcr2, CD8::GFP* driving *prosRNAi, KK* at 29 °C led to a number of DAL cells that was impossible to count, this genotype raised at 25 °C or 22 °C led to accountable cell numbers, in a proportion of Dpn+/Dpn- (average of 485/1174 or 19/502, respectively) comparable to that of *prosRNAi, LH* at 29 °C or 25 °C (average of 819/1737 or 30/1222, respectively). This data has now been added as panel EV2C (to be compared with Fig. 2B).

Notwithstanding, we also made every effort to add *in vitro* data to this, by driving both RNAis with the pan-neural driver *elav-GAL4* and quantifying relative Pros levels by Western Blot analysis. In agreement with the *KK* line being stronger than the *LH*, *elav>Dcr2, prosRNAi, KK* brains were visibly enlarged compared with *elav>Dcr2, prosRNAi, LH* or *elav>Dcr2, cherryRNAi* controls. Normalization with a variety of usual loading control genes proved unsuitable. For example, probing the same blot simultaneously for Actin and Tubulin showed disparate proportions of these across genotypes, indicating that at least one if not both were unsuitable for normalization. Indeed, "normalization" attempts with a variety of proteins led to increased ratio of Pros in RNAi samples relative to WT, whereas we know there is less Pros per cell in RNAi genotypes (very clear by immunohistochemistry using the same anti-Pros antibody). We can only speculate about the reason for this. *elav>Dcr2, prosRNAi* broadly induces a fate transformation such that the WT low-NSC/high-neuron numbers ratio is reversed (high-NSC/low-neuron numbers) and if neurons and NSCs express different amounts of the genes used as loading controls they are unsuitable as controls.

Minor concerns:

-Figure 3D: the authors state that Chinmo is expressed with the proper temporal order in "LH" knock-down cells. However the image shows persistence of Chinmo at 12h APF in LH conditions, but not wild type conditions.

Reply: The image showed Chinmo expression in **neurons** (Mira-negative cells) at 12 h APF in LH conditions. Indeed, in both WT and expanded lineages Chinmo persists in early-born **neurons** but not in late **NSCs**. The image chosen for LH happened to show Chinmo+ neurons whereas the WT did not, which may have created confusion so we have replaced the LH image with one where deeper/early cells are not in the plane of view.

-Figures 4D and 5: while the measurements show that the additional neurons have calcium transients similar to wild type cells and - interestingly enough - do not hamper locomotor activity, these are fairly crude measures of neuronal function and the statements about that in the results and discussion section need to be toned down accordingly.

Reply: We have toned down the results and discussion section. The relevant results section is now subtitled "**Supernumerary R neurons are physiologically active**" and we changed the end of the section as follows: "Regardless of their position within the GCaMP6f-labelled pool of cells, all recorded neurons showed robust response to picrotoxin (**Figure 4E single cell traces**). Together these data suggest that downregulation of Pros in Ppd5-derived DAL NSCs can be tuned to expression levels that result in non-tumorigenic supernumerary progenitors which in turn generate increased numbers of lineage-specific GABAergic interneurons that are physiologically active." We also modified the discussion section entitled "Cloned neurons..."; the first two sentences now read: " Our proof-of-principle study demonstrates *in vivo* lineage expansion as a means to generate more neurons of defined identity that can integrate into neural circuitry. We show that supernumerary GABAergic ring neurons are physiologically

active and integrate into the ellipsoid body circuit without affecting motor behavior even when the animal is exposed to sensory stimulation like mechanical shock (**Figure 5**).

-The fact that extra GABA-ergic neurons have no dramatic effects on circuit function has important implications for neuronal circuit wiring: it strongly suggests a degree of developmental plasticity that can tolerate significant changes in neuronal number with minor effects on circuit wiring. This means wiring specificity in neuronal circuits cannot possibly be solely mediated by deterministic recognition cues. The authors may wish to discuss these broader implications of their findings.

Reply: This is an interesting possibility and we now added to the discussion: “This demonstrates that the nervous system of *Drosophila* can show considerable hysteresis in tolerating substantial changes in neuron number whilst maintaining network properties and functional output. A possible implication of this work is that the neural circuits studied may not wire together solely by deterministic recognition cues, but may be influenced by other currently unknown factors that might be even stochastic in nature (Hassan and Hiesinger, 2015). We believe our results can however be rationalized by...”

