nature portfolio

Peer Review File

Mis-splicing of a neuronal microexon promotes CPEB4 aggregation in ASD

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

Version 1:

Reviewer comments:

Referee #1

(Remarks to the Author)
Garcia-Cabau et al have studied how the inclusion of microexon 4 in neuronal CPEB4 alters the protein properties and phase transition. They find that heterotypic interactions between the microexon and a cluster of His residues are necessary for the CPEB4 condensates to dissolve upon neuronal depolarization, thus allowing translation activation. The authors describe the exact kinetics of the interactions between the microexon and the His residues by a mechanism, akin to heterotypic buffering. They provide evidence that competition between homotypic interaction of aromatic clusters in CPEB4 that drive aggregation, and heterotypic interactions between the aromatic clusters and the microexon ensures the proper regulation of nCPEB4 in neurons and explain why a reduction in its degree of inclusion in ASD could result in a dominant-negative decrease in CPEB4 activity.
Despite the already known role of CPEB4 in translation regulation via phase separation, this study provides an exact molecular mechanism of how this occurs and is thus very interesting and novel. The manuscript is very well written, in a way that is apprehensible to a broader audience, of different scientific background.
Overall, the methodology used and the statistical analyses are appropriate, however, there are specific minor comments to be addressed before publication:
1. The authors should perform qPCR and WB on the N2A cell with the different constructs, in order to see the effects of their deletions on the actual ASD-related mRNAs and proteins.
Those experiments will strengthen the biological context of the work, by connecting all the herein observed phenotypes with the previously reported functions of CPEB4 (e.g. in Parras et al, 2018), and will tight the knot.
2. A short description of the statistical analysis per experiment should appear in the figure legends (type of test, sample size, p-value, etc)
3. In Figure 1, where the authors show the dissolution of particles, the image resolution is not optimal. Better, high-resolution images should be obtained.

Referee #2

(Remarks to the Author)
In their submitted manuscript, Salvatella, Mendez and colleagues focus on characterizing the mechanism by which misregulation of the neuronal-specific CPEB4 microexon impacts CPEB4 function and potentially contributes to idiopathic autism spectrum disorder (ASD). Genetic and genomic studies from several labs have provided evidence of a causal relationship between disruption of the neuronal-specific microexon program and ASD. Previous work from Mendez and colleagues revealed that CPEB4 microexon splicing affects poly(A) tail length and translation of ASD-risk genes, and perturbation of its overall inclusion levels result in ASD-reminiscent phenotypes in mouse models. Related to the authors submitted study, previous work from others has revealed that disruption of microexons in eIF4G translation initiation factors affects synaptic protein expression and animal behavior, likely through the loss of microexon-promoted phase separation with neuronal granule components, including CPEB4 (see below).

In their current study, the authors show that CPEB4 condensates are dissolved following neuronal depolarization through pH-dependent intermolecular interactions. These interactions involve histidine and arginine residues, with the latter being enriched in the CPEB4 microexon. The authors further show that both a histidine cluster, as well as the amino acids encoded by the neuronal microexon, are required for efficient phase separation of truncated CPEB4 proteins both in vitro and in cells following overexpression. However, the CPEB4 condensates formed in the absence of the microexon are more resistant to neuronal depolarization in cells and the corresponding droplets tend to transition into aggregates in vitro, likely through interactions involving the histidine cluster. Interestingly, the degree of CPEB4 aggregation depends on the ratio of microexon containing isoforms, while the level of inclusion observed in ASD individuals corresponds to high-predicted aggregation propensity.

Overall, this is an interesting study that provides insight into how microexon splicing levels can affect protein function in vitro, with implications for contributing to autistic phenotypes. In particular, the NMR experiments using urea to observe an increase in signal for resonances involved in interactions within the CPEB4 "multimers" are very clever (see below). This is an excellent approach and provides detailed site-specific interaction information that the authors exploited, including studying the pH dependence of interactions, and supporting the interactions between the Arg-rich microexon and His-rich cluster. Moreover, the authors' CALVADOS simulations have impressive agreement with experimental Csat values and provide support for the experimentally-defined key interactions contributing to phase separation.

However, while the biochemical and biophysical experiments in the study are strong, some of the overall conclusions of the authors' work are not well supported and additional experiments and analyses would be needed to strengthen their study. Specific comments follow:

1. The authors' model suggests that mis-regulation of the CPEB4 microexon may cause idiopathic forms of autism by promoting CPEB4 aggregation. However, the in vivo data to support this model are not strong. For example, does heterozygosity of the CPEB4 microexon in the GFP knock-in model result in CPEB4 aggregates in neurons? Furthermore, does inducing aggregation of endogenous CPEB4 in mouse brains through genetic manipulation result in autistic-like phenotypes?

2. Related to the above comment, most of the experiments in this study are performed in vitro or through overexpressing CPEB4 isoforms where intermolecular interactions seem to play a critical role for CPEB4 phase transition and aggregation (see comments below). However, in vivo CPEB4 is known to be present in condensates that contain additional proteins and RNA molecules. Although the authors have attempted to address this through some experiments in Xenopus these are limited in scope. Their study would be more conclusive if the effects of the microexon were studied in neurons at endogenous protein levels. This is also important when considering the existence of additional microexons regulated by the same molecular pathway, that are present in proteins known to exist in the same cellular condensates as CPEB4, and that when misregulated also result in ASD-like phenotypes, such as the aforementioned eIF4G microexons. In this regard, related and possible convergent mechanisms of CPEB4 and eIF4G microexons should at the very least be discussed by the authors.

3. What prevents the aggregation of CPEB4 in non-neuronal cells where the microexon is mostly excluded?

4. Do any of the identified PTMs overlap the microexon residues?

5. The phosphorylation study was briefly described and the results for the phosphomimetic (which did not form condensates) suggests that there is some impact of phosphorylation, which is inconsistent with the interpretation of the data for the mutants designed to remove phosphorylation, which is that phosphorylation is not significantly regulating the phase separation. The authors should elaborate and clarify their interpretation of these results.

6. There is clear methylation on Arg350 in non-stimulated cells that is missing in stimulated cells. It is known that Arg methylation significantly reduces Arg-dependent interactions and reduces phase separation. The authors could address how this PTM potentially partly explains how stimulation leads to disassembly of the condensates. Relevant work from others on the role of Arg methylation in condensate formation should be referenced.

7. p9: The lack of role of RNA in differences in phase behavior in cells for nCPEB4-NTD vs nCPEB4delta4-NTD is not convincing. This experiment should be more fully described and explained in the text.

8. The manuscript could be clarified by first outlining interactions (including pH dependence) in the microexon-containing sequence and then looking at the delta-microexon (as well as delta-His cluster) to confirm the interactions and provide insights potentially relevant to an ASD context. The expected impact of cellular pH on changing the interactions and thus changing phase behavior should be more explicitly stated. The current text is somewhat confusing.

9. p2 intro/Fig 6: "Neuronal CPEB4 (nCPEB4) binds to the transcripts encoded by ASD risk genes". This needs a reference and clarification, since not all mRNAs bound by CPEB4 are "ASD risk genes". This leads to confusion in Figure 6 where only "ASD risk gene" levels are indicated as going up and down. The actual proteins impacted by CPEB4 microexon 4 inclusion levels are not all necessarily ASD risk genes.

10. The in vitro phase separation studies are generally well-done, but the conclusion that the role of the microexon is only to prevent irreversible aggregation misses the data/point that it also contributes to phase separation in the first place (see also

comments above). The language of "homo-" vs "heterotypic" phase separation is confusing and possibly incorrect, since this term is used to describe interactions between CPEB4 whereas heterotypic implies involvement of different proteins (which is certainly the case in cells).

11. p4: "... high ionic strength (Fig. 1j), suggesting that the droplets are in part stabilized by hydrophobic interactions". High salt inducing phase separation doesn't necessarily mean hydrophobic interactions. It could be shielding repulsive electrostatic interactions. Given the results later in the paper, it seems that this interpretation is more likely.

12. p6-7: "conditions are insufficient for phase separation". While there were no visible droplets in the microscope, ~55 nm "particles" are small "droplets" or condensates. Cellular condensates such as paraspeckles can be as small as 50 nm in diameter. This should be acknowledged rather than referring to them as "intermediates" along the pathway to droplets. The language of referring to these as "multimers" that give rise to "droplets" is confusing and raises the question of whether there are different interactions for these two "states". The interactions seen in the NMR for these small droplets must be the same as in big droplets as there is no transition between the intermediate "multimers" and "droplets" in the performed experiments. They are just getting bigger.

13. Is the His cluster sequence LARK-like? It would be valuable for the authors to comment on sequence features that may be expected to lead to fibers.

14. SRRM4-dependent regulation of CPEB4 microexon in N2A cells and neurons was reported previously (Gonatopoulos-Pournatzis et al., Mol Cell, 2020) and should be referenced in the section of the authors' manuscript where they confirm this observation.

Referee #3

(Remarks to the Author)
In this study Garcia-Cabau et al. investigate the mechanism of how the microexson4 regulate the properties of CPEB4 condensates, which is important for translational regulation during depolarization of neurons. They combined cell culture, in vitro reconstitution and molecular simulations to show that the interactions between His clusters and positive Arg residues in exson4 stabilize condensates and prevent the irreversible aggregation.

The functional role of condensation has been a matter of debate for years, especially how the different properties of condensates matter. Previous studies have reported that CPEB4 condensates are important for translation repression in neurons and needs to be regulated properly. Exclusion of exson4 causes autism spectrum disorder. This study used multiple methods to show that in neurons CPEB4 forms condensates that dissolve upon KCl-mediated depolarization. The expression of CPEB4 containing microexon 4 mediates the capability of these condensates to dissolve upon depolarization, by providing an arginine-rich positively charged domain that interacts with a hydrophobic histidine-rich region in the N-terminal domain, while preventing homotypic interactions of the histidine-rich region. Reducing the fraction of CPEB4 containing microexon 4 results in an impairment of these condensates to dissolve, providing a mechanism for translational dysregulation in ASD.

Unfortunately, the study falls shorts in providing a strong connection between the properties of nCPEB4 and nCPEB4Δ4 condensates and their cellular behaviour and functions, especially in the pH regulation scenario during cell depolarization. Specifically, the central argument of this paper rests on the assumption that depolarization leads to a relevant change in pH that directly affects condensate dissolution. For publication of this article in the journal Nature, it would be required that this central claim has to be experimentally addressed and verified, as outlined in the comments below. Building on these measurements the supporting in vitro assays should then directly test if condensate dissolution kinetics are indeed different between full-length, wild type and CPEB4Δ4. In addition, the in vitro experiments were carried out with the disordered NTD. However, as an RNA-binding protein, the behavior of CPEB4 might be significantly influenced by RNA. In my view, in vitro data with purified full-length proteins with RNA have to be acquired to prove their key discoveries.

In summary, this study provides a collection of data that adds to our understanding of condensation regulation but the manuscript in its current form does not yet significantly advance the field because the data do not strongly support the claimed conclusions.

General comments:

1) To avoid confusion when labeling graph axes, brackets should ideally be used to indicate units, rather than backslashes. I.e. Time (s), rather than Time / s. The authors should revise their axis description for Fig. 2 a, d, f; Fig. 3 a, c, d, l (y-axis label completely absent); Fig. 4 b, d, h, i, j.

2) Figure panel layout needs be rearranged, so that A, B, D and onwards are in order, especially Figs 3, 4 and 5.

3) The figure legends is generally over-simplified, not reader-friendly. The author should provide some experimental details, especially for condensates experiments in vitro. Additionally, information concerning the number of replicates should be provided in the figure legend.

Major points:

1) Several claims based on cell-imaging appear to lack any quantification, and are only supported by images of a single, or handful of cells. This concerns data shown in Fig 1D,H. Ext data 1D, E, I. Fig 2B,C. Ext data fig 2B,C. Additionally, micrographs of cells would benefit from zoomed inserts of the cytoplasmic structures formed by nCPEB4.

2) In line with the previous comment, another more physiological means of depolarizing the cells would strenghten the claim of depolarization-induced dissolution of the condensates. KCl increased the membrane potential drastically, and results in a brief period of neuronal excitation (minutes), followed by a prolonged period of neuronal depression. However, it floods the cells the positively-charged potassium, which can lead to conflicting results regarding CPEB4 phase separation and dissolution. Options for more physiological stimulation are glutamate, kainate or NMDA. Even if the effect size varies from KCl treatment conditions, these should be quantified and reported (altered number of foci / altered condensate properties assessed by FRAP).

