molecular systems biology

A model of spatially restricted transcription in opposing gradients of activators and repressors

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Transaction Report:

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

1st Editorial Decision 25 June 2012

Thank you again for submitting your work to Molecular Systems Biology and apologies for the delay. We have now heard back from the two of the three referees who accepted to evaluate the study. Since their recommendations are similar, I prefer to make a *preliminary decision* now rather than delaying further the process. We will forward the third report as well as soon as we receive it.

As you will see, the referees find the topic of your study of potential interest. They raise however a series of concerns and make suggestions for modifications, which we would ask you to convincingly address in a revision of the present work. The recommendations provided by the reviewers are very clear in this regard.

Please note that Molecular Systems Biology strongly encourages authors to upload the 'source data'- for example, tables of individual numerical values and measurements-that were used to generate figures. *In the case of this study, we would kindly ask you to include source data for figure 4C.

These files are separate from the traditional supplementary information files and are submitted using the "figure source data" option in the tracking system. Source data are directly linked to specific figure panels so that interested readers can directly download the associated 'source data' (see, for example, <http://tinyurl.com/365zpej>), for the purpose of alternative visualization, re-analysis or integration with other data. Additional formatting guidelines for 'source data' are available for download [http://www.nature.com/msb/authors/source-data.pdf].

Thank you for submitting this paper to Molecular Systems Biology.

Best wishes,

Editor
Molecular Systems Biology

Referee reports:

Reviewer #2 (Remarks to the Author):

Review of White et al., "A model of spatially restricted transcription in opposing gradients of activators and repressors". Submitted to MSB.

Summary
This paper presents a thermodynamic model to analyze the role of low affinity and high affinity binding sites in determining expression boundaries in opposing gradients of activators and repressors (OARGs). The model is tested by manipulation of the wg enhancer in vivo. The central result is that differential cooperativity between repressors and activators leads to a threshold of gene expression; the position of this threshold can move in a restricted portion of the gradient by changing TF binding site affinities. The model is simple to understand and provides some nice intuition about how the system works. It is well-motivated by their elegant previous work in this system (Parker et al., 2011), and I think it will be of broad interest to MSB readers.

I do think there are some areas to improve the manuscript in terms of its clarity of presentation and its scope of discussion. I've detailed these suggestions as major and minor points below.

Major points

1. Clarification of image quantification for validation of the model in vivo.

The description of the imaging method and quantification wasn't clear to me. I've broken down my questions by topic to make them easier to address in the revision.

1a. Re: the statistics of the imaging. How many embryos were imaged? Why was data on only 2 parasegments shown even though many more are present in the embryo (and presumably available for analysis)? And why were they presented separately rather than averaged and presented with appropriate errors? This seems straightforward to do with their existing data, even if it is from a single embryo. Also, was the image presented a maximum intensity image from a confocal stack? How deep was the stack and thus how many cells are summed in the Z-direction?

1b. Re: the position of the measurements. Since the embryo is curved, how is the relevant A/P position of the parasegment accurately determined? What section of the parasegment was actually used in the quantification (e.g. was it the entire thing or a lateral portion)? Because the wg expression pattern isn't smooth, this is relevant for interpreting their results. This could be indicated on the image itself.

The way that the data were collapsed to 1D was explained on p.9:
"All pixel intensities at the same A/P position were summed together."

Does this mean that at a single A/P position all pixel values along the dorsal ventral axis were summed? If so, it seems more appropriate to average them rather than sum them to get a representation of the response at this position. And again, it matters quite a bit how much of the dorsal-ventral expression domain was included in this measurement.

1c. Re: the normalization. I'm sure that the authors did something sensible here, but it was difficult

to understand from the text. On p. 9 they state:
"Anti-GFP immunofluorescence intensity was normalized against the peak hh-lacZ immunofluorescence signal."

Does this mean that the peak hh-lacZ signal was scaled to 1 and that GFP was scaled relative to it? These two signals are in different fluorescence channels and they may or may not be correlated depending on the source of greatest variability in the experiment. If the greatest source of variability in experiment is from penetrance of the staining reagents, then one would expect the two signals to be consistently correlated, but the overall signal to vary embryo to embryo. This scenario would be appropriate for the normalization they performed (as I understand it). Alternatively, each staining reagent could suffer from different sources of variability from embryo to embryo (such as changing levels of their targets), and a different type of normalization would be required. And was that normalization done between embryos, or in a single embryo along the dorsal-ventral axis? Was it conducted before or after averaging/summing? I suggest adding a more detailed description of their process and their justification for it would be useful.

I'd like to emphasize that for comparing experimental data to their model performance, the absolute values are not necessary. Therefore the way that they are normalizing their data doesn't change the value of their results unless it changes the overall shape of the curve (which could occur depending on how the values are being averaged/summed over the D/V axis).

2. The term "enhancer affinity" is used throughout the manuscript, but I found this awkward. Enhancers are composed of multiple TF binding sites, each of which can have a different affinity. How is "enhancer affinity" then calculated (either in this paper or by enhancers themselves)? Is it a simple sum of all binding sites, or do some sites not contribute to output? I suggest using "TF binding sites" to increase clarity, especially since their model primarily deals with just a pair of binding sites as a representation of an enhancer.

