Sobp modulates the transcriptional activation of Six1 target genes and is required during craniofacial development

Andre L. P. Tavares, Karyn Jourdeuil, Karen M. Neilson, Himani D. Majumdar and Sally A. Moody

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MS TITLE: Sobp modulates Six1 transcriptional activation and is required during craniofacial development

AUTHORS: Andre Luiz Pasqua Tavares, Karyn Jourdeuil, Karen Neilson, Himani Majumdar, and Sally A Moody

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

As you will see, the referees express considerable interest in your work, but have some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision.

We are aware that you may be experiencing disruption to the normal running of your lab that make experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

This paper analyzes the function of SOBP, a protein previously predicted to interact with Six1, for the development of cranial placodes in Xenopus. Using Co-IP and immunofluorescence assays in a mammalian cell line, the paper shows that SOBP interacts with both Six1 and Eya1. Like Six1, SOBP localizes to the nucleus depending on a NLS, while Eya1 only translocates to the nucleus when binding to Six1 and this can be abolished by high levels of SOBP, suggesting that the latter competes with Eya1 for Six1 binding. Luciferase assays suggest that SOBP-Six1 complexes then repress target genes, which are activated by Six1-Eya1. Finally, functional experiments in Xenopus embryos (Morpholinos and CRISPR/Cas9) reveal that SOBP plays a role during patterning of the early neural plate border region and the otic vesicle in Xenopus. The paper also begins to explore the role of the R651X mutation of SOBP in Xenopus, which in humans leads to mental retardation and mild hearing loss.

The paper provides important new information on how the activity of Six1, a central regulator of cranial placode development, is modulated during embryogenesis. The paper is well written and the experiments are clearly described and well documented. I have only a few minor comments.

Comments for the author

- In the abstract and elsewhere, the authors state that SOBP represses "Six1+Eya1 transcriptional activation". This wording is ambiguous since it could also mean that SOBP represses the activation of Six1 and Eya1. What the authors mean and show, however, is that SOBP interferes with the transcriptional activation of Six1+ Eya1 target genes. Please clarify and reword!
- On p. 5 and elsewhere in the manuscript, the authors state that SOBP expression is complementary to Six1 expression but this is an oversimplification. In fact, their data show that SOBP and Six1 are largely expressed in the same domains but with a complementary bias of expression
(higher levels of SOBP where levels of Six1 are low and vice versa)
- P.8 and Fig. 2A. The authors state that the "amount of Six1 bound to Eya1 was diminished at a 5-fold or 10-fold increase in Sobp". However, Fig. 2 A shows that the amount of Six1 bound to Eya1 is actually higher in the presence of low levels of SOBP than in their absence (compare second to first lane of the Western blot). This should be discussed and it needs to be clarified, how this fits with the model proposed.
- P.13, The role of SOBP in the otic vesicle remains a bit unclear since the effects shown are very mild and not always convincing. For example, the decrease of Six1 in Fig.5B and of Dlx5 and Pax2 in Fig. 5F and I are not very clear. These should be replaced by better images, if possible. Altranatively this section and the corresponding part of the discussion should be toned down.
- On P. 19, the authors argue that the expansion of neural markers after SOBP loss of function and the increase of epidermal markers after SOBP gain of function suggest that SOBP acts on neural plate and epidermis independent of Six1, since the latter is not expressed in neural plate or epidermis. I'm puzzled by this argument, since SOBP and Six1 are coexpressed in the PPE which is intercalated between these territories and so a model in which SOBP+Six1 inhibit neural plate but promote epidermal fates within the PPE is compatible with the authors findings.

Reviewer 2

Advance summary and potential significance to field

In this manuscript, the authors show that Sobp is a Six1 binding factor and can influence gene induction normally produced by a Six1/Eya1 complex. The authors also show that altering levels of Sobp deferentially affect neural plate border development (including gene expression) and show in vivo that otic vesicle development is affected when Sobp is knocked down by transient CRISPR or overexpression. These findings provide an exciting new avenue with which to understand Six1 function in development and potentially explain cases of BOR in which SIX1 mutations are not observed, providing a new avenue for variant discovery.

