

The autosomal deubiquitylase Trabid, drives sex-divergent neurodegeneration in Drosophila.

Corresponding Author: Professor Petros Ligoxygakis

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

This manuscript by Xia et al demonstrates an interesting interaction between sex and function of the DUB Trabid relating to a neurodegeneration-like phenotype in drosophila. It includes some nice work characterizing Trabid in the fly and its relationship to human Trabid, and clearly shows a sex-specific impact of the mutant in several measures. However, a few data interpretation/ visualization details obscure interpretation of the manuscript, and improvement of these features would, I believe, vastly improve the interpretability of the manuscript. Points below:

- 1) In many cases, the authors include statistical analysis of only a subset of comparisons mentioned in the paper. This is especially relevant when noting no significant difference between conditions. Although this may have been assessed in statistical testing (and it looks like authors used ANOVAs for most of these appropriately) it is difficult for the reader to understand whether there has been an appropriate post hoc comparison unless it is depicted in the data visualization. Especially as the authors are looking at two key differences - those between wt and mut as well as those between females and males - they should take care to include all tests that are relevant to the interpretation.
- 2) In the figure referencing sleep health particularly (also in fig 6), but also across the paper, it is somewhat opaque to display data as bar charts with SEM error bars. It would be very helpful for interpretation to plot the data points themselves overlaid with the bars, and wherever possible it is helpful to include data points directly.
- 3) Using lifespan as an example, there are a couple of claims that end up being relevant for the direction of the paper that are a little tough to interpret. For example, the authors suggest that there is no difference between the non-mutant female and male lifespans; however, the lack of a statistically significant difference does not necessarily prove sameness. In this particular case, I would suggest the authors just soften their claims and focus on the more obvious difference between the mutants.
- 4) However, this brings up a larger point about how the paper discusses male and female differences. In several cases, the female animals do display some deficit or tendency but are just not as severe as the males, or there is an independent effect of background/mutant as in the sleep data. Likely including more careful comparisons across all conditions as mentioned above will aid in the interpretation of these experiments for the reader, but the narrative of the paper generally suggests that problems are unique to male animals to perhaps a larger extent than they are. With the exception of the claim of no difference between female mutant and wild type animals in figure 3 B+C, where it seems there are in fact observed differences, everything seems largely correct. Overall, the science seems sound, and I think this is more of a descriptive problem for manuscript writing than a need to do additional experimentation. In fact, I find it quite interesting that similar problems arise in male and female animals with different severity and timelines. Take also as an example lesion size - it's clear the females are affected but to a lesser extent. These differences are used as motivation for identifying Hsc-70, but those experiments are still worthwhile in a case where both sexes are affected but males are affected to a greater extent.
- 5) Figure 4 is a little opaque to interpret based on the normalization and genetic strategies. Could the increase in repo be based on anything other than raw cell number? And does the eventual decrease in males actually reflect cell loss, or is it just a return to baseline after perhaps changes in reporter expression? It's a little difficult to interpret what these data actually mean, and especially whether this correlates to cell death without a marker for cell death or change in neural integrity in some way. For this reason I found the lesion size quantification to be a little more clear, and wonder whether the rest could

just move to supplemental. It would be excellent to show another measure of cell loss or dysfunction if possible, but the paper could be published without it if it proves impossible. I found that the glial rescue experiments from earlier in the paper were more valuable for a mechanistic interpretation, so if I had to pick extended figure content to include in the main figures I would swap for those data. This could also help the authors narratively motivate the choice to use astrocytes in the final figures of the paper more easily.

6) Figure 5 data visualization is difficult to read, especially with names overlayed on top of each other. The addition of GO/DME terms in A and B was also hard to interpret and ought to be displayed separately.

7) The authors demonstrate nicely that Hsc70 knockdown synthesizes with Trabid C518A in female flies, suggesting a mechanism for the sex-specific effect. These data are clear and impactful, but authors would benefit from including male flies with similar conditions. We would expect that if the interaction between Hsc70 and Trabid is responsible for the male specific effect that the difference in males would not be exacerbated or would be minimally exacerbated by knockdown of Hsc70. Passing rate of the climbing assay is one of the more clear sex specific effects also, so I am curious whether the female knockdown of Hsc70 with C518A Trabid recapitulates the Eosin-measured lesion size of the males. Those two experiments if added I think would really hammer home the points made by the authors!

Overall, the manuscript is well done and will benefit from mostly minor changes.

Reviewer #2

(Remarks to the Author)

Reviewer's remarks

Xia and colleagues investigate the role of Trabid, a deubiquitylase, in driving sex-specific neurodegeneration in *Drosophila*. Studying the *Drosophila* homologue, they determine it has the same enzymatic activity as the human Trabid by assaying hydrolysis products of diubiquitin chains. They then assay health indicators in catalytically dead Trabid and measure locomotor and lifespan in these flies. Trabid has been shown to influence wnt signaling (Tran et al 2008), Imd pathway (Fernando et al 2014), NF- κ B (Kounatidis et al 2017), among others. This manuscript builds on their previous work on Trabid, NF- κ B and assaying health markers like longevity and locomotor function to assign a role for Trabid in neurodegeneration. Mechanistically, or so I am assuming, they isolate Hsc70-4 as the downstream effector of the phenotype they get from Trabid LoF.

This work is an addition to the knowledge base of Trabid function specifically and to DUBs widely and address the complexity present in pinpointing functions from organismal assays. This manuscript brings together their previous work on Trabid, TAK1, IMD-NF- κ B and pirk with an emphasis in tying the NF- κ B function to Trabid and specifically sex-specific neurodegeneration that results in locomotor and lifespan deficits. While they have laboriously shown some great data on Trabid function, the following key issues should be resolved so as to improve the quality of the manuscript before it is formally accepted for publication.

General concerns

1. The current lifespan assay using only 20 flies falls short of previous numbers (240 in the NF- κ B paper) and is too low for behavioral assessment. In Fernando et al 2014, they showed loss of Trabid leads to a compromised lifespan. The main readout in this manuscript is sex-specific neurodegeneration driven by Trabid which is novel and of interest to the field. Of concern is that neuronal Gal4-UAS intervention does not rescue Trbd loss of function. More problematic is the eosin assay they use. While they cite Jäkel S & Dimou L (2017), there is no mention of eosin as an acceptable assay for neurodegeneration here. A proper citation here would be Cao et al 2013 or any other of their previous publications. Additionally, they use area as opposed to volume and pair it with immunostaining to determine brain size.

2. The LC-MS data specifically Fig EV5B is a copy of EV5A compared to the analysed data in Fig 5. The filtering of Hsc70-4 does not have a strong rationale tied to it as it is not the most up or downregulated target. Table EV1 alludes to muscle function as the potential targets and function of Trabid. The predictive use of ColabFold to "confirm" protein-protein interaction between Hsc70-4 and Trabid is curious since they perform co-IP experiments. The WB is a little hard to follow. For example, why is there a large GFP signal in Lane 3 (mCherry-AnkOTUC518A)? Would a reverse probe with anti-mCherry yield similar conclusions?

Major revisions

1. Line 106 The homology figure in 1A would be more informative if presented the classical way showing the amino acids being compared as shown in Fig EV7
2. In Fig 1D there is hydrolysis of diubiquitin chains 6 and 48 though very faint by-product is observed. I believe the authors should mention why this phenomenon is observed in this case. In Fig EV1 it appears as if there is activity in C518A Lys63 as the samples are incubated for longer than 30 minutes. This needs to be explained.
3. At this point I would like to reiterate the importance of labeling the figures (top right corner) which would help the reviewer in following the manuscript.
4. Line 132 How are these two constructs biochemically and functionally different?
5. Line 133 This claim though sensible requires a citation to support it since there is body of research that shows DUBs exhibit specificity. Would a small molecule inhibitor experiment clarify this position? I would presume that a target that is promiscuous would tend not to be phenotypically robust.
6. Line 141 While locomotor dysfunction can and is used as readout for neurodegeneration, it is important to clarify that not all neurodegeneration results in this defect.
7. Line 168 When making this cross you can mention the line was balanced to specify how you differentiated the

homozygotes from the heterozygotes.

8. It is eluding me how the rescue in Line 175 using the Gal4/UAS took place as I do not see any information on a UAS-trbd construct or stock number in the M&M. Additionally, this rescue (since the flies were already present) should have been extended to lifespan experiments.

9. Is the locomotor deficit originating from Trabid function in glia? Since rescue in neurons is not sufficient to suppress the phenotype, I would ask to see rescue data using a muscle specific GAL4 driver since the main readout is locomotor deficit.

10. I am not sure if the sleep phenotype is as a result of lower activity in mutant flies (since sleep is measured as inactivity in flies). As these flies already have a robust locomotor deficit wouldn't it follow they will move less frequently? Column comparisons should prioritize mutant male to wt male, mut female to wt female and mut male to mut female. Since sleep is regulated at the neuronal level, would the UAS-trbd rescue this sleep phenotype?