3. Clarification of the results regarding cooperativity of activators and repressors
While reading the main body of the text, I assumed that when both activators and repressors were equally cooperative that the system is symmetric and you lose the ability to tune the activation/repression threshold with binding site affinity. But the sentence in the main text contradicts this intuition because it is overly general:

p. 3 "In the Supplementary Information and Supplementary Figure 1, we show that the results described below still apply when there are more than two binding sites, when activators and repressors do not bind with equal affinity, and when activators also exhibit cooperativity."

In the Supplement the result is stated more precisely.

Supp p. 3 "If activators and repressors are equally cooperative, then the resulting model is identical to the non-cooperative model, and binding affinity does not determine the boundaries of gene expression. However, in cases of asymmetric cooperativity, when activators and repressors both act cooperatively, but repressors exhibit stronger cooperativity, then the middle gradient zone exists, and affinity-based gene expression boundaries can occur."

This result should be explained more specifically in the main body of the text to avoid confusing readers.

Also regarding this analysis, what about the case where activators exhibit more cooperativity than repressors? This could be analyzed using eq. S4, but isn't discussed in the Supplement.

4. How are low affinity sites predicted to affect expression when intermixed with high-affinity sites?

This isn't immediately obvious, but relevant given the discussion at the end of conserved low affinity Gli sites proximal to higher affinity Gli sites in mouse enhancers. A full analysis of this case is clearly beyond the scope of this work, as it is a substantial undertaking. However, an acknowledgement of the complexities of other enhancers and perhaps some qualitative discussion of how intermixed sites may behave would be useful.

Minor points

1. Figure 1

It may be hard for readers to follow is how both concentration and affinity are influencing binding site occupancy and therefore expression. Perhaps it would be useful to include two rows of sites in Figure 1 - one row of low affinity, and one of high affinity?

2. Figure 2

Panel C was uninformative for me. It is clear from the equations that when w=1 that there is no tunable window in the system. I suggest eliminating it.

3. More explanation of the logic behind eq. 1, 2 and 3

Though the authors have recently written a comprehensive review on this topic, it would be useful to present more context for these type of models for readers of this particular manuscript. The terms are explained, but the intuition behind their construction is left out. For example, what molecular state does each term represent (2 sites bound by two activators, an activator and repressor, etc.)? And what is the purpose of the partition function? A sentence or two to address these questions would make the manuscript more broadly accessible.

4. A potentially interesting point of discussion is whether there are distance constraints on TF binding sites to maintain their cooperativity. The position of the Gli sites in the wg enhancer are shown in the supplement and they occur in 2 pairs. In their comparison of sites across the 12 Drosophila species, is this spacing maintained? Though not an essential point for their paper, it may give some insight into the physical constraints on cooperativity.

5. I was curious about the bump in the experimental data at 70% parasegment width for the low affinity constructs in Figure 4C. Any ideas what this might be due to? Perhaps this would be answered by addressing which part of the parasegment is quantified (as in Major point 1, above).

6. Add conclusions/interpretation to the supplement

For eq S2 and S4, a sentence or two about the interpretation of the equation would be useful. Right now, the sections end with the equations, without any discussion of their meaning.

7. No details on the numerical simulation were presented in the supplement. How exactly was it simulated?

8. Description of the model fit to the wg enhancers should be moved to the main text. In the current version of the manuscript, this model is described in a single sentence on p.7:
"To make a quantitative comparison between our model and the wg results, we built a specific model of the wg enhancer and trained it on the GFP data."

I suggest that the first two paragraphs of the materials and methods are moved to the main body of the text to give readers a sense of what this modeling framework entails.

The fact that the model recapitulates the shape of the curve, but not the magnitude of activation and repression may be due to co-regulation of other TFs as the authors state, but could also be due to the way that the data has been normalized, especially given that GliR and GliA are not being directly measured.

Reviewer #3 (Remarks to the Author):

The authors of this manuscript describe a "general" thermodynamic model to explain the role of Gli binding site affinity in response to the Hh morphogen gradient. The idea is based on a previous report by the same authors (Sci. Signal. 2011) where they documented a "paradoxical" behavior of the system: enhancers with low affinity sites are actually able to drive reporter expression in broader domains (in a Hh morphogen field) than enhancers with high affinity sites. In the current manuscript, the authors extend their modeling studies and provide additional insights into how this might be achieved at a thermodynamic level. The authors also present new experimental data using another Hh-responsive system in early embryos. The topic of this study is important and timely, and the authors have done a good job in explaining some of their findings. However, there are several issues that need to be addressed and more details are needed for further evaluation.

