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Genetic deletion of *hspa8* leads to selective tissue malformations in zebrafish embryonic development

Caixia Wang, Xin Zhang, Xinyu Wang, Yanpeng Zhai, Mengjiao Li, Jun Pan, Yan Bai, Xiaozhi Rong and Jianfeng Zhou
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MS TITLE: Genetic deletion of hspa8 leads to selective tissue malformations via induction of unfolded protein response in zebrafish development

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We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers raise a number of substantial criticisms that prevent me from accepting the paper at this stage. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

Please 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. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

This paper is well written and the data appear to be of good quality interpreted in a generally logical way. The study has the potential to reveal novel mechanisms of hspa8 in zebrafish development.

Comments for the author

However, there are some issues with the details of the methods used and the mutants generated that should be addressed. In addition validation of the main conclusion would greatly improve the confidence of the major conclusion. With the current status I do not think that the title fully represents the finding of the study since it is unclear to which extent the mutant are a loss of function or not.

1) The heat shock proteins are a family of proteins that are highly conserved. Are the anti-sense probes used specific for hspa8 or could they detect other members as well, such as hspa8b?
2) To which extent the hspa8 mutant zebrafish are null-mutants is not clear. According to Figure S2b, all mutations should generate protein truncations in the N-terminal domain. However, analysis of both mRNA and especially at protein level still show that protein is produced, although at lower levels. Since the antibody used bind to an epitope at amino acids 580-601 at the C-terminus of the human HSC 70 protein it is likely that the mutations generate hypomorph zebrafish or that express alternative splice variants with the antibody epitope remaining. To conclude that this is a null-mutation is therefore to over-interpret the data. Since this is central for all further conclusions, the authors should examine in detail how the mutations affect the hspa8 transcript.
3) It is not clear how many generations the CRISPR/Cas9 generated mutant lines have been outcrossed. It is common to outcross at least to F2 to eliminate off target effects. It would be good if the authors could clarify this.
4) The authors speculate that the remaining mRNA or protein in mutant embryos may be maternally derived. However, incrossing mutant zebrafish would be an easy way to elucidate the contribution of maternally derived mRNA.
5) The presence of the paralogue hsap8b is not addressed. How similar are these proteins and could the gRNA and antibodies target this gene and protein as well?
6) During the last years, growing evidence point towards the activation of compensatory mechanisms induced by premature termination codons (PTC). Since the hspa8 gene has several paralogues, it is therefore possible that the mutations introduced upregulate the expression of other family members that mask the true function of hspa8? Can the authors address question to clarify this issue?
7) According to the title, the authors draw the conclusion that hspa8 induce PA malformation due to an activated UPR response. The authors hypothesize that UPR activation is induced by PERK/p-eIF2α/ATF4 activation and use the ISRIB to inhibit this pathway. However, it is unclear if the phenotype rescue is a result of reduced UPR response. Could the authors address if ISRIB treatment of hspa8 mutants reduce UPR and E

Reviewer 2

Advance summary and potential significance to field

The report by Wang and Zhang et al. investigates the function of the ubiquitous chaperone Hspa8 in the early development of zebrafish embryos. The function of this protein has been difficult to access before due to its essentiality in cell lines. This study reports the generation of mutant lines for hspa8 gene in zebrafish and provides mechanistic evidence supporting the function of the chaperone in the induction of the unfolded protein response. The mutant lines generated and the information provided will be useful to several areas of research including understanding Hspa8 protein function at the cellular level and during the development of pharyngeal arches as well as on the link between the unfolded protein response and early vertebrate morphogenesis.

