The RNA helicase Ddx52 functions as a growth switch in juvenile zebrafish

Tzu-Lun Tseng, Ying-Ting Wang, Chang-Yu Tsao, Yi-Teng Ke, Yi-Ching Lee, Hweijan Hsu, Kenneth Poss and Chen-Hui Chen
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MS TITLE: The RNA helicase Ddx52 functions as a growth switch in juvenile zebrafish

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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 criticisms and suggestions for improvements. If you are able to revise the manuscript along the lines suggested, I will be happy receive a revised version of the manuscript. 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

The manuscript of Tzu-Lun Tseng and colleagues entitled "The RNA helicase Ddx52 functions as a growth switch in juvenile zebrafish" addresses the impact of RNA helicase on the growth trajectories of zebrafish larvae and juveniles. They describe the identification of a mutant and the causative mutated gene that they characterise in detail. They report a temperature sensitive allele of a RNA helicase that impacts on global RNA transcription in a reversible manner which causes reversible developmental arrest arguing for a mechanism that modulates the transition of developmental states.
The identification of a temperature sensitive allele of pan/Ddx52 provides a powerful tool for addressing developmental progression and the authors deliver a clear, well written and well controlled manuscript presenting a complete story. There are a few minor points I would like to see addressed before publication.

Comments for the author

Minor points:
Where is ddx52 expressed in regeneration and during juvenile development?

While the flow of the story is overall very smooth, the transition from regeneration to developmental progression in the results part is rather abrupt. I recommend polishing this transition.

Along the same lines: Since the authors test for an involvement in organismal growth, they can't possibly be surprised that pan acts there.

Points to be discussed: Is there a link between pan activity and nutrition? Does malnutrition impact on pan expression? Is pan the one an only bottleneck or just one in many?

In the second paragraph of the results section, where temperature sensitive experiments are described analysing sde1, it is not clearly stated if the analysis was performed under the restrictive or permissive conditions.

Are the ddx52 Crispr alleles temperature sensitive as well?

Fig 3D shows an AA sequence to indicate a premature STOP. The reading frame should be shown here to support the point.

The first two sentences of the Drosophila paragraph unnecessarily re-introduce ddx52. This should be avoided.

Since one aspect of the manuscript is a potential evolutionary conservation of the proposed mechanism, the Drosophila data might be represented as main figure rather than in the supplementary information.

Since mice/mammals do not regenerate as fish do, it would be an interesting experiment to analyse murine regeneration under a ddx25 overexpression regime.

Reviewer 2

Advance summary and potential significance to field

The major advance of this manuscript is the identification of a single gene that influences the transition from juvenile growth to adult growth in what appears to be a conserved manner. This is significant because this gene seems to act as a switch during this transition. It is of interest to understand mechanistically how this switch actually functions.

Comments for the author

The article “The RNA helicase Ddx52 functions as a growth switch in juvenile zebrafish,” by Tseng et al. describes a temperature-sensitive mutation in the gene coding for Ddx52. The mutation is interesting because of its specific impact on the transition from juvenile to adult in zebrafish, and because of its conserved effects in Drosophila and possibly mice. The Ddx52 protein is responsible for maintaining transcription of the 47S pre-rRNA for which the authors attribute the phenotype. Overall I find the data analyses to be carefully done, and the idea of a molecular switch for growth to be interesting. However, the conclusion that the underlying cause of the phenotype is due to reduced bulk RNA transcription may be premature since the authors do not assess the impact of the mutation on ribosome biosynthesis/function. Below I provide comments and suggestions to strengthen the manuscript.

1. It may be an overstatement to say that suppressing global RNA transcription is the conserved function since the functional impact of reduced rRNA transcription may be related to protein synthesis. “Global” suppression of RNA transcription may also be an overstatement since the affected RNAs seem to be transcribed by RNA Pol I and not by RNA Pol II.

2. Please clarify why the original pan allele is ts – is it b/c of differences in splicing that depend on temperature (which could be novel?), or is it b/c the mutant form of the Ddx52 protein is more functional at the permissive temperature than the higher temperature? I suspect the latter but this could be more clearly stated for the reader (page 5 and page 10).