1) While the authors have provided useful, new insights into the boundary position of Hh-responsive reporters in this manuscript, the expression level remains unsatisfactorily addressed. There are two issues here. The first is the fact that (as shown in their 2011 paper), while the expression domain of a reporter driven by high affinity sites is narrower, their expression level is actually higher. Why/how this occurs is not clear. In their 2011 paper, the authors used a "basal" reporter lacking Gli sites; but this basal reporter itself exhibited activities indicative of responsiveness to Hh-encoded positional information. Whether/how this spatially non-uniform basal expression may impact Gli site-dependent expression level (or boundary position) is unclear. Since the current manuscript aims to have a "general" model (the basic idea was already presented in their 2011 paper), the authors need to model (or at a minimum provide satisfactory discussions) how binding site affinity impacts reporter expression level (and boundary?) as seen in their experimental system. The second issue here is related to their data shown in Fig. 4. Here, it appears that binding site affinity affects predominantly expression level, rather than boundary position (see below for further comments on Fig 4). Thus the distinction between expression level and expression boundary position becomes blurred; further clarification is necessary.

2) The current manuscript does not provide adequate details necessary for a thorough evaluation. For example, Fig. 4 only shows the "basal-subtracted" reporter expression level. How does the basal profile itself look like? What is the standard deviation of the values at each position? Are these data from "representative" transgenic lines or are they "pooled"? Fig. S3 shows that reporters driven by high affinity sites have a higher expression level, on average, than "basal". But Fig. 3C shows that high affinity sites never induce expression level above basal. What is going on here? At what position were the data in Fig. S3 extracted from? Why were PS6 and PS7 chosen for their analyses, as opposed to using data from all the segments? Clarification and additional data/details are needed for evaluation.

3) On page 4, the authors suggest that K in Eq. 5 must be a positive value in a "physically realistic" system. Do the authors imply anything about the "activator-dominant" and "repressor-dominant" parts of the disc? Are they not physically realistic? Improved description may avoid potential confusion. The stability of the activator and repressor forms of Gli is assumed to be the same so that total Gli level remains spatially constant in their model. While stability may not significantly affect their modeling conclusion, it is useful to discuss this issue. In addition, the authors seem to imply/assume that biochemical affinity is the same as in vivo affinity (at least on a relative basis). I do not think this can be assumed automatically; if it is assumed, the authors need to explicitly make this point with justification.

4) I do not think Fig. 5 is necessary or particularly useful. Most reporter studies deal with expression level, as opposed to threshold response-and it is the latter that is the primary focus of the presented "general" model (see #1). In addition, it is not clear what ChIP data are really measuring in most

cases from a biochemical standpoint. Thus inclusion of Fig. 5 may lead to further confusion, unless the authors successfully disentangle and model the level/boundary problem (see #1).

1st Revision - authors' response 17 July 2012

Response to reviews:

Reviewer 2
Major point #1a: "How many embryos were imaged?"

Response: Three embryos, each representing an independent transgenic line. were imaged for each of the three *wg* enhancer types. We replaced the bar graph in Supplementary Figure 3 with a new graph showing data from each of these lines. The data shown in Figure 4C are from individual embryos. We have revised the Materials and Methods section to clarify:

- on p. 10, Materials and Methods, first paragraph we have indicated the number of embryos imaged and noted that results shown are from individual embryos.

"Why was data on only 2 parasegments shown even though many more are present in the embryo (and presumably available for analysis)?"

Response: Because the germ band of the embryo is curved, segmental stripes occur at different angles in the XY plane, which presents a problem when collapsing stripes into a single dimension of expression data: stripes at the ends of the embryo, which will be presented at an angle, would appear to be spread out across the X dimension (anterior-posterior). In order to circumvent this problem, we chose to analyze the two parasegments that are most vertical when the embryo is presented horizontally.

- We clarified this point on p. 10, Materials and Methods, 3rd paragraph, "Quantitation of transgenic expression data," in the sentence beginning "For embryo quantitation experiments..."

"And why were they presented separately rather than averaged and presented with appropriate errors?"

Response: We have revised Figure 4C to show how the model compares to the averaged data from parasegments 6 (original Figure 4C) and 7 (originally Supplemental Figure 2, now removed), with error bars indicating standard error. We have updated the figure legend to reflect the changes. We have updated the file 'Source Data for Figure 4C' to include data for parasegments 6 and 7, and the mean and standard error values.

"*Also, was the image presented a maximum intensity image from a confocal stack? How deep was the stack and thus how many cells are summed in the Z-direction?"*

Response: One advantage of the embryonic presumptive epidermis for quantitative analysis is that it is a monolayer epithelium. Only one cell is imaged along the Z axis. In order to capture the epidermis of half of each embryo (which curves in the Z dimension), we took z-stacks of 29 images spaced at 1.5 μm. The image presented is a maximum-intensity image from a stack.

- We have added these details to the Materials and Methods, p. 10, 2nd paragraph under "Whole mount staining and microscopy."

Major point #1b: Since the embryo is curved, how is the relevant A/P position of the parasegment accurately determined? What section of the parasegment was actually used in the quantification?

Response: The reviewer is correct that these are important points. See our response to point 1a above: we tried to circumvent problems arising from the curvature of the embryo (in the XY plane) by analyzing only those stripes that appear vertical (parallel to Y axis) when the embryo is presented

horizontally. The Hh-lacZ signal was used as an internal control to determine A/P position in each embryo: the peak of the Hh-lacZ signal was used as the reference point on the x-axis to align data.

