Traip controls mushroom body size by suppressing mitotic defects

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Original submission

First decision letter

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MS TITLE: Traip Mitotic Function Controls Brain Size

AUTHORS: Ryan S O'Neill and Nasser M Rusan

I have now received all the referees' reports on the above manuscript; apologies for the delay, but it can be challenging to secure reviews during the summer holiday period. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees express considerable interest in your work, but have some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy to receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. I encourage you to pay particular attention to the point raised by two reviewers around the wording of brain/MB size, the requests to strengthen the conclusion that Traip has a role in mitosis (and possible links with apoptotic cell death). 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

using drosophila to better understand human microcephaly disease genes

Comments for the author

Using Traip mutants (one of which the authors generate here), the authors describe a brain undergrowth phenotype that manifests in adult animals, a hallmark of human microcephaly. The quality of the data is mostly outstanding, however the phenotype of Traip mutants is not necessarily surprising or unexpected given what is known about Traip from other studies (ie human microcephaly gene, defects in fibroblasts lead to cell cycle delays and defects in DNA repair). Nevertheless, this is an outstanding manuscript with a solid story line and a clear phenotype, which is a reduced mushroom body neuropil, the key learning center in Drosophila. Below are a few specific comments and suggestions that could improve the manuscript.

1. The title should be more specific to mushroom body and not so general as brain size, unless authors provide data that Traip mutants have reductions in brain regions outside the mushroom body (minor point).
2. For Figure 1 panels L-M, the number of Kenyon cells should be quantified in rescue experiments
(major point). This applies to experiments in Fig. 4 as well, in which apoptosis is inhibited using H99.
3. For Figure 2B, the authors should specify what they are quantifying for the 3rd instar larvae and separate the quantification from the rest of the developmental ages (minor point).
4. Can amount of cell death be quantified in Traip mutants? Could increases in cell death in GMCs and early born daughter cells also contribute to phenotype? (minor point)
5. Figure 4 title, "Traip suppresses MB-NB cell death" should be rewritten to "Traip prevents (or suppresses) premature MB-NB cell death". In the text the authors state, ‘Traip mutant MB neuroblasts are lost via Caspase-dependent cell death’. This is not particularly convincing based on the data presented.
Can the authors identify activated-caspase in Traip mutant mushroom body neuroblasts? Could MB-NB be lost via an alternative means? ie terminal differentiation as is case for some other brain neuroblasts or non-apoptotic cell death? (major point)
6. For Figure 5H, can timelapse panels of neuroblast cell division in Traip- rescue animals be shown as well? Are bridge defects rescued as well as cell cycle timing? Results reports in Supplemental Fig S3, C D do not match with Fig 5H. Fig. 5H clearly shows a mitotic delay in Traip mutants, but quantification of timing in supp fig reports no significant difference. This should be clarified. (major point)
7. For Figure 6D, needs a label for the y-axis.
8. It would be helpful to list genotypes (as per figure panels) in a separate table.

Reviewer 2

Advance summary and potential significance to field

Microcephaly is a severe condition that may have different underlaying causes with genetic mutations being the most common one. There is a growing body of evidence that several microcephaly-linked genes are associated with mitotic failures, by impairing multiple known pathways for mitotic fidelity (kinetochores centrosomes, chromosome compaction, etc). Hence, uncovering a novel mitotic function for an additional microcephaly-linked gene is of prime interest with broad impact in development and human disease.

In this interesting manuscript, the authors characterize mutants for the Drosophila Traip, a E3 ubiquitin ligase whose human orthologue has well-established roles in response to replication stress. The authors established a new Drosophila Traip allele and reveal a mild decrease in central brain size associated with major defects in MB morphology. Using various rescue strategies the authors convincingly show that Traip is required during the proliferative stages of MB development.

These are very interesting findings that highlight a particular sensitivity of MB-NBs to Traip function, that although not fully exploited provide an interesting insight into why microcephaly genes affect particularly brain development.
Overall, this initial characterization of Traip-associated defects in MB development is very solid, interesting and novel. However, when trying to go deeper into the mechanism behind it and the cellular description of the phenotype the experiments (and conclusions) did not match the same standards. In particular I found that several of the claims are not fully substantiated (see details below). It remains unclear whether the phenotypes are indeed associated with a mitotic-specific function of traip (which is the main claim of the manuscript).

I would thus advice the authors to clarify several major issues before I can recommend it for publication (.ee below),

1. The claim that Traip holds a mitotic function is not very convincing. The description of mitotic phenotypes appears a bit confusing, particularly with regard to how these reflect a mitosis-specific function. The well-established role for Traip in replication could lead to unreplicated DNA and/or DNA inter-strand cross links, that is then seen as anaphase bridges during mitosis. Yet, this does not prove that Traip acts during mitosis. Persistent DNA linkages would remain even if the traip only acts during interphase. The authors' claim appears even more confusing when placed together with the mitotic localization reported, as Traip is mostly concentrated at centrosomes and/or spindle, which would actually call for some role in spindle formation. Is traip also found to co-localize at UFB at mitotic exit, if authors induce mild replication stress? This is a critical point, as model presented in the last figure depicts Traip acting on inter-strand cross-links in late mitosis but there is not a single data to support this throughout the paper. Also, the authors should quantify in more detail the nature of mitotic defects (is anaphase bridges the most common phenotype? Or are multipolar divisions often observed?) And why do the authors use all larval NB to study mitotic fidelity rather than MB NBs? Are these defects also detected in MB NBs (and potentially at higher frequency, which would explain why phenotypically the MB is the most affected brain part?)

2. Admittedly, uncovering a mitotic role for a protein that has (putative) non-mitotic functions is not experimentally trivial, as one requires to manipulate protein function specifically during mitotic stages. The deltaNLS construct is presented as a valuable tool to achieve this challenging aim, as it is not observed on chromatin during interphase. This approach has indeed its own caveats (see point #3). Yet, even if proven to be a mitosis-specific impairment condition this tool is not fully exploited in a way that could support the most novel claim of the work (a mitotic specific function for traip). For example: are DNA bridges rescued in these conditions? Is the number of MB NBs also rescued?

