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Tau protein liquid-liquid phase separation can initiate tau aggregation

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 11th September 2017

Thanks for submitting your manuscript to The EMBO Journal. Your study has now been seen by two referees and their comments are provided below.

It is clear that both referees appreciate the findings. However, there are also several issues that have to be sorted out in order to consider publication here. This concerns both the in vitro and in vivo data, but the referees offer constructive comments on how to resolve the points.

Should you be able to address the comments raised then I would like to invite you to submit a suitably revised manuscript.

Let me know if we need to discuss anything further

REFEREE REPORTS

Referee #1:

Wegmann et al present results suggesting that the MT binding full length phosphorylated tau, best known for its role in tangle formation in Alzheimer's Disease. They present very interesting evidence that tau is competent for phase separation both in vitro as well as in cells. The live cellular microscopy images of tau collections/assemblies are interesting and intriguing, though my concern is that overexpression of fluorescent protein tagged tau is not representative of the state in cells.

More importantly for this review, the manuscript makes many biophysical claims based on in vitro experiments that in my opinion are either not well designed (they cannot be used to make these conclusions) or explained and therefore the claims are not justified. I see these as critical issues that need to be addressed to support the conclusions that tau LLPS is fact regulated in the way described in the results and discussion and physiologically relevant.

Major concerns
1. In vitro tau LLPS. Chris Dobson demonstrated that effectively any protein could form amyloid (even super stable lysozyme, except perhaps very proline rich or repeat proteins) if the correct solution conditions where found. Certainly, truly any protein can form amorphous assemblies if the solvent conditions are chosen correctly. Therefore, it is reasonable to assume that essentially any IDP can phase separate. (Even folded proteins phase separate in crystallography trays.) Therefore, tau phase separation should be demonstrated first in cells in the manuscript and the emphasis should be placed there.
Additionally, in vitro phase separation here is always initiated with polymeric crowding agents. This not acceptable, despite what has been previously reported. Here it is unclear if tau can phase separate and it has a well described MT binding function unlike the LC domains that have largely dominated the attention of phase separation reports. The authors here need to show that tau can phase separate without addition of polymers that may cause phase separation by mediating multivalent weak interactions with tau instead of excluded volume effects which have been assumed here but not demonstrated. If the authors wish to show data with ficol, they should show that fluo-tagged ficol doped into the 12.5% ficol does not partition into the droplets with tau. If it does partition into the droplets, then how can we know if tau is forming tau-tau interactions? In addition, if tau needs excluded volume effects to phase separate, they should either raise the tau concentration (after all tau is very soluble as they say) and phase separate without crowders or they should crowd with monomeric crowders - instead of ficol they should use sucrose (ficol is a sucrose polymer) or glucose (instead of dextran which is glucose polymer) or ethylene glycol (PEG is the ethylene glycol polymer) (see Pielak's work http://www.pnas.org/content/113/7/1725.abstract) as these monomers in ~10% w/v will have the same crowding effect but will not be substrates for multivalent interactions. The authors may also wish to look at the edge of an evaporating solution drop (without crowders) where protein molecules are concentrated to see if such superconcentration will result in phase separation (or simply aggregation).

2. Tau concentration in droplets
The authors state that "at 2uM starting p-tau441 concentration, the addition of 10% PEG led to a concentration of p-tau441 in the droplets of ~20uM". There are no details about this calculation presented. The droplets appear to be >200x the background in the right hand inset - what is the concentration left in the supernatant as determined by other means - that will at least provide some baseline information even if the fluorescence in the background is not signal but rather is noise. The authors should show their calibration curve against free Alexa. The authors should not use free Alexa as a control because dye photo properties (even emission wavelengths) change upon conjugation. They should at least use the tau in non LLPS state at variety of concentrations to form a multipoint calibration curve. Yet: what happens to the fluorescence inside droplets - is quantum yield expected to change in this dense environment? The authors need to quantify the number of fluorophores attached per molecule, the dilution factor (fluo-label %of p-tau441). The authors should specifically state the concentration of the fluorophore in the droplets - do fluorophore-fluorophore photophysical (dipolar) interactions affect the expected fluorescence in this state? The authors should also calculate the mg/ml of this assembly and discuss if this is biophysically reasonable. There is one report from Brangwynne of low uM range droplets by ucFCS, but that report is subject to the same questions as those brought up above.

3. Viscosity in droplet. The authors use AquaVis sensitive to molecular rotation to measure viscosity. I cannot find any information on AquaVis in the methods section, nor a reference in the paper (perhaps I missed it) nor even online Google search. Second, the authors appear to conclude that the high fluorescence in the droplet suggests the viscosity is higher there. I certainly believe the viscosity is higher, but the increased fluorescence likely comes from a) partitioning of the dye into this hydrophobic-protein-containing droplet and b) binding to the high concentration of hydrophobes to slow presumable rotation about the carbon-carbon bond - rather than a higher viscosity slowing rotation.

4. "The hexanediol sensitivity or p-tau441 phase separation can likely be attributed to beta strand interactions". This claim is not supported, The reference Panas is a review article on stress granules that (correctly, as far as I understand the consensus) suggests that "1,6-hexanediol, an aliphatic alcohol that disrupts weak hydrophobic interactions, dissolves liquid droplets without affecting insoluble aggregates". It is insoluble aggregates (at least the amyloid fibril variety) that are typically thought to be stabilized by beta-sheet interactions. Hexane diol does not block the hydrogen bonds associated with beta sheet formation. The authors here present no evidence that beta-sheets are involved in LLPS. They could make variants that introduce proline residues that break beta sheets but are common in phase separating domains to try to isolate the contribution of beta sheets, but this would be a challenging task.

5. In cell LLPS: "Notably because overexpression of GFP-tau leads to intense overall fluorescence of the entire cell body and processes, GFP-tau droplets can only be identified in cells with low GFP-tau expression". To me this seems to argue against LLPS in cells as clusters should form leaving the regions without LLPS "granules" the same concentration (the critical concentration at the cell condition) regardless of expression level.

6. Droplet size - on page 13, the authors compare variants based on droplet size. The droplet size is a kinetic effect as droplets will fuse over time and will also undergo Ostwald ripening - which may be halted by gel-formation. The authors should use the approach of Mackensie et al Neuron 2017 on TIA-1 variants to estimate the critical concentration for droplet formation (the left side/arm of the phase diagram) to see if more protein is left in the cleared supernatant for different variants.

7. Full droplet bleach. The partial droplet bleach results are clear. The change in full droplet bleach do not indicate "that the liquid droplets matured rapidly into hydrogels of high viscosity" - indeed the small portion droplet bleach experiments do not support the "maturation" or gel formation until 60 minutes. The full droplet bleach lack of recovery may arises due to suppression of exchange kinetics across the interface which could be due to decrease in the availability of free monomers (the droplets get bigger and so the transport across the boundaries slows) or some type of surface hardening on the interface. The authors cannot distinguish these effects, but it cannot be due to maturation as the small portion bleach results demonstrate clearly.

8. thioflavin s. The authors claim that thioflavin S reports on beta sheet formation. Yes, it and tht are used for amyloid fibril detection, yet thioflavins are rotor dyes also that fluoresce due to quenched rotation when bound. Indeed, thioflavins also fluoresce when in high viscosity solvent http://pubs.acs.org/doi/full/10.1021/jp805822c so this expt is very much like the AquaVis experiment and cannot tell the presence of beta sheets. X-ray diffraction and TIRF could be use to indicate beta-sheet structures as demonstrated by McKnight.

9. phosphorylation effect on tau LLPS. The authors should add dephospho-tau to Figure 6 so that the differences are not due to source material rather due to phosphostate

10. the authors suggest a "conformational shift when negative charges are introduced". What are they referring to? No experiments are conformations are presented.

11. The model of phase separation via the nterminal domain would suggest that tau on the surface of the MT would be locally phase separated, confined to a 1 dimensional fiber. The authors should discuss this. This would hyperconcentrate locally the nterminal domain. The reference to membraneless organelles here as polyelectrolyte hydrogels or brushes is not consistent with LLPS organization, but could be consistent with a brush structure on an MT - though that may or may not be phase separated.

12. A number of imprecisions and misrenderings of the literature should be addressed:
Page 3 - change "comprise" to "contain"
Page 3/4 - the sentence says "The members of this protein family (presumably IDPs) ... also aggregate in protein aggregation diseases" this is not generally true, most IDPs/IDRs are not aggregation prone.
Page 4 - prion protein in prion disease is not based on an IDD
Page 4 - "accounted for" - rephrase
Page 4 - inhomogenous charge - this is not clear

Page 5 and many places - McKnight and coworkers generated hydrogels by super concentrating solubilized forms of LC domains (mCherry attached for example) and then waiting days at cold temperature for amyloid polymerization. These are distinct from gel-like forms created by "aging" of essentially instantaneous LLPS/demixing of proteins below the coexistence line for LLPS. I suggest that the authors not use the term "hydrogel" for these gel-like forms created by incubation of LLPS granules (for example, change "visoelastic hydrogel state" to "gel-like state"
Page 5 "By analogy to hnRNPA1, TDP-43, ... C9orf72" the authors should say C9orf72 derived DPRs, not the gene
Page 6 "can also manifest" should be change and the sentence is confusing
Page 6 How are "These domain charges, however, ...altered by phosphorylation, which introduces negative charges" - phosphorylation seems to occur in the negative charged domains already
Page 10 "Tau LLPS seems to be driven by electrostatic interactions in the unstructured N-terminal domain and stabilized by beta-sheet interactions in the repeat domain" - This sentence is worded to imply kinetic steps yet no kinetic experiments are presented.
Page 13 "this is characteristic for the maturation of liquid droplets into hydrogels (Kato et al 2012) and similar Kato reference on page 23: This paper does talk about hydrogels, but not about maturation of droplets. Rosen/Parker 2015 Mol Cell describes maturation of in vitro droplets.
Page 13 "the aggregation of tau in the droplets appears to release enough free energy to enable symmetry breaking of the droplets". This statement is unclear. Do the authors mean that aggregates are solid and of non spherical shape?
Page 14 first sentence. Conicella and Burke describe in vitro experiments and not stress granules. Patel describes primarily FUS granules caused by overexpression, and it is not clear this is the best reference for granules. The DPR work by Paul Taylor seems a more appropriate choice for documentation of altered stress granule dynamics
Page 14 - again, the reference to Kato is incorrect as they do not examine LLPS droplets in that work
Page 15 - define deltaK280/PP - it is not defined anywhere that I can find

Referee #2:

In their study, Wegmann and colleagues address the recent concept of liquid-liquid phase separation (LLPS) as an intriguing pathogenic mechanism for tau aggregation in tauopathies, using in vitro and cellular systems, which are complemented by the analysis of human and mouse tissue. I generally like the study that used a vast range of complementary techniques that makes a convincing point that LLPS can initiate tau aggregation in AD.
My major concerns are about the analysis/claims of tau concentration and phosphorylation pattern and the in vivo part that in my view is less convincing (the claim in the abstract that droplets are not only observed in culture but also in the intact brain). Besides that I am suggesting a few experiments that can be easily done and would clarify a few issues. The figures are well-crafted but occasionally would benefit from additional information in the corresponding legends.