For quantitation we used the ventral half of the embryo, in the region of parasegments 6 and 7. Following the reviewer's suggestion, we have indicated this with a box in Figure 4B and updated the corresponding figure legend.

- on p. 10, Materials and Methods, under "Quantitation of transgenic reporter data", we have included more detail on how A/P positions were determined.

"The way that the data were collapsed to 1D was explained on p.9: "All pixel intensities at the same A/P position were summed together." Does this mean that at a single A/P position all pixel values along the dorsal ventral axis were summed? If so, it seems more appropriate to average them."

Response: The reviewer's description of our summing method is accurate. Because all data were collected from image files of the same size and pixel resolution, there is no practical difference between summing and averaging, for the purposes of comparison. To get an average, the sums would be divided by the number of pixels in the Y dimension, which would not change the relative magnitudes of the measurements since the number of pixels is the same in every case.

- on p. 10, Materials and Methods, under "Quantitation of transgenic reporter data", we explained that images were of the same size and pixel resolution.

Major point #1c: The normalization. I'm sure that the authors did something sensible here, but it was difficult to understand from the text.

Response: We normalized the GFP signal by dividing GFP intensity by the peak Hh-lacZ intensity, in order to compare GFP signal across embryos. This was done in an attempt to correct for variable reagent penetration from embryo to embryo, which is the most significant source of variability in this type of experiment. To minimize staining differences between samples, all embryos were treated with antibody simultaneously in a single experiment, using the same batches of reagents. All images analyzed were collected in one microscopy session, using the same confocal settings, with great care being taken to ensure that the signals were not saturated and hence within the dynamic range of the instrument.

We performed an additional rescaling step to compare the data with the model. Because the maximum expression value in the model equals 1, we set the peak normalized GFP signal of the low-affinity version of the enhancer equal to 1, and rescaled all other data accordingly.

- To clarify our normalization and alignment procedures, we expanded our description of methods on p.10, Materials and Methods, 3rd paragraph under "Quantitation of transgenic reporter expression data."

Major Point # 2: The term "enhancer affinity" is used throughout the manuscript, but I found this awkward... I suggest using "TF binding sites" to increase clarity...

Response: We have taken the reviewer's suggestion and specifically referred to the affinity of TF binding sites in an enhancer. "Enhancer affinity" was only meant to indicate the association constant of the individual binding sites, and not an estimated total affinity for the enhancer. The reviewer is correct that this phrase is imprecise. We made the following changes:

In the main text:
- p. 2 in the Abstract, changed "enhancer affinities" to "enhancer binding site affinities"; changed "affinity of enhancers" to "affinity of binding sites in enhancers."

- p. 3, Introduction, 2nd paragraph, changed "differences in enhancer affinity" to "differences in enhancer binding site affinity"; Results, first sub-heading, changed "role of enhancer affinity" to "role of enhancer binding site affinity"; 3rd paragraph, changed "causes enhancer affinity to play"

to “causes TF binding site affinity in enhancers to play”, and changed “relationship between enhancer affinity” to “relationship between TF binding site affinity.”

- p. 4, first paragraph, changed “driven by enhancers of various affinities” to “driven by enhancers with TF binding sites of various affinities”; 4th paragraph (after equation 3), changed “how enhancer affinity establishes” to “how enhancer binding site affinity establishes.”

- p. 5, 3rd paragraph (after equation 7), changed “differences in enhancer affinity will produce” to “differences in enhancer TF binding site affinity”; changed “thus an enhancer of affinity *K*” to “thus an enhancer with binding sites of affinity *K*”; changed “Enhancers with affinity greater than *K*” to “Enhancers with TF binding sites of affinity greater than *K”*; changed “while enhancers with affinity lower than *K*” to “while enhancers with binding sites of affinity lower than *K*”; changed “Differences in enhancer affinity thus” to “Differences in enhancer TF binding site affinity thus”; changed “differences in enhancer affinity cannot produce” to “differences in TF binding site affinity cannot produce”; 4th paragraph, changed “all genes, regardless of enhancer affinity” to “all genes, regardless of TF binding site affinity”; 5th paragraph, changed “cooperativity, enhancer affinity, and activator-” to “cooperativity, TF binding site affinity, and activator.”

- p. 6, 2nd paragraph, changed “as the affinity of an enhancer is decreased” to “as the affinity of binding sites in an enhancer is decreased”; changed “As enhancer affinity decreases, overall occupancy” to “As binding site affinity decreases, overall occupancy”; 3rd paragraph, changed “enhancers with affinity greater than” to “enhancers with binding sites of affinity greater than”; changed “while enhancers with affinity weaker than” to “while enhancers with binding sites of lower affinity than”; changed “in the gradient where their enhancer affinity equals” to “in the gradient where TF binding site affinity equals”.
- p. 7, 1st paragraph, changed “occur at enhancers of different affinities” to “occur at enhancers with different binding site affinities”; 3rd paragraph changed “examined the role of enhancer affinity” to “examined the role of Gli binding site affinity.”

- p. 8, last paragraph, Discussion, changed “The affinity of the enhancer then determines” to “The affinities of the enhancer TF binding sites then determine.”

