nature portfolio

Peer Review File

# CELF2 Promotes 4R Tau Splicing via Nuclear Clustering and Drives Cognitive Dysfunction in Tauopathy Models

Corresponding Author: Dr Lizhen Chen

**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)
In this manuscript, the authors investigate the role of the RNA-binding protein CELF2 in regulating tau exon 10 alternative splicing and its functional consequences. Through a combination of genetic, cell-based, biochemical, and in vivo approaches, they conclude that CELF2 promotes exon 10 inclusion via its intrinsically disordered hinge domain and cooperates with other splicing regulators to control tau isoform balance. This is an elegant and thorough study that combines genetics, cell biology, proteomics, and structure–function analyses to dissect the role of CELF2 in tau exon 10 splicing. The experimental work is carefully executed and provides compelling evidence for IDR-dependent regulation and functional cooperation with other RBPs. Overall, this is a strong and interesting study. The main concern is that the authors overinterpret their data in mechanistic terms related to phase separation and condensate-mediated recruitment. These issues could potentially be addressed by revising the language throughout the manuscript, or alternatively by providing additional biophysical characterization to support the proposed mechanism.

Major comments

1. (Related to Fig. 2)
The manuscript repeatedly frames CELF2 function in terms of a “condensate-based” or phase-separation–driven mechanism (including in section titles and abstract). While the data convincingly demonstrate that the CELF2 IDR mediates multivalent self-interaction and condensate-like clustering, the evidence provided does not directly establish that these assemblies represent bona fide phase-separated condensates with liquid-like properties. In particular, the optoDroplet and LacO/LacI assays rely on forced concentration, and the in vitro droplet assays under crowding conditions do not distinguish phase separation from other forms of aggregation or coacervation. Given that the proposed mechanism hinges on phase separation, additional biophysical characterization would be required to support this conclusion, or alternatively the language throughout the manuscript should be revised to reflect a more cautious interpretation.

2. (Related to Fig. 3)
The CRISPR–dCas13 imaging experiments are interpreted as evidence that CELF2 condensates play a functional role in tau splicing regulation. However, these data primarily demonstrate partial spatial colocalization between CELF2 puncta and tau minigene RNA. Given prior evidence that CELF2 binds tau RNA, it is unclear what additional mechanistic insight this experiment provides. In particular, the data do not establish that RNA recruitment or splicing regulation depends on condensate formation.

3. (Related to Fig. 4)
While the TurboID and LacO/LacI assays convincingly support IDR-dependent interactions between CELF2 and NOVA2/SFPQ, the imaging data provide limited evidence for recruitment of these proteins into pre-existing CELF2-containing structures. In the Results, the authors state that “this suggests that CELF2 might facilitate to recruit NOVA2 and SFPQ to those nuclear condensates.” However, the data presented support heterotypic interactions and co-clustering, but do not establish directional recruitment or enrichment of NOVA2/SFPQ within CELF2 assemblies. Static colocalization does not distinguish recruitment from coincidental proximity, and additional evidence would be required to support this

mechanistic interpretation. Clarifying this distinction would strengthen the conclusions.
Consistent with this, the Results section later states that the authors have “shown that CELF2 interacts with NOVA2 and SFPQ in nuclear condensates.” However, the data support IDR-dependent interactions and co-clustering, but do not establish that these interactions occur within functionally defined nuclear condensates or involve directional recruitment into pre-existing CELF2 assemblies. Clarifying this point would improve the logical flow and mechanistic interpretation of the Results.

4. In addition to the concerns raised above, the manuscript repeatedly uses the term “condensates” to describe CELF2-containing assemblies, including heterotypic assemblies involving NOVA2 and SFPQ. While the data support IDR-dependent interactions and co-clustering, they do not distinguish condensates from dense interaction hubs or demonstrate that condensate formation is required for function.
This issue is particularly evident in the interpretation of Fig. 5. The functional data convincingly demonstrate that CELF2 cooperates with SFPQ and antagonizes NOVA2 to regulate tau exon 10 splicing. However, these experiments do not assess localization or condensate dependence, yet the Results text attributes these interactions to “biomolecular condensates.” The data are fully explained by functional cooperation and competition between RBPs, without requiring a condensate-based mechanism. Revising the language to decouple these functional conclusions from condensate terminology would improve clarity and better align interpretation with the evidence presented.

5. The in vivo experiments in C. elegans provide a fantastic validation of the structure–function relationships established in vitro and in cultured cells. The use of CELF2 WT, ΔIDR, and D388 mutants convincingly demonstrates that the CELF2 IDR and the conserved charged residue D388 are required for CELF2-mediated regulation of tau exon 10 splicing and for the resulting locomotor and cognitive phenotypes in vivo. However, the Results repeatedly conclude that these phenotypes demonstrate a requirement for “CELF2 condensation capacity.” While the genetic and functional requirements are clearly established, condensate formation, puncta, or phase behavior are not directly assessed in the animal model. Moreover, attributing the in vivo effects specifically to condensation relies on interpretations of earlier in vitro and cell-based assays, which do not directly establish bona fide condensate formation or phase separation. Extending the condensate-based mechanism to the in vivo setting is therefore not directly supported by the data presented. Rephrasing these conclusions to reflect the experimentally tested requirements would improve precision and consistency without diminishing the impact of these strong in vivo findings.

Minor comments

Line 118: “promote axon 10 inclusion” should read “promote exon 10 inclusion.”

Line 316: The text refers to “milder defects (Fig. 8D–G),” but panels D–E assess locomotion, while panels F and G correspond to chemotaxis and gentle touch assays. Clarifying the figure panel references would improve accuracy.