Comments for the author

Major comments
1. The authors state, “Remarkably, Sobp was able to bind to (Fig. S3M) and partially translocate Eya1 to the nucleus (Fig. S3C, F, I, L) without Six1 co-expression.”. This seems like important data that should be presented. Further, while acknowledging this, the authors move quickly in the next two sentences to describe how elevated levels of Sobp leads to Eya1 localization in the cytosol. What is the balance between Sobp binding to Eya1 and translocating it into the nucleus and competition of Six1-Eya1 though binding of Eya1 by Sobp and keeping it in the cytosol? By not putting the first set of data in the paper, it is easier to not discuss this point, though it is important and mentioned in the discussion. Thus, it should be shown and described in the Results.

2. In Figure 2, the 5x transfection of HA-Sobp (D, H, L, P) is shown in nuclei that appear much larger than nuclei in other panels, even though a measurement bar is included. This is a little misleading. Further, the cytosolic staining of Myc-Eya1 looks more like green background that real staining (compare D vs E); it appears that the gain on the green channel has been increased to make the green more apparent. Again, part of this is due to the size of the nuclei vs the size of the panel, as it is not possible to see what the background looks like. The authors need to show new nuclei so that they more closely resemble the other panels and leave enough space so the that it is clear that background is black and not slightly green.

3. The authors speculate that the ZF or SIM domains could account for the function of Sobp in altering the activity of the Siz1+Eya1 complex. This seems like such low-hanging fruit and would address the functional mechanism of Sobp function; it is not clear why the authors did not do these experiments. The biochemistry of this manuscript is the very clean and convincing and these experiments would be straightforward and informative.

4. The quantification and statistics in this manuscript are very good. All stats are given in good detail in the text, though nothing is shown on the figure. For example, in Figure 1M, is Six1 + Eya1 significantly different from Six1 + Eya1+Sobp? How about Six1 + Eya1 + Sobp vs control? The numbers presented in the paper can get lost in translation and the reader should not have to switch back and forth between the figure and text to understand significance. These need to be added to the bar graphs throughout the paper.

5. In the text on Page 11, the authors state, “To ensure MO specificity, embryos were injected with Sobp MO plus an MO-insensitive sobp mRNA and assessed for foxd3 expression (Fig. 4B, Q).”. This cannot be right; the authors had already presented Fig. 4B and not said anything about this condition. Further, the data they reference in Fig. S4C-D do not match what is shown in Fig. 4B, Q. The authors need to fix this.

6. On Page 11-12, the authors state that Sobp overexpression resulted in rather random changes in sox2, foxd3 and krt12.4 expression, though six1 expression appeared reduced. Using qPCR, they find that foxd3 and six1 were decreased and krt12.4 was increased. They conclude that, “These data demonstrate that altering the levels of Sobp disrupts the relative sizes and gene expression pattern of the embryonic ectodermal domains. There is no attempt to reconcile these two disparate findings; rather, the authors used the qPCR data as the basis for the assertion, presumably because it fits their hypothesis. This needs to be fixed.

7. Likewise, the presentation of the R651X mutant is needlessly complicated. The authors present each of the genes as reduced or increased, with the relative percentage stated. Before going into the qPCR, which like the above section is only partially supportive of the ISH analyses, the authors insert a sentence or two that compares full length SOBP vs R651X overexpression, in which some of similar while others are different. This presentation makes it almost impossible to keep track of the changes (I made a chart as I kept forgetting which condition was being discussed). In the end, the authors state that gene expression is changed by the R651X mutant. I can see that but not all expression was changed. This is an example where the quantification and ISH are different and while the authors are correct to check it, the findings do not corroborate each other so the authors go with the data that supports their hypothesis.

