July 22, 2010

Dear Editorial Board Member:

Editor Erik Alexandersson has graciously given us the opportunity to respond to your reviews of our manuscript entitled “Spatio-temporal regulation of Wnt and retinoic acid signaling by tbx16/spadetail during zebrafish mesoderm differentiation.” This opportunity is in response to our concerns that the materials we submitted at several points during revision were not given a thorough peer review.

Both initial reviewers agreed that our paper was an interesting and important finding appropriate for publication in BMC Genomics. The crux of the disagreement between ourselves and one initial reviewer (Reviewer #1), which resulted in advice being sought from the Editorial Board (Reviewer #3), lies in the appearance of certain panels in Figure 1. We feel that the extensive responses we provided regarding Reviewer #1’s concerns about Figure 1 were not thoroughly evaluated by Reviewer #1 or Reviewer #3. As just one example of this lack of thorough review, Reviewer #3 commented that “the lateral view … (see Fig 1 i, j) make evaluation of data nearly impossible.” However, we submitted dorsal views of the embryos in Figure 1 i and j. These dorsal views (Supplementary Figure 1) were specifically designed to address the same concern of dorsal view versus lateral view previously raised by Reviewer #1. Reviewer #3’s comment led us to conclude that Supplementary Figure 1 was not considered by Reviewer #3, perhaps because of some confusion about multiple versions of figures, etc. Reviewer #1 did not mention these dorsal views, either; thus, we were forced to conclude that neither reviewer thoroughly evaluated these figures.

Furthermore, the main conclusion of this study is that Wnt and retinoic acid signaling pathways lie downstream of the tbx16 transcription factor. This is the conclusion that was considered interesting and important by both initial reviewers. Any one or two genes can be removed from Figure 1 without impacting the conclusion of Figure 1, and certainly without impacting this main conclusion of our study, and we would be happy to do this. We successfully addressed all the other concerns from both Reviewers 1 and 2, and we ask that we and the Editorial Board work together towards successfully identifying and resolving any remaining issues surrounding certain panels in Figure 1.

Reviewer #3 also raised two additional concerns that we address in detail at the end of this document; these, too, indicate a non-thorough review. For example, Reviewer #3 wrote that we did not make our microarray data accessible. However, these data were made public by the GEO team on March 1, 2010, (before Reviewer #3 received our manuscript), and the accession
number was in the manuscript, as outlined in the Instructions for Authors. Thus, we fear there may have been some confusion about manuscript versions, etc., that we would like to address.

Below, we have compiled relevant reviewer comments about Figure 1, and our associated responses, in chronological order; thus, Reviewer #1’s comments come first, followed by Reviewer #3’s comments. We point out where we can demonstrate a non-thorough review in order to show that we have, in fact, addressed all reviewer concerns about Figure 1 over the course of our revisions. We provide an updated version of Figure 1 where Supplementary dorsal views of all genes are now integrated into the same figure for clarity. We end by responding to Reviewer #3’s other two comments in detail. We remain committed to publication of our manuscript in *BMC Genomics* and reiterate that no reviewer has disagreed with the main conclusions of our paper or their importance.

**Reviewer #1, Round One comments about Figure 1:** Several of the pictures are completely overstained. In situ pictures for C,D, G, H, I,J, K and L need to be replaced with less stained more telling pictures.

(Please note, if you would like to reference the reviewer’s original report, that we changed the organization of the manuscript following round one reviewer recommendations, so Figure 1 was initially called Figure 2).

**Our response:** We decreased staining levels for panels C and D (gene = *cebpa*) and G and H (gene = *hmbsa*). For the other 2 genes (panels I, J, K, and L), we explained in our cover letter, and in the revised manuscript, that the expression levels in domains outside of the intermediate mesoderm were high relative to expression levels within the intermediate mesoderm. Because the intermediate mesoderm is the only domain relevant to our study, we had to develop the in situps until the non-intermediate mesoderm expression domains were quite dark in order to visualize the relevant intermediate mesoderm expression. Note that for stringent comparison, the *spt* embryo was always developed for exactly the same amount of time as the wild-type embryo. We provided Illustrative Example 1 to show that the intermediate mesoderm expression is not visible with shorter developing times. We emphasize that the only thing that is relevant (but not critical) to our paper is the intermediate mesoderm expression, and the fact that it is higher in wt than in *spt* (see arrows in Figure 1). We reiterate that there is no way to see the intermediate mesoderm expression in these cases without also visualizing the other expression domains.