- p. 9, 3rd paragraph, changed “in addition to enhancer affinity” to “in addition to enhancer binding site affinity”; changed “Although enhancer affinity influences'” to “Although TF binding site affinity influences”; changed “observed when enhancer affinity” to “observed when binding site affinity.”

In the Supplemental Material:

- p. 2, 2nd paragraph, changed “boundaries depend on enhancer affinity” to “boundaries depend on enhancer binding site affinity”; 3rd paragraph, changed “All enhancers, regardless of affinity” to “All enhancers, regardless of TF binding site affinity”; changed “to repression depends on enhancer affinity” to “to repression depends on binding site affinity.”

- p. 5, Supp. Fig. 1 legend, changed “regardless of enhancer affinity” to “regardless of enhancer binding site affinity”; changed “Higher affinity enhancers (larger *K*)” to “Enhancers with higher affinity binding sites (larger *K*)”; changed “while lower affinity enhancers” to “while enhancers with lower affinity sites”; changed “repressed regardless of enhancer affinity” to “repressed regardless of TF binding site affinity.”

Major Point # 3: While reading the main body of the text, I assumed that when both activators and repressors were equally cooperative that the system is symmetric and you lose the ability to tune the activation/repression threshold with binding site affinity. But the sentence in the main text contradicts this intuition because it is overly general...

Response: To make it clear that affinity-based spatial patterning in OARGs depends, most generally, on differential cooperativity, we have changed the main text in two places. We also now discuss in the Supplementary Information the case when activators and repressors are equally cooperative. (See our response to the reviewer's next point, below). Changes in the main text:

- p.3, 2nd paragraph, changed "only in a cooperative system" to "only in a differentially cooperative system"; 1st paragraph of the Results, changed "and when activators also exhibit cooperativity" to "and when activators also exhibit some cooperativity, but less than repressors."

- p. 5, 4th paragraph, changed "The results above show that cooperativity" to "The results above show that differential cooperativity"; changed "In the non-cooperative model" to "When activators and repressors are both non-cooperative"; added a new final sentence: "The same result holds when activators and repressors are both equally cooperative (Supplementary Information).

" Also regarding this analysis, what about the case where activators exhibit more cooperativity than repressors?"

Response: We have expanded our discussion of cooperative activators in the Supplementary information.

- p. 3 We re-titled the section labeled "Cooperative activators and repressors" to "Cooperative activators"

- After equation S4 (p. 3 of the Supplement) we have added two paragraphs discussing the implications of equation S4 when activators are both more and less cooperative than repressors, beginning with "When $\omega > \varepsilon$, i.e. when repressors".

- We have referenced this additional information in the main text: p. 3, 1st paragraph of the Results section, added the final sentence: "Corresponding results hold when activators are cooperative and repressors are non-cooperative (Supplementary Information)."

Major Point #4: "How are low affinity sites predicted to affect expression when intermixed with high-affinity sites?..."

Response: We have added a brief section to the Supplementary Information titled 'Enhancers with both low- and high-affinity sites.' (p. 3-4). In this section, we describe a model of a two-site enhancer with one low and one high affinity site, and we compare the expected spatial expression pattern with models of enhancers with two low- or two high-affinity sites, or two sites with a K that is the average of the high and low sites.

- The results are shown in a new Supplementary Figure 2. (Note that the original Supplementary Figure 2 has been removed, described in our response to major point 1a above.)

- We referenced these results in the main text: p. 8, 1st paragraph of the discussion, we have added two sentences, beginning at "Enhancers with high-affinity sites produce restricted expression,"

Minor point # 1: "Perhaps it would be useful to include two rows of sites in Figure 1 - one row of low affinity, and one of high affinity?"

Response: We find that two rows of sites in Figure 1 showing high and low affinity sites is potentially confusing. Figure 1 is presented in the text before we introduce the role of differential cooperatiity and affinity in OARGs. Showing two rows of sites with differences in TF occupancy would involve the introduction of too much unexplained detail into Figure 1, or would require simplifications that contradict the results we present later in the paper.

The purpose of Figure 1 is to contrast the increasing enhancer occupancy along a single factor gradient with the competition between factors in an OARG. We find that a single row of sites best serves this purpose.

Minor point #2: "Figure 2 Panel C was uninformative for me. It is clear from the equations that

when w=1 that there is no tunable window in the system. I suggest eliminating it."

Response: While we readily agree that Figure 2 Panel C provides no additional information beyond what is explained in the text, when presenting this work to colleagues we have found that the visual presentation of the non-cooperative model often helps avoid confusion. We therefore retained Panel C.

Minor point #3: "More explanation of the logic behind eq. 1, 2 and 3. Though the authors have recently written a comprehensive review on this topic, it would be useful to present more context for these type of models for readers of this particular manuscript..."

Response: We have added a brief explanation of the terms in eqns 1-3 on p.4, in the paragraph after equation 2, beginning with "The individual terms in the numerator".

Minor point #4: "The position of the Gli sites in the wg enhancer are shown in the supplement and they occur in 2 pairs. In their comparison of sites across the 12 Drosophila species, is this spacing maintained?"