Comments for the author

In my opinion there are some points in the results section and the discussion that need clarification to improve the comprehension of the manuscript.
1. Regarding the remaining wild type Hspa8 protein levels in the homozygous mutant embryos, the authors attribute this effect to the deposition of maternal mRNA/protein in mutant embryos (lines 147-149). Did these embryos come from mutant homozygous or heterozygous mothers? The results about remaining wild type protein should differ among these two settings. Is viability affected if embryos come from mutant homozygous mothers? Please, provide more information about this point in the text and discuss the results taking it into account.
2. The authors refer on several occasions to a “PA decrease” or “reduction of PA” when mentioning the PA morphological defects they observed (for example lines: 152, 154, 190). I recommend to include in every case the biological variable you are referring to (width, area, length, fluorescence intensity). A commentary is made in line 197 and 249 about “a reduction in area of the PA region” but is not accompanied by a quantification. Thus, I suggest to include a more detailed description and quantification of the morphological defects observed in PA development: is the length or width of the arches affected? would it be possible to have an improved image of the arches? In vivo confocal imaging, nuclei counterstaining or histological analysis of wt and mutant embryos may provide a better image and the possibility to make a better description/quantification of the defects mentioned by the authors. Could you provide some background on the expression of fli:GFP in the pharyngeal arches or a publication as reference? it is usually used to evidence blood vessels, does it label other structures within PAs? Please, also provide information on the fli:GFP line used and an associated publication.
3. If high levels of wild type maternal transcript is remaining in the embryos used for ISH of NCC markers, then the timing of developmental action of Hspa8 that is obtained from analysis of the mutant embryos would be mostly due to the early presence of the maternal protein and not a specific late action of Hspa8.
I suggest clarifying the origin of embryos (homozygous vs heterozygous mother) and/or discussing this point in the text, revising the conclusion mentioned in lines 187-188.
4. Evidence on UPR activation comes from a RNAseq experiment and was confirmed by qPCR analysis and western blot for some of the genes. However, the conclusion in line 302 suggests a link between UPR activation and proliferation. I could not find the results or previous published evidence supporting this affirmation.
Please, reformulate the sentence to avoid misunderstandings.
5. The activation of p53 by UPR is supported by the RNAseq results and also from transcript detection of p53 by ISH in UPR inhibition experiments (fig9E - though this last piece of evidence is quite subjective). However, p53 overexpression or enhanced signaling seems not to be involved in PA developmental defects since p53 inactivation does not rescue the morphological defect, as shown in the section between lines 242-263 (even though data on morphological defects in fli:GFP embryos or transcript levels of sox9a were not quantified, the results from the experiment with alcian blue staining are pretty clear). Taking into account these results, p53 upregulation and decrease in proliferation seems to be a separate pathway, induced by UPR that does not relate to morphological defects in PA development. Even though the authors clearly state that p53 overexpression is not involved in PA defects in the discussion, p53 is introduced in a linear working model in figure 9F. In my opinion, this point deserves a deeper discussion to avoid misunderstandings and the working model in figure 9F should be revised to incorporate a still unknown pathway triggered by Hspa8-UPR affecting PA development.
6. Minor points to review:
- Please reformat images and plots to incorporate colorblind-friendly combinations (for example: green and magenta)
- The sentence in line 238-239 is truncated.
- Please, review the redaction of the sentence in lines 257-259.
- In figure 8B, the color code of vertical lines and the reference indicating the corresponding biological processes do not match. Please, review it.

Reviewer 3

Advance summary and potential significance to field

This paper investigates the effect of loss of a heat shock protein on craniofacial development. It shows that in the mutants studied, p53 signaling is upregulated and this occurs through the Perk/Atf4 pathway. The fact that a protein with a 'housekeeping' function has such tissue and pathway specific roles is potentially interesting. However, I feel more needs to be determined about the exact nature of the mutants studied as this affects the interpretation of the phenotype and the claims in the paper.

Comments for the author

My main concern for this paper is the nature of the mutants, as alluded to above. The paper claims that these are null mutants. However, Western blots at 3 and 4 days post fertilisation (dpf) show substantial levels of protein. Quantification has not been done but 50% would seem a reasonable estimate. This would not be consistent with the mutants being null alleles.
The authors state "Hspa8 protein levels were severely reduced in hspa8 mutants both at 72 and 96 hpf (Fig. 1C). Therefore, we concluded that the alleles are null alleles. Furthermore, these results revealed that the maternal Hspa8 protein was abundantly deposited since the protein was evenly detected in mutant embryos at 96 hpf". This is a circular argument and begs the question: are these mutants null alleles? The genetic alterations (severe truncation of the coding sequence) would suggest they should be. However, the Western blot data is not obviously consistent with the genotype.
The explanation that this is due to maternal deposition of mRNA and/or protein would need quantitative evidence and precedence. The WISH's do show strong signal from fertilisation but the authors need to show that this can account for levels found in the mutants 4 days later. Perhaps, quantitative Western blots from the 1-cell stage onwards could provide this data. Morpholinos targetted to the start codon would inhibit translation of maternal mRNA in the mutants and this would contribute to understanding the relative contribution of the maternal mRNA to the mutant and the protein's role in development.
Related to this point, is there another hspa8 gene in zebrafish? Given the whole genome duplication event that zebrafish shares with many teleosts, that is a distinct possibility. If there is another hspa8-like gene, would their antibody also recognise it? Furthermore, could the antibody be binding the the N-terminal truncated protein product?
If the authors can address these matters, then I feel the manuscript would be worth reviewing again. There is a lot of data here. The WISH, wholemount immunofluorescence and embryo stainings have been performed very well and a mechanistic link between genotype and phenotype has been provided. However, the nature of the mutant needs determining, I feel. I hope the authors can provide that data so the paper can be reviewed again.

First revision

Author response to reviewers' comments

Point-to-point response:

We thank the reviewers for their comments and suggestions. In response to these comments and suggestions, the following changes have been made. Also, a formatted pdf of the response was attached as supplementary information.