Reviewer #2

(Remarks to the Author)
This study report the important role of the RNA-binding protein CELF2 in enhancing tau exon 10 inclusion. The intrinsically disordered region (IDR) within the CELF2 hinge domain is shown to drives protein condensation and splicing activity, though this issue remains to be consolidated (see points below). Proteomic analyses identify NOVA2 and SFPQ as CELF2 interactors, though the importance of CELF2 interaction for the function of these proteins remains to be demonstrated. A conserved negatively charged residue (D388) within the IDR is shown to be critical for condensate formation, but its role in protein interactions that would lead to the proposed condensate-dependent splicing function remains unclear. The authors should adjust the strength of their interpretation and discuss alternative interpretations where appropriate, and address the points listed below:
ΔIDR variant has much lower abundance (see comments to Figure 1), therefore current findings on their own are limited in interpretation. Therefore the D388A mutant should be used for all of the same measurements where the ΔIDR is used, from Fig2 onwards, to consolidate findings.

One of the primary conclusions is that the CELF2-IDR-mediated recruitment of SFPQ and NOVA2 to condensates containing tau mRNA is important for their respective functions in tau splicing. This needs to be directly demonstrated, for example by modulating expression of SFPQ and NOVA2 in cells with CELF2 knocked down (or only expressing delta IDR), and demonstrating that altered expression of these proteins no longer impacts tau splicing if they are not part of the the CELF2 condensates.

The authors’ model on the function of CELF2/NOVA2 condensate is hard to understand. Findings from Fig5 demonstrate that the two proteins seem to compete and therefore have opposite effects on tau exon 10 inclusion. If the proteins compete, why would CELF2 be needed to recruit NOVA2 to the tau mRNA? This is quite counterintuitive, as it would mean that depletion of CELF2 should make no difference on splicing (as it would also remove the capacity of NOVA2 to repress the

exon) - this clearly is not the case. It appears that NOVA2's repressive function becomes more pronounced if CELF2 is removed. Authors need to present further evidence to validate the model of NOVA2 tau binding & function being dependent on CELF2.

The text states that the effect of SFPQ overexpression on 4R : 3R ratio is less than CELF2wt overexpression but in their quantification plots there isn't a significant difference, they look the same, which argues against the CELF2-IDR being the nexus of tau splicing. The increased impact of CELF2/SFPQ co-transfection could be due to additive effect, rather than coopeartive effect within a condensate. Also, as explained in point1, it's crucial that D388A mutant is used for such experiments.

Minor requests:

FIGURE 1
The authors should post the code for how they processed their CLIP data. They briefly describe the steps in their 'KEGG pathway analysis of CELF2 targets' section, but this would not be sufficient to reproduce their results as it stands.
Figure 1A :
the text says nuclear fraction CLIP data is used for KEGG analysis but the 'KEGG pathway analysis of CELF2 targets' methods say whole cell lysate data is used – not sure which one is correct
Figure S1A :
In the text they refer to cyto CELF2 binding 3' UTR of mRNA and nuclear CELF2 binding to introns making it sound generalised, but only show the tau mRNA as evidence of this, guess they mean to only describe the distribution of binding on tau mRNA?
Figure 1E:
Typo in text describing the figure, 'axon 10' rather than 'exon 10'.
Figure 1K:
Statistical significance stars missing off graph

FIGURE 3
Figure 3C / S3B
The delta IDR mutant has much lower expression than WT in S3B, which creates caveats for interpretation. The no CELF-delta IDR could have deficient splicing functon(and condensation) simply due to lower stability/abundance in the transient transfection experiment? The authors should discuss this. It is reassuring that the TAF15 IDR mutant also has low expression but does restore splicing function, though gapdh loading control is a lot lower in this lane. This should be redone and quantified across replicates.

FIGURE 5
Figure S5D/S5E:
The splicing ratio looks very similar on the gel in the last 4 conditions in D, but are very different according to their quantification of the bands in E, which could this be?

FIGURE 6
Fig. S6F:
No loading control is present on the WB. Here the author acknowledge equivalent expression levels are important for this experiment, but not for the initial delta IDR experiment in 3C.

FIGURE 7
By eye, co-localisation between NOVA2/SFPQ and the D388A mutant looks preserved in the cell images, but perhaps there is a smaller number of larger condensates? Could the authors address this by quantifying co-localisation relative to the size of condensates?

FIGURE 8
Analysis of in vivo protein expression of the CELF2 WT and mutants should be provided.

Reviewer #3

(Remarks to the Author)
In the manuscript entitled "CELF2 Promotes Tau 1 Exon 10 Inclusion via Hinge Domain-Mediated Nuclear Condensation, 2 Driving Cognitive Dysfunction in Tauopathy Models", the authors investigated the role of CELF2 in promoting tau (MAPT) exon 10 inclusion. Age-associated inclusion of MAPT exon 10 increases the 4R:3R ratio and is widely considered a feature of tauopathy, but the mechanism underlying exon 10 splicing remains incompletely understood. Li et al showed that Celf2 binds to the upstream intron of Mapt exon 10 in the nucleus (CLIP-Seq), and postnatal deletion of Celf2 significantly decreased exon10 inclusion in young and aged adult mouse brains. Interestingly, the Celf2 protein formed condensates through its IDR in the hinge domain, and the IDR was required for its function in promoting the MAPT 4R isoform. The authors utilized TurboID to identify binding partners of Celf2 and narrowed the targets down to Nova2 and Sfpq, both of which showed reduced interaction with the Celf2 IDR mutant form but displayed opposite effects on exon10 splicing when co-expressed with Celf2. The authors dived deeper into the IDR and showed that D388 was critical for Celf2 condensate

formation and splicing function. To test the CELF2 effect in vivo, the authors showed that human Tau 4R overexpression is toxic in C. elegans and developed a clever minigene strategy to test the splicing effect of wild-type and mutant CELF2 in promoting 4R toxicity.

Overall, this study addresses a central question about tau isoforms, reveals important roles of Celf2 in promoting the 4R:3R ratio, and reports molecular insights into Celf2 IDR. The integration of molecular, cellular, biochemical, and genetic approaches, along with rigorous controls, convincingly supported their conclusions. The reviewer raised a few minor questions to enhance the rigor of this study.

1. While the authors showed a Celf2 CLIP-Seq peak in Mapt intron 9, it would be helpful to determine whether the peak was statistically significant and associated with the Celf2 binding motif. The Mapt exon organizations are not consistent between Fig. S1A and S1B.

