# Ratiometric sensing of Pnt and Yan transcription factor levels confers ultrasensitivity to photoreceptor fate transitions in *Drosophila*

Sebastian M. Bernasek, Suzy S.J. Hur, Nicolás Peláez-Restrepo, Jean-Francois Boisclair Lachance, Rachael Bakker, Heliodoro Tejedor Navarro, Nicelio Sanchez-Luege, Luís A. N. Amaral, Neda Bagheri, Ilaria Rebay and Richard W. Carthew
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MS TITLE: Ratiometric sensing of Pnt and Yan transcription factor levels confers ultrasensitivity to photoreceptor fate transitions in Drosophila

AUTHORS: Sebastian M Bernasek, Suzy SJ Hur, Nicolas Pelaez-Restrepo, Jean-Francois Boisclair Lachance, Rachael Bakker, Heliodoro Tejedor Navarro, Nicelio Sanchez-Luege, Luis AN Amaral, Neda Bagheri, Ilaria Rebay, and Richard W Carthew

I have now received all the referees' reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees express considerable interest in your work, but have some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. Each of the referees asks for various issues to be clarified and makes constructive comments to improve the study. The key point to address is the evidence that the change in Pnt/Yan ratio is directly affecting the transition to R7 identity - this is articulated in Referee 3 Points 7 and 8. I would also highlight the various points that ask for more precision about how you describe the results and the interpretation of the statistical analyses. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision. If it would be helpful, you are welcome to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating your plans for addressing the referee’s comments, and we will look over this and provide further guidance.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost

in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

*Advance summary and potential significance to field*

The differentiation of specific photoreceptor (R) and accessory cells from undifferentiated progenitors during eye development is an almost clock-work process that proceeds under the control of short distance RTK signaling. The transduction of this signaling pathway depends on two ETS-binding transcription factors (TFs) with antagonistic functions: Pnt (the activator) and Yan (the repressor). An activator and repressor TF pair regulating (successive) transitions from progenitors into differentiated cell states would easily suggest a bi-stable type of control, in which a Yan1:Pnt0 progenitor state would transit into a Yan 0: Pnt1 R state. However, work from the authors' labs in 2015 using quantitative imaging tools, had already shown that the regulation of this transition would be subtler. In this paper, the authors carry out a detailed quantification of Pnt and Yan expression at single cell resolution in situ and, together with experimental manipulations and the aid of a biophysical model, propose a mechanism by which Pnt and Yan robustly control the progenitor to R cell transition.
Quantification of the Pnt and Yan nuclear levels (reported by a GFP:Pnt protein fusion and by an anti-Yan antibody) show that while progenitors maintain a stable Pnt:Yan ratio, their transit into R cells is associated to a "modest" two-fold increase in this ratio. Interestingly, when examined with single-cell resolution, this increase is associated to an increased heterogeneity in the ratio, as if the system were being transiently destabilized. In any case, the two-fold ratio increase does not agree with an all-or-nothing bi-stable switch type mechanism.
The authors next functionally test two points. First: globally the Pnt and Yan temporal expression profiles are correlated. Is there any cross-regulation among them? As the dose of Pnt increases from 1 to two, also does Yan. Importantly, the eyes of adult flies harboring half the normal complement of Pnt alleles (1 GFP:Pnt) are indistinguishable from those harboring a normal complement (2X GFP:Pnt). Also, when Pnt levels are compared in mosaic discs with clones harboring different doses of Yan, Pnt levels correlate with the amount (number of alleles) of Yan. These experiments indicate that Pnt and Yan indeed positively cross-regulate each other. Second: the authors alter experimentally the Pnt:Yan ratio, with the expectation that modulating this ratio will either promote differentiation (increasing the Pnt:Yan ratio) or maintain the progenitor state (decreasing the ratio). The overexpression of rasV12 activates the RTK pathway causing an increase in Pnt that results in a concomitant production of supernumerary R cells. The overexpression of an activated form of Yan produces a loss of R7 differentiation. Together, these results are taken to indicate that indeed the Pnt:Yan ratio controls the progenitor-to-R cell transition, with elevated ratios promoting it.
Up to this point, the paper has shown that the cross-regulation between Pnt and Yan ensures that their relative ratio is maintained throughout eye development despite broad variations in their nuclear concentration; and that modest increases in this ratio are functionally relevant. The next question, "how does this work", the authors address by using a biophysical model of kinetic binding-unbinding of two TFs competing for the same binding motifs.
This model updates a previous one by adding a critical and specific property of Yan: Yan multimerizes cooperatively on DNA –while Pnt binds as a monomer. This fact results in an ultrasensitive system that measures the ratio between Yan and Pnt and which responds almost as a switch to small changes in this ratio –compatible with the progenitor-to-R cell state happening with 2-fold Pnt:Yan change. By introducing this model, the authors are able to go from nuclear protein concentrations (or their ratios) to enhancer DNA occupancy and transcriptional output.
Globally, the system is robust to variations in absolute concentrations, because the positive feedback between Pnt and Yan ensures maintenance of their ratio and, as its output depends non-linearly on this ratio, acts as an ultrasensitive switch. The authors speculate that this regulatory architecture could be behind some other regulatory processes in other systems and discuss the prospect of investigating how signaling alters Pnt:Yan ratios.
The paper, combining quantitative imaging, experimental manipulations and modeling is clearly written and the conclusions reached are relevant and solid. The materials and methods section is complete.

