# Mitf-family transcription factor function is required within cranial neural crest cells to promote choroid fissure closure

Katie L. Sinagoga, Alessandra M. Larimer-Picciani, Stephanie M. George, Samantha A. Spencer, James A. Lister and Jeffrey M. Gross
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**Original submission**

First decision letter

MS ID#: DEVELOP/2019/187047

MS TITLE: Mitf-family transcription factor function is required within cranial neural crest cells to promote choroid fissure closure

AUTHORS: Katie Sinagoga, Alessandra Larimer-Picciani, Stephanie George, Samantha Spencer, James Lister, and Jeffrey Gross

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.

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*

In this manuscript, Sinagoga et al. address the important question of how Mitf transcription factors function in development and disease. Human mutations in MITF have been associated with the eye

defect coloboma yet the actual mechanisms by which loss of MITF leads to coloboma are unknown. Further, Mitf is expressed by and functions in both neural crest and RPE; it is unclear where function is required. Sinagoga et al. examine a zebrafish mitf;tfec double mutant, which exhibits pigmentation defects and coloboma. Using stable transgenic lines, they identify a neural crest cell (NCC) migration defect, and then, using cell transplantation techniques, demonstrate that Mitf family member function is actually required in NCCs, and not RPE, in order to promote choroid fissure closure.

The transplantation experiment results are striking and convincing, and contribute to our general understanding of how Mitf genes and NCCs can impact choroid fissure closure. My main concern is related to the experiments and conclusions regarding migration defects; otherwise, there are mostly minor comments and questions.

*Comments for the author*

• Related to the use of the mitfa:eGFP transgenic line to assess NCC migration: Is the cellular defect specific to the subset of NCCs expressing the transgene? Is transgene expression itself altered or defective when mitfa and/or tfec are lost? Can the authors use a different transgenic line to assess NCC migration more generally?

• Figure 2C: Can the authors quantify these results? At this stage, there seem to be more mitfa:eGFP-positive cells around the eye, but in a different position compared to wild type.

• Results, "Embryos lacked cNCCs surrounding the optic cup at 48 and 72hpf, suggesting that cNCC contribution to the POM is not simply delayed in mitfa;tfec mutants." As the authors note, the mitfa:eGFP transgene marks a subset of NCCs; it is possible that other cNCCs surround the eye during these times. The language throughout this section should be edited to reflect the possibility that this could be an effect specific for the mitfa- or tfec-expressing subpopulation, and could reflect a specific function of this cell population.

• Supplementary Movies 1 and 2: it is difficult to evaluate the defect in these movies, when the defect already seems to have arisen prior to 25 hpf, when these movies start. Have the authors looked earlier to see if other NCCs migrate normally, or when the defective localization of the mitfa:eGFP population arises?

• Results, "…lack of cNCCs within the POM in mitfa;tfec mutants is primarily driven by a defect in cell migration." The results shown at this point in the manuscript are not sufficient to conclude this. Proliferation could be affected, and as noted above, transgene expression itself could be affected. The language could be edited.

• Related to Figure 3D and E: The authors note that they only analyzed embryos "in which at least 20% of the optic cup was composed of transplanted cells". Was there any positional requirement? How many of the assayed embryos had cells in the ventral portion of the eye?

• Figure 1B (minor comment): How were mild and severe phenotypes distinguished from each other? The sections do not appear very different with respect to pigmentation.

Reviewer 2

*Advance summary and potential significance to field*

In this well-written and thoughtful manuscript, Sinagoga and colleagues study the role of mitf-family transcription factors in zebrafish cranial neural crest cells (cNCC) during ocular morphogenesis. They show that mitfa;tfec mutants have disturbed retinal pigment epithelium (RPE) and cNCC development and exhibit colobomatous microphthalmia. Their subsequent experiments show the specific importance of cNCC Mitf transcription factors in mediating optic fissure closure, likely by affecting cellular migration. I think this article is of particular importance in that it highlights a role of mitf outside the RPE that has important implications for human disease.