2. The results from Celf2 conditional homozygous KO mice are convincing. Does heterozygous Celf2 KO also reduce the 4R:3R ratio? This may have interesting indications of Celf2 dose in regulating MAPT splicing.

3. The important roles of Celf2 in human and mouse brain development should be introduced, such as PMID 34107259 and PMID 38829512. The developmental roles of Celf2 would pose a challenge to intervening in its MAPT splicing function, and this should be discussed.

4. Other splicing regulators, such as MBNL (PMID: 38772368), have been reported to regulate MAPT exon 10 inclusion. This should be discussed.

Reviewer #4

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
I thank the authors for their thorough and careful revision. My primary concern in the initial review was that the manuscript systematically overinterpreted the data in mechanistic terms related to phase separation and condensate-mediated recruitment, while the experimental evidence supported a more cautious interpretation. The authors have addressed this concern satisfactorily. The language has been revised consistently throughout the manuscript, including the title, abstract, results, and discussion. Furthermore, the FRAP data added in response to this concern independently supports the revised interpretation. All other specific points raised in my initial review have also been adequately addressed. The experimental work remains strong and the conclusions as revised are well supported by the data. I recommend acceptance.

Reviewer #2

(Remarks to the Author)
The authors have addressed all the requests, and I find the manuscript ready for publications. Congratulations on this very valuable study!

Reviewer #3

(Remarks to the Author)
The authors have adequately addressed my comments. Congratulations on the great work!

Reviewer #4

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

## GENERAL RESPONSE TO REVIEWERS

We appreciate the general enthusiasms from reviewers about our work. Here we highlight some of their positive comments:

*Reviewer 1:* This is an **elegant and thorough** study that combines genetics, cell biology, proteomics, and structure-function analyses to dissect the role of CELF2 in tau exon 10 splicing. The experimental work is **carefully executed and provides compelling evidence** for IDR-dependent regulation and functional cooperation with other RBPs. Overall, this is a **strong and interesting** study.

*Reviewer 3:* Overall, this study addresses a central question about tau isoforms, reveals important roles of Celf2 in promoting the 4R:3R ratio, and reports molecular insights into Celf2 IDR. The **integration** of molecular, cellular, biochemical, and genetic approaches, along with **rigorous controls, convincingly** supported their conclusions.

We are highly grateful to all reviewers for providing us the critical comments and constructive suggestions for strengthening our manuscript. We have made every effort to carry out extensive experiments to address all the comments and concerns from reviewers. We have conducted the following major experiments to strengthen our findings:

1. We performed additional informatic analyses of CELF2 CLIP-seq datasets. These analyses show that cytoplasmic CELF2 binding is enriched in 3′UTRs, whereas nuclear CELF2 preferentially associates with intronic regions. We provide comprehensive Excel tables listing CELF2 targets and CLIP-seq peaks (Table S1). Additionally, we show that CELF2 binding peak within intron 9 of the *Mapt* gene is highly significant and is associated with a canonical CELF2 binding motif (**Fig. 1, Fig. S1**).

2. We conducted FRAP analyses of droplets formed by CELF2 IDR variants. These experiments demonstrate that CELF2 condensates exhibit solid-like properties, as evidenced by slow fluorescence recovery dynamics (**Fig. 2, Fig. S6**).

3. We compared tau splicing in brains from +/+ and +/- newborn mice and found that haploinsufficiency of *Celf2* is sufficient to alter tau splicing, indicating dosage sensitivity *in vivo* (**Fig. S1**).

4. We performed additional western blotting and *in vivo* GFP imaging to assess expression levels of CELF2 variants, confirming that all constructs are expressed at comparable levels (response letter and **Fig. S7**).

5. We carried out new experiments to evaluate the D388A mutant in parallel with the ΔIDR construct across multiple assays, including FRAP, CRY2 optodroplet formation, RT-PCR analysis of tau splicing, as well as western blotting and fluorescence imaging to assess protein expression. These data provide a more comprehensive functional characterization of this mutant (**Figs. 7C-D, S6, S7**).

We thank reviewers for their insightful comments and questions, permitting this revised manuscript to be significantly strengthened. Please note that we have labeled the revised part of main text in blue color. As follows please see our point-to-point responses to review comments.

## POINT-BY-POINT RESPONSES TO REVIEW COMMENTS

Reviewer #1 (Remarks to the Author):

In this manuscript, the authors investigate the role of the RNA-binding protein CELF2 in regulating tau exon 10 alternative splicing and its functional consequences. Through a combination of genetic, cell-based, biochemical, and in vivo approaches, they conclude that CELF2 promotes exon 10 inclusion via its intrinsically disordered hinge domain and cooperates with other splicing regulators to control tau isoform balance. This is

an elegant and thorough study that combines genetics, cell biology, proteomics, and structure–function analyses to dissect the role of CELF2 in tau exon 10 splicing. The experimental work is carefully executed and provides compelling evidence for IDR-dependent regulation and functional cooperation with other RBPs. Overall, this is a strong and interesting study. The main concern is that the authors overinterpret their data in mechanistic terms related to phase separation and condensate-mediated recruitment. These issues could potentially be addressed by revising the language throughout the manuscript, or alternatively by providing additional biophysical characterization to support the proposed mechanism.

Major comments

1. (Related to Fig. 2)
The manuscript repeatedly frames CELF2 function in terms of a "condensate-based" or phase-separation–driven mechanism (including in section titles and abstract). While the data convincingly demonstrate that the CELF2 IDR mediates multivalent self-interaction and condensate-like clustering, the evidence provided does not directly establish that these assemblies represent bona fide phase-separated condensates with liquid-like properties. In particular, the optoDroplet and LacO/LacI assays rely on forced concentration, and the in vitro droplet assays under crowding conditions do not distinguish phase separation from other forms of aggregation or coacervation. Given that the proposed mechanism hinges on phase separation, additional biophysical characterization would be required to support this conclusion, or alternatively the language throughout the manuscript should be revised to reflect a more cautious interpretation.

