# FMRP protects the lung from xenobiotic stress by facilitating the integrated stress response

Deblina Sain Basu, Rital Bhavsar, Imtiyaz Gulami, Saraswati Chavda, Sai Manoz Lingamallu, Ravi Muddashetty, Chandrakanth Veeranna, Sumantra Chattarji, Rajesh Thimmulappa, Aditi Bhattacharya and Arjun Guha
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**Editor**: Kathleen Green

**Review timeline**

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**Original submission**

First decision letter

MS ID#: JOCES/2021/258652

MS TITLE: The Fragile X Mental Retardation Protein protects the lung from xenobiotic stress by facilitating the Integrated Stress Response

AUTHORS: Deblina Sain Basu, Rital Bhavsar, Imtiyaz Gulami, Saimanoz Lingamallu, Ravi Muddashetty, Chandrakanth Veeranna, Sumantra Chattarji, Rajesh Thimmulappa, Aditi Bhattacharya, and Arjun Guha
ARTICLE TYPE: Research Article

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.)

We were finally able to obtain a second review for the paper, and as you will see the referee felt the paper would make an important contribution to JCS. This reviewer did note the concern that the human cell line used does not have some features of human lung tissue in vivo. I would also note that the use of a single line also limits the ability to know how generalizable the results are. While I am not familiar with availability of other lines, demonstrating the general applicability in another line would be important to ensure the rigor of the study. I would also refer you to the other review and concerns regarding stress granules, which we discussed previously.

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.

*We are aware that you may be experiencing disruption to the normal running of your lab that makes 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 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*

The authors report a novel finding that links expression of FMRP to stress tolerance and cellular damage in both in vivo and in vitro models of environmentally-induced lung injury. The finding that loss of FMRP may somehow sensitize the airway epithelium to environmentally-induced damage is interesting. I also appreciate the development and validation of the C22 cell line as a model of Nap-induced lung injury. The data showing increased cell damage and oxidative stress, as well as activation of ATF4 translation, are compelling. However I have significant concerns about the claims that these findings are at all related to the formation of stress granules. The data showing what are argued by the authors to be SGs are not at all convincing, and are not properly controlled for artifacts. Therefore I cannot recommend publication of the manuscript in its current form.

*Comments for the author*

Major Criticisms
First and foremost, there is no positive control for SG formation. The positive control is 250 - 500uM arsenite for 1 hour, and it should be included every SG experiment.
Also critically, there is no prior evidence of any kind that Nap triggers the formation of bonafide SGs. Therefore, it will fall upon these authors to do the full panel of assessments for SG formation before they can claim that the bodies they are pointing to in Figures such as 3A and 5C are SGs. Most important among these is pre-treating cells with cycloheximide, which blocks the formation of SGs because it inhibits polysome disassembly. This control proves that the bodies are translation-dependent. Also, SGs should have small but not large ribosomal subunit proteins, should contain polyA+ mRNA, and should have initiation factors such as eIF3B and eIF4G. Without these controls and with no prior published evidence, the authors cannot say these puncta are SGs.
I find the staining of TIA-1 and G3BP1 to also be problematic. G3BP1 expression is diffuse and easily detectable throughout the cytoplasm in the absence of stress, and condenses into SGs upon stress. The fact that G3BP1 staining is invisible except in the presence of Nap in WT cells (Fig S4Eiii) is not at all what I would expect. Likewise TIA-1 is predominantly nuclear at steady state in most cell lines with some weak cytoplasmic signal, which is not what I see if Figure 3A.
Finally, in all of the SG figures, there is a dashed white line, though I could not in any of the legends find what that line indicates. Is that meant to mark the boundary between the nucleus and the cytoplasm? If yes (and I assume so based on the size bars) this is even more problematic for the identity of these bodies, because relative to the size of the nucleus these bodies are too small to be SGs – they look more the size of P-bodies. SGs would be clearly visible when viewing the entire cell (e.g. as presented in Figure 3F). It is not necessary to zoom to the level below the size of the nucleus to see them. SGs that are considered small are about 200nm across, which is larger than the bodies indicated in these images. Large SGs (cells usually end up with a mix of large and small over the course of stress) can be 1um across. The size of these puncta are not consistent with SGs. The dependence of their process on the PKR pathway cannot be assessed without also assessing the other eIF2a kinases. Often, a stress will cause phosphorylation of multiple ISR kinases, however

