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Phosphorylation of the FUS low-complexity domain disrupts phase separation, aggregation, and toxicity

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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 26 January 2017

Thank you for submitting your manuscript to The EMBO Journal. Three referees have now seen your study and their comments are provided below.

As you can see from the comments there is an interest in the study, but significant revisions are needed. The referees raise good and constructive points. What is clear is that some of the key findings need to be extended to mammalian cells. However, we don't need analysis of the phosphorylation status of FUS in ALS/FTD patients (referee #1 and 3) this issue can be addressed in the discussion. If you have such data then please go ahead and include it as this would clearly strengthen the findings and some of the concerns raised by the referees. We also don't need data on potential phosphatases involved (referee #1). Regarding referee #3 point #2: if DNA-PK is acting as a chaperone and not a kinase. If this issue is straightforward to address then please resolve it (can you use a kinase dead DNA-PK mutant?), but otherwise OK to discuss this issue.

Should you be able to address the raised concerns keeping in mind the specific comments raised above then I would be happy to consider a revision. I should add that it is EMBO Journal policy to allow one single major round of revision only and it is therefore important to address the raised concerns at this stage.

Let me know if we need to discuss things further - happy to do so.

When preparing your letter of response to the referees' comments, please bear in mind that this will

form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://emboj.embopress.org/about#Transparent_Process.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE REPORTS

Referee #1:

Summary:
Monahan et al. report that phase separation and aggregation of FUS, a key RNA-binding protein linked to the neurodegenerative diseases ALS and FTD, are altered by serine phosphorylation in the protein's N-terminal low complexity domain. The so-called LC domain has previously been shown to drive phase separation of FUS into liquid "droplets" or solid "hydrogels" in vitro as well as FUS aggregation in vitro or model organisms, including yeast and C. elegans. It has been speculated before that post-translational modifications (PTMs), e.g. serine or tyrosine phosphorylation, may affect phase transition of FUS, but defined modification sites and their effect on phase transition were unknown so far.
Monahan et al. now show that the FUS LC domain can be phosphorylated in vitro by DNA-PK at 12 S/TQ sites and also identify a few of these phospho sites, as well as some other S/T phospho sites, on cellular FUS after treatment of HEK cells with DNA damage-inducing drugs (previously shown to cause FUS phosphorylation). When they mutate all 12 S/TQ sites to glutamine to mimic the negative charge introduced by phosphorylation (12E mutant), the FUS LC domain does no longer form liquid droplets or fibrous aggregates anymore, due to electrostatic repulsion. Full-length FUS 12E still phase separates, but not at high salt or upon addition of RNA, and FUS 12E droplets do not convert into fibrous aggregates over time, as do FUS-WT droplets. The authors then use NMR to dissect the molecular changes caused by the phosphomimetic 12E mutation and find that it decreases transient intra- and intermolecular LC domain interactions. Finally, they overexpress a series of S/T-to-E FUS variants in yeast (e.g. FUS 2E, 4E, 6E and 12E) to examine FUS aggregation and toxicity in a simple in vivo model, as ectopically expressed human FUS-WT forms aggregates and is toxic in yeast. They found that both 6E and 12E FUS form fewer aggregates and reduce toxicity compared to FUS-WT or variants with only 2 or 3 E substitutions. They also report reduced insolubility of FUS in HEK cells treated with DNA damage-inducing drugs, suggesting that phosphorylated FUS is also less aggregation-prone in mammalian cells.

The finding that a PTM (S/T phosphorylation) in the LC domain reduces phase separation and aggregation of FUS is novel and significant, as it reveals important mechanistic insights into the molecular interactions that drive phase separation/aggregation of FUS, and even suggests that modulation of FUS phosphorylation could be further explored as a novel therapeutic strategy to prevent FUS aggregation. The claims are convincing and well-supported by the in vitro experiments with purified FUS LC and full-length FUS and the yeast model. The findings should be of interest to a wider audience, e.g. researchers in the field of neurodegeneration and those interested in phase transition, an exciting new topic in cell biology that has received a lot of attention lately.
The major shortcoming of the study, however, is that it does not go beyond these simple model systems (e.g. into mammalian cells) and does not offer any insights as to when and how phosphorylation or dephosphorylation of FUS occurs under physiological or pathophysiological conditions. If the authors could provide further insights into these interesting questions, the paper would be a significantly stronger candidate for publication in EMBO Journal.