On the next pages, please find the revised Figure 1 and the Illustrative Example 1 we submitted in response to review round one:
**Figure 1**, submitted in response to the first round of reviews. Panels C, D, G, and H were modified and improved to reflect reviewer comments. Panels I, J, K, and L show dark staining in expression domains outside of the intermediate mesoderm (intermediate mesoderm indicated by arrows), which is unavoidable because the relevant expression in the intermediate mesoderm is relatively low.
Illustrative Example 1, submitted in response to review round one. Arrowheads demonstrate that the intermediate mesoderm staining is not clearly visible without extending staining time even further, which would result in even darker staining in expression domains outside of the intermediate mesoderm. Our goal in submitting this figure was to show the reviewer that what s/he deemed overstaining in the domains outside of the intermediate mesoderm was an accurate reflection of biological reality that was unavoidable, given the relatively low expression in the relevant expression domain of the intermediate mesoderm.

Reviewer #1, Review Round Two comments about revised Figure 1: The early in situis in Figure (now) 1 are of extremely poor quality and should not be published as is. Especially here dorsal views could help to better show the somites. Also arguing that the quality of the in situis can not be improved is somewhat not acceptable in my eyes since the quality is rather poor for the early in situ.

Why this is a non-thorough response to our revisions: 1) The request that dorsal views of these embryos be provided in order to “better show the somites.” These genes are not expressed in the somites, and this figure is to show intermediate mesoderm staining (and not somite staining), so this request shows an inattentive review. 2) The statement that the in situis are “poor quality” and that “arguing that the quality cannot be improved is unacceptable since the quality is rather poor.” This response is non-specific, and it fails to make any reference to our Illustrative Example 1 or our point that expression outside of the intermediate mesoderm is high relative to expression within the intermediate mesoderm, so visualizing the intermediate mesoderm requires dark staining of other expression domains. Also, this response does not mention the four panels we modified in accordance with the reviewer’s requests and whether this was an improvement. In sum, this response conveys no evidence that our attempts to address the previous comments were considered, and it fails to give any specific insight into what the reviewer views as the remaining problem. Taken together, this shows a non-thorough review. Our best guess is that the reviewer does not believe that there is expression outside of the intermediate mesoderm for some of these genes and thinks that the staining outside of the intermediate mesoderm is noise (that
could be removed by modifying experimental design) rather than true expression. We acknowledge that this may or may not be the reviewer’s true concern – but it was our best guess.

**Our response:** We flat-mounted embryos stained for *hmbsa* and *gtpbp1* (representing what we presumed to be the “problematic” genes although, in review round two, the reviewer did not specify) and photographed them at higher magnification in dorsal view (Supplementary Figure 1). Our goals were to 1) provide a view of these genes’ expression domains that more clearly demonstrated the conclusion of Figure 1: that the intermediate mesoderm expression of these genes is reduced in *spt* compared to the wild type, and 2) show that the goal of better showing the somites isn’t relevant for these genes. We also explained in our cover letter why we are confident that the staining outside of the intermediate mesoderm is an accurate reflection of biological reality rather than an experimental artifact. Below, please find Supplementary Figure 1, submitted in response to review round two:

**Supplementary Figure 1 submitted in response to review round two.** Dorsal views are presented for the two genes with the most extra-intermediate mesoderm expression: *hmbsa* and *gtpbp1*. Intermediate mesoderm expression (arrowheads) is higher in wt than in *spt*. This is apparent despite the expression of these genes both inside and outside of the intermediate mesoderm.
At this point, our manuscript was sent back to Reviewer #1 a third time. Following his/her response, our manuscript was also sent to a member of the Editorial Board (Reviewer #3).

**Reviewer #1, Review Round Three comments about revised Figure 1:** The authors have not improved the ISH.

**Why this is a non-thorough response to our revisions:** This response makes no acknowledgement of our flat-mounted Supplementary Figure 1, nor does it address whether dorsal views make comparison of intermediate mesoderm expression more feasible. Finally, this response does not comment on our explanation of why we can’t “improve” the in situ hybridization — because there is expression both within and outside the intermediate mesoderm. Note again that we are assuming here that the reviewer’s issue, which unfortunately s/he never actually clarifies, has to do with the staining outside of the intermediate mesoderm. We feel that this reviewer did not take the time to understand 1) the expression patterns of these genes, which we explained several times both in the manuscript and in the cover letter, and/or 2) the relationship of this figure to the overall conclusions of the manuscript.

**Reviewer #3 (Editorial Board Member), comments about revised Figure 1:** I also find the figure quality of the Figure 1 not satisfactory. the lateral view and the spotted appearance of signal (see Fig 1 i, j) make evaluation of data nearly impossible.