*Comments for the author*

Minor comments:
1- P9. Noted that in the description of the RasV12 overexpression experiment, the authors describe their results as producing "a modest two-fold elevation of the Pnt:Yan ratio". Because the normal ratio between Pnt and Yan during normal R differentiation is two-fold also, this sentence is a bit confusing. Stressing the fact that this Ras1-induced two-fold ratio is specifically in progenitors may help. The authors should also mention that the model in which activation of the EGFR pathway leads to Yan turn-over (i.e. elimination) (Figure 4A) does not work, or very weakly during eye differentiation.

2- The transition from progenitors to R cells happens associated with a transient increase in heterogeneity (cell-based quantity) in the Pnt:Yan ratio followed by a reduction in heterogeneity. Any idea of why this is so? Relate to the process of induction? Or just an artifact derived from variation in where cells experience the transition that appear as "noise" in the measurements?

3- The experiment in which the Pnt:Yan ratio is decreased, by overexpression of a stable Yan seems less conclusive to me than the experiment in which the ratio is increased (i.e. RasV12). The number of resultant R7 cells is dramatically decreased (Figure 5C and Fig S7C) and one would expect these cells being still in the cluster in a "confused" or progenitor state. The authors could quantify in situ the number of cells remaining in the ommatidial cluster and check whether a cell corresponding to R7 is there but not differentiated (the experiment in S7C, in which a lack of Pros-positive R7 cells do not address the point of whether this cell is still there or has been lost. I guess that this loss/mis-specification should be noticeable as an abnormal phenotype in the adult eye?).

Reviewer 2

*Advance summary and potential significance to field*

In this work, Bernasek et al., investigate how cell fate switching occurs in the Drosophila eye. They are argue that the ratio in signal between two key transcription factors, Pnt and Yan, is critical in determining photoreceptor fate.
Quantitative imaging of both wild-type and mutant eye discs combined with detailed analysis is used to support this claim. They finish with a thermodynamic model to show that a relative small change in Pnt::Yan ratio is sufficient to induce ultrasensitive switching behaviour.

Overall, the central model of the paper is interesting and generally well supported.
Though the idea of ratiometric signalling is not new (e.g., Henderson et al., PLoS Biology 2019), this is a very clear, quantitative example within a developmental context. Therefore, it is likely to have significant impact on the field – both in terms of signal readout and robust eye formation.

*Comments for the author*

Below I outline comments to improve the manuscript.

1. My main issue is with regards the value of the ratio. The authors compare the intensity signals from two different readouts (GFP and antibody), which are normalised by the measured nuclear intensity. While the relationship between concentration and intensity is reasonable (and I believe the authors have justified in previous work), the constant of proportionality is likely different for Yan and Pnt even after normalising by nuclear intensity. Therefore, the reported ratio value of "2" is likely only qualitative. Indeed, the authors themselves highlight at the end of the results that they are not measuring absolute concentrations. This issue is important and should be highlighted much sooner in the manuscript. In essence the reporting of the changes in ratio values needs to be more circumspect, as there is an inherent uncertainty that is unknown.

2. Measuring ratios in biological systems is very challenging, as errors are compounded. For example, see the Knop lab's work on a ratiometric age sensor (Khmelinskii et al. Nat Biotech 2012). This should be further compounded by the use of log scales (e.g. Fig. 3B). Yet, the errors reported here in the ratios are remarkably small. I assume that they are calculating the "n" based on

number of cells, not replicas. As a minimum, the authors need to present the average ratio curves for each independent disc – how similar are the curve profiles? Showing the sample-sample variation would make clearer how reproducible the results are.
Currently, the errors seem almost too small to be believable.

3. The constant velocity of the furrow is used to infer time from the fixed images.
This seems a reasonable strategy, but I am somewhat surprised by the apparent temporal fineness in the resulting figures, e.g., Figure 1D. Looking at the spread of points would imply that the authors can infer time to subminute accuracy – I find that doubtful, given that even in the highly stereotypic early Drosophila embryo age can only be inferred to about 2 minute accuracy in fixed tissues. What is the typical error on a time estimate for each cell? This should be clearly stated and accounted for in calculating errors.

3. Pnt over-expression and Yan mutants were used to explore changes in cell fate. Is there a technical reason why Yan over-expression or Pnt knock-down was not investigated? At the moment, the results Figure 2 seem to be lacking the alternative scenarios – which would be important to fully test the ratiometric hypothesis.

4. “In contrast, R3/R4 cell fate specification was not impaired even though the Pnt/Yan ratio was slightly lower than normal (Figure 5E)”. I can’t see the evidence that any difference is significant (or in Figure S9). No statistical tests provided and they look very similar for the claim that the ratio dropped.

