Shadow enhancers modulate distinct transcriptional parameters that differentially effect downstream patterning events

Peter H. Whitney, Bikhyat Shrestha, Jiahan Xiong, Tom Zhang and Christine A. Rushlow
DOI: 10.1242/dev.200940

Editor: Thomas Lecuit

Review timeline
Original submission: 12 May 2022
Editorial decision: 12 July 2022
First revision received: 10 August 2022
Accepted: 6 October 2022

Original submission

First decision letter

MS ID#: DEVELOP/2022/200940

MS TITLE: Differential integration of activation and repression signals in a multi-enhancer system

AUTHORS: Peter H Whitney, Bikhyat Shrestha, Jiahan Xiong, Tom Zhang, and Christine A Rushlow

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.

The overall evaluation is positive and we would like to publish a revised manuscript in Development, provided that the referees' comments can be satisfactorily addressed. Please attend to all of the reviewers' comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so. 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.

Reviewer 1

Advance summary and potential significance to field

In this manuscript the authors use CRISPR, imaging approaches and modeling to test the roles of 2 shadow enhancers in the activation of the sog gene in the Drosophila embryo. They test properties of the enhancers in driving transcriptional activation, and link differences in their activity/timing to Sog function in formation of the Dpp gradient. Modelling suggests that together the 2 enhancers have a lower than predicted output in the mesoderm, which the authors propose is due to a repressive interaction of the distal enhancer on the proximal one.

Overall, I found the data to be of high quality and the paper is well written. I like the idea proposed that shadow enhancers could evolve based on their ability to modulate distinct transcriptional parameters.

Comments for the author

Major comments:

1) My main criticism relates to the interpretation of the data in Fig 6. In line 335, for Fig 6C the authors write 'Due to the continued production of Sog protein, we see a narrowing of the pMAD domain'. However Wang and Ferguson (Nature) provided good evidence that Sog establishes a shallow BMP gradient early then pMad refinement is due to bistability of BMP receptor interactions as a result of positive feedback.

As positive feedback appears to be the main driver of refinement, rather than continued production of Sog, I am not totally convinced by the authors' claim that the pMad staining results are 'well explained' by the MS2 data on the timings of sog transcription (this idea is repeated in the paragraph starting on line 456). Staining for eiger and cv-2, which are key to positive feedback, would help the authors clarify the extent to which positive feedback is also disrupted.

2) I also found the description/interpretation of the target gene expression pattern changes in Fig 6E confusing. The legend says nc14 embryos – are these mid or late nc14? I am assuming the latter since hnt is expressed and all the embryos appear to be around the same stage, except the wildtype control embryo for hnt in Fig. 6E which looks a little older than the others. In line 367 for hnt the authors write that 'with deltaPsogMS2 patterns appearing nearly wildtype, and deltaDsogMS2 embryos showing a pattern similar to, but stronger than deltaPdeltaDsogMS2'. To me, hnt looks similar in deltaDsogMS2 and deltaPsogMS2 embryos, and neither is nearly wildtype. Is the interpretation not that ush is active first so the expression patterns observed at late nc14 in Fig 6E reflect the mid nc14 pMad widths for deltaP and deltaD (so deltaP is broader) due to previous activation at mid nc14 and mRNA stability? In contrast, the hnt pattern reports the late nc14 pMad widths in in Fig 6D as it is only activated late, so deltaD is broader due to lower pMad refinement? The authors may be arguing this for ush but it wasn't stated very clearly to me. Also, it may be possible to gain support for this hypothesis by staining for ush with intronic and exonic probes at late nc14, as active transcription (visualized by intronic probes) may refine to be more similar to the late nc14 pMad widths, despite a broad domain of stable mRNAs (exonic probes) based on earlier activation.

I also have the following minor concerns:

1) For all figures with quantitation, the authors should report in the legends how many nuclei are analysed and from how many embryos? I could not find this information. Also please clearly state the embryo ages where appropriate.

2) In the paragraph starting on line 108 the authors describe how they replace the proximal enhancer with a previously described neutral DNA that is depleted for binding sites for early fly TFs. The main text does not mention replacement of the distal enhancer with RFP, although the legend says that this was to aid screening. The authors should mention this replacement in the main text, with the lengths of the distal enhancer deleted and the inserted RFP, after describing the proximal enhancer replacement.

3) Does the addition of a promoter (within 3x3P-RFP) in place of the distal enhancer complicate the findings? It seems plausible that the RFP promoter could compete with the sog promoter for the proximal enhancer reducing sog transcription. Is RFP RNA detectable in a sog-like expression pattern?

4) Line 118 - deltaPdeltaDsogMS2 failed to produce any homozygous flies – do the authors mean that no male flies were viable?

5) Fig 2D – I think the y-axis should have negative values rather than 2 sets of positive values.

6) From the data in Fig. 5C, the authors conclude that in the ventral bins the rate of activation but not deactivation is well predicted by the model. Is the rate of activation/deactivation the steepness of the slope on each side of the curve? If so, for the ventral most bin it seems that the

rates of activation and deactivation are both predicted well by the model, but it is the proportion of active nuclei that does not reach the level predicted. Can the authors comment on this?
6) In line 371 the authors say 'changes in the onset and rate of transcription of sog have specific and defined consequences in the selection of dorsal fates'. This statement is too strong as the authors have not actually looked at dorsal fates, they have only stained for 2 target genes. Dunipace et al (PLOS Gen) have quantitated amnioserosa fate when each of these enhancers is deleted – the authors should cite this paper, which also showed pMad data for the deletions. Do the amnioserosa counts from the enhancer deletions fit with the results presented in this study?