Response: Spacing of these pairs is not strongly conserved across the 12 sequenced species genomes, as shown in the following table:

species	pair 1-2	pair 3-4
mel	33	69
sim	33	69
sec	33	69
yak	33	72
ere	33	69
ana	48	109
pse	56	53
per	56	53
wil	45	134
vir	38	65
moj	60	65
gri	60	65

This is about the level of spacing conservation we typically see for pairs of binding sites in enhancers, unless the spacing is much closer (0-15 bp), in which case spacing is sometimes very highly conserved. Since we have not seen anything unusual in these spacing results, we have not discussed them in the text.

Minor point #5: "I was curious about the bump in the experimental data at 70% parasegment width for the low affinity constructs in Figure 4C. Any ideas what this might be due to? Perhaps this would be answered by addressing which part of the parasegment is quantified (as in Major point 1, above)."

Response: As mentioned above, we quantified fluorescence from the ventral half of the parasegment. We, too, are curious about the "bump"; looking at Figure 4B, some GFP-positive cells can be observed that are one or perhaps two cell-diameters anterior to the stripe of hh-lacZ expression. These cells probably account for the "bump", but we don't know the significance of this aspect of the wg expression pattern—except that it does follow what is predicted by the thermodynamic model: it is dependent on wild-type, low-affinity Ci sites, and expression in these cells is significantly reduced or lost when the Ci sites are converted to optimal Ci consensus sites.

Minor point #6: "For eq S2 and S4, a sentence or two about the interpretation of the equation would be useful..."

Response: We have added discussion of interpretation after eq S2 and S4 (p. 3-4 in the Supplementary Information) – see our response to major points 3 and 4 above.

Minor point #7: "No details on the numerical simulation were presented in the supplement. How

exactly was it simulated?"

Response: Numerical simulations for the results shown in Figures 2, 3, S1, and S2 were performed by putting TF concentration and affinity values directly into equations 1, 2, 3, S4, etc. We performed this with Matlab.

- We have added a new paragraph to the Methods section describing this (p. 11, "Thermodynamic model of two-site enhancers").

- We have included as a supplemental file a simple Matlab script ('twositethermomodel.m', in the folder containing the other submitted Matlab scripts) that performs these calculations for hypothetical two-site enhancers, so that readers can perform their own simulations. The script is simple enough to be easily ported to other programming languages.

Minor point #8: "Description of the model fit to the wg enhancers should be moved to the main text..."

Response: We have moved some of the material from the Materials and Methods to the main text, and have elaborated on the *wg* model.

- p. 7, we expanded the final paragraph, beginning with "The model was applied following the approach..."

- p. 11, we have altered the Materials and Methods section ("Thermodynamic model of *wg* enhancer"), to avoid redundancy with the material moved to p. 7.

"The fact that the model recapitulates the shape of the curve, but not the magnitude of activation and repression may be due to co-regulation of other TFs as the authors state, but could also be due to the way that the data has been normalized, especially given that GliR and GliA are not being directly measured."

Response: As seen in Figure 4C, the model generally does capture the magnitude of activation and repression, except for the small region of maximum activation near the A/P boundary. Our original statement on p. 7 that the model did not accurately capture the magnitude of activation and repression overemphasized this region, and so we have revised this sentence to emphasize that model generally does recapitulate expression levels:

- on p. 8, 2nd paragraph, we changed the sentence beginning "Our specific model..." to read "Our model of the wg enhancer also largely captured the magnitude of activation and repression across the parasegments."

There are several possible explanations for why the model does not capture the full level of activation near the A/P boundary, but these explanations, such as the effects of additional factors not explicitly included in the model, are generally applicable to almost any model of a biological system, and thus we have removed any discussion of them here. Our goal was to create a model with the minimum number of parameters necessary to capture the regulatory logic of the system. We have tried alternate normalization procedures (such as not rescaling the data relative to peak expression from the low-affinity construct), and we did not find any improvement in the model fit.

Reviewer 3

Point #1: "*The expression level remains unsatisfactorily addressed. There are two issues here. The first is the fact that (as shown in their 2011 paper), while the expression domain of a reporter driven by high affinity sites is narrower, their expression level is actually higher. Why/how this occurs is not clear."*

Response: The higher expression levels driven by high-affinity sites is easily explained by the thermodynamic model, and is a straightforward consequence of total TF occupancy. The total

transcription factor occupancy of enhancers with high-affinity sites is always greater than the total occupancy of enhancers with low-affinity sites. Therefore, when high-affinity enhancers are activated, *i.e.*, bound predominantly by activators, they will produce high expression levels compared to enhancers with low-affinity sites. This is why, in our 2011 paper cited by the reviewer, our thermodynamic model captured both the narrower domain and higher expression levels driven by high affinity sites.

In the current manuscript, the reason for higher expression level from high-affinity sites is illustrated in Figure 3A, red curve, which shows that, under activating conditions, high-affinity enhancers exhibit higher activator occupancy than low-affinity enhancers.