5. The strategy of using fixed images is necessary here. But, it does mean that potential oscillations within cells could be missed – especially if such oscillations were asynchronous with their neighbours, thereby being washed out by any average taking. Is there evidence to discount oscillatory behaviour, particularly in Yan?

6. At times, find the wording to describe results somewhat arbitrary. For example, “the overall trend of induction and decay was highly similar for the two proteins” – yet, looking at Figures 1D-F, the curves do have distinct behaviour, especially in the degradation part (i.e. not "highly similar"). Later, “modest” is frequently used to describe changes. But, this is ill-defined – the changes look reasonably large in the figures. Also, a “modest” two-fold change in ratio could represent a large change in concentration.

7. The cell fate change appears to be dynamic. Yet, from the presented data it is unclear on what timescale the actual cell-fate decision can occur. In the Discussion around “will bring about a compensatory change in the other factor, maintaining dynamic but approximately constant stoichiometry”, it would be good to include more detail on the predicted timescales for such dynamic behaviour.

Reviewer 3

*Advance summary and potential significance to field*

In this manuscript the authors explore the role that the levels of PnT and Yan play in the photoreceptor fate transitions in Drosophila. Along the paper the authors measure and perturb the levels of both transcription factors measuring the effect in the resulting differentiated cells in time. While these changes do not result in a natural change in the direction and details of the differentiation they provide interesting perturbations that point towards a requirement on the ratio between PnT and Yan for a healthy differentiation of the tissue.

Finally the authors sketch a mechanistic model to relate the observations with ultrasensitivity of differentiation to the ratio of PnT and Yan. The model is based on a previous model described in Hope 2017 that already included an ultrasensitivity switch. The new contribution of the authors to the model is the incorporation of PnT and the analysis of the resulting occupancy for different Yan/PnT ratios.

*Comments for the author*

1) The technique of translating space into time as the furrow advances is a smart way to obtain dynamical observations that otherwise would require live cell imaging. Nevertheless, it is hard to make statements on the dynamics of single cells, rather than an ensemble level. For instance when the authors claim that during the transition "R-cells maintained this elevated PnT-GFP for 2-4 hours" would it be possible that individual cells show non-constant differentiation dynamics of PnT but they are differentiating asynchronously? i.e. the plateau is an artifact of different number of cells differentiating at different times?
Similarly later on in the down-regulation of Yan" "R-cells also maintained this diminished level of Yan for 2 – 4 hours". Is it possible to make a more precise claim by actually measuring numbers of cells at different time points?

2) Along the text, the Kolmogorov Smirnov test is used in different instances to evaluate changes in the ratios. The KS test identifies if 2 sets of points come from different underlying distribution. Thus the test does not evaluate if the average has changed, but if actually the full distribution has changed (e.g. by changes in variance). The statements that the authors make with respect to these tests could be more accurate along the text. e.g. when the authors claim that there is no significance in the ratios, or when the claim that "greater cell-to-cell variation in the ratio coincided with the times when Pnt and Yan levels peaked", or "the median ratio values were significantly different".

3) In addition, this is important when it is claimed there is no statistical significance between ratios. If the number of points is too low, a non-significant KS result could mean that the number of points is too low to actually claim that there is a difference in the distributions. Which is different to claim that there is no change in the ratios PnT/Yan. One particular case is the figure 2E. I believe that the authors could make their case stronger by indicating the number of points included at each time point showed and/or plotting those points in the boxplot.

4) There is some apparently contradiction in how the speed of the transitions is reported. On one hand the authors claim that the transitions are very fast because the levels of Pnt in R cells is very different than the progenitor level.
Nevertheless, when the authors study the transitions (Fig. 3 C-G) they claim that cells with those levels of PnT and Yan are already available in the progenitors.
At which timescale are both claims compatible?

5) Along the whole document there is a continuous reference to a "stable" Pnt/Yan ratio e.g.: "The rapid shift in the Pnt/Yan ratio during cell state transitions suggested that the progenitor ratio becomes destabilized at the onset of these transitions". Similarly, in the discussion the authors discuss "robustness" of this ratio coming from the cross-activation. While the authors show a similar ratio preserved in different conditions, I am not sure that there is enough proof of stability claims. e.g. If it is the case that there is a GRN in charge of maintaining this ratio, could the authors show a strong correlation in the natural fluctuations of Pnt(Yan) e.g. in Figs. 3C-G?

6) Coming from outside the Drosophila expertise, I found the manipulations of ratios a bit confusing. When is the sevenless promoter expected to be active? What cells should it affect? Why are the levels of PnT expected in Sev>Ras to be affected only in the second wave of differentiation but not in the first one?
Similarly for Yan, the authors report a difference in the levels after the differentiation peaks ("Yan levels were weakly reduced in progenitor cells during the second transition period"), but in Fig 4C that difference is also observable before the peaks.

7) The pattern of R7 differentiation in Sev>Ras was interesting. The authors claim that the change in ratio is affecting the transition towards R7. Nevertheless, the ratio of cells progenitor/R7 is the same in both cases (around 4.5). So the increase in the ratio does not induce additional R7 cells, just creates messed up cells with unidentified expression patterns (while keeping the same progenitor/R7 ratio). Therefore can the authors still claim that the Yan/PnT ratio controls this particular transition?