7) Line 676 – 'Foci were assigned to single nuclei by finding the nearest nucleus in 3D space to each focus' – please clarify how this was done.

Reviewer 2

Advance summary and potential significance to field

Review: Whitney et al., "Differential integration of activation and repression signals in a multi-enhancer system"

The authors investigate how multiple seemingly redundant enhancers co-regulate transcription.

For this, they use the sog locus as a model and quantitative imaging in early Drosophila embryos. At this stage, the expression of sog is regulated by two distinct enhancers, located 20kb upstream of the sog promoter (called sog distal) and a proximal enhancer that lies within the first intron (proximal enhancer). This locus is interesting as it is dynamically regulated along the dorsal-ventral axis, with a repression in the mesoderm.

To disentangle the role of each enhancer at the sog locus, the authors used Crispr gene editing to delete each enhancer separately or both, as well as to insert an MS2 cassette. First, they score the ability of these enhancer deleted alleles to sustain sog expression and full development. Next, they investigate sog nascent transcription when the promoter is regulated by the primary, the distal alone or both, and this in various domains along the dorso-ventral axis. For this they use fixed imaging and quantify the number of active nuclei in the mesoderm and in 3 other regions in the neurogenic ectoderm. This analysis is nicely complemented by live imaging with the MS2 reporter to extract transcription dynamics upon deletion of the proximal or distal enhancer. Finally, the authors assess the functional consequences of tuning sog spatio-temporal expression with enhancer deletions on dpp signaling pathway.

Major strengths of the manuscript:

1) the model gene is an exciting choice as it allows to decipher the role of a given enhancer in different locations of the pattern.
2) From a biological point of view, it's exciting to see manipulation of enhancers at the endogenous locus.
It's fantastic to be able to relate quantitative aspects of transcriptional control to phenotypic consequences in terms of signaling.
3) The main fining is that two seemingly redundant enhancers, act in a complementary manner, with different sensitivities to repression. The combination of these two actions is necessary to shape the final sog pattern.

This is an exciting paper with conclusions well supported by the data. The paper is clearly written and the figure well organized. Conclusions of this paper are of immediate interest to many people in the field of gene expression. I recommend this paper to be published with minor corrections.

Comments for the author

Here are my specific questions and recommendations (very minor):

1.To better rationalize why the primary enhancer is more sensitive to sna-mediated repression in the mesoderm than the other enhancer, it would be interesting to show Sna binding profiles to the two enhancers.
Such data should be available from published chip-seq (in wt or ventralized embryos).

2.Did the author keep track of the parental origin of the MS2 allele?
It would be interesting to mention whether the parental origin of the MS2 allele (maternal or paternal) influences the results. I assume the authors only focus on female embryos (with 2 dots) but it would clearer to state this in the methods.

3. To compare the overall activation dynamic, I recommend showing the % of activation of the pattern in the various conditions, averaged over all nuclei in the main manuscript.
These results are currently shown in Figure S4, but not within the same graph. Comparing the % of activation curves between the different alleles would inform on whether the initiation lag time (between mitosis and 1st activation) is different between the two enhancers.
It would be interesting to examine if the distribution of these waiting times exhibits the same shape between the different alleles (see Lammers et al., 2020; Dufourt et al., 2018).
Moreover, the slope of such curves could give the speed by which the pattern is filled (synchrony). Comparing these slopes should be informative as well.

4. To quantity the activity of each enhancer, the authors use ton*loading rate for each nucleus (Figure 4F). It would be interesting to back-up these estimates with results already obtained by the authors with the different sog alleles. Either the smFISH presented in Figure 2: by counting single ‘’cell’’ mRNA numbers.
However, single RNA counting might be challenging if the imaging did not include the entire ‘pseudo-cellular’ volume and if the numerous sog transcripts form clusters. An alternative could be the quantification of single nuclear mRNA output through the integral amplitude from live imaging data.

5. While I agree with the main conclusions and appreciate the discussion, I believe that speculating about the chronology of events is challenging. A title as ‘first come, first serve’ could be nuanced, or a sentence could be added to highlight the need for a dynamic observation of enhancer-promoter interactions (with live imaging). The analysis of the lag time between mitosis to first activation (see point 2) might help understanding if the action of a given enhancer comes is faster than the other one.

Technical points

- The accuracy of Ton detection (Figure 3) depends on the detection limit with the imaging settings used.
Could the author estimate this detection limit and discuss its influence on the main conclusions of the live imaging data?

- line 232: when the authors mention ‘loading rate’, do they mean ‘initiation rate’ (kini)?
If these metrics refer to the same process, the authors should mention it for clarity.

- Figure 2: The authors mention that they focus on nuclei with only one clear TS and discard those with 2. How to the authors deal with sister chromatids?

Figures:
- Figure2. To facilitate the reading of panel D of Figure 2, the figure should include the metric: ‘’Intensity TS1/Intensity TS2’’. Some ‘-‘ signs are missing in the y axis of Figure 2D.

- Figure 3, panel A: if splicing is co-transcriptional, I would expect the scheme of the transcript positioned at exon 2 and 3 (above the text number 3) not to have MS2/MCP signal.
If the authors have evidence of a different splicing dynamics, it should be mentioned in the text.

- Figure legends should include statistics: number of nuclei, movies, embryos, different FISH experiments etc.

Minor points

- typo line 160: '…the two enhancers regulate transcriptional output differentially active across sog''. A word seems missing before 'active'
- typo line 220 : reference to fig 4 instead of fig3
- potential typo line 261: 'Curiously, the only point where WTsogMS2….'