Major points that would have to be addressed:

(1) It would be interesting to examine a few "partial" phosphomimetic mutants, e.g. 6E, as they do in the yeast model, for their ability to suppress phase separation in vitro. It does not seem very likely that all 12 S/TQ sites are fully phosphorylated, e.g. upon DNA damage, in vivo, one rather expects a partial phosphorylation of only a few residues (as supported by their mass spec analysis of cellular FUS). Therefore, it would be important to check if also such partial phosphomimetic mutants (e.g.

those that still show an aggregation-suppressing effect in yeast or those affecting the phosphorylated S/T residues identified by MS), indeed have a reduced tendency to phase separate in vitro.

(2) The figures and text describing the NMR experiments (Fig. 3) and molecular simulations (Fig. 2B) are not understandable to cell/molecular biologists, which make up a large part of the EMBO J readership. The authors should make an effort to significantly rephrase / simplify and shorten the text and explain the figures better (e.g. what are the colorful stretches in Fig. 2B?) to make this part of the paper accessible to the general readership of EMBO J as well as ALS/FTD researchers.

(3) As mentioned above, it remains unclear under which conditions FUS phosphorylation (and potentially dephosphorylation) occurs in vivo, except for the few S/T residues that they identified after treatment of HEK cells with DNA damage inducing drugs. Some key point to address (or at least to discuss) would be: What is the phosphorylation status of FUS in the cells, both under physiological conditions and in ALS/FTD patients (altered?). (b) Are there phosphatases that are able to dephosphorylate FUS at the examined S/TQ sites and thus directly promote FUS phase transition/aggregation? (c) The LC domain also contains numerous Y residues shown to be important for hydrogel formation and stress granule localization of FUS (Kato et al., 2012) - are they also phosphorylated upon DNA damage (or other conditions in vivo) and does Y-phosphorylation affect phase transition of FUS in a similar way as does S/T phosphorylation? Y mutations in ALS patients?

(4) Further work (beyond the in vitro work and yeast model) in mammalian cells would significantly strengthen the manuscript, e.g. do the FUS-6E/12E mutants (or cytosolic dNLS versions thereof) also alter FUS solubility (S/P partitioning, as examined in Fig. 4E) and how do these mutations affect RNP granule (e.g. stress granule) recruitment or dynamics?

(5) The model in Fig. 6 does not exactly represent the described findings: As phosphorylated full-length FUS still forms liquid droplets (Fig. 2), it would be more correct to show in the model figure that P-FUS still undergoes phase transition / droplet formation, although to a lesser degree, but then does not further convert into solid aggregates (as does FUS-WT).

Minor points:
- Fig. 1 C/D: Legends are swapped
- Fig. 2A: Under which conditions do the LC droplets convert to "fibrous aggregates" (over time? Which time scale? How frequent?) This is not mentioned in the text or figure legend.
- Text to Fig. 2E: It is mentioned that the 2-day old 12E droplets appear more static, as judged by lack of droplet fusion and FRAP. Is this data not shown? It would be nice to show this data in the supplement or at least say "data not shown".
- Some terms are used a bit sloppily, e.g. RNP is the abbreviation for "ribonucleoprotein", not "ribonuclear particles" (p.1 of introduction), so it would be better to say "cytoplasmic ribonucleoprotein (RNP) granules, including stress granules, P-bodies or transport granules". Instead of using "ribonuclear protein" it would be better to just say "RNA-binding protein" or RBP.
- Results: First paragraph: Fig. 1A shows gray lines, not grey bars.
- Results: bottom of first page: provide a reference for the fact that DNA-PK autophosphorylates and thus deactivates itself
- Fig. 4C: would be better to remove "low" and "high" from the x-axis and define what is meant by the white/grey bar in the legend.

Referee #2:

The manuscript by Monahan et al. focuses on the question how the self-association of the protein FUS is regulated by multi-site phosphorylation. The authors map phosphorylation sites in the Low-complexity (LC) domain of FUS generated by DNA-dependent protein kinase phosphorylation in vitro. While these sites fully fit the consensus sites expected to be phosphorylated by DNA-PK, they were not (and had not been) identifiable by MS. The authors instead used NMR spectroscopy to identify these sites. They go on to identify in cell phosphorylation sites generated by DNA damage. The authors then characterize the effect of phosphorylation on these 12 sites (or of phosphomimetic mutations in the same sites) on liquid-liquid phase separation and aggregation of FUS. WT FUS LC undergoes LLPS and aggregates over time. Multi-site phosphorylation and phosphomimics both