8) In the YanACT model the authors observe a difference in the ratio for differentiated cells, but no difference in the progenitors. Is this compatible with the idea that changes in the ratio affect the transition outside of progenitors if the progenitors themselves are unchanged? In reference to question 4) Does the subpopulation of high PnT, low Yan disappear in this case?

9) The authors show that a mechanistic model is compatible with a fast transition between cell ratios that can be obtained with an increase in the cooperativity of Yan binding. This is shown in figs 6A vs 6B. Nevertheless, if a point is weakly occupied by Pnt in the non-cooperative case ([Yan 50nM, Pnt 75nM] in 6A), increasing the cooperativity of Yan should never result in a higher occupancy of Pnt (([Yan 50nM, Pnt 75nM] in 6B)). Are other parameters changed in addition to the cooperativity between both plots?

10) In the binding site description the authors include an ETS binding site. Was this essential in their result?

11) Is there any testable hypothesis of the model? e.g. ranges of binding affinities required for PnT or numbers of binding sites? The authors claim that "the model provides guidance on how to interpret the experimental data.", but I am not sure this is obvious, going back to the levels measured experimentally, the authors observe a high variability in the Yan/PnT ratios in individual cells e.g. the progenitor distributions in 6D spread far away from the 1:1 line. Is this compatible with a model that is so sensitive to these ratios?

12) In the model the authors performed stochastic simulations using a recursive graph-traversal algorithm. Can these simulations be used to show the expected noise in occupancy, or potential bistability between a Yan occupied state and a PnT occupied state? That could in principle show a certain robustness to change in external changes of Yan or PnT.

- Minor comments

13) Figures 6A and S10 seem to be duplicated.

14) Placement of occupancy probability formula is wrong, should be after "canonical ensemble:"

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**First revision**

Author response to reviewers' comments

**Response to Reviews**
All three reviewers were extremely positive about the rigor and impact of our work and offered a number of helpful suggestions. We have used these to improve the clarity and precision with which we describe and interpret our results. The changes we have made are highlighted in yellow both in the manuscript text and in the point-by-point summary below. Because Referee #3's comments 7 and 8 were singled out as the key points to address, we address them first.

**Referee 3 - Point 7.** The referee comments that the plot in Figure 4E suggests that the ratio of progenitor cells to R7 cells is the same in wild type (97/22 = 4.4) as it is in the $Ras^{V12}$ experiment (380/83= 4.6) and that therefore our conclusion that the altered ratio affects the transition towards R7 is not warranted.

We apologize for the confusion that our unfortunate use of the term "unidentified R cells" has caused and thank the referee for pointing it out. In the experiment referred to by the reviewer, the nuclear shape and position of the ectopic R cells are highly suggestive that they are extra R7s. But we decided to label them as "unidentified" R cells. We agree with the reviewer that this is confusing and in the revised version of Figure 4E, we have relabeled them "ectopic R7 cells". Affirming the rigor of this relabeling, in an independent experiment we confirmed the cell type designation by looking at expression of the R7 molecular marker Prospero (Figure S7A-C). We have added a new panel showing that on average, the $Ras^{V12}$ discs have three Pros-positive R7 cells per ommatidium (Figure S7D). This number is consistent with the numbers shown in Figure 4E.

Repeating the calculation of progenitor cells to R7 cells (endogenous + ectopic), the difference from wild type is now quite clear (380/107 = 3.6). It is also worth mentioning that the ability of Sev-Ras$^{V12}$ expression to induce ectopic R7 cells has been documented previously, as referenced in the manuscript (Fortini et al., 1992).

Thus, our data strongly support the conclusion that changing the ratio directly affects the R7 transition.
In addition to relabeling Figure 4E and adding Figure S7D, the following changes were made to the text:
p10. "When we looked at R-cell fate specification, we observed supernumerary R7 cells in the *sev>Ras$^{V12}$* eye discs (Figure 4E), consistent with the known ability of *sev>Ras$^{V12}$* expression to induce ectopic R7 photoreceptors (Fortini et al., 1992). These ectopic R7 cells were typically located beside R7 cells, expressed the R7 marker Prospero (Figure S7A,B,D) and had comparable Pnt/Yan ratios to R7 cells (Figure 4E).

Figure Legend 4E: "In *sev>Ras$^{V12}$* eye discs, there were ectopic R7 cells (blue)."

Figure Legend S7B "Some ommatidia in a *sev>Ras$^{V12}$* eye disc have ectopic R7 cells labelled "R" (white font) that are Pros-positive and located beside the endogenous R7 cells (labeled with yellow font)."

**Referee 3 - Point 8.** The referee asks whether the results of the Yan$^{ACT}$ experiment (Figure 5) tell us that changing the ratio in transitioning cells impacts fate transitions even if the ratio is unchanged in the progenitors.

Yes – this conclusion is supported by the complete loss of mature R7 cells (Figure S7) even though a handful of "young" R7 cells are initially observed at/near the window of fate transition (Figure 4). We speculate that these cells do not stably transition to mature R7 cells due to their inability to stably increase their Pnt:Yan ratios. We have added the following sentence to the paragraph on p10 to clarify: "Thus, preventing the ratio increase that normally occurs in transitioning R7 cells was sufficient to disrupt the transition, even though the ratio was unchanged in the progenitor pool."