11. The citation (Jäkel S & Dimou L (2017)) given for the use of eosin as a quantification method does not mention eosin in its text and is misleading. The method to quantify the neuron and glia content of whole brains appear insufficient as it relies on immunostaining. How was autofluorescence and off-target intensity calculated? A brain volume-based quantification would be better than area. Comparison wise, Fig 4 C to H, the Trabid flies should be compared to their wildtypes first.

12. Fig EV5B appears to be an exact match to EV5A which is a comparison between male and female Trabid with their controls. This is in contrast to the LC-MS data that shows different patterns for females and males.

13. The rationale for Hsc70 as a target for Trabid while it is not exactly the most upregulated or downregulated escapes me. Other studies show that ubiquitination of Hsc70 leads to its degradation.

14. The protein interaction experiment in Fig 8 is highly problematic. Co-expressing two synthetic constructs is not conclusive to interaction. A better but painful experiment would be to use one or the other in S2 cell or adult brain lysates to perform co-immunoprecipitation.

15. Hua et al 2022 have published a Trabid polyclonal antibody that would benefit the manuscript in staining the brain and performing Ips to show the binding of Trabid to Hsc70-4?

Minor revisions

1. Line 79 Sentence starting with "In mice," needs rewriting to make some sense and correct the in-text citation style

2. Line 87 Consistency in using either Trabid or dTrabid in the manuscript

3. Line 95 Delete most in regards to significance

4. In results, make the in-text citation style uniform

5. Line 115 Is this bacterially expressed Trabid? If so it should be mentioned in this sentence to make it clear to the reader

6. Line 117 When the concentration is increased to 600 nM it is not clear to the reader why there is a reduction in 63. Can Fig. EV1 be normalized and quantified?

7. Fig 2B The "NS" needs to be included in the figure from line 149

8. Line 223 What is unique between the two AMP10 should be described that allow rescue from one and not the other

9. Line 280 What was the rationale for 200 heads compared to say 1000? Not triplicates?

10. Line 304 How does EV6A or B, which are principal components QC plots, show regulatory processes? Same to line 305

11. Line 377 How is the claim Hsc70 a direct target of Trbd supported? Same to the claim based on ColabFold (a prediction tool). This is only a prediction not a confirmation. This claim is only supported after the immunoprecipitation experiments.

Reviewer #3

(Remarks to the Author)

Brief summary of the manuscript

In this manuscript, Xia et. al. investigate the role of the Drosophila deubiquitylase Trabid in driving sex-specific neurodegeneration. Using a CRISPR-generated enzymatic-null point mutation (trbdC518A), the authors demonstrate that inactivation of Trabid results in male-specific neurodegenerative phenotypes, including locomotion deficits, fragmented sleep, reduced lifespan, glial and neuronal loss, and increased inflammatory signaling. In contrast, female mutants show elevated ubiquitination of neuroprotective proteins like Hsc70-4, which appears to mitigate degeneration. The study uses a comprehensive combination of behavioral, histological, proteomic, and genetic approaches to support its conclusions.

Overall impression

The study addresses an important and underexplored topic: sex-specific vulnerability to neurodegeneration. The work convincingly links inflammatory regulation, ubiquitin homeostasis, and neuronal survival to the enzymatic activity of Trabid. Importantly, the authors identify Hsc70-4 as a key neuroprotective factor, whose regulation appears to differ by sex and depend on direct interaction with Trabid. However, some interpretations require clarification or stronger supporting evidence, and a few experiments or analyses would substantially improve the manuscript's rigor and clarity. In particular, the use and validation of CRISPR lines, RNAi efficiency, sample sizes, and mechanistic interpretation of proteomic data would benefit from refinement or expansion.

Specific comments

Major comments

1. In the Methods section, the authors mention that two independent trbdC518A lines were maintained and used for experiments. Please clarify whether both lines were consistently used across all key experiments, or whether one was prioritized. If only one line was primarily analyzed, I recommend replicating a subset of critical phenotypes (e.g., locomotion, lifespan) in the second line, or providing a strong justification for why this was not feasible.

The rescue experiments using wild-type trbd expression in glia and ubiquitously are well designed and support the specificity of the phenotype. However, for full rigor, please clarify whether any additional steps were taken to control for off-target CRISPR edits, such as predicted off-target site screening or backcrossing to an isogenic background.

2. Lifespan assays are highly sensitive to husbandry and environment, so key variables must be tightly controlled and explicitly reported. Please clarify in Methods (and echo key details in the relevant figure legends) how adult cohorts were age-synchronized (timed collections/eclosion window), whether sexes were housed together or separately and adults were mated or virgins, the exact diet formulation (e.g., 10% SY), temperature, humidity, and light:dark cycle, how larval density was controlled during development, and any incubator position randomization/rotation to avoid rack effects. Notably, the literature typically reports shorter lifespan in wild-type males than females under standard 10% SY at 25 °C; your data appear to show the reverse. Please reconcile this discrepancy—e.g., by clarifying diet composition/batch, genetic background, microbiota, mating regime, density, censoring/exclusion criteria, or other procedural differences. Importantly, please report the number of independent biological replicates per genotype.

3. It is unclear what the three strains designated Δ AMP10, Δ AMP10_Ch2, and Δ AMP10_Ch3 specifically comprise—i.e., which AMP genes are deleted in each and how they were generated. Why does Δ AMP10_Ch3 rescue whereas Δ AMP10_Ch2 does not? Were AMP transcripts or peptides levels measured in trbdC518A flies, and do the data implicate particular AMPs or indicate a broader AMP-dependent effect? Please clarify these points and ensure that all AMP genotypes are fully described in the Methods (composition, origin/stock IDs, and genetic background/backcrossing).

4. The authors correctly point out that the w1118 and yw backgrounds can influence climbing assays. It may be helpful to mention whether any genetic background effects were observed in the Δ AMP10 rescue experiments.

5. The eosin assay is a useful screen. Please include representative raw z-stacks.

6. Please revise the legend for Figure 4: the descriptions do not match the panels shown. Ensure each legend item corresponds to the correct panel (letters, labels, and order) and that the terminology aligns with the main text.

7. In the ubiquitylation and proteomic profiling, the authors compare trbdC518A males vs w1118 males and trbdC518A females vs w1118 females to suggest sex-specific effects; however, the within-genotype sex comparisons are missing and are necessary. Figure 5: To better visualise sex-specific differences please add volcano plots for trbdC518A males vs females and, for context, w1118 males vs females. Clearly label the axes (e.g., x = log2 fold change [male/female], y = -log10 q-value [FDR-adjusted P]). Finally, the Figure 5B legend appears to reference the female panel (Fig. 5A); please correct it so that it accurately describes the male comparison.

8. The order in which the figures are discussed in the Results section is confusing and disrupts the logical flow of the manuscript. The text first introduces Figure 5C, then shifts to a full paragraph on Figure 6A–C, then returns to Figure 5C, followed by references to Figure 5A and 5C again, and only afterward introduces Figure 6D. This back-and-forth between figures, particularly crossing figure numbers in a non-sequential way, breaks the narrative structure and makes it difficult for readers to follow the progression of the results. Figures should be discussed in numerical and conceptual order, aligning with how the data build upon each other. Consider restructuring the Results section so that all of Figure 5 is covered before moving on to Figure 6, and avoid returning to earlier figures after discussing later ones.

9. The manuscript states that Lys6/Lys27/Lys33 linkages accumulate “in females but not in males,” which overstates the contrast: Fig. 6A–C shows no significant increase in males, but mean values are comparable or only modestly lower than in females. Please soften the wording: not statistically significant does not mean no effect. The male data show a similar trend that does not reach significance. Provide the within-genotype male–female comparison (or an interaction test) to support a female-specific conclusion. It is also unclear whether multiple-testing correction was applied across linkage analyses—please clarify and report adjusted q-values. Because K29 was not measured and the in vitro substrate profile differs from the in vivo pattern (in vitro K29/K33 vs in vivo K6/K27/K33), a brief explanation or caveat in the Discussion would help. The sentence “Except M1 and Lys29 linkages, the GlyGly enrichment allowed us to compare ubiquitin linkages...” is unclear—please state which linkage types were quantified, which were not, and why (e.g., not detected/below confidence/method limitation), and indicate explicitly whether K29 was analysed. Define the y-axis (“log2 normalized value”: what is normalized, and to what reference?), specify all statistical contrasts reported (trbdC518A F vs w1118 F; trbdC518A M vs w1118 M; trbdC518A F vs trbdC518A M; w1118 F vs w1118 M), and ensure the legend/brackets match those contrasts; also correct the y-axis typo to “normalized.”

10. The manuscript states that Hsc70-4 is one of the most divergently ubiquitylated proteins between trbdC518A females and males, being upregulated in females and downregulated in males relative to sex-matched w1118 controls. Please make the contrasts explicit and, if you wish to claim sex-divergent regulation, report the within-genotype male–female comparison (or a sex $\times$ genotype interaction); otherwise avoid wording that implies a tested cross-sex difference. The conclusion currently rests on ubiquitylation readouts only; please indicate whether total Hsc70-4 (transcript or protein) also differs by sex or genotype, and whether site/protein-level ubiquitylation was interpreted relative to total abundance. The assertion that autosomal Trabid affects X-linked proteins (HIP/HIP-R) is interesting but speculative, either soften it or support it with additional analysis (e.g., considering sex-chromosome dosage/compensation or proposing a direct pathway). For Fig. 5C,

document the inclusion thresholds ($|\log_2\text{FC}|$ cutoff, adjusted q-values), state whether these were pre-specified, provide the number of biological replicates and the total background set used for the Venn, and define what you mean by “divergently ubiquitylated.” Also align the legend wording with these criteria. As a clarity aid, consider adding a volcano or table showing effect sizes and q-values for Hsc70-4 and HIP/HIP-R.