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Reply: We appreciate the reviewer's point that RNAi always raises concerns of specificity and requires validation. The two *UAS-RNAi* lines utilized in this work are well validated in that they a) abolish detectable Pros antigen when induced at temperatures optimal for GAL4 activity; b) phenocopy *pros* loss-of-function mutations. They are thus widely accepted in the field and were also employed in the cited Narbonne-Reveau *et al.* 2016 study. Concerning, the differential strength of the 2 lines, we observed differential Pros levels within the targeted lineages (regions outlined by dotted line in EV2B) between *prosRNAi,LH* and *prosRNAi,KK* if animals were reared at lower temperatures (≤ 22 °C) such that anti-Pros staining was detectable in RNAi animals. Stainings were of course carried out in parallel for this comparison and imaged under the same conditions. The dark patches devoid of signal in the *LH* panel are where NSCs (Dpn⁺ cells) reside in the section; note that **in the Dpn⁻ cells**, anti-*pros* signal is generally stronger in the *LH* panel than in the *KK*. This observation is in good agreement with our functional data to which we have now added the suggested *in vivo* experiment: showing that *prosRNAi,KK* at lower temperatures behaves more like *prosRNAi,LH* at higher temperatures: whereas *en>Dcr2,CD8::GFP* driving *prosRNAi,KK* at 29 °C led to a number of DAL cells that was impossible to count, this genotype raised at 25 °C or 22 °C led to accountable cell numbers, in a proportion of Dpn⁺/Dpn⁻ (average of 485/1174 or 19/502, respectively) comparable to that of *prosRNAi,LH* at 29 °C or 25 °C (average of 819/1737 or 30/1222, respectively). This data has now been added as panel EV2C (to be compared with Fig. 2B).

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Second, the authors show in figure 5 that supernumerary R neurons did not affect motor behavior, thus claiming that expanded GABAergic neuronal progeny is functional. To validate the behavior quantification, the authors should add a control experiment that demonstrates their experimental system is able to detect changes in these behaviors. For example, they may use flies with dysfunctional R neurons or apply optogenetics.

Reply: In consideration to this point, we silenced these neurons (driver: *en>Dcr2,CD8::GFP;tsh-GAL80*) by expressing a dominant-negative version of *Drosophila* Dynamin (*UAS-shi^{DN}*) or Tetanus-Toxin-Light-Chain (TNT), encoding an inhibitor of synaptic transmission (*UAS-TNT*). Unfortunately no adults eclosed so we were unable to perform behavioral experiments on them. However, we have indeed been able to detect significant behavioral changes upon EB circuit perturbation. Below is an example consisting of inactivation of the GABA-A receptor in EB ring neurons, which will be published elsewhere. We have now added a sentence in the results section (p.18) to this effect: “Whilst this assay can report differences in motor behavior upon EB circuit perturbations (<http://dx.doi.org/10.1101/100420>), analysis of controls and animals with supernumerary R neurons revealed no significant differences (Figure 5).”

Figure for referees not shown.

Third, while the authors speculate that Pros-regulated expansion of neurogenesis might be a mechanism for expanding neural circuits in evolution, alternative possibilities should be discussed.

Reply: We have now discussed alternative possibilities for expanding neural circuits in evolution by expanding the discussion section headed “**Cloned neurons can contribute to behaviorally relevant circuitry**” to include the following: "Several developmental and genetic mechanisms have been proposed for neural circuit evolution. These include inter-progenitor pool wiring whereby a fraction of neurons derived from one progenitor pool migrate away and integrate into a remote brain domain to establish new neuronal wiring (Suzuki and Sato, 2017). In our study, however, supernumerary ring neurons remain at their site of origin and send projections to the EB ring. These observations suggest another mechanism, namely duplication of an entire circuit module (Tosches, 2017). Lineage-related R neurons constitute layers of the ellipsoid body circuitry and thus can be regarded as ontogenetic clones that form a circuit module of the adult brain. The fact that the EB circuit can accommodate a range of cell numbers reveals a plasticity that might have promoted (and carry on doing so) evolutionary adaptation. In fact, the similarity in temporal marker expression between the DALv2 and v3 lineages lends itself to the hypothesis the two might have originated by duplication of an ancestral lineage that subsequently diversified projections and acquired different functions. Indeed, the primary tracts of DALv2 and DALv3 are juxtaposed before DALv3 bifurcates into so-called supra- and the sub-ellipsoid secondary axon tracts (Lovick et al., 2013). Such multiplication and functional reuse of an existing feature is a known process in evolution, called exaptation (Gould and Vrba, 1982). It has been suggested that whenever circuit duplication is followed by exaptation, the properties of the circuit would initially remain unaltered (Tosches, 2017). In accordance with this hypothesis, we do not observe gross alterations for the supernumerary ring neurons in their transition through the temporal cascade and the resulting molecular signature, such as GABA and Poxn expression; nor do we observe gross changes in their physiological properties or behavioral readout with the assay applied. It is therefore tempting to speculate that amplification of ontogenetic clones such as lineage-related ring neurons, followed by exaptation of the resulting circuits could be an adaptive mechanism underlying brain and behavioral evolution (Strausfeld and Hirth, 2013; Grillner and Robertson, 2016).