only one of those kinases is essential for the activation of downstream processes including the phosphorylation of eIF2a and translational arrest. The authors' finding that P-PKR is normal in the Fmr1 KD condition but P-eIF2a is affected is intriguing but incomplete without analysis of the other kinases. The authors should use their siRNAs to deplete each kinase and determine the dependence of their effects on P-eIF2a in response to Nap or PQ.
Minor points many of the labels inside the fluorescence microscopy panels are very small and difficult to read; these will be impossible to read once they are further shrunk down for publication. Please edit the labels to make them more legible.
the phrases "more widely" and "more broadly" are used throughout the manuscript to describe expression of FMRP. The context in which they are used is highly ambiguous, and the authors should rephrase to eliminate these clauses, or explain more explicitly where precisely they are claiming FMRP is expressed in the relevant context.
FMRP is described in the introduction as being expressed in humans and mammals – I believe FMR1 is conserved at least down to insects as Drosophila is a model for FXS.
The authors should be aware of the updated standards of this and many journals as well as funding agencies that when samples sizes are small (e.g. n=3), individual data points should be overlaid upon bar graphs. The authors will save themselves time by making this adjustment prior to their resubmission.

Reviewer 2

*Advance summary and potential significance to field*

The study by Basu et al. reports findings from a study examining Fragile X Mental Retardation Protein (FMRP) in stress responses in lung tissue using a Naphthalene (Nap) injury model. They look at the role of FMRP using both in vivo and cell culture in response to Nap-induced lesions. They find that FMRP deficiency impaired cellular activation of the Integrated Stress Response (ISR) Pathway, induced the expression of ATF4, and increased expression of cell stress and cell death markers. Using knockdown approaches, they replicate the FMRP KO phenotype with ATF4 deficiency. The work here adds to the information about the role of the evolutionarily conserved ISR pathway used by organisms to respond to xenobiotic stress, or in the case of this study, harmful inhaled agents. The study extends our knowledge of FMRP function beyond its role in classically studied domains (i.e., neurobiological function and cognition). The experiments are well-designed with appropriate controls. The data presented convincing, and data presented in supplemental make for a compelling case of a real immunoblotting story. This is important because many of the antibodies used in this study have been used to quantify off-target signals to make conclusions (i.e., nonspecific ATF4 antibody used, Costa-Mattioli 2007). This reviewer is satisfied that the data are accurate. The study makes a clear case for FMRP in ATF4 expression and ISR pathway activation in response to lung toxicants. The only mild reservation is that the models used have modestly limited relevance to humans (NAP uses CYP2F2, not found in humans, and the human cell line used does not have some features of human lung tissue in vivo). It is a critical study extending our knowledge of the cellular functions of FMRP beyond modalities where it is predominantly studied. It is a significant contribution and will be of broad interest to the readership of the Journal of Cell Science.

*Comments for the author*

Major
The use of a lung injury model that does not recapitulate toxicity in humans (Nap model) reduces the impact of the study. This is mitigated to a great degree by including human lung cell line experiments, but it is a human cell line lacking important human lung tissue features (i.e., CCs). This limitation should be mentioned in the discussion.

Minor
Abstract Lines 9-10 "…cells fail to actuate the Integrated Stress 9 Response Pathway (ISR) and…should be cells fail to actuate the Integrated Stress 9 Response (ISR) Pathway and….
Fmr1 KO should be Fmr1 KO throughout the text; in many cases, genes are not italicized.
How was CYP2F2 staining specificity confirmed? The CYP is highly related and generally shows a lot of cross-reactivities with other CYPs.

Figure2 The figures should include a key to the identity of the different colored bar groups. The information is in the figure legends, but it makes it easier to interpret the Figure if contained within it.
Are there any experiments in the design of Figure 2 that examine the effects of FMRP overexpression? Such data would go a long way to establish FMRP's role in lung stress responses.

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**First revision**

Author response to reviewers' comments

We would like to thank the reviewers for engaging with our manuscript and for their insightful comments. We apologize for the delay in the resubmission. This was largely due to COVID-related restrictions at our end. Please find below our detailed responses to the reviewers. We have tried our best, given limited time and resources, to address all of the concerns that have been raised.

We look forward to a positive reply.

Yours truly,

Arjun Guha

Editor's comment:

"Dear Dr. Guha,
We have now reached a decision on the above manuscript.

We were finally able to obtain a second review for the paper, and as you will see the referee felt the paper would make an important contribution to JCS. This reviewer did note the concern that the human cell line used does not have some features of human lung tissue in vivo. I would also note that the use of a single line also limits the ability to know how generalizable the results are. While I am not familiar with availability of other lines, demonstrating the general applicability in another line would be important to ensure the rigor of the study. I would also refer you to the other review and concerns regarding stress granules, which we discussed previously."