11. The claim that Hsc70-4 is neuroprotective in *trbdC518A* females rests on astrocyte RNAi worsening climbing at day 18 (Fig. 6D). Please add a sufficiency test: astrocyte-specific Hsc70-4 overexpression in *trbdC518A* males to ask whether increasing Hsc70-4 can rescue locomotion/sleep/lifespan. If development is a concern, use an adult-onset driver (GeneSwitch) to separate developmental from adult effects.

12. The manuscript does not show whether Hsc70-4 knockdown is efficient or specific in astrocytes. Please include transcript or protein-level validation of the RNAi line in the appropriate tissue context, or include a second, non-overlapping RNAi line.

13. Please document adult driver specificity and, if feasible, test an independent astrocyte driver. Clarify whether sexes were assayed in parallel and whether male astrocyte RNAi was tested (even if negative), as that would bound the sex bias.

14. The ColabFold scores are below typical confidence (pTM 0.437; ipTM 0.268), the HDOCK model uses human structures, and the co-IP is in overexpressing S2 cells with a domain fragment (AnkOTU^{C518A}). Either soften the language (“candidate interactor”) or add in-vivo support: endogenous head co-IP (or glia-enriched prep), reverse IP, substrate-trapping with catalytically dead Trbd showing increased Hsc70-4 binding, and site-level ubiquitylation changes on Hsc70-4 in heads that track with genotype/sex.

15. Please align several Discussion statements more tightly with the presented evidence. In particular, treat Hsc70-4 as a candidate interactor or neuroprotective factor, rather than a demonstrated direct *Trabid* substrate, unless in vivo substrate validation is added. Clarify whether ubiquitylation changes were interpreted independently of total Hsc70-4 protein levels, or whether such quantification was performed. Statements describing effects “in females but not in males” should be supported by within-genotype sex comparisons or formal sex $\times$ genotype interaction tests, rather than inferred from separate sex-matched contrasts.

Additionally, the observed mismatch between *Trabid*’s in vitro K29/K33 linkage preference and the in vivo accumulation of K6/K27/K33 chains in female heads should be acknowledged as a limitation. Please consider offering possible mechanistic or technical explanations (e.g., indirect effects, detection thresholds, or secondary chain editing).

Regarding the AMP/Relish axis and the ΔAMP10 rescue, the inference implicating specific AMPs like Drs or AttD remains hypothesis-level. Please either soften the mechanistic claims or support them with targeted validation (e.g., loss-of-function or rescue of single AMPs in the *trbdC518A* background, or tissue-specific AMP manipulations).

Finally, where cross-species implications are proposed (e.g., extrapolating *Trabid*/Hsc70-4 interactions to mammals), please clearly frame these as model-based or speculative, unless functional evidence is provided. These adjustments would help keep the Discussion ambitious yet well-calibrated to the presented data.

16. In the Introduction you state that tissue-specific transformer (*tra*) knockdown attenuates some sex differences, and the Methods list a *tra* RNAi line; however, I cannot find the corresponding experiments in the Results or figures. If these data exist, please present them explicitly, with context, quantification, sample sizes, and statistics, and cross-reference the exact figure/panel. If the experiments were not performed or are unavailable, please remove or soften the claim in the Introduction/Methods.

Minor comments

- The manuscript reports rescue with actin-GAL4 and repo-GAL4 but not with elav-GAL4, yet no rationale is offered. Please comment on potential reasons (driver strength/timing, transgene expression levels in heads, cell-type specificity) and, if feasible, provide supporting data (qPCR/Western for transgene expression, or an alternative neuronal driver/adult-onset driver) to clarify why neuronal expression fails to rescue.

- Line 201, please rephrased to better understand at which males authors are talking about.

- Line 291, “ $p < 0.05$ and fold change $-\log_2 > \text{or } \log_2 < -1$ ” which is unclear.

- In Methods add catalog numbers for antibodies, di-ubiquitin standards, and key reagents.

- References. Check consistency of citation style (e.g., 38–41) and ensure all database or software tools (AlphaFold2/ColabFold, HDOCK, BioRender) are cited per their guidelines.

- Line 714: “Two sample t-test was performed, and we used proteins that were $\text{FDR} > 0.05$ and $\log_2$ fold change < -1 or > 1 to perform Venn diagram analysis.” As written, this selects non-significant proteins and uses a nonsensical log-FC threshold (> -1 includes virtually everything). Please correct to $\text{FDR} < 0.05$ and a symmetrical $|\log_2\text{FC}|$ cutoff (e.g., > 1), and ensure these criteria match what is used in all figures.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have added and clarified several sections that have proved informative, particularly removing some data that were confusing to understand and adding especially 6D. I would also like to thank the authors for including original data in their bar graphs now, as these data greatly improve the interpretability of the manuscript and my confidence that their findings are accurately reported. My main concern is that the story the authors tell right now does not fully match the data they have taken great pains to acquire. It seems as if the story will remain compelling and important to the field even if certain points are toned down (notably how specific the phenotype is to males, how significant the change of Hsc70-4 is).

Below are some points the authors should address:

- After the reorganization of the text, in some places the narrative has become slightly fractured and reads rather disjointed. As an example, more description is needed to introduce the role of astrocytes in this pathway, and the jump in and out of astrocyte and AMP-related genes is a little confusing just for readability.
- Across all experiments, authors should be sure to describe strategies, strains, and acronyms/abbreviations with great attention to detail, as they have done in many cases for us as reviewers in the rebuttal (e.g. what is arlm? Which genes are impacted that make delta chr. 2 and delta chr. 3 different? What is oamean and how should the reader interpret it?)
- In (an admittedly smaller) handful of places, the authors still claim that a phenotype is male or sex-specific on occasions when females are also affected. Throughout the text, it would make interpretation less awkward to just emphasize the increased severity of several measures in males. Also, do these differences (for example female effect in trabid del but not c518A) help the authors tell their story (i.e. that differences in ubiquitination cause the male phenotype in c518A that would not be recapitulated in trabid del) perhaps?
- It is unfortunate that the authors' prediction that Hsc70-4 is differentially regulated on the protein level is not supported. However, this finding should either be more clear to the reader in the text (for example, authors should not say it is upregulated when the expression and protein levels do not change) or the authors could reevaluate the data after completing a third biological replicate of the mass spectrometry experiments. The selection of Hsc70-4 is interesting as a target regardless of the outcomes, but the authors should describe clear and correct reasons for its selection. The addition of 6D is very powerful for the narrative, and I am glad the authors completed the experiment! If the authors are able to do the add-back of Hsc70-4 in males that would be lovely, but I take the point that making an additional strain may be out of the scope at this time.

Minor:

- For Fig 2D, the authors should clarify claim that males are more affected. Instead of motivating by p value, authors should motivate by the significant difference between groups as indicated.
- Statistics are missing for EV2B and ought to be included.
- In line 295, authors say that trbd is required in astrocytes; however, they perform a sufficiency experiment. The authors should either clarify the wording or perform a necessity experiment (knockdown or mutant).
- For figure 3B, labels should clarify that the AMP10s are on the trbdC518A background
- In general, the authors could clean up some figures and labels (for example, EV4A) to make the manuscript more polished.

Reviewer #2

(Remarks to the Author)

REVIEWER REMARKS

I appreciate the efforts the authors have made in improving the quality of this manuscript and applaud the rigor as portrayed in the rebuttal. This is clearly a much-improved work that is ready for publication. In addition, I believe that the authors could make one small tweak to the final manuscript that could benefit the reader. This is only a suggestion based on my own frustration to understand the alignment figure.

Suggested revision:

1. It is still my opinion that Fig 1A would benefit the reader by having the amino acid sequences as opposed to blocks of color. Having the amino acid sequences allows the reader to make critical observations on domain conservation among other protein structural/sequence features.

It also makes it clear to the reader the protein sequence used by the authors in the case of revision to the sequence database.

The authors have put in a lot of changes, from my view, these changes reflect an improved manuscript.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have completed all necessary changes and I believe the manuscript is ready for publication.

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RESPONSE TO REVIEWERS COMMSBIO-25-7921

Reviewer #1 (Remarks to the Author):

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1) In many cases, the authors include statistical analysis of only a subset of comparisons mentioned in the paper. This is especially relevant when noting no significant difference between conditions. Although this may have been assessed in statistical testing (and it looks like authors used ANOVAs for most of these appropriately) it is difficult for the reader to understand whether there has been an appropriate post hoc comparison unless it is depicted in the data visualization. Especially as the authors are looking at two key differences - those between wt and mut as well as those between females and males - they should take care to include all tests that are relevant to the interpretation.