prevent this transition. However, full-length FUS, when released from an MBP-FUS construct through TEV cleavage, can still undergo LLPS even in the phosphomic state. LLPS of this phosphomimic full-length construct is, however, reduced at high salt or high RNA concentrations. This suggests that the interactions underlying LLPS in the full-length phosphomimic are different than in the full-length WT protein, likely based on electrostatic interactions between the RRMs and the now negatively charged LC domain. This may allow for some interesting, context-dependent regulation of LLPS, which the authors could discuss in more detail.
The authors then use NMR spectroscopy to uncover the mechanism of reduced assembly of the phosphomimic. They observe reduced intra- and intermolecular PREs, indicating reduced transient collapse (in agreement with their MD simulations) and reduced intermolecular interactions. These mechanistic observations nicely tie into the observed morphological observations.
In yeast cells, the phosphomimic mutant, and a mutant with 6 phosphomimetic sites, show reduced ability to form punctate structure in the nucleus. In mammalian cells, the phosphomimic mutant looses the ability to segregate into the insoluble fraction after DNA damage stress. The toxicity in yeast cells is also reduced for the phosphomimic mutant.
The experiments are very well designed, the data is beautiful, the question asked timely, and the conclusions well supported by the data. The manuscript should be published, in my opinion, after one major and several minor points are addressed.

Major point:
The authors use turbidity measurements as a measure of the propensity to undergo LLPS. E.g. the addition of increasing concentrations of RNA to the full length FUS 12E mutant reduces turbidity. While the observed trend seems reasonable, I am not sure one can indeed conclude that the phase separation propensity is lower in case of a lower turbidity. The turbidity is a function of the size and number of droplets in the sample, and the two parameters are non-additive. I foresee the possibility that same volume fraction of dense phase could appear as very many tiny droplets that hardly scatter the light, a medium number of droplets that are large enough to considerably scatter the light, or a small number of very large droplets, which again would not scatter the light strongly. The authors should actually quantify the propensity of their protein constructs to undergo LLPS under different conditions.

Minor points:

1. "Though FUS is primarily localized in the nucleus, familial ALS mutations in FUS most often disrupt the nuclear localization signal, resulting in FUS cytoplasmic accumulation and apparent gain-of-function toxicity." I believe that the authors may not want to use the word "though".

2. "Interestingly, in vitro phosphorylation of recombinant, isolated FUS LC reduces its ability to bind to hydrogels formed from amyloid-like fibrils of recombinant, isolated FUS LC (Han et al., 2012), though hydrogels are more static than LLPS granules." Again, the word "though" seems misplaced.

3. p6: "In total, by mass spectrometry and NMR we have putatively identified 17 phosphosites in FUS LC, suggesting phosphorylation may be critical to FUS LC function." The identification of the phosphorylation sites does not allow the conclusion that they are critical for function. The authors show functional data later, and should reserve such a statement for after these data are presented.

4. "We performed differential interference contrast microscopy under conditions where WT FUS LC undergoes liquid-liquid phase separation (LLPS) to form round micron-sized phases..." I think it would be more appropriate to call the round structures droplets, rather than phases.

5. "These 2-day old FUS 12E structures appear more static than when LLPS is initiated, as judged by lack of droplet fusion and fluorescence recovery after photobleaching, similar to previous observations for FUS and FUS LC at other conditions (Lin et al., 2015, Patel et al., 2015)." The authors should show the FRAP data they are referring to.

6. "Taken together, these data on isolated FUS LC and full-length FUS demonstrate that the addition of negatively charged phosphate groups or phosphomimetic substitutions impedes FUS self-association and subsequent aggregation." It seems that this statement is not fully warranted; the conditions under which the specific constructs assemble change, and the assemblies do not promote

further aggregation.

7. "However, transverse relaxation rate constants, R2, for wild-type is slightly higher than for 12E at both 850 and 500 MHz 1H Larmor, " I believe the missing word is "frequency".

8. "Given that FUS LC is composed of 24 degenerate tyrosine repeats, these changes in intramolecular behavior provide insight into how phosphorylation suppresses both LLPS and aggregation." The authors do not mention anywhere else that the FUS LCD is composed of 24 degenerate tyrosine repeats. It is unclear, why they mention it here, and what it has to do with the suppression of LLPS by Ser/Thr phosphorylation.
9. What was the molar ratio of spin-labeled and unlabeled protein in the intermolecular PRE experiments?

I am not able to assess whether the coarse-grained simulations that show self-association of the WT LC, but not self-association of the phosphorylated form, are suitable to come to the presented conclusions. Please make sure that a reviewer with expertise in this area can comment on these data.

