Nrf2 dictates the neuronal survival and differentiation of embryonic zebrafish harboring compromised alanyl-tRNA synthetase

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MS TITLE: Nrf2 dictates the neuronal fate of embryonic zebrafish harboring compromised tRNA synthetase

AUTHORS: Binbin Jin, Luping Zhou, Dan Zhan, Liqin Xie, Zhi Feng, Jiangyong He, Jie Qin, Congjian Zhao, Lingfei Luo, and Li Li

I have now received the reports of three referees on your manuscript and I 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, two of the three referees express great interest in your work, but all referees also have significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. In particular, all the referees criticise the poor writing of the manuscript and they request that you improve the text very substantially. They also request that you improve the quantification and statistical analysis of the data, as detailed by referee 1. Referee 1 criticises the use of morpholinos, particularly for the analysis of neurodegenerative phenotypes. Referee 2 requests that you re-analyse and re-interpret the cell death data, including which cell types die in the mutant larvae. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy to receive a revised version of the manuscript. Your revised paper will be re-reviewed by the original referees, and its acceptance will depend on your addressing satisfactorily all their 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

Jin et all have produced a very thorough characterisation of an aars1 mutant zebrafish, identified through a large ENU mutagenesis screen looking to identify novel genes required for neurogenesis. Initial findings were confirmed with secondary mutants and Morpholinos for aars1, which phenocopies the ENU mutant nicely. Single cell RNA seq on mutants suggested ER stress and protein overload could be the mechanisms behind the phenotypes. This was confirmed via secondary readouts- western and qPCR. Perk signalling was found to be specifically responsible for this, and inhibition of PERK led to a rescue of phenotypes. To tease apart the pathway further, NRF2 paralogue was found to be increased in the mutant neurons and its inhibition also rescued mutant phenotypes. Further investigation implicated p53 as a regulator of this process. This report has implications for neurogenesis, but also neurodegeneration as few LOF AARS1 models have been reported in different species, especially in such detail- as well as providing mechanistic insights on a variety of different levels, including several routes of rescue.

Comments for the author

I very much liked this paper and think is a good fit for Development. But, there are lots of revisions that are needed, however, few of these need to be experimental and is more revision of the text. There is a tremendous quantity of work in here, sometimes too much, and it becomes overkill, which at times makes the narrative somewhat what confusing. I suggest the authors go through all figures and simplify, to make the information more palatable. Some figures should be taken out entirely if their addition to the narrative is superfluous. On the other hand, some things need to be explained in greater depth to those not completely familiar with the pathways involved.

Essential revisions Major points that need to be changed for all figures- everything needs to be quantified in the text and in the legend- quite often westerns are shown without quantification and a simple increase is described, significance unknown. It is not therefore known how much change is occurring and by what significance. For example, Figs 3d, 3k, 4a, 4f, 5c and 5d to name a few. There are more in the supp figs.
Throughout the manuscript the mutants are incorrectly referred to simply as mutant. Are these heterozygous or homozygous mutant? Furthermore, are the homozygous mutants viable? If not, what is their lifespan? Survival curves would be required for this. Correct mutant nomenclature should be used throughout. When talking aout different alleles of the same gene the authors could simple write aars1ENU-/- and aars1Crispr-/- for example.
The mutants are described as not moving very much- however, do they respond to touch or other stimuli – such as light dark paradigm. This would be more informative as to how the neuronal phenotypes effect circuitry and behaviour.
Page 7- the western blot described will not show enzyme activity, merely protein levels, please change throughout the text. This should be accompanied by qpcr for the transcript in mut vs wt to demonstrate if NMD is occurring due to the mutation or not- this will have implications for genetic compensation.
Page 7 and throughout- the use of morpholinos in the field is controversial and in some cases, unnecessary in this study. Using Mos to rescue phenotypes is a valid approach as this is unlikely to be a toxic off target effect, so common in Mos (as it is neuroprotective in this case). However, where the MO is used to cause a neurodegenerative phenotype to validate, this is an inappropriate use of the technology. In the case of using, it to phenocopy the AARS1 mutant- it is also not necessary, as the authors have two different stable mutants, ENU and Crispr that both show the same phenotype. The MO work is a distraction and sends a bad message to the community. I would remove all the AARS1 MO work.
Page 11- XBP gels need quantifying

Page 11- Perk MO – KD needs quantifying, but again, if a PERK inhibiter rescues the phenotypes in the mutants, the MO work is a distraction and not necessary.
Page 11- CHOP MO KD needs quantification- if not possible, this experiment can be repeated with CRISPANTS see Kroll et al 2021 and Keatinge et al 2021. Crispant strategy will likely replace Mos for rapid loss of function studies in the future as they can be quantified easily and are far less toxic than Mos.
Page 13 figure 5I, the increase in nfe2l2b should be quantified
N numbers needed to be included for every experiment as it is not clear what they are in some, please include in the legend for each figure. Furthermore, how many clutches were used in each experiment? It is inappropriate to use a single clutch per experiment, multiple clutches, at least n=2 should be reported for each experiment. For example n= 15 from 2 independent clutches.
The stat test used and the p value also must be reported for each experiment in the main text and legend. T tests are not appropriate for all experiments here, 2 way anova should be employed when comparing 2 groups in 2 different conditions-
for example WT and MUT on a non tg and a tg background. These experiments would require re analysis.

There is a lot of information regarding the pathways- a diagram or cartoon at the end of the paper would be incredibly helpful to the reader to summarise the findings.

Minor points
Abstract
In the abstract NRF2 is mentioned as an "executor" and "employer" of PERK. These phrases need clarification.
Introduction The entire second paragraph of the introduction should be condensed and simplified.
What are sti mice? are these AARS1 mouse mutants, it is not clear.
ANKRD16 is mentioned, but the text does not explain what is it is? Is this a chemical? a gene? Etc.
"heterozygous mutations in the AARS1 gene were discovered in people with CNS disorders" this needs to be expanded and the appropriate sources citated. What CNS disorders would be helpful.
Within the paragraph CNS disorders are conflated with Neurodegenerative diseases. This is vague and should be rectified.
Final paragraph of intro “the function of nfe2l2a in ZF embryonic development was revealed recently”. Please elaborate.
Results GFAP is referred to as an astrocyte marker- this is incorrect. It is an astrocyte marker in mammalian models, but in fish it labels both astrocytes and all radial glial.
Figure 1C legend and text- Zebrafish midbrains measurements are reported in “inches” this is clearly incorrect, please clarify the correct units.
Page 7 – please list the ESNT (from Ensembl) numbers of the different aars1 transcripts described.
Page 7 – none of the data shows a reduction in AARS1 activity per se, but actually shows a loss of function- so the crispr mutant phenocopies due to loss of function as opposed to loss of activity, as the latter is hard to demonstrate unless one has a validated functional enzyme activity assay.
Page 13 aars1cq71;nfe2l2b-/- are these double homozygous mutants? It is unclear correct mutation nomenclature should be employed throughout the paper.
Page 14- typo- I think “folds” should be “fold”
Figure S2- in situs photos are not needed and are of poor quality- I would remove all except for the close up of the head in figure S2I.

Reviewer 2

Advance summary and potential significance to field

Beginning with a mutation picked up in a zebrafish forward genetic screen, this manuscript describes a pathway connecting the Aars1 tRNA synthetase and Nrf2 in brain development. For me the strength of the manuscript lies in some of the experiments that functionally connect different components of the pathway. In particular, the double mutant analyses are quite convincing and compelling. I do not work on this pathway or on cell survival, so it is a bit difficult for me to assess novelty and significance, but the findings, if supported, appear to be important. My biggest concern with this manuscript is in how many of the data are described and interpreted, resulting in, for me, considerable confusion about whether aars1 function is important in progenitors or

neurons. My specific points are detailed below. Altogether, I think this manuscript has the potential to be an important story, but it requires extensive revision to more clearly articulate the primary role of Aars1 in neural development.