RESPONSE: We have indeed performed all possible comparisons but showed only those helpful to the interpretation for sex-specific effects in mutant flies. Following this suggestion we have added “ns” (non-significant) to most multiple comparisons, including in Fig. 2C, Fig. 3, Fig. 4, Fig. 5A-C and E-F, and Fig. EV4E. For the two-way ANOVA analysis in Fig. 2B and Fig. EV2A we did not add “ns”, due to the significant number of comparisons, which would cause readers’ confusion with understanding which data are relevant to the argument. All statistical tests that we used are now annotated in the figure legends.

2) In the figure referencing sleep health particularly (also in fig 6), but also across the paper, it is somewhat opaque to display data as bar charts with SEM error bars. It would be very helpful for interpretation to plot the data points themselves overlaid with the bars, and wherever possible it is helpful to include data points directly.

RESPONSE: This is a very good point. We have changed all SEM error bars to 95%CI, including Fig 2B-C, Fig 3, Fig 4, Fig 6D-F, Fig EV2A. We checked the normality of the data before further comparison tests. Data that did not pass the normality check was subject to nonparametric comparison.

3) Using lifespan as an example, there are a couple of claims that end up being relevant for the direction of the paper that are a little tough to interpret. For example, the authors suggest that there is no difference between the non-mutant female and male lifespans; however, the lack of a statistically significant difference does not necessarily prove sameness. In this case, I would suggest the authors just soften their claims and focus on the more obvious difference between the mutants.

RESPONSE: We compared sexes within the same genotype as well as sex-matched samples across genotypes. As the reviewer suggested, the lack of differences between non-mutant females and males in some phenotypes—including lifespan, climbing pass rate, and brain lesion rate—does not imply that the two sexes are identical. This conclusion is supported by clear sex-specific differences in sleep patterns and omics data. Accordingly, in the revised text we removed the original statement and focused on sex-matched comparisons between genotypes.

4) However, this brings up a larger point about how the paper discusses male and female differences. In several cases, the female animals do display some deficit or tendency but are just not as severe as the males, or there is an independent effect of background/mutant as in the sleep data. Likely including more careful comparisons across all conditions as mentioned above will aid in the interpretation of these experiments for the reader, but the narrative of the paper generally suggests that problems are unique to male animals to perhaps a larger extent than they are. Except for the claim of no difference between female mutant and wild type animals in figure 3 B+C, where it seems there are in fact observed differences, everything seems largely correct. Overall, the science seems sound, and I think this is more of a descriptive problem for manuscript writing than a need to do additional experimentation. In fact, I find it quite interesting that similar problems arise in male and female animals with different severity and timelines. Take also as an example lesion size - it's clear the females are affected but to a lesser extent. These differences are used as motivation for identifying Hsc-70, but those experiments are still worthwhile in a case where both sexes are affected but males are affected to a greater extent.

RESPONSE: Here the reviewer made a series of constructive suggestions. We largely rewrote the manuscript adding new experiments (including eosin brain staining in *trbd*^{C518A} flies with reduced Hsc70-4 levels, new lifespan experiments and new protein-protein interaction co-IPs between Hsc70-4 and Trabid). As we mentioned in the response to question 3), we abandoned the focus on differences between control male and female. It is relevant here to note that eosin staining in brains of *trbd*^{C518A} females with reduced Hsc70-4 levels in astrocytes increased

brain lesions compared to *trbd*^{C518A} females (Fig 6E).

5) Figure 4 is a little opaque to interpret based on the normalization and genetic strategies. Could the increase in repo be based on anything other than raw cell number? And does the eventual decrease in males reflect cell loss, or is it just a return to baseline after perhaps changes in reporter expression? It's a little difficult to interpret what these data mean, and especially whether this correlates to cell death without a marker for cell death or change in neural integrity in some way. For this reason, I found the lesion size quantification to be a little clearer, and wonder whether the rest could just move to supplemental. It would be excellent to show another measure of cell loss or dysfunction, if possible, but the paper could be published without it if it proves impossible. I found that the glial rescue experiments from earlier in the paper were more valuable for a mechanistic interpretation, so if I had to pick extended figure content to include in the main figures I would swap for those data. This could also help the authors narratively motivate the choice to use astrocytes in the final figures of the paper more easily.

RESPONSE: A few reviewers pointed out issues with the brain immunofluorescent staining experiment. Since the eosin brain lesion staining is a more informative result to evaluate the cerebral damage of flies. We dropped the entire dataset and only used the eosin brain lesion experiments in the revised manuscript. For rescue experiments, as the reviewer suggested, in the revised manuscript, we only presented data using *alrm-GAL4* (astrocyte driver) (Fig. 6D-F, Fig. EV2B).

6) Figure 5 data visualization is difficult to read, especially with names overlayed on top of each other. The addition of GO/DME terms in A and B was also hard to interpret and ought to be displayed separately.

RESPONSE: We remade the volcano plots and GO/DME terms (Fig. 5A-B and Fig. EV3 A-B). Since we reduced a large amount of illustration of omics data, we currently focus only on omics that are associated with immunity and ubiquitylation. The GO/DME terms presented is only related to immunity and ubiquitylation.

7) The authors demonstrate nicely that Hsc70 knockdown synthesizes with Travid C518A in female flies, suggesting a mechanism for the sex-specific effect. These data are clear and impactful, but authors would benefit from including male flies with similar conditions. We would expect that if the interaction between Hsc70 and Travid is responsible for the male specific effect that the difference in males would not be exacerbated or would be minimally exacerbated by knockdown of Hsc70. Passing rate

of the climbing assay is one of the clearer sex specific effects also, so I am curious whether the female knockdown of Hsc70 with C518A Trabid recapitulates the Eosin-measured lesion size of the males. Those two experiments if added I think would really hammer home the points made by the authors!

RESPONSE: Here, the reviewer provided another constructive suggestion. We performed these experiments, and the results are presented in Figs 6E and F. We knocked down Hsc70-4 in astrocytes in a *trbd*^{C518A} genetic background. Females showed an increase in the number of brain lesions compared to control *trbd*^{C518A} females. In contrast, reduction of Hsc70-4 expression did not have an effect in the number of brain lesions in male *trbd*^{C518A}.

Overall, the manuscript is well done and will benefit from mostly minor changes.

Reviewer #2 (Remarks to the Author):

Xia and colleagues investigate the role of Trabad, a deubiquitylase, in driving sex-specific neurodegeneration in Drosophila. Studying the Drosophila homologue, they determine it has the same enzymatic activity as the human Trabad by assaying hydrolysis products of diubiquitin chains. They then assay health indicators in catalytically dead Trabad and measure locomotor and lifespan in these flies. Trabad has been shown to influence wnt signaling (Tran et al 2008), Imd pathway (Fernando et al 2014), NF- κ B (Kounatidis et al 2017), among others. This manuscript builds on their previous work on Trabad, NF- κ B and assaying health markers like longevity and locomotor function to assign a role for Trabad in neurodegeneration. Mechanistically, or so I am assuming, they isolate Hsc70-4 as the downstream effector of the phenotype they get from Trabad LoF.

This work is an addition to the knowledge base of Trabad function specifically and to DUBs widely and address the complexity present in pinpointing functions from organismal assays. This manuscript brings together their previous work on Trabad, TAK1, IMD-NF- κ B and pirk with an emphasis in tying the NF- κ B function to Trabad and specifically sex-specific neurodegeneration that results in locomotor and lifespan deficits. While they have laboriously shown some great data on Trabad function, the following key issues should be resolved to improve the quality of the manuscript before it is formally accepted for publication.

General concerns

1. The current lifespan assay using only 20 flies falls short of previous numbers (240 in the NF- κ B paper) and is too low for behavioural assessment. In Fernando et al 2014,

they showed loss of *Trabid* leads to a compromised lifespan. The main readout in this manuscript is sex-specific neurodegeneration driven by *Trabid* which is novel and of interest to the field. Of concern is that neuronal Gal4-UAS intervention does not rescue *Trbd* loss of function. More problematic is the eosin assay they use. While they cite Jäkel S & Dimou L (2017), there is no mention of eosin as an acceptable assay for neurodegeneration here. A proper citation here would be Cao et al 2013 or any other of their previous publications. Additionally, they use area as opposed to volume and pair it with immunostaining to determine brain size.

RESPONSE: As the reviewer pointed out, the previous version caused confusion regarding both sample size and the assessment of neurodegeneration using brain immunofluorescence (IF) and eosin staining. To address the sample size issue in lifespan, we clarify that each vial contained 20 flies and that approximately 8 vials were used per biological replicate, with a total of three biological replicates (Fig. 2D legend, lines 959–964 and Materials and Methods, line 628). We also added sample size information and statistical test details to the legends of other figures. Regarding the IF data, the reviewers raised multiple concerns; therefore, we removed these data from the revised manuscript. As suggested by the reviewer, we also cited Cao *et al.*, and Arora *et al.*, as the appropriate references.