*Comments for the author*
The authors should be applauded for their careful attention to detail on several counts. Examples include showing the range of phenotypes in double mutants, including data on the relative number of genotypes observed with their incrossing strategy, showing both serial section and whole-mount data to make quantification more believable.

SCIENCE

1. Figure S1, I am not 100% convinced of the absence of coloboma. To my eye, it appears that there is a slight indentation in the ventral optic cup, which is sometimes seen in mutant fish and mice where there edges of the fissure approximate but don't fuse. The authors have beautiful laminin staining of sagittal sections in Figure 1; it would be great to see a similar panel/inset here. The sections shown appear coronal. Please state that in the legend. Regardless, it is clear that the phenotype of the double mutants shown in Figure 1 is more severe.
2. Figure 2a. It appears that the triallelic mutant has a difference between nasal and temporal melanin pigmentation. Was this a consistent observation? Consider mentioning/commenting in the text or the figure legend.
3. Figure 3. These are very elegant transplantation experiments. It might be helpful to expand a bit on the caveat the authors give, namely, "…that our transplants could be sub-threshold for the number of cells necessary to mediate effective closure." Is an additional possibility that the cellular/molecular state of the genetically wild-type cells transplanted into the mutant embryos cease to be as "genuinely wild-type" as they would be in the context of a completely normal optic cup? Also, the authors do not comment on the fact that the transplantation seems to make the non-fusion WORSE, at least as measured by the degree to which the optic fissure is open. Could they comment a bit, please?

EDITORIAL
1. Introduction
a. Suggest "…subdivides into the neural retina and retinal pigmented epithelium…".
2. Results and discussion
a. Is the best word "apposing" rather than "opposing"? Honestly, I think I have seen it both ways.
b. If possible, could a dotted line be included in the movies to show the outline of the fish? It might help with quick/easy orientation.

Reviewer 3

*Advance summary and potential significance to field*

The essential, and novel contribution from this manuscript, is demonstration of a degree of rescue of coloboma with transplantation of neural crest cells (NCC), and gene-specific expression within NCCs. This has relevance to other tissues where incomplete closure (cleft lip/palate, neural tube defects etc.) results in a spectrum of developmental phenotypes.

*Comments for the author*

This short, and thoughtfully-written manuscript, presents evidence that two paralogous genes (Mitf and Tfec) are required for cranial neural crest function. Deficiency induces coloboma in zebrafish, and likely in patients. The results presented are of significant interest, with three areas that could be beneficially enhanced.

1. Context Scope exists to enhance the background regarding Mitf's roles, regulatory interactions, and disease phenotypes, e.g. cross-repression of Chx10 (Vsx2), mutation of which induces microphthalmia, a coloboma-associated phenotype. The unmentioned interactions with Otx1, Vax1/Vax2 and Pax6, miss the opportunity to outline the diverse mechanisms by which altered Mitf/Tfec function may induce disease.

Description of Mitf's roles in human genetics/disease are also underdeveloped. Heterozygous mutation was first shown to induce pigmentation/deafness phenotypes

(Waardenburg Syndrome), with compound Mitf mutations recently implicated in a multisystem phenotype that includes coloboma. However, this phenotype has a narrow evidence base - just two patients with biallelic Mitf mutations. The mammalian genetics is likely more complex than currently appreciated, since a single Mitf mutation can induce coloboma [Id # 305773, Decipher Database, https://decipher.sanger.ac.uk/]. Consequently, analyses in zebrafish may be particularly informative, and could be usefully discussed with enhanced description of the rationale for studying compound *mitf:tfec* mutants, rather than other paralog combinations.

Neural crest's roles in coloboma could also be outlined in more detail. The pigmentation and other phenotypes make neural crest dysfunction a very plausible hypothesis, and description in such terms would provide an excellent rationale and introduction for the subsequent experiments.

2. Data Presentation At several points in the manuscript, primary data are not provided (e.g. Fig. 3E) - inclusion of a montage as an additional supplemental figure would work well. An explicit statement regarding which experiments were repeated (and how many times), should be added.

3. Validation In contrast to the above textual edits, an additional readout for the rescue reported in Figure 3 would strengthen the manuscript.