In conclusion, the results of the Ras$^{V12}$ and Yan$^{ACT}$ experiments together make a compelling argument that the change in Pnt/Yan ratio directly affects the transition to R7 identity.

Below we address point-by-point all of the other comments made by the three referees.
**Referee 1** found the paper to be clearly written and the conclusions relevant and solid. Only three minor comments were noted.

1. The reviewer suggests clarifying that the two-fold ratio change in the Ras$^{V12}$ experiment refers specifically the progenitors, in order to distinguish it from the two-fold ratio increase that accompanies normal R differentiation.

We have adjusted the last sentence of the first paragraph on p10 to do this: "Thus, just as a two- fold ratio increase accompanies progenitor to R7 fate transitions in a wildtype eye disc, artificially inducing a two-fold elevation in the Pnt/Yan ratio in the progenitor pool causes additional progenitor cells to inappropriately transit to R-cell fates."
The reviewer also points out that the increase in signaling in the Ras$^{V12}$ experiment results in only a weak reduction in Yan levels and asks whether this means the mechanism in which EGFR signaling induces Yan degradation is not applicable (or is inefficient) in the eye.

This is an astute question that gets to the heart of why Ras$^{V12}$ "breaks" the mechanism that normally sets the ratio. First the mechanism of signal-induced Yan degradation diagrammed in Figure 4A is applicable to the eye (Rebay & Rubin, Cell 1995). Increasing signaling (with Ras$^{V12}$) should reduce Yan levels. However, increasing signaling also increases Pnt levels (as shown in Figure 4B). Under wildtype conditions, increased Pnt levels would be compensated by an increase in Yan levels to maintain the ratio (Figure 2). However, the elevated MAPK signaling induced by Ras$^{V12}$ prevents this compensatory response by increasing Yan turnover (the fact that Yan levels are lower than normal is consistent with this), and as a consequence of both increased Pnt levels and decreased Yan levels, the progenitor ratio is elevated and ectopic R7 fate transitions are triggered.

We have added this sentence on p9-10: Although Ras/MAPK signaling promotes Yan

degradation (Figure 4A) (Rebay and Rubin, 1995), the close to normal Yan levels suggests that the system "tried" to compensate for the increase in Pnt levels by increasing Yan production (Figure 2), but "failed" because the sustained MAPK activation induced by $Ras^{V12}$ prevented Yan protein accumulation.

2. The reviewer asks whether we think the transient increase in heterogeneity in the Pnt:Yan ratio that accompanies transition is related to the biological mechanism of cell fate induction.

We think the transient increase reflects the biology of how EGFR signals are transmitted and received. Photoreceptor R8 is the main source of activating ligand, and we speculate that the signal is initially received by a larger pool of neighboring progenitors and that variability in strength of signal received by these cells increases Pnt:Yan heterogeneity. We predict that from this pool, only a subset of progenitors activate EGFR signaling to sufficient levels to transit to R cells. Because the real time single cell resolution measurements of signaling levels needed to confirm these predictions are not yet technically feasible in our system, we have not explored this point further.

3. The reviewer comments that the experiments in which $Yan^{ACT}$ expression is used to reduce the Pnt:Yan ratio are less compelling than the experiments in which $Ras^{V12}$ is used to increase the ratio.

Because $Ras^{V12}$ induces ectopic transitions that can be followed at single cell resolution, we can document the appearance of ectopic R7-like photoreceptors. However with $Yan^{ACT}$, as discussed above in our response to Point 8 from Referee #3, we can document transitioning cells with aberrant Pnt/Yan ratios and the subsequent loss of R7 fates, but we cannot identify the "confused progenitors", as the referee refers to the cells that have failed to transition. There is simply no way to identify those few cells within the larger progenitor pool. The reviewer suggests counting the number of progenitors, with the expectation that there should be one additional cell per ommatidium, but given the progenitor pool is in vast excess relative to the number of specified R cells and is not made up of a stereotyped number of cells, the difference of one extra progenitor cell per ommatidium would be impossible to detect.

As the reviewer correctly guesses, the $Yan^{ACT}$-induced failure to negotiate R7 fate transitions produces adult eye defects in which this photoreceptor type is absent. In addition to Supplementary Figure S7C which uses a molecular marker of R7 fate to show the loss of the R7 cell type in the $Yan^{ACT}$ experiment, we have added the reference to the original paper reporting this phenotype in the paragraph on p.10: "If the serines and threonines targeted by MAPK are mutated in Yan, then signaling-induced Yan turnover is blocked (Figure 5A) and as a result photoreceptor fates are not induced (Rebay and Rubin, 1995)."

**Referee 2** found the central model of the paper interesting and commented that our work provides a clear quantitative example of ratiometric signaling in a multicellular developmental context that will have significant impact on the field. The reviewer made several helpful comments and suggestions.