3) The authors should try to asses to which extent neuronal, intracellular pH (pHi) changes upon depolarization, possibly by employing small molecule fluorophores or genetically encoded pH sensors. Does cytosolic alkalinization / change in pHi exceed the previously reported 0.05 units? (PMID: 3353214)

4) From the molecular dynamic simulations, the authors suggested that His clusters work as a sensor for pH, i.e., at pH6 the His clusters interact with negative charges at positions 72-147, and at pH8 the His clusters interacts with Arg in exon 4. Any experimental data to support this? Are these predictions consistent with the salt effects on nCPEB4 condensation at pH6 and pH8? If possible / reasonable a change in hydrodynamic diameter, depending on pH, as determined by DLS or any other technique would be convincing.

5) What is the relationship between nCPEB4 condensates and RNA in cells and in vitro?

6) Are the multimers of nCPEB4 and nCPEB4Δ4 regulated by pH? And what is the driving force for the multimer formation of nCPEB4Δ4?

7) The authors used overexpression system to show the formation and dissolution of nCPEB4 and nCPEB4Δ4, in which the expression levels are heterogeneous. However, condensation process is concentration-dependent. How the authors carried out quantification and generated the conclusion with the heterogeneous system?

8) Since the dissolution of nCPEB4 condensates upon neuron depolarization caused by intracellular pH increases, the authors should study how the pH influences the properties of the individual nCPEB4 condensates, nCPEB4Δ4 condensates and co-condensates of nCPEB4 and nCPEB4Δ4 in vitro.

9) Fig 4E: The lack of ‘reversibility of condensation’ in cells expressing CPEB4Δ4 is resulting in remaining CPEB4Δ4 structures in the cytoplasm upon depolarization. However, the material properties of these structures have not been investigated. Do they remain liquid-like or transition to an aggregate-like state, like the in vitro experiments? In the cartoon in Fig 6 even the term ‘irreversible aggregation’ is mentioned, which is not demonstrated at all in cells. More data should be obtained in cells to evaluate physical properties of remaining CPEB4Δ4 structures, for example FRAP as a starting point. Also concerning Fig. 4F - quantification of the key experiment of this paper, supporting the claim of indicated in the title - it would strengthen the manuscript, if the authors quantified ‘% cells with foci’ prior to stimulus application - additionally the ‘#foci per cell’ for these two time points could lend additional support.

10) Fig 4I: The authors should characterize the aggregates formed by nCPEB4Δ4 in vitro, for example by FRAP.

11) In Fig 5A, the authors should characterize the properties of co-condensates of nCPEB4 and nCPEB4Δ4 with FRAP, and investigate their dissolution upon cell depolarization.

12) The authors claimed that homotypic interactions of His clusters in nCPEB4Δ4 accelerate the condensation to aggregation transition. The authors should give stronger evidence to support the key discovery.

13) The nCPEB4 and nCPEB4Δ4 are co-expressed in neurons, and the ratio of these two proteins determines normal (Δ4~30%) and ASD (Δ4~45%). The authors found a sigmoidal pattern for the properties of the co-condensates. The authors should show the ratios of these two isoforms also cause similar properties changes in cells, and potentially correlate these properties to its function. One suggestion might be to perform photobleaching analyses on experiments, shown in Fig. 5A and Fig. 5E.

14) In the discussion it is stated: “In nCPEB4 the changes in phase separation propensity and activity that occur upon depolarization are caused by local changes in intracellular pH.” However, this statement is not backed up by any data. In vitro pH was shown to alter phase separation behavior, and condensation patterns (in one single cell) shown in Fig 2C correlate with published data on pH changes upon depolarization, but a causal link is not shown. A true mechanistic insight in how KCl depolarization results in condensate dissolution in cells is still lacking. The claim of pH dependence could be strenghted by co-imaging with commercially available pH sensors, but further evidence is needed for causality.

Minor points:

1) Please give the domain-wise structure of nCPEB4, and indicate the localization of microexon4.

2) Lines 48-60: the introduction of nCPEB4 should be more logic, i.e., introducing CPEBs family (line 53-55), then nCPEB4.

3) Please show FRAP data in the endogenous nCPEB4 in mice striatal neurons, correlating to Fig 1C. Also, in Fig 1C, the label should be mEGFP-nCPEB4.

4) Highlight the nerve system in Extended Fig 1A.

5) Several bargraphs in Extended Data 1F and G lack error bars. Clarification is needed on why: is this a single replicate, error bar is too small to see or otherwise.

6) If the phosphorylation does not play roles in dissolution of nCPEB4 condensates, the authors should remove the data from Fig 2 into Extended Fig 2. In addition, the authors should explain the discripancy between their data and previous research.

7) Please clarify the pH for the contact maps in Fig 2G? Is it pH6 or pH8?

8) Fig 2E is far too small to see which residues were changed from histidine to serine.

9) In Extended Fig 2F, the Csat from the MD at pH6, 7 and 8 are similar. From the experimental data, Csat should be the lowest at pH6. Please address this.

10) Are the multimer of nCPEB4 regulated by pH?

11) In Extended Fig 3A, the figure legends mentions the temperature is from 5 to 60oC, however, the the figure only shows up to 35oC.

12) All the DLS measurements were with volume fraction except for Extended Fig 3C with intensity fraction. It will be better to be consistent.

13) Line 222: please clarify ca350 in the figure legend.

14) Lines 235-239: please clarify.

15) For Fig 4C, the authors should compare full-length nCPEB4 and nCPEB4Δ4 in cells.

16) Fig 4I and and Extended Fig 4I use the same images. Please change it.

17) nCPEB4 and nCPEB4Δ4 ratios in control and ASD patients are given as 0.3 and 0.45, respectively, but no reference is provided for this data.

Version 2:

Reviewer comments:

Referee #1

(Remarks to the Author)
Garcia-Cabau et al have studied how the inclusion of microexon 4 in neuronal CPEB4 alters the protein properties and phase transition. They find that heterotypic interactions between the microexon and a cluster of His residues are necessary for the CPEB4 condensates to dissolve upon neuronal depolarization, thus allowing translation activation. The authors describe the exact kinetics of the interactions between the microexon and the His residues by a mechanism, akin to heterotypic buffering. They provide evidence that competition between homotypic interaction of aromatic clusters in CPEB4 that drive aggregation, and heterotypic interactions between the aromatic clusters and the microexon ensures the proper regulation of nCPEB4 in neurons and explain why a reduction in its degree of inclusion in ASD could result in a dominant-negative decrease in CPEB4 activity. Despite the already known role of CPEB4 in translation regulation via phase separation, this study provides a molecular mechanism of how this occurs, and is thus interesting and novel. The manuscript is well written, in a way that is apprehensible to a broader audience, of different scientific backgrounds.
In the re-submitted version the authors have made an effort to adequately address most of the reviewers' comments. They have added in vivo experimental evidence that correlate their findings with ASD, and thus strengthen the biological context of their work (Fig.5). Re-arrangement of some of the findings improved the flow and the readers' understanding of the work (Fig.2, 4 and 5).
Overall, the revisions significantly improved the manuscript.

Referee #2

(Remarks to the Author)
The authors have submitted an improved manuscript that addresses many of the reviewers' comments. A major concern of the three reviewers was whether the in vitro demonstration of aggregation of nCPEB4Δ4 represents an accurate in vivo model. The authors now demonstrate aggregates in brain homogenates from their mouse model that recapitulate the 'ASD ratio' (but see comment below) of nCPEB4/nCPEB4Δ4. However, some the reviewer comments could have been better addressed. Consequently, the results as they stand are not as conclusive as the authors portray. Some remaining concerns and suggestions for further revision are summarized below.

1. The authors assert that their original mouse model (relying on over-expression of nCPEB4Δ4 to alter CPEB4 isoform ratios) represents a better model for ASD than a clean heterozygous knockout of the CPEB4 neuronal microexon. This view is based largely on data from their previous manuscript (Parras et al Nature, 2018). For example, the authors state that their model "recapitulates ASD conditions: microexon inclusion/skipping differences in ASD are more subtle than a 50% ratio, with values changing from 70/30 % in 'normal individuals' to 55/45 % in patients with ASD". However, the data in their previous paper shows that there is extensive variability in the ratios of the microexon included versus skipped isoforms across patient samples, and it is anyone's guess as to how these variable ratios correspond to protein level changes in patient brains, or which specific ratios might be the most physiologically/autism-relevant when an entire program of microexons is disrupted by altered activity.

This makes it difficult to appreciate the rationale behind not deleting the CPEB4 microexon in the GFP-tagged line (which is hardly used in the authors' study), or in a WT mouse line. More specifically, according to the biochemical data presented (Fig 5c, S5d), a more pronounced increase in the Δex isoform ratio than observed in ASD individuals (i.e. ~65% skipping instead of ~45% skipping in autism and ~30% in control animals) should not reduce aggregate formation compared to the ASD-associated ratio. Therefore, a heterozygous endogenous microexon deletion mouse would be expected to result in ASD-like phenotypes and display CPEB4 aggregates. While this reviewer fully appreciates the technically demanding and time-consuming nature of such experiments, such an analysis would have helped address concerns related to the overexpression of nCPEB4Δ4, which was also a limitation of their previous paper. The argument that 'readjustment' of the overall CPEB4 levels is also questionable as it is possible that temporal overexpression (i.e. not detected at the time of harvesting) could have detrimental developmental effects. These issues should at the very least be discussed by the authors and mentioned as a limitation of their work.

2. The authors also do not fully address a request from Reviewer 3 (which this reviewer agrees with) to perform more physiological treatments for activity-stimulation, given the issues with the extreme KCl treatment conditions used. The authors attempt depolarizing N2a cells with increasing concentrations of glutamate and NMDA, but these failed to cause depolarization as measured using immediate early gene expression markers. However, this is not surprising since it is known that N2a cells do not express glutamate receptors. It is unclear why the authors did not attempt more experiments using primary neurons.

3. Reviewer 1, Comment 3: To address the issue of obtaining a high-resolution demonstration of the dissolution of particles, as mentioned by the authors, attaching a movie demonstrating events before and after stimulation would be much more valuable than the inset, which doesn't really help.

4. Reviewer 2, Comment 5: A follow-up question to the authors' explanation here is whether they think that the difference in the cells with foci remaining after stimulation between the 11A and 11D is significant (Fig ED 2e). If it is significant, then there could potentially be an effect of phosphorylation in addition to pH.

5. Reviewer 2, Comment 7: RNA impact on phase behavior is not the same as RNA binding, with affinities measured in dilute solution. More specifically, lack of folded RNA-binding domains in the protein does not preclude an impact of RNA on phase separation. An in vitro experiment addressing the impact of RNA on phase separation propensity (or further characterization of droplets) of nCPEB4 vs nCPEB4Δ4 may be appropriate.

Additional comments

1. In some places in the manuscript the authors refer to conclusions relating to CPEB4 microexon function 'in neurons' when they are referring to experiments performed in N2A cells. They also make some general conclusions that are not well supported by their data; for example, at the end of the Introduction: "In summary, we show how microexon 4 ensures the proper regulation of nCPEB4 in neurons and explain why a reduction in its degree of inclusion in ASD results in a dominant negative decrease in CPEB4 activity". I suggest toning down and replacing 'explain' with 'providing evidence suggesting'….

2. Avoid referring to autism under the umbrella of 'disease': For example, in the Abstract, the authors should change 'neurodevelopmental diseases' to 'neurodevelopmental or brain disorders'.

3. Reviewer 3, Comment 9 (second part): How many cells (or what percentage) have foci before stimulation?

4. Methods section: It is not clear how the quantification of aggregation was performed. What is meant by "fluorescence signal not stemming from the condensates"? Is this background or non-spherical structures?

5. Main Figure 4m legend: Please clarify in the legend what the two curves in the right panel indicate. Also check if the left

panel is nCPEB4 or nCPEB4Δ4.

Referee #3

(Remarks to the Author)
I have had a chance to look the responses in detail, and thank the authors for doing an excellent job of addressing my comments.