Although the expanded number of GABAergic neurons may be functional as an ensemble, some of these neurons might not be functional. It is unclear to this reviewer how many neurons were quantified and how they were selected. It seems that there are only 5 samples in Figures 4D and 4E. The quantification and statistical analysis for data shown in these figures need to be strengthened.

Reply: The spatial extent and density of the population, as well as the resolution of the acquired images, made it impossible to measure the activity of every neuron in the population. Individual cells were thus picked randomly from amongst the most visually accessible. No data were excluded from the analysis and representative examples were shown in the figures. In total we analysed between 50 and 59 random cells per brain for the control condition and 53 to 74 for *prosRNAi* brains, which is now also stated in the Methods (p. 29). However, for better clarity we only show 5 example neurons each in Figure 4E. We now show an additional figure with neuronal responses in a further pair of brains in EV5. There is biological variability in both control and *pros* knock-down brains, which we tried to convey in the figures by depicting a range of responses. We did not observe any non-responding cells in either control or mutant brains, but the referee is correct in saying that we cannot exclude that some neurons did not respond.

N~5 brains is customary in electrophysiology experiments (see for example Frank *et al.* (2017) *Curr Biol* 27:2381-8 and Enoki *et al.* (2017) *PNAS* 114:E2476-85; in flies and mice, respectively) and all the neurons in the brains consistently responded within a minute to picrotoxin. Figure 4D thus shows the average population response of 5 pairs of brains to the picrotoxin treatment demonstrating their similarity. We tested for normality using the KS test and since both columns passed ($p>0.1$) used an unpaired two-tailed t-test: $p=0.6068$ (Mann Whitney test also gives $p=0.4206$).

We have also added to to Figure 4 legend that “solid line is mean, shaded area is S.E.M.” and “unpaired two-tailed t-test: $p=0.6068$ ”.

Thank you for submitting your revised manuscript to The EMBO Journal. I have now heard back from the referees. As you can see below, the referees appreciate the introduced changes and I am therefore very happy to accept the manuscript for publication here. Before we can transfer the manuscript to our publisher there are a few things we should sort out. I have provided a link below so that you can upload the revised version.

REFeree REPORTS

Referee #1:

Overall, I am satisfied with the revisions the authors have made and their efforts to address the concerns of the reviewers experimentally and by appropriate textual adjustments. I have no further concerns to raise.

Referee #2:

The revised manuscript and point-by-point response have addressed my concerns. I support its publication in EMBO J.

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Rita Sousa-Nunes

Journal Submitted to: The EMBO Journal

Manuscript Number: EMBOJ-2017-98163R

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 authorship 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 χ^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?	Sample size was arbitrary
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	n/a
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No data was excluded from analyses.
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.	No randomization was used.
For animal studies, include a statement about randomization even if no randomization was used.	Control and experimental genotypes were selected by markers and treated in equivalent ways.
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.	Behavioural experiments were performed on 3 genotypes blind.
4.b. For animal studies, include a statement about blinding even if no blinding was done	Behavioural experiments were performed on 3 genotypes blind.
5. For every figure, are statistical tests justified as appropriate?	Yes. Data was checked for normalcy. When normal, significance was tested through an unpaired student t-test; when not, either with Mann-Whitney or Kruskal-Wallis tests (which is indicated in figure legends).
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes. Data was checked for normalcy. When normal, significance was tested through an unpaired student t-test; when not, either with Mann-Whitney or Kruskal-Wallis tests (which is indicated in figure legends).
Is there an estimate of variation within each group of data?	Standard deviation and standard error of the mean were calculated for each data set.
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-tur>

<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://jil.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).	Details provided for all antibodies used. Antibodies made for this study available upon request.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	n/a

* 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.	Strains of <i>Drosophila melanogaster</i> used are indicated and so are husbandry details.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	n/a
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.	n/a

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	n/a
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.	n/a
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	n/a
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	n/a
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	n/a
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.	n/a
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.	n/a

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	n/a
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)).	n/a
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).	n/a
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.	n/a

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.	n/a
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