Referee #3:

In this study, Monahan et al. assess how phosphorylation of the low-complexity domain (LCD) of FUS might impact its phase transition behavior and aggregation propensity, which is of interest for several neurodegenerative disorders (e.g. ALS and FTD) where FUS aggregates accumulate in the cytoplasm of dying neurons. They identify several serines and threonines, including T7, T11, T19, S26, S42, S61, S84 in the FUS LCD that can be phosphorylated by DNA-PK in vitro. In HEK293T cells, upon DNA damage ~17 sites in the FUS LCD become phosphorylated. Pure protein experiments reveal that DNA-PK antagonizes FUS LCD phase transitions and aggregation, as does mutation of 12 putative DNA-PK phosphosites in the FUS LCD to glutamate (FUS12E). In the context of full-length protein, FUS12E undergoes liquid-liquid phase separation like FUS, but fails to fibrillize and displays different sensitivity to salt and RNA. In yeast, FUS12E is less aggregation prone and less toxic. Although several aspects of the study are interesting, I have a number of concerns that reduce my enthusiasm for this study:

1. The detailed opening to this paper tries to resolve which residues in the FUS LCD are actually phosphorylated by DNA-PK in vitro and in cells. However, a clear picture fails to emerge of exactly what phopsho-forms of FUS become populated, and it is not clear whether FUS ever bears multiple DNA-PK phosphorylated serines or threonines. Nonetheless, multiple phosphorylations are subsequently mimicked with the FUS12E variant in the rest of the paper. The lack of clarity here makes it difficult to assess the physiological relevance of the subsequent FUS12E studies. Moreover, what might happen in terms of FUS LCD phosphorylation in a relevant cell type (e.g. motor neurons) or in disease (e.g. ALS/FTD patient samples) is not clear and not addressed in the present study, which is a major weakness.

2. DNA-PK is shown to inhibit FUS LCD phase transition and aggregation (Fig. 2). However, it is not clear what concentration of DNA-PK was added. It is possible that DNA-PK is acting as a chaperone and not a kinase in this assay. A control is needed with a kinase-dead DNA-PK mutant or with a DNA-PK small-molecule inhibitor to demonstrate that the effect is due to kinase activity. Likewise, a FUS LCD that cannot be phosphorylated (e.g. S/T to A or even Q/N [to retain the uncharged polar character of the side chain) would be a valuable control. Finally, these DNA-PK experiments should be repeated with full-length FUS as it is not clear whether the full-length FUS would be similarly affected by DNA-PK activity.

3. There is a heavy reliance on the phosphomimetic FUS12E variant to ascribe effects of FUS phosphorylation. However, it is not clear whether the glutamate mutations truly mimic phosphoserine or phosphothreonine. Ideally, phosphorylated versions of FUS or FUS LCD would be purified and assessed in terms of phase transition and aggregation behavior. This could be achieved by further fractionation of FUS/FUS-LCD after DNA-PK phosphorylation in vitro, or via introduction of phosphoserine using technology developed by the Chin lab (Rogerson et al. Nat Chem. Biol. 2015. DOI: 10.1038/nchembio.1823), or via semisynthetic approaches. I raise this issue as phosphorylation mimics (S129E/D) do not reproduce the effect of phosphorylation on the

structural and aggregation properties of alpha-synuclein in vitro (Paleologou et al. JBC 2008. DOI: 10.1074/jbc.M800747200), and a similar concern applies to FUS. Indeed, phosphorylation extends the ensemble of conformations populated by α-synuclein close to its fully random coil-like dimensions, but this effect is not observed with the S129E/D substitutions. This issue could also apply to FUS, and experiments using phosphorylated FUS are needed to address this issue.

4. While the observations in yeast (Fig. 4 & 5) are intriguing they need to be validated in more complex model systems, e.g. in primary neurons or in an animal model (e.g. fly), to confirm their relevance to ALS or FTD.

5. While phosphorylation of the FUS LCD by DNA-PK is proposed to inhibit aggregation and phase transitions it is not clear whether this phosphorylation can promote the reversal of these events. Experiments should be added to determine whether FUS LCD phosphorylation reverses aggregation or phase transitions, as these would provide further support for increasing FUS LCD phosphorylation as a potential therapeutic. For example, do FUS LCD droplets disperse if DNA-PK is added?

6. Finally, FUS that is phosphorylated by DNA-PK appears to accumulate in the cytoplasm (Deng et al. J. Neurosci. 2014. doi: 10.1523/JNEUROSCI.0172-14.2014). Thus, increasing FUS phosphorylation is unlikely to restore nuclear FUS in disease. This issue needs to be discussed.