3. Does keeping the larvae at 34C for long term monitoring have any impact on fish health or viability (i.e. not only for pan, but in general)?

Either way, this should be noted in the Methods.

4. For the analysis in Drosophila, it would be good to demonstrate that the expression of ddx52 is actually reduced in the progeny from the GAL4 x UAS-RNAi cross.

5. The authors show that bulk RNA translation is reduced, but also indicate that this is because rRNAs make up the majority of the cellular RNA. So this effect could be due almost entirely to less 47S pre-rRNA, which is essential for ribosome biogenesis. Reduced ribosome formation or function would have a stronger impact on rapidly dividing tissues. At a minimum reduced ribosome function should be considered as the underlying cause of the observed phenotypes. It would be better to test if ribosome number/activity is impacted, or try to assess the level of protein translation following the block in Ddx52 function.

Reviewer 3

Advance summary and potential significance to field

The authors discover a very interesting heat sensitive mutation that causes developmental and growth arrest in zebrafish. This could give novel insights into developmental checkpoints and how developmental progress is regulated.

Comments for the author

General comments:
This is an extremely interesting and important study, where the authors discover a very exciting heat sensitive mutation that causes developmental and growth arrest in zebrafish. I have a high level of enthusiasm for the work, and I think that the work could be greatly strengthened by including a careful delineation between the process of growth and the process of development. There are different ways to alter growth rates in zebrafish, including hypoxia, low temperature, and high density, and release from these low growth conditions is followed by a period of catch-up growth. These treatments and the concept of catch-up growth should be discussed in the manuscript. Growth rates can also be suppressed by mutating growth hormone or blocking thyroid hormone, these should also be included. What is striking and unique about the pan mutant is that it appears to be not just small, but legitimately developmentally arrested: the pan mutant at 10 wpf is not just a miniature version of the control: the mutant is actually arrested at an earlier developmental stage. The mutants maintain the proportions and features of more immature fish.

To me, this is the most exciting component of the phenotype, and I think that it should be unpacked more thoroughly. There are many ways to inhibit growth; consider the gh1 mutant: growth is severely inhibited, but development proceeds essentially normally and the small fish show proportions and features of adults. Inhibiting Ddx52 is far more interesting, because not only is growth inhibited, but developmental progression is blocked.

The data from flies are far more compelling to me than the data from mice, because the flies show what appears to be a legitimate developmental arrest and a failure to progress to the next stage of development. The mice simply show growth retardation, which, to me, Is not nearly as interesting. (I imagine there are lots of drugs you can inject a mouse with to slow down its development, and I imagine mice will often show catch-up growth after cessation of treatment)

Specific comments:
Introduction page 4: The 4 stages of zebrafish development are not, in my view, distinct, rather more like a gradient. They transition smoothly from one to the other rather than having discrete transitions.

Fig 1: In order to assess development, apart from simply growth, the authors should stage the fish.

Figure 1 clearly shows that the pan mutants show delays in their external feature, but this can be assessed quantitatively. Lateral images of the fish can be used for developmental staging, according to the postembryonic normal table. This should be relatively straightforward to do with the images the authors have already taken at 8, 12, 16 and 20 wpf. Are the mutants legitimately arrested, and by how much? Do the mutants show the expected features for their sizes? Is there a decoupling between the processes of development and growth?

Also the pan mutant in Fig 1 E seems to show an extremely long pectoral fin. Is this a real phenotype that merits further description?

In supplementary Fig S2, it seems like it would be more informative to show absolute SL rather than relative growth normalized to SL. “relative growth by SL” should be more clear, and should be consistent among figures (either show relative or absolute SL in all). This could be more clear. Do the pan individuals really catch up to the hets in their size? Fig 2C suggests that they do, but the pan individuals in Fig S2 all look smaller than the het, and it is impossible to tell from the graph.

The authors speculate that overexpression of ddx52 does not increase adult body size perhaps because it functions in a switch-like manner or acts synergistically with other components. My interpretation would be more along the lines of: there are physiological limits on body growth, and it is probably impossible to surpass these limits in this manner.