My specific comments are as follows:

1. Page 7, Fig 1C: A significant part of this paper is based on making claims about the role of phosphorylation of tau in driving LLPS. The authors cite a previous paper of tau preparation in insect cells that has shown phosphorylation of tau at 18 sites. How can they be sure that their current preparation shows the same pattern? Moreover, it is not only important which sites are phosphorylated (most likely using more sensitive methods would pick up more sites), but what the stochiometry is. Both the phosphorylation sites and the stochiometry (ratio of moles of phosphate per mole of tau) need to be determined or the statement needs to be modified.

2. Page 8: What is a physiologically critical tau concentration and how is this being determined? The authors claim (Fig. 2F) that 1.5 uM is 'critical' but they only tested 0, 1, 2 ...uM so this is really difficult to say. I guess it also needs a better explanation in the legend and what the green and black labeling of the dots means. The statement on this page that tau is restricted to the axon is not entirely correct and the question is whether the cited 30-50% of unbound free tau (2005 ref) still holds up considering that tau binds to and interacts with so many proteins.

3. Page 9: Estimation of intraneuronal tau concentrations: This is an interesting approach but we are only given the end results without the figures for the intermediate steps. I find this entire paragraph problematic, especially as even a correct estimate would not inform on local (i.e. subcellular) protein concentration.

4. Page 9, Figure S3: The C-terminal half of tau should be added as a 'negative' control for p-tau256.

5. Page 10, Figure S3I: Please add non-reduced, unfractionated brain lysate such as to provide an estimate of the relative abundance of the N-terminal fragments in human brain.

6. Page 10, Section: 'Tau phase separation in neurons'. Here, I have several comments: The authors want to test 'whether tau can form droplets under physiological conditions' and use an over-expression of GFP-tagged tau. I think it would be better to drop the reference to 'physiological conditions' and refer to this as 'in neurons'. The claim of 'droplet-like tau accumulations' to me is an over-statement as there is simply a tau accumulation which could be anything. Wegmann and colleagues use FRAP to assess the behavior of the 'droplets' but FRAP should also be applied to 'droplet-free areas'. If the recovery rate is the same this would not substantiate the claim. What is also ignored is that in any area of bleaching there can be a mixture of tau populations. Further down the text, 'droplet-like' is replaced by 'droplets' without any proof that droplets actually form in vivo. It can also not be claimed that the droplets in the cell are 'in a viscoelastic hydrogel state'. This can be speculated but should go into the discussion. The Lim et al. reference and statement are out of place.

7. Page 12, Figure 4: Fig 4A: The phosphorylation pattern is mostly assumed but not shown. Fig 4B: There is a huge variability in total tau (between blots and between transfectants) indicating massive differences in tau levels so the observed effects could be because of phosphorylation and levels.

Minor comments:

1. M. Materials and methods are presented after the figure legends and before the figures. There is some inconsistency in the referencing style.

2. M. Bottom of page 4: Low complexity domain (LCD): The authors make the point that tau is a protein with low amino acid variance, intrinsic disorder and inhomogenous charge or polarity distribution. This led them to postulate that tau could undergo phase separation. But then they argue that tau has no defined LCD domain. This discrepancy needs to clarified.

3. M. Page 7, Fig 1E: Is this in the presence of Ficoll? If so, I assume tau does not form such a pattern without Ficoll.

4. M. Figure 2C: It is unclear to me what is shown in the top and bottom row of the figure.

5. M. Page 9: 'in contrast to most other LLPS proteins' - please provide info which ones do and which ones don't.

6. M. Page 14. Some statements should be toned down to 'this SUGGESTED that the exchange ... ; this MAY ALSO EXPLAIN... Figure 5A,B: There seems to be a mix-up as the actual droplets look the same and the bleached area whereas in A the entire field (i.e. the entire drop) is bleached. Fig. 5C: This is impressive. Is this because tau forms precipitates on the surface of the droplets?

7. M. page 16: TauRD construct needs to be explained: page 17: anti-aggregation mutation PP needs to be explained.

8. M. Page 19: This text needs to go into the discussion.

9. M. Page 37: Labeling of panel Figure 6: C) used twice.

1st Revision - authors' response 26th October 2017

We thank the reviewers for the critical reading and the constructive comments for our manuscript on tau liquid-liquid phase separation. We addressed all concerns raised and performed a set of additional experiments in order to do so. We also changed the manuscript structure and, where needed, the text in order to be more clear in our description and discussion of the data. We hope that the revised version of our manuscript now fulfills the scientific criteria to be published in EMBO Journal.
In the following you find our point-by-point answers to the reviewer comments including a short description of the new data and the changes made in the manuscript.

Referee #1:

Wegmann et al present results suggesting that the MT binding full length phosphorylated tau, best known for its role in tangle formation in Alzheimer's Disease. They present very interesting evidence that tau is competent for phase separation both in vitro as well as in cells. The live cellular microscopy images of tau collections/assemblies are interesting and intriguing, though my concern is that overexpression of fluorescent protein tagged tau is not representative of the state in cells. More importantly for this review, the manuscript makes many biophysical claims based on in vitro experiments that in my opinion are either not well designed (they cannot be used to make these conclusions) or explained and therefore the claims are not justified. I see these as critical issues that need to be addressed to support the conclusions that tau LLPS is fact regulated in the way described in the results and discussion and physiologically relevant.
Authors: At first, we want to thank the reviewer for the very constructive and scientifically outstanding review of our manuscript. We truly appreciate that the reviewer pointed out several the weak points and discussed the concerns in the reviewer's comments, and we agree that some major points describing the biophysical process of LLPS needed to be further investigated and clarified. We tried to address all (major and minor) concerns raised by performing additional experiments, and we think that the manuscript benefitted a lot from the additional data we added and the way we restructured and re-phrased the text in *Results* and *Discussion* to better reflect our observations .

Major concerns
1. In vitro tau LLPS. Chris Dobson demonstrated that effectively any protein could form amyloid (even super stable lysozyme, except perhaps very proline rich or repeat proteins) if the correct solution conditions where found. Certainly, truly any protein can form amorphous assemblies if the solvent conditions are chosen correctly. Therefore, it is reasonable to assume that essentially any IDP can phase separate. (Even folded proteins phase separate in crystallography trays.) Therefore, tau phase separation should be demonstrated first in cells in the manuscript and the emphasis should be placed there.
Authors: We restructured the manuscript and now start with showing that p-tau LLPS can be observed in in neurons *in vitro,* and that droplet-shaped tau accumulations can be found in the cytosol in the living brain of mice. We also made clear that this is happening (for now) in tau overexpressing experimental systems, although the data from AD brain suggest that a similar mechanism of tau LLPS may also be possible at physiological tau levels in the human brain, or more specifically, when the cytosolic phsopho-tau concentration is abnormally elevated in AD.

This is also further supported by the the conditions we chose to investigate tau LLPS *in vitro* in more detail: instead of harsh conditions needed to assemble soluble proteins into amyloids, we used near physiological tau protein concentrations (1-10 mM) and buffer conditions (50-150 mM NaCl, pH7.4) to initiate tau LLPS and subsequent aggregation. It is accepted that tau is an amyuloid forming pritein both in vivo and in vitro, and we also show that under the usually mild conditions used to trigger tau assembly into amyloid-like fibrils in vitro, which is the addition of the organic polyanionic polymers heparin or RNA, tau LLPS can readily be observed. We thus would argue that tau LLPS and aggregation can be compared to a general phenomenon of any given protein under harsh conditions. And in the case of LLPS, it may even be interesting to challenge the idea that LLPS could be a general (functional or dysfunctional) common mechanism of IDPs?

Additionally, in vitro phase separation here is always initiated with polymeric crowding agents. This not acceptable, despite what has been previously reported. Here it is unclear if tau can phase separate and it has a well described MT binding function unlike the LC domains that have largely

dominated the attention of phase separation reports. The authors here need to show that tau can phase separate without addition of polymers that may cause phase separation by mediating multivalent weak interactions with tau instead of excluded volume effects which have been assumed here but not demonstrated.
If the authors wish to show data with ficol, they should show that fluo-tagged ficol doped into the 12.5% ficol does not partition into the droplets with tau. If it does partition into the droplets, then how can we know if tau is forming tau-tau interactions?
In addition, if tau needs excluded volume effects to phase separate, they should either raise the tau concentration (after all tau is very soluble as they say) and phase separate without crowders or they should crowd with monomeric crowders - instead of ficol they should use sucrose (ficol is a sucrose polymer) or glucose (instead of dextran which is glucose polymer) or ethylene glycol (PEG is the ethylene glycol polymer) (see Pielak's work http://www.pnas.org/content/113/7/1725.abstract) as these monomers in ~10% w/v will have the same crowding effect but will not be substrates for multivalent interactions. The authors may also wish to look at the edge of an evaporating solution drop (without crowders) where protein molecules are concentrated to see if such superconcentration will result in phase separation (or simply aggregation).
Authors: We thank the reviewer for being critical and pointing out these concerns about the driving forces behind tau LLPS in crowdning conditions, and for the constructive suggestion of experiments to address the open questions. We peformed several experiments to answer these questions:

First, to shed light on the question if the polymeric crowders cause tau LLPS by templating multivalent interactions and co-seperation into the tau droplets, we spiked fluorescently labeled dextranes of different molecular weights (3 kDa, 20 kDa, 70 kDa) into dextran (70 kDa) induced p-tau LLPS setups and imaged the droplets by confocal microscopy over a time of 5 to 60 min. We observed that immediately after tau LLPS initiation by dextran, all three dextranes were excluded from the tau droplets, which did not change over time. This suggested that tau LLPS triggered by the polymeric crowder dextran relies on tau-tau interactions induced by excluded volume effects that lead to high local tau concentrations, rather than on interactions of dextran molecules with tau. The results are shown in Supplementary Figure S4C.

Then, we tested if crowding induced by the monomeric entities of the polymeric crowders dextran and PEG can initiate tau LLPS as well, as was suggested by the reviewer. Indeed, at 10% (w/v), we only observed crowding after the addition of dextran and PEG, but not in presence of glucose or ethylene glycol (Supplemental Figure S 4F), suggesting that excluded volume effects caused by the polymeric crowders are needed to supersaturate tau and thereby initiate tau LLPS.