In their revised manuscript Garcia-Cabau et al. address all the points raised by this reviewer in March 2023 in an exemplary and highly satisfactory manner.
As an example of the major advances achieved by the authors over the last few months should here serve the following addition (requested by this reviewer in Major point 3) of Fig. 2 b and c of the revised manuscript:

Here the authors, by carefully quantifying the condensed fraction of CPEB4 and changes in intracellular pH in a time-resolved manner, can successfully correlate these two parameters and reveal the underlying physicochemical basis most likely at the heart of the regulation of many biomolecular condensates throughout biology.

Version 3:

Reviewer comments:

Referee #2

(Remarks to the Author)
The authors have provided a significantly improved manuscript that satisfactorily addresses our main comments. We commend them for an exciting study the contributes important and timely insight into the mechanism by which mis-splicing of microexon 4 of Cpeb4 may contribute to altered regulation of gene expression in the context of ASD.

When finalizing their manuscript, we recommend the following (minor) changes:

1. P6: “the switch of CPEB4 from translational repression to activation, is regulated neither by post translational modifications nor by calcium mobilization.”

Since the evidence for PTM regulation is only via a specific set of mass spec experiments, PTMs that are not easily detected via mass spec could potentially be regulating the phase behavior. This includes the O-GlcNAc modification (often not seen in standard mass spec data) and some kinds of oxidation. Thus, the evidence shows that phosphorylation is not a regulator, but the statement should be less general and not imply that the data rule out all PTMs. Obviously, pH is shown to regulate phase behavior, so it is likely that PTMs are not involved, but the sentence should be changed to be more precise.

2. The authors could add a representative image of unstimulated primary neurons in Extended Data Figure 1h. Additionally, it is recommended that they clarify the Extended Data Figure 1i legend: what does the y axis indicate, and how were the first, max and last time frames defined?

3. P7: “The precise effect of missplicing will be determined by the identity of the microexon, causing changes in phase transition, as seen with CPEBs, or binding to cofactors, as observed with eIF4G.”

The eIF4G microexon also changes the phase behavior of the host protein. The authors’ concluding and summarizing statement should acknowledge this and reference the relevant paper in the sentence.

Referee #1 (alternative splicing, translation regulation, neurobiology):

Garcia-Cabau et al have studied how the inclusion of microexon 4 in neuronal CPEB4 alters the protein properties and phase transition. They find that heterotypic interactions between the microexon and a cluster of His residues are necessary for the CPEB4 condensates to dissolve upon neuronal depolarization, thus allowing translation activation. The authors describe the exact kinetics of the interactions between the microexon and the His residues by a mechanism, akin to heterotypic buffering. They provide evidence that competition between homotypic interaction of aromatic clusters in CPEB4 that drive aggregation, and heterotypic interactions between the aromatic clusters and the microexon ensures the proper regulation of nCPEB4 in neurons and explain why a reduction in its degree of inclusion in ASD could result in a dominant-negative decrease in CPEB4 activity.

Despite the already known role of CPEB4 in translation regulation via phase separation, this study provides an exact molecular mechanism of how this occurs and is thus very interesting and novel. The manuscript is very well written, in a way that is apprehensible to a broader audience, of different scientific background.

Overall, the methodology used and the statistical analyses are appropriate, however, there are specific minor comments to be addressed before publication:

> We thank the Reviewer for her/his comments and specific points. We have included the requested clarifications/experiments as specified in the point-by-point responses. We trust that (s)he will consider that they have been addressed satisfactorily in the attached revised version.

1. The authors should perform qPCR and WB on the N2A cell with the different constructs, in order to see the effects of their deletions on the actual ASD-related mRNAs and proteins. Those experiments will strengthen the biological context of the work, by connecting all the herein observed phenotypes with the previously reported functions of CPEB4 (e.g. in Parras et al, 2018), and will tight the knot.

> This suggestion is logical but technically more complex than what it may appear initially.
>
> First, to maintain the CPEB4 levels close to the endogenous ones we keep the % of N2a cells transfected cells low: these analyses, therefore, have to be carried out in a single-cell manner, which is technically challenging. Since translational differences must be studied as a result of depolarization, not at steady-state levels in unstimulated cells, we encountered a second technical difficulty: the transient nature of depolarization ($\Delta t < 1$ h) and, therefore, of the translational differences, unlikely to be reflected in total RNA or protein levels. The scenario analyzed in our previous work[1] is different because, in brains, total RNA and protein levels are the result of the accumulation of a large number of depolarization events over the life of the animal. Despite these challenges we have attempted to perform co-immunofluorescence of CPEB4 and several of its targets in a single cell manner: however, we were unable, as expected, to measure changes upon depolarization, let alone changes due to CPEB4 aggregation.

To strengthen the biological context of the work and connect all the observed phenotypes reported in this work with the functions previously reported[1] we have analyzed the presence of CPEB4 aggregates, revealed in this work, in the ASD mouse model used previously.

This was indeed a request of Reviewer #2, which we addressed as follows:

"To strengthen the link between the ASD-like imbalance of isoforms and CPEB4 aggregation, we have analyzed the formation of CPEB4 aggregates in the mouse model described above[1]. This model recapitulates the 55/45 inclusion/skipping ratio observed in ASD patients and has an extensively characterized ASD phenotype, while control animals express the 70/30 ratio.

For this, we have used two complementary approaches.

i - We have fractionated samples enriched in CPEB4, extracted from brain homogenates of Control and TgCPEB4Δ4 littermates by size exclusion chromatography. In them we have detected, by semi-denaturing detergent agarose gel electrophoresis (SDD-AGE), Proteinase-K resistant CPEB4 aggregates in the void volume of the latter, that can seed the aggregation of monomeric CPEB4 extracted from Control mice. These results are in agreement with our conclusions (Fig. 5f,g,h and ED Fig. 5e,f,g).

ii - We have studied the presence of CPEB4 aggregates in striatal neurons of Control and TgCPEB4Δ4 mice brain slices. By using the dye Proteostat, which is specific for protein aggregates, we have found that the number of CPEB4 condensates showing signs of aggregation is higher in TgCPEB4Δ4 than in Control mice (Fig. 5i,j), again in agreement with our conclusions."

2. A short description of the statistical analysis per experiment should appear in the figure legends (type of test, sample size, p-value, etc)

A description of the statistical analysis has been added to the Figure legends of the revised version.

3. In Figure 1, where the authors show the dissolution of particles, the image resolution is not optimal. Better, high-resolution images should be obtained.

Please, note that these are not images of fixed cells but, rather, snapshots of live cell imaging movies and, therefore, of intrinsic lower resolution. Tracking the changes in morphology by recording movies is more appropriate than taking images of fixed cells due to the dynamic nature of the phenotypes studied and, especially, because cell fixation can alter the properties of condensates. To improve the images, we have inverted their colour and added zoomed in inserts of the condensates.

Referee #2 (microexons, neurobiology):

In their submitted manuscript, Salvatella, Mendez and colleagues focus on characterizing the mechanism by which misregulation of the neuronal-specific CPEB4 microexon impacts CPEB4 function and potentially contributes to idiopathic autism spectrum disorder (ASD). Genetic and genomic studies from several labs have provided evidence of a causal relationship between disruption of the neuronal-specific microexon program and ASD. Previous work from Mendez and colleagues revealed that CPEB4 microexon splicing affects poly(A) tail length and translation of ASD-risk genes, and perturbation of its overall inclusion levels result in ASD-reminiscent phenotypes in mouse models. Related to the authors submitted study, previous work from others has revealed that disruption of microexons in eIF4G translation initiation factors affects synaptic protein expression and animal behavior, likely through the loss of microexon-promoted phase separation with neuronal granule components, including CPEB4 (see below).

In their current study, the authors show that CPEB4 condensates are dissolved following neuronal depolarization through pH-dependent intermolecular interactions. These interactions involve histidine and arginine residues, with the latter being enriched in the CPEB4 microexon. The authors further show that both a histidine cluster, as well as the amino acids encoded by the neuronal microexon, are required for efficient phase separation of truncated CPEB4 proteins both in vitro and in cells following overexpression. However, the CPEB4 condensates formed in the absence of the microexon are more resistant to neuronal depolarization in cells and the corresponding droplets tend to transition into aggregates in vitro, likely through interactions involving the histidine cluster. Interestingly, the degree of CPEB4 aggregation depends on the ratio of microexon containing isoforms, while the level of inclusion observed in ASD individuals corresponds to high-predicted aggregation propensity.

Overall, this is an interesting study that provides insight into how microexon splicing levels can affect protein function in vitro, with implications for contributing to autistic phenotypes. In particular, the NMR experiments using urea to observe an increase in signal for resonances involved in interactions within the CPEB4 "multimers" are very clever (see below). This is an excellent approach and provides detailed site-specific interaction information that the authors exploited, including studying the pH dependence of interactions, and supporting the interactions between the Arg-rich microexon and His-rich cluster. Moreover, the authors' CALVADOS simulations have impressive agreement with experimental Csat values and provide support for the experimentally-defined key interactions contributing to phase separation.

However, while the biochemical and biophysical experiments in the study are strong, some of the overall conclusions of the authors' work are not well supported and additional experiments and analyses would be needed to strengthen their study.

> We thank the reviewer for his/her comments and specific points. We have included the requested clarifications/experiments as specified in the point-by-point responses. We trust that (s)he will consider that they have been addressed satisfactorily in the attached revised version.

Specific comments follow:

1. The authors' model suggests that mis-regulation of the CPEB4 microexon may cause idiopathic forms of autism by promoting CPEB4 aggregation. However, the in vivo data to support this model are not strong. For example, does heterozygosity of the CPEB4 microexon in the GFP knock-in model result in CPEB4 aggregates in neurons?

We apologize for not making this relevant point more apparent in the original submission. We initially considered the experimental set-up proposed by the Reviewer as it would indeed have been the standard approach to study a loss-of-function mutation. However, the results of our 2018 study indicate that the nCPEB4Δ4 overexpression model better recapitulates ASD conditions: microexon inclusion/skipping differences in ASD are more subtle than a 50% ratio, with values changing from 70/30 % in "normal individuals" to 55/45 % in patients with ASD[1]. Please note that, *in vitro*, those subtle differences are sufficient to change the material properties of the translation-repression condensates, in a dominant-negative manner (Fig. 5 of the revised manuscript).

Therefore, we have now done experiments with our previously described TgCPEB4Δ4 mouse model, which exactly recapitulates the ratio of patients with ASD[1] whereas, presumably, a heterozygous mouse would have a 35/65 ratio. Moreover, a CPEB4 western blot from brain homogenates (ED Fig. 5e) shows that, despite the additional copy, total CPEB4 levels (re)adjust to the same steady state in Control and the TgCPEB4Δ4 models. In addition, combining this model with the GFP knock-in would have a major technical limitation, that would prevent us from observing the CPEB4 condensates: unfortunately, it is impossible to perform live detection with a single labelled copy of the gene/protein (i.e. in heterozygosis).

Furthermore, does inducing aggregation of endogenous CPEB4 in mouse brains through genetic manipulation result in autistic-like phenotypes?

To strengthen the link between the ASD-like imbalance of isoforms and CPEB4 aggregation, we have analyzed the formation of CPEB4 aggregates in the mouse model described above[1]. This model recapitulates the 55/45 inclusion/skipping ratio observed in ASD patients and has an extensively characterized ASD phenotype, while control animals express the 70/30 ratio.

For this, we have used two complementary approaches.

i - We have fractionated samples enriched in CPEB4, extracted from brain homogenates of Control and TgCPEB4Δ4 littermates by size exclusion chromatography. In them we have detected, by semi-denaturing detergent agarose gel electrophoresis (SDD-AGE), Proteinase-K resistant CPEB4 aggregates in the void volume of the latter, that can seed the aggregation of monomeric CPEB4 extracted from Control mice. These results are in agreement with our conclusions (Fig. 5f,g,h and ED Fig. 5e,f,g).

ii - We have studied the presence of CPEB4 aggregates in striatal neurons of Control and TgCPEB4Δ4 mice brain slices. By using the dye Proteostat, which is specific for protein aggregates, we have found that the number of CPEB4 condensates showing signs of aggregation is higher in TgCPEB4Δ4 than in Control mice (Fig. 5i,j), again in agreement with our conclusions.