On page 12/13, the authors say that “since invertebrates and vertebrates split from a common ancestor 700 mya… the major protein domains are preserved” This seems like odd phrasing. Would it be more clear to say that “despite the fact that invertebrates and vertebrates” diverged 700 mya the domains are preserved?

The quantifications in S5D might be more clear if presented in a single chart showing colored bars broken down by % of animals in each stage. Alternatively, pie charts might make it more clear that the CG5589 knockdowns are developmentally arrested. I think that such a graph would merit inclusion in one of the main figures, and could strengthen the entire story. Frankly, I find the fly data more compelling than the mouse data presented in Fig 6, since the flies show an unambiguous developmental arrest, and I recommend including these data in the main paper.

The authors state that inactivation of Ddx52 has no significant impacts on the levels of shh and lef1, but is a 6 hour window long enough to see the read-out of any potential effects? I recommend the authors to temper their interpretation, saying just that inactivation has no effect on mRNA levels of these genes after 6 hours.

Fig 5: I would like to see a quantification of the regenerate sizes at 4dpa and at 21dpa in Act D-treated fins in Fig 5. I think a bar graph showing controls, Act D treatments and HU treatments

showing size of regenerate as a proportion of the original size at both 4 and 21 dpa will be considerably more compelling than the categorical quantification shown.

Did the authors try treating larvae with HU as they did with Act D? It would be interesting to see how the effects of blocking DNA replication compare with blocking RNA transcription.

I was also unclear why H and I are presented as % increase. It would be more consistent with Figs 1 and 2 to show the absolute measurements for SL and HAA (or else show % increase in Figs 1 and 2)

Fig 6: The authors show convincingly that Act D treatment in mice depresses growth in a reversible manner. However, this looks like it could be simply a growth deficiency, which is unsurprising given that DNA synthesis is blocked. It is also unsurpsing that the mice show catch-up growth after the treatment ends. I would be considerably more interested in evidence of a developmental arrest, rather than simply a growth arrest. At 11 dpt, do the ActD mice show any evidence of developmental arrest? Presumably WT mice show some isometric growth during this period. Could a morphometric analysis of the skull, or even a simple femur/tibia ratio measurement before and after treatment in controls vs. Act D treated reveal that development is actually inhibited, in addition to growth being inhibited? To me, this would be much more compelling that the growth changes alone.

Does the permanent change in the femur/tibia ratio in the Act D treated mice reflect more juvenile proportions? If so, does this suggest that a developmental pause in mice is in fact NOT reversible?

First revision

Author response to reviewers' comments

Reviewer 1 Advance Summary and Potential Significance to Field:

The manuscript of Tzu-Lun Tseng and colleagues entitled "The RNA helicase Ddx52 functions as a growth switch in juvenile zebrafish" addresses the impact of RNA helicase on the growth trajectories of zebrafish larvae and juveniles. They describe the identification of a mutant and the causative mutated gene that they characterise in detail. They report a temperature sensitive allele of a RNA helicase that impacts on global RNA transcription in a reversible manner which causes reversible developmental arrest arguing for a mechanism that modulates the transition of developmental states.
The identification of a temperature sensitive allele of pan/Ddx52 provides a powerful tool for addressing developmental progression and the authors deliver a clear, well written and well controlled manuscript, presenting a complete story. There are a few minor points I would like to see addressed before publication.

Reviewer 1 Comments for the Author:

Minor points:
Where is ddx52 expressed in regeneration and during juvenile development?
Response (1.1): We thank the reviewer for the encouraging comments on the study. We apologize for not clearly presenting the expression data. The expression domain of ddx52 during regeneration is restricted to the distal part of the mesenchymal compartment, according to our RNA in situ hybridization assay (Fig. S6D). We have now highlighted the ddx52 expression domain in the figure with a red arrowhead.
During juvenile development, we failed to detect a specific signal by RNA in situ hybridization, which could be due to either a low expression level during development or that expression may temporally fluctuate during juvenile stages. We hope the reviewer will allow us to report a more thorough analysis of ddx52 expression in future studies.