Next, we tested if p-tau can phase separate in the absence of molecular crowding agents at very high concentrations by imaging an air-exposed droplet of 100 mM p-tau. In this setting, the droplet surface was exposed to evaporation, which led to a gradual local increase of the tau concentration and a supersaturation of tau on the droplet-air interface. We observed that immediately after droplet deposition, tau LLPS started to occur from the outside to the inside of the droplet, suggesting that tau can undergo phase separation at high concentration of $\geq$50 mM. These results are shown in Supplemental Figure S4E and reported in the text. The supersaturation of tau as a cause for tau aggregation aggregation under disease conditions in the aging and AD brain have previously been suggested by Christopher Dobson and Michele Vendrusculolo (Ciryam *et al*, 2013, 2015). Tau LLPS, as a precursor for amyloid-like aggregation, agrees with this theory and adds an additional phase separation step into the process.
Furthermore, a similar tau LLPS at high concentrations but in the absence of crowders has very recently been reported for the microtubule binding domain of 4R tau by the lab of Markus Zweckstetter (Ambadipudi *et al*, 2017).
In the revised version of the manuscript, all of the above results are mentioned in the text and the effect of supersaturation as a possible initiator of tau LLPS and subsequent aggregation is mentioned in the *Discussion*.

2. Tau concentration in droplets
The authors state that "at 2uM starting p-tau441 concentration, the addition of 10% PEG led to a concentration of p-tau441 in the droplets of ~20uM". There are no details about this calculation presented. The droplets appear to be >200x the background in the right hand inset - what is the concentration left in the supernatant as determined by other means - that will at least provide some

baseline information even if the fluorescence in the background is not signal but rather is noise. The authors should show their calibration curve against free Alexa.
The authors should not use free Alexa as a control because dye photo properties (even emission wavelengths) change upon conjugation. They should at least use the tau in non LLPS state at variety of concentrations to form a multipoint calibration curve.
Yet: what happens to the fluorescence inside droplets - is quantum yield expected to change in this dense environment? The authors need to quantify the number of fluorophores attached per molecule, the dilution factor (fluo-label %of p-tau441).
The authors should specifically state the concentration of the fluorophore in the droplets - do fluorophore-fluorophore photophysical (dipolar) interactions affect the expected fluorescence in this state?
The authors should also calculate the mg/ml of this assembly and discuss if this is biophysically reasonable. There is one report from Brangwynne of low uM range droplets by ucFCS, but that report is subject to the same questions as those brought up above.
Authors: We apologize for the previously incomplete presentation of the data on the tau concentration in PEG induced droplets *in vitro*. In the revised version of the manuscript, we added detailed information about the experimental procedure to the *Methods* part and report all the calibration and measurement steps in an additional supplemental Figure panel (Supplemental Figure S4B).
To reassure our previous results, we redid the entire experiment with the proper calibration of the fluorescence intensity in the recorded images against different concentrations of Alexa568-labeled p-tau441 in the absence of LLPS (no PEG). We assured that the Alexa568 fluorophore does not majorly change its fluorescence characteristics in the presence of 0-20% PEG. We determined the average number of Alexa568 fluorophores per molecule p-tau441 as [tau:Alexa568] = [1:1.45]. With this information and the imaging of >100 p-tau441-a568 droplets, we come to the very similar conclusion that at a starting concentration fo 5 mM p-tau441, the tau concentration in the droplets (as measured by fluorescence intensity of p-tau441-a568 droplets by image analysis) is ~22 mM (~ 1.25 mg/ml p-tau441-a568; min=0.64mg/ml; max=2.01mg/ml). The concentration of the remaining tau in the non-droplet phase is ~3 mM (~0.17 mlg/ml). This new data are presented in the text and in Figure 2B and the data are discussed in the *Discussion* part.

3. Viscosity in droplet. The authors use AquaVis sensitive to molecular rotation to measure viscosity. I cannot find any information on AquaVis in the methods section, nor a reference in the paper (perhaps I missed it) nor even online Google search. Second, the authors appear to conclude that the high fluorescence in the droplet suggests the viscosity is higher there. I certainly believe the viscosity is higher, but the increased fluorescence likely comes from a) partitioning of the dye into this hydrophobic-protein-containing droplet and b) binding to the high concentration of hydrophobes to slow presumable rotation about the carbon-carbon bond - rather than a higher viscosity slowing rotation.
Authors: We apologize for the sloppy referencing for the viscosity measurement dye Viscous Aqua^{TM} (Ursa Bioscience, Maryland, USA). However, after considering the interpretation of the reviewer, that the dye becomes concentrated in the tau droplets due to hydrophobic interactions of the concentrated protein, we agree that we can at this point not conclude or proof the viscosity in the tau droplets. We thus decided to take the Viscous Aqua data out of the manuscript and deleted the statement about the increased viscosity in the droplets, also because the FRAP data actually already shows that point.
In fact, both the Viscous Aqua and Thioflavine-S fluorescence in the freshly formed droplets both indicate the retention of these hydrophobic dyes in the droplets; and we further verified this idea with results showing also the retention of Methylene Blue in the droplets immediately after formation. These results are included in the revised version of the manuscript in Supplemental Figure S4D.

4. "The hexanediol sensitivity or p-tau441 phase separation can likely be attributed to beta strand interactions". This claim is not supported, The reference Panas is a review article on stress granules that (correctly, as far as I understand the consensus) suggests that "1,6-hexanediol, an aliphatic alcohol that disrupts weak hydrophobic interactions, dissolves liquid droplets without affecting insoluble aggregates". It is insoluble aggregates (at least the amyloid fibril variety) that are typically thought to be stabilized by beta-sheet interactions. Hexane diol does not block the hydrogen bonds associated with beta sheet formation. The authors here present no evidence that beta-sheets are

involved in LLPS. They could make variants that introduce proline residues that break beta sheets but are common in phase separating domains to try to isolate the contribution of beta sheets, but this would be a challenging task.
Authors: We agree with the reviewer that, although we observe the inhibiting effect of hexanediol on full-length p-tau LLPS, we do not know if beta-strand interactions *per se* are effected. This is a speculation based on previous publications (Eckermann *et al*, 2007; Von Bergen *et al*, 2005, 2001) that showed that hydrophobic beta-strand interactions in the tau repeat domain play a major role for the aggregation of the tau. Based on this knowledge we speculated that these interactions may also drive or at least influence tau LLPS. Because we cannot clearly proof the relation between beta-strand interaction and tau LLPS at this point, we now mention this interpretion as one possible mechanism of the inhibiting effect of haxanediol, which is supported by the finding that in mutants with enhanced beta-strand propensity in the repeat domain (e.g DeltaK280; von Bergen *et al*, 2000) LLPS can occur even in the absence of phsophoylation, and that mutants breaking this enhanced beta-strand propensity due to proline insertions in the beta-strand motif (e.g. DeltaK280/PP; Eckermann *et al*, 2007) abolish tau LLPS. We hope that this idea is more precisely described and better discussed in the revised version of the manuscript. We also made clear that further experiments will be needed to examine the role of particular intra- and intermolecular tau:tau interactions in the process of tau LLPS.

5. In cell LLPS: "Notably because overexpression of GFP-tau leads to intense overall fluorescence of the entire cell body and processes, GFP-tau droplets can only be identified in cells with low GFP-tau expression". To me this seems to argue against LLPS in cells as clusters should form leaving the regions without LLPS "granules" the same concentration (the critical concentration at the cell condition) regardless of expression level.
Authors: We agree with the reviewer that this statement is somewhat confusing when assuming that tau LLPS is dependent mainly on tau concentration. However, not only is the tau distribution in the neurons is *per se* very heterogenous (normally in axons by far higher than in dendrites and the soma; and *de novo* tau transcription was reported to exist in dendrites upon Abeta exposure (Zempel & Mandelkow, 2015; Li & Götz, 2017) but also dependent on stress conditions and highly dependent on local and transient phosphorylation. The dependency of tau LLPS on phosphorylation becomes clear from the data in this manuscript, it is an entire separate and very complex issue to understand the dynamics of phosphroylation in the neuron, a problem we and multiple research groups currently work on.
To remove the confusion caused by the statement pointed out by the reviewer, we added a sentence explaining this issue of local tau concentration and phosphorylation and the dependency of tau LLPS on these parameters.

6. Droplet size - on page 13, the authors compare variants based on droplet size. The droplet size is a kinetic effect as droplets will fuse over time and will also undergo Ostwald ripening - which may be halted by gel-formation. The authors should use the approach of Mackensie et al Neuron 2017 on TIA-1 variants to estimate the critical concentration for droplet formation (the left side/arm of the phase diagram) to see if more protein is left in the cleared supernatant for different variants.
Authors: We absolutely agree with the reviewer that the droplet growth is a result of various contributing and overlapping effects, including Ostwald ripening, fusion/fission, and gelation. The detected growth differences between the phosphorylation states of tau are thus likely also due to a combination of these factors. We also agree that it would be of interest to sort out more in detail which biophysical parameters during the LLPS and the subsequent ripening of the droplets change with alterations in the phosphorylation. However, at this point in our studies, the complexity of the biophysical mechanisms behind LLPS and droplet phase transitions as well as the complex biology behind tau phosphorylation and its relevance for tau function and molecular behavior prevents us from analyzing all the details behind the observation that phosphorylation obviously has a large impact on tau LLPS.
To clarify the raised question of the critical LLPS concentration of the phosphorylation forms of tau, we followed the reviewer's advice and performed experiments in analogy to *Mackensie et al Neuron 2017 on TIA-1*, in which we aimed to separate formed droplets from the non-droplet phase by simple centrifugation (20000 g for 15 min at room temperature). Measuring the p-tau441 concentration in the soluble phase before (no PEG) and after LLPS (+10% PEG, supernatant), we were indeed able to detect a reduction of tau in the soluble non-droplet phase (~3-fold reduction at starting concentration of 10 mM p-tau441) by protein determination at 280 nm. Unfortunately we were unable to detect changes in the other tested phospho-tau constructs (E17, MARK tau441), and when

checking the droplet phase (pellet after centrifugation) and non-droplet phase by microscopy, no enrichment of tau droplets in the pellet could be detected for these constructs. This inefficient separation could be explained by the large difference in droplet sizes between p-tau441 and E17 and MARK-tau4, which drastically changes their sedimentation behavior; also the droplet phase stability may be different between the phospho-tau constructs.
As much as we want to sort out these questions, we believe that it will take an extended amount of time and that such aim can be seen as a whole new project in itself. In fact, we are already performing a complex experimental setup with multiple different approaches to precisely evaluate the impact of individual tau phosphorylation - and other PTMs - on tau LLPS behavior. For example, we are currently preparing a mutant tau library, in which all phosphorylation sites relevant for function and misfunction/aggregation of tau are systematically disabled or pseudophosphorylated, and plan to carefully characterize these mutants with different biophysical and protein biochemical methods to sort out the role of cellular PTMs on tau LLPS.
However, to account for the raised concerns of the reviewer in the manuscript, we added a sentence to the text discussing the issue and analyzed the number of droplets per volume and the volume fraction in z-stacks recorded for each construct (Figure 3D) in order to have at least a qualitative idea about the amount of tau phase separating into droplets.