2. Related to the above comment, most of the experiments in this study are performed in vitro or through overexpressing CPEB4 isoforms where intermolecular interactions seem to play a critical role for CPEB4 phase transition and aggregation (see comments below). However, in vivo CPEB4 is known to be present in condensates that contain additional proteins and RNA molecules. Although the authors have attempted to address this through some experiments in Xenopus these are limited in scope. Their study would be more conclusive if the effects of the microexon were studied in neurons at endogenous protein levels. This is also important when considering the existence of additional microexons regulated by the same molecular pathway, that are present in proteins known to exist in the same cellular condensates as CPEB4, and that when misregulated also result in ASD-like phenotypes, such as the aforementioned eIF4G microexons. In this regard, related and possible convergent mechanisms of CPEB4 and eIF4G microexons should at the very least be discussed by the authors.

We agree with the reviewer that the absence of differences in the composition of the translation repressive CPEB4 condensates formed in *Xenopus* oocytes in the presence and absence of microexon 4 does not entirely rule out the possibility of a change in interactions in neurons. We have now immunoprecipitated nCPEB4 and nCPEB4Δ4 from N2A cells (please note that we had to use ectopically expressed proteins as we do not have a microexon-specific antibody). The co-immunoprecipitates were analyzed by mass spectrometry, and we did not find significant differences in the relevant interactomes (ED Fig. 4f).

Indeed, both CPEB4 and eIF4G are part of an SRRM4-coordinated neuron-specific program that is dysregulated in ASD. Which is, mechanistically, the functional interplay between eIF4G and CPEB4, with and without microexon? Which is the mRNA target overlap, etc? These are key questions that will certainly need specific combinatorial experiments to be addressed. Considering that the microexon inclusion in eIF4G promotes global translation repression, whereas in CPEB4 microexon skipping promotes mRNA-specific translational repression, further studies will be required before a model can be suggested. We have now added a comment on this topic in the discussion of the manuscript.

3. What prevents the aggregation of CPEB4 in non-neuronal cells where the microexon is mostly excluded?

We apologize for not addressing this topic in the original submission; we have further expanded this aspect in the discussion of the revised version. Briefly, there are three parameters to consider: concentration, time, and re-assembly of the repressive dynamic aggregates.

During the cell cycle, CPEB4 is synthesized during the G2/M transition, as the result of CPEB1 activation, and shortly after, in metaphase, activated by CdK1/ERK phosphorylation, which promotes the dissolution of the translation-repression condensates[2,3]. Then, upon cytokinesis, CPEB4 is diluted and, as far as we can tell, the translation-repression condensates in the subsequent cell division are composed of newly synthesized CPEB4, i.e. there is no reassembly. During chronic unfolded protein response (UPR), CPEB4 is synthesized, as the result of eIF2a phosphorylation, concomitantly with its phosphorylation by ERK: the repressive dynamic aggregates therefore never assemble[4,5].

Those scenarios are clearly different from the scenario in neurons, which express much higher levels of CPEB4 than any other cell type, and in which this protein forms translation-repression condensates that need to be kept available for very long periods to be eventually transiently dissolved (by pH changes) and subsequently reassembled. For this reason, neurons are the only cell type where CPEB4 condensates can undergo a liquid-to-solid transition leading to CPEB4 aggregation in the absence of microexon 4.

4. Do any of the identified PTMs overlap the microexon residues?

As we now indicate in Extended Data Fig. 2a none of the identified PTMs are found in the microexon.

5. The phosphorylation study was briefly described and the results for the phosphomimetic (which did not form condensates) suggests that there is some impact of phosphorylation, which is inconsistent with the interpretation of the data for the mutants designed to remove phosphorylation, which is that phosphorylation is not significantly regulating the phase separation. The authors should elaborate and clarify their interpretation of these results.

We apologize for not being clear enough in explaining the results shown in Fig. 2b and Extended Data Fig. 2b of the original submission. We have expanded the interpretation of these experiments in the revised manuscript.

Briefly, we detect eleven phosphorylated residues in the NTD of nCPEB4 in N2a cells, but the phosphorylated/non-phosphorylated ratio was low and, most importantly, not altered upon depolarization (Fig. 2a of the original submission). Thus, nCPEB4 is mostly not phosphorylated in N2a cells, regardless of its condensation status. Critically, a non-phosphorylable nCPEB4 variant, where the identified residues were mutated to alanine, still dissolved upon depolarization. Altogether these results indicate that nCPEB4 condensation is not regulated by phosphorylation following depolarization.

To further compare these results with the CPEB4 phase transition regulation during cell cycle, which is regulated by CdK1/ERK phosphorylation[3], we mutated the residues identified in N2a cells, even if modified at very low levels, to Asp (mutant 11D). These negative charges decreased condensation, indicating that the presence of the microexon does not abolish a potential regulation by phosphorylation. Although phosphorylation is not implicated in the regulation of nCPEB4 upon depolarization, it could play a role in

neuronal stress responses, like ischemia[6], and therefore we thought it was informative to include the 11D mutant result.

6. There is clear methylation on Arg350 in non-stimulated cells that is missing in stimulated cells. It is known that Arg methylation significantly reduces Arg-dependent interactions and reduces phase separation. The authors could address how this PTM potentially partly explains how stimulation leads to disassembly of the condensates. Relevant work from others on the role of Arg methylation in condensate formation should be referenced.

We did not study this (de)methylation further because it was not statistically significant across replicates (please, see fig below).

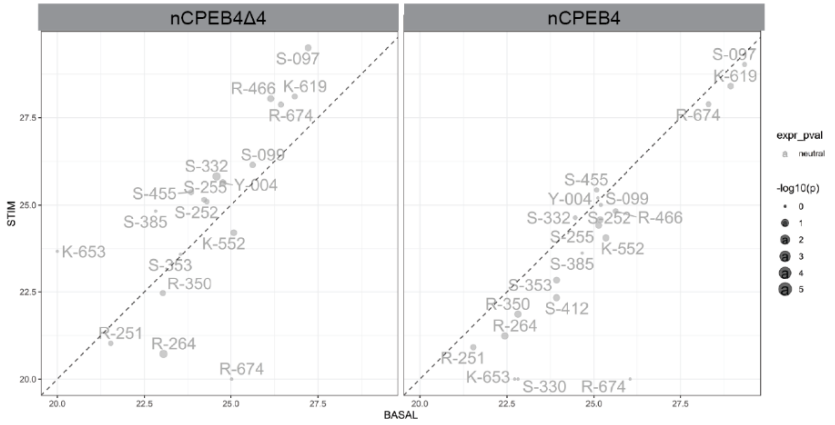

Fig. 1. Post-translational modifications in nCPEB4 and nCPEB4Δ4 observed before and after stimulation, annotated according to the statistical significance of the differences. Scatter plots representing the log2 Intensities of PTM sites in stimulated cells (y-axis) versus PTM sites in non-stimulated cells (x-axis). The facet grid breaks down the particular protein, named nCPEB4 and nCPEB4Δ4. PTM sites with all values missing in one condition were imputed at 20 only for visualization reasons.

Also, demethylation would not be consistent with the dissolution of the condensates upon depolarization: as the reviewer indicates, demethylation should stabilize the condensates, not promote their dissolution[7–10].

7. p9: The lack of role of RNA in differences in phase behavior in cells for nCP4EB4-NTD vs nCPEB4delta4-NTD is not convincing. This experiment should be more fully described and explained in the text.

The sentence in page 9 highlighted by the Reviewer "To confirm that microexon … nor with RNA (Extended Data Fig. 4c)" is indeed confusing as neither nCPEB4-NTD nor nCPEB4Δ4-NTD contain RNA binding domains: we apologize for this. We have now compared the effect of the microexon on the phase separation capacity of the full-length protein (Fig. 4c,d): briefly, loss of microexon 4 leads to a small but significant decrease in the number of phase-separated foci formed by nCPEB4-NTD, and the same effect can be

observed in the context of the FL protein but to a lesser extent: the relevant sentence has been amended.

The competition assay that we used to show that the microexon has no effect on the affinity that CPEB4 has for RNA, described in detail in reference 10 of our original submission,[11] is based on the out-competition of endogenous CPEBs by transfected proteins.

As positive controls we transfected truncated protein variants containing only the RRMs or the ZZ domain (RRM12ZZ): these variants bind to RNA via the tandem RRMs but do not recruit the cofactors directly responsible for polyadenylation (such as CPSF, Symplekin, or GLD2).

Now, in this revised work, we tested whether the presence or absence of the microexon in CPEB4 affects its binding to RNA obtaining as shown in ED Fig. 4g that the microexon has no effect.

8. The manuscript could be clarified by first outlining interactions (including pH dependence) in the microexon-containing sequence and then looking at the delta-microexon (as well as delta-His cluster) to confirm the interactions and provide insights potentially relevant to an ASD context. The expected impact of cellular pH on changing the interactions and thus changing phase behavior should be more explicitly stated. The current text is somewhat confusing.

We have added new panels to Fig. 1, rearranged Figs. 2, 4 and 5 as well as added new data to Figs. 4 and 5, trusting that this makes the manuscript clearer.

9. p2 intro/Fig 6: "Neuronal CPEB4 (nCPEB4) binds to the transcripts encoded by ASD risk genes". This needs a reference and clarification, since not all mRNAs bound by CPEB4 are "ASD risk genes". This leads to confusion in Figure 6 where only "ASD risk gene" levels are indicated as going up and down. The actual proteins impacted by CPEB4 microexon 4 inclusion levels are not all necessarily ASD risk genes.

Indeed, while the mRNAs of most ASD risk genes are bound by CPEB4, not all CPEB4 target mRNAs are ASD genes[1]. We did not intend to imply that nCPEB4 *exclusively* binds transcripts encoded by ASD risk genes and have modified the text and Fig. 6e of the revised version accordingly.

10. The in vitro phase separation studies are generally well-done, but the conclusion that the role of the microexon is only to prevent irreversible aggregation misses the data/point that it also contributes to phase separation in the first place (see also comments above). The language of "homo-" vs "heterotypic" phase separation is confusing and possibly incorrect, since this term is used to describe interactions between CPEB4 whereas heterotypic implies involvement of different proteins (which is certainly the case in cells).

Please, note that the loss of microexon 4 decreases the phase separation of nCPEB4-NTD *in vitro* and in N2a cells but does not significantly change the phase separation propensity of full-length nCPEB4 in N2a cells: it therefore has, if at all, a modest effect on the thermodynamic stability of the translation-repression condensates.

We would like to emphasize that even if the loss of microexon 4 were to substantially decrease the phase separation propensity of nCPEB4, this would not explain the decrease in nCPEB4 function observed in ASD models and patients: the condensates formed by CPEB4 are repressive of translation, and their destabilization by loss of microexon 4 should lead, if anything, to the activation of translation.

With regards to the language "homo-" vs "heterotypic" we think our usage is correct and in line with current usage in the literature[12] because the two motifs correspond to two different regions of sequence that in the context of the condensate will belong to different protein molecules. Nevertheless, we have added text to the revised version to define these two terms precisely.

11. p4: "... high ionic strength (Fig. 1j), suggesting that the droplets are in part stabilized by hydrophobic interactions". High salt inducing phase separation doesn't necessarily mean hydrophobic interactions. It could be shielding repulsive electrostatic interactions. Given the results later in the paper, it seems that this interpretation is more likely.

We have modified the text accordingly.

12. p6-7: "conditions are insufficient for phase separation". While there were no visible droplets in the microscope, ~55 nm "particles" are small "droplets" or condensates. Cellular condensates such as paraspeckles can be as small as 50 nm in diameter. This should be acknowledged rather than referring to them as "intermediates" along the pathway to droplets. The language of referring to these as "multimers" that give rise to "droplets" is confusing and raises the question of whether there are different interactions for these two "states". The interactions seen in the NMR for these small droplets must be the same as in big droplets as there is no transition between the intermediate "multimers" and "droplets" in the performed experiments. They are just getting bigger.

We agree with this interpretation and consider that the multimers are likely small droplets/condensates. We would like to point out, however, that their small size precludes us from confirming their liquid character by fluorescence recovery after photobleaching (FRAP) experiments as well as limits the extent to which we can observe fusion events by microscopy: for this reason, we refrained from making a strong statement about them having the same physical properties as the liquid droplets characterized in Figures 1 and 2. To address this point, we have added text to explain this in the revised manuscript.

13. Is the His cluster sequence LARK-like? It would be valuable for the authors to comment on sequence features that may be expected to lead to fibers.