3. Related with the previous point, the deltaNLS tool has indeed a great potential. But further analysis is needed to ensure the interphase function is fully prevented here. It is conceivable that, for example, reduced nuclear levels are sufficient to fulfil Traip functions. Alternatively, subcellular localization may not be determinant for its interphase role (e.g. the E3 ubiquitin ligase reaction could take place efficiency in the cytoplasm). Thus, as it stands, it is still possible some residual interphase activity. The authors should exclude this experimentally as this is the main argument to support that Traip activity is required solely during mitosis. Otherwise, these potential caveats should be openly discussed in the discussion.

4. In a reciprocal line of thought to the claimed mitotic function, the authors provide evidence that Traip is not involved in DDR in interphase. Yet, the γH2Av experiments are missing a positive control (conditions where DNA damage is artificially induced (e.g. irradiation) to test whether or not this assay is efficient at detecting DNA damage in NBs). This would also be a good assay to test the role of traip in these more challenging conditions. The complementary RNAi

experiments, considering their negative nature, are also difficult to interpret without further validating the tools.

5. The authors claim that reduced neuron numbers in traip- were explained by premature caspase-dependent cell death of MB-NBs). However, the data in figure 4 is not very convincing as a rather mild effect on MB-NBs cell number is seen upon Df(3L)H99 and only at 24hrs APF. And for the alternative method used (TraipΔ ; DRICE RNAi), no MB NBs counts is presented. The manuscript is written under the assumption that aneuploidy/mitotic failure will lead to cell death, which is actually a rather controversial topic in the field (see prior studies on the fate of aneuploid neuroblasts: Gogendeau D et al Nat Comm 2015; Poulton et al JCB 2017; Mirkovic et al, Plos Biol 2019). In line with this, the authors themselves present evidence for aneuploid clones growing, which would also suggest that mitotic failure does not trigger immediate cell death. Hence, the authors should either provide further evidence to substantiate these claims (evidence of apoptosis activation specifically in MB NBs, a more convincing rescue on MB NBs cell number by preventing apoptosis (e.g. p35 expression)) or alternatively tone down their claims and discuss their findings in the context of current literature.
6. Statistics need to be revised throughout the manuscript: The authors refer to the use of t-test or Mann-witney in graphs with more than two datasets. One-way ANOVA (or Kruskal-Wallis test if non-parametric) should be used instead, to account for the variance of all datasets on each comparison.

Comments for the author

1. The claim that Traip holds a mitotic function is not very convincing. The description of mitotic phenotypes appears a bit confusing, particularly with regard to how these reflect a mitosis-specific function. The well-established role for Traip in replication could lead to unreplicated DNA and/or DNA inter-strand cross links, that is then seen as anaphase bridges during mitosis. Yet, this does not prove that Traip acts during mitosis. Persistent DNA linkages would remain even if the traip only acts during interphase. The authors' claim appears even more confusing when placed together with the mitotic localization reported, as Traip is mostly concentrated at centrosomes and/or spindle, which would actually call for some role in spindle formation. Is traip also found to co-localize at UFB at mitotic exit, if authors induce mild replication stress? This is a critical point, as model presented in the last figure depicts Traip acting on inter-strand cross-links in late mitosis but there is not a single data to support this throughout the paper. Also, the authors should quantify in more detail the nature of mitotic defects (is anaphase bridges the most common phenotype? Or are multipolar divisions often observed?) And why do the authors use all larval NB to study mitotic fidelity rather than MB NBs? Are these defects also detected in MB NBs (and potentially at higher frequency, which would explain why phenotypically the MB is the most affected brain part?)
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4. In a reciprocal line of thought to the claimed mitotic function, the authors provide evidence that Traip is not involved in DDR in interphase. Yet, the γH2Av experiments are missing a positive control (conditions where DNA damage is artificially induced (e.g. irradiation) to test

whether or not this assay is efficient at detecting DNA damage in NBs). This would also be a good assay to test the role of traip in these more challenging conditions. The complementary RNAi experiments, considering their negative nature, are also difficult to interpret without further validating the tools.
5. The authors claim that reduced neuron numbers in traip- were explained by premature caspase-dependent cell death of MB-NBs). However, the data in figure 4 is not very convincing as a rather mild effect on MB-NBs cell number is seen upon Df(3L)H99 and only at 24hrs APF. And for the alternative method used (TraipΔ ; DRICE RNAi), no MB NBs counts is presented. The manuscript is written under the assumption that aneuploidy/mitotic failure will lead to cell death, which is actually a rather controversial topic in the field (see prior studies on the fate of aneuploid neuroblasts: Gogendeau D et al Nat Comm 2015; Poulton et al JCB 2017; Mirkovic et al, Plos Biol 2019). In line with this, the authors themselves present evidence for aneuploid clones growing, which would also suggest that mitotic failure does not trigger immediate cell death. Hence, the authors should either provide further evidence to substantiate these claims (evidence of apoptosis activation specifically in MB NBs, a more convincing rescue on MB NBs cell number by preventing apoptosis (e.g. p35 expression)) or alternatively tone down their claims and discuss their findings in the context of current literature.
6. Statistics need to be revised throughout the manuscript: The authors refer to the use of t-test or Mann-witney in graphs with more than two datasets. One-way ANOVA (or Kruskal-Wallis test if non-parametric) should be used instead, to account for the variance of all datasets on each comparison.