Reviewer 3

Advance summary and potential significance to field

The authors use live imaging to measure the regulation of a key patterning gene, Sog, by two separate enhancers in the early Drosophila embryo.

Comments for the author

The authors provide an interesting analysis of how two enhancers interact and collectively regulate the transcription of a single target gene. The paper uses CRISPR-Cas9 manipulations of the endogenous Sog locus to delete enhancers and simultaneously insert MS2 loops to measure the impact on transcription. The analysis of live cell imaging is rigorous and provides new insights concerning enhancer interactions. For example, the authors present evidence that the Sog distal and proximal enhancers drive synergistic repression by Snail in the ventral-most regions of the embryo. They also explain how changes in Sog transcription influence the dorso-ventral patterning network.

The manuscript is clearly written and the authors do a good job of describing their findings in a developmental context. They nicely describe where the relevant signals are located within the embryo and how these signals are integrated at the level of the enhancers and Sog transcription. Some of the findings were recently reported by the Stathopoulos lab, however, I believe this paper merits publication in Development since it contains better quality data, better analysis and a better story.

First revision

Author response to reviewers' comments

We thank the reviewers for their support of our study and insightful suggestions, and have addressed their comments and suggestions below (see black type).

Reviewer 1 Advance Summary and Potential Significance to Field:
In this manuscript the authors use CRISPR, imaging approaches and modeling to test the roles of 2 shadow enhancers in the activation of the sog gene in the Drosophila embryo. They test properties of the enhancers in driving transcriptional activation, and link differences in their activity/timing to Sog function in formation of the Dpp gradient. Modelling suggests that together the 2 enhancers have a lower than predicted output in the mesoderm, which the authors propose is due to a repressive interaction of the distal enhancer on the proximal one.

Overall, I found the data to be of high quality and the paper is well written. I like the idea proposed that shadow enhancers could evolve based on their ability to modulate distinct transcriptional parameters.

We thank the reviewer for supporting our study. We have changed the title of the paper from "Differential integration of activation and repression signals in a multi-enhancer system" to "Shadow enhancers modulate distinct transcriptional parameters that differentially effect downstream patterning events," as the reviewer nicely summarizes our last Discussion point.

Reviewer 1 Comments for the Author:

Major comments:

1) My main criticism relates to the interpretation of the data in Fig 6. In line 335, for Fig 6C the authors write 'Due to the continued production of Sog protein, we see a narrowing of the pMAD domain'. However, Wang and Ferguson (Nature) provided good evidence that Sog establishes a shallow BMP gradient early, then pMad refinement is due to bistability of BMP receptor interactions as a result of positive feedback. As positive feedback appears to be the main driver of refinement, rather than continued production of Sog, I am not totally convinced by the authors' claim that the pMad staining results are 'well explained' by the MS2 data on the timings of sog transcription (this idea is repeated in the paragraph starting on line 456). Staining for eiger and cv-2, which are key to positive feedback, would help the authors clarify the extent to which positive feedback is also disrupted.

We take the point that much of the refinement is driven by a bistable feedback loop. We believe that our data supports a critical aspect of the feedback model by demonstrating that peak pMAD levels, regardless of *sog* enhancer genotype and therefore the width of the pMAD domain, eventually reach the same level of intensity. One would expect that regardless of the starting conditions of Dpp, i.e., whether it is allowed to diffuse broadly, or if it is concentrated at the dorsal midline, eventually the genetic circuitry that induces bi-stability will drive the highest pMAD+ cells to the same level.

Indeed, Wang and Ferguson (2005) acknowledge that *sog* will shape the distribution of Dpp, following their observations of *sog* and *screw* mutant embryos: "We propose that in the wild type, Scw diffuses freely in the perivitelline space and forms a complex with Dpp homodimers after transport by Sog, but before receptor- mediated endocytosis." In their model, there is nothing to prevent Sog from acting continuously in the process of pMAD domain refinement, further narrowing the domain of high pMAD cells over time through continued concentration of Dpp ligand. We believe that our paper convincingly demonstrates that this is indeed the case, by showing that refinement occurs differently when *sog* expression is delayed (in the case of *ΔPsogMS2* embryos), or diminished and terminated prematurely (in the case of *ΔDsogMS2* embryos).

We thought about adding some explanation of this to the manuscript, but since discussing the feedback mechanism and how this integrates into our model would require more than a couple of sentences, we decided against adding it, as well as attempting further experiments to address it. However, to avoid confusion, we deleted the sentence (that was on line 335), "Due to the continued production of Sog protein, we see a narrowing of the pMAD domain".

2) I also found the description/interpretation of the target gene expression pattern changes in Fig 6E confusing. The legend says nc14 embryos – are these mid or late nc14? I am assuming the latter since hnt is expressed and all the embryos appear to be around the same stage, except the wildtype control embryo for hnt in Fig. 6E which looks a little older than the others.

We swapped out the wildtype and *ΔDsogMS2* mid 14 embryos for late 14 embryos.

In line 367 for hnt the authors write that 'with deltaPsogMS2 patterns appearing nearly wildtype, and deltaDsogMS2 embryos showing a pattern similar to, but stronger than deltaPdeltaDsogMS2'. To me, hnt looks similar in deltaDsogMS2 and deltaPsogMS2 embryos, and neither is nearly wildtype. Is the interpretation not that ush is active first so the expression patterns observed at late nc14 in Fig 6E reflect the mid nc14 pMad widths for deltaP and deltaD (so deltaP is broader) due to previous activation at mid nc14 and mRNA stability? In contrast, the hnt pattern reports the late nc14 pMad widths in in Fig 6D as it is only activated late, so deltaD is broader due to lower pMad refinement? The authors may be arguing this for ush but it wasn't stated very clearly to me.