2. The LC-MS data specifically Fig EV5B is a copy of EV5A compared to the analysed data in Fig 5. The filtering of Hsc70-4 does not have a strong rationale tied to it as it is not the most up or downregulated target. Table EV1 alludes to muscle function as the potential targets and function of *Trabid*. The predictive use of ColabFold to “confirm” protein-protein interaction between Hsc70-4 and *Trabid* is curious since they perform co-IP experiments. The WB is a little hard to follow. For example, why is there a large GFP signal in Lane 3 (mCherry-AnkOTUC518A)? Would a reverse probe with anti-mCherry yield similar conclusions?

RESPONSE: In the previous version, Fig. EV5 essentially duplicated Fig. 5. In the revised manuscript, Fig. EV3 now correctly presents the proteomic data. We selected Hsc70-4 for further analysis because we observed sexually dimorphic, neurodegeneration-related phenotypes in *trbd*^{C518A}. Although Hsc70-4 was not the strongest hit, it was the only candidate that showed sex-specific regulation in both the ubiquitinome and proteome datasets.

Functionally, astrocyte-specific knockdown of Hsc70-4 in *trbd*^{C518A} females reduced climbing pass rates and increased brain lesion frequency, despite *trbd*^{C518A} females having a lower baseline lesion rate than males (Fig. 6E). In contrast, Hsc70-4 knockdown in *trbd*^{C518A} males did not further increase brain lesion rates beyond the male baseline.

As the reviewer noted, the original ordering of the *in-silico* analysis before the co-immunoprecipitation (Co-IP) data was awkward. In the revised version, we

present the *in-silico* predictions immediately prior to the new IP experiments (Fig. 7). The *in-silico* analysis predicted a potential interaction between Travid-AnkOTU and Hsc70-4. Consistent with this prediction, in the IP experiments, mCherry-Travid-AnkOTU^{C518A} was used as bait to immunoprecipitate endogenous Hsc70-4. In the left blot of Fig. 7E, the mCherry-Travid-AnkOTU^{C518A} signal (~90 kDa) was enriched following immunoprecipitation with mCherry-Trap. In the right blot of Fig. 7E, endogenous Hsc70-4 was detected in the IP eluate using an Hsc70-4-specific antibody.

Major revisions

1. Line 106 The homology figure in 1A would be more informative if presented the classical way showing the amino acids being compared as shown in Fig EV7

RESPONSE: Fig. 1A presents an alignment of the Travid amino acid sequences from three species. The schematic at the top illustrates the domain organization of Travid, while the alignment occupancy shown below summarizes the degree of sequence conservation across species. We carefully considered the reviewer's suggestion to present the alignment in a conventional residue-by-residue format, as shown in Fig. EV6 (previously Fig. EV7). However, we found that this alternative presentation was less concise and did not substantially improve the clarity or informativeness of the figure.

2. In Fig 1D there is hydrolysis of diubiquitin chains 6 and 48 though very faint by-product is observed. I believe the authors should mention why this phenomenon is observed in this case. In Fig EV1 it appears as if there is activity in C518A Lys63 as the samples are incubated for longer than 30 minutes. This needs to be explained.

RESPONSE: The chase-pause DUB assay started at 0 min and reactions were stopped at 5 and 30 min. The mono-ubiquitin bands in Lys 6 and 48 lanes were originally from the diubiquitin preparation, which can be regarded as background. The mono-ubiquitin intensity of these two samples did not increase at 5 and 30 mins.

3. At this point I would like to reiterate the importance of labelling the figures (top right corner) which would help the reviewer in following the manuscript.

RESPONSE: We made the labelling of the figures as the reviewer suggested.

4. Line 132 How are these two constructs biochemically and functionally different?

RESPONSE: The differences between the two constructs were previously

explained and are now explained in lines 133-140.

5. Line 133 This claim though sensible requires a citation to support it since there is body of research that shows DUBs exhibit specificity. Would a small molecule inhibitor experiment clarify this position? I would presume that a target that is promiscuous would tend not to be phenotypically robust.

RESPONSE: We agree with the reviewer that many deubiquitinases (DUBs) exhibit specificity toward ubiquitin linkages, and we have revised the text to reflect this, with appropriate citations supporting linkage specificity among DUBs. Substrates themselves can be modified by different ubiquitin chain types, and substrate conformation can further influence protein–protein interactions between a DUB and its targets.

In principle, a small-molecule inhibitor could be used to test the functional consequences of inhibiting DUB activity. However, at present there are no small-molecule inhibitors that selectively and effectively target TRABID without potentially affecting other DUBs or pathways, limiting the interpretability of such an approach.

Although Trabid can bind a broad range of substrates modified with Lys29- or Lys33-linked ubiquitin chains, this does not preclude phenotypic robustness. As shown in our manuscript, within the same genetic background, *trbd*^{C518A} males exhibit strong and reproducible phenotypes, including climbing defects, reduced lifespan, and increased brain lesion rates. Moreover, our genetic interaction experiments demonstrate that modulation of AMPs or Hsc70-4 in the *trbd*^{C518A} background can rescue these defects, supporting the conclusion that specific downstream pathways mediate the observed phenotypes despite the broader substrate-binding capacity of Trabid.

6. Line 141 While locomotor dysfunction can and is used as readout for neurodegeneration, it is important to clarify that not all neurodegeneration results in this defect.

RESPONSE: We appreciate the reviewer’s reasonable suggestion. In the *Drosophila* field, climbing assays are commonly used as an initial screening tool for neurodegenerative phenotypes. In our study, we further substantiated the presence of neurodegeneration in *trbd*^{C518A} flies using additional, more specific assays, including the DAM assay and brain lesion analyses (Fig. 3 and Fig. 4B). As recommended by the reviewer, we have revised the text at line 143-144 (previously line 141) to read: “Locomotor defects are characteristic symptoms of neurological

dysfunction and are widely used as assessment measures for conditions associated with neurodegeneration”

7. Line 168 When making this cross you can mention the line was balanced to specify how you differentiated the homozygotes from the heterozygotes.

RESPONSE: The $+/\text{trbd}^{C518A}$ heterozygotes were generated by crossing homozygous trbd^{C518A} with homozygous w^{1118} -none of these had any balancers. The $+/\text{trbd}^{C518A}$ heterozygotes were only used for Fig 2C to test that trbd^{C518A} is a recessive allele. Repeats were performed by crossing again homozygous trbd^{C518A} with homozygous w^{1118} .

8. It is eluding me how the rescue in Line 175 using the Gal4/UAS took place as I do not see any information on a UAS-trbd construct or stock number in the M&M. Additionally, this rescue (since the flies were already present) should have been extended to lifespan experiments.

RESPONSE: As the reviewer recommended, we added info on the *UAS-trbd* in Materials and Methods (lines 518-521 and 587-591). We also implemented the advice for lifespan experiments using trbd^{C518A} ; *alrm-Gal4/UAS-trbd* (Fig. EV2B).

9. Is the locomotor deficit originating from Trabad function in glia? Since rescue in neurons is not sufficient to suppress the phenotype, I would ask to see rescue data using a muscle specific GAL4 driver since the main readout is locomotor deficit.

RESPONSE: As the reviewer mentioned in 8 above, we performed the rescue in lifespan expressing *trbd* in astrocytes (Fig EV2B). This indicates the type of cell involved in the phenotype. We thus removed the climbing passing rate data in neurons and glia. In this context, performing the UAS rescue with a muscle *GAL4* line will divert focus from the role of the brain.

10. I am not sure if the sleep phenotype is because of lower activity in mutant flies (since sleep is measured as inactivity in flies). As these flies already have a robust locomotor deficit wouldn't it follow they will move less frequently? Column comparisons should prioritize mutant male to wt male, mut female to wt female and mut male to mut female. Since sleep is regulated at the neuronal level, would the UAS-trbd rescue this sleep phenotype?

RESPONSE: The reviewer provided a very constructive suggestion here. For the

sleep assay, we implemented “aomean” in Fig. 3D and H. It seems that *trbd*^{C518A} male flies had locomotor defects, but they moved more frequently during time awake.

We implemented all the multiple comparisons throughout the manuscript. The idea of doing the rescue experiment in every assay is constructive. We do not want to overwhelm the readers with rescue experiments and in this context, we have shown that reducing AMPs will rescue some defects in *trbd*^{C518A} males while reduction of *Hsc70-4* exaggerated climbing defects and increased brain lesions in *trbd*^{C518A} females

11. The citation (Jäkel S & Dimou L (2017)) given for the use of eosin as a quantification method does not mention eosin in its text and is misleading. The method to quantify the neuron and glia content of whole brains appear insufficient as it relies on immunostaining. How was autofluorescence and off-target intensity calculated? A brain volume-based quantification would be better than area. Comparison wise, Fig 4 C to H, the Trabad flies should be compared to their wildtypes first.

RESPONSE: We have implemented the citation of Cao *et al.* and referred to our previous work using this technique (Arora *et al*, 2025 Brain Comms, line 248). All reviewers raised questions about the IF experiment. We removed the data, since eosin staining is sufficient to indicate the pathogenesis in the mutant flies at histology level and can be used for quantification.