We thank the reviewer for this important comment. We agree that distinguishing bona fide liquid-liquid phase separation from other forms of biomolecular assembly requires careful biophysical characterization.
In response to this concern, we performed fluorescence recovery after photobleaching (FRAP) experiments to probe the material properties of CELF2 IDR droplets. These analyses revealed minimal fluorescence recovery over the measured timescale (**Figs. 2, S6**), indicating that the condensates are relatively immobile and exhibit solid- or gel-like behavior rather than liquid-like dynamics. This observation is consistent with the possibility that CELF2 assemblies represent more stable, less dynamic condensates, or potentially a continuum of material states rather than classical liquid droplets.

We agree that the optoDroplet and LacO/LacI systems, as well as *in vitro* crowding assays, rely on elevated local concentrations and do not, on their own, definitively establish liquid-liquid phase separation. In light of this, we have revised the language throughout the manuscript to adopt a more cautious interpretation. Specifically, we now refer to these structures as "condensates" or "assemblies" rather than strictly "phase-separated liquid droplets," and we clarify that our data support multivalent, IDR-driven clustering with condensate-like properties, without asserting fully liquid-like behavior.

We have incorporated the FRAP data into the revised manuscript (**Figs. 2, S6**) and updated the Discussion to highlight that CELF2 assemblies may occupy a more solid-like or gel-like regime within the broader spectrum of biomolecular condensates. We also note that further biophysical characterization will be valuable in future work to more precisely define their material state. For the reviewer's convenience, we have pasted the added discussion below:
*"A key challenge in the biomolecular condensate field is distinguishing bona fide liquid-liquid phase separation from other forms of protein assembly, as condensates can exist across a continuum of material states ranging from highly dynamic liquids to more rigid gels and solids. To further characterize the nature of CELF2 assemblies, we performed FRAP on CELF2 IDR droplets (**Figs. 2, S6**). These analyses revealed minimal fluorescence recovery over the measured timescale, indicating limited molecular mobility within the droplets. These findings suggest that CELF2 assemblies do not exhibit the highly dynamic behavior typically associated with classical liquid condensates, but instead occupy a more solid-like or gel-like regime within the broader spectrum of biomolecular condensates. Such material properties may reflect the formation of stable intermolecular interaction networks that support CELF2 function in RNA regulation. More broadly, our results underscore the importance of evaluating condensate material properties experimentally rather than inferring liquid behavior solely from the appearance of microscopically visible droplets. Future studies employing additional biophysical approaches will be valuable for more precisely defining the material state of CELF2 assemblies and determining how their physical properties contribute to their biological function."*

2. (Related to Fig. 3)
The CRISPR–dCas13 imaging experiments are interpreted as evidence that CELF2 condensates play a functional role in tau splicing regulation. However, these data primarily demonstrate partial spatial colocalization between CELF2 puncta and tau minigene RNA. Given prior evidence that CELF2 binds tau RNA, it is unclear what additional mechanistic insight this experiment provides. In particular, the data do not establish that RNA recruitment or splicing regulation depends on condensate formation.

We thank the reviewer for this thoughtful comment and agree that the CRISPR-dCas13 imaging experiments primarily demonstrate partial spatial colocalization between CELF2 puncta and tau minigene RNA, rather than directly establishing a functional requirement for condensate formation in tau splicing regulation. We now present these experiments as supportive evidence that tau RNA can localize to CELF2-enriched compartments, which is consistent with prior CLIP-seq data that CELF2 binds tau RNA, but does not on its own demonstrate a functional dependence on condensate formation. To further reflect this more cautious interpretation, we have moved the CRISPR-dCas13 imaging data to the Supplementary Figure (**Fig. S3C-E**) and adjusted the main text accordingly.

3. (Related to Fig. 4)
While the TurboID and LacO/LacI assays convincingly support IDR-dependent interactions between CELF2 and NOVA2/SFPQ, the imaging data provide limited evidence for recruitment of these proteins into pre-existing CELF2-containing structures. In the Results, the authors state that "this suggests that CELF2 might facilitate to recruit NOVA2 and SFPQ to those nuclear condensates." However, the data presented support heterotypic interactions and co-clustering, but do not establish directional recruitment or enrichment of NOVA2/SFPQ within CELF2 assemblies. Static colocalization does not distinguish recruitment from coincidental proximity, and additional evidence would be required to support this mechanistic interpretation. Clarifying this distinction would strengthen the conclusions.
Consistent with this, the Results section later states that the authors have "shown that CELF2 interacts with NOVA2 and SFPQ in nuclear condensates." However, the data support IDR-dependent interactions and co-clustering, but do not establish that these interactions occur within functionally defined nuclear condensates or involve directional recruitment into pre-existing CELF2 assemblies. Clarifying this point would improve the logical flow and mechanistic interpretation of the Results.

We thank the reviewer for this insightful comment and agree that our imaging data do not establish directional recruitment of NOVA2 or SFPQ into pre-existing CELF2 assemblies. Rather, as the reviewer notes, the data support IDR-dependent heterotypic interactions and co-clustering, but cannot distinguish recruitment from coincidental spatial proximity based on static colocalization alone.

In response, we have revised the text throughout the Results to down-tone our interpretation and avoid implying directional recruitment. Specifically, we have modified statements such as "CELF2 might facilitate recruitment of NOVA2 and SFPQ to nuclear condensates" to "our data demonstrate co-localization and co-clustering consistent with multivalent interactions", without establishing causality or directionality.

4. In addition to the concerns raised above, the manuscript repeatedly uses the term "condensates" to describe CELF2-containing assemblies, including heterotypic assemblies involving NOVA2 and SFPQ. While the data support IDR-dependent interactions and co-clustering, they do not distinguish condensates from dense interaction hubs or demonstrate that condensate formation is required for function.
This issue is particularly evident in the interpretation of Fig. 5. The functional data convincingly demonstrate that CELF2 cooperates with SFPQ and antagonizes NOVA2 to regulate tau exon 10 splicing. However, these experiments do not assess localization or condensate dependence, yet the Results text attributes these interactions to "biomolecular condensates." The data are fully explained by functional cooperation and competition between RBPs, without requiring a condensate-based mechanism. Revising the language to decouple these functional conclusions from condensate terminology would improve clarity and better align interpretation with the evidence presented.