Minor points:
1. The mitotic function would call for a strong reduction in cell bodies of the NBs. Yet, the more convincing phenotypic analysis of MB morphological defects appears as the area of the α lobe cross section, which I trust at this stage could arise from other defects (e.g. pruning). Complementing this analysis with MB neuron counts (cell bodies, as shown in figure 1 M,L) would make the message a bit clearer on how the two sections of the manuscript are indeed interdependent.
2. Statistical analysis is missing for the Central Brain size between traip- and rescued condition
3. The experiments describing that human traip do not rescue the observed phenotype are interesting. It would be nice to complement this figure with a comparison between the two proteins.
4. Two reports have previously described sckl syndrome: Silengo et al. (2001), and Harley et al. (2016)
In summary, I found the manuscript very interesting and with great potential for novel discoveries. Yet, as it stands, several additional experiments would need to be performed to support the major novel claim, that Traip is active during mitotic stages. Or alternatively, the claim of a mitosis-specific function should be toned down.

Reviewer 3

Advance summary and potential significance to field

O'Neill and Rusan have demonstrated in this ms that a DNA damage repair gene called TRAIP causes reduced central brain volume in the adult CNS. The authors nicely show that TRAIP mutant MBs produced smaller lineages, attributed partly by reduced MB neuroblasts, caused by cell death. Traip is localised to centrosomes and the mitotic spindle, Traip mutant MB NBs demonstrate polyploidy, and chromosomal bridge defects. The data produced are of a high quality and demonstrate a novel role of Traip in MB NB regulation. I have several queries that I hope the authors can address in the revision process.

Comments for the author

1. Since Traip is expressed in the CB NBs (i.e. both type I and type II NBs), the reduced volume decrease in the adult CB could be due to defects in type II or type I NB proliferation other

than MB NBs. The authors should check if CB type I or II lineage cell death/proliferation or lineage size is affected.
Did Wor-GAL4 UASGFP::Traip rescue also rescue type II and type I lineage size in the mutant?
2. H99 driven brain size rescue is not complete. MB NBs have also been shown to undergo autophagy prior to terminal differentiation. Therefore the possibility that increased autophagy may also contribute to reduced CB volume should be addressed.
3. Since Traip is localised to the centrosomes, are there ectopic centrosomes?
5. Although mitotic progression is not affected, is asymmetric division machinery affected? The mislocalisation of asymmetric division machinery often accompanies centrosome defects, or mitotic spindle defects.
6. The use of the wording "MB size" should be revised, as it is confusing whether the authors are referring to the cell size of MB NBs or MB lineage.

First revision

Author response to reviewers' comments

Reviewer Responses

> ***Reviewer 1 Advance Summary and Potential Significance to Field:***
> *Using drosophila to better understand human microcephaly disease genes*
>
> ***Reviewer 1 Comments for the Author:***
> *Using Traip mutants (one of which the authors generate here), the authors describe a brain undergrowth phenotype that manifests in adult animals, a hallmark of human microcephaly. The quality of the data is mostly outstanding, however the phenotype of Traip mutants is not necessarily surprising or unexpected, given what is known about Traip from other studies (ie human microcephaly gene, defects in fibroblasts lead to cell cycle delays and defects in DNA repair). Nevertheless, this is an outstanding manuscript with a solid story line and a clear phenotype, which is a reduced mushroom body neuropil, the key learning center in Drosophila. Below are a few specific comments and suggestions that could improve the manuscript.*

We thanks the reviewer for their overall support of the manuscript. We have provided our detailed response to your comments and have attempted to address all your concerns.

> *1. The title should be more specific to mushroom body and not so general as brain size, unless authors provide data that Traip mutants have reductions in brain regions outside the mushroom body (minor point).*

We agree with the reviewer that our study focuses on a prominent defect in the Mushroom Body. Therefore, we have changed the title of the manuscript from:

> "Traip Mitotic Function Controls Brain Size"

To:

> "Traip Controls Mushroom Body Size by Suppressing Mitotic Defects".

> 2. *For Figure 1 panels L-M, the number of Kenyon cells should be quantified in rescue experiments (major point). This applies to experiments in Fig. 4 as well, in which apoptosis is inhibited using H99.*

We have repeated the Kenyon cell counting experiments, which now include the full length GFP::Traip rescue (Figure 1L and 1M), the *Df(3L)H99* suppression (Figure 4F and 4G), and the GFP::Traip$^{\Delta NLS}$ rescue (Figure 7J and 7K).

3. *For Figure 2B, the authors should specify what they are quantifying for the 3rd instar larvae and, separate the quantification from the rest of the developmental ages (minor point).*

We have now separated the larval and pupal data into separate graphs (now Figure 2B and 2C). We also changed the text on page 6 to include a brief description of the differences between the larval and pupal MB structures:

> "In the 3rd instar larval stage, when only γ lobes are present, *traip*- MB size was not significantly reduced compared to controls (Figure 2B). However, as development progressed through the pupal stages, there was a significant reduction in MB size of *traip*- compared to controls (Figure 2C). Note that the large reduction in MB cross sectional area between larval and 24 hours APF stages is due to extensive developmental remodelling of the short, thick larval γ lobes during metamorphosis, which are replaced by longer, thinner α lobes during the early pupal stages (Lee et al., 1999)."

4. *Can amount of cell death be quantified in Traip mutants? Could increases in cell death in GMCs and early born daughter cells also contribute to phenotype? (minor point)*

We appreciate this line of thinking, and have tried several times to address this experimentally. Specifically, we have stained for cleaved caspase Dcp-1, which labels cells with activated caspases. We did not detect a significant difference in the number of cleaved caspase-positive KCs at the 24 hour APF stage between controls and *traip*- (although there was a very slight trend towards higher death in *traip*-). This suggests that caspase-dependent cell death in KCs is not a major contributor to the phenotype. We detected one cleaved caspase-positive MB-NB in *traip*- (1/48 MB-NBs), compared to none detected in controls (0/85 MB-NBs). We reasoned that the low number of observed cleaved caspase-positive MB-NBs in the mutant could be due to rapid death and clearing of cells, thus making them difficult to detect. Further, looking at the 24 hours APF stage meant that there were on average ~2 MB-NBs per hemisphere, making a possibly rare event more difficult to capture. Alternatively, as the reviewer points out in the next comment, and consistent with the only partial and temporary suppression of MB-NB loss via *Df(3L)H99*, other mechanisms could be contributing to the premature loss of MB-NBs.