7. Full droplet bleach. The partial droplet bleach results are clear. The change in full droplet bleach do not indicate "that the liquid droplets matured rapidly into hydrogels of high viscosity" - indeed the small portion droplet bleach experiments do not support the "maturation" or gel formation until 60 minutes. The full droplet bleach lack of recovery may arises due to suppression of exchange kinetics across the interface which could be due to decrease in the availability of free monomers (the droplets get bigger and so the transport across the boundaries slows) or some type of surface hardening on the interface. The authors cannot distinguish these effects, but it cannot be due to maturation as the small portion bleach results demonstrate clearly.

Authors: Thank you for pointing out that our simplified interpretation. We changed this statement in the text. We agree with reviewer and would also interpret the lack of recovery after full-droplet bleach as some kind of surface hardening on the interface; this idea is to some part supported by the observed shape changes of droplets (deviation from spherical shape) as shown for the p-tau constructs in Figure 3D, and by the growth of tau aggregates on the interface of the droplets (Figure 4D). However, we now mention these possible explanations in the *Discussion*.

8. Thioflavin s. The authors claim that thioflavin S reports on beta sheet formation. Yes, it and tht are used for amyloid fibril detection, yet thioflavins are rotor dyes also that fluoresce due to quenched rotation when bound. Indeed, thioflavins also fluoresce when in high viscosity solvent http://pubs.acs.org/doi/full/10.1021/jp805822c so this expt is very much like the AquaVis experiment and cannot tell the presence of beta sheets. X-ray diffraction and TIRF could be use to indicate beta-sheet structures as demonstrated by McKnight.
Authors: We absolutely agree with the reviewer that Thioflavine S can not only function as a beta-sheet detection compound, but also partitions into droplets (likely due to hydrophobic interactions) and hence produces enhanced fluorescence in viscous droplets even in the absence of beta-sheet structures (see Figure 4E). However, Thioflavine-S has a high preference to bind to amyloid and amyloid-like beta-sheet rich macromolecular assemblies and exhibits a ~10-fold increase in fluorescence upon binding these structures.
It became thus the classic amyloid detection dye used in neurological research over multiple decades, and has undoubtfully proven its usefulness to specifically detect amyloid plaques as well as tau aggregates in neurofibrillary tangles, both *in vitro* as well as in the brain. While being aware of the “unspecific” partitioning of ThioS into tau droplets, we would argue that the very intense ThioS fluorescence of the tau aggregates that form on the droplet surfaces is a strong indication for b-sheets in these tau aggregates.
The b-sheet content of tau aggregates remains difficult to detect even in pure paired helical filament preparations assembled *in vitro*. This is likely because the actually beta-strand motifs are very short (a hexapeptide motif in the beginning of repeat 3), and beta-sheets formed by this motif are detectable by CD, FTIR and X-ray in “clean” amyloid fibrils made from tau constructes containing only the motif or the repeat domain (Von Bergen *et al*, 2005), but barely in full-length tau amyloid assemblies.

In the current version of the manuscript, we made sure to better explain the compromise between ThioS fluorescence intensity in viscous droplets and in beta-sheet rich aggregates, and we toned down our statement of the b-sheet content in the tau aggregates on tau droplets.
We also added a Supplemental Figure S4D, in which we show that also other dyes like Methylene Blue and Viscous Aqua co-partition into fresh tau droplets ; likely due to hydrophobic interactions, as the reviewer pointed out.

9. phosphorylation effect on tau LLPS. The authors should add dephospho-tau to Figure 6 so that the differences are not due to source material rather due to phosphostate
Authors: Figure 6 shows data on non-phosphorylated (E. coli derived) tau proteins that carry FTD-mutations, which can initiate tau LLPS, even in the absence of any phosphorylation. The source of all tau proteins is in this case is the same (E coli). deP-tau441 from Sf9 cells still carries residual phosphorylation (average of 4 phosphates per tau molecule), which appears to be sufficient to initiate tau LLPS. To avoid any confusion of the reader, we stated that more clearly in the text as well as in the legend of this Figure.
The de-phosphorylated Sf9 cell tau we used to show the effect of decreased phosphorylation on tau LLPS still carries residual phosphorylation of in average 4 phosphates per molecule (Mair *et al*, 2016; Tepper *et al*, 2014).

10. the authors suggest a "conformational shift when negative charges are introduced". What are they referring to? No experiments are conformations are presented.
Authors: We apologize for this unreferenced statement in the manuscript. Hyperphosphorylation of tau found in Alzheimer's disease brains - for example by kinase GsK3beta at multiple sites – has long been reported to preceed pathological conformational changes of tau that lead to aggregation (Iqbal *et al*, 2005; Stoothoff & Johnson, 2005; Mondragón-Rodríguez *et al*, 2008). And more recently, Time-Resolved ElectroSpray Ionization Mass Spectrometry (TRESI-MS) suggested a molecular conformational shift towards more amyloidogenic structure upon phosphorylation (Zhu *et al*, 2015). We added these references as well as a sentence of better explanation to the text.

11. The model of phase separation via the nterminal domain would suggest that tau on the surface of the MT would be locally phase separated, confined to a 1 dimensional fiber. The authors should discuss this. This would hyperconcentrate locally the nterminal domain. The reference to membraneless organelles here as polyelectrolyte hydrogels or brushes is not consistent with LLPS organization, but could be consistent with a brush structure on an MT - though that may or may not be phase separated.

Authors: Thank you for pointing out this very interesting aspect of possible roles of tau phase separation. We already did some Gedankenexperiments on this previously, and now added a sentence to the *Discussion* part of the revised manuscript.

12. A number of imprecisions and misrenderings of the literature should be addressed:
Page 3 - change "comprise" to "contain"
Page 3/4 - the sentence says "The members of this protein family (presumably IDPs) ... also aggregate in protein aggregation diseases" this is not generally true, most IDPs/IDRs are not aggregation prone.
Page 4 - prion protein in prion disease is not based on an IDD
Page 4 - "accounted for" - rephrase
Page 4 - inhomogenous charge - this is not clear
Page 5 and many places - McKnight and coworkers generated hydrogels by super concentrating solubilized forms of LC domains (mCherry attached for example) and then waiting days at cold temperature for amyloid polymerization. These are distinct from gel-like forms created by "aging" of essentially instantaneous LLPS/demixing of proteins below the coexistence line for LLPS. I suggest that the authors not use the term "hydrogel" for these gel-like forms created by incubation of LLPS granules (for example, change "visoelastic hydrogel state" to "gel-like state"
Page 5 "By analogy to hnRNPA1, TDP-43, ... C9orf72" the authors should say C9orf72 derived DPRs, not the gene
Page 6 "can also manifest" should be change and the sentence is confusing
Page 6 How are "These domain charges, however, ...altered by phosphorylation, which introduces negative charges" - phosphorylation seems to occur in the negative charged domains already

Page 10 "Tau LLPS seems to be driven by electrostatic interactions in the unstructured N-terminal domain and stabilized by beta-sheet interactions in the repeat domain" - This sentence is worded to imply kinetic steps yet no kinetic experiments are presented.
Page 13 "this is characteristic for the maturation of liquid droplets into hydrogels (Kato et al 2012) and similar Kato reference on page 23: This paper does talk about hydrogels, but not about maturation of droplets. Rosen/Parker 2015 Mol Cell describes maturation of in vitro droplets.
Page 13 "the aggregation of tau in the droplets appears to release enough free energy to enable symmetry breaking of the droplets". This statement is unclear. Do the authors mean that aggregates are solid and of non spherical shape?
Page 14 first sentence. Conicella and Burke describe in vitro experiments and not stress granules. Patel describes primarily FUS granules caused by overexpression, and it is not clear this is the best reference for granules. The DPR work by Paul Taylor seems a more appropriate choice for documentation of altered stress granule dynamics
Page 14 - again, the reference to Kato is incorrect as they do not examine LLPS droplets in that work
Page 15 - define deltaK280/PP - it is not defined anywhere that I can find

Authors: We thank the reviewer for the careful and critical reading and pointing out these potentially conflicting choices of our wording. We changed the text at all mentioned points to correct the mistakes and improve the manuscript.

Referee #2:

In their study, Wegmann and colleagues address the recent concept of liquid-liquid phase separation (LLPS) as an intriguing pathogenic mechanism for tau aggregation in tauopathies, using in vitro and cellular systems, which are complemented by the analysis of human and mouse tissue. I generally like the study that used a vast range of complementary techniques that makes a convincing point that LLPS can initiate tau aggregation in AD.
My major concerns are about the analysis/claims of tau concentration and phosphorylation pattern and the in vivo part that in my view is less convincing (the claim in the abstract that droplets are not only observed in culture but also in the intact brain). Besides that I am suggesting a few experiments that can be easily done and would clarify a few issues. The figures are well-crafted but occasionally would benefit from additional information in the corresponding legends.
Authors: First, we want to thank the reviewer for the careful and critical reading of our manuscript and for his/her constructive critics and providing ideas for experiments to remove some issues that needed clarification. We tried to address most points experimentally or changed the way we presented and described the data in the text in order to be more specific. We believe the manuscript benefitted a lot from these changes. We also toned down our statement of tau LLPS in the intact brain to not overstate.

My specific comments are as follows:

1. Page 7, Fig 1C: A significant part of this paper is based on making claims about the role of phosphorylation of tau in driving LLPS. The authors cite a previous paper of tau preparation in insect cells that has shown phosphorylation of tau at 18 sites. How can they be sure that their current preparation shows the same pattern? Moreover, it is not only important which sites are phosphorylated (most likely using more sensitive methods would pick up more sites), but what the stochiometry is. Both the phosphorylation sites and the stochiometry (ratio of moles of phosphate per mole of tau) need to be determined or the statement needs to be modified.
Authors: We absolutely agree that some more effort was needed to verify the phosphorylation state of the used p-tau preparation. Since the source of the protein derived from Sf9 insect cells is the same as the one analyzed in great detail by Mass Spectrometry in Tepper et al. and Mair et al. (Tepper *et al*, 2014; Mair *et al*, 2016), - meaning the protein was produced in the same laboratory using the same expression system, cell line, passage number, and purification protocol – we think that is is okay to assume a very similar phosphorylation pattern in the p-tau we used in this study. To verify the phosphorylation state of the used protein, we went back to the previously determined phosphorylation pattern of p-tau441 (Mair *et al*, 2016) and verified the existence of the described phsophorylation sites by Western Blot analysis. Using 16 different phosphorylation site specific antibodies, we could verify the presence of all except of two previously described sites in p-tau441.