While the flow of the story is overall very smooth, the transition from regeneration to developmental progression in the results part is rather abrupt. I recommend polishing this transition. Along the same lines: Since the authors test for an involvement in organismal growth, they can't possibly be surprised that pan acts there.

Response (1.2): As suggested, we have revised the first sentence on page 7, paragraph 2 to make a smoother transition. Also, we changed "Surprisingly" to "Intriguingly" in the text where we first described the pan phenotype in growth.

Points to be discussed: Is there a link between pan activity and nutrition? Does malnutrition impact on pan expression? Is pan the one an only bottleneck or just one in many?

Response (1.3): As suggested, we have added a paragraph in the discussion section to address these points; page 19, paragraph 2:

> "Besides specific genetic factors, the growth of zebrafish may be affected by certain rearing conditions, including nutrient supply, oxygen levels, temperature, and aquarium density (Kamei et al., 2018; Kimmel et al., 1995; Wills et al., 2008a). Upon release from adverse, low growth conditions, affected individuals may enter a catch-up period of rapid growth until the adult form is reached. Thus, it will be interesting to determine whether *ddx52* or other DEAD-box RNA helicase family members (Zhang et al., 2011) might influence growth progression under known low growth conditions, acting as a downstream effector of the environmental influences on animal growth and development. Of note, when manipulating Ddx52 activity, the progression of growth and development remains coupled in pan mutants (Fig. S3). This key feature is distinct from growth mutants that are defective in hormonal signaling, such as the gh1 and duox mutants (Chopra et al., 2019; McMenamin et al., 2013). In both of those mutants, growth is severely inhibited, but the animals still display many developmental characteristics of adults."

In the second paragraph of the results section, where temperature sensitive experiments are described analysing sde1, it is not clearly stated if the analysis was performed under the restrictive or permissive conditions.

Response (1.4): We added "under the restrictive temperature" to the end of the sentence to clearly state the condition used in the experiment (page 8, paragraph 1).

Are the ddx52 Crispr alleles temperature sensitive as well?

Response (1.5): Because the ddx52 crisper mutant embryos display severe developmental phenotypes at room temperature, we did not further examine their phenotypes at a higher temperature. We added the temperature information to the sentence (page 11, paragraph 2) and the figure legend (Fig. 3F).

Fig 3D shows an AA sequence to indicate a premature STOP. The reading frame should be shown here to support the point.

Response (1.6): As suggested, we have updated Fig. 3D to indicate the reading frame and the exact location of the premature stop codon.

The first two sentences of the Drosophila paragraph unnecessarily re-introduce ddx52. This should be avoided.

Response (1.7): As suggested, we have removed the first two sentences of the Drosophila paragraph to avoid the re-introduction of ddx52.

Since one aspect of the manuscript is a potential evolutionary conservation of the proposed mechanism, the Drosophila data might be represented as main figure rather than in the supplementary information.

Response (1.8): We appreciate the reviewer's comment and have revised the figures as suggested. The Drosophila data are now shown as a main figure (new Figure 4).

Since mice/mammals do not regenerate as fish do, it would be an interesting experiment to analyse murine regeneration under a ddx25 overexpression regime.

Response (1.9): Yes, we fully agree with the reviewer that further study of ddx52 function in regeneration with mouse models would be very interesting. We will pursue this direction in future studies.

Reviewer 2 Advance Summary and Potential Significance to Field:

The major advance of this manuscript is the identification of a single gene that influences the transition from juvenile growth to adult growth in what appears to be a conserved manner. This is significant because this gene seems to act as a switch during this transition. It is of interest to understand mechanistically how this switch actually functions.

Reviewer 2 Comments for the Author:

The article "The RNA helicase Ddx52 functions as a growth switch in juvenile zebrafish," by Tseng et al. describes a temperature-sensitive mutation in the gene coding for Ddx52. The mutation is interesting because of its specific impact on the transition from juvenile to adult in zebrafish, and because of its conserved effects in Drosophila and possibly mice. The Ddx52 protein is responsible for maintaining transcription of the 47S pre-rRNA, for which the authors attribute the phenotype. Overall I find the data analyses to be carefully done, and the idea of a molecular switch for growth to be interesting. However, the conclusion that the underlying cause of the phenotype is due to reduced bulk RNA transcription may be premature since the authors do not assess the impact of the mutation on ribosome biosynthesis/function. Below I provide comments and suggestions to strengthen the manuscript.