We thank the reviewer for this thoughtful and important comment. We agree that our current use of the term "condensates" may overextend the interpretation of the data. While our results support IDR-dependent interactions

and co-clustering of CELF2 with partner RBPs, they do not directly demonstrate that these assemblies meet the criteria of biomolecular condensates or that condensate formation is required for function. As mentioned above, we performed FRAP experiments on CELF2-containing assemblies and observed very limited fluorescence recovery, indicating that these assemblies are relatively solid rather than dynamic liquid-like condensates. In response, we have revised the manuscript to use more neutral terminology (e.g., "assemblies" and "clustering").

Specifically for Fig. 5, we have removed language attributing the observed functional interactions to "biomolecular condensates" and now describe the results in terms of cooperative and antagonistic regulation of tau exon 10 splicing by CELF2, SFPQ, and NOVA2. This revision ensures that the interpretation remains fully supported by the presented data without invoking a condensate-based mechanism. We believe these changes improve the precision and clarity of the manuscript and better align our conclusions with the experimental evidence.

5. The in vivo experiments in C. elegans provide a fantastic validation of the structure–function relationships established in vitro and in cultured cells. The use of CELF2 WT, ΔIDR, and D388 mutants convincingly demonstrates that the CELF2 IDR and the conserved charged residue D388 are required for CELF2-mediated regulation of tau exon 10 splicing and for the resulting locomotor and cognitive phenotypes in vivo. However, the Results repeatedly conclude that these phenotypes demonstrate a requirement for "CELF2 condensation capacity." While the genetic and functional requirements are clearly established, condensate formation, puncta, or phase behavior are not directly assessed in the animal model. Moreover, attributing the in vivo effects specifically to condensation relies on interpretations of earlier in vitro and cell-based assays, which do not directly establish bona fide condensate formation or phase separation. Extending the condensate-based mechanism to the in vivo setting is therefore not directly supported by the data presented. Rephrasing these conclusions to reflect the experimentally tested requirements would improve precision and consistency without diminishing the impact of these strong in vivo findings.

We agree that our current *in vivo* data do not directly measure condensate formation, puncta, or phase behavior, and that our conclusions regarding condensation *in vivo* are inferred from prior *in vitro* and cell-based assays. To address this, we have revised the manuscript to more explicitly frame our *in vivo* findings rather than making direct claims about condensate formation. Specifically, we have adjusted the language in the Results: *"Together, these data demonstrate that CELF2's in vitro clustering capacity correlates with its function in regulating tau isoform expression and neuronal function in vivo, highlighting the functional importance of CELF2 clustering."*

Minor comments

Line 118: "promote axon 10 inclusion" should read "promote exon 10 inclusion."

Thank you for catching the error. This has now been corrected.

Line 316: The text refers to "milder defects (Fig. 8D–G)," but panels D–E assess locomotion, while panels F and G correspond to chemotaxis and gentle touch assays. Clarifying the figure panel references would improve accuracy.

Thank you for pointing this out. We apologize for the confusion. The text has been updated so that the description accurately reflects the figure panels.

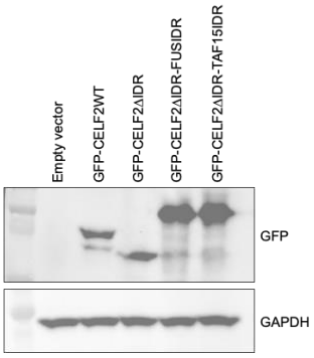
Reviewer #2 (Remarks to the Author):

This study report the important role of the RNA-binding protein CELF2 in enhancing tau exon 10 inclusion. The intrinsically disordered region (IDR) within the CELF2 hinge domain is shown to drives protein condensation and splicing activity, though this issue remains to be consolidated (see points below). Proteomic analyses identify NOVA2 and SFPQ as CELF2 interactors, though the importance of CELF2 interaction for the function of these proteins remains to be demonstrated. A conserved negatively charged residue (D388) within the IDR is shown to be critical for condensate formation, but its role in protein interactions that would lead to the proposed condensate-dependent splicing function remains unclear. The authors should adjust the strength of their interpretation and discuss alternative interpretations where appropriate, and address the points listed below:

We have carefully revised the manuscript to moderate the strength of our interpretations and to present a more balanced discussion. Specifically, we have tempered statements that were previously assertive and added discussion of alternative interpretations where appropriate. We believe these changes improve the clarity and rigor of our conclusions.

ΔIDR variant has much lower abundance (see comments to Figure 1), therefore current findings on their own are limited in interpretation.

We thank the reviewer for raising this point and apologize for any confusion. While the western blot in Fig. S3B suggested that the ΔIDR variant has lower abundance, the data in Fig. S4A show comparable expression levels between full-length CELF2 and ΔIDR. Also, GFP fluorescence intensity was similar across all GFP-tagged CELF2 variants, supporting comparable expression of the constructs. Moreover, transgenic *C. elegans* strains exhibit similar GFP-CELF2 levels across different CELF2 variants (**new Fig. S7**). These observations suggest that the difference in Fig. S3B likely reflects experimental variation rather than differences in protein expression or stability. To address this directly, we provide the western blot data from a repeated experiment below, confirming that ΔIDR is expressed at levels equivalent to WT.




Therefore the D388A mutant should be used for all of the same measurements where the ΔIDR is used, from Fig2 onwards, to consolidate findings.

We thank the reviewer for this suggestion. In response, we have now included new D388A experiments where ΔIDR is used:

- Fig. 2 related, FRAP analysis, showing that WT and D388A droplets have similarly slow recovery after photobleaching, despite the smaller droplet size for d388A (**Fig. S6**).
- Fig. 5, tau splicing, CELF2D388A + SFPQ and CELF2D388A + NOVA2 data are provided (**Fig. 7C-D**).
- Fig. 8 related, GFP-CELF2 variants showing similar GFP intensity, suggesting comparable expression levels (**Fig. S7**).