Comments for the author

1. The cells that the gene expression probes (transgenic reports, in situ RNA hybridization probes, and antibodies) need to be defined. Although some markers such as HuC (which should be called Elavl3), are widely known, others are not making it difficult to know if the authors are referring to progenitors, neuronal precursors, neurons, or specific glial cell types.
2. The authors refer to gfap as a "typical astrocyte marker" (p. 5) but in zebrafish at these stages it probably marks mostly radial glia.
3. The authors refer to olig2 as "labeling oligodendrocytes" (p. 6) but it also marks progenitors and neurons. The authors use olig2 expression to assert that oligodendrocytes "were similarly diminished in the cq71 mutant". However, the cells that the authors highlight in Figures 1A and S1A and not oligodendrocytes.
Instead, the highlighted cells are probably eurydendroid neurons of the cerebellum (McFarland KA, Topczewska JM, Weidinger G, Dorsky RI, Appel B. Hh and Wnt signaling regulate formation of olig2+ neurons in the zebrafish cerebellum. Dev Biol. 2008 Jun 1;318(1):162-71. doi: 10.1016/j.ydbio.2008.03.016. Epub 2008 Mar 21. PMID: 18423594; PMCID: PMC2474464. Bae YK, Kani S, Shimizu T, Tanabe K, Nojima H, Kimura Y, Higashijima S, Hibi M. Anatomy of zebrafish cerebellum and screen for mutations affecting its development. Dev Biol. 2009 Jun 15;330(2):406-26. doi: 10.1016/j.ydbio.2009.04.013. Epub 2009 Apr 14. PMID: 19371731.
4. The authors state that "the WT sibling midbrains of 3 dpf were ~23.72 inches in size." Other midbrain measurements are similarly stated. The measurements are not sufficiently described in the Methods for me to understand how the authors arrived at those numbers, and I can't understand how they could be so large.
5. I have a significant concern about how the Acridine Orange (AO) data for assessing cell death are interpreted. The authors present the data as a percentage of the total area that is fluorescent. It appears to me from the images that the AO+ signals are "larger" in mutant larvae compared to wild type, but it is not so apparent that there is a difference in the number, which would be indicative of a difference in number of dying cells. An alternative explanation for these data are that microglia an unable to clear dying cells in mutant larvae, resulting in greater AO signal, but there is no difference in the amount of cell death. To their credit, the authors perform two other assays using TUNEL and anti-Caspase 3.
However, the TUNEL staining is not quantified and, although the anti-Caspase 3 staining is quantified, the accompanying image in Figure S1J is too small to use for verification by a reader. Altogether, I do not think the data that are provided are sufficient to support the interpretation that mutant larvae have increased cell death.
6. I have a very significant concern about interpretation and description of the data regarding the cell types that die in mutant larvae, and how this is used to set up the rescue experiments. On page 6 the authors state that the apoptosis markers are increased "especially in the Sox2+ and HuC+ cells…", which would suggest that both progenitors and neurons die. However, the selected images make it appear that it is mostly the Sox2+ progenitors that die. Indeed, the authors conclude this section on page 6 by stating that "indicated an increased apoptosis of neuronal progenitors in the cq71 mutant brains." Apart from the somewhat imprecise description of the data, the conclusion that progenitors cells die seems fine. However, the authors then go on to do a double labeling experiment to show that HuC+ neurons express aars1 and a qPCR experiment to show that aars1 transcripts are enriched in HuC+ but not coro1a+ cells. The authors do not define the cell type marked by coro1a, so I do not know what the comparison is, but in any case the authors conclude that neurons express aars1. The authors then use these data as rationale to perform a rescue experiment by using HuC regulatory DNA to drive aars1 expression in neurons of mutant larvae, and claim that this suppresses cell death. They also used a cell transplantation experiment to claim that mutant HuC-EGFP+ neurons have elevated levels of cell death in wild type larvae. Based on these data the authors assert that "aars1 was intrinsically required and functionally conserved in neural survival and development." For me these observations do not entirely align. My main question is this – if Sox2+ neural progenitors are the primary cell type that undergoes apoptosis, which the data seem to indicate, then how is it that neurons are enriched for aars1 expression and neuronal expression of aars1 rescues cell death? I consider this to be a major point requiring clarification.

7. The Annexin V staining needs to be explained better in the Methods.
8. The image panels and print in the Figures are so small they are impossible to evaluate on a printed page.
9. Single cell RNA-seq analysis of mutant and wild-type larvae show that the largest gene expression differences occur in neural progenitors. Related to #6 this again raises the question of whether aars1 gene function is required in primarily in progenitors rather than neurons. Indeed, treating mutant embryos with cycloheximide rescued Sox2+ progenitor number in mutant larvae.
10. The Figures, in general, and not sufficiently well annotated. As noted above, markers are not defined, nor are some experimental manipulations explained
(e.g. “Tg” and “Tn”).

Reviewer 3

Advance summary and potential significance to field

The manuscript explores the link between tRNA synthetase deficienc, nrf2 and p53 in neurogenesis in the larval zebrafish brain.

Comments for the author

Unfortunately the paper is written with a language so confusing that it's hard to understand. Many imprecision and mistakes make impossible to follow the logic of the work as it is presented. I list below some specific points but that is far from being a complete assessment of all the work that should be done on the manuscript and all the experimental criticism that should be addressed.
1)the title doesn't reflect the main message of the paper as the main topic here is not neuronal fate but neuronal survival and differentiation.
2)the introduction start with a first paragraph completely disconnected from the rest of the manuscript about neurogenesis and gliogenesis without any link to the following parts of the manuscript.
3)in the introduction the authors define protein folding a post-traslational modification. This definition is debatable and not supported by the review that they cite at this point (Deribe et al 2010) where post translational modification are classically defined as the "addition of a modifying chemical group or another protein to one or more of its amino acid residues". I don't see why the authors insist twice in the introduction in calling folding a post-translational modification.
4) The manuscript contains some words that are not found in standard English such as "cytomic, distichously etc" or used in the wrong contest such as "phenomenons" (?) in place of data or results.
5) Throughout the manuscript the authors refer as measuring protein "activity" when what they do is simply doing western blots to quantify protein levels.
6) "...were significantly recovered in the aars1cq71;nfe2l2b-/- compounds compared to either the siblings or nfe2l2b-/- variants". Here the words "compounds" and "variants" do not make sense.
7)"and disability (?) of ATF6 inhibitor Ceapin A7 to attenuate cell apoptosis".
8)When describing cell transplantation experiments the authors use the term of "intrinsically required" as "cell autonomous".
9)"Over-providing" is used in place of "over-expressing"
These ares just very few examples but almost every sentence in the manuscript should be corrected or rephrased.
From a conceptual point the choice of Nrf2 as putative downstream factor of the PERK-CHOP axes seems not well justified as it is the choice of p53 as Nrf2 target.
Experimentally, for what is possible to understand, there are some strange points such as the use of HSP promoter to drive transgene expression without mentioning in the text any heat shock.
Cell transplantation should be done as the gold standard in the zebrafish community by blastomere transplantation and not as done in the paper. They refer to a protocol adapted to transplant hematopoietic kidney marrow stem cells, which is not the same as transplanting neuronal progenitors integrated in the neuroepithelial tissue.

First revision

Author response to reviewers' comments

Reviewer 1:
Reviewer 1 Advance Summary and Potential Significance to Field:
Jin et all have produced a very thorough characterisation of an aars1 mutant zebrafish, identified through a large ENU mutagenesis screen looking to identify novel genes required for neurogenesis. Initial findings were confirmed with secondary mutants and Morpholinos for aars1, which phenocopies the ENU mutant nicely. Single cell RNA seq on mutants suggested ER stress and protein overload could be the mechanisms behind the phenotypes. This was confirmed via secondary readouts- western and qPCR. Perk signalling was found to be specifically responsible for this, and inhibition of PERK led to a rescue of phenotypes. To tease apart the pathway further, NRF2 paralogue was found to be increased in the mutant neurons and its inhibition also rescued mutant phenotypes. Further investigation implicated p53 as a regulator of this process. This report has implications for neurogenesis, but also neurodegeneration as few LOF AARS1 models have been reported in different species, especially in such detail- as well as providing mechanistic insights on a variety of different levels, including several routes of rescue.

Reviewer 1 Comments for the Author:
I very much liked this paper and think is a good fit for Development. But there are lots of revisions that are needed, however, few of these need to be experimental and is more revision of the text.
There is a tremendous quantity of work in here, sometimes too much, and it becomes overkill, which at times makes the narrative somewhat what confusing. I suggest the authors go through all figures and simplify, to make the information more palatable. Some figures should be taken out entirely if their addition to the narrative is superfluous. On the other hand, some things need to be explained in greater depth to those not completely familiar with the pathways involved.
We deeply appreciate your great efforts in reviewing and justifying our manuscript. Your comments and suggestions are quite valuable for improving our manuscript.

Essential revisions
1. Major points that need to be changed for all figures- everything needs to bequantified in the text and in the legend- quite often westerns are shown withoutquantification and a simple increase is described, significance unknown. It is not therefore known how much change is occurring and by what significance. For example, Figs 3d, 3k, 4a, 4f, 5c and 5d to name a few. There are more in the suppfigs.
Answer: We are grateful to your careful estimation and apologize for lacking the quantification of several results, including westerns. To deal with these issues and improve the data quality, wequantified the original data by using the imageJ software according to the methods in previous reports (Shin et al., 2017). The revised figures of western blot were included in the present **Figures 2C, 3J, 4A, 4F, 5C, 5D, 7H**, **S2C, S4I, S5A, S5H, S5J, and S6A**. The quantification method descriptions were added in the revised manuscript accordingly (lines 805-813 in page 25, marked by yellow background). Thanks again for your kind comments!

2. Throughout the manuscript the mutants are incorrectly referred to simply as mutant. Are these heterozygous or homozygous mutant? Furthermore, are thehomozygous mutants viable? If not, what is their lifespan? Survival curves would be required for this. Correct mutant nomenclature should be used throughout. When talking about different alleles of the same gene the authors could simple writeaars1ENU-/- and aars1Crispr-/-for example.
Answer: Thank you for valuable advices. We would like to apologize for the rough and incorrect reference of various mutants in the previous manuscript. We corrected the nomenclature of the homozygous mutant types to *aars1*$^{ENU-/-}$, *aars1*$^{CRISPR-/-}$, *aars1*$^{ENU/CRISPR}$, *tars1*$^{ENU-/-}$, *nfe2l2b*$^{CRISPR-/-}$, or *aars1*$^{ENU-/-}$;*nfe2l2b*$^{CRISPR-/-}$ and heterozygous mutants to *nfe2l2b*$^{CRISPR+/-}$ and etc in the corresponding figures and text throughout. We evaluated the viable ability of *aars1*$^{-/-}$ homozygous mutants. The results indicated the *aars1*$^{ENU-/-}$ homozygous mutants could not live beyond 7 dpf. The survival curve was included in the revised **Figure S1D**.