To their credit, the authors employed two independent approaches, however both assess anatomical closure of the choroid fissure. Can expression of wild type *tfec* within cranial neural crest be repeated and other endpoints analyzed? Partial rescue of either patterning, laminin within the CF, retinal ganglion cell projection (DiI/DiO), ocular size, or a dose-response effect, would be highly persuasive. The choice of readout is the authors, with a single experiment to validate the manuscript's central conclusion, all that is required.

Minor points:

Neural crest cells per 1000um$^2$ are quantified to 4 decimal places (sometimes)

"cNCCs in …. mutants appeared to explode" Since the relevant two hour section of the video does not depict high velocity fragmentation, perhaps an alternative term might be used?

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**First revision**

Author response to reviewers' comments

We'd like to thank the Reviewers for their enthusiasm about our work and the constructive comments they provided to improve it. Indeed, these suggestions were helpful in strengthening the story. Below, we provide point-by-point responses to these comments (in purple) and, when applicable, indicate specific text or figures revisions that have been made to address the concerns. Changes in the revised manuscript are also colored purple to make clear revisions from the original submission.

While we were able to address each comment, there remain a couple of additional small changes that we intended to make but due to the SARS-CoV-2 shutdown at the University of Pittsburgh, we have been unable to access the laboratory since early March and it appears that we will remain closed at least through the end of May. Unfortunately, while our animals are being maintained by a centralized care staff, we have also been unable to breed new generations and fear that by the time we regain access to the lab, our breeding pairs will no longer be sufficient to generate new embryos

and will need to be regenerated, requiring several additional months. Thus, following guidance from the editorial staff at *Development*, we are resubmitting our manuscript in its current form with nearly all issues addressed. Importantly, these last open comments are relatively minor and we are confident that we have addressed the main concerns raised in review and that the manuscript and our data strongly support a model in which Mitf activity is required within cranial neural crest cells (cNCCs) during CF closure. Moreover, our data are exciting and relevant to the Special Issue in *Development* on the Origins of Developmental Disorders, for which we would still like it considered. Indeed, they shed light on the cellular mechanisms underlying colobomas in patients with *MITF* mutations and identify a novel role for Mitf function in cNCCs during CF closure. With this in mind, we hope that the manuscript is now acceptable for publication.

Reviewer 1 Advance Summary and Potential Significance to Field:
In this manuscript, Sinagoga et al. address the important question of how Mitf transcription factors function in development and disease. Human mutations in MITF have been associated with the eye defect coloboma, yet the actual mechanisms by which loss of MITF leads to coloboma are unknown. Further, Mitf is expressed by and functions in both neural crest and RPE; it is unclear where function is required. Sinagoga et al. examine a zebrafish mitf;tfec double mutant, which exhibits pigmentation defects and coloboma. Using stable transgenic lines, they identify a neural crest cell (NCC) migration defect, and then, using cell transplantation techniques, demonstrate that Mitf family member function is actually required in NCCs, and not RPE, in order to promote choroid fissure closure.

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• Related to the use of the mitfa:eGFP transgenic line to assess NCC migration: Is the cellular defect specific to the subset of NCCs expressing the transgene? Is transgene expression itself altered or defective when mitfa and/or tfec are lost? Can the authors use a different transgenic line to assess NCC migration more generally?
This was a helpful suggestion and agree that another NCC transgenic line should be utilized to address this question. In response to this, we have quantified *sox10*:eGFP$^{+}$ cells in 4dpf embryos. We additionally show that expression of wild-type *tfec* into cNCCs significantly increases the number of *sox10*:eGFP$^{+}$ cells (Fig. 3F). Thus, this experiment demonstrates both that other lineages of NCCs are affected by loss of mitfa and tfec and that reintroducing *tfec* into the cNCC lineage also rescues cNCC phenotypes.