The following D388A experiments were already included in the original submission:

- Fig. 2D-E, *in vitro* droplet formation assay, D388A data already included in **Fig. 6C-D**.
- Fig. 2F-H, LacO array assay, D388A data already included in **Fig. 7A-B**.
- Fig. 3A-B, CELF2 puncta in cells, D388A data already included in **Fig. 6E-F**.
- Fig. 3C-D, tau splicing, D388A data already included in **Fig. 6G-H**.
- Fig. 6-8, already included D388A in the original submission (**Fig. 6-8**).

We did not include D388A variant in the following experiment:

- Fig. 4, TurboID, which requires several months to complete. Importantly, the interacting proteins identified by TurboID have been independently confirmed using the LacO array assay. And we performed this assay for D388A (**Fig. 7A-B**).

One of the primary conclusions is that the CELF2-IDR-mediated recruitment of SFPQ and NOVA2 to condensates containing tau mRNA is important for their respective functions in tau splicing. This needs to be directly demonstrated, for example by modulating expression of SFPQ and NOVA2 in cells with CELF2 knocked down (or only expressing delta IDR), and demonstrating that altered expression of these proteins no longer impacts tau splicing if they are not part of the the CELF2 condensates.

We thank the reviewer for this insightful comment and agree that our imaging data do not demonstrate directional recruitment of NOVA2 or SFPQ into pre-existing CELF2 assemblies. Rather, the data are consistent with IDR-dependent heterotypic interactions and co-clustering, but do not distinguish active recruitment from coincidental spatial proximity based on static colocalization alone. This concern was also raised by Reviewer #1 (see above). In response, we have revised the Results section throughout to temper our interpretation and avoid implying directional recruitment.

The authors' model on the function of CELF2/NOVA2 condensate is hard to understand. Findings from Fig5 demonstrate that the two proteins seem to compete and therefore have opposite effects on tau exon 10 inclusion. If the proteins compete, why would CELF2 be needed to recruit NOVA2 to the tau mRNA? This is quite counterintuitive, as it would mean that depletion of CELF2 should make no difference on splicing (as it would also remove the capacity of NOVA2 to repress the exon) - this clearly is not the case. It appears that NOVA2's repressive function becomes more pronounced if CELF2 is removed. Authors need to present further evidence to validate the model of NOVA2 tau binding & function being dependent on CELF2.

We thank the reviewer for this insightful comment, which has helped us refine and clarify our model. We agree that our original presentation was confusing and not fully supported by our data, particularly in implying a requirement for CELF2 in recruiting NOVA2.

There are examples in the literature where proteins with opposing regulatory functions coexist within the same biomolecular condensates, enabling rapid and dynamic regulation. For instance, in Wnt signaling, components like Dishevelled and Axin, which serve opposite roles in regulating protein degradation, gather within the same signalosome condensates (PMID: 36342472). In this context, our data support a model in which CELF2 and NOVA2 co-cluster via IDR-dependent interactions, while exerting antagonistic effects on tau exon 10 inclusion. We have now included this in the Discussion.

We agree that our current data support co-localization/co-clustering but do not demonstrate directional recruitment of NOVA2 by CELF2. We have therefore revised the text throughout the manuscript to remove this implication. This revised framework is consistent with the reviewer's observation that NOVA2-mediated repression becomes more pronounced upon CELF2 depletion, which may reflect the loss of a competing or buffering activity rather than loss of recruitment.

The text states that the effect of SFPQ overexpression on 4R : 3R ratio is less than CELF2wt overexpression but in their quantification plots there isn't a significant difference, they look the same, which argues against the CELF2-IDR being the nexus of tau splicing. The increased impact of CELF2/SFPQ co-transfection could be due to additive effect, rather than coopeartive effect within a condensate. Also, as explained in point1, it's crucial that D388A mutant is used for such experiments.

We thank the reviewer for carefully examining this point and for highlighting the discrepancy. Upon re-evaluation, we agree that CELF2 and SFPQ overexpression produce comparable effects on the 4R:3R tau splicing ratio, and that the difference between them is not statistically significant in our quantification. We have corrected the text accordingly to avoid overstating this comparison.

We also agree that the enhanced effect observed upon co-transfection of CELF2 and SFPQ may reflect an additive, rather than cooperative, mechanism. We have revised the manuscript to explicitly acknowledge this alternative interpretation and to temper our conclusion regarding the role of the CELF2 IDR as a central nexus for tau splicing regulation.

Also, as suggested, we have incorporated the D388A mutant into these experiments. We find that the D388A mutation, but not D388E, attenuates the 4R-promoting effect of CELF2 (Fig. 6G-H). Furthermore, co-expression of CELF2-D388A with SFPQ results in a reduced 4R tau ratio compared to co-expression of wild-type CELF2 with SFPQ (**new Fig. 7C-D**). And co-expression of CELF2-D388A with NOVA2 further reduced 4R tau ratio relative to wild-type CELF2 co-expressed with NOVA2. Overall, the D388A mutation produced a phenotype similar to that of ΔIDR, although the effect was less pronounced. These results further support a functional contribution of this residue.

Minor requests:

FIGURE 1
The authors should post the code for how they processed their CLIP data. They briefly describe the steps in their 'KEGG pathway analysis of CELF2 targets' section, but this would not be sufficient to reproduce their results as it stands.

We use command environment for data analysis but didn't generate any new code. We agree with the reviewer that more detailed parameters description could improve the reproduction of our results. We made our changes in the revised method section.

*"Piranha 1.2.1 was used to identify regions of statistically significant read enrichment, with parameters "-s -b 500 -u 501" to cover the broad binding regions. ChIPseeker was used to annotate and associate the peaks with genes by default parameters. The whole cell lysate CLIP-seq data was used to identify the potential target genes (4279 genes), which were then subjected to DAVID (https://davidbioinformatics.nih.gov) KEGG pathway analysis."*

Figure 1A :
the text says nuclear fraction CLIP data is used for KEGG analysis but the 'KEGG pathway analysis of CELF2 targets' methods say whole cell lysate data is used – not sure which one is correct.