3. The mutants are described as not moving very much- however, do they respond to touch or other stimuli – such as light dark paradigm. This would be more informative as to how the neuronal phenotypes effect circuitry and behaviour.

Answer: Thank you very much for the suggestions. We performed the stimuli assays of needle touch and light-dark paradigm (Marquez-Legorreta et al., 2020; Sztal et al., 2016) to measure the reactions of 5 dpf *aars1*$^{ENU-/-}$ homozygous mutant larvae. The data indicated that WT larvae rapidly responded to the external stimuli. However, the mutant larvae occasionally acted to response to the stimuli. But they remained freezing at most time. And the mobility and speeds of *aars1*$^{ENU-/-}$ mutant larvae reduced drastically relative to their WT siblings. The data were added in the revised **Figures S1G-S1L** and the descriptions were included accordingly (lines134-140 in page 5, marked by yellow background).

4. Page 7- the western blot described will not show enzyme activity, merely protein levels, please change throughout the text. This should be accompanied by qpcr for the transcript in mut vs wt to demonstrate if NMD is occurring due to the mutation or not- this will have implications for genetic compensation.
Answer: We are grateful to your suggestion and comments. We corrected the description of "enzyme activity" to "protein levels" throughout the text. We performed qPCR to examine the transcription levels of several NMD-related genes(Cheruiyot et al., 2021). The transcription levels of *upf1, upf3b* and *smg* factors increased significantly. But *upf3a* exhibited a slight reduction in the *aars1*$^{ENU-/-}$ homozygous mutant relative to their WT siblings. These data indicated the nonsense-mediated mRNA attenuation (NMD) (El-Brolosy et al., 2019; Ma et al., 2019) in promoting rapid degradation of *aars1* mRNA in *aars1*$^{ENU-/-}$ mutants. The data were included in the revised **Figure S2D**. The information was described in the revised manuscript (lines 178-182 in page 7, marked by yellow background).

5. Page 7 and throughout- the use of morpholinos in the field is controversial and, in some cases, unnecessary in this study. Using Mos to rescue phenotypes is a valid approach as this is unlikely to be a toxic off target effect, so common in Mos (as it is neuroprotective in this case). However, where the MO is used to cause a neurodegenerative phenotype to validate, this is an inappropriate use of the technology. In the case of using, it to phenocopy the AARS1 mutant- it is also not necessary, as the authors have two different stable mutants, ENU and Crispr, that both show the same phenotype. The MO work is a distraction and sends a bad message to the community. I would remove all the AARS1 MO work.
Answer: We appreciate your great suggestions! These issues were also raised by referee
2. We removed the work of *aars1* MOs and replaced it by using the CRISPANTS strategyas suggested (Kroll et al., 2021). Application of the CRISPANTS successfully knocked out *aars1* gene in the F0 generation. The new data were included in the revised manuscript (lines 199-204 in page 7, marked by yellow background).

6. Page 11- XBP gels need quantifying.
Answer: We quantified the XBP gels by using the imageJ software to measure the gray intensities of different bands. The values were added in the revised **Figures 7I, S4D**.The quantification descriptions were added in the revised manuscript accordingly (lines 805-813 in page 25, marked by yellow background). Many thanks!

7. Page 11- Perk MO – KD needs quantifying, but again, if a PERK inhibiter rescuesthe phenotypes in the mutants, the MO work is a distraction and not necessary.
Answer: The work of *perk* MO was removed as suggested. And the data regarding PERK-specific inhibitor GSK2656157 (Jia et al., 2015) was quantified in the revised **Figures 4G, 7J** accordingly.

8. Page 11- CHOP MO KD needs quantification- if not possible, this experiment can be repeated with CRISPANTS see Kroll et al 2021 and Keatinge et al 2021. Crispant strategy will likely replace Mos for rapid loss of function studies in the future, as they can be quantified easily and are far less toxic than Mos.
Answer: We deeply appreciate your valuable advice. We used the CRISPANTS strategy to successfully knockout *chop* gene in the F0 generation. We substituted the previous *chop* MO data by the CRISPANTS thoroughly in the revised **Figures 4I, 4J, 5K, 6J, S4G, and S4H.**

9. Page 13 figure 5I, the increase in nfe2l2b should be quantified.
Answer: Many thanks to your kind suggestions. We determined the mean fluorescence intensity (AFI) of the *nfe2l2b* FISH signals by using imageJ software, according to a previous report (Bankhead, 2014). The quantification data were included in revised **Figure 5I**.

10. N numbers needed to be included for every experiment as it is not clear what they are in some, please include in the legend for each figure. Furthermore, how manyclutches were used in each experiment? It is inappropriate to use a single clutch per experiment, multiple clutches, at least n=2 should be reported for each experiment. For example, n= 15 from 2 independent clutches.
Answer: We deeply appreciate your valuable advices and comments. We included the information regarding N numbers of each experiment and the number of clutches in the revised figures and legends thoroughly.

11. The stat test used and the p value also must be reported for each experiment in the main text and legend. T tests are not appropriate for all experiments here, 2-wayanova should be employed when comparing 2 groups in 2 different conditions-for example WT and MUT on a non tg and a tg background. These experiments would require re analysis.
Answer: Thank you very much for your instructive suggestions. We are sorry for the inappropriate statistical methods and lacking p values in the previous manuscript. In the revised version, we corrected the statistical methods as follows: unpaired t-test was used when only two groups were compared; one-way ANOVA was conducted to compare more than two groups; two-way ANOVA was applied when two groups were compared under two different conditions. The information was included in the text and figure legends. The P values were exhibited in the figures accordingly. Thank you again!

12. There is a lot of information regarding the pathways- a diagram or cartoon at the end of the paper would be incredibly helpful to the reader to summarise the findings.
Answer: Thanks very much to your wonderful suggestions. We simplified the corresponding pathway information by a summarized diagram at the end of each section. Please refer to **Figures 1G, 3M, 4K, 5S, 6O and 7N**. Thanks again!

Minor points
Abstract
13. In the abstract NRF2 is mentioned as an "executor" and "employer" of PERK. These phrases need clarification.
Answer: We are very sorry to incorrectly use "executor" and "employer". We correct **"employer"** to **"executor"** in the revised abstract.

Introduction
14. The entire second paragraph of the introduction should be condensed and simplified. Answer: We appreciate your kind suggestion. To make the manuscript easily to be followed, we conducted a substantial revision. The second paragraph of introduction was simplified and included in the current first paragraph.

15. What are *sti* mice? are these AARS1 mouse mutants, it is not clear.
Answer: We are very sorry for the unclear introduction of *sti/sti* mutant mice. It is an *Aars1* mutant mouse that harbored a C-to-A mutation at amino acid 734 in the editing domain of aminoacyl-tRNA synthetase (Aars1). We added this information in our revised manuscript (lines 80-84 in page 3, marked by yellow background).

16. ANKRD16 is mentioned, but the text does not explain what is it is? Is this a chemical? a gene? Etc.
Answer: We are sorry that we did not explain ANKRD16 previously. *Ankrd16* is a gene that encodes vertebrate-specific protein containing ankyrin repeats. As a modifier, the lysine side chains of ANKRD16 (Ankryin Repeat Domain 16) can capture the mis-charged serine by the hypomorphic *Aars1* to decrease the amount of serine mis-incorporation in the nascent peptides, which alleviate the loss of Purkinje cell in *Aars1*$^{sti/sti}$ mice. We included this information in the revised manuscript (lines 84-87 in pages 3 and 4, marked by yellow background).

17. "Heterozygous mutations in the AARS1 gene were discovered in people with CNS disorders" this needs to be expanded and the appropriate sources citated. What CNS disorders would be helpful. Within the paragraph CNS disorders are conflated with neurodegenerative diseases. This is vague and should be rectified.
Answer: We apologized for the unclear and conflated introduction about "heterozygous mutations

in the *AARS1* gene in human CNS disorders". We expanded the information. And the references were included in the revised manuscript accordingly (lines 87-91 in page 4, marked by yellow background). Thank you for your comments and suggestions.

18. Final paragraph of intro "the function of nfe2l2a in ZF embryonic development was revealed recently". Please elaborate.
Answer: A recent study reported that activation of *nfe2l2a* could attenuate the ER stress in *pmm2*-deficient larval liver (Mukaigasa et al., 2018). We added this information in the revised manuscript (lines 68-69 in page 3, marked by yellow background). Thanks again for your advice!

Results
19. GFAP is referred to as an astrocyte marker- this is incorrect. It is an astrocyte marker in mammalian models, but in fish it labels both astrocytes and all radial glial.
Answer: Many thanks to your kind suggestion. We corrected this error in the revised manuscript (line 124 in page 5, marked by yellow background).

20. Figure 1C legend and text- Zebrafish midbrains measurements are reported in "inches" this is clearly incorrect, please clarify the correct units.
Answer: We apologize for this error. The units of zebrafish midbrain area were corrected to "**μm^2**" in the revised manuscript.

21. Page 7 – please list the ESNT (from Ensembl) numbers of the different aars1 transcripts described.
Answer: The ESNT (from Ensembl) numbers of different *aars1* transcripts were added in the revised manuscript accordingly (lines 168-170 in page 6, marked by yellow background).