1. It may be an overstatement to say that suppressing global RNA transcription is the conserved function since the functional impact of reduced rRNA transcription may be related to protein synthesis. "Global" suppression of RNA transcription may also be an overstatement since the affected RNAs seem to be transcribed by RNA Pol I and not by RNA Pol II.

Response (2.1): As suggested, we have removed the term "global" and the related sentences from the text.

2. Please clarify why the original pan allele is ts - is it b/c of differences in splicing that depend on temperature (which could be novel?), or is it b/c the mutant form of the Ddx52 protein is more functional at the permissive temperature than the higher temperature? I suspect the latter but this could be more clearly stated for the reader (page 5 and page 10).

Response (2.2): We apologize for not making this part clear. Yes, we think the mutant form of the Ddx52 protein is more functional at the permissive temperature than at the restrictive temperature. We have added a sentence for clarification; page 11, paragraph 2:

> "Taken together, these findings suggest that the truncated form of the Ddx52 protein likely acts as a hypomorph at the restrictive temperature to impair adult tailfin regeneration and juvenile growth".

3. Does keeping the larvae at 34C for long term monitoring have any impact on fish health or viability (i.e. not only for pan, but in general)? Either way, this should be noted in the Methods.

Response (2.3): As suggested we have now included the larvae survival rate data in the methods on page 23, paragraph 2.

> "Of note, when shifted to 34°C at 5 wpf, *pan*/+ and *pan* have similar survival rates by 9 wpf (wild- type, 80%; *pan*/+, 73%; *pan* 73%; n = 30 each)."

4. For the analysis in Drosophila, it would be good to demonstrate that the expression of ddx52 is actually reduced in the progeny from the GAL4 x UAS-RNAi cross.

Response (2.4): As suggested, we performed experimental validation of ddx52 expression in the

progeny from the GAL4 x UAS-RNAi cross. We determined that the RNAi lines are effective at reducing the level of ddx52 expression at larval stages (decrease of 36%). The new RT-qPCR data are included as Fig. 4C; the primer information is given in Table S1, and the results are described on page 13, paragraph 1.

5. The authors show that bulk RNA translation is reduced, but also indicate that this is because rRNAs make up the majority of the cellular RNA. So this effect could be due almost entirely to less 47S pre- rRNA, which is essential for ribosome biogenesis. Reduced ribosome formation or function would have a stronger impact on rapidly dividing tissues. At a minimum, reduced ribosome function should be considered as the underlying cause of the observed phenotypes. It would be better to test if ribosome number/activity is impacted, or try to assess the level of protein translation following the block in Ddx52 function.

Response (2.5): We thank the reviewer for the comment. As suggested, we determined the level of protein translation in regenerating tailfins following the block in Ddx52 function. Here, we chose the induction of tenascin C, an ECM component, as a marker. We conducted immunostaining for tenascin C upon tailfin amputation at 1 dpa. As expected, we found that the level of tenascin C protein is markedly reduced following the block on Ddx52 function. We have now included the experimental scheme and result in Fig. S6I and S6J, and the finding is described on page 16, paragraph 1.

Reviewer 3 Advance Summary and Potential Significance to Field:

The authors discover a very interesting heat sensitive mutation that causes developmental and growth arrest in zebrafish. This could give novel insights into developmental checkpoints and how developmental progress is regulated.