This indicates that the phosphorylation between the proteins used here and in the previously studies is indeed very similar. The data are included in the manuscript in Supplemental Figure S3C.
Of course we cannot exclude small variations in the stoichiometry of the PTMs. This would ask for another detailed Mass spectrometry analysis, which at this time would unfortunately exceed our time line for the manuscript revision. However, we are in the process of planning and performing experiments using tau constructs with local single amino acid mutantions to inhibit and/or pseudo-phsophorylate single phospho-sites in order to figure out the importance and contribution of individual pshophorylation sites for tau LLPS. We hope that this data will be available in near future and inform about the biological function of neuronal tau LLPS.

2. Page 8: What is a physiologically critical tau concentration and how is this being determined? The authors claim (Fig. 2F) that 1.5 uM is 'critical' but they only tested 0, 1, 2 ...uM so this is really difficult to say. I guess it also needs a better explanation in the legend and what the green and black labelling of the dots means. The statement on this page that tau is restricted to the axon is not entirely correct and the question is whether the cited 30-50% of unbound free tau (2005 ref) still holds up considering that tau binds to and interacts with so many proteins.
Authors: We apologize for the unclear description of the phase diagram of tau LLPS under different conditions and added this information to the Figure Legend. We also performed additional experiments on p-tau441 LLPS around the critical concentration (at 0.5, 1.0, and 2.0 uM pTau in the presence of 10% and 20% PEG) to determine the critical concentration in more detail. We found that tau LLPS, as visible my light microscopy using a 40x objective - can occur even at ~1 uM p-tau441. Since this is a microscopic and not molecular evaluation of the LLPS process, it may be well possible that LLPS can occur at even lower concentration but is missed in this analysis due to the insensitivity of the method caused by the diffraction limit. At this point, there is – to our knowledge – no better way of determining LLPS because chemical shifts in NMR upon tau LLPS are almost not detectible and turbidity measurements are rather insensitive. Since this is a critical issue also for almost all previously published studies claiming concentration limits for protein LLPS, we point out this issue in the text to make the reader aware of this topic.
We also added a sentence to the text mentioning the fact that neuronal tau has multiple binding partners in the cell, which likely causes large fluctuations in the local intraneuronal tau concentration available for LLPS. We also mentioned in the text (page 10) that 30-50% of tau has been reported to be unbound (free). At an assumed average intraneuronal concentration of ~ 2 mM tau that would give a concentration of ~0.7-1 mM free tau detached from microtubules, and this free tau is usually phosphorylated, which increases the local concentration of p-tau species, potentially leading to a higher local LLPS propensity.

3. Page 9: Estimation of intraneuronal tau concentrations: This is an interesting approach but we are only given the end results without the figures for the intermediate steps. I find this entire paragraph problematic, especially as even a correct estimate would not inform on local (i.e. subcellular) protein concentration.
Authors: Thank you for this thorough understanding of tau biology and the critical comment on the issue of intraneuronal tau concentration – a topic that is most of the time under-appreciated as well as a longstanding open question. We tried to tackle the issue to relate the tau concentration range critical for in vitro LLPS to brain tau content. However, we are aware that the global tau content we estimated in the human brain becomes rather irrelevant when talking about precise local tau concentrations in neuronal sub-compartments.
We also apologize for the lack of intermediate data presentation.
In the revised version of our manuscript, we mention this brain tau level in the text to present the relevance of the tau concentration in LLPS to the reader (also without tau biology background knowledge) and present all the data and Method of how we estimated the human brain tau content in a separate Supplementary Information file.
We hope that this now clarifies the question about tau brain concentration and tau LLPS relevance without confusing the reader.

4. Page 9, Figure S3: The C-terminal half of tau should be added as a ‘negative’ control for p-tau256.
Authors: Thank you for pointing out this critical lack of data! We designed and expressed a tau construct in insect cells that contains the C-terminal half of full-length human tau (amino acids 242 to 441) fused to GFP (p-tauCt-GFP). We found that this conctruct can actually undergo LLPS in the presence of molecular crowding and that p-tauCt-GFP LLPS was insensitive to high salt

concentrations as well as the presence of hexanediol. These data is now included in the revised version of the manuscript, as it is menstioned in the text and included in an extra Supplemental Figure S5. Similarly, LLPS of the tau microtubule binding domain has very recently been presented also by others (Ambadipudi *et al*, 2017).

5. Page 10, Figure S3I: Please add non-reduced, unfractionated brain lysate such as to provide an estimate of the relative abundance of the N-terminal fragments in human brain.
Authors: A Wetsern Blot of non-reduced whole brain lysates from the same AD and Control brains has been added to the Figure S6H .

6. Page 10, Section: 'Tau phase separation in neurons'. Here, I have several comments: The authors want to test 'whether tau can form droplets under physiological conditions' and use an over-expression of GFP-tagged tau. I think it would be better to drop the reference to 'physiological conditions' and refer to this as 'in neurons'. The claim of 'droplet-like tau accumulations' to me is an over-statement as there is simply a tau accumulation which could be anything. Wegmann and colleagues use FRAP to assess the behavior of the 'droplets' but FRAP should also be applied to 'droplet-free areas'. If the recovery rate is the same this would not substantiate the claim.
Authors: We agree with the reviewer that stating “physiological conditions” in overexpressing neurons and tau LLPS in the “intact brain” may have been slightly overstated in the manuscript. We followed the reviewers advice and toned down these statements accordingly. We also shifted the data on Dendra2-tau LLPS in the mouse cortex by two-photon imaging into the Supplemental Figure S3A.
Regarding the FRAP data, all data has been normalized to FRAP of droplet-free adjacent areas in the same cell during the data analysis. To make this clear, we added this information to the figure legend and explained it in the *Methods* part more explicitly.

What is also ignored is that in any area of bleaching there can be a mixture of tau populations. Further down the text, 'droplet-like' is replaced by 'droplets' without any proof that droplets actually form in vivo. It can also not be claimed that the droplets in the cell are 'in a viscoelastic hydrogel state'. This can be speculated but should go into the discussion.
Authors: Thank you for pointing out this imprecise description of our data. We did not intend to claim droplets *in vivo* at this point without having the undoubtful proof. We changed the wording as suggested by the reviewer and removed the speculation of an viscoelastic state of intraneuronal tau droplets.

7. Page 12, Figure 4: Fig 4A: The phosphorylation pattern is mostly assumed but not shown.
Authors: As described in more detailed in our answer to Major Concern #1, we tested the existence of the phosphorylation sites that we reported previously for p-tau441 using tau specific Mass Spectrometry here now by extensive Western Blot analysis and present the data in Supplmenetal Figure S3C.
This Analysis showed that almost all (two exceptions) phsophorylation sites tested were present in the current p-tau441 preparation sa well. deP-tau441, the Alkaline Phosphatase treated p-tau441, showed a clear reduction in Molecular Weight down to the size of non-phosphorylated E. coli tau441, indicating the efficient removal of most phosphorylation; this also becomes clear in the massive reduction of Western Blot signal using the antibodies PHF1 (pS396/S404) as well as 12e8 (pS262/S356) in Figure 4B.
The phsophorylation of tau by MARK2 *in vitro* has been analyzed in great detail previously in our lab (Schwalbe *et al*, 2013; Timm *et al*, 2003). And although the in vitro phosphorylation efficiency may vary from batch to batch, Western Blot analysis of the two main tau target sites of MARK (pS262 and pS356, antibody 12e8) shows excellent phosphorylation efficiency in our current preparation.
We believe that with the new Western Blot analysis of the tested phsopho-tau versions, the data became more convincing and clear. Our previous studies on these tau phosphorylations are explicitly cited in the text as well, in order to ensure more transparency and easier context access for the reader.

Fig 4B: There is a huge variability in total tau (between blots and between transfectants) indicating massive differences in tau levels so the observed effects could be because of phosphorylation and levels.

Authors: We apologize for the previously poor quality of the Western Blots and repeated the blots using protein from the same batch used for the LLPS experiments and loading the same amount of protein (protein concentration was deteremined by BCA). The new data are now included in the Figure and show the differences in phosphorylation at equal total tau protein amounts.

Minor comments:

1. M. Materials and methods are presented after the figure legends and before the figures. There is some inconsistency in the referencing style.

2. M. Bottom of page 4: Low complexity domain (LCD): The authors make the point that tau is a protein with low amino acid variance, intrinsic disorder and inhomogenous charge or polarity distribution. This led them to postulate that tau could undergo phase separation. But then they argue that tau has no defined LCD domain. This discrepancy needs to clarified.

3. M. Page 7, Fig 1E: Is this in the presence of Ficoll? If so, I assume tau does not form such a pattern without Ficoll.

4. M. Figure 2C: It is unclear to me what is shown in the top and bottom row of the figure.

5. M. Page 9: 'in contrast to most other LLPS proteins' - please provide info which ones do and which ones don't.

6. M. Page 14. Some statements should be toned down to 'this SUGGESTED that the exchange ... ; this MAY ALSO EXPLAIN... Figure 5A,B: There seems to be a mix-up as the actual droplets look the same and the bleached area whereas in A the entire field (i.e. the entire drop) is bleached. Fig. 5C: This is impressive. Is this because tau forms precipitates on the surface of the droplets?

7. M. page 16: TauRD construct needs to be explained: page 17: anti-aggregation mutation PP needs to be explained.

8. M. Page 19: This text needs to go into the discussion.

9. M. Page 37: Labelling of panel Figure 6: C) used twice.

Authors: We corrected and changed all mentioned issues in the manuscript in order to address all minor comments of the reviewer. We hope, that the revised version is now suitable for publication in EMBO Journal.

2nd Editorial Decision 4th December 2017

Thank you for submitting your revised manuscript to The EMBO Journal. Your manuscript has now been re-reviewed by the two referees and their comments are provided below.

Both referees appreciate the introduced changes and find that the analysis has been strengthened. Referee #1 has some remaining concerns with the technical aspects that I would like to ask you to take into consideration in a final revision. Some of the issues should be straightforward to address. Some of the issues (concentration of the droplets) are more difficult to sort out. The referee also suggests that as this aspect is not key to the overall message that this part could also be removed from the manuscript. Either option is fine with me - happy to discuss further if helpful.