22. Page 7 – none of the data shows a reduction in AARS1 activity per se, but actually shows a loss of function- so the crispr mutant phenocopies due to loss of function as opposed to loss of activity, as the latter is hard to demonstrate unless one has a validated functional enzyme activity assay.
Answer: Thank you for your valuable suggestion. In the present manuscript, we revised the Aars1 "**activities**" to "**protein levels**".

23. Page 13 aars1cq71;nfe2l2b-/- are these double homozygous mutants? It is unclear, correct mutation nomenclature should be employed throughout the paper.
Answer: We clarified the mutation nomenclature throughout in the revised manuscript. For example, we correct "***aars1^{cq71};nfe2l2b$^{-/-}$***" to "***aars1$^{ENU-/-}$;nfe2l2b$^{CRISPR-/-}$***".

24. Page 14- typo- I think "folds" should be "fold"
Answer: We are quite sorry for the mistake. We corrected "**folds**" to "**fold**" in the revised manuscript.

25. Figure S2- in situs photos are not needed and are of poor quality- I would remove all except for the close up of the head in figure S2I.
Answer: We removed the in-situ photos except for the close up of the head as suggested in the revised **Figure S2I**. Many thanks again!

Reviewer 2:
Reviewer 2 Advance Summary and Potential Significance to Field:
Beginning with a mutation picked up in a zebrafish forward genetic screen, thismanuscript describes a pathway connecting the Aars1 tRNA synthetase and Nrf2 inbrain development. For me the strength of the manuscript lies in some of theexperiments that functionally connect different components of the pathway. Inparticular, the double mutant analyses are quite convincing and compelling. I donot work on this pathway or on cell survival, so it is a bit difficult for me toassess novelty and significance, but the findings, if supported, appear to beimportant. My biggest concern with this manuscript is in how many of the data aredescribed and interpreted, resulting in, for me, considerable confusion aboutwhether aars1 function is important in progenitors or neurons. My specific pointsare detailed below. Altogether, I think this manuscript has the potential to be animportant story, but it requires extensive revision to more clearly articulate theprimary role of Aars1 in neural development.

We deeply appreciate your great efforts in evaluating our manuscript. The comments and suggestions are critical for improving our story.

Reviewer 2 Comments for the Author:

1. The cells that the gene expression probes (transgenic reports, in situ RNA hybridization probes, and antibodies) need to be defined. Although some markers, such as HuC (which should be called Elavl3), are widely known, others are not, making it difficult to know if the authors are referring to progenitors, neuronal precursors, neurons, or specific glial cell types.

Answer: We are grateful to the suggestions. We defined the markers, including NBT, HuC, *neurod1*, *neurogenin1* (*ngn1*), *map2*, *tuba1b*, *sox2*, *gfap* and *olig2*, in the revised manuscript according to the referred literatures (lines119-124 in page 5, marked by light blue background)

2. The authors refer to gfap as a "typical astrocyte marker" (p.5) , but in zebrafish at these stages it probably marks mostly radial glia.

Answer: Thank you very much for your kind suggestion. This point was also mentioned by referee 1. We apologized for the mistake. We corrected the description of *gfap*-labeled cell types, according to your suggestions and the literatures (line 124 in page 5, marked by yellow background). Thank you again!

3. The authors refer to olig2 as "labeling oligodendrocytes" (p. 6) but it also marks progenitors and neurons. The authors use olig2 expression to assert that oligodendrocytes "were similarly diminished in the cq71 mutant". However, the cells that the authors highlight in Figures 1A and S1A and not oligodendrocytes. Instead, the highlighted cells are probably eurydendroid neurons of the cerebellum (McFarland KA, Topczewska JM, Weidinger G, Dorsky RI, Appel B. Hh and Wnt signaling regulate formation of olig2+ neurons in the zebrafish cerebellum. DevBiol. 2008 Jun 1;318(1):162-71. doi: 10.1016/j.ydbio.2008.03.016. Epub 2008 Mar 21. PMID: 18423594; PMCID: PMC2474464. Bae YK, Kani S, Shimizu T, Tanabe K, Nojima

Answer: We are sorry for the incorrect description of *olig2*$^{+}$ cells. We carefully read the literatures of McFarland KA et al (McFarland et al., 2008; Weber et al., 2020) and realized the signatures of *olig2*$^{+}$ cells in different locations. We corrected the cell types of *olig2*$^{+}$ cells in the revised manuscript (lines 123-124 in page 5, marked by light blue background). Thanks very much for this important point.

4. The authors state that "the WT sibling midbrains of 3 dpf were ~23.72 inches in size." Other midbrain measurements are similarly stated. The measurements are not sufficiently described in the Methods for me to understand how the authors arrived at those numbers, and I can't understand how they could be so large.

Answer: This issue was also concerned by referee 1. We deeply apologize for the mistakes in the description of brain size measurements. We carefully re-measured the midbrain area using imageJ software and corrected the units of "**inches**" to "**μm^2**" in our revised manuscript. The information was included accordingly (lines 128-130 and 791-798 in pages 5 and 24-25, marked by light blue background).

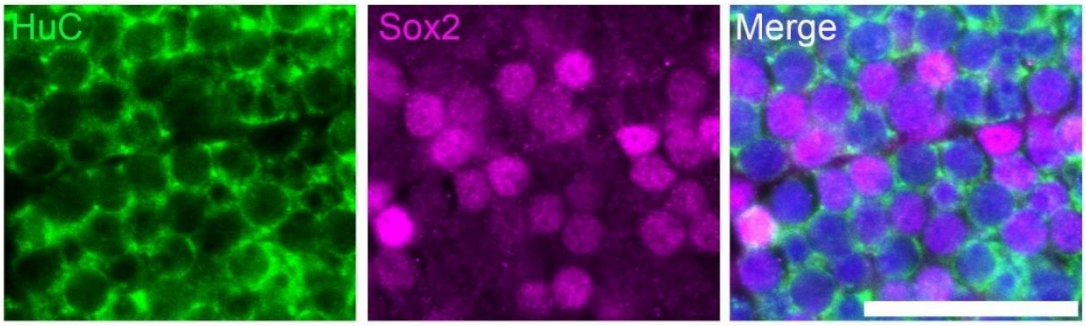
5. I have a significant concern about how the Acridine Orange (AO) data for assessing cell death are interpreted. The authors present the data as a percentage of the total area that is fluorescent. It appears to me from the images that the AO+ signals are "larger" in mutant larvae compared to wild type, but it is not so apparent that there is a difference in the number, which would be indicative of a difference in number of dying cells. An alternative explanation for these data are that microglia an unable to clear dying cells in mutant larvae, resulting in greater AO signal, but there is no difference in the amount of cell death. To their credit, the authors perform two other assays using TUNEL and anti-Caspase 3. However, the TUNEL staining is not quantified and, although the anti-Caspase 3 staining is quantified, the accompanying image in Figure S1J is too small to use for verification by a reader. Altogether, I do not think the data that are provided are sufficient to support the interpretation that mutant larvae have increased cell death.

Answer: We are grateful to your careful estimation. Indeed, the most significant phenotypes of AO^{+} signals were their much larger volumes in the *aars1*$^{-/-}$ mutant larvae than that in the WT siblings. This is also an interesting phenomenon attracting our attention. Your kind suggestion led us to explore the actions of microglia. We conducted an imaging on the *Tg(coro1a:DsRed)* line that labels microglia in the brain (Li et al., 2012). The results indicated

that the AO^+ signals were intensively aggregated in the coro1a-$Dsred^+$ cells (**revised Figure S1S**), implying the enlarged AO^+ signals was caused by a defect clearance of dying cells by the mutant microglia. The microglia phenotypes warranted future investigation. However, there still existed tremendous AO^+ signals that were not merged with coro1a-$Dsred^+$ cells in *aars1*$^{-/-}$ mutants in compared to WT siblings (**revised Figure S1S**). This result indicated an increased cell death in the *aars1*$^{-/-}$ mutant larvae. To provide more support evidence, we repeated the staining of Caspase-3 and TUNEL. The images and statistical results displayed a significant increment of $TUNEL^+$ and Caspase-3 signals in the $Sox2^+$ cells of *aars1*$^{-/-}$ mutant midbrains than that in WT siblings (**revised Figures 1D-1F, S1P-1Q**). All these results indicated that the *aars1*$^{-/-}$ mutant larvae had an increased apoptosis of neuronal progenitors. The data and description were included in the revised manuscript accordingly (lines 148-155 in page 6, marked by light blue background)

6. I have a very significant concern about interpretation and description of the data regarding the cell types that die in mutant larvae, and how this is used to set up the rescue experiments. On page 6 the authors state that the apoptosis markers are increased "especially in the Sox2+ and HuC+ cells…", which would suggest that both progenitors and neurons die. However, the selected images make it appear that it is mostly the Sox2+ progenitors that die. Indeed, the authors conclude this section on page 6 by stating that "indicated an increased apoptosis of neuronal progenitors in the cq71 mutant brains." Apart from the somewhat imprecise description of the data, the conclusion that progenitor cells die seems fine. However, the authors then go on to do a double labeling experiment to show that HuC+ neurons express aars1 and a qPCR experiment to show that aars1 transcripts are enriched in HuC+ but not coro1a+ cells. The authors do not define the cell type marked by coro1a, so I do not know what the comparison is, but in any case, the authors conclude that neurons express aars1. The authors then use these data as rationale to perform a rescue experiment by using HuC regulatory DNA to drive aars1 expression in neurons of mutant larvae, and claim that this suppresses cell death. They also used a cell transplantation experiment to claim that mutant HuC-EGFP+ neurons have elevated levels of cell death in wild type larvae. Based on these data the authors assert that "aars1 was intrinsically required and functionally conserved in neural survival and development." For me, these observations do not entirely align. My main question is this – if Sox2+ neural progenitors are the primary cell type that undergoes apoptosis, which the data seem to indicate, then how is it that neurons are enriched for aars1 expression and neuronal expression of aars1 rescues cell death? I consider this to be a major point requiring clarification.