We thank the reviewer for pointing out the inaccuracies and confusion in this Results section. We rectified this by revising the text as follows (line 373): "In *ΔPsogMS2* embryos, the *ush* pattern is much wider than wildtype, while *hnt* is only slightly wider than wildtype (Fig. 6E), matching the changes in pMAD over time (Fig. 6D). In *ΔDsogMS2* embryos, both *ush* and *hnt* are somewhat wider than wildtype, matching the unchanging pMAD domain over time, but not as wide as *ush* in

ΔPsogMS2 embryos. These results suggest that the changes observed in pMAD stainings functionally impact the subsequent patterning steps, and that changes in the onset and rate of transcription of *sog* have specific and defined consequences for downstream signaling events."

Also, it may be possible to gain support for this hypothesis by staining for ush with intronic and exonic probes at late nc14, as active transcription (visualized by intronic probes) may refine to be more similar to the late nc14 pMad widths, despite a broad domain of stable mRNAs (exonic probes) based on earlier activation.

The suggestion to use *ush* intronic probes is a good one; live imaging of *ush* MS2 transcription would be even better, but for practical reasons (the current data - that the *ush* domain is initially set at a broader width in *ΔPsogMS2* - is the main takeaway we wish to focus on), as well as logistical reasons (all but the senior author has moved on to other positions and all three reviews are very favorable), we prefer to move the paper along without further wet bench experiments.

I also have the following minor concerns:
1) For all figures with quantitation, the authors should report in the legends how many nuclei are analysed and from how many embryos? I could not find this information. Also please clearly state the embryo ages where appropriate.

We have added this information to the figure legends (lines 896, 913, 923, and 956).

2) In the paragraph starting on line 108 the authors describe how they replace the proximal enhancer with a previously described neutral DNA that is depleted for binding sites for early fly TFs. The main text does not mention replacement of the distal enhancer with RFP, although the legend says that this was to aid screening. The authors should mention this replacement in the main text, with the lengths of the distal enhancer deleted and the inserted RFP, after describing the proximal enhancer replacement.

We have added this information to the revised text (line 111-119).

3) Does the addition of a promoter (within 3x3P-RFP) in place of the distal enhancer complicate the findings? It seems plausible that the RFP promoter could compete with the sog promoter for the proximal enhancer reducing sog transcription. Is RFP RNA detectable in a sog-like expression pattern?

3x3P Enhancer is definitely off, because we do not see a difference in RFP background levels in the live imaging in the H2aV-RFP channel. We have not stained for RFP transcripts, however we also made crispants with the distal enhancer deleted without a replacement and find no difference in *sog* output between the two genotypes in our fixed imaging experiment. We added a sentence to the methods to include these observations (line 529): "3x3P-RFP was used as a marker for screening of *ΔD* crispants; there was no apparent RFP fluorescence in early embryos of flies carrying this allele. Moreover, this allele behaved transcriptionally identically to a straight deletion of the enhancer in colorimetric *in situ* hybridization assays (data not shown)."

4) Line 118 - deltaPdeltaDsogMS2 failed to produce any homozygous flies – do the authors mean that no male flies were viable?

Yes, we never saw any male flies that lacked the balancer chromosome. We changed the sentence in the manuscript to (line 127): "produced neither homozygous adult females nor hemizygous adult males."

5) Fig 2D – I think the y-axis should have negative values rather than 2 sets of positive values.

We have fixed this typo, thank you.

6) From the data in Fig. 5C, the authors conclude that in the ventral bins the rate of activation but not deactivation is well predicted by the model. Is the rate of activation/deactivation the steepness of the slope on each side of the curve? If so, for the ventral most bin it seems that the rates of activation and deactivation are both predicted well by the model, but it is the proportion

of active nuclei that does not reach the level predicted. Can the authors comment on this?
You are correct in pointing out that the total number of nuclei is different, while the slopes on both ends of the curves are similar. The reason it is described as "rate of deactivation" rather than "proportion of nuclei active" is that the model assesses activity by picking t_{on} and t_{off} values for each nucleus, then assigning the "active" state at each time point "t" if ($t_{on} < t < t_{off}$). For any given nucleus, the t_{off} value has the possibility to be less than the t_{on} value, in which case the condition ($t_{on} < t < t_{off}$) is never satisfied at any timepoint for that nucleus.

Alternatively, it would have been possible to include an additional parameter that describes the binary possibility that a nucleus will become active or not, which supersedes both the t_{on} and t_{off} value. However, our goal with the model was to describe the collective action of all nuclei with the fewest number of parameters, and adding a binary activity parameter would have increased the complexity of our model.

In the ventral bins for all genotypes, the number of nuclei that become active is related directly to the t_{off} values chosen. Therefore, the combined model appears to fail in this region because the number of nuclei with t_{off} values less than their t_{on} values is lower than expected given the observations in the *WTsogMS2* data. Having said this, we understand that the distribution of t_{off} values is technically not a "rate of deactivation." We added additional language in the revised text to address this nuance (line 315): "While in all D/V bins the rate of activation was remarkably well predicted by the model (Fig. 5C, see overlap between initial rise in curves), the t_{off} values generated by the combined model were much higher than the distribution of t_{off} values measured by experiment, leading to a dramatic overactivation of the model output compared to the data in the ventral bins (Fig. 5C, note different curve heights)."