Reviewer 1 Advance Summary and Potential Significance to Field:
This paper is well written and the data appear to be of good quality interpreted in a generally logical way. The study has the potential to reveal novel mechanisms of hspa8 in zebrafish development.

Reviewer 1 Comments for the Author:

However, there are some issues with the details of the methods used and the mutants generated that should be addressed. In addition, validation of the main conclusion would greatly improve the confidence of the major conclusion. With the current status I do not think that the title fully represents the finding of the study since it is unclear to which extent the mutant are a loss of function or not.

1) The heat shock proteins are a family of proteins that are highly conserved. Are the anti-sense probes used specific for hspa8 or could they detect other members as well, such as hspa8b?

We thank the reviewer for this suggestion. We agree that the heat shock proteins are a family of proteins that are highly conserved. To ensure the anti-sense probe we used was *hspa8* specific, we analyzed the aligned whole cDNA sequences of *hspa8* and *hspa8b* and found that the lowest conserved region between them is in the 3′ UTR (41% identity) (Fig. S1A). Therefore, we made a new plasmid DNA containing a partial ORF and 3′ UTR of zebrafish *hspa8* to generate *hspa8* specific sense and antisense riboprobes and performed whole-mount *in situ* hybridization experiments with the new *hspa8* riboprobes. The new data were included as Fig. S2D in the revised MS. It is in consistent with previous results. It was not surprise. As mentioned in below 5), the mRNA of *hspa8b* was not detectable throughout embryogenesis.

2) To which extent the hspa8 mutant zebrafish are null-mutants is not clear. According to Figure S2b, all mutations should generate protein truncations in the N-terminal domain. However, analysis of both mRNA and especially at protein level still show that protein is produced, although at lower levels. Since the antibody used bind to an epitope at amino acids 580-601 at the C-terminus of the human HSC 70 protein it is likely that the mutations generate hypomorph zebrafish or that express alternative splice variants with the antibody epitope remaining. To conclude that this is a null-mutation is therefore to over-interpret the data. Since this is central for all further conclusions, the authors should examine in detail how the mutations affect the hspa8 transcript.

Thanks for this comment. We agree with the concern raised by the reviewer. To examine the effects on *hspa8* transcripts in detail after knockout of *hspa8*, we performed a series of new experiments to address this question. We generated two constructs which contain the full ORF and 3′ UTR of *hspa8* and *hspa8b*, respectively. We then performed semi-quantitative RT-PCR to examine the transcripts of *hspa8* and *hspa8b* at different stages using these two constructs as positive controls. As shown in the revised Fig. S2A, the transcripts of *hspa8* were easily detected in the indicated developmental stages we examined. While transcripts of *hspa8b* were not detectable by semi-quantitative RT-PCR analysis. These results supported by the RNA-seq data in the above indicated stages (*hspa8*: 286 to 809 FPKM v.s. *hspa8b*: 0 to 2 FPKM) (https://www.ebi.ac.uk/gxa/experiments/E-ERAD-475). Therefore, transcripts of *hspa8* were abundant, while transcripts of *hspa8b* were extremely low throughout embryogenesis. In addition, we examined protein levels of *hspa8* at different stages with an antibody against aa 580-601 at the C-terminus of human HSPA8 protein. Since the amino acid identity of HSPA8, Hspa8, and Hspa8b is high (Fig. S1B), the GFP-tagged Hspa8 and Hspa8b were generated and transfected into HEK293T cells to validate this antibody. Indeed, GFP-tagged Hspa8 or Hspa8b was detected by this antibody, suggesting that this antibody recognizes endogenous Hspa8 and Hspa8b in zebrafish embryos (Fig. S2B). Then, we examined protein levels of Hspa8 at different stages with this antibody. Given that mRNAs of *hspa8b* were not detectable, we believe that the protein we detected was most likely Hspa8. As shown in Fig. S2C, Hspa8 protein was expressed abundantly during embryogenesis. Taken together, we concluded that Hspa8 was maternally deposited, while mRNA levels of *hsp8b* were extremely low. We subsequently examined mRNA levels of *hspa8* with the above validated *hspa8* specific primers and protein levels of Hspa8 in the sibling and mutant embryos at 72 hpf and 96 hpf. The new data have been included as Fig. 1B and 1C in the revised MS. The *hspa8* transcripts were dramatically decreased in *hspa8* mutants both at 72 and 96 hpf. Likewise, the Hspa8 protein levels were also severely reduced. It should be noted that only a single protein band was detected in *hspa8* mutants, suggesting that alternative splicing variants are unlikely produced after knockout of *hspa8*. Collectively, these results suggested that Hapa8 protein are maternally deposited as well as *hspa8* mutants are null-mutants.