12. Fig EV5B appears to be an exact match to EV5A which is a comparison between male and female Trabad with their controls. This contrasts with the LC-MS data that shows different patterns for females and males.

RESPONSE: We apologise for this error. We have now included the proteome result in Fig EV3.

13. The rationale for HSC70 as a target for Trabad while it is not exactly the most upregulated or downregulated escapes me. Other studies show that ubiquitination of Hsc70 leads to its degradation.

RESPONSE: In our analysis of ubiquitinome and proteome, Hsc70-4 was the only protein that was divergently ubiquitylated in different sexes. It is the only candidate that may explain the sex divergent phenotype observed in *trbd*^{C518A} male and female. Canonically, Lys 48 ubiquitin chain is the signal for proteasome degradation. As we explained in question 5, a substrate can be post-transcriptionally modified (including ubiquitylation, phosphorylation etc.) to

different status. For example, depending on the ubiquitylase that modifies the substrate, the substrate can be ubiquitylated by Lys 48 chain, Lys 29 chain or a mix of Lys 48 and 29 chains. It also can be modified at different positions. This multilayer regulation provides different signals to subsequent biochemical reactions. For example, reference 62 that we newly cited in our discussion phosphorylation of Hspa8 (human homolog of Hsc70-4) regulates Hspa8's localization and activates Sting driven innate immunity.

14. The protein interaction experiment in Fig 8 is highly problematic. Co-expressing two synthetic constructs is not conclusive to interaction. A better but painful experiment would be to use one or the other in S2 cell or adult brain lysates to perform co-immunoprecipitation.

RESPONSE: All reviewers raised questions about the Co-IP result. These suggestions were very helpful. We used mCherry-Trabid-AnkOTUC518A, which potentially can lock ubiquitylated substrate, to IP endogenous Hsc70-4 using mcherryTrap (Fig 7E).

15. Hua et al 2022 have published a Trabid polyclonal antibody that would benefit the manuscript in staining the brain and performing Ips to show the binding of Trabid to Hsc70-4?

RESPONSE: Please refer to question 14.

Minor revisions

1. Line 79 Sentence starting with “In mice,” needs rewriting to make some sense and correct the in-text citation style

RESPONSE: The sentence was rewritten, and the citation style was changed to the correct style.

2. Line 87 Consistency in using either Trabid or dTrabid in the manuscript

RESPONSE: The requested changes have been made, clarifying that Trabid is the protein and *trbd* is the gene.

3. Line 95 Delete most in regards to significance

RESPONSE: This has been done

4. In results, make the in-text citation style uniform

RESPONSE: We have fixed the in-text citation style.

5. Line 115 Is this bacterially expressed Trabid? If so it should be mentioned in this sentence to make it clear to the reader

RESPONSE: “Expressed *E. coli*” was added.

6. Line 117 When the concentration is increased to 600 nM it is not clear to the reader why there is a reduction in 63. Can Fig. EV1 be normalized and quantified?

RESPONSE: Licchesi et al. (2012) previously reported that Trabid is predominantly active toward Lys29- and Lys33-linked diubiquitin, whereas cleavage of Lys63-linked chains occurs only at higher enzyme concentrations. Consistent with these findings, the reduction in the Lys63 signal observed at 600 nM reflects reduced substrate specificity at elevated Trabid concentrations rather than a preferential activity toward Lys63 linkages. The assay conditions and concentrations used in our experiments follow well-established protocols in the field. Because this experiment is intended to demonstrate relative linkage preference rather than kinetic efficiency, we present representative blots rather than normalized quantification.

7. Fig 2B The “NS” needs to be included in the figure from line 149

RESPONSE: We have added “ns” to most multiple comparisons, including Fig 2C, Fig 3, Fig 4, Fig 5 A-C and E-F, and Fig EV 4E. In the two-way ANOVA analysis in Fig 2B and Fig EV2A we did not implement “ns”, due to the significant amount comparisons, which would cause readers’ confusion. All statistical tests used are annotated in the figure legends.

8. Line 223 What is unique between the two AMP10 should be described that allow rescue from one and not the other

RESPONSE: Information on the differences of the two chromosomes from the Δ AMP10 strain is given in line 260-266.

9. Line 280 What was the rationale for 200 heads compared to say 1000? Not

triplicates?

RESPONSE: Approximately 200 fly heads represent the optimised minimum number required to obtain sufficient protein for LC–MS analysis. Due to limited funding, we were unable to perform experiments in triplicate for LC–MS and therefore conducted two biological replicates.

10. Line 304 How does EV6A or B, which are principal components QC plots, show regulatory processes? Same to line 305.

RESPONSE: The problem has now been fixed. The new EV3 refers to proteomic results.

11. Line 377 How is the claim Hsc70 a direct target of Trbd supported? Same to the claim based on ColabFold (a prediction tool). This is only a prediction not a confirmation. This claim is only supported after the immunoprecipitation experiments.

RESPONSE: This was a logical error on our part. We thank the reviewer for making a critical comment. In the new context we used prediction first (lines 400-427) and then used IP experiment to verify the prediction.

Reviewer #3 (Remarks to the Author):

Brief summary of the manuscript

In this manuscript, Xia et. al. investigate the role of the *Drosophila* deubiquitylase Trbid in driving sex-specific neurodegeneration. Using a CRISPR-generated enzymatic-null point mutation (trbdC518A), the authors demonstrate that inactivation of Trbid results in male-specific neurodegenerative phenotypes, including locomotion deficits, fragmented sleep, reduced lifespan, glial and neuronal loss, and increased inflammatory signaling. In contrast, female mutants show elevated ubiquitination of neuroprotective proteins like Hsc70-4, which appears to mitigate degeneration. The study uses a comprehensive combination of behavioral, histological, proteomic, and genetic approaches to support its conclusions.

Overall impression

The study addresses an important and underexplored topic: sex-specific vulnerability to neurodegeneration. The work convincingly links inflammatory regulation, ubiquitin homeostasis, and neuronal survival to the enzymatic activity of Trabd. Importantly, the authors identify Hsc70-4 as a key neuroprotective factor, whose regulation appears to differ by sex and depend on direct interaction with Trabd. However, some interpretations require clarification or stronger supporting evidence, and a few experiments or analyses would substantially improve the manuscript's rigor and clarity. In particular, the use and validation of CRISPR lines, RNAi efficiency, sample sizes, and mechanistic interpretation of proteomic data would benefit from refinement or expansion.

Specific comments

Major comments

1. In the Methods section, the authors mention that two independent trbdC518A lines were maintained and used for experiments. Please clarify whether both lines were consistently used across all key experiments, or whether one was prioritized. If only one line was primarily analyzed, I recommend replicating a subset of critical phenotypes (e.g., locomotion, lifespan) in the second line or providing a strong justification for why this was not feasible.

RESPONSE: We received four CRISPR lines and carried out the backcrosses with the *w¹¹¹⁸* background before lifespan, rescue, sleep and climbing experiments. Two lines were confirmed by Sanger sequence. Due to COVID restrictions (the project started in 2019) only one line was maintained (lines 579-586).

The rescue experiments using wild type trbd expression in glia and ubiquitously are well designed and support the specificity of the phenotype. However, for full rigor, please clarify whether any additional steps were taken to control for off-target CRISPR edits, such as predicted off-target site screening or backcrossing to an isogenic background.

RESPONSE: We mentioned the backcrosses (see above) in Materials and Methods (lines 585-586). As reviewer 1 recommended, in the revision, we only use an astrocyte-specific *GAL4* line (*alrm-GAL4*) for all overexpression experiments to

support the specificity of the phenotype.

2. Lifespan assays are highly sensitive to husbandry and environment, so key variables must be tightly controlled and explicitly reported. Please clarify in Methods (and echo key details in the relevant figure legends) how adult cohorts were age-synchronized (timed collections/eclosion window), whether sexes were housed together or separately and adults were mated or virgins, the exact diet formulation (e.g., 10% SY), temperature, humidity, and light:dark cycle, how larval density was controlled during development, and any incubator position randomization/rotation to avoid rack effects. Notably, the literature typically reports shorter lifespan in wild-type males than females under standard 10% SY at 25 °C; your data appear to show the reverse. Please reconcile this discrepancy—e.g., by clarifying diet composition/batch, genetic background, microbiota, mating regime, density, censoring/exclusion criteria, or other procedural differences. Importantly, please report the number of independent biological replicates per genotype.

RESPONSE: The reviewer proposed very valuable suggestions most of which we had already adhere to. We have now clarified the methodology of the cohort environment for lifespan (lines 623-630). The incubation room environment was mentioned in climbing assays (lines 609-610). We also reported the number of independent biological replicates per genotype in all fly experiments in the figure legends. Specifically, for the Fig 2D lifespan information can be found in lines 957-962.

3. It is unclear what the three strains designated Δ AMP10, Δ AMP10_Chr2, and Δ AMP10_Chr3 specifically comprise—i.e., which AMP genes are deleted in each and how they were generated. Why does Δ AMP10_Chr3 rescue whereas Δ AMP10_Chr2 does not? Were AMP transcripts or peptides levels measured in *trbdC518A* flies, and do the data implicate AMPs or indicate a broader AMP-dependent effect? Please clarify these points and ensure that all AMP genotypes are fully described in the Methods (composition, origin/stock IDs, and genetic background/backcrossing).