• Figure 2C: Can the authors quantify these results? At this stage, there seem to be more mitfa:eGFP- positive cells around the eye, but in a different position compared to wild type.
We thank the reviewer for this comment and note that this is one of the experiments we were unable to complete due to SARS-CoV-2 shutdowns. However, while we did intend to do this because it is quite straightforward (quantification at 48hpf), we note two things that make us feel it is unnecessary. 1) We quantified of the NCCs at 24hpf (Figure 2B and Supplemental Figure 3B), which we feel is a more relevant time point for assessing differences in cNCC numbers and shows that a phenotype is already present at this earlier time. 2) We additionally provide *in vivo* imaging movies (~25-40hpf), which track the progression of NCC migration in relation to eye position and further support this early reduction and absence of cells migrating around the eye and into the CF.

• Results, “Embryos lacked cNCCs surrounding the optic cup at 48 and 72hpf, suggesting that cNCC contribution to the POM is not simply delayed in mitfa;tfec mutants.” As the authors note, the mitfa:eGFP transgene marks a subset of NCCs; it is possible that other cNCCs surround the eye during these times. The language throughout this section should be edited to reflect the possibility that this could be an effect specific for the mitfa- or tfec-expressing subpopulation, and could reflect a specific function of this cell population.
We have edited this to specify the *mitfa*:GFP population of cNCCs.

• Supplementary Movies 1 and 2: it is difficult to evaluate the defect in these movies, when the

defect already seems to have arisen prior to 25 hpf, when these movies start. Have the authors looked earlier to see if other NCCs migrate normally, or when the defective localization of the mitfa:eGFP population arises?
We thank the reviewer for this comment and agree that earlier time points may shed some additional light on whether there are earlier cNCC defects in *mitfa;tfec* mutants. However, the purpose of this experiment was to supplement section analyses and further test the hypothesis that cNCC defects were present at 25hpf as well as to determine whether cNCCs migrate to the eye field, as this was a possible mechanism underlying the colobomas in *mitfa;tfec* mutants. These data support that model and we hope to address exactly what/how mitfa and tfec affect cNCCs and optic cup morphogenesis in future experiments.

• Results, "…lack of cNCCs within the POM in mitfa;tfec mutants is primarily driven by a defect in cell migration." The results shown at this point in the manuscript are not sufficient to conclude this. Proliferation could be affected, and as noted above, transgene expression itself could be affected. The language could be edited.
We agree with the author and have edited the language to amend the specific statement here and we have also softened the migration language throughout other portions of the Results/Discussion section.

• Related to Figure 3D and E: The authors note that they only analyzed embryos "in which at least 20% of the optic cup was composed of transplanted cells". Was there any positional requirement? How many of the assayed embryos had cells in the ventral portion of the eye?
We apologize for the confusion here. All transplanted embryos had some portion of the ventral eye transplanted with wildtype cells, with the proportion varying slightly from embryo to embryo (which is common with these types of cell mosaics). We have amended our materials and methods section to further clarify this point and how the transplants were performed.

• Figure 1B (minor comment): How were mild and severe phenotypes distinguished from each other? The sections do not appear very different with respect to pigmentation.
We assessed mild and severe phenotypes via the severity of the angle of opening in the retina. We have amended the Materials and Methods text to clarify this point.

Reviewer 2 Advance Summary and Potential Significance to Field:
In this well-written and thoughtful manuscript, Sinagoga and colleagues study the role of mitf-family transcription factors in zebrafish cranial neural crest cells (cNCC) during ocular morphogenesis. They show that mitfa;tfec mutants have disturbed retinal pigment epithelium (RPE) and cNCC development and exhibit colobomatous microphthalmia. Their subsequent experiments show the specific importance of cNCC Mitf transcription factors in mediating optic fissure closure, likely by affecting cellular migration. I think this article is of particular importance in that it highlights a role of mitf outside the RPE that has important implications for human disease.

Reviewer 2 Comments for the Author:

The authors should be applauded for their careful attention to detail on several counts. Examples include showing the range of phenotypes in double mutants, including data on the relative number of genotypes observed with their incrossing strategy, showing both serial section and whole-mount data to make quantification more believable.