The sequence of HClust is not identified as LARK-like by LRKSdb (https://servicesn.mbi.ucla.edu/LARKSdb/index.py). However, an alignment of nCPEB4-NTD with Orb2, the *Drosophila melanogaster* homologue of human CPEBs 2-4, indicates that the cluster aligns with the region of sequence, rich in His and Gln residues, that stabilises the fibrils formed by this protein in the fly brain[13]. We have added the sequence alignment in ED Fig. 4m and text to the discussion of the paper to provide this information.

14. SRRM4-dependent regulation of CPEB4 microexon in N2A cells and neurons was reported previously (Gonatopoulos-Pournatzis et al., Mol Cell, 2020) and should be referenced in the section of the authors' manuscript where they confirm this observation.

We apologize for this error: we cited this work in the introduction (Reference 3 of the original submission) but failed to cite it in the relevant part of the Results section. We have added this reference to the revised manuscript.

Referee #3 (Remarks to the Author):

In this study Garcia-Cabau et al. investigate the mechanism of how the microexson4 regulate the properties of CPEB4 condensates, which is important for translational regulation during depolarization of neurons. They combined cell culture, in vitro reconstitution and molecular simulations to show that the interactions between His clusters and positive Arg residues in exson4 stabilize condensates and prevent the irreversible aggregation.

The functional role of condensation has been a matter of debate for years, especially how the different properties of condensates matter. Previous studies have reported that CPEB4 condensates are important for translation repression in neurons and needs to be regulated properly. Exclusion of exson4 causes autism spectrum disorder. This study used multiple methods to show that in neurons CPEB4 forms condensates that dissolve upon KCl-mediated depolarization. The expression of CPEB4 containing microexon 4 mediates the capability of these condensates to dissolve upon depolarization, by providing an arginine-rich positively charged domain that interacts with a hydrophobic histidine-rich region in the N-terminal domain, while preventing homotypic interactions of the histidine-rich region. Reducing the fraction of CPEB4 containing microexon 4 results in an impairment of these condensates to dissolve, providing a mechanism for translational dysregulation in ASD.

Unfortunately, the study falls shorts in providing a strong connection between the properties of nCPEB4 and nCPEB4Δ4 condensates and their cellular behaviour and functions, especially in the pH regulation scenario during cell depolarization. Specifically, the central argument of this paper rests on the assumption that depolarization leads to a relevant change in pH that directly affects condensate dissolution. For publication of this article in the journal Nature, it would be required that this central claim has to be experimentally addressed and verified, as outlined in the comments below. Building on these measurements the supporting in vitro assays should then directly test if condensate dissolution kinetics are indeed different between full-length, wild type and CPEB4Δ4. In addition, the in vitro experiments were carried out with the disordered NTD. However, as an RNA-binding protein, the behavior of CPEB4 might be significantly influenced by RNA. In my view, in vitro data with purified full-length proteins with RNA have to be acquired to prove their key discoveries.

In summary, this study provides a collection of data that adds to our understanding of condensation regulation but the manuscript in its current form does not yet significantly advance the field because the data do not strongly support the claimed conclusions.

> We thank the reviewer for his/her comments and specific points. We have included the requested clarifications/experiments as specified in the point-by-point responses. We trust that (s)he will consider that they have been addressed satisfactorily in the attached revised version. Before addressing the specific points, we would like to highlight that, to the best of our knowledge, this is the first time that the regulation of CPEB4 condensation in neurons has been addressed, even more so considering the implications of the neuron-specific microexon. While previous works from our laboratories described regulation by phosphorylation of non-neuronal CPEB4 (i.e. without the microexon) in mitosis/meiosis[3],

here we show a novel neuron-specific regulation mechanism (distinct from cell cycle regulation) that explains the role of the neuron specific microexon in the ASD phenotype.

General comments:

1) To avoid confusion when labeling graph axes, brackets should ideally be used to indicate units, rather than backslashes. I.e. Time (s), rather than Time / s. The authors should revise their axis description for Fig. 2 a, d, f; Fig. 3 a, c, d, I (y-axis label completely absent); Fig. 4 b, d, h, i, j.

We have modified the graph axes accordingly (for Fig. 1 f, g, i, j; ED Fig. 1 k, l; ED Fig. 2 d, e, f; Fig. 3 e; ED Fig. 3 a, b, c, d, e, f, g, h, i, j, k; ED Fig. 4 a, e, g, i; Fig. 5 c, d, i; ED Fig. 5 a, c, d; ED Fig. 6 a,b). Please note that in Fig. 3i the plots are one-dimensional nuclear magnetic resonance spectra where the x dimension represents the chemical shift (ppm) and the y dimension represents signal intensity, which in this technique is customarily represented without units.

2) Figure panel layout needs be rearranged, so that A, B, D and onwards are in order, especially Figs 3, 4 and 5.

We have rearranged the panels accordingly.

3) The figure legends is generally over-simplified, not reader-friendly. The author should provide some experimental details, especially for condensates experiments in vitro. Additionally, information concerning the number of replicates should be provided in the figure legend.

We have included all the requested information in the figure captions and legends.

Major points:

1) Several claims based on cell-imaging appear to lack any quantification, and are only supported by images of a single, or handful of cells. This concerns data shown in Fig 1D,H. Ext data 1D, E, I. Fig 2B,C. Ext data fig 2B,C. Additionally, micrographs of cells would benefit from zoomed inserts of the cytoplasmic structures formed by nCPEB4.

In the revised version, we provide quantifications of the results shown in Figs. 1D and 1H, ED Figs. 1D, 1E and 1I, Figs. 2B and 2C and ED Figs. 2B and 2C and zoomed inserts of the cytoplasmic structures formed by nCPEB4. In particular, regarding the quantification of condensate dissolution upon neuron stimulation, we now include quantifications of the condensed fraction of the cell over time (fraction of the cell occupied by condensates, in area), on top of the percentage of cells with remaining foci after stimulation.

2) In line with the previous comment, another more physiological means of depolarizing the cells would strenghten the claim of depolarization-induced dissolution of the condensates. KCl

increased the membrane potential drastically, and results in a brief period of neuronal excitation (minutes), followed by a prolonged period of neuronal depression. However, it floods the cells the positively-charged potassium, which can lead to conflicting results regarding CPEB4 phase separation and dissolution. Options for more physiological stimulation are glutamate, kainate or NMDA. Even if the effect size varies from KCl treatment conditions, these should be quantified and reported (altered number of foci / altered condensate properties assessed by FRAP).

We have attempted depolarizing the N2a cells with increasing concentrations of glutamate and NMDA, but these failed to cause depolarization as measured by the early depolarization marker cFos (see figure below, equivalent results were obtained for JunB).

Regarding the effects on CPEB4 condensates of changes in ionic strength during depolarization by KCl we would like to point out that, at the pH where dissolution takes place, an increase of ionic strength would promote condensation, not condensate dissolution (Fig. 2l).

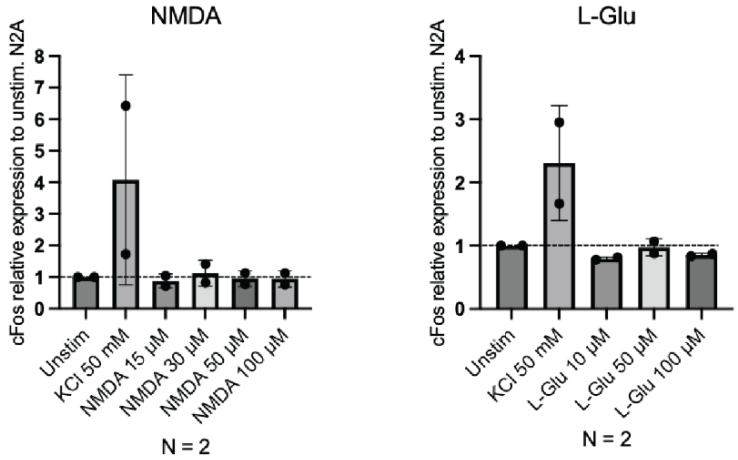

Fig. 2. Fold change increase in transcript levels of early depolarization marker cFos in N2a cells exposed to KCl (KCl 50 mM) or increasing concentrations of NMDA (left) or glutamate (right), relative to unstimulated cells (Unstim.). Dots represent independent experiments (n = 2).

To address this we have introduced a clarification in the relevant part of the results section.

3) The authors should try to asses to which extent neuronal, intracellular pH (pHi) changes upon depolarization, possibly by employing small molecule fluorophores or genetically encoded pH sensors. Does cytosolic alkalinization / change in pHi exceed the previously reported 0.05 units? (PMID: 3353214)

We have measured the changes in pHi by using the SNARF-5F dye. We found that in most N2a cells the pHi decreases by slightly more than 0.05 units during the 5 to 10 min that follow depolarization, in agreement with previous reports[14], and that it then

progressively increases by approximately 0.3 units (Fig. 2c). These changes follow the transient increase in phase separation propensity and subsequent dissolution of nCPEB4 foci (Fig. 2a,b).

To strengthen our conclusion that these pH changes can change the phase equilibrium of nCPEB4 we measured the pKa of the His side chains *in vitro* by solution NMR: we obtained a value of approximately 7.1, which is higher than expectations, presumably due to sequence context, and close to the physiological pH (Fig. 2d).

We have introduced changes in the manuscript to describe these results.

4) From the molecular dynamic simulations, the authors suggested that His clusters work as a sensor for pH, i.e., at pH6 the His clusters interact with negative charges at positions 72-147, and at pH8 the His clusters interacts with Arg in exon 4. Any experimental data to support this? Are these predictions consistent with the salt effects on nCPEB4 condensation at pH6 and pH8? If possible / reasonable a change in hydrodynamic diameter, depending on pH, as determined by DLS or any other technique would be convincing.

We would like to emphasize that we carried out the molecular simulations because, even under conditions where the multimers are least stable i.e. low temperature and NaCl, the concentration of monomer is ca 0.5 μM, too low to measure interresidue interactions by NMR, especially when the resonances of the relevant residues are extremely weak.

Measurements of the hydrodynamic diameter by DLS are also very challenging. This is because the low concentration of monomer renders the estimation of its value unreliable and, especially, because increasing the strength of interresidue interactions by decreasing the pH stabilizes the multimers, precluding the observation of the monomer (Fig. 3a).

Therefore, to address this point, we imaged the nCPEB4-NTD condensates at pH 6 and varying NaCl concentrations. As shown in Fig. 2l increasing ionic strength destabilizes the condensates, unlike at pH 8 (original Fig. 1j) indicating that the interactions stabilizing the condensates are different at pH 6 and 8, in agreement with the simulations.

To complement these observations we measured, at pH 6 and 8, the C_{sat} of nCPEB4-NTD at 0, 100 and 500 mM NaCl (40 °C): at pH 8 we observed no phase separation without added NaCl and a progressive decrease in C_{sat} as the NaCl concentration increased whereas at pH 6 we observed the opposite (ED Fig. 2o).

5) What is the relationship between nCPEB4 condensates and RNA in cells and in vitro?

The first part of the question is closely related to point 7 of Reviewer #2, which we addressed as follows:

"The sentence in page 9 highlighted by the Reviewer "To confirm that microexon … nor with RNA (Extended Data Fig. 4c)" is indeed confusing as neither nCPEB4-NTD nor nCPEB4Δ4-NTD contain RNA binding domains: we apologize for this. We have now

compared the effect of the microexon on the phase separation capacity of the full-length protein (Fig. 4c,d): briefly, loss of microexon 4 leads to a small but significant decrease in the number of phase-separated foci formed by nCPEB4-NTD, and the same effect can be observed in the context of the FL protein but to a lesser extent: the relevant sentence has been amended.

The competition assay that we used to show that the microexon has no effect on the affinity that CPEB4 has for RNA, described in detail in reference 10 of our original submission,[11] is based on the out-competition of endogenous CPEBs by transfected proteins.

As positive controls we transfected truncated protein variants containing only the RRMs or the ZZ domain (RRM12ZZ): these variants bind to RNA via the tandem RRMs but do not recruit the cofactors directly responsible for polyadenylation (such as CPSF, Symplekin, or GLD2).

Now, in this revised work, we tested whether the presence or absence of the microexon in CPEB4 affects its binding to RNA obtaining as shown in ED Fig. 4g that the microexon has no effect."