To address the review's comment in the manuscript, we have added a figure and quantification of KC and MB-NB stained for Dcp-1 (Figures 4I-L). We have changed the concluding paragraph of the respective results section on page 8 to back off from suggesting only one cell death pathway is in play here, from:

> "Together, these data support a model where Traip prevents premature caspase-dependent cell death of MB-NBs to ensure proper KC number and MB morphology."

To:

> "Together, these data support a model where Traip prevents premature loss of MB-NBs and possibly neurons and/or GMCs, and that in the absence of Traip these cells are lost in part via caspase-dependent cell death."

We have also added a statement to the discussion on page 15 to address the possibility of caspase-dependent cell death in GMCs or KCs contributing to the MB size defect:

> "Further, it is likely that loss of KCs and/or GMCs also contributes some to *traip*- MB size defects."

5. *Figure 4 title, "Traip suppresses MB-NB cell death" should be rewritten to "Traip prevents (or suppresses) premature MB-NB cell death". In the text the authors state, 'Traip mutant MB neuroblasts are lost via Caspase-dependent cell death'. This is not particularly convincing based on the data presented. Can the authors identify activated-caspase in*

Traip mutant mushroom body neuroblasts? Could MB-NB be lost via an alternative means? ie terminal differentiation as is case for some other brain neuroblasts or non-apoptotic cell death? (major point)

As suggested, we have now changed the title of Figure 4 to "Traip suppresses premature MB-NB cell death" to reflect the partial phenotypic rescue via *Df(3L)H99*.

Related to our response to reviewer comment #4, our experiments staining for cleaved caspase Dcp-1 provided only weak support for mutant MB-NBs with activated caspase. We suspect, but cannot prove, that caspase-positive cells are a rare event because the MB-NB cells die quickly. Alternatively, as the reviewer suggests, several other possible mechanisms of cell loss could account for the missing *traip*$^{-}$ MB-NBs. Thus, we added a brief discussion of the possibilities tothe Discussion on page 15:

> "However, our caspase-inhibition experiments did not fully suppress *traip*$^{-}$ MB phenotypes, suggesting that redundant mechanisms in addition to caspase-dependent cell death may play a role in MB-NB loss. For example, when caspase- dependent apoptosis is inhibited, MB-NBs are primarily lost via autophagy (Pahlet al., 2019). Alternatively, the irregular, crenellated nuclear envelope morphology of some *traip*$^{-}$ MB-NBs (Figure 5C) could point to non-apoptotic celldeath pathways (Kutscher and Shaham, 2017). Finally, aneuploidy-induced cellcycle exit in *traip*$^{-}$ MB-NBs could lead to loss via premature differentiation(Gogendau et al., 2015)."

6. *For Figure 5H, can timelapse panels of neuroblast cell division in Traip- rescue animals be shown as well? Are bridge defects rescued as well as cell cycle timing? Results reports in Supplemental Fig S3, C D do not match with Fig 5H. Fig. 5H clearly shows a mitotic delay inTraip mutants, but quantification of timing in supp fig reports no significant difference. This should be clarified. (major point)*

We have added time lapse panels and a new Movie of Traip rescue NB mitosis (Figure 5K and Movie 4).

The reviewer is correct that there seems to be a discrepancy between the quantification and the movies shown in 5H. However, the quantification was based on anaphase onset to complete furrow constriction (shown by new blue arrow), which we define as initial time at which the opposing cortical regions meet in center of the cell. We used a combination of GFP::Tubulin and moesin::GFP markers (previously shown in the Movies but not in the Figure) to mark these two timepoints. Additionally, we were previously showing the most extreme example of *traip*$^{-}$ mitotic bridge defects, which also happened to have the longest mitotic duration. Thus, we havereplaced the timelapse panels in Figure 5 to be more to reflect the average duration of mitosis,and included a second *traip*$^{-}$ example to show an extreme bridge defect.

The figure now rearranged such that the first panel shows metaphase, the second panel shows the moment of anaphase onset (with T = 0), the third panel always shows mid-anaphase (to highlight the bridge), and the fourth panel always shows the moment of complete furrow constriction. We also included an inset with both colors for clarity. Finally, we moved the mitotic timing data from the supplement to the main Figure 5 in order to more clearly show that mitotic timing was not a defect.

7. *For Figure 6D, needs a label for the y-axis.*

We added "Surviving offspring genotype (%)" to the y-axis.

8. *It would be helpful to list genotypes (as per figure panels) in a separate table.*

We have now made sure that all fly strains used are listed in a table in the Methods section.

Reviewer 2 Advance Summary and Potential Significance to Field:
Microcephaly is a severe condition that may have different underlaying causes, with genetic mutations being the most common one. There is a growing body of evidence that several microcephaly-linked genes are associated with mitotic failures, by impairing multiple known pathways for mitotic fidelity (kinetochores, centrosomes, chromosome compaction, etc). Hence, uncovering a novel mitotic function for an additional microcephaly-linked gene is of prime interest with broad impact in development and human disease.

Reviewer 2 Comments for the Author:
In this interesting manuscript, the authors characterize mutants for the Drosophila Traip, a E3 ubiquitin ligase whose human orthologue has well-established roles in response to replication stress. The authors established a new Drosophila Traip allele and reveal a mild decrease in central brain size, associated with major defects in MB morphology. Using various rescue strategies, the authors convincingly show that Traip is required during the proliferative stages of MB development.

These are very interesting findings that highlight a particular sensitivity of MB-NBs to Traip function, that although not fully exploited provide an interesting insight into why microcephaly genes affect particularly brain development. Overall, this initial characterization of Traip- associated defects in MB development is very solid, interesting and novel. However, when trying to go deeper into the mechanism behind it and the cellular description of the phenotype, the experiments (and conclusions) did not match the same standards. In particular,
I found that several of the claims are not fully substantiated (see details below). It remains unclear whether the phenotypes are indeed associated with a mitotic-specific function of traip (which is the main claim of the manuscript).