SCIENCE

1. Figure S1, I am not 100% convinced of the absence of coloboma. To my eye, it appears that there is a slight indentation in the ventral optic cup, which is sometimes seen in mutant fish and mice where there edges of the fissure approximate but don't fuse. The authors have beautiful laminin staining of sagittal sections in Figure 1; it would be great to see a similar panel/inset here. The sections shown appear coronal. Please state that in the legend. Regardless, it is clear that the phenotype of the double mutants shown in Figure 1 is more severe.
We thank the reviewer for the comment and have additionally sectioned more *tfec* mutant embryos to confirm our absence of a coloboma. We assessed an additional clutch of wildtype and sibling tfec single mutant fish at 4dpf and confirmed that none displayed a ventral indentation or any sign of

mild coloboma. This is our other SARS-CoV-2 compromise; we intended to stain some of these for laminin-1, but were not able to complete this before shutdown. That said, we have looked at numerous mutant alleles that possess subtle colobomas over the years in our laboratory and are confident that CF closure in *tfec* mutant is normal. We did take a more representative whole mount image of the *tfec* mutant to better represent our findings in the paper, included in a revised Fig. S1. The section plane was transverse, and we have indicated this as well as provided orientation in this (and all) figures to make that clearer.

2. Figure 2a. It appears that the triallelic mutant has a difference between nasal and temporal melanin pigmentation. Was this a consistent observation? Consider mentioning/commenting in the text or the figure legend.
This is an interesting point, but something that we haven't followed closely enough to have data on which to comment. There is certainly pigment variability amongst the various *mitfa;tfec* mutant alleles. We haven't noticed an obvious nasal-temporal difference in pigmentation in any specific allelic combination, but, again, we were not directly following this in our analyses so we can't say for sure whether there is or is not. We will certainly track this in future analyses but highlight here that, while interesting, it doesn't impact our findings on CF closure and coloboma.

3. Figure 3. These are very elegant transplantation experiments. It might be helpful to expand a bit on the caveat the authors give, namely, "…that our transplants could be sub- threshold for the number of cells necessary to mediate effective closure." Is an additional possibility that the cellular/molecular state of the genetically wild-type cells transplanted into the mutant embryos cease to be as "genuinely wild-type" as they would be in the context of a completely normal optic cup? Also, the authors do not comment on the fact that the transplantation seems to make the non-fusion WORSE, at least as measured by the degree to which the optic fissure is open. Could they comment a bit, please?
We agree with the additional possibility and have addressed it, as well as the exacerbation of CF closure defects with additional text in the Results/Discussion.

EDITORIAL
1. Introduction
a. Suggest "…subdivides into the neural retina and retinal pigmented epithelium…". Amended
2. Results and discussion
a. Is the best word "apposing" rather than "opposing"? Honestly, I think I have seen it both ways.
We looked closely at our use….we use "appose" to indicate the two closely associated sides of the CF and "opposed" to indicate the opposite facing sides. We also have seen it both ways in papers but have tried to use these definitions throughout our work.
b. If possible, could a dotted line be included in the movies to show the outline of the fish? It might help with quick/easy orientation.
We have added these.

Reviewer 3: This short, and thoughtfully-written manuscript, presents evidence that two paralogous genes (Mitf and Tfec) are required for cranial neural crest function. Deficiency induces coloboma in zebrafish, and likely in patients. The results presented are of significant interest, with three areas that could be beneficially enhanced.

1. Context Scope exists to enhance the background regarding Mitf's roles, regulatory interactions, and disease phenotypes, e.g. cross-repression of Chx10 (Vsx2), mutation of which induces microphthalmia, a coloboma- associated phenotype. The unmentioned interactions with Otx1, Vax1/Vax2 and Pax6, miss the opportunity to outline the diverse mechanisms by which altered Mitf/Tfec function may induce disease. Description of Mitf's roles in human genetics/disease are also underdeveloped. Heterozygous mutation was first shown to induce pigmentation/deafness phenotypes (Waardenburg Syndrome), with compound Mitf mutations recently implicated in a multisystem phenotype that includes coloboma. However, this phenotype has a narrow evidence base - just two patients with biallelic Mitf mutations. The mammalian genetics is likely more complex than currently appreciated, since a single Mitf mutation can induce coloboma [Id # 305773, Decipher Database, https://decipher.sanger.ac.uk/ ]. Consequently, analyses in zebrafish may be particularly informative, and could be usefully discussed with enhanced description of the rationale for studying compound mitf:tfec mutants, rather than other paralog combinations. Neural crest's roles in coloboma could also be outlined in more detail. The pigmentation and other