Reviewer 3 Comments for the Author:

General comments:

This is an extremely interesting and important study, where the authors discover a very exciting heat sensitive mutation that causes developmental and growth arrest in zebrafish. I have a high level of enthusiasm for the work, and I think that the work could be greatly strengthened by including a careful delineation between the process of growth and the process of development. There are different ways to alter growth rates in zebrafish, including hypoxia, low temperature, and high density, and release from these low growth conditions is followed by a period of catch-up growth. These treatments and the concept of catch-up growth should be discussed in the manuscript. Growth rates can also be suppressed by mutating growth hormone or blocking thyroid hormone, these should also be included. What is striking and unique about the pan mutant is that it appears to be not just small, but legitimately developmentally arrested: the pan mutant at 10 wpf is not just a miniature version of the control: the mutant is actually arrested at an earlier developmental stage. The mutants maintain the proportions and features of more immature fish. To me, this is the most exciting component of the phenotype, and I think that it should be unpacked more thoroughly. There are many ways to inhibit growth; consider the gh1 mutant: growth is severely inhibited, but development proceeds essentially normally and the small fish show proportions and features of adults. Inhibiting Ddx52 is far more interesting, because not only is growth inhibited, but developmental progression is blocked.

Response (3.1): First of all, we thank the reviewer for the enthusiastic comments. As suggested, we have added a paragraph in the discussion section to address these points; page 19, paragraph 2:

> "Besides specific genetic factors, the growth of zebrafish may be affected by certain rearing conditions, including nutrient supply, oxygen levels, temperature, and aquarium density (Kamei et al., 2018; Kimmel et al., 1995; Wills et al., 2008a). Upon release from adverse, low growth conditions, affected individuals may enter a catch-up period of rapid growth until the adult form is reached. Thus, it will be interesting to determine whether *ddx52* or other DEAD-box RNA helicase family members (Zhang et al., 2011) might influence growth progression under known low growth conditions, acting as a downstream effector of the environmental influences on animal growth and development. Of note, when manipulating the Ddx52 activity, the progression of growth and development remains coupled in pan mutants

(Fig. S3). This key feature is distinct from growth mutants that are defective in hormonal signaling, such as the gh1 and duox mutants (Chopra et al., 2019; McMenamin et al., 2013). In both of those mutants, growth is similarly inhibited, but the animals still display many developmental characteristics of adults."

The data from flies are far more compelling to me than the data from mice, because the flies show what appears to be a legitimate developmental arrest and a failure to progress to the next stage of development. The mice simply show growth retardation, which, to me, Is not nearly as interesting. (I imagine there are lots of drugs you can inject a mouse with to slow down its development, and I imagine mice will often show catch-up growth after cessation of treatment)

Response (3.2): As suggested, we now show the Drosophila data as a main figure (new Figure 4).

Specific comments:
Introduction page 4: The 4 stages of zebrafish development are not, in my view, distinct, rather more like a gradient. They transition smoothly from one to the other rather than having discrete transitions.

Response (3.3): We thank the reviewer for the comment. We have changed the word "distinct" to "major" in the sentence on page 4, paragraph 2.

Fig 1: In order to assess development, apart from simply growth, the authors should stage the fish. Figure 1 clearly shows that the pan mutants show delays in their external feature, but this can be assessed quantitatively. Lateral images of the fish can be used for developmental staging, according to the postembryonic normal table. This should be relatively straightforward to do with the images the authors have already taken at 8, 12, 16 and 20 wpf. Are the mutants legitimately arrested, and by how much? Do the mutants show the expected features for their sizes? Is there a decoupling between the processes of development and growth?

Response (3.4): As suggested we have now included an additional figure (Fig. S3) to comment on whether *pan* mutants show the expected developmental features for their sizes. We matched images taken at 8, 12, 16 and 20 wpf with the descriptions of normally developing zebrafish at the respective Standard Lengths given in the postembryonic normal development table (Parichy et al., 2009). Based on the progression of the "tailfin development" and the "pigment pattern formation", we conclude that the processes of development and growth remain coupled in pan mutants. We have updated the text on page 9, paragraph 2:

"Of note, key developmental features and growth remain coupled in pan mutants during the recovery phase, as determined by comparing the mutant phenotype with the normal table of postembryonic zebrafish development (Parichy et al., 2009) (SL and images of tailfin development and pigment pattern formation are shown in Fig. S3)."

Also the pan mutant in Fig 1 E seems to show an extremely long pectoral fin. Is this a real phenotype that merits further description?