REFEREE REPORTS

Referee #1:

Wegmann and coworkers have thoroughly updated their manuscript and have answered the great majority of the critical questions brought up by reviewers. Their new manuscript focuses on dynamic tau self assembly in cells and possibly even in tissues and then evaluates the role of phosphorylation on tau self assembly in detail in vitro. Particularly helpful are the new experiments with fluorescent crowding agents which show exclusion and the experiments demonstrating phase separation in the absence of crowders. Also helpful is the detail provided regarding the biophysical experiments which facilitates interpretation. A number of issues are now clarified and hence questions are outstanding that need to be addressed to support the claims as written.

Comments

MAJOR
In their experiments in cells, the authors should comment on the possibility of GFP autorecovery from photobleaching due to noncovalent darkening of fluorophore GFP - the authors should do control FRAP on GFP fusions that form aggregates (so they should not recover FRAP) with same imaging and FRAP parameters to show that the recovery observed is much smaller than with the GFP-tau441. As these effects are extremely dependent on imaging parameters and can be up to majority of the recovery observed in some cases, it is important to show in parallel in same setup. See this report:
doi: 10.1016/j.bpj.2012.02.029
The authors should also include images from the FRAP timecourse in Fig 1D.

The authors suggest that electrostatic interactions are important for phase separation but then show some data for LLPS vs salt concentration that they say suggests salt concentration does not affect LLPS. First, these need to be reconciled. Second, the experiments showing salt concentration effects in Figures 2G and EV3C are not convincing, as there are droplets at all conditions. The authors should present a two dimensional "phase diagram" (like in Figure 2G, left) or at least perform the experiments at lower tau concentration - either way, so that they pass through a transition to no droplets like in EV4F.

CFP appears to show punctate distribution alone. Is this an artifact of aggregation? I suggest high centrifuge spinning (14000rpm in microcentrifuge tube) of the CFP stock before use as that can clear aggregates. It is important to show this negative control has no assemblies if it is to be shown at all.

Droplet volumes are presented but no information is presented about how they are calculated - the droplets imaged on the glass slide surface are not spherical and no z-stack quantification is described or presented, hence it is difficult to understand. Also, is this the maximum volume observed as there are different sizes of droplets? What is the error bar derived from? Similarly, the volume fraction measurement is not described. The volume fraction on a glass slide will be dependent on the droplets that have fallen on to the surface - how much height is being integrated? It is not clear to me what this is measuring or if it will be helpful. Perhaps % area of slide covered is more straightforward and more precise - and will make clear that the property measured is a function of the experimental setup (extensive) in a way that "volume fraction" (an intensive property) does not imply. It is possible I have misunderstood these measurements and missed the explanation in the methods, but then I suggest additional explanation in the methods and also in the results.

Concentration in the droplet. The authors have now explained their measurements. However, this causes concern for at least three reasons and additional caution is still warranted in my opinion. The authors nicely show a calibration curve with ptau441a568 showing linearity and low uncertainty. Presumably this was conducted with the confocal volume/image placed far away from the coverslip, so the entire confocal volume is filled with solution and not partially in the air or in the glass. But is the confocal volume in the droplet containing pixels filled with droplet or does it extend to the glass or tau-depleted phase above the droplets? 1) for the droplets imaged on the coverslip, it is not clear if the focus is adjusted too close to the glass - in other words is the confocal volume going beneath the surface - a z-stack showing a clear decrease going down into the glass and then above it a

uniform fluorescence intensity with increasing z is required to demonstrate that the focus is not too low. 2) it is not clear if the confocal volume extends above the droplet into the tau depleted solution above the droplet. In my understanding, the confocal resolution is poor in z and the volume can in fact extend approximately 2 microns or more in z. The "gelled" droplets observed by AFM presented here are not bigger than this height. What evidence do the authors show that the confocal volume is filled? The droplet cross sections highlight this significant concern - the fluorescence vs x/y (at a constant height z) appear rounded (indeed the highest one is a near perfect semi-circle profile - Figure 2C) directly suggesting that the confocal volume is not filled (at least it cannot be filled at any point except the max height- and still point 1 above would have to be shown not to be too low). If the volume is not filled and extends above the droplet, then the authors are averaging in the tau-depleted phase above, which decreases the concentration estimate. The authors should look for the highest, biggest droplet forming conditions and show a z-stack with a clear z slice that is demonstrated not at the bottom but has a sharp, flat profile in an x vs z plot, not the rounded profile shown in this version.
3) Additionally, to rule out dye-dye "self quenching" interactions (https://www.nature.com/articles/srep20237), the authors should show linearity with decreasing fraction of fluo-labeled peptide. Are these experiments conducted with samples made of 100% tau labeled with fluorophore and 0% unlabeled tau? I remain cautious that fluorophore-fluorophore proximity in the droplet may alter photophysical properties and strongly suggest qualifying any concentration determined in this way unless the linearity in total fluorescence (e.g. no effect of fluorescence label concentration) as a function of fluolabeled concentration is demonstrated. This would also control for dye effects on phase separation. 4) However, even fluorophore dilution does not control for fluorophore quenching by being brought into greater proximity with amino acids (aromatic, histidine, methionine) as is well known though poorly understood (http://pubs.acs.org/doi/abs/10.1021/ja100500k) and for this I can see no easy control experiment - if the droplets are as concentrated as reported for Ddx4 (100-350mg/ml of protein) the fluorophore is effectively in a >100mM concentration of quenching amino acids. In other words, the inside of the droplet is effectively a different solvent condition that could (dramatically) change fluorescence intensity.
The authors would also have to rationalize their other observations -
A) if the droplets are approximately 20uM in concentration, how could 20uM tau in the absence of crowders remain completely 1 low-tau phase - indeed LLPS at 20uM (Fig 2G) requires the same 5% PEG to see phase separation at 2uM and 20uM, suggesting they are both far from the saturation concentration. Indeed, no tau phase separation is seen at much higher concentration (50-100uM) except at the evaporating edge of a solution drop where protein concentration (and buffer concentration?) are again much higher than 50uM. In other words, if a droplet inside has only 22uM, how can the solution support 50uM ptau441? No crowding agent should be needed to push the tau together since it is already above the droplet concentration and hence the system should actually be crossed all the way into a single tau-rich phase regime (all tau rich "droplet") with no droplets.
B) Furthermore, if the 5uM solution demixes into 3uM in tau depleted phase and 22uM in tau concentrated phase, this is a concentration factor of only 11x (2uM of the protein goes into assemblies that are 22uM dense). Therefore, the phase separated material should make up about 9% of the total volume. That means that for a water drop on a cover slip that is approximately 1 millimeter in height, there should be a uniform layer 90 microns tall of tau dense "droplet" protein that falls to the bottom. It does not appear to have this much volume of phase separated material formed based on the image in 2C, indeed the height is much less - about 2 micron height for 5 micron width droplet 4F.

In my reading, the concentration is not a critical finding and I would suggest further investigation of these aspects as described above and a second method to confirm the concentration of the droplet material is required if they wish to keep this claim, or much more easily, removing the claim and the related part in the discussion.

To summarize the major concerns listed - I think they are major issues in the manuscript as written but can be easily addressed by simple experiments or removal of claims (concentration)

MINOR
Introduction - FUS, TDP-43, hnRNPA1 are not best described as "LLPS proteins" - they are prion-like domain containing proteins (King et al Brain research - "tip of the iceberg") and they are a subset of proteins shown to be directed to cellular RNP granules and phase separate in vitro.

please correct spelling of hnRNPA1 throughout - (not "hnRNP1", "hRNP-A1", or "hRNP1" as it is abbreviated differently in nonstandard fashion in three places). Or clarify if they mean a different protein by spelling out the name before using the abbreviation.

substitute "β-sheet" for "beta-pleated sheet" - though the authors may be more precise in saying amyloid fibril cross-β structure as these dyes are not thought to be sensitive to globular β-sheet proteins, rather they detect amyloid fibrils.

I again believe that the authors should change the statement "whereas the C-terminal MT binding domain can stabilized tau droplets through β-sheet interactions" to "whereas the C-terminal MT binding domain can stabilized tau droplets through hydrophobic interactions, possibly made up of β-sheet structures". The authors do not conclusively show β-sheet assembly so I think it would be best to temper the conclusion, though I think it is an exciting possibility and hence it should remain in the discussion and in qualified statements as suggested above.

Results:
Rephrase the sentence at the top of pg 6 starting with "In LLPS proteins,". Perhaps just say "Several proteins including Ddx4 are known to phase separate via electrostatic interactions between clusters of positive and negative residues distributed in a low complexity sequence domain". Cite Nott et al Mol Cell 2015 for Ddx4

"anterogate" - do the authors mean "anterograde"?

GFP-tau256 - define

Dendra2-tau441 - define

Page 7 - "au LLPS" - remove or replace "au" (tau?)

Page 8 - 2 kDa is listed here but 3kDa in the figure - which is it?

EV2D - it is difficult to see if ThioflavineS partitions into the droplet. First, in the previous version, I questioned if ThioS signal is simply from slower motions. As ThT is known to change fluorescence with viscosity, the increase in fluorescence may just be from slowed motion. Secondly, the assemblies are very difficult to see when the figure is printed.
EV2F - are the round features droplets or artifacts of imaging?

Page 8 - reference to figure 1E - is that supposed to be 2E?

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Change "The aggregation of tau in the droplets appears to release enough free energy to enable symmetry breaking of the droplets, which can be detected as droplet deformation and the growth of non-spherical solid aggregates." to be more simple and precise as no thermodynamic measurements are performed: "The aggregation of tau can be detected as droplet deformation and the growth of non-spherical solid aggregates."

What happened to heparin or RNA added p-tau441 droplets over time - did aggregates emerge?

In the description of cellular assemblies on pg 16, the authors should call them "rounded" or "spherical" instead of "droplet-shaped" to be more precise.

Pg 20 - change extend to extent

Referee #2:

Susanne Wegmann, Brad Hyman and colleagues have satisfactorily addressed my concerns, in particular with regards to statements for the in vivo situation. This is a really nice study.

The authors may wish to check that all figure panels are properly references, as my specific comment 1 was addressed by the authors in Figure EV1C, not S3C. My specific comment 4 was addressed in Figure EV3, not S5. My specific comment 5 was addressed in E4H, not S6H.

I have one final (minor) comment regarding point 5:

"5. Page 10, Figure S3I: Please add non-reduced, unfractionated brain lysate such as to provide an estimate of the relative abundance of the N-terminal fragments in human brain. Authors: A Wetsern Blot of non-reduced whole brain lysates from the same AD and Control brains has been added to the Figure S6H"

Thank you for adding these blots but still, because separate fractions have been analysed (SEC fraction 3 for HMW species showing only full-length tau, and SEC fraction 14 for LMW, showing only N-terminal fragments) the relative abundance of the N-terminal fragments in human brain cannot be determined.