We thank the reviewer for pointing out this inconsistency. We carefully re-examined our data and confirm that the KEGG pathway analysis of CELF2 targets was performed using the whole cell lysate CLIP-seq dataset. We have now corrected the text to accurately state that the whole cell CLIP data was used for KEGG analysis. We apologize for the confusion and appreciate the reviewer's attention to this detail.

Figure S1A :
In the text they refer to cyto CELF2 binding 3' UTR of mRNA and nuclear CELF2 binding to introns making it sound generalised, but only show the tau mRNA as evidence of this, guess they mean to only describe the distribution of binding on tau mRNA?

We thank the reviewer for this helpful comment. We apologize for the lack of clarity in our original wording. Our intention was to describe a general binding pattern of CELF2, rather than a conclusion based solely on tau mRNA. To address this, we have now included a pie chart summarizing transcriptome-wide binding site distributions, showing that cytoplasmic CELF2 predominantly binds to 3′ UTR regions, whereas nuclear CELF2 preferentially binds to intronic regions (**new Fig. 1A**). We have also revised the text to clarify that this represents a global observation and is not specific to tau mRNA.

Figure 1E:
Typo in text describing the figure, 'axon 10' rather than 'exon 10'.

Thank you for catching the error. This has now been corrected.

Figure 1K:
Statistical significance stars missing off graph

Thank you for catching the error. Statistical significance stars are now included in the graph.

FIGURE 3
Figure 3C / S3B
The delta IDR mutant has much lower expression than WT in S3B, which creates caveats for interpretation. The no CELF-delta IDR could have deficient splicing functon(and condensation) simply due to lower stability/abundance in the transient transfection experiment? The authors should discuss this. It is reassuring that the TAF15 IDR mutant also has low expression but does restore splicing function, though gapdh loading control is a lot lower in this lane. This should be redone and quantified across replicates.

We thank the reviewer for raising this point. As stated above, the western blot in Fig. S3B suggested that the ΔIDR variant has lower abundance, but the data in Fig. S4A show comparable expression levels between full-length CELF2 and ΔIDR. Moreover, transgenic *C. elegans* strains exhibit similar GFP-CELF2 levels across different CELF2 variants (**new Fig. S7**). These observations suggest that the difference in Fig. S3B likely reflects experimental variation rather than differences in protein expression or stability. We also provide the western blot data from a repeated experiment below, confirming that ΔIDR is expressed at levels equivalent to WT (see above).

FIGURE 5
Figure S5D/S5E:
The splicing ratio looks very similar on the gel in the last 4 conditions in D, but are very different according to their quantification of the bands in E, which could this be?

We apologize for the confusion. In the gel of Figure S5D, the last 4 samples are in fact two conditions, with 2 shRNA for each condition. The effects of shRNA#1 and shRNA#2 on tau splicing were very consistent with each other. We observed a consistent reduction in 4R tau in CELF2ΔIDR + shNOVA2 (compared to CELF2WT + shNOVA2), although the difference is subtle on the gel image. The way of quantification makes the subtle difference quite obvious on the bar graph. The bar graph is generated using data from 3 different gel images (3 biological replicates).

FIGURE 6
Fig. S6F:
No loading control is present on the WB. Here the author acknowledge equivalent expression levels are important for this experiment, but not for the initial delta IDR experiment in 3C.

We apologize for the confusion. The WB in Fig. S6B was performed using purified recombinant proteins. These recombinant proteins were generated for the *in vitro* droplet formation assay, and the WB was included to confirm protein purification. Therefore, no loading control was used. The text in the Results section has also been corrected accordingly.

FIGURE 7
By eye, co-localisation between NOVA2/SFPQ and the D388A mutant looks preserved in the cell images, but perhaps there is a smaller number of larger condensates? Could the authors address this by quantifying co-localisation relative to the size of condensates?

We apologize for the confusion. In Figure 7 images, an artificial system was used to assess the multivalent interactions between CELF2 (WT or mutant) and NOVA2/SFPQ. Through specific LacI-LacO interaction, eYFP-LacI-tagged protein is recruited to the LacO array, generating a concentrated interaction hub. mCherry-tagged protein is brought to the hub through multivalent interactions and the intensity of mCherry at the eYFP hub can be measured to quantify the potency of these multivalent interactions. In this experiment, we only measure the intensity of mCherry at that one eYFP hub, not the overall co-localization at any other puncta.

FIGURE 8
Analysis of in vivo protein expression of the CELF2 WT and mutants should be provided.

Thank you for this important suggestion. We agree that assessing *in vivo* protein expression levels is important. However, as the transgenic worms used in this study carry extrachromosomal arrays rather than integrated transgenes, performing quantitative western blot analysis would require collecting a very large number of animals

carrying the transgene and is technically challenging. To address this, we instead assessed expression by imaging GFP-labeled CELF2 WT and mutant proteins *in vivo*. These analyses show that the different CELF2 variants are expressed at comparable levels across lines. We have now included representative images and quantification (**Fig. S7**), and clarified this point in the revised manuscript.

Reviewer #3 (Remarks to the Author):

In the manuscript entitled "CELF2 Promotes Tau 1 Exon 10 Inclusion via Hinge Domain-Mediated Nuclear Condensation, 2 Driving Cognitive Dysfunction in Tauopathy Models", the authors investigated the role of CELF2 in promoting tau (MAPT) exon 10 inclusion. Age-associated inclusion of MAPT exon 10 increases the 4R:3R ratio and is widely considered a feature of tauopathy, but the mechanism underlying exon 10 splicing remains incompletely understood. Li et al showed that Celf2 binds to the upstream intron of Mapt exon 10 in the nucleus (CLIP-Seq), and postnatal deletion of Celf2 significantly decreased exon10 inclusion in young and aged adult mouse brains. Interestingly, the Celf2 protein formed condensates through its IDR in the hinge domain, and the IDR was required for its function in promoting the MAPT 4R isoform. The authors utilized TurboID to identify binding partners of Celf2 and narrowed the targets down to Nova2 and Sfpq, both of which showed reduced interaction with the Celf2 IDR mutant form but displayed opposite effects on exon10 splicing when co-expressed with Celf2. The authors dived deeper into the IDR and showed that D388 was critical for Celf2 condensate formation and splicing function. To test the CELF2 effect in vivo, the authors showed that human Tau 4R overexpression is toxic in C. elegans and developed a clever minigene strategy to test the splicing effect of wild-type and mutant CELF2 in promoting 4R toxicity.