Response (3.5): We thank the reviewer for pointing this out. After a close examination of the pan mutant images, we conclude that the long pectoral fin phenotype is not as evident as it may seem from the image. Thus, we believe the feature may not merit further description.

In supplementary Fig S2, it seems like it would be more informative to show absolute SL rather than relative growth normalized to SL. "relative growth by SL" should be more clear, and should be consistent among figures (either show relative or absolute SL in all). This could be more clear. Do the pan individuals really catch up to the hets in their size? Fig 2C suggests that they do, but the pan individuals in Fig S2 all look smaller than the het, and it is impossible to tell from the graph.

Response (3.6): As suggested, we now include "absolute SL" in Figure S2. We keep the panels showing "Relative growth by SL", as this normalization can highlight changes that occur in each pan individual. Also, despite the fact that pan individuals may appear small in Fig. S2 by 20 wpf, we expect that these animals would eventually catch up with the hets at later time points, based on our findings shown in Fig. 2C.

The authors speculate that overexpression of ddx52 does not increase adult body size perhaps because it functions in a switch-like manner or acts synergistically with other components. My interpretation would be more along the lines of: there are physiological limits on body growth, and it is probably impossible to surpass these limits in this manner.

Response (3.7): We thank the reviewer for the comment. We included the reviewer's point on page 12, paragraph 2:

> "Thus, we speculated that ddx52 may either function in a switch-like manner or it might act synergistically with yet-to-be-identified components in order to regulate regeneration and growth. It is equally possible that rapid growth during juvenile stages may have physiological limits that are difficult if not impossible to surpass."

On page 12/13, the authors say that "since invertebrates and vertebrates split from a common ancestor 700 mya… the major protein domains are preserved" This seems like odd phrasing. Would it be more clear to say that "despite the fact that invertebrates and vertebrates" diverged 700 mya the domains are preserved?

Response (3.8): Yes, it is more clear to say it that way. As suggested, we have rephrased the sentence on page 12, paragraph 3.

> "Of note, the major protein domains encoded by ddx52 orthologues are conserved (Fig. 4A,B), despite the fact that invertebrates and vertebrates diverged 700 million years ago (Kumar et al., 2017; Wray, 2015)."

The quantifications in S5D might be more clear if presented in a single chart showing colored bars broken down by % of animals in each stage. Alternatively, pie charts might make it more clear that the CG5589 knockdowns are developmentally arrested. I think that such a graph would merit inclusion in one of the main figures, and could strengthen the entire story. Frankly, I find the fly data more compelling than the mouse data presented in Fig 6, since the flies show an unambiguous developmental arrest, and I recommend including these data in the main paper.

Response (3.9): Yes, it is clearer to show the results with pie charts. As suggested, we have now included the fly data as a main figure (new Figure 4), and pie charts were included as Fig. 4E.

The authors state that inactivation of Ddx52 has no significant impacts on the levels of shh and lef1, but is a 6 hour window long enough to see the read-out of any potential effects? I recommend the authors to temper their interpretation, saying just that inactivation has no effect on mRNA levels of these genes after 6 hours.

Response (3.10): As suggested, we have revised the text on page 15, paragraph 1.

Fig 5: I would like to see a quantification of the regenerate sizes at 4dpa and at 21dpa in Act D-treated fins in Fig 5. I think a bar graph showing controls, Act D treatments and HU treatments showing size of regenerate as a proportion of the original size at both 4 and 21 dpa will be considerably more compelling than the categorical quantification shown.

Response (3.11): As suggested, we have now added a quantification of the fin regenerate sizes as a proportion of the original size at 4 dpa and at 21 dpa in Act D- and HU-treated fins (Fig. 6F).

Did the authors try treating larvae with HU as they did with Act D? It would be interesting to see how the effects of blocking DNA replication compare with blocking RNA transcription.