I would thus advice the authors to clarify several major issues before I can recommend it for publication (.ee below),

1. The claim that Traip holds a mitotic function is not very convincing. The description of mitotic phenotypes appears a bit confusing, particularly with regard to how these reflect a mitosis- specific function. The well-established role for Traip in replication could lead to unreplicated DNA and/or DNA inter-strand cross links, that is then seen as anaphase bridges during mitosis. Yet, this does not prove that Traip acts during mitosis. Persistent DNA linkages would remain even if the traip only acts during interphase. The authors' claim appears even more confusing when placed together with the mitotic localization reported, as Traip is mostly concentrated at centrosomes and/or spindle, which would actually call for some role in spindle formation.

We completely agree with the reviewer that the conclusion of a definitive mitotic function was pushed too strongly. While it was strongly suggested by other studies that TRAIP is activated at mitosis to unload stalled replication machinery, our data do not directly show this. We have now clarified several points in the paper to highlight mitotic phenotypes, with appropriate caveats and remain agnostic to whether the primary mutant defects arise due to loss of an interphase function or mitotic function.

1) We changed to title from:

"Traip Mitotic Function Controls Brain Size"

To:

"Traip Controls Mushroom Body Size by Suppressing Mitotic Defects"

2) In the Results, page 13, we changed:

"We next tested whether **Traip primarily controls MB development through a mitotic**

function."

To:

"We next tested whether **mitotic localization of Traip was sufficient for proper MB development."**

3) In the Results, page 13, we changed:

"Nonetheless, *GFP::Traip*$^{\Delta NLS}$ fully rescued *traip*$^{-}$ MB size (Figure 7G and 7H), indicating that a **mitotic Traip function is critical for MB-NBs**."

To:

"Nonetheless, *GFP::Traip*$^{\Delta NLS}$ fully rescued *traip*$^{-}$ adult MB lobe size (Figure 7G and 7H), 24 hrs APF MB-NB number (Figure 7I), KC number (Figure 7J and 7K) and nearly completely rescued mitotic chromosome bridging (22/23, Figure 7L, Movie 7), indicating that **Traip function during mitosis is sufficient for MB-NBs**."

4) In the Discussion page 14, we changed:

"Instead, we discovered the presence of mitotic DNA bridges, sensitivity to inter-strand crosslinking agents, RING domain-dependence, **and requirement for mitotic localization only,** and therefore conclude that the primary function for Traip in MB-NBsis to ubiquitylate and remove stalled replication machinery during mitosis (Figure 8A;Deng et al., 2019; Priego Moreno et al., 2019; Sonneville et al., 2019)."

To:

"Instead, we discovered the presence of mitotic DNA bridges, sensitivity to inter-strand crosslinking agents, and RING domain-dependence, consistent with the well-established role of TRAIP in unloading stalled replication machinery to initiate repair (Figure 8A;Deng et al., 2019; Priego Moreno et al., 2019; Sonneville et al., 2019). **Further, GFP::Traip**$^{\Delta NLS}$ **rescue experiments suggest either that Traip primarily performs this unloading function during mitosis (Deng et al., 2019), or else that Traipnormally functions during interphase but is able to unload stalled forks during mitosis if necessary."**

Is traip also found to co-localize at UFB at mitotic exit, if authors induce mild replication stress?This is a critical point, as model presented in the last figure depicts Traip acting on inter-strandcross-links in late mitosis but there is not a single data to support this throughout the paper.

We spent considerable time over the past 4 months attempting to do live imaging of either GFP::Traip or controls vs *traip*$^{-}$ with the addition of replication stress-inducing drugs (both aphidicolin and cisplatin) – however, we were never able to find ideal conditions for these experiments: we either saw no obvious effects at lower concentrations (ie: no DNA bridges induced), or we caused a complete arrest of all mitosis at higher concentrations.

We also tried extensive live imaging of GFP::Traip$^{\text{RING mutant}}$, reasoning that a non-functional Traip might get stuck at DNA bridges and thus show localization. Although we saw a few instances of GFP::Traip$^{\text{RING mutant}}$ puncta that seemed to be following bridges, they were not especially convincing. Thus, since we were never able to detect clear localization of Traip at thebridge, we have removed that aspect from the Figure 8 diagram.

Also, the authors should quantify in more detail the nature of mitotic defects (is anaphasebridges the most common phenotype? Or are multipolar divisions often observed?)

We never observed any other obvious mitotic defects apart from the anaphase bridges. We have added a statement to clarify this in the Results, page 9.

> *And why do the authors use all larval NB to study mitotic fidelity rather than MB NBs? Are these defects also detected in MB NBs (and potentially at higher frequency, which would explainwhy phenotypically the MB is the most affected brain part?)*

We tried to image larval brains live with His2Av::mRFP and *OK107-GAL4 > UAS-mCD8::GFP* to directly observe MB-NBs in control and mutant conditions, but the MB-NB and KC clustersare positioned within the larval brain such that they remain distant from the objective lens regardless of whether we orient the brains dorsal or ventral side up. Despite our efforts and extensive experience in imaging brains, we still are unable to get reliable high resolution time-lapse movies of MB-NBs.

Thus, instead of directly imaging the MB-NBs, we reasoned that the DNA bridge defects occurin all NBs, and that the MBs are most strongly affected due to their increased number of celldivisions compared to other NBs, which could allow either small defects to accumulate orincreased probability of rare severe defects occurring (see Discussion, page 14). We have addeda statement stating our inability to directly image MB-NBs live to the Results, page 9.

> *2. Admittedly, uncovering a mitotic role for a protein that has (putative) non-mitotic functions is not experimentally trivial, as one requires to manipulate protein function specifically during mitotic stages. The deltaNLS construct is presented as a valuable tool to achieve this challengingaim, as it is not observed on chromatin during interphase. This approach has indeed its own caveats (see point #3). Yet, even if proven to be a mitosis-specific impairment condition, this tool is not fully exploited in a way that could support the most novel claim of the work (a mitotic specific function for traip). For example: are DNA bridges rescued in these conditions? Is the number of MB NBs also rescued?*

We quantified MB-NB number, KC number, and DNA bridging rescue for the ΔNLS rescue, and have included them in Figure 7 (I, J, K, L).