RESPONSE: The Δ AMP10 stock was generated in Bruno Lemaitre's lab by Mark Hanson and Mickael Poidevin (Hanson *et al* eLife 2019). It comprises a deletion of 10 out of 14 AMPs in *Drosophila melanogaster*. The deletions are in chromosome 2 (Chr_2) and chromosome 3 (Chr_3). The latter deletion comprises *Attacin-D* and the *Drosomycin* locus while the former deletion includes all others (*diptericiens*, *attacinsA-C*, *metchnikowin*, *defensin*). Combining Chr_2 from the Δ AMP10 stock with *trbd*^{C518A} or Chr_3 from the Δ AMP10 stock with *trbd*^{C518A}, we generated

double mutants and tested locomotion (lines 258-262, 592-597).

4. The authors correctly point out that the w¹¹¹⁸ and yw backgrounds can influence climbing assays. It may be helpful to mention whether any genetic background effects were observed in the Δ AMP10 rescue experiments.

RESPONSE: The Δ AMP10 stock was generated in the w¹¹¹⁸ background (through the DrosDel toolbox, Hanson *et al*, 2019 eLife). In this context, reference to yw is not relevant. No difference in locomotion between the genetic background and the Δ AMP10 deletion was observed (Fig. 4B).

5. The eosin assay is a useful screen. Please include representative raw z-stacks.

RESPONSE: See EV2C and EV5C.

6. Please revise the legend for Figure 4: the descriptions do not match the panels shown. Ensure each legend item corresponds to the correct panel (letters, labels, and order) and that the terminology aligns with the main text.

RESPONSE: Other reviewers raised questions about the old Fig 4. We removed the dataset.

7. In the ubiquitylation and proteomic profiling, the authors compare trbdC518A males vs w¹¹¹⁸ males and trbdC518A females vs w¹¹¹⁸ females to suggest sex-specific effects; however, the within-genotype sex comparisons are missing and are necessary. Figure 5: To better visualise sex-specific differences please add volcano plots for trbdC518A males vs females and, for context, w¹¹¹⁸ males vs females. Clearly label the axes (e.g., x = log₂ fold change [male/female], y = -log₁₀ q-value [FDR-adjusted P]). Finally, the Figure 5B legend appears to reference the female panel (Fig. 5A); please correct it so that it accurately describes the male comparison.

RESPONSE: We had performed the within-genotype sex comparisons. However, most significant differential hits are related to sex-specific proteins, such as the Yp family proteins. Presenting the comparison doesn't help the reader understand the difference. However, we implemented the comparison result in Table S1. The volcano plots were remade and labelled (Fig. 5 and Fig. EV3). The legend of Fig. 5 was rewritten (lines 1016-1026).

8. The order in which the figures are discussed in the Results section is confusing and disrupts the logical flow of the manuscript. The text first introduces Figure 5C, then

shifts to a full paragraph on Figure 6A–C, then returns to Figure 5C, followed by references to Figure 5A and 5C again, and only afterward introduces Figure 6D. This back-and-forth between figures, particularly crossing figure numbers in a non-sequential way, breaks the narrative structure and makes it difficult for readers to follow the progression of the results. Figures should be discussed in numerical and conceptual order, aligning with how the data build upon each other. Consider restructuring the Results section so that all of Figure 5 is covered before moving on to Figure 6 and avoid returning to earlier figures after discussing later ones.

RESPONSE: We conducted a major rewrite of the manuscript. We have avoided returning to earlier figures after discussing later ones.

9. The manuscript states that Lys6/Lys27/Lys33 linkages accumulate “in females but not in males,” which overstates the contrast: Fig. 6A–C shows no significant increase in males, but mean values are comparable or only modestly lower than in females. Please soften the wording: not statistically significant does not mean no effect. The male data show a similar trend that does not reach significance. Provide the within-genotype male–female comparison (or an interaction test) to support a female-specific conclusion. It is also unclear whether multiple-testing correction was applied across linkage analyses—please clarify and report adjusted q-values. Because K29 was not measured and the in vitro substrate profile differs from the in vivo pattern (in vitro K29/K33 vs in vivo K6/K27/K33), a brief explanation or caveat in the Discussion would help. The sentence “Except M1 and Lys29 linkages, the GlyGly enrichment allowed us to compare ubiquitin linkages...” is unclear—please state which linkage types were quantified, which were not, and why (e.g., not detected/below confidence/method limitation), and indicate explicitly whether K29 was analysed. Define the y-axis (“log2 normalized value”: what is normalized, and to what reference?), specify all statistical contrasts reported (trbdC518A F vs w1118 F; trbdC518A M vs w1118 M; trbdC518A F vs trbdC518A M; w1118 F vs w1118 M), and ensure the legend/brackets match those contrasts; also correct the y-axis typo to “normalized.”

RESPONSE: In lines 344–346, we now explicitly state the six ubiquitin linkage types that were quantified. As explained in lines 345–346, M1- and Lys29-linked ubiquitin chains could not be quantified due to limitations of GlyGly peptide detection under our experimental conditions.

The normalization method used for the ubiquitinome analysis is described in Materials and Methods to improve transparency (lines 683–687).

With respect to sex differences, our analysis focuses on sex-matched genotype comparisons, which we consider the most appropriate framework for addressing

sex-dependent effects in this study. We have therefore softened the wording throughout the manuscript to reflect that males show a similar trend that does not reach statistical significance, rather than implying an absence of effect.

10. The manuscript states that Hsc70-4 is one of the most divergently ubiquitylated proteins between trbdC518A females and males, being upregulated in females and downregulated in males relative to sex-matched w1118 controls. Please make the contrasts explicit and, if you wish to claim sex-divergent regulation, report the within-genotype male–female comparison (or a sex $\times$ genotype interaction); otherwise avoid wording that implies a tested cross-sex difference. The conclusion currently rests on ubiquitylation readouts only; please indicate whether total Hsc70-4 (transcript or protein) also differs by sex or genotype, and whether site/protein-level ubiquitylation was interpreted relative to total abundance. The assertion that autosomal Trabad affects X-linked proteins (HIP/HIP-R) is interesting but speculative, either soften it or support it with additional analysis (e.g., considering sex-chromosome dosage/compensation or proposing a direct pathway). For Fig. 5C, document the inclusion thresholds ($|\log_2\text{FC}|$ cutoff, adjusted q-values), state whether these were pre-specified, provide the number of biological replicates and the total background set used for the Venn, and define what you mean by “divergently ubiquitylated.” Also align the legend wording with these criteria. As a clarity aid, consider adding a volcano or table showing effect sizes and q-values for Hsc70-4 and HIP/HIP-R.

RESPONSE: We implemented multiple comparisons with an interaction effect (post-hoc correction). There was no significant difference in protein expression for Hsc70-4 (lines 360). The presentation of HIP and HIP-R were only clues that led us to test the genetic interaction of Hsc70-4 with Trabad in later experiments. We have taken out the mention of the two proteins in that section and retained it in the figure legend for Fig 5 (line 1025). The pathway search was conducted for *Drosophila melanogaster*. The information was included in line 680-682.

11. The claim that Hsc70-4 is neuroprotective in trbdC518A females rests on astrocyte RNAi worsening climbing at day 18 (Fig. 6D). Please add a sufficiency test: astrocyte-specific Hsc70-4 overexpression in trbdC518A males to ask whether increasing Hsc70-4 can rescue locomotion/sleep/lifespan. If development is a concern, use an adult-onset driver (GeneSwitch) to separate developmental from adult effects.

RESPONSE: We appreciate the reviewer’s suggestion and agree that an astrocyte-specific overexpression experiment would be a strong sufficiency test. However, testing the sufficiency of Hsc70-4 is beyond the scope of the current manuscript, which is focused on a more general understanding of sex-specific differences in neurodegeneration rather than establishing a gain-of-function framework for Hsc70-4.

Instead, we have further strengthened the evidence for a neuroprotective role of Hsc70-4 by quantifying brain lesions following astrocyte-specific Hsc70-4

knockdown in the *trbd*^{C518A} background, thus providing a more direct measure of neurodegeneration (Fig. 6E and F). We believe these data address the underlying concern by demonstrating that loss of Hsc70-4 exacerbates neurodegenerative pathology in this context.

12. The manuscript does not show whether Hsc70-4 knockdown is efficient or specific in astrocytes. Please include transcript or protein-level validation of the RNAi line in the appropriate tissue context, or include a second, non-overlapping RNAi line.

RESPONSE: We appreciate the reviewer's concern regarding RNAi efficiency and astrocyte specificity. In this study, astrocyte-specific Hsc70-4 knockdown produced neurodegenerative phenotypes only in the *trbd*^{C518A} background and not when expressed alone, indicating a strong genetic interaction rather than a general toxic effect of the RNAi.

While we did not include direct transcript- or protein-level validation of Hsc70-4 knockdown in astrocytes in this revision, we strengthened the functional relevance of this interaction by adding neuropathological analyses, including brain lesion quantification, which provide a direct measure of neurodegeneration.