Regarding the second part of the question we would like to highlight that our previous work[3] as well as our experiments in N2a cells show that nCPEB4-NTD recapitulates the condensation properties of the full-length protein, including the dissolution of the condensates upon neuronal stimulation (original Fig. 1e,h).

To nevertheless address this, we expressed and purified full-length nCPEB4 from insect cells but found that this protein, even in the presence of RNA, had high aggregation propensity, rendering the experiments challenging.

Finally, we also would like to highlight that we have validated the conclusions of our *in vitro* work, carried out by using nCPEB4-NTD as background, in experiments in N2a cells by using full-length nCPEB4 protein as background (Fig. 4e,f and k).

There is little doubt that the condensation properties of full-length nCPEB4 will be influenced by interactions with RNA mediated by the RRMs but consider that our experimental approach allows separating these from the effects of mis-splicing, which was the goal of this work.

6) Are the multimers of nCPEB4 and nCPEB4Δ4 regulated by pH? And what is the driving force for the multimer formation of nCPEB4Δ4?

To address these points, we have carried out DLS experiments equivalent to those presented in the original Fig. 3a for nCPEB4Δ4-NTD, and for both isoforms at pH 7.5. We obtained that the stability of the multimers formed by nCPEB4Δ4-NTD depends on solution conditions as that of those formed by nCPEB4-NTD, and that decreasing the pH increases the stability of both types of multimers (Fig. 3a and ED Fig. 4b).

From these results, we conclude that the multimers are stabilised by interactions equivalent to those stabilising the condensates, as discussed by Reviewer #2. We have also studied the multimers formed by nCPEB4Δ4-NTD by solution NMR (after assigning this other variant) and obtained results equivalent to those obtained for nCPEB4-NTD, confirming that the former are equivalent to the latter, albeit less stable due to the loss of microexon 4 (ED Fig. 4c,d).

7) The authors used overexpression system to show the formation and dissolution of nCPEB4 and nCPEB4Δ4, in which the expression levels are heterogeneous. However, condensation process is concentration-dependent. How the authors carried out quantification and generated the conclusion with the heterogeneous system?

We would like to highlight that in Fig. 1 we show the condensates and depolarization-induced dissolution of the endogenous GFP-tagged CPEB4 (i.e. at physiological levels), with similar morphological properties that the analyzed overexpressed proteins. Further, in the experiments with overexpression in N2a cells, which we carried out to compare the phase separation properties of nCPEB4 and nCPEB4Δ4, we ensured that the expression levels of these two proteins were equivalent according to the mean fluorescence intensity.

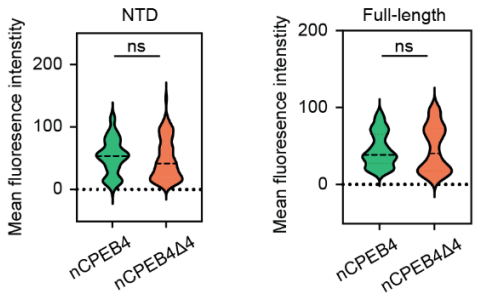

Fig. 3. Mean fluorescence intensities of cells expressing GFP-tagged nCPEB4 and nCPEB4Δ4 used to compare the phase separation properties of these two proteins.

8) Since the dissolution of nCPEB4 condensates upon neuron depolarization caused by intracellular pH increases, the authors should study how the pH influences the properties of the individual nCPEB4 condensates, nCPEB4Δ4 condensates and co-condensates of nCPEB4 and nCPEB4Δ4 in vitro.

In the original manuscript we studied the effect of pH on the cloud points of pure solutions of nCPEB4-NTD (original Fig. 2d) and nCPEB4Δ4-NTD (original ED Fig. 4e) as well as the cloud points as a function of their composition of solutions containing both isoforms (original Fig. 5c) but we did not investigate the effect of pH on the cloud point of mixtures.

We have now carried out these experiments for the two key compositions in this work (Ctrl and ASD, original Fig. 5c,d) and have obtained that they phase-separate with the

same dependence on pH as the pure samples (as expected as microexon 4 does not contain His residues and its ionisation state should thus not be altered in the pH range relevant to this work, ED Fig. 5c).

Additionally, we have assessed the effect of pH on the aggregation propensity of the samples (individual and co-condensates) and obtained aggregation only above pH 7, in agreement with the pKa of His residues (Fig. 4j and ED Fig. 5d). This indicates that at physiological pH aggregation can indeed take place.

9) Fig 4E: The lack of 'reversibility of condensation' in cells expressing CPEB4Δ4 is resulting in remaining CPEB4Δ4 structures in the cytoplasm upon depolarization. However, the material properties of these structures have not been investigated. Do they remain liquid-like or transition to an aggregate-like state, like the in vitro experiments? In the cartoon in Fig 6 even the term 'irreversible aggregation' is mentioned, which is not demonstrated at all in cells. More data should be obtained in cells to evaluate physical properties of remaining CPEB4Δ4 structures, for example FRAP as a starting point.

We would like to emphasize that our results *in vitro* indicate that the differences in the properties of the condensates formed by nCPEB4-NTD and nCPEB4Δ4-NTD become apparent only after incubation or after multiple cycles of condensation and dissolution.

N2a cells cannot, unfortunately, undergo multiple depolarization cycles, which limits the extent to which aggregation can occur and be characterized in cell culture: to investigate how the observations made *in vitro* relate to the behavior of the proteins *in vivo* we investigated whether CPEB4 aggregates accumulate in the brains of mice expressing the two CPEB4 isoforms in the relevant ratio (ASD model TgCPEB4Δ4[1]), obtaining the results shown in Fig. 5.

This point is in fact equivalent to point 1 of Reviewer #2, which we addressed as follows:

"To strengthen the link between the ASD-like imbalance of isoforms and CPEB4 aggregation, we have analyzed the formation of CPEB4 aggregates in the mouse model described above[1]. This model recapitulates the 55/45 inclusion/skipping ratio observed in ASD patients and has an extensively characterized ASD phenotype, while control animals express the 70/30 ratio.

For this, we have used two complementary approaches.

i - We have fractionated samples enriched in CPEB4, extracted from brain homogenates of Control and TgCPEB4Δ4 littermates by size exclusion chromatography. In them we have detected, by semi-denaturing detergent agarose gel electrophoresis (SDD-AGE), Proteinase-K resistant CPEB4 aggregates in the void volume of the latter, that can seed the aggregation of monomeric CPEB4 extracted from Control mice. These results are in agreement with our conclusions (Fig. 5f,g,h and ED Fig. 5e,f,g).

ii - We have studied the presence of CPEB4 aggregates in striatal neurons of Control and TgCPEB4Δ4 mice brain slices. By using the dye Proteostat, which is specific for protein aggregates, we have found that the number of CPEB4 condensates showing signs of aggregation is higher in TgCPEB4Δ4 than in Control mice (Fig. 5i,j), again in agreement with our conclusions."

In summary, we now provide evidence of the accumulation of CPEB4 aggregates *in vivo,* in the brains of 6-month-old mice expressing the two CPEB4 isoforms in the ratio associated with ASD (TgCPEB4Δ4), which justifies our use of the term irreversible phase transition in Fig. 6, corresponding to Fig. 6e in the revised manuscript. "

Also concerning Fig. 4F - quantification of the key experiment of this paper, supporting the claim of indicated in the title - it would strengthen the manuscript, if the authors quantified '% cells with foci' prior to stimulus application - additionally the '#foci per cell' for these two time points could lend additional support.

We would like to clarify that in this experiment we monitored individual cells, all of which had foci prior to stimulation; we have introduced text to emphasize this in the revised version.

10) Fig 4I: The authors should characterize the aggregates formed by nCPEB4Δ4 in vitro, for example by FRAP.

To address this point, we have performed FRAP experiments on an aggregated sample of nCPEB4Δ4 and observed no recovery (MF = 0.06 ± 0.02), indicating that the condensates mature into a less dynamic solid-like state, which is consistent with the new *in vivo* data presented in Figure 5f,g,h and ED Fig. 5e,f,g. The results are presented in ED Fig. 4l of the revised version and discussed in the text.

11) In Fig 5A, the authors should characterize the properties of co-condensates of nCPEB4 and nCPEB4Δ4 with FRAP, and investigate their dissolution upon cell depolarization.

nCPEB4 and nCPEB4Δ4 behave similarly in FRAP experiments immediately after transfection (ED Fig. 5a).

We would like to emphasize that our results *in vitro* indicate that the properties of the condensates are determined by their composition, i.e. in the molar fraction of the two isoforms and, as explained in our response to point 9 of this Reviewer, the differences in the properties of the condensates formed by the two isoforms become apparent only after incubation or after multiple cycles of condensation and dissolution.

In N2a cells, it is not possible to control the composition of the CPEB4 condensates in an overexpression system, even to quantify it *a posteriori*: we therefore studied the dissolution upon depolarization of the condensates formed by either nCPEB4 or nCPEB4Δ4 in N2a cells, where the composition is certain, obtaining the results shown in Fig. 4.

As mentioned in our response to point 9 to investigate how the observations made *in vitro* relate to the behavior of the proteins *in vivo* we investigated whether CPEB4 aggregates accumulate in the brains of mice expressing the two CPEB4 isoforms in the relevant ratio (ASD model TgCPEB4Δ4[1]), obtaining the results shown in Fig. 5 and ED Fig. 5.

12) The authors claimed that homotypic interactions of His clusters in nCPEB4Δ4 accelerate the condensation to aggregation transition. The authors should give stronger evidence to support the key discovery.

We would like to emphasize that deleting the motif HClust prevents aggregation *in vitro* (original Fig. 4 i,j,k) and allows the reversibility of the phase transition *in cells* (original ED Fig. 4j).

To provide more robust evidence for this, we took advantage of the ionizable character of His side chains, with pKa ≈ 7, by carrying out experiments equivalent to those presented in original Fig. 4i and ED Fig. 4i with nCPEB4Δ4-NTD but at pHs 7.5, 7 and 6. In agreement with our conclusions, we found that aggregation did not occur at pH 7 and 6, because His side chain protonation weakens homotypic interactions between His clusters due to repulsive electrostatic interactions between protonated His side chains (Fig. 4j).

Finally, as discussed in our response to a point raised by Reviewer #2, we aligned the sequence of nCPEB4Δ4 to that of the *Drosophila melanogaster* ortholog of CPEB4, Orb2 and found homology between HClust and the region of sequence of this protein that is responsible for its aggregation and represents the core of the endogenous fibrils according to the cryo-EM structure[13] (ED Fig. 4m).

13) The nCPEB4 and nCPEB4Δ4 are co-expressed in neurons, and the ratio of these two proteins determines normal (Δ4~30%) and ASD (Δ4~45%). The authors found a sigmoidal pattern for the properties of the co-condensates. The authors should show the ratios of these two isoforms also cause similar properties changes in cells, and potentially correlate these properties to its function. One suggestion might be to perform photobleaching analyses on experiments, shown in Fig. 5A and Fig. 5E.

Please see above (points 9 and 11).

14) In the discussion it is stated: "In nCPEB4 the changes in phase separation propensity and activity that occur upon depolarization are caused by local changes in intracellular pH." However, this statement is not backed up by any data. In vitro pH was shown to alter phase separation behavior, and condensation patterns (in one single cell) shown in Fig 2C correlate with published data on pH changes upon depolarization, but a causal link is not shown. A true mechanistic insight in how KCl depolarization results in condensate dissolution in cells is still lacking. The claim of pH dependence could be strenghted by co-imaging with commercially available pH sensors, but further evidence is needed for causality.

Please see above (point 3).

Minor points:

1) Please give the domain-wise structure of nCPEB4, and indicate the localization of microexon4.

We have added the domain organization of the protein to ED Fig. 1a.

2) Lines 48-60: the introduction of nCPEB4 should be more logic, i.e., introducing CPEBs family (line 53-55), then nCPEB4.

We have changed the text in the introduction accordingly.

3) Please show FRAP data in the endogenous nCPEB4 in mice striatal neurons, correlating to Fig 1C. Also, in Fig 1C, the label should be mEGFP-nCPEB4.

Performing FRAP in these cells with our experimental settings was unfortunately impossible due to excessive photobleaching of the surroundings of the bleached areas. We have added an explanation to the relevant results section.

4) Highlight the nerve system in Extended Fig 1A.