We have made changes that should clarify this issue. As described above in the response to Reviewer 2's minor point #8, we have expanded the discussion of the details of the thermodynamic model of the *wg* enhancer in the main text:

- p. 3, 2nd paragraph, we emphasize our focus on gene expression boundaries (as opposed to levels) in the current manuscript, changing "the role of binding site number and affinity is the opposite" to "the effect of binding site number and affinity on the boundary of gene expression is the opposite".

- p. 7, we expanded the last paragraph, beginning with "This model was applied".

"In their 2011 paper, the authors used a "basal" reporter lacking Gli sites; but this basal reporter itself exhibited activities indicative of responsiveness to Hh-encoded positional information. Whether/how this spatially non-uniform basal expression may impact Gli site-dependent expression level (or boundary position) is unclear..."

Response: Non-uniform basal expression does not impact Gli site-dependent effects on expression level or boundary position in the model because they are represented by mathematically independent terms. The varying basal expression level is captured in the model by the RNA polymerase (RNAP)-DNA interaction parameter, which is independent of the parameters that describe Gli interaction with DNA and Gli interaction with RNAP. How these parameters are determined is described in the Methods section (p. 11-12), as well as in the methods of our 2011 paper, which we reference in the Methods of the current manuscript.

The effect of Gli in the model is to cause activation or repression relative to the level of basal expression, regardless of what basal expression level happens to be at a given position in the gradient. The boundary between Gli-dependent activation and Gli-dependent repression is determined solely by the effects of Gli, and not by the basal expression term. The changing basal expression across the gradient in the reporter gene experiments represents the effects of all other factors acting on the enhancer (such as *engrailed* in the case of *dpp* in our 2011 paper).

To clarify, we have extended our discussion of the *wg* model in the main text:

- p. 7, 2nd full paragraph, added material beginning with "This basal enhancer reveals", and extended the discussion in the 3rd full paragraph;

- p. 12, Materials and Methods, added three sentences to the final paragraph immediately before "Genome sequences and alignments section", beginning "This term captures the effects"; see also our response to Reviewer 2, major point #8, above).

"The second issue here is related to their data shown in Fig. 4. Here, it appears that binding site affinity affects predominantly expression level, rather than boundary position (see below for further comments on Fig 4). Thus the distinction between expression level and expression boundary position becomes blurred; further clarification is necessary."

Response: The reviewer raises an important point regarding the *wg* results shown in Figure 4C – these data show only one expression boundary, the one produced by the wild-type, low-affinity version of the enhancer which switches from repression to activation at ~ 60% parasegment width.

The high-affinity version of the enhancer does not exhibit a boundary – it is always repressed below basal level. The explanation for this is that the parasegment Hh gradients exhibit only a limited range of Hh signal, producing a gradient that corresponds only to the middle zone and repressor dominant regions of the gradient shown in Figure 2 A and B.

The key result in Figure 4C is that, contrary to what is expected for single activator gradients, low-affinity sites produce activation in the same region where high-affinity sites produce repression. This is exactly what our model predicts should happen in the middle zone of an OARG, but could never happen in a single activator gradient. Thus, Figure 4C is not about absolute expression level; it is primarily about where activation and repression occur.

- To clarify this point, we have added a sentence in the main text, p. 7, 3rd full paragraph, beginning with "The observation that the high-affinity version".

Point #2: "*How does the basal profile itself look like? What is the standard deviation of the values at each position? ... Why were PS6 and PS7 chosen for their analyses, as opposed to using data from all the segments?* "

Response: We have added a new Supplementary Figure 5 to show the normalized *wg* expression data before basal subtraction, presented in a way that is comparable to the *dpp* data presented in Figure 2C of our previous paper (Parker, et al. 2011). This figure shows how basal expression changes over the Hh gradient. We have referenced this figure in the main text:

- p. 10, Materials and Methods, under the subheading "Quantitation of transgenic reporter data", beginning with "Plots of the data".

As described in our response to Reviewer 2's major point 1a above, we have averaged data for parasegments 6 and 7 originally shown in Figure 3C and the original Supplementary Figure 2. Figure 3C now shows mean values and standard error.

We quantified only parasegments 6 and 7 because they were the parasegments least affected by embryo curvature – see our response to the same question in Reviewer 2's major point 1a, above.

"Are these data from "representative" transgenic lines or are they "pooled"? Fig. S3 shows that reporters driven by high affinity sites have a higher expression level, on average, than "basal". But Fig. 3C shows that high affinity sites never induce expression level above basal... At what position were the data in Fig. S3 extracted from?"

Response: Each *wg* enhancer construct was tested in three independent transgenic lines, and the data shown in Figure 3C are from individual embryos. The bar graph in the original version of Fig. S3 indicated mean peak GFP signal in all lines for each enhancer type, but one of the high-affinity enhancer lines was an outlier relative to the other two high-affinity lines, and this raised the mean of the high-affinity category above the mean of the basal category.

To better show the variability among transgenic lines, we have replaced the bar graph in Figure S3 with a new plot showing data from parasegments 6 and 7 in each of the nine transgenic lines tested. Data are taken from the position of peak GFP signal in each parasegment. We have indicated the median peak GFP signal on the graph, which shows that the median high-affinity signal is lower than the median basal signal, consistent with the results shown in Figure 3C.