1st Revision - authors' response 31 May 2017

Referee #1:

Summary:

Monahan et al. report that phase separation and aggregation of FUS, a key RNA-binding protein linked to the neurodegenerative diseases ALS and FTD, are altered by serine phosphorylation in the protein's N-terminal low complexity domain. The so-called LC domain has previously been shown to drive phase separation of FUS into liquid "droplets" or solid "hydrogels" in vitro as well as FUS aggregation in vitro or model organisms, including yeast and C. elegans. It has been speculated before that post-translational modifications (PTMs), e.g. serine or tyrosine phosphorylation, may affect phase transition of FUS, but defined modification sites and their effect on phase transition were unknown so far.

Monahan et al. now show that the FUS LC domain can be phosphorylated in vitro by DNA-PK at 12 S/TQ sites and also identify a few of these phospho sites, as well as some other S/T phospho sites, on cellular FUS after treatment of HEK cells with DNA damage-inducing drugs (previously shown to cause FUS phosphorylation). When they mutate all 12 S/TQ sites to glutamine to mimic the negative charge introduced by phosphorylation (12E mutant), the FUS LC domain does no longer form liquid droplets or fibrous aggregates anymore, due to electrostatic repulsion. Full-length FUS 12E still phase separates, but not at high salt or upon addition of RNA, and FUS 12E droplets do not convert into fibrous aggregates over time, as do FUS-WT droplets. The authors then use NMR to dissect the molecular changes caused by the phosphomimetic 12E mutation and find that it decreases transient intra- and intermolecular LC domain interactions. Finally, they overexpress a series of S/T-to-E FUS variants in yeast (e.g. FUS 2E, 4E, 6E and 12E) to examine FUS aggregation and toxicity in a simple in vivo model, as ectopically expressed human FUS-WT forms aggregates and is toxic in yeast. They found that both 6E and 12E FUS form fewer aggregates and reduce toxicity compared to FUS-WT or variants with only 2 or 3 E substitutions. They also report reduced insolubility of FUS in HEK cells treated with DNA damage-inducing drugs, suggesting that phosphorylated FUS is also less aggregation-prone in mammalian cells.

The finding that a PTM (S/T phosphorylation) in the LC domain reduces phase separation and aggregation of FUS is novel and significant, as it reveals important mechanistic insights into the molecular interactions that drive phase separation/aggregation of FUS, and even suggests that modulation of FUS phosphorylation could be further explored as a novel therapeutic strategy to prevent FUS aggregation. The claims are convincing and well-supported by the in vitro experiments

with purified FUS LC and full-length FUS and the yeast model. The findings should be of interest to a wider audience, e.g. researchers in the field of neurodegeneration and those interested in phase transition, an exciting new topic in cell biology that has received a lot of attention lately.

The major shortcoming of the study, however, is that it does not go beyond these simple model systems (e.g. into mammalian cells)

We thank the reviewer for the positive feedback, and for investing time to very thoroughly evaluate our manuscript. We agree using a mammalian experimental system is a reasonable extension of our discoveries. Based on this suggestion, we now present data on an ALS-causing mutation (R495X, which leads to cytoplasmic accumulation of FUS in diseased motor neurons). The cytoplasmic aggregation of FUS(R495X) expressed in human cells is dramatically decreased by phosphomimetic substitution (both 6E and 12E, see Figure 5). Importantly, this shows that phosphomimetic substitutions can alter the aggregation of an established pathological variant of FUS. These findings are consistent with the other experimental models (yeast, recombinant proteins in vitro, and endogenously phosphorylated FUS in mammalian cells). We're very excited that all our experimental models systems support the same theme: introduction of charge into FUS's prion-like LC domain disfavors phase separation and solid aggregate formation.

and does not offer any insights as to when and how phosphorylation or dephosphorylation of FUS occurs under physiological or pathophysiological conditions. If the authors could provide further insights into these interesting questions, the paper would be a significantly stronger candidate for publication in EMBO Journal.

We very strongly agree that determining the extent of when and how phosphorylation or dephosphorylation of FUS occurs is important and we hope to spend the next several years addressing these questions. As such, we hope the reviewer will agree that these questions are outside the scope of the current contribution. Here, we have confirmed that FUS is phosphorylated following DNA damage/stress, and we have provided the highest resolution characterization to date of post-translational modification of FUS's prion-like domain. Our identification of several non-DNA-PK/ATM phosphorylation sites also hints at the complexity of FUS phosphorylation/dephosphorylation. However, our contribution to the field is to demonstrate that post translational modification reduces phase separation and aggregation. This is significant because phase separation in dynamic assemblies is integral to many diverse cellular processes, but perhaps more importantly, FUS aggregation underlies untreatable disease. We hope our findings now justify further stand-alone efforts (by our group and others) to describe the detailed phosphorylation/dephosphorylation pathway(s) of FUS.