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Minor comments:
1. In figure 3B, the table inside the panel is almost unreadable at a decent magnification. The same is true for the qPCR graph labels in Figs. 4 and 5.
2. The authors need to use the correct human nomenclature for their variants. Note that the correct term is variant, not mutation. Further, the correct human nomenclature is SOBP p.R661X, not R661X (HUGO). Further, the authors should include “Sobp” and not just R661X so that it is clear in the figures which protein variant is being tested.
3. The authors have been very frugal in the use of words. In some places, the reasoning and results and conclusions for an experiment span only two sentences. This can, at times, limit the understanding and impact of the writing. One example is the paragraph on Page 8 that introduces Pa2g4. While the authors state that Pa2g4 repressed Six1 + Eya1 activity and provide a reference, there is no description of what Pa2g4 is, whether it is known to be involved in any disease phenotype, how it was found, etc. I have not done a word count so I don’t know how close to the limit the authors are, but this is but one of multiple examples where the authors inadequately or too quickly proceed through experiments without adequate background or explanation. Also, when listing two or three conditions, the panels associated with each condition should follow the condition, not be lumped together with other panel lettering at the end of the sentence. Too much time is wasted going back and forth with the text and figures.

First revision

Author response to reviewers' comments

Reviewer 1 Advance Summary and Potential Significance to Field...

This paper analyzes the function of SOBP, a protein previously predicted to interact with Six1, for the development of cranial placodes in Xenopus. Using Co-IP and immunofluorescence assays in a mammalian cell line, the paper shows that SOBP interacts with both Six1 and Eya1. Like Six1, SOBP localizes to the nucleus depending on a NLS, while Eya1 only translocates to the nucleus when binding to Six1 and this can be abolished by high levels of SOBP, suggesting that the latter competes with Eya1 for Six1 binding. Luciferase assays suggest that SOBP-Six1 complexes then repress target genes, which are activated by Six1-Eya1. Finally, functional experiments in Xenopus embryos (Morpholinos and CRISPR/Cas9) reveal that SOBP plays a role during patterning of the early neural plate border region and the otic vesicle in Xenopus. The paper also begins to explore the role of the R651X mutation of SOBP in Xenopus, which in humans leads to mental retardation and mild hearing loss.

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Reviewer 1 Comments for the Author...

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We thank the reviewer for pointing out this ambiguity. We have changed the wording to that suggested by the reviewer throughout the text, including the Title, Summary Statement, Abstract (line 8); Introduction (para 4), Results (section 2 title, para 3, 4, 5, 6), Discussion (para 1, 2, 4, 8) and Figure legends 1, 2, 3 and 7.

- On p. 5 and elsewhere in the manuscript, the authors state that SOBP expression is complementary to Six1 expression, but this is an oversimplification. In fact, their data show that SOBP and Six1 are largely expressed in the same domains but with a complementary bias of expression (higher levels of SOBP where levels of Six1 are low and vice versa)

We agree with the reviewer that our statement was an oversimplification. We added a better description of the expression patterns for Six1 and Sobp in the introduction (para 4). We did not find another location in the paper in which we used similar language to describe their expression patterns.

- P.8 and Fig. 2A. The authors state that the "amount of Six1 bound to Eya1 was diminished at a 5-fold or 10-fold increase in Sobp". However, Fig. 2 A shows that the amount of Six1 bound to Eya1 is actually higher in the presence of low levels of SOBP than in their absence (compare second to first lane of the Western blot). This should be discussed and it needs to be clarified, how this fits with the model proposed.

We also noticed that low levels of Sobp resulted in more Six1 bound to Eya1. Interestingly, this is a reproducible finding and not an artifact of one single experiment. Patrick et al. (2009) showed that Six1 protein stability is increased in the presence of the co-factor Eya2. It is possible that low levels of Sobp increase Six1 protein stability without inhibiting its transcriptional function. It is also possible that at low levels, as Sobp is able to translocate Eya1 into the nucleus, there is more Eya in the cell nucleus and therefore more bound Six1. This is still in agreement with our dose-dependent competition mechanism. We now report this finding in the Results section (p8, para2, lines 8-9), the Figure 2 legend and added a brief explanation to the Discussion (p18, para2).