1. The reviewer points out that because we are measuring Pnt and Yan levels with two different readouts (GFP and antibody), the constant of proportionality could be different even after normalizing for nuclear intensity, and that therefore the reported ratio values are likely only qualitative.

We agree that this is a limitation of our approach and as suggested we have highlighted it earlier in the text. While we do not claim that a ratio of 2 is a quantitative measurement, the most critical measurements and results center on the relative two-fold changes in the ratio that accompany fate transitions. Those remain valid quantitative metrics of the system, even without knowing the absolute protein concentrations that drive them.

We have modified a sentence on p5 where we first describe the approach to emphasize this point: "This enabled us to measure nuclear Pnt-GFP and Yan protein abundance simultaneously in individual cells; however it is important to note that our approach cannot measure absolute protein concentrations."

We also added a sentence to p8 to explain that ratio values are likely only qualitative: "Because we are not measuring absolute Pnt and Yan protein concentrations, and because even after normalizing by nuclear intensity the constant of proportionality may be different for Pnt (measured with GFP) and Yan (measured with antibody), our measurements are limited to assessing how the ratio values are changing relatively between sampled populations and between

time points."

2. The reviewer comments that measuring ratios in biological systems is challenging because errors are compounded, and that the errors we report are surprisingly small, in fact so small that they are difficult to believe.

The reviewer is correct that the reported sample sizes (in the thousands) refer to total number of cells aggregated across a handful of distinct eye discs (usually 3 or 4). In the Figure 3A example they mention, the black lines are the moving averages across all cells aggregated across all discs. No confidence intervals are displayed in that panel, but as an example, in Figure 3B we show the 95% confidence interval for the mean for each cell type. These intervals were computed by aggregating all measurements from all replicate eye discs, then performing 10k-fold bootstrapping within each frame of the sliding window to estimate the $2.5^{th}$ and $97.5^{th}$ percentile of the moving average. The reviewer correctly points out that this approach potentially obscures some variation between discs, so we have added two new panels, Figure S4E and S4F, to confirm that our conclusions are not affected by our visualization approach and to show the disc-to-disc reproducibility. Using the same data in Figure 3B, we used a two-stage hierarchical resampling approach that first subsamples the independent discs, then subsamples the cell measurements within them. All of the same trends are apparent in Figure S4F, albeit with wider confidence intervals. In Figure S4E, we plotted the moving averages separately for each replicate disc in Figure 3B. We feel these representative examples sufficiently demonstrate the rigor and reproducibility of our measurements and analysis, and so have not included disc-to-disc comparisons or changed the way we plot the data in the other experiments.

3. The reviewer comments that the way we plot our data gives the mistaken impression that we can infer time to subminute accuracy and asks us to state explicitly the temporal resolution. We have added the following sentence to the legend for Figure 1D: Although time appears continuous in these plots (see experimental procedures), temporal resolution is about two hours, i.e., the time between specification of each column of R8 cells.

4. The reviewer asked us to provide statistical tests supporting the statement "In contrast, R3/R4 cell fate specification was not impaired even though the Pnt/Yan ratio was slightly lower than normal (Figure 5E)" and in Figure S9E and S9F because the data shown do not appear strikingly different by eye.
In this experiment the data suggest that the Pnt:Yan ratio increases ~10 hours earlier among wildtype R3/R4 cells than in their Sev>$Yan^{ACT}$ counterparts. To support this conclusion further, we compared R3/R4 cells between $15 < t < 20$ h (the initial "delay" in upregulation of Pnt:Yan) as well as $30 < t < 40$ h (more mature R3/R4 cells). In both cases, a KS 2-sample test on the $\log_2$Pnt:Yan ratio yields $p<1e-5$.
This new analysis has been added to Figure S9 as panels S9I and S9J.
5. The reviewer comments that our use of fixed images means that potential oscillations within cells will be removed. This is a valid point that we discussed in our earlier paper (Peláez et al., 2015). We have opted not to repeat the discussion here.

6. The review finds our use of qualitatively subjective terms such as "similar" and "modest" was at times somewhat arbitrary. We have carefully reread the paper and reduced the use of the term "modest" and "similar". For example: we removed "modest" from the following sentence on p.9 : "Together these measurements suggest that a subset of progenitor cells elevate their Pnt/Yan ratio by approximately two- fold at transition points, and that this ~~modest~~ increase is sufficient to confer molecular competency for transitioning to a photoreceptor state" ; and on p6 we changed "the overall trend of induction and decay was highly similar" to "an overall trend of induction and decay was apparent for both proteins. "

7. The reviewer comments that the presented data do not make clear the timescale on which cell fate decisions occur. We modified the Figure 3A legend to clarify this: Cells marked as "Young R7" are the initial cohort of R7 cells that are present in the timespan of the first ten identifiable R7 cells ("young" R7s become "mature" Elav-positive R7s in about two hours). We opted not to elaborate further in the discussion.

**Referee 3** found several of our descriptions, interpretations and comparisons of results confusing

and lacking in accuracy as written and requested clarification of multiple points. The numerous adjustments we have made to address these are listed below.