13. Please document adult driver specificity and, if feasible, test an independent astrocyte driver. Clarify whether sexes were assayed in parallel and whether male astrocyte RNAi was tested (even if negative), as that would bound the sex bias.

RESPONSE: Following this suggestion, in Fig. 6E and F, we checked brain lesion occurrence for *alrm*>Hsc70-4 in both mutant females and males.

14. The ColabFold scores are below typical confidence (pTM 0.437; ipTM 0.268), the HDock model uses human structures, and the co-IP is in overexpressing S2 cells with a domain fragment (AnkOTU^{C518A}). Either soften the language ("candidate interactor") or add in-vivo support: endogenous head co-IP (or glia-enriched prep), reverse IP, substrate-trapping with catalytically dead Trbd showing increased Hsc70-4 binding, and site-level ubiquitylation changes on Hsc70-4 in heads that track with genotype/sex.

RESPONSE: All reviewers raised questions about the Co-IP result. These suggestions were very helpful. We used mCherry-Trabid-AnkOTUC518A, which potentially can lock ubiquitylated substrate, to IP Hsc70-4 using mCherryTrap (Fig 7E).

15. Please align several Discussion statements more tightly with the presented evidence.

Treat Hsc70-4 as a candidate interactor or neuroprotective factor, rather than a demonstrated direct Trabid substrate, unless in vivo substrate validation is added. Clarify whether ubiquitylation changes were interpreted independently of total Hsc70-4 protein levels, or whether such quantification was performed. Statements describing effects “in females but not in males” should be supported by within-genotype sex comparisons or formal sex $\times$ genotype interaction tests, rather than inferred from separate sex-matched contrasts.

RESPONSE: Following the reviewer’s suggestion, we rewrote most of the manuscript. As we illustrated in previous questions, we have carried out sex $\times$ genotype interaction effect in multiple comparisons test.

Additionally, the observed mismatch between Trabid’s in vitro K29/K33 linkage preference and the in vivo accumulation of K6/K27/K33 chains in female heads should be acknowledged as a limitation. Please consider offering possible mechanistic or technical explanations (e.g., indirect effects, detection thresholds, or secondary chain editing).

RESPONSE: We implemented the suggestion and discuss our approach in the context of *in vitro* vs. *in vivo* Ub-type accumulation in lines 455-460.

Regarding the AMP/Relish axis and the Δ AMP10 rescue, the inference implicating specific AMPs like Drs or AttD remains hypothesis-level. Please either soften the mechanistic claims or support them with targeted validation (e.g., loss-of-function or rescue of single AMPs in the trbdC518A background, or tissue-specific AMP manipulations).

RESPONSE: Mechanistic claims were toned down in the discussion.

Finally, where cross-species implications are proposed (e.g., extrapolating Trabid/Hsc70-4 interactions to mammals), please clearly frame these as model-based or speculative, unless functional evidence is provided. These adjustments would help keep the Discussion ambitious yet well-calibrated to the presented data.

RESPONSE: The discussion was rewritten entirely with guidance from the reviewers’ suggestions.

16. In the Introduction you state that tissue-specific transformer (tra) knockdown attenuates some sex differences, and the Methods list a tra RNAi line; however, I cannot find the corresponding experiments in the Results or figures. If these data exist, please

present them explicitly, with context, quantification, sample sizes, and statistics, and cross-reference the exact figure/panel. If the experiments were not performed or are unavailable, please remove or soften the claim in the Introduction/Methods.

RESPONSE: We have amended the introduction/methods to delete the *tra* part.

Minor comments

- The manuscript reports rescue with actin-GAL4 and repo-GAL4 but not with elav-GAL4, yet no rationale is offered. Please comment on potential reasons (driver strength/timing, transgene expression levels in heads, cell-type specificity) and, if feasible, provide supporting data (qPCR/Western for transgene expression, or an alternative neuronal driver/adult-onset driver) to clarify why neuronal expression fails to rescue.

RESPONSE: Following comments by Reviewer 1, and to focus on cell-specific brain rescue we only present rescue experiments using astrocyte-specific *alrm-GAL4*.

- Line 201, please rephrased to better understand at which males authors are talking about.

RESPONSE: This part was rewritten.

- Line 291, “ $p < 0.05$ and fold change $-\log_2 >$ or $\log_2 <$ ” which is unclear.

RESPONSE: Please refer to line 289 for the change FDR-adjusted p -value < 0.05 and $|\log_2 \text{ fold change}| > 1$)

- In Methods add catalog numbers for antibodies, di-ubiquitin standards, and key reagents.

RESPONSE: This information can be found in the material list.

- References. Check consistency of citation style (e.g., 38–41) and ensure all database or software tools (AlphaFold2/ColabFold, HDOCK, BioRender) are cited per their guidelines.

RESPONSE: The change was made (line 692).

- Line 714: “Two sample t-test was performed, and we used proteins that were $FDR > 0.05$ and $\log \text{ fold change} < -1$ or > 1 to perform Venn diagram analysis.” As written,

this selects non-significant proteins and uses a nonsensical log-FC threshold (> -1 includes virtually everything). Please correct to $\text{FDR} < 0.05$ and a symmetrical $|\log_2\text{FC}|$ cutoff (e.g., >1) and ensure these criteria match what is used in all figures.

RESPONSE: These changes were implemented (line 678-679).

RESPONSE TO REVIEWERS Xia *et al*_COMMSBIO-25-7921-R2

Reviewer 1

- After the reorganization of the text, in some places the narrative has become slightly fractured and reads rather disjointed. As an example, more description is needed to introduce the role of astrocytes in this pathway, and the jump in and out of astrocyte and AMP-related genes is a little confusing just for readability.

Response: We have gone over the transitions and tried to make them better. In the example of the reviewer, we introduced fly astrocytes in one sentence along with the *alrm-GAL4* driver (lines 188-192).

- Across all experiments, authors should be sure to describe strategies, strains, and acronyms/abbreviations with great attention to detail, as they have done in many cases for us as reviewers in the rebuttal (e.g. what is arlm? Which genes are impacted that make delta chr. 2 and delta chr. 3 different? What is oamean and how should the reader interpret it?)

Response: The term oamean, which means activity counts per awake minute, is explained in line 214 and 216.

- In (an admittedly smaller) handful of places, the authors still claim that a phenotype is male or sex-specific on occasions when females are also affected. Throughout the text, it would make interpretation less awkward to just emphasize the increased severity of several measures in males. Also, do these differences (for example female effect in trabid del but not c518A) help the authors tell their story (i.e. that differences in ubiquitination cause the male phenotype in c518a that would not be recapitulated in trabid del) perhaps?

Response: The reviewer is right that we have inadvertently left some of this language throughout the manuscript. We have now modified the claims and any sex-specific language used to present results is now presented as sex-dimorphic changes (see lines 103-104, line 439, line 444, line 449, lines 458-459, line 492).

- It is unfortunate that the authors' prediction that Hsc70-4 is differentially regulated on the protein level is not supported. However, this finding should either be clearer to the reader in the text (for example, authors should not say it is upregulated when the expression and protein levels do not change) or the authors could reevaluate the data after completing a third biological replicate of the mass spectrometry experiments. The

selection of Hsc70-4 is interesting as a target regardless of the outcomes, but the authors should describe clear and correct reasons for its selection. The addition of 6D is very powerful for the narrative, and I am glad the authors completed the experiment! If the authors can do the add-back of Hsc70-4 in males that would be lovely, but I take the point that making an additional strain may be out of the scope at this time.

Response: In lines 363-364, we explain that ubiquitylation is not limited to protein expression or degradation but also involved in cellular regulatory signalling as is the case for Hsc70-4.

Minor:

- For Fig 2D, the authors should clarify claim that males are more affected. Instead of motivating by p value, authors should motivate by the significant difference between groups as indicated.
- Statistics are missing for EV2B and ought to be included.

Response: NS was added to EV2A. The statistics results of EV2B were included in the legends.

- In line 295, authors say that trbd is required in astrocytes; however, they perform a sufficiency experiment. The authors should either clarify the wording or perform a necessity experiment (knockdown or mutant).

Response: Does the reviewer mean 195 instead of 295? We have now added a preamble to that section to explain astrocytes and the use of the *alrm-GAL4* line (lines 188-190). As Trbd is a negative regulator, sufficiency experiments would be too complex to achieve and beyond the scope of this study.

- For figure 3B, labels should clarify that the AMP10s are on the trbdC518A background

Response: We think the reviewer means figure 4B? We have labeled the AMP10 deletions were done under trbdC518A background.

- In general, the authors could clean up some figures and labels (for example, EV4A) to make the manuscript more polished.

Response: This has been done.

Reviewer 2

It is still my opinion that Fig 1A would benefit the reader by having the amino acid sequences as opposed to blocks of color. Having the amino acid sequences allows the reader to make critical observations on domain conservation among other protein structural/sequence features.

It also makes it clear to the reader the protein sequence used by the authors in the case of revision to the sequence database.

Response: We added the amino acid sequences comparison in Fig EV1A.