**Why this is a non-thorough response to our revisions:** This response doesn’t mention our flat-mounted Supplementary Figure 1 and specifically mentions problems with the lateral view for a gene (gtpbp1) that we also presented in dorsal view (Supplementary Figure 1C and D). From this, we infer that this reviewer perhaps was not able to access Supplementary Figure 1. Additionally, Reviewer #3’s interpretation of our figures seems fundamentally different from that of Reviewer #1. We can’t be certain of this, since both reviewers gave non-specific comments, but our interpretation is the following: Reviewer #3 mentions “the spotted appearance of the signal,” which we interpret to mean that s/he agrees with our assessment that the staining outside of the intermediate mesoderm is true signal (expression) rather than noise. Thus, this reviewer seems to fundamentally disagree with Reviewer #1’s apparent major issue with our manuscript. Based on this, we are confused as to why Reviewer #3 suggested rejection. If Reviewer #3 thinks that the expression outside of the intermediate mesoderm is true expression, but that it makes comparison within the intermediate mesoderm difficult in lateral view, we can address this in one of two ways, both of which we present on the following pages: we can 1) integrate flat-mounted dorsal views into Figure 1, or 2) eliminate from Figure 1 the two genes with the greatest non-intermediate mesoderm expression (hmbsa and gtpbp1) altogether, since this does not impact the main conclusion of our study. We ask that the Editorial Board clarify the problem with Figure 1 so that we might address it a different way, if neither of the two possible figures included here successfully address the concern.
Figure 1, now with de-yolked, flat-mounted embryos in dorsal view for all panels (A’ to L’) with corresponding letters for embryos of the same genotype and probe (A and A’, etc). All embryos are oriented tail to the right, and all panels demonstrate that intermediate mesoderm expression in the wild-type (arrowheads) is stronger than that in the spt embryos.
If the provided dorsal views fail to resolve the issues, another option is to remove analysis of these genes from the entire manuscript. Below is an example of how Figure 1 would look without *hmbsa* and *gtpbp1*.

**Figure 1**, now with analyses for *hmbsa* and *gtpbp1*, the two genes with the greatest non-intermediate mesoderm expression, removed. This figure still demonstrates that our study has identified individual genes whose intermediate mesoderm expression is reduced in *spt* compared to the wild type, but simply reduces the number of genes identified by two.
Reviewer #3 had two additional concerns that we respond to below:

**1.** *The authors do not present the data of their Affymetric microarrays. This experiment was used to obtain GESA data.*

In my opinion, it is not possible to judge the quality of the experiment if the array data as well as global analysis of the primary data are not available.

We are committed to providing our data in whatever format reviewers would like. This comment is confusing to us. We followed the “Instructions for Authors” and deposited our microarray data in GEO under accession # GSE19955. This GEO number appeared in the submission following review round one (submitted on January 19, 2010). We contacted the GEO personnel, who informed us that the data were made public on March 1, so Reviewer #3 should have had full access to all of our data. (Please note that we have since asked the GEO personnel to make our data private again while the manuscript remains unpublished, but reviewers and editors may access the data by clicking on http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=lrglvoqouswiote&acc=GSE19955).

From this, we can only infer that Reviewer #3 perhaps mistakenly read our first submission of the manuscript (submitted on October 21, 2009) rather than the manuscript submitted after two rounds of revision (submitted on March 15, 2010). Alternatively, perhaps there is a different format in which Reviewer #3 would like to see the data. In this case, please let us know what it is and we are happy to provide it.

**2.** *The authors performed RiboAmp HS RNA Amplification - which is a valid procedure, but distortions of the global profile may only be analyzed by comparison of amplified versus non amplified data from control materials.*

We were surprised by this comment, as this comparison is not routinely performed in association with RNA amplification studies. It is not clear to us what the appropriate control material would be in this case, as any other materials would have different gene expression profiles; thus, we wonder how generalizable the conclusions about presence/absence of distortion would be from the control to experimental material. Out of curiosity, in early July, we queried the most recently published articles in *BMC Genomics* that included amplified RNA to see whether they included amplified vs. non-amplified data from control materials, and we found that none of them did. We feel that rejecting our manuscript because this experiment was not performed, when this experiment was not performed in many other studies published in *BMC Genomics*, is unusual.

Thank you for taking the time to review this review process. Please do not hesitate to contact us if we can provide any additional information. Please know that we are committed to improving our manuscript in accordance with your expectations, and we hope that you can clarify the remaining issues and whether either of our updated figures addresses them satisfactorily.

Best wishes,

Rachel Mueller, Cheng Huang, and Robert Ho