We have highlighted the nerve system in ED Fig. 1a of the original manuscript.

5) Several bargraphs in Extended Data 1F and G lack error bars. Clarification is needed on why: is this a single replicate, error bar is too small to see or otherwise.

We have corrected this in the revised version.

6) If the phosphorylation does not play roles in dissolution of nCPEB4 condensates, the authors should remove the data from Fig 2 into Extended Fig 2. In addition, the authors should explain the discripancy between their data and previous research.

This point is equivalent to one raised by Reviewer #2, which we addressed as follows:

"We apologize for not being clear enough in explaining the results shown in Fig. 2b and Extended Data Fig. 2b of the original submission. We have expanded the interpretation of these experiments in the revised manuscript.

Briefly, we detect eleven phosphorylated residues in the NTD of nCPEB4 in N2a cells, but the phosphorylated/non-phosphorylated ratio was low and, most importantly, not altered upon depolarization (Fig. 2a of the original submission). Thus, nCPEB4 is mostly not phosphorylated in N2a cells, regardless of its condensation status. Critically, a non-phosphorylable nCPEB4 variant, where the identified residues were mutated to alanine, still dissolved upon depolarization. Altogether these results indicate that nCPEB4 condensation is not regulated by phosphorylation following depolarization.

To further compare these results with the CPEB4 phase transition regulation during cell cycle, which is regulated by CdK1/ERK phosphorylation[3], we mutated the residues identified in N2a cells, even if modified at very low levels, to Asp (mutant 11D). These negative charges decreased condensation, indicating that the presence of the microexon does not abolish a potential regulation by phosphorylation. Although phosphorylation is not implicated in the regulation of nCPEB4 upon depolarization, it could play a role in neuronal stress responses, like ischemia[6], and therefore we thought it was informative to include the 11D mutant result."

We have moved Fig. 2a,b to ED Fig. 2.

7) Please clarify the pH for the contact maps in Fig 2G? Is it pH6 or pH8?

The contact map shows the ratio of contacts between q_{His} = 0.50 and q_{His} = 0.01, where q_{His} is the charge of the histidine residues. We have now added this information to the figure legend too.

8) Fig 2E is far too small to see which residues were changed from histidine to serine.

We have made changes to this panel to make it possible to see which residues were substituted, and we have added the protein sequences corresponding to each mutant in the materials and methods.

9) In Extended Fig 2F, the Csat from the MD at pH6, 7 and 8 are similar. From the experimental data, Csat should be the lowest at pH6. Please address this.

In the simulations performed in this work the pH is solely determined by the protonation state of His side chains. To investigate the effect of their protonation on the phase separation capacity of nCPEB4 and, especially, to identify changes in the interactions occurring in the condensates, we carried out simulations at pH values flanking the nominal pKa of His side chains (6) corresponding to q_{His} = 0.50 (pH 6), q_{His} = 0.1 (pH 7) and q_{His} = 0.01 (pH8). As discussed in our response to major point 3 of this Reviewer we have now measured the pKa experimentally and obtained that they are distributed around an average value of 7.1. We think, therefore, that it is more appropriate to label these simulations according to the exact parameter used in the simulation, that is the charge of the His side chain, rather than to an approximate parameter such as pH. Finally, concerning the point raised by Reviewer 3, we would like to emphasize that, although the C_{sats} at q_{His} = 0.50, 0.1 and 0.01 are indeed similar, the values of C_{con} are in qualitative agreement with the experimental results; nevertheless, we have removed the relevant sentence from the text.

10) Are the multimer of nCPEB4 regulated by pH?

Please see our response to major comment 6 asking the same question, which we addressed as follows:

"To address these points, we have carried out DLS experiments equivalent to those presented in the original Fig. 3a for nCPEB4Δ4-NTD, and for both isoforms at pH 7.5. We obtained that the stability of the multimers formed by nCPEB4Δ4-NTD depends on solution conditions as that of those formed by nCPEB4-NTD, and that decreasing the pH increases the stability of both types of multimers (Fig. 3a and ED Fig. 4b).

From these results, we conclude that the multimers are stabilised by interactions equivalent to those stabilising the condensates, as discussed by Reviewer #2. We have also studied the multimers formed by nCPEB4Δ4-NTD by solution NMR (after assigning this other variant) and obtained results equivalent to those obtained for nCPEB4-NTD, confirming that the former are equivalent to the latter, albeit less stable due the loss of microexon 4 (ED Fig. 4c,d)."

11) In Extended Fig 3A, the figure legends mentions the temperature is from 5 to 60oC, however, the the figure only shows up to 35oC.

We have corrected this in the revised version.

12) All the DLS measurements were with volume fraction except for Extended Fig 3C with intensity fraction. It will be better to be consistent.

We would like to clarify that we showed the intensity deconvolution in ED Fig. 3c to emphasize that no oligomeric species larger than the multimers are detected in these samples: in our experience a volume deconvolution can mask the presence of large oligomeric species if these are present at low population. As suggested by Reviewer #3, we have added to the plot the volume deconvolution.

13) Line 222: please clarify ca350 in the figure legend.

We used *ca* as an abbreviation of the Latin word *circa* but have replaced it with *approximately* in line 222, that does not correspond to a figure legend. We have checked for the usage of this abbreviation throughout the manuscript and replaced this abbreviation accordingly.

14) Lines 235-239: please clarify.

We have made changes to the text to make it more clear.

15) For Fig 4C, the authors should compare full-length nCPEB4 and nCPEB4Δ4 in cells.

We have modified this figure and now show images and quantifications of nCPEB4 and nCPEB4Δ4 both for the NTD and the FL constructs. For reference, we include here our response to Reviewer #2 on this matter:

"The sentence in page 9 highlighted by the Reviewer "To confirm that microexon … nor with RNA (Extended Data Fig. 4c)" is indeed confusing as neither nCPEB4-NTD nor nCPEB4Δ4-NTD contain RNA binding domains: we apologize for this. We have now compared the effect of the microexon on the phase separation capacity of the full-length protein (Fig. 4c,d): briefly, loss of microexon 4 leads to a small but significant decrease in the number of phase-separated foci formed by nCPEB4-NTD, and the same effect can be observed in the context of the FL protein but to a lesser extent: the relevant sentence has been amended.

The competition assay that we used to show that the microexon has no effect on the affinity that CPEB4 has for RNA, described in detail in reference 10 of our original submission,[11] is based on the out-competition of endogenous CPEBs by transfected proteins.

As positive controls we transfected truncated protein variants containing only the RRMs or the ZZ domain (RRM12ZZ): these variants bind to RNA via the tandem RRMs but do not recruit the cofactors directly responsible for polyadenylation (such as CPSF, Symplekin, or GLD2).

Now, in this revised work, we tested whether the presence or absence of the microexon in CPEB4 affects its binding to RNA obtaining as shown in ED Fig. 4g that the microexon has no effect."

16) Fig 4I and and Extended Fig 4I use the same images. Please change it.

We have modified these figures and there are no repeated images in the revised version.

17) nCPEB4 and nCPEB4Δ4 ratios in control and ASD patients are given as 0.3 and 0.45, respectively, but no reference is provided for this data.

The reference was cited at the beginning of the relevant paragraph but not where these values were stated. We have corrected this in the revised version.

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2. Igea, A. & Méndez, R. Meiosis requires a translational positive loop where CPEB1 ensues its replacement by CPEB4. *EMBO J.* **29**, 2182–2193 (2010).
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13. Hervas, R. *et al.* Cryo-EM structure of a neuronal functional amyloid implicated in memory persistence in *Drosophila*. *Science* **367**, 1230–1234 (2020).
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Referee #1 (alternative splicing, translation regulation, neurobiology):

Garcia-Cabau et al have studied how the inclusion of microexon 4 in neuronal CPEB4 alters the protein properties and phase transition. They find that heterotypic interactions between the microexon and a cluster of His residues are necessary for the CPEB4 condensates to dissolve upon neuronal depolarization, thus allowing translation activation. The authors describe the exact kinetics of the interactions between the microexon and the His residues by a mechanism, akin to heterotypic buffering. They provide evidence that competition between homotypic interaction of aromatic clusters in CPEB4 that drive aggregation, and heterotypic interactions between the aromatic clusters and the microexon ensures the proper regulation of nCPEB4 in neurons and explain why a reduction in its degree of inclusion in ASD could result in a dominant-negative decrease in CPEB4 activity. Despite the already known role of CPEB4 in translation regulation via phase separation, this study provides a molecular mechanism of how this occurs, and is thus interesting and novel. The manuscript is well written, in a way that is apprehensible to a broader audience, of different scientific backgrounds.
In the re-submitted version the authors have made an effort to adequately address most of the reviewers' comments. They have added in vivo experimental evidence that correlate their findings with ASD, and thus strengthen the biological context of their work (Fig.5). Re-arrangement of some of the findings improved the flow and the readers' understanding of the work (Fig.2, 4 and 5).
Overall, the revisions significantly improved the manuscript.

We thank the reviewer for her/his comments and previous suggestions to improve our manuscript.

Referee #2 (microexons, neurobiology):

The authors have submitted an improved manuscript that addresses many of the reviewers' comments. A major concern of the three reviewers was whether the in vitro demonstration of aggregation of nCPEB4Δ4 represents an accurate in vivo model. The authors now demonstrate aggregates in brain homogenates from their mouse model that recapitulate the 'ASD ratio' (but see comment below) of nCPEB4/nCPEB4Δ4. However, some the reviewer comments could have been better addressed. Consequently, the results as they stand are not as conclusive as the authors portray. Some remaining concerns and suggestions for further revision are summarized below.

> We thank the reviewer for her/his re-evaluation of the manuscript and the new suggestions. We have addressed the specific points raised and trust that she/he will find the revised manuscript suitable for publication in *Nature*.

1. The authors assert that their original mouse model (relying on over-expression of nCPEB4Δ4 to alter CPEB4 isoform ratios) represents a better model for ASD than a clean heterozygous knockout of the CPEB4 neuronal microexon. This view is based largely on data from their previous manuscript (Parras et al Nature, 2018). For example, the authors state that their model "recapitulates ASD conditions: microexon inclusion/skipping differences in ASD are more subtle than a 50% ratio, with values changing from 70/30 % in 'normal individuals' to 55/45 % in patients with ASD". However, the data in their previous paper shows that there is extensive variability in the ratios of the microexon included versus skipped isoforms across patient samples, and it is anyone's guess as to how these variable ratios correspond to protein level changes in patient brains, or which specific ratios might be the most physiologically/autism-relevant when an entire program of microexons is disrupted by altered activity.

This makes it difficult to appreciate the rationale behind not deleting the CPEB4 microexon in the GFP-tagged line (which is hardly used in the authors' study), or in a WT mouse line. More specifically, according to the biochemical data presented (Fig 5c, S5d), a more pronounced increase in the Δex isoform ratio than observed in ASD individuals (i.e. ~65% skipping instead of ~45% skipping in autism and ~30% in control animals) should not reduce aggregate formation compared to the ASD-associated ratio. Therefore, a heterozygous endogenous microexon deletion mouse would be expected to result in ASD-like phenotypes and display CPEB4 aggregates. While this reviewer fully appreciates the technically demanding and time-consuming nature of such experiments, such an analysis would have helped address concerns related to the overexpression of nCPEB4Δ4, which was also a limitation of their previous paper. The argument that 'readjustment' of the overall CPEB4 levels is also questionable as it is possible that temporal overexpression (i.e. not detected at the time of harvesting) could have detrimental developmental effects. These issues should at the very least be discussed by the authors and mentioned as a limitation of their work.