- P.13, The role of SOBP in the otic vesicle remains a bit unclear since the effects shown are very mild and not always convincing. For example, the decrease of Six1 in Fig.5B and of Dlx5 and Pax2 in Fig. 5F and I are not very clear. These should be replaced by better images, if possible. Alternatively, this section and the corresponding part of the discussion should be toned down.

We performed new ISH analyses and replaced Fig. 5B, F and I with better images and by adding insets showing a higher magnification of the otic vesicles present more convincing phenotypes. Regarding Fig. 5F and I, we did state in the Results section that effects on the otic vesicle of R651X injection are mild, with most analyzed embryos showing no change (p15, para2, lines 2-3). We modified the description of these images in the Figure 5 legend for clarity. It now states: "Images in each box are the control and injected sides of the same embryo, and are representative of the most frequent phenotype for CRISPR (A, D, G) and *sobp* mRNA (B, E, H). Images for p.R651X mRNA represent a less frequently observed phenotype (C, F, I)."

- On P. 19, the authors argue that the expansion of neural markers after SOBP loss of function and the increase of epidermal markers after SOBP gain of function suggest that SOBP acts on neural plate and epidermis independent of Six1, since the latter is not expressed in neural plate or epidermis. I'm puzzled by this argument, since SOBP and Six1 are coexpressed in the PPE which is intercalated between these territories and so a model in which SOBP+Six1 inhibit neural plate but promote epidermal fates within the PPE is compatible with the authors findings.

We thank the reviewer for pointing out this inconsistency. We incorporated the reviewer's comments in the Discussion (p20, last sentence to top of p21).

Reviewer 2 Advance Summary and Potential Significance to Field...

In this manuscript, the authors show that Sobp is a Six1 binding factor and can influence gene induction normally produced by a Six1/Eya1 complex. The authors also show that altering levels of Sobp deferentially affect neural plate border development (including gene expression) and show in vivo that otic vesicle development is affected when Sobp is knocked down by transient CRISPR or overexpression. These findings provide an exciting new avenue with which to understand Six1 function in development and potentially explain cases of BOR in which SIX1 mutations are not observed, providing a new avenue for variant discovery.

Reviewer 2 Comments for the Author...

Major comments
1. The authors state, “Remarkably, Sobp was able to bind to (Fig. S3M) and partially translocate Eya1 to the nucleus (Fig. S3C, F, I, L) without Six1 co-expression.”. This seems like important data that should be presented. Further, while acknowledging this, the authors move quickly in the next two sentences to describe how elevated levels of Sobp leads to Eya1 localization in the cytosol. What is the balance between Sobp binding to Eya1 and translocating it into the nucleus and competition of Six1-Eya1 though binding of Eya1 by Sobp and keeping it in the cytosol? By not putting the first set of data in the paper, it is easier to not discuss this point, though it is important and mentioned in the discussion. Thus, it should be shown and described in the Results.

We agree with the reviewer that the data presented in Figure S3 are important and therefore added them to revised Figure 2. The description of the Figure S3 results (now part of revised Figure 2) had already been included in the Results section, and we now also include it in the explanation of the dose-dependent competition mechanism for Sobp in the Discussion (p18 last line to top of p19).

2. In Figure 2, the 5x transfection of HA-Sobp (D, H, L, P) is shown in nuclei that appear much larger than nuclei in other panels, even though a measurement bar is included. This is a little misleading. Further, the cytosolic staining of Myc-Eya1 looks more like green background that real staining (compare D vs E); it appears that the gain on the green channel has been increased to make the green more apparent. Again, part of this is due to the size of the nuclei vs the size of the panel, as it is not possible to see what the background looks like. The authors need to show new nuclei so that they more closely resemble the other panels and leave enough space so the that it is clear that background is black and not slightly green.