Response (3.12): As suggested, we tested the effect of HU on juvenile zebrafish. However, we found that a 5-day treatment scheme markedly reduced the survival rate of the animals (decrease of 47%; n = 15), and yet the animal growth was not significantly affected at times up to 21 days post-treatment when compared with the vehicle control. We suspect that prolonged treatment of HU at juvenile stages may have pleiotropic effects or the doses need to be further optimized. We hope the reviewer to allow us to omit these inconclusive results.

I was also unclear why H and I are presented as % increase. It would be more consistent with Figs 1 and 2 to show the absolute measurements for SL and HAA (or else show % increase in Figs 1 and 2).

Response (3.13): As suggested, we now show the absolute measurements for SL and HAA in Fig. 6I and 6J and Fig. 7J and 7K for consistency.

Fig 6: The authors show convincingly that Act D treatment in mice depresses growth in a reversible manner. However, this looks like it could be simply a growth deficiency, which is unsurprising given that DNA synthesis is blocked. It is also unsurpsing that the mice show catch-up growth after the treatment ends. I would be considerably more interested in evidence of a developmental arrest, rather than simply a growth arrest. At 11 dpt, do the ActD mice show any evidence of developmental arrest? Presumably WT mice show some isometric growth during this period. Could a morphometric analysis of the skull, or even a simple femur/tibia ratio measurement before and after treatment in controls vs. Act D treated reveal that development is actually inhibited, in addition to growth being inhibited? To me, this would be much more compelling that the growth changes alone.

Response (3.14): We thank the reviewer for this insightful comment. As suggested, we measured the "skull length" and the "skull size" from micro-CT images captured at 11 dpt. We determined that the Act D-treated mice indeed show evidence of developmental arrest at 11 dpt (i.e., a smaller head). Intriguingly, we found that both readouts support the conclusion that development had caught up at 46 dpt, consistent with the growth progression of the animals. We have now included these results as Fig. 7D-F and described the findings on page 18, paragraph 1:

> "To determine whether the treatment indeed causes a developmental pause, we performed a high-resolution micro-CT scan of the growth-paused mice and their respective vehicle-treated siblings at 11 dpt. A significant reduction in skull length and size was observed (Fig. 7D-F), supportive of a bona fide developmental arrest."

Does the permanent change in the femur/tibia ratio in the Act D treated mice reflect more juvenile proportions? If so, does this suggest that a developmental pause in mice is in fact NOT reversible?

Response (3.15): As suggested, we determined the femur/tibia ratio in the Act D-treated mice at 11 and 46 dpt. We found the ratios are not significantly different from the controls at either time point. Thus, we conclude that the measurements of the skull are a better readout for a developmental *pause. The skull measurements support the idea that the developmental pause in the Act D- treated mice is reversible. We have now updated the text on page 18, paragraph 1.*

Second decision letter

MS ID#: DEVELOP/2021/199578

MS TITLE: The RNA helicase Ddx52 functions as a growth switch in juvenile zebrafish

AUTHORS: Tzu-Lun Tseng, Ying-Ting Wang, Chang-Yu Tsao, Yi-Teng Ke, Yi-Ching Lee, Hweijan Hsu, Kenneth Poss, and Chen-Hui Chen

ARTICLE TYPE: Research Article

I am happy to tell you that the referees are happy with your revisions and your manuscript has been accepted for publication in Development, pending our standard ethics checks. The referee reports on this version are appended below.

Reviewer 1

Advance summary and potential significance to field

The manuscript presents a well written, convincing and carefully controlled story touching an interesting novel aspect of global developmental/regeneration control.
Comments for the author

The authors have addressed the key points raised by this referee.
There are no open issues left.

Reviewer 2

Advance summary and potential significance to field

The authors have addressed my concerns.

Comments for the author

None.

Reviewer 3

Advance summary and potential significance to field

The authors have identified a temperature sensitive allele that causes a reversible pause to developmental progression in zebrafish. The affected gene is required for RNA transcription, and the authors demonstrate that inhibiting transcription in other species (flies and mice) similarly halts developmental progression. This represents a significant advance and opens new horizons for investigating the regulation of developmental progression in vertebrates.

Comments for the author

The authors have adequately addressed all of my concerns, and I find this to be an excellent and greatly improved manuscript.