Major points that would have to be addressed:
(1) It would be interesting to examine a few "partial" phosphomimetic mutants, e.g. 6E, as they do in the yeast model, for their ability to suppress phase separation in vitro. It does not seem very likely that all 12 S/TQ sites are fully phosphorylated, e.g. upon DNA damage, in vivo, one rather expects a partial phosphorylation of only a few residues (as supported by their mass spec analysis of cellular FUS). Therefore, it would be important to check if also such partial phosphomimetic mutants (e.g. those that still show an aggregation-suppressing effect in yeast or those affecting the phosphorylated S/T residues identified by MS), indeed have a reduced tendency to phase separate in vitro.

We also think that intermediate and/or partial phosphorylation of FUS may occur in cells. Based on the reviewer's suggestion, we have now completed characterization of full length FUS bearing 6 phosphomimetic variants (6E) in vitro (See the new Figure 2). We've evaluated full-length FUS with 0, 6 and 12 phosphomimetic substitutions under several conditions in vitro, including varying RNA and salt levels. We see that 6 phosphomimetic substitutions in FUS's LC domain are sufficient to achieve many of the same effects observed with 12 substitutions. Likewise, as mentioned above, we've included characterization of 6E in mammalian cells, as well as wild-type and 12E FUS.

(2) The figures and text describing the NMR experiments (Fig. 3) and molecular simulations (Fig. 2B) are not understandable to cell/molecular biologists, which make up a large part of the EMBO J readership. The authors should make an effort to significantly rephrase / simplify and

shorten the text and explain the figures better (e.g. what are the colorful stretches in Fig. 2B?) to make this part of the paper accessible to the general readership of EMBO J as well as ALS/FTD researchers.

We thank the reviewer for pointing out this issue and we have simplified the section on molecular simulation and NMR experiments, and we have better explained the figures. We have also added language to simplify spectral density mapping to make it more accessible to a general audience. Regarding the colorful sketches in Fig. 2B, we've added the following text, "*Snapshots of simulated FUS LC polypeptides are shown within Figure 2B to illustrate the change from a single uniform phase to a phase separated condition; wild-type is phase separated at temperatures below the phase transition (upper left), while 12E remains dispersed, not phase separated (bottom).*"

(3) As mentioned above, it remains unclear under which conditions FUS phosphorylation (and potentially dephosphorylation) occurs in vivo, except for the few S/T residues that they identified after treatment of HEK cells with DNA damage inducing drugs. Some key point to address (or at least to discuss) would be: What is the phosphorylation status of FUS in the cells, both under physiological conditions and in ALS/FTD patients (altered?). (b) Are there phosphatases that are able to dephosphorylate FUS at the examined S/TQ sites and thus directly promote FUS phase transition/aggregation? (c) The LC domain also contains numerous Y residues shown to be important for hydrogel formation and stress granule localization of FUS (Kato et al., 2012) - are they also phosphorylated upon DNA damage (or other conditions in vivo) and does Y-phosphorylation affect phase transition of FUS in a similar way as does S/T phosphorylation? Y mutations in ALS patients?

We thank the reviewer for this suggestion. A) We have clarified that under normal unstressed conditions, FUS is not known to be phosphorylated in cell culture. We've added the following text, "No FUS LC phosphopeptides were identified in control treatments with DMSO, which is consistent with none being previously reported under un-stressed conditions (Deng et al., 2014, Gardiner et al., 2008)." Concerning phosphatases, cytoplasmic phospho-FUS is presumably dephosphorylated. Consistent with this, phosphomimetic FUS has retarded nuclear import, thus dephosphorylation by cytoplasmic phosphatases of normal phospho-FUS is probable (Deng, Kukar et al. 2014 and our unpublished data). As far as we know, the phosphorylation state of FUS in FUS-ALS/FTD patients is not known. We agree the phosphorylation status of FUS in diseased brains is a critically important question. We have added the following text in the discussion: "Therefore, it will be of importance to determine the detailed FUS phosphorylation state in ALS-FUS cell models and FUS-associated ALS patient tissue as well as healthy controls." Toward these efforts, we have initiated a collaboration with Dr. Dan Perl, the Director of the Center for Neuroscience and Regenerative Medicine Brain Tissue Repository in Bethesda, MD. In future work, we plan to probe FUS neuronal inclusions with phospho-specific antibodies we're developing.
B) The capacity of phosphatases to promote FUS phase transition seems plausible. Currently, FUS appears to be un-phosphorylated in its default form, so under this regime, phosphatases wouldn't necessarily be immediately necessary to initiate phase transition.
C) To our knowledge, the tyrosine residues of FUS have not been shown to be phosphorylated. Further, our experimental approach to identify phosphopeptides is designed to find phosphoY sites as well as pT and pS, but we find no pY sites. To our knowledge, no missesnse mutations altering Y have been reported. We do find it curious that FUS LC has 24 Y and 0 F residues; investigating this question might be an interesting direction to take future work.