> *3. Related with the previous point, the deltaNLS tool has indeed a great potential. But further analysis is needed to ensure the interphase function is fully prevented here. It is conceivable that,for example, reduced nuclear levels are sufficient to fulfil Traip functions. Alternatively, subcellular localization may not be determinant for its interphase role (e.g. the E3 ubiquitin ligase reaction could take place efficiency in the cytoplasm). Thus, as it stands, it is still possiblesome residual interphase activity. The authors should exclude this experimentally as this is the main argument to support that Traip activity is required solely during mitosis. Otherwise, these potential caveats should be openly discussed in the discussion.*

As mentioned above, we have significantly reduced the claim that Traip directly functions during mitosis and instead lean towards being agnostic about when Traip functions to suppress mitoticDNA bridges. Additionally, we included a statement of the caveats raised by the reviewer abovein the Discussion, page 14:

> "Alternatively, we cannot rule out that there may be residual GFP::Traip$^{\Delta NLS}$ in the nucleus to properly function during interphase, or else nuclear localization of Traip is not required for an interphase function."

> *4. In a reciprocal line of thought to the claimed mitotic function, the authors provide evidence that Traip is not involved in DDR in interphase. Yet, the γH2Av experiments are missing a positive control (conditions where DNA damage is artificially induced (e.g. irradiation) to testwhether or not this assay is efficient at detecting DNA damage in NBs). This would also be a good assay to test the role of traip in these more challenging conditions. The complementary RNAi experiments, considering their negative nature, are*

also difficult to interpret without further validating the tools.

Over the past two months we have tried to perform positive controls by incubating brains with MMS or cisplatin, which resulted in γH2Av staining that more closely resembles that seen in several papers mentioned below (eg: Gogendeau et al Nat Comm 2015; Poulton et al JCB 2017; Mirkovic et al, Plos Biol 2019) where some cells are clearly "γH2Av-positive" with strong and more uniform nuclear staining. In other words, our *traip*$^{-}$ MB-NBs with high numbers of puncta looked weaker in comparison to these positive controls with extremely strong γH2Av staining.

However, we are not fully convinced that our original staining failed to work properly because we actually did observe a small number of *traip*$^{-}$ cells with significantly higher puncta (which were never observed in controls). Although we initially suggested that these *traip*$^{-}$ outliers represent apoptosing cells, we have since realized that their crenellated nuclear envelope morphology is not consistent with apoptosis. Further, after thinking more carefully about how to interpret the experiment, we agree with the reveiwer that it does not indicate a lack of interphase function for Traip. Thus, we have removed our assertions that the γH2Av indicates a lack of interphase function. Instead, our current interpretation of our γH2Av data is that most MB-NBs have a very low base-level of DNA damage (represented by a small number of γH2Av puncta per nucleus), and the small subset of *traip*$^{-}$ MB-NBs with elevated numbers of puncta represent cells that might have experienced DNA damage during mitosis (eg: due to DNA bridging).
Accordingly, we now present these data in Supplemental Figure S3F-I, mentioned briefly on page 10, at the end of the Results section on DNA bridges. However, given the different appearance of our staining compared to some published literature, we could remove the data altogether if necessary since they are not required to support any major claims.

5. The authors claim that reduced neuron numbers in traip- were explained by premature caspase-dependent cell death of MB-NBs). However, the data in figure 4 is not very convincing as a rather mild effect on MB-NBs cell number is seen upon Df(3L)H99 and only at 24hrs APF. And for the alternative method used (TraipΔ ; DRICE RNAi), no MB NBs counts is presented. The manuscript is written under the assumption that aneuploidy/mitotic failure will lead to cell death, which is actually a rather controversial topic in the field (see prior studies on the fate of aneuploid neuroblasts: Gogendeau D et al Nat Comm 2015; Poulton et al JCB 2017; Mirkovic etal, Plos Biol 2019). In line with this, the authors themselves present evidence for aneuploid clones growing, which would also suggest that mitotic failure does not trigger immediate cell death. Hence, the authors should either provide further evidence to substantiate these claims (evidence of apoptosis activation specifically in MB NBs, a more convincing rescue on MB NBs cell number by preventing apoptosis (e.g. p35 expression)) or alternatively tone down their claims and discuss their findings in the context of current literature.

We have now included experiments staining for cleaved caspase Dcp-1 (Figure 4I-L). As mentioned in the responses to Reviewer 1, these experiments provided limited support for mutant MB-NBs and KCs with activated caspase. Given that our caspase-inhibiting experiments only ever partially rescue mutant phenotypes, and, as Reviewer 1 suggests, several other possible mechanisms of cell loss could account for the missing *traip*$^{-}$ MB-NBs, therefore we have toned down claims of caspase-dependent cell death being the main mechanism of cell loss. For example, on the conclusion of the relevant Results section on page 8, we now state:

> "Together, these data support a model where Traip prevents premature loss of MB-NBs and possibly KCs and/or GMCs, and that in the absence of Traip these **cells are lost in part via caspase-dependent cell death**."

As mentioned previously, we added a brief discussion of alternate possibilities to the Discussion on page 15:

> "Further, our caspase-inhibition experiments did not fully suppress *traip*$^{-}$ MB phenotypes, suggesting that redundant mechanisms in addition to caspase-dependent cell death may play a role in MB-NB loss. For example, when caspase- dependent apoptosis is inhibited,

> MB-NBs are primarily lost via autophagy (Pahl et al., 2019). Alternatively, the irregular, crenellated nuclear envelope morphology of some *traip*$^{-}$ MB-NBs (Figure 5C) could point to non-apoptotic cell death pathways (Kutscher and Shaham, 2017). Finally, aneuploidy-induced cell cycle exit in *traip*$^{-}$ MB-NBs could lead to loss via premature differentiation (Gogendau et al., 2015)."