I found one typo in the Fig 5Dpanel: Alzheimer's disease tau FROMS droplets

2nd Revision - authors' response 20th December 2017

We are glad that we could eliminate most of the reviewer's concerns in our manuscript in the first round of revisions, and we appreciate that our work got another round of critical reviewing to give us the opportunity of submitting another revised version. In the current version (EMBOJ-2017-98049RR), we have addressed all the concerns raised by the reviewers and made all the minor changes that were listed. The changes made are highlighted in the manuscript.doc. We hope that the manuscript is now in an acceptable shape and ready for acceptance.

In the following, please find the point-by-point answers to the reviewer's concerns.

Referee #1:

Wegmann and coworkers have thoroughly updated their manuscript and have answered the great majority of the critical questions brought up by reviewers. Their new manuscript focuses on dynamic tau self-assembly in cells and possibly even in tissues and then evaluates the role of phosphorylation on tau self-assembly in detail in vitro. Particularly helpful are the new experiments with fluorescent crowding agents which show exclusion and the experiments demonstrating phase separation in the absence of crowders. Also helpful is the detail provided regarding the biophysical experiments which facilitates interpretation. A number of issues are now clarified and hence questions are outstanding that need to be addressed to support the claims as written.

Comments

MAJOR
1) In their experiments in cells, the authors should comment on the possibility of GFP autorecovery from photobleaching due to noncovalent darkening of fluorophore GFP - the authors should do control FRAP on GFP fusions that form aggregates (so they should not recover FRAP) with same imaging and FRAP parameters to show that the recovery observed is much smaller than with the GFP-tau441. As these effects are extremely dependent on imaging parameters and can be up to majority of the recovery observed in some cases, it is important to show in parallel in same setup. See this report:
doi: 10.1016/j.bpj.2012.02.029
The authors should also include images from the FRAP time course in Fig 1D.

Authors: Thank you for this critical comment regarding our FRAP experiments. We agree that the mentioned controls of mature aggregates are necessary to show the difference between tau droplets versus mature aggregates. Unfortunately, GFP-tau does not form aggregates in primary neurons even after extended time of expression or treatment with pre-aggregated "seeding-competent" tau. To overcome this problem, we 1) expressed aggregate forming GFP-polyQ(79) in primary neurons and FRAP-ed the emerging polyQ aggregates, and 2) FRAP-ed TauRD aggregates in HEK TauRDP301S-CFP/YFP cells. Both controls did not show any recovery. We also performed more FRAP experiments on GFP-tau441 expressing neurons and show the very fast recovery of cytosolic soluble tau and a little slower recovery of microtubule bound tau. All this data is now added to Extended View Figure 1 (Figure EV1B-C).
We also added time course images of a GFP-tau441 droplet FRAP experiments (Figure EV1A). The additional data is now also mentioned in the text.

2) The authors suggest that electrostatic interactions are important for phase separation but then show some data for LLPS vs salt concentration that they say suggests salt concentration does not affect LLPS of p-tauCt. First, these need to be reconciled. Second, the experiments showing salt concentration effects in Figures 2G and EV3C are not convincing, as there are droplets at all conditions. The authors should present a two dimensional "phase diagram" (like in Figure 2G, left) or at least perform the experiments at lower tau concentration - either way, so that they pass through a transition to no droplets like in EV4F.

Authors: Indeed, the salt insensitivity of p-tau441 LLPS was also to us quite puzzling, especially because it seems very unusual for proteins that undergo LLPS and because it has, accordingly, not been reported previously for other LLPS proteins. In order to shed more light on this unusual behavior of p-tau, we extended the range of NaCl in the buffer up to 3M and tested extreme pH values of the buffer (pH 3.0 and 9.5). We also tested high concentrations of KCl and MgCl2; all these conditions still allowed for p-tau LLPS. We then tested the sensitivity of p-tau LLPS to salts with different "salting-in/out" characteristics according to the Hofmeister series (Hofmeister, 1888; Sawyer & Puckridge, 1973), *and found remarkable differences between the chaotropic salts guanidinium hydrochloric and sodium thiocyanate, which inhibited p-tau LLPS, compared to the cosmotropic salt ammonium sulfate, which seemed to enhance p-tau LLPS. We also show that p-tau441 LLPS is sensitive for the presence of urea at 1-3M, conditions that unfold secondary structures in proteins without major impact on electrostatic interactions. Our new data somewhat supports the idea that hydrophobic interactions, likely due to secondary structure in p-tau, but not electrostatic interactions are needed to facilitate p- tau441 LLPS.*
The new data has been added to the main Figure 2 (panel G and H) and is presented in Extended View Figure EV3).

3) CFP appears to show punctate distribution alone. Is this an artifact of aggregation? I suggest high centrifuge spinning (14000rpm in microcentrifuge tube) of the CFP stock before use as that can clear aggregates. It is important to show this negative control has no assemblies if it is to be shown at all.

Authors: We repeated the control experiments with a fresh stock of GFP, which we sonicated and centrifuged at 21000x g before the experiment. We did not see any evidence of LLPS at 2 mM GFP in the presence of 10% PEG. The new data is included in Figure EV2A.

4) Droplet volumes are presented but no information is presented about how they are calculated - the droplets imaged on the glass slide surface are not spherical and no z-stack quantification is described or presented, hence it is difficult to understand. Also, is this the maximum volume observed as there are different sizes of droplets? What is the error bar derived from? Similarly, the volume fraction measurement is not described. The volume fraction on a glass slide will be dependent on the droplets that have fallen on to the surface - how much height is being integrated? It is not clear to me what this is measuring or if it will be helpful. Perhaps % area of slide covered is more straightforward and more precise - and will make clear that the property measured is a function of the experimental setup (extensive) in a way that "volume fraction" (an intensive property) does not imply. It is possible I have misunderstood these measurements and missed the explanation in the methods, but then I suggest additional explanation in the methods and also in the results.

Authors: We apologize for the lack of explanation of our methods applied for the droplet volume analysis. To clarify, the droplets imaged were not attached to the surface but free floating in the solution. We collected Z-stacks over larger volumes (z= ~20-40 mm) at a confocal plane thickness of 2 mm. In each plane of the z-stack, only droplets that were in focus in the focal plane were analyzed by fitting a circle on their phase boundary and calculating their volume (assuming spherical shape). The data represent mean droplet volume ±SD; this is mentioned in the figure legend. We removed the data of the droplet volume fraction since we agree that it does not add additional information.

5) Concentration in the droplet. The authors have now explained their measurements. However, this causes concern for at least three reasons and additional caution is still warranted in my opinion. The authors nicely show a calibration curve with ptau441a568 showing linearity and low uncertainty. Presumably this was conducted with the confocal volume/image placed far away from the coverslip, so the entire confocal volume is filled with solution and not partially in the air or in the glass. But is the confocal volume in the droplet containing pixels filled with droplet or does it extend to the glass or tau-depleted phase above the droplets?

1) for the droplets imaged on the coverslip, it is not clear if the focus is adjusted too close to the glass - in other words is the confocal volume going beneath the surface - a z-stack showing a clear decrease going down into the glass and then above it a uniform fluorescence intensity with increasing z is required to demonstrate that the focus is not too low.

2) it is not clear if the confocal volume extends above the droplet into the tau depleted solution above the droplet. In my understanding, the confocal resolution is poor in z and the volume can in fact extend approximately 2 microns or more in z. The "gelled" droplets observed by AFM presented here are not bigger than this height. What evidence do the authors show that the confocal volume is filled? The droplet cross sections highlight this significant concern - the fluorescence vs x/y (at a constant height z) appear rounded (indeed the highest one is a near perfect semi-circle profile - Figure 2C) directly suggesting that the confocal volume is not filled (at least it cannot be filled at any point except the max height- and still point 1 above would have to be shown not to be too low). If the volume is not filled and extends above the droplet, then the authors are averaging in the tau-depleted phase above, which decreases the concentration estimate. The authors should look for the highest, biggest droplet forming conditions and show a z-stack with a clear z slice that is demonstrated not at the bottom but has a sharp, flat profile in an x vs z plot, not the rounded profile shown in this version.
3) Additionally, to rule out dye-dye "self quenching" interactions (https://www.nature.com/articles/srep20237), the authors should show linearity with decreasing fraction of fluo-labeled peptide. Are these experiments conducted with samples made of 100% tau labeled with fluorophore and 0% unlabeled tau? I remain cautious that fluorophore-fluorophore proximity in the droplet may alter photophysical properties and strongly suggest qualifying any concentration determined in this way unless the linearity in total fluorescence (e.g. no effect of fluorescence label concentration) as a function of fluolabeled concentration is demonstrated. This would also control for dye effects on phase separation.
4) However, even fluorophore dilution does not control for fluorophore quenching by being brought into greater proximity with amino acids (aromatic, histidine, methionine) as is well known though poorly understood (http://pubs.acs.org/doi/abs/10.1021/ja100500k) and for this I can see no easy control experiment - if the droplets are as concentrated as reported for Ddx4 (100-350mg/ml of protein) the fluorophore is effectively in a >100mM concentration of quenching amino acids. In other words, the inside of the droplet is effectively a different solvent condition that could (dramatically) change fluorescence intensity.
The authors would also have to rationalize their other observations -
A) if the droplets are approximately 20uM in concentration, how could 20uM tau in the absence of crowders remain completely 1 low-tau phase - indeed LLPS at 20uM (Fig 2G) requires the same 5% PEG to see phase separation at 2uM and 20uM, suggesting they are both far from the saturation concentration. Indeed, no tau phase separation is seen at much higher concentration (50-100uM) except at the evaporating edge of a solution drop where protein concentration (and buffer concentration?) are again much higher than 50uM. In other words, if a droplet inside has only 22uM, how can the solution support 50uM ptau441? No crowding agent should be needed to push the tau together since it is already above the droplet concentration and hence the system should actually be crossed all the way into a single tau-rich phase regime (all tau rich "droplet") with no droplets.

B) Furthermore, if the 5uM solution demixes into 3uM in tau depleted phase and 22uM in tau concentrated phase, this is a concentration factor of only 11x (2uM of the protein goes into assemblies that are 22uM dense). Therefore, the phase separated material should make up about 9% of the total volume. That means that for a water drop on a cover slip that is approximately 1 millimeter in height, there should be a uniform layer 90 microns tall of tau dense "droplet" protein that falls to the bottom. It does not appear to have this much volume of phase separated material formed based on the image in 2C, indeed the height is much less - about 2 micron height for 5 micron width droplet 4F.

In my reading, the concentration is not a critical finding and I would suggest further investigation of these aspects as described above and a second method to confirm the concentration of the droplet material is required if they wish to keep this claim, or much more easily, removing the claim and the related part in the discussion.

To summarize the major concerns listed - I think they are major issues in the manuscript as written but can be easily addressed by simple experiments or removal of claims (concentration).