Before moving on to the point-by-point response to the comments, we would like to provide an overview of the changes that we have made. This revision contains three significant changes. **First**, we have validated the use of the murine bronchial epithelial C22 cell line for the analysis of Naphthalene (Nap) injury *ex vivo*. In the earlier version of this manuscript we reported that the cytochrome Cyp2f2, the cytochrome that confers sensitivity to Nap, is expressed at modest levels in the C22. We now show that the siRNA-based knockdown of Cyp2f2 in C22 abolishes Nap sensitivity in this line, just as the deletion of Cyp2f2 in mice does *in vivo*. **Second**, we have generalized the findings in the human airway cell line BEAS-2B to another epithelial cell line derived from the human lung. We show that A549 cells, which are alveolar in origin, express FMRP and are dependent on FMRP for the activation of the Integrated Stress Response Pathway in response to 9,10-Phenanthrenequinone exposure (Supplementary Figure S5). **Third**, we have established that the markers that we have used to stress-induced condensates can detect canonical stress granules and that canonical stress granules are not generated in FMRP-deficient cells. These findings have been presented in the response to the reviewers but have been excluded from the manuscript itself due to the lack of space. We have replaced the term "stress granule" with "stress-induced granule" to avoid any confusion.

First Reviewer's comment:

"Reviewer 1 Advance Summary and Potential Significance to Field:

The authors report a novel finding that links expression of FMRP to stress tolerance and cellular damage in both in vivo and in vitro models of environmentally-induced lung injury. The finding that loss of FMRP may somehow sensitize the airway epithelium to environmentally-induced damage is interesting. I also appreciate the development and validation of the C22 cell line as a model of Nap-induced lung injury. The data showing increased cell damage and oxidative stress, as well as activation of ATF4 translation, are compelling. However I have significant concerns about the claims that these findings are at all related to the formation of stress granules. The data showing what are argued by the authors to be SGs are not at all convincing, and are not properly controlled for artifacts. Therefore I cannot recommend publication of the manuscript in its current form.

We thank the reviewer for appreciating the novelty of this work.

Reviewer 1 Comments for the Author:

Major Criticisms
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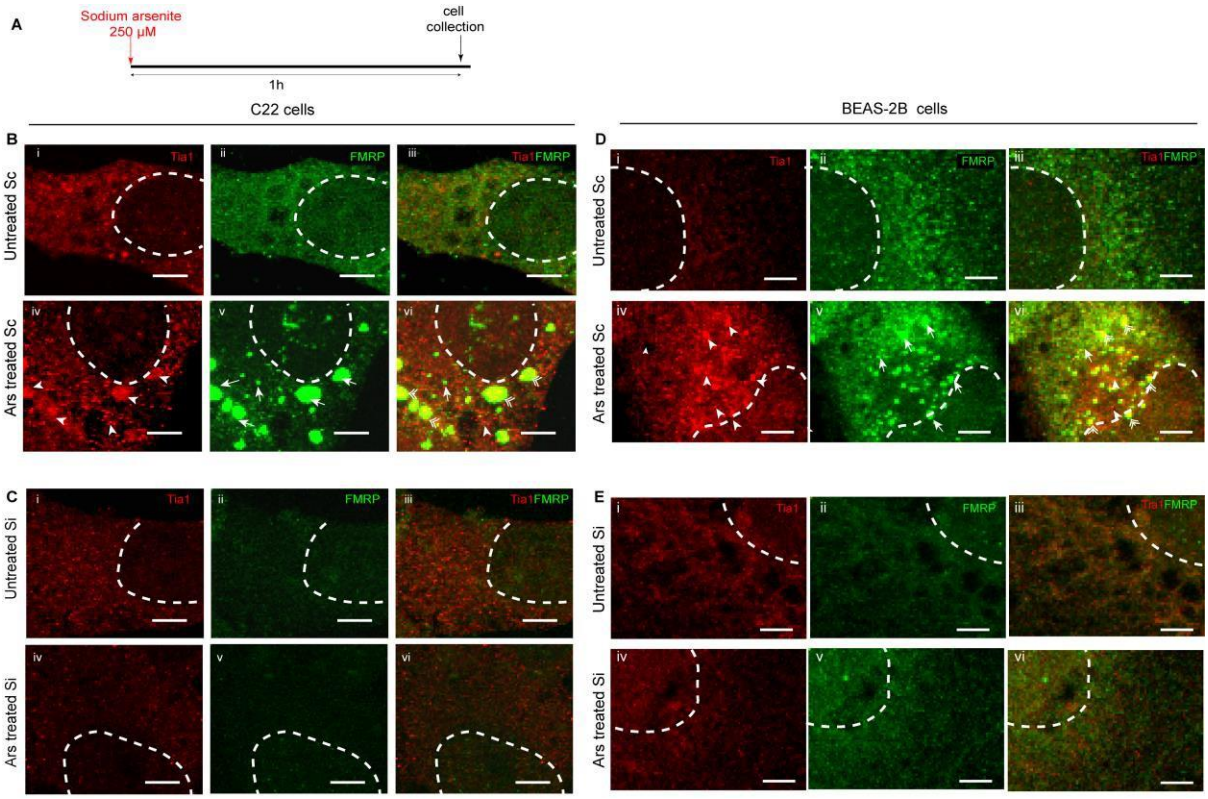
I find the staining of TIA-1 and G3BP1 to also be problematic. G3BP1 expression is diffuse and easily detectable throughout the cytoplasm in the absence of stress, and condenses into SGs upon stress. The fact that G3BP1 staining is invisible except in the presence of Nap in WT cells (Fig S4Eiii) is not at all what I would expect. Likewise TIA-1 is predominantly nuclear at steady state in most cell lines with some weak cytoplasmic signal, which is not what I see if Figure 3A. Finally, in all of the SG figures, there is a dashed white line, though I could not in any of the legends find what that line indicates. Is that meant to mark the boundary between the nucleus and the cytoplasm? If yes (and I assume so based on the size bars) this is even more problematic for the identity of these bodies, because relative to the size of the nucleus these bodies are too small to be SGs – they look more the size of P-bodies. SGs would be clearly visible when viewing the entire cell (e.g. as presented in Figure 3F). It is not necessary to zoom to the level below the size of the nucleus to see them. SGs that are considered small are about 200nm across, which is larger than the bodies indicated in these images. Large SGs (cells usually end up with a mix of large and small over the course of stress) can be 1um across. The size of these puncta are not consistent with SGs.