We agree with the reviewer that the cell nuclei in the original Figure 2D, H, L, P are slightly larger than the nuclei in other panels. We believe this was a consequence of small differences in cell confluency between transfected slides since we have observed these nuclei-size differences in previous, unrelated experiments. We performed additional culture experiments and replaced images D, H, L, P with new ones in revised Figure 2 that show cells with nuclear sizes that are comparable to the other images and contain untransfected cells in the field that have a clear background in the green and red channels – i.e., no one turned up the gain. As this panel was rearranged in response to Comment #1, these images are now labeled Figure 2Q, U, X, B’.

3. The authors speculate that the ZF or SIM domains could account for the function of Sobp in altering the activity of the Siz1+Eya1 complex. This seems like such low-hanging fruit and would address the functional mechanism of Sobp function; it is not clear why the authors did not do these experiments. The biochemistry of this manuscript is the very clean and convincing and these experiments would be straightforward and informative.

We very much agree with the reviewer, and a complete understanding to the functional domains and protein interaction sites is a next goal in this project. However, the time it would take to make the deletion constructs and perform all of the analyses reported herein, particularly during this Covid time when our laboratory is not able to work at full capacity, is not practical. We have revised para4 of the Discussion to make it clear that we intend to do the suggested analyses in the future. This also was previously and remains the last sentence of the manuscript.

4. The quantification and statistics in this manuscript are very good. All stats are given in good detail in the text, though nothing is shown on the figure. For example, in Figure 1M, is Six1 + Eya1 significantly different from Six1 + Eya1+Sobp? How about Six1 + Eya1 + Sobp vs control? The numbers presented in the paper can get lost in translation and the reader should not have to switch back and forth between the figure and text to understand significance. These need to be added to the bar graphs throughout the paper.

As requested by the reviewer, we added pairwise comparisons between all groups in Figure 1M. As Figure 3K and 3T have an extra experimental group, the graphs became too busy and confusing when we added all pairwise comparisons. In addition, most of the comparisons were already shown in Figure 1M; thus, we added only the pairwise comparisons between the most relevant groups to Figure 3K and 3T.

5. In the text on Page 11, the authors state, “To ensure MO specificity, embryos were injected with Sobp MO plus an MO-insensitive sobp mRNA and assessed for foxd3 expression (Fig. 4B, Q).”. This cannot be right; the authors had already presented Fig. 4B and not said anything about this condition. Further, the data they reference in Fig. S4C-D do not match what is shown in Fig. 4B, Q. The authors need to fix this.

We thank the reviewer for identifying this error. The sentences have been revised to make it clear that Fig. S3C-D (previously Fig. S4C-D) depict the rescue experiment (29.4% show reduced *foxd3*), whereas Fig, 4B, Q depict the MO alone experiment (90.2% show reduced *foxd3*). They also have been moved to the Methods section (p27) due to edits to the paragraph on p11, as explained in point #6 below.

6. On Page 11-12, the authors state that Sobp overexpression resulted in rather random changes in sox2, foxd3 and krt12.4 expression, though six1 expression appeared reduced. Using qPCR, they find that foxd3 and six1 were decreased and krt12.4 was increased. They conclude that, “These data demonstrate that altering the levels of Sobp disrupts the relative sizes and gene expression pattern of the embryonic ectodermal domains. There is no attempt to reconcile these two disparate findings; rather, the authors used the qPCR data as the basis for the assertion, presumably because it fits their hypothesis. This needs to be fixed.

We thank the reviewer for pointing out the confusion caused by the style of our presentation, which was driven by the challenge of the journal’s 7000 word limit, not an attempt to fit our hypothesis.

It is not surprising that the qPCR data and the ISH results do not fully match because qPCR measured changes from whole embryos, whereas ISH showed spatial changes in specific ectodermal domains. We have rewritten the paragraphs describing the Sobp KD and mRNA experiments. Each paragraph now leads with the qPCR data from whole embryos, and then presents the spatial domain changes observed by ISH.