3) It is not clear how many generations the CRISPR/Cas9 generated mutant lines have been outcrossed. It is common to outcross at least to F2 to eliminate off target effects. It would be good if the authors could clarify this.

Thanks for this comment. We regret that we did not write this information clearly to make readers easily to understand. The adult F0 fishes were outcrossed with WT fish to obtain the F1 generation, which was genotyped and confirmed by sequencing of targeting sites. To exclude off-target effects, the heterozygous F1 zebrafish were outcrossed with WT zebrafish for two generations. Then, the heterozygous adult fish were incrossed to obtain embryos for phenotypic analysis. This information has been added in the revised MS p 12, line 494-496, 501-503.

4) The authors speculate that the remaining mRNA or protein in mutant embryos may be maternally derived. However, incrossing mutant zebrafish would be an easy way to elucidate the contribution of maternally derived mRNA.

Thanks for your helpful suggestion. We regret that we did not present the information of mutant fish clearly. Indeed, *hspa8* homozygous embryos died around 10-12 dpf. We could not obtain adult homozygous fish to generate maternal-zygotic mutant embryos to eliminate maternally derived mRNA or protein for phenotypic analysis. We had to analyze the phenotype in zygotic mutant embryos. We have added this information in the result of revised MS p 4, line 155-158.

5) The presence of the paralogue hsap8b is not addressed. How similar are these proteins and could the gRNA and antibodies target this gene and protein as well?

Thanks for your comments and suggestion. At the beginning of this project, we have already noticed that the zebrafish genome contains two *hspa8* genes, *hspa8* and *hspa8b*. Although amino acid sequence identity of the two proteins is high (94.2%), the RNA-seq data of zebrafish embryos at different stages suggested that the mRNA levels of *hspa8b* were very low during embryogenesis (0 to 2 FPKM) (https://www.ebi.ac.uk/gxa/experiments/E-ERAD-475). Hence, we did not investigate the contribution of Hspa8b throughout embryogenesis. Following this comment, we performed semi-quantitative RT-PCR analysis with *hspa8b* specific primers to examine mRNA levels of *hspa8b* during embryogenesis. The mRNA of *hspa8b* was not detectable (Fig. S2A). Additionally, we tested whether the gRNAs for *hspa8* we used could target *hspa8b*. To this purpose, we performed sequencing analysis of the most probable targeting site in *hspa8b* within *hspa8* mutant embryos. Within the sequenced region, we did not observe that *hspa8b* had been targeted by the gRNAs for *hspa8*. Therefore, the gRNAs we used were *hspa8* specific.
For the antibody we used, the GFP-tagged Hspa8 and Hspa8b were generated and transfected into HEK293T cells to validate this antibody since the amino acid sequence identity of HSPA8, Hspa8, and Hspa8b is high (Fig. S1B). Both GFP-tagged Hspa8 and Hspa8b were detected by this antibody, suggesting that this antibody recognizes endogenous Hspa8 and Hspa8b in zebrafish embryos (Fig. S2B). Then, we examined protein levels of Hspa8 in zebrafish embryos at different stages with this antibody. Given that *hspa8b* mRNA was not detectable, we speculated that the protein we detected was most likely Hspa8.

6) During the last years, growing evidence point towards the activation of compensatory mechanisms induced by premature termination codons (PTC). Since the hspa8 gene has several paralogues, it is therefore possible that the mutations introduced upregulate the expression of other family members that mask the true function of hspa8? Can the authors address question to clarify this issue?

We thank this reviewer for this suggestion. To address this issue, we analyzed the RNA-seq data to test whether the expression of other Hsp70 family members were upregulated in PAs of *hspa8* mutants at 48 hpf. The mRNA levels of some Hsp70 family members were upregulated (Table S1). Among them, we noticed that *hspa8b* was listed as having significantly higher mRNA levels than the other members. In addition, we performed qRT-PCR to verify whether mRNA levels of *hspa8b* in PAs were upregulated in *hspa8* mutants at 48 hpf. Likewise, conventional RT-PCR was performed to measure the mRNA levels of *hspa8b* in the whole *hspa8* mutant embryos at 72 and 96 hpf. The new data were shown as Fig. S4A-C in the revised MS. The mRNA levels of *hspa8b* in PAs were markedly induced in *hspa8* mutants at 48 hpf (Fig. S4B, C). Likewise, the mRNA levels of

hspa8b were also markedly increased in whole *hspa8* mutant embryos at 72 and 96 hpf (Fig. S4A). Since the antibody we used recognized

expressed GFP-tagged Hspa8 and Hspa8b, the endogenous protein we detected in *hspa8* mutant embryos at 72 and 96 hpf in Fig. 1C may contain Hspa8b. These findings indicated that upregulated *hspa8b* might compensate loss of Hspa8. Thus, genetic loss of Hspa8 likely induced the activation of a compensatory network, which might compromise the phenotype after *hspa8* was deleted. This issue was discussed in the revised MS p 10, line 399-401.