phenotypes make neural crest dysfunction a very plausible hypothesis, and description in such terms would provide an excellent rationale and introduction for the subsequent experiments.
We thank the Reviewer for the comments/thoughts and completely agree. We have added some additional text about *MITF* in human disease and neural crest in coloboma. We'd happily add more; however, the word limit for Research Reports is 3000 words and our revised manuscript was at 3700 words and returned by the editorial staff for us to cut to 3400. Thus, we've tried to retain a focus in the Introduction and Discussion aspects of the manuscript on colobomas and optic cup morphogenesis, and roles for cNCCs therein, as well as broad background on Mitf in development and disease. While we have added some text addressing these diverse roles, without editorial guidance/approval from *Development*, we can't easily add additional text without cutting relevant results text.

2. Data Presentation At several points in the manuscript, primary data are not provided (e.g. Fig. 3E) - inclusion of a montage as an additional supplemental figure would work well. An explicit statement regarding which experiments were repeated (and how many times), should be added.
We have included a montage of serial sections from both cNCC and retina/RPE transplants in Supplement Figure 3. We have additionally added a statement in the Materials and Methods section to clarify that the embryos analyzed were taken from 3 independent transplantation experiments and include n's in Results and legends.

3. Validation In contrast to the above textual edits, an additional readout for the rescue reported in Figure 3 would strengthen the manuscript. To their credit, the authors employed two independent approaches, however both assess anatomical closure of the choroid fissure. Can expression of wild type tfec within cranial neural crest be repeated and other endpoints analyzed? Partial rescue of either patterning, laminin within the CF, retinal ganglion cell projection (DiI/DiO), ocular size, or a dose-response effect, would be highly persuasive. The choice of readout is the authors, with a single experiment to validate the manuscript's central conclusion, all that is required.
We have performed additional analyses on *tfec*-injected embryos to show an additional readout of rescue. Here, we have quantified the number of *sox10*:eGFP$^{+}$ cells in 4dpf embryos. Our data show that expression of wild-type *tfec* in *mitfa*$^{-/-}$;*tfec*$^{-/-}$ mutants also rescued localization of endogenous *sox10*:eGFP$^{+}$ cNCCs within the cranial field (Fig. 3F). These data indicate that cNCCs outside of the *mitfa* lineage are affected by loss of *mitfa* and *tfec*, but also that their localization can be rescued by the presence of wild-type *tfec*.

Minor points: Neural crest cells per 1000um2 are quantified to 4 decimal places (sometimes) "cNCCs in …. mutants appeared to explode" Since the relevant two hour section of the video does not depict high velocity fragmentation, perhaps an alternative term might be used?
We have taken these points into consideration and altered the text accordingly.

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Second decision letter

MS ID#: DEVELOP/2019/187047

MS TITLE: Mitf-family transcription factor function is required within cranial neural crest cells to promote choroid fissure closure

AUTHORS: Katie Sinagoga, Alessandra Larimer-Picciani, Stephanie George, Samantha Spencer, James Lister, and Jeffrey Gross
ARTICLE TYPE: Research Report

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*

This is unchanged from my previous review.

*Comments for the author*

In this revised manuscript, the authors have addressed all of my major concerns, and I agree that the last of my questions that remain unaddressed are minor. I appreciate the efforts they have put into this work especially during this difficult time.

Reviewer 2

*Advance summary and potential significance to field*

The advances remain unchanged from my previous review.

*Comments for the author*

The authors have addressed my previous comments/concerns, especially under the circumstance of the current pandemic. I have no further comments.

Reviewer 3

*Advance summary and potential significance to field*

-

*Comments for the author*

Thank you for sending across the revised manuscript - I am happy for it to now move forward to publication