For the KD data, we address the inconsistency for *sox2* and *krt14.2* in the last three sentences of the paragraph (p11, end of para1):
“The results from the two different assays are summarized in Table S1. The qPCR and ISH approaches both showed reductions in *foxd3* and *six1* expression, but the ISH assay was more sensitive to changes in the spatial domains of *sox2* and *krt14.2*. Whereas qPCR did not detect a change in *sox2* levels in the whole embryo, the *sox2* domain was broader and fainter, which would likely lead to no significant overall change as assessed by qPCR. Whereas qPCR did not detect a change in *krt14.2* levels in the whole embryo, the *krt14.2* domain was obviously reduced along the anterior neural plate (Fig. 4G, K); this may be too subtle a change to be detected by qPCR of a whole embryo covered by epidermis.”

For the sobp mRNA data, we address the inconsistency for *sox2* and *krt14.2* in the last three sentences of the paragraph (p12, end of para1):
“Similar to the qPCR data, the six1 domain was smaller in the majority of the embryos (86.9%, Fig. 4N, V). Even though the krt12.4 domain was increased in some embryos with ectopic expression of this gene overlapping the lineage tracer (33.3%, Fig. 4O, V), consistent with the qPCR results, for most embryos the domain of this gene was not detectably changed (54.2%, Fig. 4V). Overall, these data, summarized in Table S1, demonstrate that increasing the levels of Sobp disrupts the relative

sizes of the gene expression domains of foxd3 and six1 and can induce ectopic expression of krt14.2."

For both sets of experiments we summarize the qPCR and ISH data in a single table for easier comparison (new Table S1). The offending sentence on page 12 now reads:
"Overall, these data, summarized in Table S1, demonstrate that increasing the levels of Sobp disrupts the relative sizes of the gene expression domains of *foxd3* and *six1* and can induce ectopic expression of *krt14.2*." This sentence incorporates results from the ISH and qPCR data without mentioning increase or decrease for the expression of these genes.

7. Likewise, the presentation of the R651X mutant is needlessly complicated. The authors present each of the genes as reduced or increased, with the relative percentage stated. Before going into the qPCR, which like the above section is only partially supportive of the ISH analyses, the authors insert a sentence or two that compares full length SOBP vs R651X overexpression, in which some of similar while others are different. This presentation makes it almost impossible to keep track of the changes (I made a chart as I kept forgetting which condition was being discussed). In the end, the authors state that gene expression is changed by the R651X mutant. I can see that but not all expression was changed. This is an example where the quantification and ISH are different and while the authors are correct to check it, the findings do not corroborate each other so the authors go with the data that supports their hypothesis.

As above, we have rewritten the paragraph describing the R651X variant (p13-14), leading with the qPCR, and specifically addressing the differences between the qPCR and ISH and variant versus full-length Sobp. These data are summarized for the reader's convenience in Table S1. We apologize if we gave the impression that we were simply picking data that supported our hypothesis. On the contrary, we were trying to be concise in light of the journal's 7000 word limit. The reviewer's point, however, it well taken, and by being given the opportunity to revise this section and provide Table S1 we are able to more accurately present the data.

8. The authors show that dlx5 and pax2 are downregulated in embryos following knockdown of sobp by CRISPR. They fortunately show qPCR, since the reduction in staining is not very convincing in D and G. The authors should try to improve this data or provide better pictures/insets.

We replaced figures 5D (ISH for dlx5) and 5G (ISH for pax2) and added insets showing a higher magnification of the otic vesicles to make it easier to see the differences in size of the expression domains.

9. The alcian blue preps of embryos are not good. While the CRISPR is okay, the sobp and R651X cartilage staining is not at all clear; it just looks like a mush of blue. While the models are obviously clear, it would be far more beneficial if one could actually see the detail in of the cartoon in the staining of the embryo.