7) According to the title, the authors draw the conclusion that hspa8 induce PA malformation due to an activated UPR response. The authors hypothesize that UPR activation is induced by PERK/p-eIF2a/ATF4 activation and use the ISRIB to inhibit this pathway. However, it is unclear if the phenotype rescue is a result of reduced UPR response. Could the authors address if ISRIB treatment of hspa8 mutants reduce UPR and ER Stress.

We thank this reviewer for this helpful suggestion. Following this suggestion, we performed western blot analysis with the manually dissected PAs to examine the alteration of Atf4 protein levels after mutant embryos were treated by DMSO or ISRIB from 24 to 96 hpf. The new data were shown as Fig. 8H in the revised MS. Treatment with ISRIB blunted Hspa8 depletion- induced Atf4 protein levels in PAs. These results suggested that ISRIB treatment reduced the activated UPR response after Hspa8 was depleted.

Reviewer 2 Advance Summary and Potential Significance to Field:

The report by Wang and Zhang et al. investigates the function of the ubiquitous chaperone Hspa8 in the early development of zebrafish embryos. The function of this protein has been difficult to access before due to its essentiality in cell lines. This study reports the generation of mutant lines for hspa8 gene in zebrafish and provides mechanistic evidence supporting the function of the chaperone in the induction of the unfolded protein response. The mutant lines generated and the information provided will be useful to several areas of research including understanding Hspa8 protein function at the cellular level and during the development of pharyngeal arches as well as on the link between the unfolded protein response and early vertebrate morphogenesis.

Reviewer 2 Comments for the Author:

In my opinion there are some points in the results section and the discussion that need clarification to improve the comprehension of the manuscript.

1. Regarding the remaining wild type Hspa8 protein levels in the homozygous mutant embryos, the authors attribute this effect to the deposition of maternal mRNA/protein in mutant embryos (lines 147-149). Did these embryos come from mutant homozygous or heterozygous mothers? The results about remaining wild type protein should differ among these two settings. Is viability affected if embryos come from mutant homozygous mothers? Please, provide more information about this point in the text and discuss the results taking it into account.

We thank this reviewer for this comment. We regret that we did not present the information of the mutant embryos clearly. All mutant embryos we analyzed are from incrossing of heterozygous adult fish. Homozygous embryos died from 10 to 12 dpf. Therefore, we could not obtain homozygous adult fish to generate maternal-zygotic mutant embryos to eliminate maternal mRNA/protein for phenotypic analysis. The mutants we obtained are indeed zygotic mutant embryos. We have added this information and discussed this issue in revised MS p 4, line 155-158 and p 9-10, line 393-399.

2. The authors refer on several occasions to a "PA decrease" or "reduction of PA" when mentioning the PA morphological defects they observed (for example lines: 152, 154, 190). I recommend to include in every case the biological variable you are referring to (width, area, length, fluorescence intensity). A commentary is made in line 197 and 249 about "a reduction in area of the PA region" but is not accompanied by a quantification. Thus, I suggest to include a more detailed description and quantification of the morphological defects observed in PA development: is the length or width of the arches affected? Would it be possible to have an

improved image of the arches? In vivo confocal imaging, nuclei counterstaining or histological analysis of wt and mutant embryos may provide a better image and the possibility to make a better description/quantification of the defects mentioned by the authors. Could you provide some background on the expression of fli:GFP in the pharyngeal arches or a publication as reference? it is usually used to evidence blood vessels, does it label other structures within PAs? Please, also provide information on the fli:GFP line used and an associated publication.

We thank this reviewer for this helpful suggestion. To make the description of PA alteration more detailed, we quantified the morphological traits observed in PAs from multiple views. For the morphological images of PAs in embryos at 96 hpf with each genetic background, we quantified the area of PAs (Fig. 1E, 5C and 8B). For the Alcian blue-stained PAs with each genetic background, we quantified the number of PAs (Fig. 1I, 5G and 8E). Following suggestions by this reviewer, we performed additional experiments and obtained better confocal images. The PAs were immunostained with an anti-Col2 antibody to indicate the number of pharyngeal arches and nuclei were counterstained with DAPI. The number of PAs was quantified again (Fig. 1G). The quantified results were included as new Figures in the revised MS to support our view.
The *Tg* (*fli1:EGFP*)^{y1} transgenic line was originally generated to visualize endothelial cells (Lawson and Weinstein, 2002; Dev Biol 248, 307-318). Additionally, this line expresses GFP in post-migratory cranial neural crest cells in the pharyngeal arches. Thus, it has been widely used to image cranial neural crest cells within the pharyngeal arches. This information and associated publications have been added in the result of revised MS p 4, line 173-176.