6) In line 371 the authors say 'changes in the onset and rate of transcription of sog have specific and defined consequences in the selection of dorsal fates'. This statement is too strong as the authors have not actually looked at dorsal fates, they have only stained for 2 target genes. Dunipace et al (PLOS Gen) have quantitated amnioserosa fate when each of these enhancers is deleted – the authors should cite this paper, which also showed pMad data for the deletions. Do the amnioserosa counts from the enhancer deletions fit with the results presented in this study?

We agree that this was overstated and changed the sentence in the revised text to (line 378): "…changes in the onset and rate of transcription of *sog* have specific and defined consequences for downstream signaling events. We had not cited the Dunipace paper here (though we cited it in four other sections) because: 1) our analysis better quantified pMAD (Fig. 6), 2) their pMAD images were from stage 6 embryos, while ours focused on early vs. late stage 5 embryos, and 3) our point was the that there is a temporal difference in early vs. late *ΔPsogMS2* embryos. Though our late stage 5 results are consistent with their stage 6 results, the point in this section was about the changes in domain width in *ΔPsogMS2* embryos.

With respect to amnioserosa counts, we did not do that analysis because we felt (from previous experience) that counting amnioserosa cells in images of lateral views of embryos undergoing germ band elongation is not accurate enough to measure subtle differences. The embryos must be in a perfectly lateral position and at the exact same stage - not too elongated and not starting germ band retraction - to avoid mis-counting cells at the dorsal midline. Thus we focused on pMAD and Dpp target expression patterns as more informative measures of *sog* enhancer activity.

7) Line 676 – 'Foci were assigned to single nuclei by finding the nearest nucleus in 3D space to each focus' – please clarify how this was done.

After spot calling, the data is exported as a .csv file and imported into R, where it is converted to a matrix. The matrix has 3 columns, each containing the x, y, and z coordinates of each object, with each row representing a single object. Both nuclei and foci positions are contained within their own separate matrices. Assignment of a focus to its corresponding nucleus is done by calculating the Euclidean distance between that focus and all nuclei, using the following formula:

$$distance = \sqrt{((focus_x - nucleus_x)^2 + (focus_y - nucleus_y)^2 + (focus_z - nucleus_z)^2)}$$

From this calculation, each focus generates a set of distances. The nucleus that produced the minimum distance is assumed to be the correct nucleus for that focus. Nuclei that are assigned an impossible number of foci, (i.e. any number > 2) are discarded, assuming to be either spot detection errors (one focus erroneously called as two), or assignment errors (a focus belonging to one nucleus assigned to another).

During the creation of this pipeline, accuracy was additionally assessed by visual inspection. Each nucleus and its assigned foci were plotted on 2D graph with their x and y coordinates and given a unique color, making it easy to spot any errors not picked up by the filtering step.

Several methods for filtering errors were attempted, such as a more advanced version of the assignment that attempted to resolve any conflicts examining the DAPI signal intensity along the chord created by the focus point and the nucleus point. This method assumed that a correct assignment would be demonstrated by a continuously high signal in the DAPI channel along this chord, and incorrect assignment would produce a characteristic drop in signal intensity along this chord.

Ultimately, errors in assignment only accounted for <1% of the total data, and no conflict resolving methods ever changed the results of the analysis. Discarding obvious errors was therefore adopted as the simplest and least computationally intensive method.

We condensed this explanation and added this to the Methods section of the revised text (line 691): “Assignment of a focus to its corresponding nucleus was done by calculating the Euclidean distance between that focus and all nuclei, using the following formula:

$$\text{distance} = \sqrt{((\text{focus}_x - \text{nucleus}_x)^2 + (\text{focus}_y - \text{nucleus}_y)^2 + (\text{focus}_z - \text{nucleus}_z)^2)}$$

Nuclei with more than two assigned foci were excluded from the analysis, and represented less than 1% of the data. During the creation of this pipeline, accuracy was additionally assessed by visual inspection. Each nucleus and its assigned foci were plotted on 2D graph with their x and y coordinates and given a unique color, making it easy to spot any errors not picked up by the filtering step.”

Reviewer 2 Advance Summary and Potential Significance to Field:
Review: Whitney et al., “Differential integration of activation and repression signals in a multi-enhancer system”

The authors investigate how multiple seemingly redundant enhancers co-regulate transcription. For this, they use the sog locus as a model and quantitative imaging in early Drosophila embryos. At this stage, the expression of sog is regulated by two distinct enhancers, located 20kb upstream of the sog promoter (called sog distal) and a proximal enhancer that lies within the first intron (proximal enhancer). This locus is interesting as it is dynamically regulated along the dorsal-ventral axis, with a repression in the mesoderm.
To disentangle the role of each enhancer at the sog locus, the authors used Crispr gene editing to delete each enhancer separately or both, as well as to insert an MS2 cassette. First, they score the ability of these enhancer deleted alleles to sustain sog expression and full development. Next, they investigate sog nascent transcription when the promoter is regulated by the primary, the distal alone or both, and this in various domains along the dorso-ventral axis. For this they use fixed imaging and quantify the number of active nuclei in the mesoderm and in 3 other regions in the neurogenic ectoderm. This analysis is nicely complemented by live imaging with the MS2 reporter to extract transcription dynamics upon deletion of the proximal or distal enhancer. Finally, the authors assess the functional consequences of tuning sog spatio-temporal expression with enhancer deletions on dpp signaling pathway.

Major strengths of the manuscript:
1) the model gene is an exciting choice as it allows to decipher the role of a given enhancer in different locations of the pattern.
2) From a biological point of view, it’s exciting to see manipulation of enhancers at the endogenous locus.