We agree with the reviewer that the cartilage staining preps in Figure 6E and 6F were not ideal because the blue color looked faded. Alcian blue staining of Xenopus tadpoles is not as clear as staining of other species because of soft tissue background; this is why we added the line drawings of what we observed with the dissecting microscope so we could better communicate the state of the cartilaginous elements. We have replaced both figures with cartilage preps that show a better contrast of the cartilages, particularly the otic capsule.

Minor comments:
1. In figure 3B, the table inside the panel is almost unreadable at a decent magnification. The same is true for the qPCR graph labels in Figs. 4 and 5.

Thank you for pointing this out. We rearranged Figures 3A and 3B so that 3B is now larger. We increased the font size for all labels in the qPCR graphs in Figures 4 and 5.

2. The authors need to use the correct human nomenclature for their variants. Note that the correct term is variant, not mutation. Further, the correct human nomenclature is SOBP p.R661X, not R661X (HUGO). Further, the authors should include "Sobp" and not just R661X so that it is clear in the figures which protein variant is being tested.

Thank you for pointing this out. We replaced the word “mutation” with “variant” throughout the manuscript. We added the correct nomenclature for the variants: p.R661X and p.R651X. We also added p.R651X variant of sobp/p.R651X Sobp whenever this variant was first mentioned in a section and in the legends for Figures 3, 4, 5 and 6.

3. The authors have been very frugal in the use of words. In some places, the reasoning and results and conclusions for an experiment span only two sentences. This can, at times, limit the understanding and impact of the writing. One example is the paragraph on Page 8 that introduces Pa2g4. While the authors state that Pa2g4 repressed Six1 + Eya1 activity and provide a reference, there is no description of what Pa2g4 is, whether it is known to be involved in any disease phenotype, how it was found, etc. I have not done a word count so I don’t know how close to the limit the authors are, but this is but one of multiple examples where the authors inadequately or too quickly proceed through experiments without adequate background or explanation. Also, when listing two or three conditions, the panels associated with each condition should follow the condition, not be lumped together with other panel lettering at the end of the sentence. Too much time is wasted going back and forth with the text and figures.

Although we agree many aspects of our findings would benefit from fuller details, indeed there is a 7000 word limit for this journal that makes fuller explanations a challenge. By adding the supplemental data in previous Supplemental Figure 3 into the main text (Major Comment #1 above), and adding other details as requested by both reviewers, the word number is now already over the word limit. We have provided a small amount of additional detail to the sentence describing Pa2G4 (p8, para2), but there also is reference that provides extensive detail about this protein. The main point is that it also is a Six1 cofactor.

With regards to the last concern, we have complied to the reviewer’s request by separating out the references to each condition rather than lumping them together at the end of sentences, even though this adds to the word count of the text.

Second decision letter

MS ID#: DEVELOP/2021/199684

MS TITLE: Sobp modulates the transcriptional activation of Six1 target genes and is required during craniofacial development

AUTHORS: Andre Luiz Pasqua Tavares, Karyn Jourdeuil, Karen Neilson, Himani Majumdar, and Sally A Moody
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

See my review of the first version

Comments for the author

All my comments have been satisfactorily addressed

Reviewer 2

Advance summary and potential significance to field

The authors show that Sobp is a Six1 cofactor capable of binding to Six1 and disrupting the activation of Six1/Eya1-induced genes. Further, introduction of the Sobp mutation into embryos results in craniofacial defects. These findings illustrate that Sobp likely acts be sequestering Six and as such, is crucial for Six1-driven PPE and craniofacial development. Sobp may therefore indeed be considered a candidate gene for BOR syndrome.

Comments for the author

The authors have done a very good job at addressing reviewer concerns. The addition of statistics to several figures is notable. In addition, the authors have made several figures legible while also providing new images of stained embryos for several sections (ISH and alcian blue among them). As revised, this is a much improved manuscript.