We thank the reviewer for alerting us to the distinction between Stress Granules and other stress-induced condensates, a highly heterogeneous population of "granules" that are induced in response to stress.

We have examined the distributions of Tia-1 and FMRP in response to arsenite treatment (Guarino, A. M., Di Mauro, G., Ruggiero, G., Geyer, N., Delicato, A., Foulkes, N. S., Calabrò, V. (2019). YB-1 recruitment to stress granules in zebrafish cells reveals a differential adaptive response to stress. *Sci. Rep.* 9(1). 1-14.) in control and FMRP-deficient C22 and BEAS-2B cells (see Figure below). These stainings clearly show the following. **First**, that the granules induced in response to arsenite exposure are significantly larger than granules induced in response to either Naphthalene or 9,10- Phenanthrenequinone (compare with punctae in Figures 3A, 5B). Thus, it is plausible that Stress Granules and the granules induced in response to Naphthalene or 9,10-Phenanthrenequinone are distinct from each other. **Second**, we tested whether granule formation

in response to arsenite is perturbed in FMRP-deficient cells to find that this is indeed the case. This corroborates the earlier findings that FMRP (Didiot MC et al., 2009) is required for Stress Granule formation and for the formation of other stress-induced condensates (stress-induced granules).




(A) Schematic showing regimen for sodium arsenite exposure. (B-C) Tia1 (red) and FMRP (green) immunostaining prior to and post arsenite in control and FMRP-depleted C22 cells (Scrambled siRNA treated, Sc and *Fmr1* siRNA treated, Si). (B i- B iii) Tia1 and FMRP staining in uninjured Sc. (B iv- B vi) Tia1 and FMRP immunostaining in Sc post arsenite exposure. Tia1 granules indicated with white arrowheads, FMRP granules indicated with white arrow, Tia1 and FMRP co-expressing granules shown with double arrowheads. Note the presence of granules with overlapping and non-overlapping expression of these markers (n=3 experiments). (C i- C iii) Tia1 and FMRP staining in uninjured Si. (C iv- C vi) Tia1 and FMRP immunostaining in Si post arsenite exposure. Note absence of both Tia1 and FMRP granules in Si cells (n=2 experiments). (D-E) Tia1 (red) and FMRP (green) immunostaining prior to and post arsenite exposure in control and FMRP-depleted BEAS-2B cells (Scrambled siRNA treated, Sc and *Fmr1* siRNA treated, Si). ( (D i- D iii) Tia1 and FMRP staining in uninjured Sc. (D iv- D vi) Tia1 and FMRP immunostaining in Sc post arsenite exposure. Tia1 granules indicated with white arrowheads, FMRP granules indicated with white arrow, Tia1, FMRP co-expressing granules shown with double arrowheads. Note the presence of granules with overlapping and non-overlapping expression of these markers (n=3 experiments). (E i- E iii) Tia1 and FMRP staining in uninjured Si. (E iv- E vi) Tia1 and FMRP immunostaining in Si post arsenite. Note absence of both Tia1 and FMRP granules in Si cells (n=3 experiments). Scale bar= 5µm.