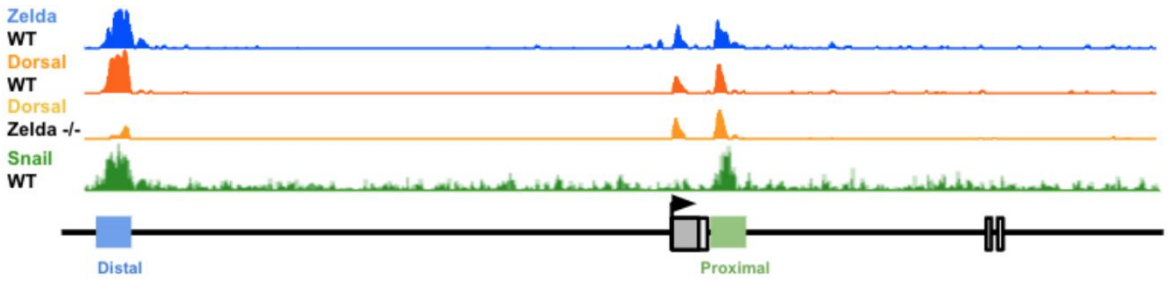
It's fantastic to be able to relate quantitative aspects of transcriptional control to phenotypic consequences in terms of signaling.
3) The main fining is that two seemingly redundant enhancers, act in a complementary manner, with different sensitivities to repression. The combination of these two actions is necessary to shape the final sog pattern.

This is an exciting paper with conclusions well supported by the data. The paper is clearly written and the figure well organized. Conclusions of this paper are of immediate interest to many people in the field of gene expression. I recommend this paper to be published with minor corrections.

Reviewer 2 Comments for the Author:
Here are my specific questions and recommendations (very minor):

1. To better rationalize why the primary enhancer is more sensitive to sna-mediated repression in the mesoderm than the other enhancer, it would be interesting to show Sna binding profiles to the two enhancers. Such data should be available from published chip-seq (in wt or ventralized embryos).

We also thought that might be the case, but it is not. ChIP binding of Snail is higher at the distal enhancer, as is Dorsal and Zelda binding (Sun et al., 2015, PMID: 26335633; Zeitlinger et al., 2007, PMID: 17322397). There are more Zelda binding sites in the distal enhancer, likely giving a stronger Zelda peak, and hence stronger Dorsal and Snail binding peaks, as their binding is dependent on Zelda's pioneering activity. Since we cannot make any conclusion about Snail binding as it relates to Snail repressor activity, we did not add it to the original manuscript.



2. Did the author keep track of the parental origin of the MS2 allele?
It would be interesting to mention whether the parental origin of the MS2 allele (maternal or paternal) influences the results. I assume the authors only focus on female embryos (with 2 dots) but it would clearer to state this in the methods.

Every embryo in the FISH and MS2 experiments is female. We added a statement to the methods section to make it more explicit (line 512) : "Both fixed and live experiments were performed using female embryos, with the MS2 allele always coming from the male flies. Females were selected for either by imaging embryos carrying two *sog* alleles in the fixed experiments, or by looking for MS2 expression in the live experiments."

3. To compare the overall activation dynamic, I recommend showing the % of activation of the pattern in the various conditions, averaged over all nuclei in the main manuscript.
These results are currently shown in Figure S4, but not within the same graph. Comparing the % of activation curves between the different alleles would inform on whether the initiation lag time (between mitosis and 1st activation) is different between the two enhancers.
It would be interesting to examine if the distribution of these waiting times exhibits the same shape between the different alleles (see Lammers et al., 2020; Dufourt et al., 2018).

Average waiting times were quantified (shown in Fig. 4C), and indeed there are differences between the genotypes, with *ΔPsogMS2* showing much higher ton times than either *WTsogMS2* or *ΔDsogMS2*. However, we took the suggestion to plot distributions of ton times (waiting times) and % active over time for all genotypes together and made an additional supplemental figure (now Figure S2). To accommodate this new supplemental figure, we added to the revised text (line 249):

”Notably, *ΔPsogMS2* embryos exhibited a much broader distribution of ton times than either *WTsogMS2* or *ΔDsogMS2* embryos, giving rise to a lack of synchrony of activation across the *sog* domain (Fig. S2A-C).”

Moreover, the slope of such curves could give the speed by which the pattern is filled (synchrony). Comparing these slopes should be informative as well.

Although we did not include this in Figure S2, as we believe the above metrics are sufficient to describe this lack of synchrony, the slopes of the % of activation are different, and again appear to reflect the synchrony of the various genotypes. Again, *ΔPsogMS2* has the lowest slopes across the domain, and this is confirmed when looking at the movies - *ΔPsogMS2* shows a high degree of stochasticity in activation between nuclei.

4. To quantity the activity of each enhancer, the authors use ton*loading rate for each nucleus (Figure 4F). It would be interesting to back-up these estimates with results already obtained by the authors with the different sog alleles. Either the smFISH presented in Figure 2: by counting single ‘’cell’’ mRNA numbers. However, single RNA counting might be challenging if the imaging did not include the entire ‘pseudo-cellular’ volume and if the numerous sog transcripts form clusters. An alternative could be the quantification of single nuclear mRNA output through the integral amplitude from live imaging data.

This was actually part of the original design of the study, but unfortunately we discovered that this was impossible for two reasons:

1. Counting individual *sog* transcripts is very difficult in our system. In early NC14, transcripts are detectable as individual spots, and we can perform counting reasonably well. However, as NC14 proceeds, transcripts appear to accumulate preferentially on the apical side of nuclei. The high number of transcripts in a concentrated volume causes the spot counting to become increasingly inaccurate (individual spots are no longer clearly separated).