Answer: We appreciated your careful estimation and valuable comments! We realized that we did not well define the cell types that were regulated by *aars1*. We apologized for the unclear and confused explanation of the cell types that died in the *aars1*$^{-/-}$ mutant larvae in previous manuscript. These days, we fortunately obtained the sox2-GFP line (Shin et al., 2014), neurod1-EGFP line (Lu et al., 2019) and olig2-DsRed2 line (Kim et al., 2008). We examined the transcript levels of *aars1* in various neural cells via performing qPCR on the isolated cells. The results indicated that *aars1* levels were the most highly detected in the *NBT*$^+$, *HuC*$^+$ and *Sox2*$^+$ cells but limited present in *neurod1*$^+$ and *olig2*$^+$ cells (revised **Figure S2O**). However, almost all the $Sox2^+$ signals were merged with NBT^+ and HuC^+ cells according to the double staining data (see **attached Figure and revised Figure S2P**). These results suggested the enrichment of *aars1* in the neural progenitor cells and explained the previous transient rescue assay using HuC-aars1. Concordantly, the drastic appearance of apoptotic signals in $Sox2^+$ cells were observed in *aars1*$^{-/-}$ mutants (**revised Figure 1D**). And the results of both single-cell sequence analysis and the co-staining of *aars1* with sox2 (**revised Figures 2H, 3E**) supported the regulatory functions of *aars1* in the neural progenitor cells. These days, we acquired a stable *Tg(NBT:aars1)* allele, which provided *aars1* in the pan-neuronal cells including $sox2^+$ cells. The results indicated that *Tg(NBT:aars1)* well rescued the neuronal phenotypes in *aars1*$^{-/-}$ mutant. To simplify our data, we used *Tg(NBT:aars1)* and removed the previous Huc-aars1 results in the revised manuscript. The information was included accordingly (lines 148-150, 212-223 in pages 6 and 8, marked by light blue background).



The immunofluorescent staining images of merged HuC with Sox2.

7. The Annexin V staining needs to be explained better in the Methods.
Answer: Thank you for the suggestions! The methods of Annexin V staining were further explained in the revised manuscript (lines 638-647 in page 21, marked by light blue background).

8. The image panels and print in the Figures are so small they are impossible to evaluate on a printed page.
Answer: This point is also raised by referees 1 and 3. Accordingly, we removed several superfluous data to simplify the panel organizations, which enable the clear presentation of important images with high resolutions in the revised manuscript.

9. Single cell RNA-seq analysis of mutant and wild-type larvae show that the largest gene expression differences occur in neural progenitors. Related to #6, this again raises the question of whether aars1 gene function is required in primarily in progenitors rather than neurons. Indeed, treating mutant embryos with cycloheximide rescued Sox2+ progenitor number in mutant larvae.
Answer: Thanks very much for the comments and suggestions. We clarified our conclusion in the revised manuscript accordingly.

10. The Figures, in general, and not sufficiently well annotated. As noted above, markers are not defined, nor are some experimental manipulations explained (e.g. “Tg” and “Tn”). Answer: We apologize for the rough annotation and explanation of the images, markers and experimental methods. We made a substantial revision throughout to improve the qualities of the manuscript. Thapsigargin (Tg) and tunicamycin (Tn) are the chemical inhibitors of calcium pump and protein glycosylation, respectively. The information was included (lines 282-285 in page 10, marked by light blue background). Thank you very much again for all the generous comments and valuable suggestions!

Reviewer 3:
Reviewer 3 Advance Summary and Potential Significance to Field:
The manuscript explores the link between tRNA synthetase deficienc, nrf2 and p53 in neurogenesis in the larval zebrafish brain.

Reviewer 3 Comments for the Author:
Unfortunately the paper is written with a language so confusing that it's hard to understand. Many imprecision and mistakes make impossible to follow the logic of the work as it is presented. I list below some specific points but that is far from being a complete assessment of all the work that should be done on the manuscript and all the experimental criticism that should be addressed.
We apologized for the poor presentation of previous manuscript. We simplified the data and made a substantial revision, including the language editing. We tried our best to address all the concerns and improve the manuscript quality, making it accessible. Thank you very much to your great effects and constructive comments on our manuscript!

1. the title doesn't reflect the main message of the paper as the main topic here is not neuronal fate but neuronal survival and differentiation.
Answer: We are grateful to your kind suggestions. We have changed the title to “**Nrf2 dictates the neuronal survival and differentiation of embryonic zebrafish harboring compromised alanyl-tRNA synthetase**” as suggested.

2. the introduction start with a first paragraph completely disconnected from the rest of the manuscript about neurogenesis and gliogenesis without any link to the following parts of the manuscript.
Answer: We apologized for the poor introduction of the first paragraph. We made a dramatic revision to well introduce the background. Thanks very much!

3. In the introduction the authors define protein folding a post-traslational modification. This definition is debatable and not supported by the review that they cite at this point (Deribe et al 2010) where post translational modification are classically defined as the "addition of a modifying chemical group or another protein to one or more of its amino acid residues". I don't see why the authors insist twice in the introduction in calling folding a post-translational modification.
Answer: We sincerely apologize for the incorrect definition of protein folding in the previous manuscript. We read several literatures carefully and realized our mistakes. We corrected this error and changed the **"post-translational modification"** into **"protein folding"**in the revised manuscript accordingly (lines 49-51 in page 2, marked by green background). We deeply appreciate your comments!

4. The manuscript contains some words that are not found in standard English such as "cytomic, distichouslyetc" or used in the wrong contest such as "phenomenons" (?) in place of data or results.
Answer: We are sorry for the poor language. We corrected the words and tried our best to improve the manuscript substantially (lines 48, 155, 446, 457 and 509 in pages 2, 6, 16 and 18, marked by green background).

5. Throughout the manuscript the authors refer as measuring protein "activity" when what they do is simply doing western blots to quantify protein levels.
Answer: This point was also mentioned by referee 1. We are quite sorry for the incorrect description about protein **"activity"**. We revised the **"activity"** to **"level"** throughout the revised manuscript.

6. "...were significantly recovered in the aars1cq71;nfe2l2b-/- compounds compared to either the siblings or nfe2l2b-/- variants". Here the words "compounds" and "variants" do not make sense.
Answer: Thanks very much for your comments and we removed "**compounds**" and "**variants**" in the revised manuscript.

7. "and disability (?) of ATF6 inhibitor Ceapin A7 to attenuate cell apoptosis".
Answer: We corrected the **"disability"** into **"inability"** (line 302 in page 11, marked by green background).

8. When describing cell transplantation experiments the authors use the term of "intrinsically required" as "cell autonomous".
Answer: Thanks very much for your suggestions. The "**intrinsically required**" was changed to "**cell autonomously required**" in the revised manuscript accordingly (line 232 in page 8, marked by green background).

9. "Over-providing" is used in place of "over-expressing".
Answer: Thanks very much for your suggestions. It is done accordingly (line 313 in page 11, marked by green background).

10. These are just very few examples but almost every sentence in the manuscript should be corrected or rephrased.
Answer: We sincerely apologize for the poor language in the previous description. We made a substantial rephrase to improve the quality of our revised manuscript.

11. From a conceptual point the choice of Nrf2 as putative downstream factor of the PERK-CHOP axes seems not well justified as it is the choice of p53 as Nrf2 target.
Answer: Thank you for raising this interesting point. NRF2 is a master factor in response to the environmental stress, in particular oxidative and metabolism stress. However, its roles in ER stress were noticed these days. Our study supported the crucial roles of NRF2 as a downstream factor of PERK-CHOP axes in the ER stress, based on the alleviation of the neuronal phenotypes in the *aars1*$^{ENU-/-}$*;nfe2l2b*$^{CRISPR-/-}$ double mutants than *aars1*$^{ENU-/-}$mutants. However, the effects of

NRF2 in the stress were still debatable. Although a major opinion is NRF2 promotes cell survival but others proposed an opposite view (Kang et al., 2019). Our study indicated that *nfe2l2b*, a novel paralogue NRF2, promoted cell apoptosis when over-expressed. We are curious that how *nfe2l2b* regulates cell apoptosis in our study. Considering NRF2 was a transcriptional factor, we searched its downstream targets and observed *p53* was a strong candidate. The promoter study and genetic analysis further verified the choice of *p53* as Nrf2b target and explained its roles in cell survival. We noticed that recent reports also documented the involvement of *p53* in the NRF2 functions in ER stress. For example, it is reported that the upregulation of tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) death receptor DR5 required the co-regulation of *p53* and C/EBP homologous protein (CHOP) (Dilshara et al., 2019; Jung et al., 2015), but the upstream and downstream relationship between the two has not been revealed. A recent study documented the P53 and NRF2 against Acetaminophen (APAP) hepatotoxicity through the key docking molecule Sirtuin 6 (SIRT6) (Zhou et al., 2021). One work identified a novel long noncoding RNA (named Nrf2-activating lncRNA) that controlled the cell fate by modulating p53-dependent Nrf2 activation (Joo et al., 2019). Meanwhile, the interaction of NRF2 with P53 to promotecancer cell death was unveiled (Kang et al., 2019). Our study extended the realization that "*nfe2l2b* probably functioned downstream of the Perk-Chop axis in the regulation of neural progenitor cellssurvival via targeting p53". We hope this study could provide clues regarding NRF2 functions in cell survival.