To reiterate, we appreciate the fact that the cytoplasmic condensates induced in response to stress are indeed heterogeneous and the condensates formed in response to Naphthalene or 9,10-Phenanthrenequinone are significantly smaller in size than Stress Granules. In order to avoid any confusion, we have replaced the term Stress Granule in the manuscript with Stress- induced granules (SIGs).

The dependence of their process on the PKR pathway cannot be assessed without also assessing the other eIF2a kinases. Often, a stress will cause phosphorylation of multiple ISR kinases, however

only one of those kinases is essential for the activation of downstream processes including the phosphorylation of eIF2a and translational arrest. The authors' finding that P- PKR is normal in the Fmr1 KD condition but P-eIF2a is affected is intriguing, but incomplete without analysis of the other kinases. The authors should use their siRNAs to deplete each kinase and determine the dependence of their effects on P-eIF2a in response to Nap or PQ.

While the data with PKR and P-PKR suggests that the role of FMRP in the activation of the Integrated Stress Response is downstream to kinase activation, we agree that the data is preliminary. However, we also think that exploring the role of FMRP in the activation of each of the stress responsive kinases upon Nap or 9,10-PQ is beyond the scope of this manuscript. Our aim here is to report that FMRP is required for the induction of the Integrated Stress Response. Future studies will investigate the role of the four stress responsive kinases in the Nap and 9,10-PQ models.

Minor points

Many of the labels inside the fluorescence microscopy panels are very small and difficult to read; these will be impossible to read once they are further shrunk down for publication. Please edit the labels to make them more legible.

We have edited the labels. All labels are 12 pt-18 pt as per the Journal of Cell Science guidelines.

The phrases "more widely" and "more broadly" are used throughout the manuscript to describe expression of FMRP. The context in which they are used is highly ambiguous, and the authors should rephrase to eliminate these clauses, or explain more explicitly where precisely they are claiming FMRP is expressed in the relevant context.

We have qualified these phrases in the text wherever applicable or appropriate.

FMRP is described in the introduction as being expressed in humans and mammals – I believe FMR1 is conserved at least down to insects as Drosophila is a model for FXS.

Noted.

The authors should be aware of the updated standards of this and many journals as well as funding agencies that when samples sizes are small (e.g. n=3), individual data points should be overlaid upon bar graphs. The authors will save themselves time by making this adjustment prior to their resubmission."

Each figure has been revised and the individual data points have been indicated wherever necessary.

Second reviewer's comment:

"Reviewer 2 Advance Summary and Potential Significance to Field:

The study by Basu et al. reports findings from a study examining Fragile X Mental Retardation Protein (FMRP) in stress responses in lung tissue using a Naphthalene (Nap) injury model. They look at the role of FMRP using both in vivo and cell culture in response to Nap-induced lesions. They find that FMRP deficiency impaired cellular activation of the Integrated Stress Response (ISR) Pathway, induced the expression of ATF4, and increased expression of cell stress and cell death markers. Using knockdown approaches, they replicate the FMRP KO phenotype with ATF4 deficiency. The work here adds to the information about the role of the evolutionarily conserved ISR pathway used by organisms to respond to xenobiotic stress, or in the case of this study, harmful inhaled agents. The study extends our knowledge of FMRP function beyond its role in classically studied domains (i.e., neurobiological function and cognition). The experiments are well-designed with appropriate controls. The data presented convincing, and data presented in supplemental make for a compelling case of a real immunoblotting story. This is important because

many of the antibodies used in this study have been used to quantify off-target signals to make conclusions (i.e., nonspecific ATF4 antibody used, Costa-Mattioli 2007). This reviewer is satisfied that the data are accurate. The study makes a clear case for FMRP in ATF4 expression and ISR pathway activation in response to lung toxicants. The only mild reservation is that the models used have modestly limited relevance to humans (NAP uses CYP2F2, not found in humans, and the human cell line used does not have some features of human lung tissue in vivo). It is a critical study extending our knowledge of the cellular functions of FMRP beyond modalities where it is predominantly studied. It is a significant contribution and will be of broad interest to the readership of the Journal of Cell Science.