3. If high levels of wild type maternal transcript is remaining in the embryos used for ISH of NCC markers, then the timing of developmental action of Hspa8 that is obtained from analysis of the mutant embryos would be mostly due to the early presence of the maternal protein and not a specific late action of Hspa8. I suggest clarifying the origin of embryos (homozygous vs heterozygous mother) and/or discussing this point in the text, revising the conclusion mentioned in lines 187-188.

Thank you for this suggestion. The mutant embryos are from incrossing of heterozygous adult fish. This information has been added in the result in the revised MS. We agree that the phenotype in mutants was mostly due to early presence of the maternal protein. The conclusion mentioned in lines 187-188 was changed into "Hspa8 is critical for PA maturation during embryonic development". In addition, this issue was also discussed in the revised MS p 9-10, line 393-399.

4. Evidence on UPR activation comes from a RNAseq experiment and was confirmed by qPCR analysis and western blot for some of the genes. However, the conclusion in line 302 suggests a link between UPR activation and proliferation. I could not find the results or previous published evidence supporting this affirmation. Please, reformulate the sentence to avoid misunderstandings.

Thank you for this suggestion. This sentence has been changed into "these results suggested that loss of Hspa8 in PA region likely triggers ER stress and the UPR" in the revised MS p 8, line 327-328, to avoid misunderstandings.

5. The activation of p53 by UPR is supported by the RNAseq results and also from transcript detection of p53 by ISH in UPR inhibition experiments (fig9E - though this last piece of evidence is quite subjective). However, p53 overexpression or enhanced signaling seems not to be involved in PA developmental defects since p53 inactivation does not rescue the morphological defect, as shown in the section between lines 242-263 (even though data on morphological defects in fli:GFP embryos or transcript levels of sox9a were not quantified, the results from the experiment with alcian blue staining are pretty clear). Taking into account these results, p53 upregulation and decrease in proliferation seems to be a separate pathway, induced by UPR that does not relate to morphological defects in PA development. Even though the authors clearly state that p53 overexpression is not involved in PA defects in the discussion, p53 is introduced in a linear working model in figure 9F. In my opinion, this point deserves a deeper discussion to avoid misunderstandings and the working model in figure 9F should be revised to incorporate a still unknown pathway triggered by Hspa8-UPR affecting PA development.

Thanks for your comments and suggestion. We agree with the reviewer that p53 induction is not involved in PA defects and p53 is introduced in a linear working model in Fig. 9F is not suitable. We have removed the p53 from the working model to avoid misunderstandings. Instead, an unknown pathway was incorporated in the working model to make the description more clearly. The revised working model was shown as Fig. 8I in the revised MS.

6. Minor points to review:
- Please reformat images and plots to incorporate colorblind-friendly combinations (for example: green and magenta)

Thank you for this suggestion. We regret that we did not consider images and plots colorblind-friendly. The images and plots are now reformatted in the revised figures.

- The sentence in line 238-239 is truncated.

Changed. Thank you.

- Please, review the redaction of the sentence in lines 257-259.

Changed. Thank you.

- In figure 8B, the color code of vertical lines and the reference indicating the corresponding biological processes do not match. Please, review it.

Changed. Thank you.

Reviewer 3 Advance Summary and Potential Significance to Field:
This paper investigates the effect of loss of a heat shock protein on craniofacial development. It shows that in the mutants studied, p53 signaling is upregulated and this occurs through the Perk/Atf4 pathway. The fact that a protein with a 'housekeeping' function has such tissue and pathway specific roles is potentially interesting. However, I feel more needs to be determined about the exact nature of the mutants studied as this affects the interpretation of the phenotype and the claims in the paper.

Reviewer 3 Comments for the Author:
My main concern for this paper is the nature of the mutants, as alluded to above. The paper claims that these are null mutants. However, Western blots at 3 and 4 days post fertilisation (dpf) show substantial levels of protein. Quantification has not been done but 50% would seem a reasonable estimate. This would not be consistent with the mutants being null alleles. The authors state "Hspa8 protein levels were severely reduced in hspa8 mutants both at 72 and 96 hpf (Fig. 1C). Therefore, we concluded that the alleles are null alleles. Furthermore, these results revealed that the maternal Hspa8 protein was abundantly deposited since the protein was evenly detected in mutant embryos at 96 hpf". This is a circular argument and begs the question: are these mutants null alleles? The genetic alterations (severe truncation of the coding sequence) would suggest they should be. However, the Western blot data is not obviously consistent with the genotype.
The explanation that this is due to maternal deposition of mRNA and/or protein would need quantitative evidence and precedence. The WISH's do show strong signal from fertilisation but the authors need to show that this can account for levels found in the mutants 4 days later. Perhaps, quantitative Western blots from the 1-cell stage onwards could provide this data. Morpholinos targetted to the start codon would inhibit translation of maternal mRNA in the mutants and this would contribute to understanding the relative contribution of the maternal mRNA to the mutant and the protein's role in development.
Related to this point, is there another hspa8 gene in zebrafish? Given the whole genome duplication event that zebrafish shares with many teleosts, that is a distinct possibility. If there is another hspa8-like gene, would their antibody also recognise it? Furthermore, could the antibody be binding the the N-terminal truncated protein product?