We changed "apoptosis" to "cell loss" in several parts of the discussion on page 17. Additionally, we included the reference Mirkovic et al 2019 in the discussion on page 17as it is consistent with our observation of both tolerated and lethal aneuploidies:

> "Similarly, one recent study found that, while many karyotypes are permitted in NBs, loss of both copies of any of the three major *Drosophila* chromosomesresulted in a failure to proliferate and likely elimination"

We did attempt p35 expression using OK107-GAL4, but it was lethal.

> *6. Statistics need to be revised throughout the manuscript: The authors refer to the use of t-test or Mann-witney in graphs with more than two datasets. One-way ANOVA (or Kruskal-Wallis test if non-parametric) should be used instead, to account for the variance of all datasets on each comparison.*

We revised the statistics for Figures 1B, 1C, 1I, 1J, 1M, 3D, 3F, 4B, 4E, 6F, 7H, S1C, S1D, S1E, S1G, S1H, and S3C to ordinary one-way ANOVA, and 5L to Kruskal-Wallis.

> *Minor points:*
>
> *1. The mitotic function would call for a strong reduction in cell bodies of the NBs. Yet, the more convincing phenotypic analysis of MB morphological defects appears as the area of the a lobecross section, which I trust at this stage could arise from other defects (e.g. pruning). Complementing this analysis with MB neuron counts (cell bodies, as shown in figure 1 M,L) would make the message a bit clearer on how the two sections of the manuscript are indeed interdependent.*

We repeated the KC counting experiments to now include the ΔNLS rescue as Figure 7J/K.

> *2. Statistical analysis is missing for the Central Brain size between traip- and rescued condition*

We added the stat bars to this graph (not significant), as well as adding additional stat bars to graphs in Figures 1I, 1J, 3D, 3F, 5L, S1C, S1G, and S1H.

> *3. The experiments describing that human traip do not rescue the observed phenotype are interesting. It would be nice to complement this figure with a comparison between the twoproteins.*

We now include a protein alignment of Traip and TRAIP in Figure S6B, including their major known domains and sequence identity/similarity. Additionally, we added a statement to theResults, page 12:

> "Human GFP::TRAIP also failed to rescue *traip*$^{-}$ MB size (Figure S6A); while the domain structures of Traip and TRAIP are conserved, they are fairly divergent at the protein level (22%identical and 62% similar; Figure S6B)."

> *4. Two reports have previously described sckl syndrome: Silengo et al. (2001), and Harley et al. (2016)*

We have included Harley et al., 2016, as it is the first clear clinical study linking Seckel Syndrome to a mutation in TRAIP. However, we chose not to include Silengo et al., 2001 andother publications relating to Seckel Syndrome where there was no link made to a specific gene.

In summary, I found the manuscript very interesting and with great potential for novel discoveries. Yet, as it stands, several additional experiments would need to be performed to support the major novel claim, that Traip is active during mitotic stages. Or alternatively, theclaim of a mitosis-specific function should be toned down.

We agree with the criticism of our strong claim of a mitotic function for Traip, and hope that softening the language to be more agnostic about when Traip functions will alleviate some of the reviewer concerns.

Reviewer 3 Advance Summary and Potential Significance to Field:
O'Neill and Rusan have demonstrated in this ms that a DNA damage repair gene called TRAIPcauses reduced central brain volume in the adult CNS. The authors nicely show that TRAIP mutant MBs produced smaller lineages, attributed partly by reduced MB neuroblasts, caused bycell death. Traip is localised to centrosomes and the mitotic spindle, Traip mutant MB NBs demonstrate polyploidy, and chromosomal bridge defects. The data produced are of a high quality and demonstrate a novel role of Traip in MB NB regulation. I have several queries that Ihope the authors can address in the revision process.

Reviewer 3 Comments for the Author:

1. Since Traip is expressed in the CB NBs (i.e. both type I and type II NBs), the reduced volume decrease in the adult CB could be due to defects in type II or type I NB proliferation other than MB NBs. The authors should check if CB type I or II lineage cell death/proliferation or lineage size is affected. Did Wor-GAL4 UASGFP::Traip rescue also rescue type II and type I lineage sizein the mutant?

In line with the Reviewer's thoughts in this comment, we suspected that all central brain NBs likely exhibit the same defects – in fact, our live imaging of mitotic DNA bridges was performed on central brain NBs rather than MB-NBs specifically (see response to Reviewer 2 regarding imaging). However, we also predicted that non-MB parts of the brain are likely only weakly affected due to the relatively small number of divisions most central brain NBs undergo compared to the more proliferative MB-NBs. Thus, we have not extensively analyzed the lineages of type 1 and type 2 NB, as we predicted that defectsin the type I and type II NBs will be relatively rare and possibly more subtle compared to the MB-NB defects. We slightly modified our Discussion on page 14 to make this explanation more clear:

"While most NBs have a limited window of proliferation, MB-NBs divide continuously from embryogenesis into late pupal stages (Ito and Hotta, 1992; Truman and Bate, 1988), potentially allowing more accumulation of rare or small effects over many cell cycles. ….. Additionally, while many tissues can make up for lost cells via compensatory proliferation (Haynie and Bryant, 1977; Pfau et al., 2016), no such process appears to exist for replacing lost NPCs. Thus, we speculate that mutations in microcephaly genes likely affect all CB-NBs to some degree due to their inability to replace lost cells via compensatory proliferation, however the MB-NBs are especially sensitive to these mutations as a consequence of their relatively prolonged period of proliferation. Further, we speculate that a similar explanation may account for the sensitivity to microcephaly gene mutation in the human cortex."

2. H99 driven brain size rescue is not complete. MB NBs have also been shown to undergo autophagy prior to terminal differentiation. Therefore the possibility that increased autophagymay also contribute to reduced CB volume should be addressed.