We thank the reviewer for appreciating the novelty of this work.

Reviewer 2 Comments for the Author:

Major
The use of a lung injury model that does not recapitulate toxicity in humans (Nap model) reduces the impact of the study. This is mitigated to a great degree by including human lung cell line experiments, but it is a human cell line lacking important human lung tissue features (i.e., CCs). This limitation should be mentioned in the discussion.

We appreciate the criticism. In this revised manuscript we have extended our findings in the murine model /human bronchial epithelial cell line (BEAS-2B) cells to human alveolar epithelial cell line A549 cells (using the same 9,10-Phenanthrenequinone injury model). We agree that the use of these human cell lines only indicate that the findings are relevant to humans, and that other more physiologic assays are necessary to establish the validity of the reported findings. This has been highlighted in the last paragraph of the Discussion.

Minor
Abstract Lines 9-10 “...cells fail to actuate the Integrated Stress 9 Response Pathway (ISR) and...should be cells fail to actuate the Integrated Stress 9 Response (ISR) Pathway and....

Noted.

Fmr1 KO should be Fmr1 KO throughout the text; in many cases, genes are not italicized.

Corrected.

How was CYP2F2 staining specificity confirmed? The CYP is highly related and generally shows a lot of cross-reactivities with other CYPs.

We have shown that the Cyp2f2 staining is greatly decreased in cells in which Cyp2f2 expression is knocked down using siRNAs (Supplementary Figure S2). We have also shown that the sensitivity to Nap is abolished in cells in which Cyp2f2 expression is knocked down using siRNAs (Supplementary Figure S2). Taken together, we think these new experiments validate the C22 model of Naphthalene injury.

Figure2 The figures should include a key to the identity of the different colored bar groups. The information is in the figure legends, but it makes it easier to interpret the Figure if contained within it.

Figures have been revised for greater clarity.

Are there any experiments in the design of Figure 2 that examine the effects of FMRP overexpression? Such data would go a long way to establish FMRP's role in lung stress responses.”

This experiment is currently beyond our scope but we expect to investigate this in the future.

Second decision letter

MS ID#: JOCES/2021/258652

MS TITLE: The Fragile X Mental Retardation Protein protects the lung from xenobiotic stress by facilitating the Integrated Stress Response

AUTHORS: Deblina Sain Basu, Rital Bhavsar, Imtiyaz Gulami, Saraswati Chavda, Saimanoz Lingamallu, Ravi Muddashetty, Chandrakanth Veeranna, Sumantra Chattarji, Rajesh Thimmulappa, Aditi Bhattacharya, and Arjun Guha
ARTICLE TYPE: Research Article

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, while one of the referees is satisfied with the revised paper, the other still raises a major concern about how you have dealt with the stress granule issue. They state "I maintain there are critical problems with the granule/condensate-related data in this paper which should prohibit its publication in the present form. In summary, I believe the authors have satisfactorily addressed all issues previously raised by myself and the other reviewer EXCEPT with relation to the granule/condensate data. I strongly recommend that the authors simply remove this data from the manuscript, upon removal of which I would recommend publication of this work." The referee follows up with some specific examples that illustrate their concerns.

Based on the strength of their arguments I cannot accept the paper in its present form. If you were to address the concerns to their satisfaction, however, I would be amenable to considering one last version of the paper.

*We are aware that you may be experiencing disruption to the normal running of your lab that makes 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 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*

I maintain there are critical problems with the granule/condensate-related data in this paper which should prohibit its publication in the present form. In summary I believe the authors have satisfactorily addressed all issues previously raised by myself and the other reviewer EXCEPT with relation to the granule/condensate data. I strongly recommend that the authors simply remove this data from the manuscript, upon removal of which I would recommend publication of this work.