Thank you for your comments and helpful suggestion. We realized that the concern raised by the reviewer was caused mostly by “western blot data is not obviously consistent with the genotype”. To address the concern raised by this reviewer, we performed additional experiments to examine the mutants. Because the band of Hspa8 protein we detected in *hspa8* mutant embryos was likely from two distinct contribution, we answered these questions together to make the explanation more clearly.

The zebrafish genome contains two *hspa8* genes, *hspa8* and *hspa8b*. Transcriptomic analysis of zebrafish embryos at different stages showed that the transcriptional levels of *hspa8b* were extremely low during embryogenesis (0 to 2 FPKM) (https://www.ebi.ac.uk/gxa/experiments/E-ERAD-475). Hence, we did not consider the contribution of Hspa8b during embryogenesis when we started this project, despite amino acid sequence identity of Hspa8 and Hspa8b is high (94.2%) (Fig. S1B). To address the concern raised by this reviewer, we examined the expression of Hspa8 and Hspa8b. We first generated two plasmids which contain the full ORF and 3′ UTR of *hspa8* and *hspa8b*, respectively. We next examined the transcriptional levels of *hspa8* and *hspa8b* at different stages by semi- quantitative RT-PCR using the above constructs as positive controls. The transcripts of *hspa8* were abundant, while the transcripts of *hspa8b* were not detectable (Fig. S2A). These results are in consistent with the RNA-seq data in the above indicated stages (*hspa8*: 286 to 809 FPKM v.s. *hspa8b*: 0 to 2 FPKM). Collectively, transcriptional levels of *hspa8b* were extremely low during embryogenesis. We subsequently generated a new *hspa8* specific anti-sense riboprobe which contains partial ORF and the whole 3′ UTR and then performed whole-amount *in situ* hybridization again. The spatiotemporal distribution of *hspa8* transcripts is in consistent with the previous result. This is not surprising because the mRNA levels of *hspa8* are extremely low. To better understand the maternal contribution of Hspa8 protein, we also examined protein levels of Hspa8 at different stages with an antibody against aa 580-601 at the C-terminus of human HSPA8. Since the amino acid sequence identity of HSPA8, Hspa8, and Hspa8b is high (Fig. S1B), we first validate this antibody with cell lysates from GFP-tagged Hspa8- or Hspa8b- overexpressing HEK293T cells. Indeed, GFP-tagged Hspa8 or Hspa8b was detected by this antibody, suggesting that this antibody recognizes endogenous Hspa8 and Hspa8b in zebrafish embryos (Fig. S2B). Following your suggestion, we examined the protein levels of Hspa8 from 1-cell stage to 96 hpf with this antibody. The new data were included as Fig. S2C in the revised MS. Hspa8 protein was abundantly expressed even at 1-cell stage. Given that mRNA of *hspa8b* was not detectable, we believe that the protein we detected was most likely Hspa8. Taken together, we concluded that Hspa8 protein was maternally deposited, while *hsp8b* transcripts were extremely low.

After we obtained the above basic transcriptional and translational information of *hspa8* and *hspa8b* during embryogenesis, we subsequently examined mRNA levels of *hspa8* with validated *hspa8* specific primers as well as protein levels of Hspa8 in the sibling and mutant embryos at 72 hpf and 96 hpf. These new data were included as Fig. 1B, C in the revised MS. The *hspa8* transcripts were dramatically decreased in *hspa8* mutants both at 72 and 96 hpf. Likewise, the protein levels were also severely reduced. Following your suggestion, we particularly noticed whether the antibody could bind to the N-terminal truncated Hspa8 protein products. Indeed, only a single 75 kDa band was detected in *hspa8* mutants, suggesting that N- terminal truncated Hspa8 protein products are unlikely recognized. Collectively, these results suggested that Hapa8 protein is maternally deposited as well as *hspa8* mutants are null-mutants. We also agree with this reviewer that “translation-blocking Morpholinos would inhibit translation of maternal mRNA in the mutants and this would contribute to understanding the relative contribution of the maternal mRNA to the mutant and the protein's role in development”. Because abundant Hspa8 protein was detected at 1-cell stage and Morpholinos might act later which could not alter the already deposited protein, we did not perform knockdown analysis in
the zygotic mutant embryos.