2. It is not clear how to directly convert any estimate of total number of nascent transcripts to MS2-GFP foci intensity. Ideally, we could compare the total number of nascent transcripts calculated using the exonic *sog* probe to intensity of the intronic MS2 probe, then attempt to relate intronic probe intensity to MS2-GFP intensity. However, we found that there is no clear correlation between intronic and exonic probe intensities. This is likely due to the fact that the density of actively transcribing RNA polymerase is not evenly distributed across the gene body.

When transcription is first initiated, the first few RNA polymerase molecules have transcribed far enough to have their nascent transcripts labeled with the 5’ exonic probe, but are not labeled with the intronic probe. As transcription proceeds, those transcripts become labeled with both probe sets, but as co-transcriptional splicing occurs, intronic labeling is lost. For a brief window, when the gene body is fully saturated at steady-state transcription, we expect there to be good correlation. However this is lost when loading of new transcripts stops, and all remaining polymerase molecules leave the intronic region, but remain labeled by the 5’ probe.

5. While I agree with the main conclusions and appreciate the discussion, I believe that speculating about the chronology of events is challenging. A title as ‘first come, first serve’ could be nuanced, or a sentence could be added to highlight the need for a dynamic observation of enhancer-promoter interactions (with live imaging). The analysis of the lag time between mitosis to first activation (see point 2) might help understanding if the action of a given enhancer comes is faster than the other one.

This is a good point, and what originally motivated us to attempt a modeling approach. We wanted to develop a way to rigorously test the hypothesis that the enhancers can be thought of as acting in a mechanistic vacuum, independent of their counterpart. However, you are correct in suggesting that “first come, first serve” is not backed up by any underlying biological mechanism investigated by our study. Any discussion of the physical nature of the enhancers in the onset of activation, whether it be looping to move in close physical proximity to the promoter or any other such model is deliberately omitted, largely because we are not entirely convinced that any meaningful

consensus has been achieved by the field. Therefore we are hesitant to add any additional language speculating that measurements of physical enhancer-promoter interactions would be sufficient to validate the model we propose.

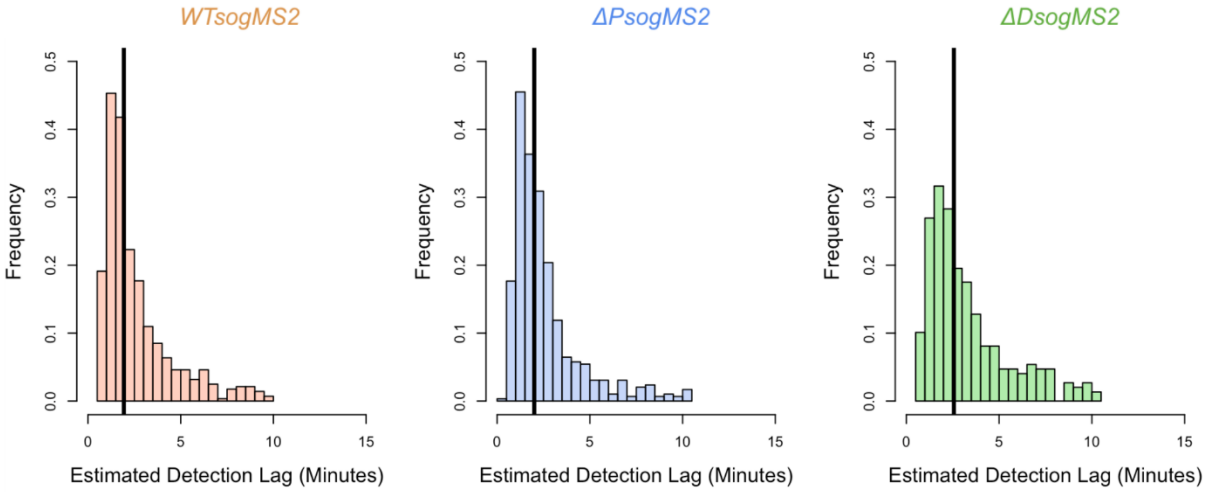
What we wished to convey by “first come, first serve” was that enhancer activation time for our pair of shadow enhancers is purely additive, as in, given the distribution of potential enhancer activation times for both enhancers, one can accurately predict the wildtype enhancer pair activation time, and it is unnecessary to assume any synergy. Therefore, we conclude that in any given nucleus the first enhancer that *does* activate transcription dictates the activation time of the wildtype system for that nucleus. We do concede that the use of this phrase does conjure up the image of enhancers “arriving” at the promoter, and therefore may not be well suited for the discussion of our results. For this reason, we have omitted the phrase “first come first serve” from this Discussion section (both title and text; see section starting on line 439). To end the paragraphe, we added (line 447): “This form of additivity is similar to the additivity seen in the domain of activation from our fixed experiments, where saturation of activation occurs in the central region of the *sog* domain.”

Technical points

-The accuracy of Ton detection (Figure 3) depends on the detection limit with the imaging settings used. Could the author estimate this detection limit and discuss its influence on the main conclusions of the live imaging data?

Because of the above mentioned limitations regarding the conversion of the fixed imaging data (to estimate exact number of transcripts) to the live imaging data, we instead attempted to look for bias in the detection of GFP foci above background fluorescence. It is likely the case that detection of foci can be accomplished faster when loading rates are higher, as fluorescence at a focus will climb above background levels sooner. This introduces a particular complication for *ΔDsogMS2* embryos, as loading rates are generally lower and therefore we may be artificially assigning larger t_{on} values.