1. Our description of fate transitions are based on our measurements of population dynamics. When we state that "R cells maintained this elevated Pnt-GFP for 2-4 hours", the reviewer asks whether the apparent plateau of elevated Pnt might be an artifact of different numbers of cells differentiating at different times.

We agree with the reviewer's assessment that it is difficult to make statements about single-cell dynamics given our measurement strategy. To directly answer the question posed, it is possible (and perhaps likely) that individual cells transition asynchronously. This amounts to measurement error in the "time" coordinate of each data point and contributes to our measurement error overall. Because we typically only plot dynamics for a given cell type (e.g. R1/R6), all data points within a given contour are visibly differentiating. Consequently, the leading plateau in Pnt/Yan levels is unlikely to reflect an increasing fraction of cells differentiating as time elapses. However, it is possible that relatively sparse sampling of R cells at very early developmental times precludes us from accurately measuring the macroscopic trend in those regions. Because measuring single-cell dynamics is beyond the scope of our study, we have not discussed this in the text.

2. The reviewer requests that our statements in the text with respect to the KS tests be more accurate -e.g. when we claim that there is no significance in the ratios, or when the claim that "greater cell-to-cell variation in the ratio coincided with the times when Pnt and Yan levels peaked", or "the median ratio values were significantly different". As the reviewer points out, a KS test does not evaluate if an average has changed, but if the full distribution has changed. For the analysis in Figure 3H and in Figure S8 we have replaced the KS test with a one-tailed Mann-Whitney U test; use of the KS test is appropriate for Figure 2E and has not been changed.
p.9 Nevertheless, the median ratio values were significantly greater in R cells and in all other cells (Figure 3H, $P$<0.001, one-tailed Mann-Whitney U test).
Figure 3H Legend : Asterisks indicate significant differences (P<0.001, one-tailed Mann-Whitney U test).

Figure S8D Legend: The *sev>Yan*$^{ACT}$ R7 cells show higher Yan levels (P<0.001, one-tailed Mann-Whitney U test), while Pnt levels are indistinguishable from wildtype (P=0.4, KS 2-sample test).

3. Related to point 2 above, the reviewer commented that when we interpret a non-significant KS result to indicate no statistically significant difference between ratios (Figure 2E) that we may just be sampling too few points.
KS tests are very sensitive when dealing with large numbers; because our experiments measure Pnt and Yan levels in hundreds and thousands of cells, the confidence in concluding differences/no differences in ratio is high.

4. The reviewer comments that our statement that R state transitions are rapid because the ratio is already elevated when we can first distinguish "young" R cells seems inconsistent with a subsequent statement in which we explain that in addition to the young R cells with high ratios, we can identify a subset of other cells at comparable time points that have similarly elevated ratios.

We have made a few modifications to clear up the confusion – briefly, and using R7 as an example, for each R7 state transition, signaling is received simultaneously in more than one progenitor cell. This means a few cells will increase their ratio, but only one of them, the one that sustains the ratio, will transition to a stable R7 state. Because our measurements use population-level analysis to infer individual cell behaviors, and because the spatial distribution of R8 cells that we use to convert to time axis gives us about 2hr resolution, we cannot define these events with greater precision.

p9. "Together, these measurements suggest that in response to inductive signaling, a subset of progenitor cells elevate their Pnt/Yan ratio by approximately two-fold relative to the average progenitor pool ratio, and that this increase is sufficient to confer molecular competency for

transitioning to a photoreceptor state. From this pool of competent progenitors, only the cell that sustains the elevated ratio will complete the transition to an R state."

5. The referee comments that our suggestion that progenitors maintain a dynamically stable Pnt/Yan ratio and that destabilization of the ratio marks the onset of a transition may not be adequately supported by the data. We respectfully disagree. We admittedly do not yet understand the mechanism (GRN as the referee refers to it) for the cross-regulation between Pnt and Yan that maintains comparable progenitor ratios under different conditions, and we agree it is an important future direction; however, it will involve extensive experiments that fall well beyond the scope of this study. Because we feel our suggestion that destabilization of the progenitor ratio marks the onset of transitions is strongly supported by the analysis shown in Figures 3A and S5, we have left the paragraph in question unchanged (p8 "The rapid shift in the Pnt/Yan ratio during cell state transitions suggested that the progenitor ratio becomes destabilized at the onset of these transitions. …").

6. The referee asks for further explanation of the experimental manipulations used to perturb the ratio. We have made a number of minor adjustments to clarify the points mentioned.

p9. $Ras^{V12}$ was transiently expressed in subsets of progenitor cells during the R3/R4 and R7 transition points by driving it with the *sevenless* (*sev*) promoter (Figure S6; (Fortini et al., 1992)). In addition to driving expression in subsets of progenitors during the second wave of R fate transitions, the *sev* promoter also drove stable expression in R3/R4 and R7 cells (Figure S6).

We also adjusted the Figure 4 and 5 Legends: Figure 4 "Red arrow marks time during which cells express $Ras^{V12}$", and Figure 5 "Red arrow marks time during which cells express $Yan^{ACT}$."