Point #3: *"On page 4, the authors suggest that K in Eq. 5 must be a positive value in a "physically realistic" system. Do the authors imply anything about the "activator-dominant" and "repressor-dominant" parts of the disc?..."*

Response: Equation 5 defines the conditions under which it is possible for the affinity of TF binding sites to determine the boundary of gene expression within an OARG. Thus, only when equation 5 is true are affinity-based boundaries of gene expression possible. Since association constants K can never be negative numbers by definition, equation 5 is not true under those conditions where it

implies a negative number for K. When equation 5 is not true, affinity-based gene expression boundaries are not possible. These are not physically unrealistic scenarios, they are scenarios in which changes in binding site affinity cannot produce changes in gene expression boundaries.

We have clarified the main text as follows:

- on p. 5, 2nd paragraph, to clarify, we have replaced the phrase "Since a physically realistic K" with "Since by definition *K*"; 3rd paragraph (after eq. 7), we expanded the sentence beginning "Outside of this middle region" to read "Outside of this middle region equation 5 is not true, because"

"The stability of the activator and repressor forms of Gli is assumed to be the same so that total Gli level remains spatially constant in their model. While stability may not significantly affect their modeling conclusion, it is useful to discuss this issue."

Response: When we model the *wingless* reporter gene data, we assume for the sake of simplicity that total Gli levels are constant throughout the gradient. It is possible to alter the model to incorporate changing Gli levels, but as we previously discussed in our 2011 paper (p. 6), as long as total Gli levels change slowly relative to the steepness of the Hh gradient, the model will produce essentially the same results.

To clarify, made the following change to the text:

- p. 11, 2nd paragraph, added a new final sentence describing our assumption of constant Gli levels, beginning with "As described previously (Parker *et al*, 2011)..."

We do not make assumptions about Gli stability in the theoretical results presented in the first half of the paper. The thermodynamic model equations 1-3 take as input the level of activator and repressor at a single position in the gradient, and present as output the activator and repressor occupancy levels on the enhancer at that gradient position. As long as the levels of activator and repressor at each point in the gradient are specified, and as long as total Gli levels are such that high-affinity sites are highly occupied compared to low-affinity sites, the model will produce the expression patterns described.

For simplicity of visual presentation, the hypothetical Gli OARG shown in Figures 1, 2, and 3 are given as opposing linear gradients with constant total Gli level.

"In addition, the authors seem to imply/assume that biochemical affinity is the same as in vivo affinity (at least on a relative basis)."

Response: *In vivo* affinity is, in part, a function of biochemical affinity. Our model incorporates important factors that govern the relationship between biochemical affinity and *in vivo* binding. *In vivo*, whether a TF is bound to a site or not depends on the intrinsic TF-DNA binding energy (biochemical affinity), as well as the presence of other TFs, chromatin state, the concentration of TF, the modification state of the TF, etc. While we do not model all factors that influence *in vivo* binding, the model predicts what *in vivo* binding should be based on the intrinsic TF affinity for DNA, as well as concentration of TF and cooperative interactions with other TFs, and with RNAP.

Point #4: "I do not think Fig. 5 is necessary or particularly useful. Most reporter studies deal with expression level, as opposed to threshold response-and it is the latter that is the primary focus of the presented "general" model (see #1)..."

Response: We have removed Figure 5 and the associated text and references. The purpose of Figure 5 was only to show that demonstrably functional and/or conserved low-affinity Gli sites exist in mammalian enhancers, suggesting an important role for low-affinity sites in the mammalian Gli response. The reviewer is correct that most of the Gli sites shown in Figure 5 have been studied with respect to expression level, not threshold level. In the text, we retain references to the literature to highlight the importance of low-affinity Gli sites in mammals, and suggest that our model offers a possible explanation for the presence of these low-affinity sites.

Specific changes to the text:

- p. 8, 2nd full paragraph, removed references to Figure 5 and replaced them with the appropriate literature references. We then moved this entire paragraph to the Discussion, beginning with the 4th paragraph on p. 8, at "This suggests that the role of affinity".

- p. 12, in the Materials and Methods, removed the section "Gli binding model", and removed references to mouse and human alignments in the "Genome sequences and alignments" section.

- Removed the figure legend for Figure 5

Additional changes:

We corrected the activator and repressor occupancy equations S1 and S3 (Supplementary Information, p. 2-3) by adding the left side term to each:

- for equation S1 we added "occR ="
- for equation S3 we added "occA="

Acceptance letter 20 August 2012

Thank you again for sending us your revised manuscript. We are now satisfied with the modifications made and I am pleased to inform you that your paper has been accepted for publication.

Thank you very much for submitting your work to Molecular Systems Biology.

Sincerely,

Editor
Molecular Systems Biology

http://www.nature.com/msb

Reviewer #2 (Remarks to the Author):

The authors have addressed all of my comments. I believe the revised manuscript is much clearer and is now ready for publication.

Reviewer #3 (Remarks to the Author):

The authors have addressed my concerns.