12. Experimentally, for what is possible to understand, there are some strange points such as the use of HSP promoter to drive transgene expression without mentioning in the text any heat shock.
Answer: We apologized for limited explanation on the heat shock assay. The temporal control of target genes could be achieved in *hsp*-promoter-driven transgenic zebrafish using heat shock (Wu and Wang, 2020). We therefore applied this strategy to induce *p53* over-expression at designed time points. To this end, *Tg(hsp70:p53)* embryos were placed into 38.5°C egg water and incubated for 40 minutes. This method effectively induced the intensive expression of *p53* in whole body at 3dpf (**revised Figure S6E**). The detailed information of the heat-shock experiments was included in the revised manuscript accordingly (lines 592-595 in page 20, marked by green background). Thanks very much again for the comments.

13. Cell transplantation should be done as the gold standard in the zebrafish community by blastomere transplantation and not as done in the paper. They refer to a protocol adapted to transplant hematopoietic kidney marrow stem cells, which is not the same as transplanting neuronal progenitors integrated in the neuroepithelial tissue.
Answer: Thanks very much for the comments and suggestions. These days, we conducted the blastomere transplantation according to a previous report (Zou and Wei, 2010). We utilized CRISPANTS technology (Kroll et al., 2021) as suggested by referee 1 to effectively knock out *aars1* gene in F0. This assay facilitated the transplantation. *Tg(NBT:DenNTR)* transgenic background was applied because it labels neural progenitor cells (co-localization of $Sox2^{+}$ with NBT^{+} in revised **Figure S2P**). The transplantation strategy was shown in revised **Figure 2K**. Briefly, equal numbers (30-50) of WT or *aars1* F0 blastomere donor cells in the *Tg(NBT:DenNTR)* background were transplanted into $aars1^{ENU-/-}$ mutant and WT siblings at 1K cell stage separately. Annexin V was applied as the indicators of apoptotic signals. Resultantly, Annexin V^{+} signals did not present notable alterations in the $aars1^{ENU-/-}$ mutant recipients after accepting the NBT-$DenNTR^{+}$ blastomeres cells from the WT siblings but elevated dramatically when receiving the donors of the *aars1* F0, in compared to their control groups. We included these data and description in the revised manuscripts (revised **Figures 2K-2M,** lines 223-231 and 649-655 in pages 8 and 21, marked by green background). Thank you very much again for your valuable advice and suggestive comments!

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Second decision letter

MS ID#: DEVELOP/2021/200342

MS TITLE: Nrf2 dictates the neuronal survival and differentiation of embryonic zebrafish harboring compromised alanyl-tRNA synthetase

AUTHORS: Binbin Jin, Liqin Xie, Dan Zhan, Luping Zhou, Zhi Feng, Jiangyong He, Jie Qin, Congjian Zhao, Lingfei Luo, and Li Li

I have now received the reports of two of the referees who reviewed the earlier version of your manuscript and I 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.

The overall evaluation is positive and we would like to publish a revised manuscript in Development, provided that you satisfactorily address the remaining suggestions and comments of the two referees. Please attend to all of the reviewers' comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

Jin et all have produced a very thorough characterisation of an aars1 mutant zebrafish, identified through a large ENU mutagenesis screen looking to identify novel genes required for neurogenesis. Initial findings were confirmed with secondary mutants and Morpholinos for aars1, which phenocopies the ENU mutant nicely. Single cell RNA seq on mutants suggested ER stress and protein overload could be the mechanisms behind the phenotypes. This was confirmed via secondary readouts- western and qPCR. Perk signalling was found to be specifically responsible for this, and inhibition of PERK led to a rescue of phenotypes. To tease apart the pathway further, NRF2 paralogue was found to be increased in the mutant neurons and its inhibition also rescued mutant phenotypes. Further investigation implicated p53 as a regulator of this process. This report has implications for neurogenesis, but also neurodegeneration as few LOF AARS1 models have been reported in different species, especially in such detail- as well as providing mechanistic insights on a variety of different levels, including several routes of rescue.

Comments for the author

My original comments in black

Author comments in blue.

Below in red script are my views on how the authors have tackled them. For the most part I think they have done a great job, but some tweaking still needs to be done and some comments still not have been addressed. None of these will be experimental, only re analysis and additions to the main text.

Reviewer 1:
Reviewer 1 Advance Summary and Potential Significance to Field:
Jin et all have produced a very thorough characterisation of an aars1 mutant zebrafish, identified through a large ENU mutagenesis screen looking to identify novel genes required for neurogenesis. Initial findings were confirmed with secondary mutants and Morpholinos for aars1, which phenocopies the ENU mutant nicely. Single cell RNA seq on mutants suggested ER stress and protein overload could be the mechanisms behind the phenotypes. This was confirmed via secondary readouts- western and qPCR. Perk signalling was found to be specifically responsible for this, and inhibition of PERK led to a rescue of phenotypes. To tease apart the pathway further, NRF2 paralogue was found to be increased in the mutant neurons and its inhibition also rescued mutant phenotypes. Further investigation implicated p53 as a regulator of this process. This report has implications for neurogenesis, but also neurodegeneration as few LOF AARS1 models have been reported in different species, especially in such detail- as well as providing mechanistic insights on a variety of different levels, including several routes of rescue.

Reviewer 1 Comments for the Author:
I very much liked this paper and think is a good fit for Development. But there are lots of revisions that are needed, however, few of these need to be experimental and is more revision of the text.
There is a tremendous quantity of work in here, sometimes too much, and it becomes overkill, which at times makes the narrative somewhat what confusing. I suggest the authors go through all figures and simplify, to make the information more palatable. Some figures should be taken out entirely if their addition to the narrative is superfluous. On the other hand, some things need to be explained in greater depth to those not completely familiar with the pathways involved.
We deeply appreciate your great efforts in reviewing and justifying our manuscript. Your comments and suggestions are quite valuable for improving our manuscript.

Essential revisions

1. Major points that need to be changed for all figures- everything needs to bequantified in the text and in the legend- quite often westerns are shown withoutquantification and a simple increase is described, significance unknown. It is not therefore known how much change is occurring and by what significance. For example, Figs 3d, 3k, 4a, 4f, 5c and 5d to name a few. There are more in the suppfigs.
Answer: We are grateful to your careful estimation and apologize for lacking the quantification of several results, including westerns. To deal with these issues and improve the data quality, wequantified the original data by using the imageJ software according to the methods in previous reports (Shin et al., 2017). The revised figures of western blot were included in the present Figures 2C, 3J, 4A, 4F, 5C, 5D, 7H, S2C, S4I, S5A, S5H, S5J, and S6A. The quantification method descriptions were added in the revised manuscript accordingly (lines 805-813 in page 25, marked by yellow background). Thanks again for your kind comments!
This has still not been done. On all westerns I see a ratio, but these need to be expressed as a % compared to the control with appropriate p value, both in the legend, main text and figure. For example, levels of X protein were reduced by 96% in the experimental group compared to control (P<0.005).

2. Throughout the manuscript the mutants are incorrectly referred to simply as mutant. Are these heterozygous or homozygous mutant? Furthermore, are thehomozygous mutants viable? If not, what is their lifespan? Survival curves would be required for this. Correct mutant nomenclature should be used throughout. When talking about different alleles of the same gene the authors could simple writeaars1ENU-/- and aars1Crispr-/-for example.
Answer: Thank you for valuable advices. We would like to apologize for the rough and incorrect reference of various mutants in the previous manuscript. We corrected the nomenclature of the homozygous mutant types to aars1ENU-/-, aars1CRISPR-/-, aars1ENU/CRISPR, tars1ENU-/-, nfe2l2bCRISPR-/-, or aars1ENU-/-;nfe2l2bCRISPR-/- and heterozygous mutants to nfe2l2bCRISPR+/- and etc in the corresponding figures and text throughout. We evaluated the viable ability of aars1-/- homozygous mutants. The results indicated the aars1ENU-/- homozygous mutants could not live beyond 7 dpf. The survival curve was included in the revised Figure S1D.
This has been completed and makes the paper far easier to read.

3. The mutants are described as not moving very much- however, do they respond to touch or other stimuli – such as light dark paradigm. This would be more informative as to how the neuronal phenotypes effect circuitry and behaviour.
Answer: Thank you very much for the suggestions. We performed the stimuli assays of needle touch and light-dark paradigm (Marquez- Legorreta et al., 2020; Sztal et al., 2016) to measure the reactions of 5 dpf aars1ENU-/- homozygous mutant larvae. The data indicated that WT larvae rapidly responded to the external stimuli. However, the mutant larvae occasionally acted to response to the stimuli. But they remained freezing at most time. And the mobility and speeds of aars1ENU-/- mutant larvae reduced drastically relative to their WT siblings. The data were added in the revised Figures S1G-S1L and the descriptions were included accordingly (lines134-140 in page 5, marked by yellow background).
This has been completed.

