RE: Manuscript ISCIENCE-D-18-00009

Dear Dr. Tonzani,

On behalf of all authors, please find attached a revised version of our manuscript ***“Mean-Independent Noise Control via Intermediate States”*** - ISCIENCE-D-18-00009. We thank you for your attention to our manuscript and the reviewers for their comments. We have edited the manuscript accordingly. Below we provide a point-by-point response to the reviewer comments.

Here is a brief overview of the major changes:

1. We clarified the motivation and background for RMF and made it more precise on how the model incorporates RA-RAR and downstream regulations of Crabps.
2. We added an analytical calculation of the master equation formulation for SM to show the mean-independent noise control mechanisms remain valid beyond the SDE approach.
3. We added the suggested citations by the Reviewer 2, and clarified several points suggested by the Reviewer 1.

We hope that these revisions satisfactorily address all of the comments made by the reviewers. We thank you for your attention on our manuscript.

Sincerely yours,

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**Responses to Reviewer 1**

*Point 1: The authors propose a mechanism, independent of the concentration of RA, which is able to generate an intermediate state that reduces the RA noise. This mechanism relies on the modulation of crabp-activity. They provide theoretical models concluding that proportional-reversibility enables mean-independent noise in a short time-lapse (100s). Although this is sound, it is a bit difficult to understand how this would biologically work. How a tissue under continuous challenges such as cell proliferation and morphogenesis can maintain such a refined control of crabp-activity over time? Do cells continuously compute crabp-activity? What is the minimum crabp-activity required?*

**Response:** Thank you for pointing this out. There are two Crabps (Crabp1 and Crabp2) in mammals and four in zebrafish (Crabp1a and b; Crabp2a and b). Some work in cultured cells suggests that the two Crabps in mammals serve at least partially different functions, both transport RA within the cell but Crabp1 may serve more to hold it in the cytoplasm while Crabp2 transports it into the nucleus. Our experimental results (Cai et al. 2012) have shown that only one of the four genes in zebrafish, Crabp2a, is induced by RA and appears to be required for robustness and noise attenuation. So cells can control Crabp2a production based on the RA levels they detect. This regulation is incorporated into the RMF model for which mean-independent noise control is shown to hold. We have clarified our introduction of the RMF model in the last paragraph of section one to highlight this point. There is also the dynamic on-off rate of Crabp binding to RA and its regulation, but little is known about that mechanistically and as far as we know the minimum Crabp activity required is unknown.

*Point 2: In the same line than before, which are the candidate regulators of crabp-activity? Eph/Ephrin has been extensively reported to play an important role in the sharpening of gene expression boundaries. Is crabp-activity downstream of Eph/Ephrin? Or the other way around?*

**Response:** RA regulates Crabp2a. Eph/Ephrins are initially downstream of Crabps but certain Crabps do become localized to specific rhombomeres (see Cai et al., 2012) and at later stages may also be required for Eph/Ephrin expression to remain restricted to those segments. However, this regulation would be indirect. The RMF model shows that the mean-independent noise control property is preserved when downstream signals regulate Crabps. We’re aware that Eph/Ephrin mediated cell-cell sorting is an important element in boundary sharpening as well. In the revision, we cited Wang et al. 2017 to note this point in the last paragraph of Page 8.

*Point 3: Considering that crabp proteins are expressed in a non-restricted manner in the hindbrain and RA concentration changes along the AP axis, how the authors can explain the robustness of the system in the generation of sharp gene expression in all the boundaries along the whole antero-posterior axis?*

**Response:** That is a good point. There are other factors involved, particularly for more anterior rhombomeres (r1-3) where RA levels are low and the gradient is shallow. In addition, expression of some Crabps does eventually become restricted to particular rhombomeres. We know the most about the r4/5 boundary and this is where we have looked at noise attenuation. We added a discussion point about the limitations of the model to the end of the second paragraph of the discussion section which highlights these points.

*Point 4: In Figure 3, the authors propose that changes in the amount of BP affect the sharpness of the RA signaling boundary w/o changing its location. Does this approach account for cell division? We know that at these stages cells undergo extensive proliferation. How does dynamics of cell proliferation impact in the position of the landmark locations? Can they include this analysis?*

**Response:** Thank you for pointing this out. Cell cycle length has been shown to average 3-4 hours around the time of boundary sharpening (Kimmel et al., 1994). Our model in Figure 3 looks at the variance in a 100 second snapshot of the formed gradient to match the experimental setup of Sosnik et al. 2016. The gene regulatory noise in this system is caused by quick transient interactions and thus acts on a much faster time scale than the full developmental process. To make this point clearer, we clarified the setup of this investigation in the Supplementary Material (SM) section titled “Numerical Parameter Search in Spatial Knockdown Experiments”.

**Responses to Reviewer 2**

*Point 1: The first simple model is presented using a SDE framework. Since the system is linear and a more general master equation framework should also be applicable. It would be of value to showcase that the proposed hypothesis still holds under a more general master equation framework ( and I think it should be). In X Wang, B Errede, TC Elston Biophysical journal 94 (6), 2017-2026, it is shown that mean, variance , and covariance can be calculated.*

**Response:** Thanks for the suggestion. In the revision we have added analytical calculations for the mean, variance, and covariance of the master equation form of SM and show that the mean-independent noise control mechanism remains true. We added this calculation in the Transparent Method File with a new subsection, along with a note in the revised main text.

*Point 2: More recent work on noise enabled state transitions in multistable systems should be acknowledged:Wu et al, PNAS 110 (26), 10610-10615 (2013); F Wu, RQ Su, YC Lai, X Wang, eLife 6 (2017)*

**Response:** Thank you for pointing us to these references. These citations have been added to the Introduction section as an additional motivation for studying noise control.