(4) Further work (beyond the in vitro work and yeast model) in mammalian cells would significantly strengthen the manuscript, e.g. do the FUS-6E/12E mutants (or cytosolic dNLS versions thereof) also alter FUS solubility (S/P partitioning, as examined in Fig. 4E) and how do these mutations affect RNP granule (e.g. stress granule) recruitment or dynamics?

As described above, we agree with the Reviewer on the importance of extending our experiments to a mammalian model system. We expressed the cytosolic ALS-causing ΔNLS FUS variant (FUSR495X) in human cell culture and observed extensive cytoplasmic aggregation that is dramatically reduced by phosphomimetic substitutions (FUS-6E(R495X) and FUS-12E(R495X)). We are currently designing experiments to evaluate stress granule dynamics, but we expect these studies to take many months to complete since wild-type FUS does not readily form stress granules and phosphomimetic substitutions within FUS alter its normal localization. The ALS-causing

mutation we used (R495X) favors strong cytoplasmic aggregation. The most tractable stress granule assays exploit NLS mutations that have more subtle effects on FUS localization, so we are exploring such mutations.

(5) The model in Fig. 6 does not exactly represent the described findings: As phosphorylated full-length FUS still forms liquid droplets (Fig. 2), it would be more correct to show in the model figure that P-FUS still undergoes phase transition / droplet formation, although to a lesser degree, but then does not further convert into solid aggregates (as does FUS-WT).

We thank the reviewer for the helpful suggestion and have simplified and updated the figure (currently Figure 7 in the latest draft) to more accurately reflect the findings.

Minor points:
Fig. 1 C/D: Legends are swapped
We have corrected this error, and are grateful for the Reviewer's attention.

Fig. 2A: Under which conditions do the LC droplets convert to "fibrous aggregates" (over time? Which time scale? How frequent?) This is not mentioned in the text or figure legend.
We have clarified this in the Legend of Figure 2.

Text to Fig. 2E: It is mentioned that the 2-day old 12E droplets appear more static, as judged by lack of droplet fusion and FRAP. Is this data not shown? It would be nice to show this data in the supplement or at least say "data not shown".

We thank the reviewer for this suggestion. We have clarified this section and added FRAP data about wild-type FUS in the supplementary movie1.

Some terms are used a bit sloppily, e.g. RNP is the abbreviation for "ribonucleoprotein", not "ribonuclear particles" (p.1 of introduction), so it would be better to say "cytoplasmic ribonucleoprotein (RNP) granules, including stress granules, P-bodies or transport granules". Instead of using "ribonuclear protein" it would be better to just say "RNA-binding protein" or RBP.

We have amended the text in the introduction and throughout, as suggested.

Results: First paragraph: Fig. 1A shows gray lines, not grey bars.

We have changed the reference in the text and in the caption for Figure 1A to say gray "line".

Results: bottom of first page: provide a reference for the fact that DNA-PK autophosphorylates and thus deactivates itself

We agree that a reference should have been included. Consistent with our observation that phosphorylation activity does not proceed beyond 30 minutes, we've concluded that DNA-PK is inactivated as previously reported (Carter, Vanculova et al. MCB 1990).

Fig. 4C: would be better to remove "low" and "high" from the x-axis and define what is meant by the white/grey bar in the legend.

We thank the reviewer for the helpful suggestion to clarify the figure. We have removed "low" and "high" and have clarified in the Legend that these are high and low expression vectors, indicating that the aggregation and partitioning of FUS is dependent on molecular properties and not expression level.

Referee #2:

The manuscript by Monahan et al. focuses on the question how the self-association of the protein FUS is regulated by multi-site phosphorylation. The authors map phosphorylation sites in the Low-complexity (LC) domain of FUS generated by DNA-dependent protein kinase phosphorylation in