4. Page 7- the western blot described will not show enzyme activity, merely protein levels, please change throughout the text. This should be accompanied by qpcr for the transcript in mut vs wt to demonstrate if NMD is occurring due to the mutation or not- this will have implications for genetic compensation.
Answer: We are grateful to your suggestion and comments. We corrected the description of "enzyme activity" to "protein levels" throughout the text. We performed qPCR to examine the transcription levels of several NMD-related genes(Cheruiyot et al., 2021). The transcription levels of upf1, upf3b and smg factors increased significantly. But upf3a exhibited a slight reduction in the aars1ENU-/- homozygous mutant relative to their WT siblings. These data indicated the nonsense-mediated mRNA attenuation (NMD) (El-Brolosy et al., 2019; Ma et al., 2019) in promoting rapid degradation of aars1 mRNA in aars1ENU-/- mutants. The data were included in the revised Figure S2D. The information was described in the revised manuscript (lines 178-182 in page 7, marked by yellow background).
The authors went above and beyond with this experiment. I simply meant that they should analyse the levels of the AARS transcript in the mutant and compare it to WT. If NMD is occurring there would be a large decrease in AARS mRNA in the mutants. Looking at the NMD pathway is also fine. This has therefore been completed.

5. Page 7 and throughout- the use of morpholinos in the field is controversial and, in some cases, unnecessary in this study. Using Mos to rescue phenotypes is a valid approach as this is unlikely to be a toxic off target effect, so common in Mos (as it is neuroprotective in this case). However, where the MO is used to cause a neurodegenerative phenotype to validate, this is an inappropriate use of the technology. In the case of using, it to phenocopy the AARS1 mutant- it is also not necessary, as the authors have two different stable mutants, ENU and Crispr, that both show the same phenotype. The MO work is a distraction and sends a bad message to the community. I would remove all the AARS1 MO work.
Answer: We appreciate your great suggestions! These issues were also raised by referee 2. We removed the work of aars1 MOs and replaced it by using the CRISPANTS strategyas suggested (Kroll et al., 2021). Application of the CRISPANTS successfully knocked out aars1 gene in the F0 generation. The new data were included in the revised manuscript (lines 199-204 in page 7, marked by yellow background).
I appreciate the efforts the authors have gone to here. The MO work has been removed and replaced with a F0 Crispant approach. However, the Crispr activity needs to be quantified as a % mutation rate. As is stands, they have performed headloop PCR, but this has not been properly explained or quantified making it very confusing to the reader. So the gels need to be adequately explained and a mutation rate assigned to each guide RNA. Such as – Guide 1 mutated 80% of its target loci.

6. Page 11- XBP gels need quantifying.
Answer: We quantified the XBP gels by using the imageJ software to measure the gray intensities of different bands. The values were added in the revised Figures 7I, S4D.The quantification descriptions were added in the revised manuscript accordingly (lines 805-813 in page 25, marked by yellow background). Many thanks!
Needs properly quantifying as previously mentioned.

7. Page 11- Perk MO – KD needs quantifying, but again, if a PERK inhibiter rescuesthe phenotypes in the mutants, the MO work is a distraction and not necessary.

Answer: The work of perk MO was removed as suggested. And the data regarding PERK-specific inhibitor GSK2656157 (Jia et al., 2015) was quantified in the revised Figures 4G, 7J accordingly.
Needs properly quantifying as previously mentioned.

8. Page 11- CHOP MO KD needs quantification- if not possible, this experiment can be repeated with CRISPANTS see Kroll et al 2021 and Keatinge et al 2021. Crispant strategy will likely replace Mos for rapid loss of function studies in the future, as they can be quantified easily and are far less toxic than Mos.
Answer: We deeply appreciate your valuable advice. We used the CRISPANTS strategy to successfully knockout chop gene in the F0 generation. We substituted the previous chop MO data by the CRISPANTS thoroughly in the revised Figures 4I, 4J, 5K, 6J, S4G, and S4H.
Needs properly quantifying as previously mentioned.

9. Page 13 figure 5I, the increase in nfe2l2b should be quantified.
Answer: Many thanks to your kind suggestions. We determined the mean fluorescence intensity (AFI) of the nfe2l2b FISH signals by using imageJ software, according to a previous report (Bankhead, 2014). The quantification data were included in revised Figure 5I.

10. N numbers needed to be included for every experiment as it is not clear what they are in some, please include in the legend for each figure. Furthermore, how manyclutches were used in each experiment? It is inappropriate to use a single clutch per experiment, multiple clutches, at least n=2 should be reported for each experiment. For example, n= 15 from 2 independent clutches.
Answer: We deeply appreciate your valuable advices and comments. We included the information regarding N numbers of each experiment and the number of clutches in the revised figures and legends thoroughly.

11. The stat test used and the p value also must be reported for each experiment in the main text and legend. T tests are not appropriate for all experiments here, 2-wayanova should be employed when comparing 2 groups in 2 different conditions-for example WT and MUT on a non tg and a tg background. These experiments would require re analysis.
Answer: Thank you very much for your instructive suggestions. We are sorry for the inappropriate statistical methods and lacking p values in the previous manuscript. In the revised version, we corrected the statistical methods as follows: unpaired t-test was used when only two groups were compared; one-way ANOVA was conducted to compare more than two groups; two-way ANOVA was applied when two groups were compared under two different conditions. The information was included in the text and figure legends. The P values were exhibited in the figures accordingly.
Thank you again!

12. There is a lot of information regarding the pathways- a diagram or cartoon at the end of the paper would be incredibly helpful to the reader to summarise the findings.
Answer: Thanks very much to your wonderful suggestions. We simplified the corresponding pathway information by a summarized diagram at the end of each section. Please refer to Figures 1G, 3M, 4K, 5S, 6O and 7N.
Thanks again!

Minor points
Abstract
13. In the abstract NRF2 is mentioned as an "executor" and "employer" of PERK. These phrases need clarification.
Answer: We are very sorry to incorrectly use "executor" and "employer".
We correct “employer” to “executor” in the revised abstract.
This has been completed

Introduction
14. The entire second paragraph of the introduction should be condensed and simplified.
Answer: We appreciate your kind suggestion. To make the manuscript easily to be followed, we conducted a substantial revision. The second paragraph of introduction was simplified and included in the current first paragraph.
This has been completed

15. What are sti mice? are these AARS1 mouse mutants, it is not clear.
Answer: We are very sorry for the unclear introduction of sti/sti mutant mice. It is an Aars1 mutant mouse that harbored a C-to-A mutation at amino acid 734 in the editing domain of aminoacyl-tRNA synthetase (Aars1). We added this information in our revised manuscript (lines 80-84 in page 3, marked by yellow background).
This is still very confusing in the text and makes no sense. This simple explanation put in this review rebuttal would be more than sufficient to educate the reader.

16. ANKRD16 is mentioned, but the text does not explain what is it is? Is this a chemical? a gene? Etc.
Answer: We are sorry that we did not explain ANKRD16 previously. Ankrd16 is a gene that encodes vertebrate-specific protein containing ankyrin repeats. As a modifier, the lysine side chains of ANKRD16 (Ankryin Repeat Domain 16) can capture the mis-charged serine by the hypomorphic Aars1 to decrease the amount of serine mis-incorporation in the nascent peptides, which alleviate the loss of Purkinje cell in Aars1sti/sti mice. We included this information in the revised manuscript (lines 84-87 in pages 3 and 4, marked by yellow background).
This has been completed

17. "Heterozygous mutations in the AARS1 gene were discovered in people with CNS disorders" this needs to be expanded and the appropriate sources citated. What CNS disorders would be helpful. Within the paragraph CNS disorders are conflated with neurodegenerative diseases. This is vague and should be rectified.
Answer: We apologized for the unclear and conflated introduction about "heterozygous mutations in the AARS1 gene in human CNS disorders". We expanded the information. And the references were included in the revised manuscript accordingly (lines 87-91 in page 4, marked by yellow background). Thank you for your comments and suggestions.
This has been completed

18. Final paragraph of intro “the function of nfe2l2a in ZF embryonic development was revealed recently”. Please elaborate.
Answer: A recent study reported that activation of nfe2l2a could attenuate the ER stress in pmm2-deficient larval liver (Mukaigasa et al., 2018). We added this information in the revised manuscript (lines 68-69 in page 3, marked by yellow background). Thanks again for your advice!
This has been completed

Results
19. GFAP is referred to as an astrocyte marker- this is incorrect. It is an astrocyte marker in mammalian models, but in fish it labels both astrocytes and all radial glial.
Answer: Many thanks to your kind suggestion. We corrected this error in the revised manuscript (line 124 in page 5, marked by yellow background).
This has not been completed and it still says GFAP is an astrocyte marker.

20. Figure 1C legend and text- Zebrafish midbrains measurements are reported in “inches” this is clearly incorrect, please clarify the correct units.
Answer: We apologize for this error. The units of zebrafish midbrain area were corrected to “ìm2” in the revised manuscript.
This has been completed
21. Page 7 – please list the ESNT (from Ensembl) numbers of the different aars1 transcripts described.
Answer: The ESNT (from Ensembl) numbers of different aars1 transcripts were added in the revised manuscript accordingly (lines 168-170 in page 6, marked by yellow background).
This has been completed

22. Page 7 – none of the data shows a reduction in AARS1 activity per se, but actually shows a loss of function- so the crispr mutant phenocopies due to loss of function as opposed to loss of activity, as the latter is hard to demonstrate unless one has a validated functional enzyme activity assay.
Answer: Thank you for your valuable suggestion. In the present manuscript, we revised the Aars1 “activities” to “protein levels”.
This has been completed.