*Comments for the author*

My three specific and significant objections to the granule work are as follows:
1. The first objection is the lack of clarity in differentiating bona fide stress granules (SGs) from the stress-induced condensates observed by these authors. On line 34, the authors introduce these foci as "stress-induced condensates or granules", then choose stress induced granules (SIGs) for reference. I would strongly suggest the use of "stress induced condensates (SICs)", to avoid any unintentional confusion or conflation with bona fide stress granules.
The authors present evidence for their case related to actual stress granules but refer to these as SIGs rather than SGs. For example, in lines 49-51, and again in lines 167-169, the authors are clearly referring to bona fide stress granules, and yet are referring to these as SIGs. The work by Kedersha and Anderson is about SGs, not SICs. Thus the reference is a misrepresentation of the literature. Overall, the effect of how this is written is to confuse the condensates the authors are seeing with genuine SGs, and suggests that the SICs that the authors report are the same as genuine SGs. Its just not accurate, and its misleading for readers.
When referencing the SG literature, these structures should be identified as SGs. When referencing your own observations, the puncta should be referred to as SICs. By using the same name (SIGs) to refer to both structures, you are telling the reader that you believe these are one in the same.
2. The data do not support a critical role for Nap exposure in whatever condensates are being observed here.
The authors first say on line 172: "Confocal analysis showed that Sc cells contain a few larger punctae, that the frequencies of these larger punctae increased dramatically 3, 6, and 12 h post Nap and returned to baseline by 24 h"
However they provide no quantitative support for this statement, and I disagree based on the images shown in Figures 3 and S3 that this statement is defensible.
Then the authors state on line 177: "Co-staining cells with markers of SIG and FMRP showed that FMRP has a punctate distribution both before and after Nap and that FMRP punctae were largely distinct from SIGs". The most straightforward interpretation for this observation is that there's not a significant relationship of Nap to the granules at all. FMRP staining simply looks punctate in these authors' hands, and when its knocked down there are fewer of these puncta because you've depleted FMRP.
There does not appear to be any important relationship of these puncta either to bona fide SGs, nor really to any other type of condensate. There is also no convincing relationship of these granules to Nap exposure.
3. Finally I maintain that the staining patterns for TIA-1, Atx-2 and G3BP1 are not as expected, and that the quality of these images are insufficient to draw the conclusions being made by these authors. Why, for instance, does the intensity of the TIA-1 and Atx-2 staining decrease in the Fmr1 knockdown panels in Figures 3A and S3A respectively? There should be no effect on TIA-1 levels nor Atx2, and yet the knockdown panels clearly look dimmer. In panel 3A ix there is an arrow pointing to a structure within the nucleus – however SGs are exclusively cytoplasmic. In figure 5B panel vi, my eye perceives punctate staining in this image that are positive for TIA-1 and highly similar to the untreated condition in panel ii, and yet are not labeled with arrows. There is no quantitative support for any of the claims or interpretations of these images. These anomalies in the data are problematic, and call into question the validity of the conclusions related to these images.
My strong recommendation would be to remove all reference granules/condensates from the manuscript entirely. The images that are shown are not convincing, and the existence of condensates does not add to the story at all. The authors need not link their work to granule formation in order for their observations of ISR activation to be valid. With the removal of the SG related references, I have no objections to the data presented in the remainder of the article and believe that the authors have satisfactorily addressed all other points. Therefore, with the caveat related to the SIC/SG data removal, I would recommend publication of the manuscript.

Reviewer 2

*Advance summary and potential significance to field*

The study by Basu et al. reports findings from a study examining Fragile X Mental Retardation Protein (FMRP) in stress responses in lung tissue using a Naphthalene (Nap) injury model. They look at the role of FMRP using both in vivo and cell culture in response to Nap-induced lesions. They find that FMRP deficiency impaired cellular activation of the Integrated Stress Response (ISR)

Pathway, induced the expression of ATF4, and increased expression of cell stress and cell death markers. Using knockdown approaches, they replicate the FMRP KO phenotype with ATF4 deficiency. The work here adds to the information about the role of the evolutionarily conserved ISR pathway used by organisms to respond to xenobiotic stress, or in the case of this study, harmful inhaled agents. The study extends our knowledge of FMRP function beyond its role in classically studied domains (i.e., neurobiological function and cognition).

*Comments for the author*

I think the author's made an excellent attempt to address the primary critiques of Reviewer 1 and 2. Although I would have like to see FMRP gof studies, I understand that it is perhaps out of the scope of this current study. I am satisfied with the current iteration and am pleased especially at the attempts to reconcile the SG issue raised by Reviewer 1.

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**Second revision**

Author response to reviewers' comments

In our previous correspondence Prof. Green had highlighted the following comment by Reviewer#1 (regarding Stress Granules) and asked us to revise the manuscript in a suitable manner. Reviewer #1 had stated “I strongly recommend that the authors simply remove this data from the manuscript, upon removal of which I would recommend publication of this work.” We have carefully assessed the comments of Reviewer #1 regarding the stress granule/condensate data and come to the conclusion that the concerns raised are indeed relevant. In the paragraph below, I highlight how our follow up analysis has led us to this conclusion. Pertinently, as suggested by Reviewer #1, we have removed all of the data concerning stress granules/condensates from the version we are resubmitting here.