To perform this analysis, we calculated loading rates for each focus, and used the resulting slope to back- calculate when the intensity of the focus would have intersected with the background fluorescence level of GFP in each nucleus. This gave us a distribution of values, the median of which hopefully gives insight into how much systematic error we are introducing in our detection of t_{on}.



While *WTsogMS2* and *ΔPsogMS2* embryos showed nearly identical intersection times just under 5 frames, or about 2 minutes in imaging time, our *ΔDsogMS2* embryos did appear to have an increase at just over 6 frames, which converts to a discrepancy of around 30 seconds. This would potentially remove some of the difference between the t_{on} times *WTsogMS2* and *ΔDsogMS2* embryos. It is also interesting to note that t_{on} times are nearly the same between these two genotypes in the ventral most bins, where loading times for *ΔDsogMS2* embryos were generally higher. It is therefore likely that the t_{on} times for *WTsogMS2* and *ΔDsogMS2* embryos should be considered closer than they appear in Figure 4C.

As for the top-line conclusion to our analysis that there is an apparent underactivation of *WTsogMS2* in the ventral portion of the embryo, we do not believe that an error of 30 seconds changes the validity of our conclusion. This result still holds even if we artificially assume this error, and furthermore, we believe that because of the higher loading rates of *ΔDsogMS2* embryos in this region, we believe that ton detection does not suffer significantly in those bins. Therefore, these histograms have not been added to the manuscript.

-line 232: when the authors mention 'loading rate', do they mean 'initiation rate' (kini)? If these metrics refer to the same process, the authors should mention it for clarity.
We are using the definition of RNA polymerase loading rate as taken from Garcia et al 2013, which describes how quickly new polymerases are being added to the gene body as transcription occurs. We included the words "RNA polymerase loading rate" at this line (line 230) to avoid confusion.

-Figure 2: The authors mention that they focus on nuclei with only one clear TS and discard those with 2. How to the authors deal with sister chromatids?

The imaging for Figure 2 is done at a resolution that is low enough that most sister chromatids are indistinguishable and appear as one large spot. Presumably they are below the diffraction limit, and thus measuring their intensity effectively measures the intensity of both chromatids.

Figures:
-Figure2. To facilitate the reading of panel D of Figure 2, the figure should include the metric: ''Intensity TS1/Intensity TS2''. Some '-' signs are missing in the y axis of Figure 2D.

We have amended the figure, thank you for pointing this out.

-Figure 3, panel A: if splicing is co-transcriptional, I would expect the scheme of the transcript positioned at exon 2 and 3 (above the text number 3) not to have MS2/MCP signal.
If the authors have evidence of a different splicing dynamics, it should be mentioned in the text.

We have amended the text to include a citation that directly measured co-transcriptional splicing of the first *sog* intron (Bothma et al., 2011) (lines 214 and 786). We have slightly modified the figure panel to reposition polymerase attached to the transcript undergoing splicing as well.

-Figure legends should include statistics: number of nuclei, movies, embryos, different FISH experiments etc.

We revised all figure legends (lines 896, 913, 923, and 956), thank you for pointing out this deficiency.

Minor points
-typo line 160: '…the two enhancers regulate transcriptional output differentially active across sog''. A word seems missing before 'active'
Edited to "...regulate transcriptional output differently across…" (line 166).

-typo line 220 : reference to fig 4 instead of fig3.
Fixed, thanks! (lines 227 and 229).

-potential typo line 261: 'Curiously, the only point where WTsogMS2….'
Changed "point" to "place" (line 270).

Reviewer 3 Advance Summary and Potential Significance to Field:
The authors use live imaging to measure the regulation of a key patterning gene, Sog, by two separate enhancers in the early Drosophila embryo.

Reviewer 3 Comments for the Author:
The authors provide an interesting analysis of how two enhancers interact and collectively regulate the transcription of a single target gene. The paper uses CRISPR-Cas9 manipulations of the endogenous Sog locus to delete enhancers and simultaneously insert MS2 loops to measure the

impact on transcription. The analysis of live cell imaging is rigorous and provides new insights concerning enhancer interactions. For example, the authors present evidence that the Sog distal and proximal enhancers drive synergistic repression by Snail in the ventral-most regions of the embryo. They also explain how changes in Sog transcription influence the dorso-ventral patterning network.

The manuscript is clearly written and the authors do a good job of describing their findings in a developmental context. They nicely describe where the relevant signals are located within the embryo and how these signals are integrated at the level of the enhancers and Sog transcription. Some of the findings were recently reported by the Stathopoulos lab, however, I believe this paper merits publication in Development since it contains better quality data, better analysis and a better story.

We greatly appreciate the reviewer's support for our study.

Second decision letter

MS ID#: DEVELOP/2022/200940

MS TITLE: Shadow enhancers modulate distinct transcriptional parameters that differentially effect downstream patterning events

AUTHORS: Peter H Whitney, Bikhyat Shrestha, Jiahan Xiong, Tom Zhang, and Christine A Rushlow
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

Please see my previous review.

Comments for the author

The reviewers have addressed all of my concerns and I support publication of this work.

Reviewer 2

Advance summary and potential significance to field

The authors has answered to all my comments. The paper is suitable for publication.

Comments for the author

The authors has answered to all my comments. The paper is suitable for publication.

Reviewer 3

Advance summary and potential significance to field

The authors provide a meticulous and quantitative dissection of two separate promoters regulating a common target gene, short gastrulation. The evidence for separate modes of regulation and somewhat distinct roles in embryonic patterning is quite compelling
Comments for the author

I believe the authors have addressed the major concerns raised by the referees and the revised manuscript should be published in Development without further delay.