Authors: Images for calibrating the fluorescence intensity of soluble p-tau441 were taken by confocal imaging (pin hole = 2 mm) in the center of the droplet, meaning far away from droplet surface or glass surface to avoid any surface effects on tau concentration and imaging artifacts. When imaging the sample with droplets, the imaging plane was of course chosen high enough above the glass surface to not penetrate into the glass. We agree that the confocal of 2 mm may be larger than the diameter of some of the droplets, which may cause an underestimation of the actual fluorescence intensity in these droplets because of the averaging across both the droplet phase and the soluble phase above the droplet. We now account for this artifact in our corrected measurements that analyze the maximum fluorescence intensity in each droplet and only considering large droplets with a plateau profile. Using these parameters, we now estimate a higher tau concentration in the droplets of 30.5 mM. Because these data are not essential for the manuscript but may be interesting for the reader, we removed the data from the main figure and now show it in the Supplemental Data Figure EV2B.
We also mentioned in the text that the estimated concentration of 34.3 mM in the droplets rather underestimated the actual concentration because of fluorophore:fluorophore quenching and other imaging artifacts due to the high condensation and viscosity in the droplets. We feel that the data, although presented self-critically and as an estimate, is informative enough to be reported.

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> We edited the sentence in the introduction and added the reference.

please correct spelling of hnRNPA1 throughout - (not "hnRNP1", "hRNP-A1", or "hRNP1" as it is abbreviated differently in nonstandard fashion in three places). Or clarify if they mean a different protein by spelling out the name before using the abbreviation.
> This has been corrected throughout the manuscript.

substitute "β-sheet" for "beta-pleated sheet" - though the authors may be more precise in saying amyloid fibril cross-β structure as these dyes are not thought to be sensitive to globular β-sheet proteins, rather they detect amyloid fibrils.
> We replaced β-sheet with "amyloid fibril cross-β structure" in the context of ThioS fluorescence.

I again believe that the authors should change the statement "whereas the C-terminal MT binding domain can stabilized tau droplets through β-sheet interactions" to "whereas the C-terminal MT binding domain can stabilized tau droplets through hydrophobic interactions, possibly made up of β-sheet structures". The authors do not conclusively show β-sheet assembly so I think it would be best to temper the conclusion, though I think it is an exciting possibility and hence it should remain in the discussion and in qualified statements as suggested above.
> We changed the sentence in the discussion accordingly.

Results:
Rephrase the sentence at the top of pg 6 starting with "In LLPS proteins,". Perhaps just say "Several proteins including Ddx4 are known to phase separate via electrostatic interactions between clusters of positive and negative residues distributed in a low complexity sequence domain". Cite Nott et al Mol Cell 2015 for Ddx4
> *We rephrased and added the reference.*

"anterogate" - do the authors mean "anterograde"?
> *This has been corrected.*

GFP-tau256 - define
> *We added a short definition.*

Dendra2-tau441 - define
> *We added a short definition.*

Page 7 - "au LLPS" - remove or replace "au" (tau?)
> *"au" has been replaced by "tau".*

Page 8 - 2 kDa is listed here but 3kDa in the figure - which is it?
> *The right molecular weight is 3 kDa. It has been corrected in the text.*

EV2D - it is difficult to see if ThioflavineS partitions into the droplet. First, in the previous version, I questioned if ThioS signal is simply from slower motions. As ThT is known to change fluorescence with viscosity, the increase in fluorescence may just be from slowed motion. Secondly, the assemblies are very difficult to see when the figure is printed.
> *We mentioned the viscosity dependency of ThioS fluorescence in the text and increased the contrast for ThioS droplets in Figure EV2D.*

EV2F - are the round features droplets or artifacts of imaging?
> *Yes they are lens artifacts. We mentioned this in the figure legend to avoid confusion.*

Page 8 - reference to figure 1E - is that supposed to be 2E?
>*The reference has been changed.*

Page 9 - Ab - Aβ?
> *Yes. we corrected to A-beta.*

Change "The aggregation of tau in the droplets appears to release enough free energy to enable symmetry breaking of the droplets, which can be detected as droplet deformation and the growth of non-spherical solid aggregates." to be more simple and precise as no thermodynamic measurements are performed: "The aggregation of tau can be detected as droplet deformation and the growth of non-spherical solid aggregates."
> *The sentence has been changed.*

What happened to heparin or RNA added p-tau441 droplets over time - did aggregates emerge?
> *We are currently investigating more in detail if heparin induced or mutation induced aggregation of tau follows tau LLPS and under which condition. These experiments are ongoing and too preliminary to be reported at the moment. However, extended incubation of p-tau441 with heparin leads to aggregation into fibrillary aggregates in vitro. We mentioned this also in the text and added a reference (Tepper et al, 2014).*

In the description of cellular assemblies on pg 16, the authors should call them "rounded" or "spherical" instead of "droplet-shaped" to be more precise.
> *We changed "droplet-shaped" into "spherical".*

Pg 20 - change extend to extent
> *This has been changed.*

Referee #2:

Susanne Wegmann, Brad Hyman and colleagues have satisfactorily addressed my concerns, in particular with regards to statements for the in vivo situation. This is a really nice study.

The authors may wish to check that all figure panels are properly references, as my specific comment 1 was addressed by the authors in Figure EV1C, not S3C. My specific comment 4 was addressed in Figure EV3, not S5. My specific comment 5 was addressed in E4H, not S6H.

Authors: We are glad that we could answer the reviewer's questions in a satisfying way. We double checked the Figure references in the newest version of the text again to make sure there are no errors.

I have one final (minor) comment regarding point 5:
"5. Page 10, Figure S3I: Please add non-reduced, unfractionated brain lysate such as to provide an estimate of the relative abundance of the N-terminal fragments in human brain. Authors: A Wetsern Blot of non-reduced whole brain lysates from the same AD and Control brains has been added to the Figure S6H"

Thank you for adding these blots but still, because separate fractions have been analyzed (SEC fraction 3 for HMW species showing only full-length tau, and SEC fraction 14 for LMW, showing only N-terminal fragments) the relative abundance of the N-terminal fragments in human brain cannot be determined.
> We agree with this comment and changed the word "abundance" to "presence" of N-terminal fragments in the figure legend of Figure EV5H. We also mention now that N-terminal fragments of tau appear in eth LMW but not in the HMW brain lysate SEC fractions.

I found one typo in the Fig 5Dpanel: Alzheimer's disease tau FROMS droplets
> This has been corrected.

3rd Editorial Decision 17th January 2018

Thanks for submitting your revised manuscript to The EMBO Journal. Your study has now been re-reviewed by referee #1 whose comments are provided below. The referee appreciates the introduced changes and has just a few minor comments left that can be resolved with appropriate text changes. I am therefore very happy to say that we are pleased to publish your study in The EMBO Journal.

REFEREE REPORTS

Referee #1:

Wegmann and coworkers have further improved the manuscript and answered each question, many of them with new experiments and or analysis. I appreciate the authors' careful addressing of the points brought up in the reviews.

Concentration in the droplet. The authors have addressed most of the points regarding the confocal microscopy and avoid others by their caution. However, they do not address my point "A" about the total concentration and point "B" about the total volume. Therefore, I believe the authors should instead of saying "estimate a concentration" they should change it to say they "measure an apparent concentration". And, in the results section paragraph ending that section where they describe having "50-100uM" tau in solution without crowding agents, they need to say that the droplet concentration should therefore be bigger than this concentration. The caution/qualifications they provide is useful. I still remain highly cautious about this apparent concentration.

Abstract last sentence should be more precise - I think inclusions made up of aggregated RNA binding proteins remains the hallmark of the majority of cases of ALS, not LLPS.

3rd Revision - authors' response 17th January 2018

We are very pleased to hear that we could eliminate your concerns with our manuscript and now made some last changes in order to remove the last minor issues mentioned by Reviewer #1, according to the suggestions (see below).

In detail, we (i) changed the wording from “we estimated the p-tau441 concentration in the droplets” to “we measured the apparent p-tau441 concentration in the droplets”, (ii) we added a sentence stating that the tau concentration in the spontaneously formed droplets at high tau concentrations has to be higher than the initial solution concentration and that we underestimate the concentration in the fluorescence measurements by ~30%, and (ii) we changed the last sentence of the abstract to make clear that FUS, TDP43 and hnRNP1 are aggregating proteins in ALS, that have also been shown to undergo LLPS.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Dr. Susanne Wegmann
Journal Submitted to: The Embo Journal
Manuscript Number: EMBOJ-2017-98049R2

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	In individual experiments (e.g. droplet counts, droplet FRAP, cell counts, fluorescence intensity measurements), sample sizes were chosen to enable statistical tests (minimum n=3), and large enough to gain staistical power and minimize natural variance. Starting point usually n=5. all tests use were very robust and needed no large n. Each experiment was repeated three times to give a sample size of n=3 or higher to be able to give statiscal values of mean and error.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Animal numbers (for cortical AAV injections and cortical primary neuron preparations) were kept at a minimum to repeatedly observe a phenomenon. We used n=3 mice for cortical injections and n=10 for primary neuron cultures
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	no data or animals were excluded
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	doesn't apply
For animal studies, include a statement about randomization even if no randomization was used.	sample sizes were chosen to enable statistical tests (minimum n=3), and large enough to gain staistical power and minimize natural variance. Starting point usually n=5. all tests use were very robust and needed no large n.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	the samples for phase diagrams of tau LLPS were investigated randomaized and in a blinded fashion
4.b. For animal studies, include a statement about blinding even if no blinding was done	doesn't apply
5. For every figure, are statistical tests justified as appropriate?	no comparison tests were perfomed
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	n.a.
Is there an estimate of variation within each group of data?	yes

USEFUL LINKS FOR COMPLETING THIS FORM

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http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

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http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

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http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

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http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

Is the variance similar between the groups that are being statistically compared?	doesn't apply

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	we provided a list with all antibody information in the Appendix in Table S4
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	HEK TauRDP301S-CFP/YFP cells were a gift from Marc Diamond's Lab; Neuro-2a cells (N2a) were derived from ATCC (Neuro-2a (ATCC® CCL-131™)); both cell lines were tested frequently for mycoplasma contamination

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	male C57 Black6 from Jackson Laboratories (C57BL/6J) were used for two-photon in vivo imaging; timed pregnant female CD1 mice from Charles River were used for primary neuron preparations
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All animal work was done in compliance with the MassGeneral Hospital, the NIH, and The Institutional Animal Care and Use Committee (IACUC) Wellfare act.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	n.a.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	n.a.
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	n.a.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	n.a.
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	n.a.
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	n.a.
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	n.a.
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	n.a.

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	n.a.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	n.a.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	n.a.
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	n.a.

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	n.a.