23. Page 13 aars1cq71;nfe2l2b-/- are these double homozygous mutants? It is unclear, correct mutation nomenclature should be employed throughout the paper.
Answer: We clarified the mutation nomenclature throughout in the revised manuscript. For example, we correct “aars1cq71;nfe2l2b-/-” to “aars1ENU-/-;nfe2l2bCRISPR-/-”.
This has been completed.

24. Page 14- typo- I think “folds” should be “fold”
Answer: We are quite sorry for the mistake. We corrected “folds” to “fold” in the revised manuscript.
This has been completed.

25. Figure S2- in situs photos are not needed and are of poor quality- I would remove all except for the close up of the head in figure S2I. Answer: We removed the in-situ photos except for the close up of the head as suggested in the revised Figure S2I. Many thanks again!
Answer: We removed the in-situ photos except for the close up of the head as suggested in the revised Figure S2I. Many thanks again!
This has been completed.

Reviewer 2

Advance summary and potential significance to field

see prior review

Comments for the author

This is still a tough paper to get through but the authors went to a considerable amount of effort to revise it and I think the most important points have been adequately addressed. Consequently, I think the paper will be an important contribution to the field. There are still a few things that should be cleaned up.
1. In the schematic of Figure 1, the authors are still depicting astrocyte and oligodendrocyte deficits.
However, none of the data in the paper address these cell types. This needs to be corrected.
2. Line 124, the authors are still using GFAP as an “astrocyte” marker, although reviewers pointed out that it is largely a radial glia marker at these stages in zebrafish.
3. The authors should provide allele designations for all mutant alleles following ZFIN guidelines. I actually disagree with reviewer 1 on designating these as “ENU” and CRISPR” alleles. Each allele needs a unique identifier, and that identifier should be used throughout the text. I apologize for not noting this in my prior review.
4. The authors substituted “CRISPANT” experiments for MO knockdowns. However, there are no validations provided to indicate targeting efficiency and accuracy. I really don’t want to ask for any more experiments but this concerns me. It might be helpful, though, if the authors have some data regarding targeting efficiency they could provide in the supplemental data section.

Second revision

Author response to reviewers' comments

Reviewer 1 Comments for the Author:
Below in red script are my views on how the authors have tackled them. For the most part I think they have done a great job, but some tweaking still needs to be done and some comments still not have been addressed. None of these will be experimental, only re analysis and additions to the main text.
Thanks for your great efforts in assessing our revised manuscript again. Your comments and suggestions are quite valuable for improving our manuscript.

Essential revisions

1. Major points that need to be changed for all figures- everything needs to be quantified in the text and in the legend- quite often westerns are shown without quantification and a simple increase is described, significance unknown. It is not therefore known how much change is occurring and by what significance. For example, Figs 3d, 3k, 4a, 4f, 5c and 5d to name a few. There are more in the suppfigs.

This has still not been done. On all westerns I see a ratio, but these need to be expressed as a % compared to the control with appropriate p value, both in the legend, main text and figure. For example, levels of X protein were reduced by 96% in the experimental group compared to control (P<0.005).

Answer: We are quite sorry for the insufficient revision on westerns. We have edited the expression of quantitative westerns and added corresponding P value in this version as suggested (Lines 175-177, 193-194, 202-208, 271-274, 283-286, 303-306, 312-315, 345-347, 349-351, 355-357, 375-377, 379-381, and 451-452 throughout the whole text, highlighted by black underlines). Many thanks to your suggestive advices.

5. Page 7 and throughout- the use of morpholinos in the field is controversial and, in some cases, unnecessary in this study. Using Mos to rescue phenotypes is a valid approach as this is unlikely to be a toxic off target effect, so common in Mos (as it is neuroprotective in this case). However, where the MO is used to cause a neurodegenerative phenotype to validate, this is an inappropriate use of the technology. In the case of using, it to phenocopy the AARS1 mutant- it is also not necessary, as the authors have two different stable mutants, ENU and Crispr, that both show the same phenotype. The MO work is a distraction and sends a bad message to the community. I would remove all the AARS1 MO work.

I appreciate the efforts the authors have gone to here. The MO work has been removed and replaced with a F0 Crispant approach. However, the Crispr activity needs to be quantified as a % mutation rate. As is stands, they have performed headloop PCR, but this has not been properly explained or quantified making it very confusing to the reader. So the gels need to be adequately explained and a mutation rate assigned to each guide RNA. Such as – Guide 1 mutated 80% of its target loci.

Answer: Thanks for your kind suggestions. We explained the headloop PCR gels and included the mutation rate of each guide RNA in the revised **Figure S2L** and the text accordingly (Page 8, lines 202-206, highlighted by black underlines).

6. Page 11- XBP gels need quantifying.

Needs properly quantifying as previously mentioned.

Answer: We quantify the XBP gels by "%" as suggested (Page 35, lines 1219-1220, highlighted by black underlines).

7. Page 11- Perk MO – KD needs quantifying, but again, if a PERK inhibiter rescues the phenotypes in the mutants, the MO work is a distraction and not necessary.

Needs properly quantifying as previously mentioned.

Answer: It is done in the revised **Figure S4F** and the information is included in the revised manuscript accordingly (Page 11, lines 312-315, highlighted by black underlines).

8. Page 11- CHOP MO KD needs quantification- if not possible, this experiment can be repeated with CRISPANTS see Kroll et al 2021 and Keatinge et al 2021. Crispant strategy will likely replace Mos for rapid loss of function studies in the future, as they can be quantified easily and are far less toxic than Mos.

Needs properly quantifying as previously mentioned.

Answer: It is done in the revised **Figure S4H** and corresponding legend. The description is included accordingly (Page 12, lines 324-327, highlighted by black underlines).

15. What are sti mice? are these AARS1 mouse mutants, it is not clear.

This is still very confusing in the text and makes no sense. This simple explanation put in this review rebuttal would be more than sufficient to educate the reader.

Answer: We are sorry for the simple and confusing explanation of *sti* mice. We provide more background of *Aars1*$^{sti/sti}$ mutant mice in the revised text (Lines 80-83 in page 3, marked by light blue background). We hope it give enough information to the readers.

Results
19. GFAP is referred to as an astrocyte marker- this is incorrect. It is an astrocyte marker in mammalian models, but in fish it labels both astrocytes and all radial glial.
This has not been completed and it still says GFAP is an astrocyte marker.
Answer: We are quite sorry for the mistakes. We correct the description of GFAP in page 5 (Lines 127-128, marked by yellow background). Thank you very much again for your insightful comments.

Reviewer 2 Comments for the Author:
This is still a tough paper to get through but the authors went to a considerable amount of effort to revise it and I think the most important points have been adequately addressed. Consequently, I think the paper will be an important contribution to the field. There are still a few things that should be cleaned up.
Thank you very much to your constructive comments and suggestions in improving our manuscript.

1. In the schematic of Figure 1, the authors are still depicting astrocyte and oligodendrocyte deficits. However, none of the data in the paper address these cell types. This needs to be corrected.
Answer: Many thanks to your valuable comments. We agreed your opinion and removed the astrocytes and oligodendrocytes in the schematic as suggested. The corrected pictures are included in the revised **Figures 1G, 7N**.

2. Line 124, the authors are still using GFAP as an "astrocyte" marker, although reviewers pointed out that it is largely a radial glia marker at these stages in zebrafish.
Answer: We are quite sorry for the mistakes. The information is corrected in the revised manuscript (Page 5, lines 127-128, marked by yellow background).

3. The authors should provide allele designations for all mutant alleles following ZFIN guidelines. I actually disagree with reviewer 1 on designating these as "ENU" and CRISPR" alleles. Each allele needs a unique identifier, and that identifier should be used throughout the text. I apologize for not noting this in my prior review.
Answer: Thank you very much for your valuable advices. We agree that allele designations following "ZFIN guidelines" are more convenient for the readers. We correct the allele designations of the present mutant alleles accordingly. Mut$^{cq71/cq71}$, *aars1*$^{cq71/cq71}$,*aars1*$^{\Delta 10/\Delta 10}$, *nfe2l2b*$^{\Delta 1/\Delta 1}$, *tars1*$^{cq16/cq16}$ were included in the revised manuscript.

4.The authors substituted "CRISPANT" experiments for MO knockdowns. However, there are no validations provided to indicate targeting efficiency and accuracy. I really don't want to ask for any more experiments but this concerns me. It might be helpful, though, if the authors have some data regarding targeting efficiency they could provide in the supplemental data section.
Answer: Thanks very much for your kind suggestion. We apologized for lacking the efficiency and accuracy of CRISPANT technology in the previously manuscript. We verify the efficiency of CRISPANT technology by western blot and qPCR. The data is added in **Figure S2M** and **S4I.** The information is included accordingly (Pages 8 and 12, lines 202-208 and 324-327, highlighted by black underlines).

Third decision letter

MS ID#: DEVELOP/2021/200342

MS TITLE: Nrf2 dictates the neuronal survival and differentiation of embryonic zebrafish harboring compromised alanyl-tRNA synthetase

AUTHORS: Binbin Jin, Liqin Xie, Dan Zhan, Luping Zhou, Zhi Feng, Jiangyong He, Jie Qin, Congjian Zhao, Lingfei Luo, and Li Li
ARTICLE TYPE: Research Article

I am delighted to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.