Our perception that FMRP depletion perturbs stress-induced condensate formation post Naphthalene (C22 cells) and 9,10-PQ (BEAS-2B cells) exposure is based on two observations. First, exposure to these toxicants results in increased frequencies of Tia-1-expressing puncta 3-6 h post exposure. Second, this increase is not observed in FMRP-deficient cells. In light of the comments made by the first reviewer, we analyzed the distributions of Tia-1 post exposure more carefully. We find that the vast majority of puncta that are observed post exposure in both models are in the 0.1-0.5 um size range. These puncta are significantly smaller than the Tia1-expressing structures that are induced in the same cells in response to arsenite exposure. Given the sizes of the puncta that increase in frequency post Nap or 9,10-PQ exposure, we agree with Reviewer #1 that these puncta are not Stress Granules. Moreover, it is also unclear that they are stress-induced condensates of some type. Thus, we have omitted the data linking FMRP and stress granules/stress-induced condensates from the manuscript. Nevertheless, our data suggests that Tia-1 expression is altered upon exposure to Naphthalene and 9,10-PQ and that these changes in expression pattern are not observed in FMRP-deficient cells. Future studies will probe the signification of this finding.

Omitting the data about stress-induced condensate formation in the Naphthalene or 9,10-PQ models does not impact the validity of the hypothesis nor the conclusions reached.

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Third decision letter

MS ID#: JOCES/2021/258652

MS TITLE: FMRP protects the lung from xenobiotic stress by facilitating the Integrated Stress Response

AUTHORS: Deblina Sain Basu, Rital Bhavsar, Imtiyaz Gulami, Saraswati Chavda, Saimanoz Lingamallu, Ravi Muddashetty, Chandrakanth Veeranna, Sumantra Chattarji, Rajesh Thimmulappa, Aditi Bhattacharya, and Arjun Guha
ARTICLE TYPE: Research Article

Thank you for your revised manuscript and response to the reviewer's request to remove the stress granule data. After looking over your revised paper, I see that in responding to this request, you have introduced a section in which several "data not shown" observations are reported. I recommend that you revised the rationale for looking at the role FMRP in the ISR pathway, so as not to include these unsupported observations. If you an make these revisions and highlight them clearly, I will look the paper over one last time before acceptance.

*We are aware that you may be experiencing disruption to the normal running of your lab that makes 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 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.

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**Third revision**

Author response to reviewers' comments

In our previous correspondence you had highlighted the following comment by Reviewer#1 (regarding Stress Granules) and asked us to revise the manuscript in a suitable manner. Reviewer #1 had stated “I strongly recommend that the authors simply remove this data from the manuscript, upon removal of which I would recommend publication of this work.” We have carefully assessed the comments of Reviewer #1 regarding the stress granule/condensate data and come to the conclusion that the concerns raised are indeed relevant. In the paragraph below, I highlight how our follow up analysis has led us to this conclusion. Pertinently, as suggested by Reviewer #1, we have removed all of the data concerning stress granules/condensates from the version we are resubmitting here.

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that increase in frequency post Nap or 9,10-PQ exposure, we agree with Reviewer #1 that these puncta are not Stress Granules. Moreover, it is also unclear that they are stress-induced condensates of some type. Thus, we have omitted the data linking FMRP and stress granules/stress-induced condensates from the manuscript. Nevertheless, our data suggests that Tia-1 expression is altered upon exposure to Naphthalene and 9,10-PQ and that these changes in expression pattern are not observed in FMRP-deficient cells. Future studies will probe the signification of this finding.

Omitting the data about stress-induced condensate formation in the Naphthalene or 9,10-PQ models does not impact the validity of the hypothesis nor the conclusions reached. We have revised the text to provide a justification for investigating the role of FMRP in the ISR without any references to unsubstantiated data.

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Fourth decision letter

MS ID#: JOCES/2021/258652

MS TITLE: FMRP protects the lung from xenobiotic stress by facilitating the Integrated Stress Response

AUTHORS: Deblina Sain Basu, Rital Bhavsar, Imtiyaz Gulami, Saraswati Chavda, Saimanoz Lingamallu, Ravi Muddashetty, Chandrakanth Veeranna, Sumantra Chattarji, Rajesh Thimmulappa, Aditi Bhattacharya, and Arjun Guha
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.