> Following the suggestions by the reviewer we have included the following clarifications in the text:

i) We have made it clear in the manuscript that in our previous work the ratios (*70/30 % in 'normal individuals' to 55/45 % in patients with ASD*) refer to transcript levels and represent the mean for the analysed samples: the difference between control and ASD when all samples are analysed is statistically significant but individual samples may therefore deviate from the mean. In addition, although we showed that the transcripts are translated to similar extents and the proteins are similarly stable, we cannot exclude the possibility that the ratios at the protein are slightly different[1].

ii) For this reason we would like to clarify that the observation that $\chi\Delta4_{crit}$ is flanked by the $\chi\Delta4$ values computed from the mRNA levels reported in the 2018 study is worth pointing out but should not be interpreted strictly quantitatively: as we now state more clearly in the revised manuscript the key result is the sigmoidal dependence of Agg on $\chi\Delta4$, not the specific value of $\chi\Delta4_{crit}$.

ii) We acknowledge that the net increase of the levels of CPEB4 transcripts is a limitation of our mouse model (TgCPEB4Δ4). We would like to point out, however, that this does not result in a net increase of protein levels (ED Fig. 5e). It will nevertheless be interesting, in the future, to generate a mouse model replicating the relevant ratios of CPEB4 isoforms without overexpressing CPEB4, for example by heterozygous deletion of the microexon. We have thus introduced a paragraph stating that "*In our previous study the average ratio of isoforms observed in individuals diagnosed with ASD ($\chi\Delta4$ = 0.45) was obtained in mice by overexpressing nCPEB4Δ4. At the age at which they were analyzed these mice recapitulate that specific ratio, without a net increase in total protein levels, but its dynamics during embryonic neurodevelopment have not been analyzed. It will therefore be interesting, in future studies, to study CPEB4 splicing during embryonic neurodevelopment and to generate alternative mouse models recapitulating patient events, including the dynamics of splicing ratios, during embryonic neurodevelopment.*"

2. The authors also do not fully address a request from Reviewer 3 (which this reviewer agrees with) to perform more physiological treatments for activity-stimulation, given the issues with the extreme KCl treatment conditions used. The authors attempt depolarizing N2a cells with increasing concentrations of glutamate and NMDA, but these failed to cause depolarization as measured using immediate early gene expression markers. However, this is not surprising since it is known that N2a cells do not express glutamate receptors. It is unclear why the authors did not attempt more experiments using primary neurons.

In line with the explanation of the reviewer, and following the original request of reviewer # 3, we failed to stimulate N2a cells with neurotransmitters. Now, as suggested by the reviewer, we have performed the depolarization experiments in primary neurons. Since overexpressing CPEB4 with viral vectors has deleterious effects for primary neurons and cell fixation interferes with phase transitions[2] we have relied on imaging the endogenous GFP-tagged CPEB4 in live cells: we have observed that the endogenous CPEB4 condensates dissolve upon depolarization induced by N-methyl-D-aspartate receptor (NMDA), as observed in N2a cells upon depolarization induced by KCl. In addition, in some cases it has also been possible to observe a slight increase in

condensed fraction prior to dissolution, also as observed in N2a cells. We have included this experiment in the revised manuscript (Extended Data Fig. 1h,i,j).

3. Reviewer 1, Comment 3: To address the issue of obtaining a high-resolution demonstration of the dissolution of particles, as mentioned by the authors, attaching a movie demonstrating events before and after stimulation would be much more valuable than the inset, which doesn't really help.

To address this we attach a movie as supplementary material (Supplementary Video 1).

4. Reviewer 2, Comment 5: A follow-up question to the authors' explanation here is whether they think that the difference in the cells with foci remaining after stimulation between the 11A and 11D is significant (Fig ED 2e). If it is significant, then there could potentially be an effect of phosphorylation in addition to pH.

ED Fig. 2e of the previous manuscript version was used to illustrate that the condensates formed by both 11A and 11D dissolve upon depolarization despite neither of them being phosphorylatable; we think that the slight difference between the percentage of cells with foci 60 minutes after depolarization reflects the lower propensity to condense of 11D, relative to that of 11A, which is in agreement with expectations. We have now quantified the condensed fraction of the cells 60 min post-stimulation and find no significant differences between 11A and 11D. We have included this quantification in the revised manuscript (ED Fig. 2e, right panel).

5. Reviewer 2, Comment 7: RNA impact on phase behavior is not the same as RNA binding, with affinities measured in dilute solution. More specifically, lack of folded RNA-binding domains in the protein does not preclude an impact of RNA on phase separation. An in vitro experiment addressing the impact of RNA on phase separation propensity (or further characterization of droplets) of nCPEB4 vs nCPEB4Δ4 may be appropriate.

We have now carried out experiments *in vitro* with the NTD of nCPEB4 and nCPEB4Δ4 in the presence of RNA. We have on the one hand studied the effect of RNA on phase separation propensity by measuring the cloud points of these solutions: the results show that RNA slightly increases the cloud point, likely by interacting with positively charged amino acids, which are important for intermolecular interactions stabilising the condensates (ED Fig. 4m). On the other hand, we have also measured the aggregation in the condensates for both isoforms in the presence of RNA, finding that it has no effect (ED Fig. 4n).

Additional comments

1. In some places in the manuscript the authors refer to conclusions relating to CPEB4 microexon function 'in neurons' when they are referring to experiments performed in N2A cells. They also make some general conclusions that are not well supported by their data; for example, at the end of the Introduction: "In summary, we show how microexon 4 ensures the

proper regulation of nCPEB4 in neurons and explain why a reduction in its degree of inclusion in ASD results in a dominant negative decrease in CPEB4 activity". I suggest toning down and replacing 'explain' with 'providing evidence suggesting'....

> Reviewer #1 is correct and we thank her/him for highlighting this error. We now explicitly mention 'in N2A cells' where relevant and use only 'in neurons' for the experiments carried out by using primary neurons (Figure 1).

2. Avoid referring to autism under the umbrella of 'disease': For example, in the Abstract, the authors should change 'neurodevelopmental diseases' to 'neurodevelopmental or brain disorders'.

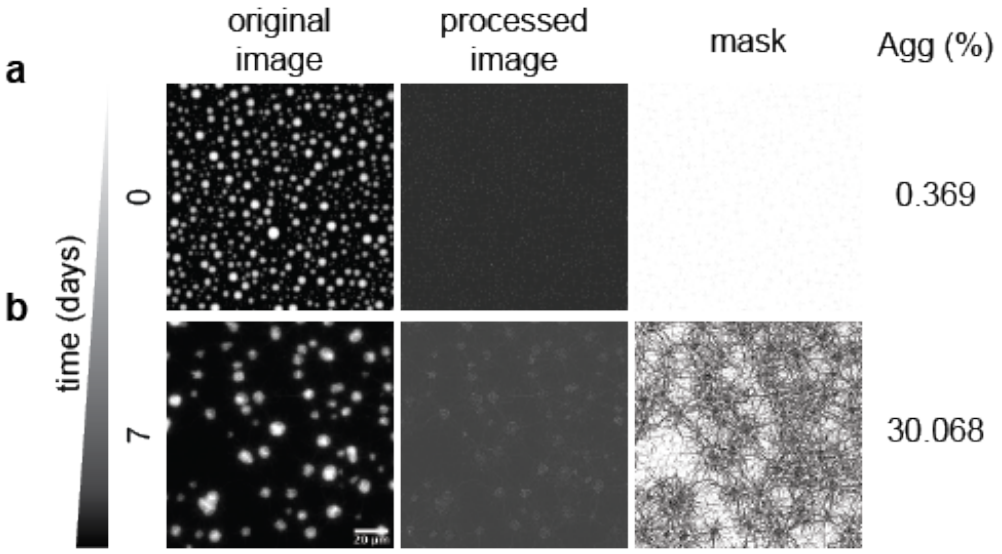
> Reviewer #2 is correct and we also thank her/him for highlighting this error. We have modified the text accordingly.

3. Reviewer 3, Comment 9 (second part): How many cells (or what percentage) have foci before stimulation?

> All the cells have foci before stimulation; we have made changes to the manuscript to clarify this point.

4. Methods section: It is not clear how the quantification of aggregation was performed. What is meant by "fluorescence signal not stemming from the condensates"? Is this background or non-spherical structures?

> It refers to fluorescence signal stemming neither from the background nor the spherical condensates; for clarification, we have prepared a figure where we explain the quantification (Reviewers Figure 1). Here one can see an image (panel a) where all the fluorescence signal arises from the droplets. By contrast, in panel b, the droplets show signs of aggregation and fibrillar species of lower fluorescence intensity are present in the sample. After image processing we can quantify the percentage of the field of view (in area) occupied by aggregated droplets and fibrilar structures.



Reviewers Figure 1. Example of the aggregation calculation of an nCPEB4Δ4-NTD sample at time 0 (a) and 7 (b) days. Scale bar represents 20 μm.

5. Main Figure 4m legend: Please clarify in the legend what the two curves in the right panel indicate. Also check if the left panel is nCPEB4 or nCPEB4Δ4.

We thank the reviewer for pointing out these errors in the caption of Fig. 4m, which we have corrected upon revision. Indeed the left panel corresponds to nCPEB4Δ4 and, as we now indicate in the figure caption, the two curves for Δ4ΔHClust represent the two most common behaviours. We decided to display them separately to differentiate the behaviour of cells where the condensates dissolve early after stimulation ($Time_{KCl}$ < 20 min) from that of cells where the condensates dissolve later ($Time_{KCl}$ > 20 min), but we still thought it was important to plot them together as these are the cells that show an increase in condensed fraction prior to dissolution.

Referee #3 (Remarks to the Author):

I have had a chance to look the responses in detail, and thank the authors for doing an excellent job of addressing my comments.

In their revised manuscript Garcia-Cabau et al. address all the points raised by this reviewer in March 2023 in an exemplary and highly satisfactory manner.
As an example of the major advances achieved by the authors over the last few months should here serve the following addition (requested by this reviewer in Major point 3) of Fig. 2 b and c of the revised manuscript:

Here the authors, by carefully quantifying the condensed fraction of CPEB4 and changes in intracellular pH in a time-resolved manner, can successfully correlate these two parameters and reveal the underlying physicochemical basis most likely at the heart of the regulation of many biomolecular condensates throughout biology.

We thank the reviewer for her/his comments and previous suggestions to improve our manuscript.

References

1. Parras, A. *et al.* Autism-like phenotype and risk gene mRNA deadenylation by CPEB4 mis-splicing. *Nature* **560**, 441–446 (2018).
2. Irgen-Gioro, S., Yoshida, S., Walling, V. & Chong, S. Fixation can change the appearance of phase separation in living cells. *Elife* **11**, (2022).

Referee #2 (microexons, neurobiology):

The authors have provided a significantly improved manuscript that satisfactorily addresses our main comments. We commend them for an exciting study the contributes important and timely insight into the mechanism by which mis-splicing of microexon 4 of Cpeb4 may contribute to altered regulation of gene expression in the context of ASD.

> We thank the reviewer for her/his comments and suggestions to improve our manuscript.

When finalizing their manuscript, we recommend the following (minor) changes:

1. P6: "the switch of CPEB4 from translational repression to activation, is regulated neither by post translational modifications nor by calcium mobilization."

Since the evidence for PTM regulation is only via a specific set of mass spec experiments, PTMs that are not easily detected via mass spec could potentially be regulating the phase behavior. This includes the O-GlcNAc modification (often not seen in standard mass spec data) and some kinds of oxidation. Thus, the evidence shows that phosphorylation is not a regulator, but the statement should be less general and not imply that the data rule out all PTMs. Obviously, pH is shown to regulate phase behavior, so it is likely that PTMs are not involved, but the sentence should be changed to be more precise.

> To address this we have changed the sentence to "our results indicate that the dissolution of the condensates formed by nCPEB4 in N2a cells and, thus, the switch from translational repression to activation, is regulated neither by phosphorylations of this protein nor by calcium mobilisation"

2. The authors could add a representative image of unstimulated primary neurons in Extended Data Figure 1h. Additionally, it is recommended that they clarify the Extended Data Figure 1i legend: what does the y axis indicate, and how were the first, max and last time frames defined?

> In these experiments we started recording images as soon as technically possible after depolarization. This time point, t_{first} = 5 min, is used to calculate the normalised condensed fraction plotted in the y axis in ED Fig. 1j as explained in Materials and Methods. We have modified the panel to clarify this as well as provided the three relevant time points in the figure caption.

3. P7: "The precise effect of missplicing will be determined by the identity of the microexon, causing changes in phase transition, as seen with CPEBs, or binding to cofactors, as observed with eIF4G."

The eIF4G microexon also changes the phase behavior of the host protein. The authors' concluding and summarizing statement should acknowledge this and reference the relevant paper in the sentence.

> To address this we have changed the sentence to "The effects of missplicing are likely to be microexon and protein-specific and can include changes in phase behavior as well as in the interaction with co-factors[5]. Ultimately, the altered translation program in ASD is due to the combined effects of all changes in neuron microexon inclusion that occur in this disorder."