Genetic deletion of *hspa8* may lead to activation of compensatory mechanisms by upregulating *hspa8b* expression. To test this possibility, we performed RT-PCR analysis to examine the mRNA levels of *hspa8b* in *hspa8* mutant embryos at 72 and 96 hpf. These new data were included as Fig. S4A in the revised MS. The transcriptional levels of *hspa8b* were indeed markedly increased in *hspa8* mutant embryos at 72 and 96 hpf. Since the antibody we used recognized GFP-tagged Hspa8 and Hspa8b, the endogenous protein we detected in *hspa8* mutant embryos at 72 and 96 hpf might contain Hspa8b (Fig. 1C). Upregulated *hspa8b* may partially compensate loss of Hspa8. Therefore, the protein we detected in *hspa8* mutant embryos likely contains Hspa8b besides the possibly remaining Hspa8.

From the above results, we concluded that *hspa8* mutants are null-mutants and genetic deletion of *hspa8* leads to activation of compensatory mechanisms by upregulating *hspa8b* expression which may compromise Hspa8-deleted phenotype. The discussion on this issue has been included in the revised MS p 9-10, line 393-401.

If the authors can address these matters, then I feel the manuscript would be worth reviewing again. There is a lot of data here. The WISH, wholemount immunofluorescence and embryo stainings have been performed very well and a mechanistic link between genotype and phenotype has been provided. However, the nature of the mutant needs determining, I feel. I hope the authors can provide that data so the paper can be reviewed again.

We highly appreciate the positive feedback from this referee.

Second decision letter

MS ID#: JOCES/2021/259734

MS TITLE: Genetic deletion of hspa8 leads to selective tissue malformations in zebrafish embryonic development

AUTHORS: Caixia Wang, Xin Zhang, Xinyu Wang, Yanpeng Zhai, Mengjiao Li, Jun Pan, Yan Bai, Xiaozhi Rong, and Jianfeng Zhou
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

The revised version of the manuscript is very well performed and address the questions raised by the reviewers. The additional work has strengthened the nature of the hspa8 mutants and also increased the transparency of the methods used. I think that the findings made by Wang;C et al. will contribute to a better understanding of hspa8 in zebrafish.

Comments for the author

The lack of specific antibodies towards Hspa8 make interpretations regarding the mutations effect on Hsap8 protein level hard. The authors claim that the mutations created are deletions of Hspa8 and that the remaining protein detected most likely is produced by Hspa8b. However, since the authors cannot rule out that the Hspa8 protein detected is produced from the hspa8 gene, I would suggest to modify statements of deletion to something that will be correct even if the mutants would be hypomorphs. An example would be to change the title from “Genetic deletion of hsap8 leads to…” to “Mutations in hsap8 leads to…..”.
The manuscript contains several other sentences claiming that a genetic deletion has been created (see below) . These would also preferable be changed to better mirror the findings made. Please look through and modify accordingly.
Line 53 Line 123 Line 132 Line 184-185 Line 192 Line 267 Line 274 Line 381 Line395 Line 407-408 Line 812 Line 837 Line 875

Reviewer 2

Advance summary and potential significance to field

This study reports the generation of mutant lines for hspa8 gene in zebrafish and provides mechanistic evidence supporting the function of the chaperone Hspa8 in the induction of the unfolded protein response. After revision the authors incorporated a detailed analysis of hspa8 and hspa8b expression through early development and uncovered a compensatory effect in the mutant line. The results presented in this work are highly relevant for the field of chaperone, UPR pathway and PA development.

Comments for the author

The authors addressed all my comments carefully and I have no further concerns.
The manuscript has greatly improved after revision so I strongly recommend its publication.

Reviewer 3

Advance summary and potential significance to field

I reviewed this paper previously. The authors have responded to my comments and very similar comments from two other reviewers. My main concerns, shared by the other two reviewers, were: that the mutants might not be null mutants; and that the proposed maternal contribution of protein and mRNA had not been demonstrated; that the presence of a related Hsp8 proteins might also explain the observations of persistent Hsp8 epitopes detected in the Western blots of the mutants. I feel these concerns have been addressed by the additional experiments performed here. The new data show a strong maternal contribution of protein and mRNA. In addition, there is another related protein, Hsp8b which is switched on in the mutant embryos and is recognised by the antibody used to detect Hsp8. There is therefore a reasonable explanation for the mutant lines giving bands on the Western blot where anti-Hsp8(a) antibody is used as probe. The authors discuss these issues succinctly in the text.

I am satisfied that my concerns have been addressed fully. I commend the authors for the experimental work peformed.

I copy here my previous comments:

This paper investigates the effect of loss of a heat shock protein on craniofacial development. It shows that in the mutants studied, p53 signaling is upregulated and this occurs through the Perk/Atf4 pathway. The fact that a protein with a 'housekeeping' function has such tissue and pathway specific roles is interesting.

Comments for the author

No minor changes.