We have added to our Discussion on page 16 details about the alternative possibilities of MB-NB loss, including autophagy:

"Further, our caspase-inhibition experiments did not fully suppress *traip*$^{-}$ MBphenotypes, suggesting that redundant mechanisms in addition to caspase-dependent celldeath may play a role in MB-NB loss. **For example, when caspase-dependent apoptosis is inhibited,**

> **MB-NBs are primarily lost via autophagy (Pahl et al., 2019).** Alternatively, the irregular, crenellated nuclear envelope morphology of some *traip*$^{-}$ MB- NBs (Figure 5C) could point to non-apoptotic cell death pathways (Kutscher and Shaham, 2017). Finally, aneuploidy-induced cell cycle exit in *traip*$^{-}$ MB-NBs could lead to loss via premature differentiation (Gogendau et al., 2015)."

However, we were not able to experimentally address these other possibilities of cell loss, including autophagy.

3. Since Traip is localised to the centrosomes, are there ectopic centrosomes?

We characterized the mitotic centrosomes of control and *traip*$^{-}$ MB-NBs at the 24 hours APF stage, finding no difference in centrosome morphology. These results are now presented in Figure S7E, and summarized at the end of the Results, page 13:

> "Given the centrosome and spindle localization and the apparent importance of proper mitotic localization, we hypothesized that *traip*$^{-}$ MB-NBs could have centrosome or spindle defects that could contribute to microcephaly phenotypes. However, we detected no abnormalities in centrosome numbers, γ-Tubulin recruitment to centrosomes (Figure S7E), or in the polarized localization of aPKC (Figure S7F) in metaphase 24 hours APF MB-NBs."

4. (missing)

5. Although mitotic progression is not affected, is asymmetric division machinery affected? The mislocalisation of asymmetric division machinery often accompanies centrosome defects, or mitotic spindle defects.

We characterized the localization of the polarity marker aPKC in control and *traip*$^{-}$ MB-NBs at the 24 hours APF stage, finding no mis-localization of polarity markers in the mutant. These results are now presented in Figure S7F, and summarized at the end of the Results, page 13 (see above).

6. The use of the wording "MB size" should be revised, as it is confusing whether the authors are referring to the cell size of MB NBs or MB lineage.

We changed "MB size" to "MB lobe size" throughout the manuscript.

Second decision letter

MS ID#: DEVELOP/2021/199987

MS TITLE: Traip Controls Mushroom Body Size by Suppressing Mitotic Defects

AUTHORS: Ryan S O'Neill and Nasser M Rusan

I have now received all the referees reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

The overall evaluation is positive and we would like to publish a revised manuscript in Development. I would however ask that you address two outstanding comments, and explain how you have done so in your point-by-point response. If you do not agree with these suggestions/requests, please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

see statement from previous review

Comments for the author

check: this sentence refers to something different that what is shown in 7J To test whether proper mitotic localization is important for Traip function, we introduced a Tom20 tag to ectopically force Traip to the mitochondria. Tom20::TagRFP::Traip localized cytoplasmically in interphase, and was absent from the spindle region in mitosis (Figure 7J).

Reviewer 2

Advance summary and potential significance to field

In this revised version, the authors have addressed most of my comments in a satisfactory manner. In most of the cases, the authors have toned down their conclusions instead of further supporting their initial claims. Yet, I trust this version better reports their interesting findings and the discussion better acknowledges the loose ends/open questions, which will be of great interest to the field. A minor comment would be to include some controls in the graph Fig. 7 I as having the rescue values alone looks very odd.

Comments for the author

In this revised version, the authors have addressed most of my comments in a satisfactory manner. In most of the cases, the authors have toned down their conclusions instead of further supporting their initial claims. Yet, I trust this version better reports their interesting findings and the discussion better acknowledges the loose ends/open questions, which will be of great interest to the field. A minor comment would be to include some controls in the graph Fig. 7 I as having the rescue values alone looks very odd.

Reviewer 3

Advance summary and potential significance to field

N/A

Comments for the author

I am satisfied with the revisions made.

Second revision

Author response to reviewers' comments

Reviewer Responses

Reviewer 1 Comments for the author
check: this sentence refers to something different that what is shown in 7J. To test whether proper mitotic localization is important for Traip function, we introduced a Tom20 tag to ectopically force Traip to the mitochondria. Tom20::TagRFP::Traip localized cytoplasmically in interphase, and was absent from the spindle region in mitosis (Figure 7J).

We corrected this incorrect Figure reference noted by the Reviewer.
Reviewer 2 Comments for the author
In this revised version, the authors have addressed most of my comments in a satisfactory manner. In most of the cases, the authors have toned down their conclusions instead of further supporting their initial claims. Yet, I trust this version better reports their interesting findings and the discussion better acknowledges the loose ends/open questions, which will be of great interest to the field. A minor comment would be to include some controls in the graph Fig. 7 I as having the rescue values alone looks very odd.

We added the control and traip- genotypes to the graph in Fig. 7I.

Reviewer 3 Comments for the author
I am satisfied with the revisions made.

Third decision letter

MS ID#: DEVELOP/2021/199987

MS TITLE: Traip Controls Mushroom Body Size by Suppressing Mitotic Defects

AUTHORS: Ryan S O'Neill and Nasser M Rusan

Thank you for making these final changes to the manuscript. Before I can formally accept it, I note that the revised graph you have provided in Fig 7I (which now includes controls, as requested by the reviewer) does not feature statistical significance bars. In the legend, there is no mention of the statistical analysis either, and the following sentence "Control and traip- are reproduced from Fig. 4B." left me wondering whether controls and rescue flies may not have been quantified in the same experiment. Could you please ensure that this dataset corresponds to a fully controlled, stand alone experiment, and document the statistics as you have done for all other datasets in this paper? Many thanks.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. 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.

Third revision

Author response to reviewers' comments

NA

Fourth decision letter

MS ID#: DEVELOP/2021/199987

MS TITLE: Traip Controls Mushroom Body Size by Suppressing Mitotic Defects

AUTHORS: Ryan S O'Neill and Nasser M Rusan
ARTICLE TYPE: Research Article

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