Overall, this study addresses a central question about tau isoforms, reveals important roles of Celf2 in promoting the 4R:3R ratio, and reports molecular insights into Celf2 IDR. The integration of molecular, cellular, biochemical, and genetic approaches, along with rigorous controls, convincingly supported their conclusions. The reviewer raised a few minor questions to enhance the rigor of this study.

1. While the authors showed a Celf2 CLIP-Seq peak in Mapt intron 9, it would be helpful to determine whether the peak was statistically significant and associated with the Celf2 binding motif. The Mapt exon organizations are not consistent between Fig. S1A and S1B.

We thank the reviewer for this insightful comment. We have now included statistical measures for the CLIP-seq peak in *Mapt* intron 9. Specifically, the peak shows a fold enrichment of 5.87 with a −log10(q value) of 13.34 in CLIP-seq performed using whole-cell lysate, and a fold enrichment of 5.92 with a −log10(q value) of 10.53 in CLIP-seq using the nuclear fraction, indicating strong statistical significance. This information is now included in Figure 1 legend and in Supplemental Table 1.

In addition, we examined the underlying sequence and found it to be enriched for TG repeats and TGTT motifs, which correspond to UG repeats and UGUU motifs in RNA. These are well-established binding motifs for CELF2. The peak sequence is now included in Fig. S1 and below, with TG repeats and TGTT motifs highlighted.

CELF2 binding motif: (UG)n and UGUU
Binding peak sequence (mm9, chr 11, 104179002 – 104179227):
GTAACTAAGTATTGTATGTGCACGCCACTGTGTTCAGAAGGCAGAAGTGGGGTTTTATGACAAGTTCTTTACCCTC
CCTGAGCCATCCCACCAGCTCAGCTCAAGACAATCTGGTTTGAAGCCTTTAGCCTTGCCACTCCCCAGCCTTGGA
CGCTCTTTGTGATAAGGTGCCTTGCACTGGTGTGTCTGTTTGGATTGTTGTTGATCTTTCTTCTCATCACTTGAC

And we apologize for the confusion on the exon organization. Fig. S1A and Fig. S1B depict different gene models: Fig. S1A shows the exon organization of *Mapt*, whereas Fig. S1B (New Fig. S1C) shows the exon organization of *Celf2*. The figure legend has now been clarified to prevent future confusion.

2. The results from Celf2 conditional homozygous KO mice are convincing. Does heterozygous Celf2 KO also reduce the 4R:3R ratio? This may have interesting indications of Celf2 dose in regulating MAPT splicing.

We thank the reviewer for this insightful point. In response, we compared WT and Celf2+/- brains for 4R:3R tau ratio. Interestingly, we observed a subtle, but consistent reduction of 4R:3R ratio in Celf2 +/- mouse brains compared to brains of WT littermates (**new Fig. S1D-E**), suggesting that one allele of Celf2 loss is sufficient to impact tau splicing.

3. The important roles of Celf2 in human and mouse brain development should be introduced, such as PMID 34107259 and PMID 38829512. The developmental roles of Celf2 would pose a challenge to intervening in its MAPT splicing function, and this should be discussed.

We appreciate this insightful suggestion. We agree that the developmental roles of CELF2 are important to consider in the context of targeting its regulation of *MAPT* splicing. In response, we have added a discussion of CELF2's functions in human and mouse brain development, incorporating the studies you highlighted (PMID: 34107259 and 38829512). We also explicitly discuss how these developmental roles may complicate therapeutic strategies aimed at modulating CELF2 activity.

"Recent work demonstrates that Celf2 plays critical roles in neuronal differentiation, maturation, and the establishment of appropriate splicing programs during brain development [72, 73]. Disruption of CELF2 has been associated with widespread alterations in alternative splicing networks that are essential for neuronal identity and function in both human and mouse systems [73, 74]. These findings suggest that CELF2 is not only a regulator of *MAPT* exon usage but also a broader orchestrator of neurodevelopmental RNA processing programs. Given its pleiotropic functions, global manipulation of CELF2 could lead to unintended consequences on neuronal health and gene expression. Therefore, effective therapeutic approaches may require more precise strategies, such as cell type-specific targeting, temporal control of intervention, or selective modulation of CELF2 interactions at the *MAPT* locus rather than broad perturbation of its activity."

This expanded discussion has been incorporated into the revised manuscript (Discussion section).

4. Other splicing regulators, such as MBNL (PMID: 38772368), have been reported to regulate MAPT exon 10 inclusion. This should be discussed.

Thank you for this helpful suggestion. We agree that additional splicing regulators of *MAPT* exon 10, including MBNL proteins, are important to consider. In response, we have incorporated a discussion of Muscleblind-like (MBNL) proteins and their reported role in regulating *MAPT* exon 10 inclusion (PMID: 38772368).

*"MBNL proteins have been shown to influence exon 10 splicing as part of a broader network of RNA-binding proteins that coordinate alternative splicing decisions in neurons [82]. This further highlights that* MAPT *exon 10 inclusion is not governed by a single factor, but rather reflects the combinatorial and context-dependent actions of multiple splicing regulators. The interplay between Celf2 and other splicing factors may be critical for fine-tuning* MAPT *isoform balance, and that perturbations in this regulatory network could contribute to disease-relevant splicing dysregulation"*

Reviewer #4 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.