9. The reviewer suggests that parameters other than cooperativity must have been changed between the two plots because if a point is weakly occupied by Pnt in the non-cooperative case in 6A (50nM Yan, 75nmPnt) then increasing Yan cooperativity should never result in high occupancy of Pnt as shown in 6B (Yan 50nm, Pnt 75nm).

As described in the Methods section on p21, when increasing Yan cooperativity we also made a compensatory increase in the non-cooperative binding affinity of Pnt. We did this to keep the transition from Yan to Pnt occupancy centered about the 1:1 Pnt:Yan ratio line. The actual value of the ratio is meaningless, and so for ease of interpretation, we felt that re-centering the system about the 1:1 Pnt:Yan ratio line made cooperativity's sensitization to the Pnt:Yan ratio more visually apparent.

10. The referee asks whether including an ETS binding sites in the model was essential to the result.

Yes, based on our prior work (Hope et al., 2017 Biophysical Journal), inclusion of an ETS site is essential for modeling cooperative Yan-Yan interactions, and therefore essential for exploring how changes in the Pnt-to-Yan ratio, with or without Yan cooperativity, impact Pnt/Yan occupancy and transcriptional output.

11. The referee asks if the model generates any testable hypotheses.

The model is intended to provide a qualitative description of the system with a resolution appropriately matched to what has been experimentally measured. Namely, Pnt and Yan compete for a common set of binding sites, and SAM-SAM interactions yield a strong cooperative effect for Yan binding those sites.

The binding model is not meant to be a quantitative representation of the biological Pnt/Yan system, because the relevant binding affinities and binding site configurations have not been measured. Our primary takeaway from the model is that competition and SAM-SAM interactions are sufficient to yield a system in which the downstream transcriptional response is sensitive to the relative abundance of two transcription factors rather than the absolute nuclear concentration of either factor.

As for cell-to-cell variation in the Pnt:Yan ratio (for example in Figure 6D as mentioned by the

reviewer), it's important to remember that we are presenting a static snapshot of cells that likely differ slightly in their progression along the developmental time course. That means that many of the "progenitor" cells in the overlapping region (near the 1:1 line in the plot) may very well be on their way to becoming young R cells, but weren't yet identifiable as such during cell type annotation. Despite the variance of the two distributions presented in Figure 6D, we interpret the apparent multimodality as consistent with our proposed model of the Pnt:Yan system. Furthermore, all downstream transcriptional activity is presumably integrated over time, which should mitigate any short-term fluctuations in the relative binding site occupancy of Pnt and Yan.

12. The referee asks whether our simulations could be used to provide further insight into DNA occupancy by Pnt and Yan.

The simulations themselves are not stochastic in the sense that the potential of each individual microstate is explicitly calculated and is therefore deterministically fixed for a given pair of Yan and Pnt concentrations. The recursive graph-traversal algorithm efficiently enumerates each of the microstates. Since we already compute the probability of each microstate, we effectively have a discrete distribution over all microstates. Using that distribution, we can compute the variance of *NP* (the number of Pnt- bound sites) across all possible microstates, e.g.

$$var(N_P) = \sum_k (N_P - \langle N_P \rangle) p_k$$

where $p_k$ is the probability of microstate *k*. We acknowledge the variance would be of interest to compute, however it would require substantial refactoring of our simulation code because (1) $\langle N_P \rangle$ is not known until all microstates have been enumerated and (2) we do not store the individual probability of each microstate in memory because there are $3^N$ unique microstates. Furthermore, we agree this could help lend additional support to the notion of robustness to changes in concentrations of Yan or Pnt, but we also feel that robustness is also apparent (and easier to interpret) in the gradual vs switch-like transitions presented in Figures S11C and S11E.

13. The referee comments that Figures 6A and S10 seem to be duplicates.
Although the landscape appears identical, the two figures were plotted with different calculations, and so they are not duplicates. This is clearly stated in the text on p12 " In the absence of stabilizing SAM-SAM interactions, the landscape of overall binding site occupancy was identical to that obtained with the simple equilibrium model described earlier (Figures 6A, S10)."

14) Placement of occupancy probability formula is wrong, should be after "canonical ensemble:"
We have corrected this formatting error.

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Second decision letter

MS ID#: DEVELOP/2022/201467

MS TITLE: Ratiometric sensing of Pnt and Yan transcription factor levels confers ultrasensitivity to photoreceptor fate transitions in Drosophila

AUTHORS: Sebastian M Bernasek, Suzy SJ Hur, Nicolas Pelaez-Restrepo, Jean-Francois Boisclair Lachance, Rachael Bakker, Heliodoro Tejedor Navarro, Nicelio Sanchez-Luege, Luis AN Amaral, Neda Bagheri, Ilaria Rebay, and Richard W Carthew
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.

Reviewer 1

*Advance summary and potential significance to field*

I woud like to, first of all, appologize for my delayed review.
In the revised version, the authors have answered the three points I raised satisfactorily.

*Comments for the author*

None.

Reviewer 2

*Advance summary and potential significance to field*

I am satisfied with the changes.

*Comments for the author*

I am satisfied with the changes.

Reviewer 3

*Advance summary and potential significance to field*

The authors have addressed successfully my comments.

*Comments for the author*

None