vitro. While these sites fully fit the consensus sites expected to be phosphorylated by DNA-PK, they were not (and had not been) identifiable by MS. The authors instead used NMR spectroscopy to identify these sites. They go on to identify in cell phosphorylation sites generated by DNA damage. The authors then characterize the effect of phosphorylation on these 12 sites (or of phosphomimetic mutations in the same sites) on liquid-liquid phase separation and aggregation of FUS. WT FUS LC undergoes LLPS and aggregates over time. Multi-site phosphorylation and phosphomimics both prevent this transition. However, full-length FUS, when released from an MBP-FUS construct through TEV cleavage, can still undergo LLPS even in the phosphomic state. LLPS of this phosphomimic full-length construct is, however, reduced at high salt or high RNA concentrations. This suggests that the interactions underlying LLPS in the full-length phosphomimic are different than in the full-length WT protein, likely based on electrostatic interactions between the RRMs and the now negatively charged LC domain. This may allow for some interesting, context-dependent regulation of LLPS, which the authors could discuss in more detail.
The authors then use NMR spectroscopy to uncover the mechanism of reduced assembly of the phosphomimic. They observe reduced intra- and intermolecular PREs, indicating reduced transient collapse (in agreement with their MD simulations) and reduced intermolecular interactions. These mechanistic observations nicely tie into the observed morphological observations.
In yeast cells, the phosphomimic mutant, and a mutant with 6 phosphomimetic sites, show reduced ability to form punctate structure in the nucleus. In mammalian cells, the phosphomimic mutant looses the ability to segregate into the insoluble fraction after DNA damage stress. The toxicity in yeast cells is also reduced for the phosphomimic mutant.
The experiments are very well designed, the data is beautiful, the question asked timely, and the conclusions well supported by the data. The manuscript should be published, in my opinion, after one major and several minor points are addressed.

We're very grateful for the Reviewer's assessment of our paper.

Major point:
The authors use turbidity measurements as a measure of the propensity to undergo LLPS. E.g. the addition of increasing concentrations of RNA to the full length FUS 12E mutant reduces turbidity. While the observed trend seems reasonable, I am not sure one can indeed conclude that the phase separation propensity is lower in case of a lower turbidity. The turbidity is a function of the size and number of droplets in the sample, and the two parameters are non-additive. I foresee the possibility that same volume fraction of dense phase could appear as very many tiny droplets that hardly scatter the light, a medium number of droplets that are large enough to considerably scatter the light, or a small number of very large droplets, which again would not scatter the light strongly. The authors should actually quantify the propensity of their protein constructs to undergo LLPS under different conditions.

The Reviewer makes an excellent point and is absolutely correct about the limitations of using turbidity as a direct reporter of phase separation. It's been our observation having done considerable qualitative assessment of phase separation by DIC microscopy that turbidity measurements generally correlate directly with phase separation. We like to use turbidity because it is much easier to measure than taking field views of all samples over time. In our updated Figure S2, we show that images from DIC microscopy (Panel B) correlate with turbidity (Panel C). Mostly, this is just a qualitative assessment of whether or not phase separation has occurred – not the more nuanced characterization proposed by the Reviewer. In the new Figure S2, we show that FUS 6E and 12E turbidity decreases are in fact due to diminished phase separation as viewed by DIC microscopy.

Minor points:

1. "Though FUS is primarily localized in the nucleus, familial ALS mutations in FUS most often disrupt the nuclear localization signal, resulting in FUS cytoplasmic accumulation and apparent gain-of-function toxicity." I believe that the authors may not want to use the word "though".

We've altered the language as follows: "Familial ALS mutations in FUS most often disrupt the nuclear localization signal, resulting in cytoplasmic accumulation and apparent gain-of-function toxicity (Scekic-Zahirovic et al., 2016, Sharma et al., 2016)."

2. "Interestingly, in vitro phosphorylation of recombinant, isolated FUS LC reduces its ability

to bind to hydrogels formed from amyloid-like fibrils of recombinant, isolated FUS LC (Han et al., 2012), though hydrogels are more static than LLPS granules." Again, the word "though" seems misplaced.

Based on the Reviewer's comment, we've simplified the sentence by removing the entire clause. Distinctions between hydrogels and LLPS are beyond the scope of this manuscript.

3. p6: "In total, by mass spectrometry and NMR we have putatively identified 17 phosphosites in FUS LC, suggesting phosphorylation may be critical to FUS LC function." The identification of the phosphorylation sites does not allow the conclusion that they are critical for function. The authors show functional data later, and should reserve such a statement for after these data are presented.

We agree our text was overly speculative. We've simplified it by passively concluding, "…we have identified 17 putative phosphosites in FUS LC, suggesting phosphorylation could have a role in FUS function."

4. "We performed differential interference contrast microscopy under conditions where WT FUS LC undergoes liquid-liquid phase separation (LLPS) to form round micron-sized phases..." I think it would be more appropriate to call the round structures droplets, rather than phases.

We've replaced "phases" with "droplets".

5. "These 2-day old FUS 12E structures appear more static than when LLPS is initiated, as judged by lack of droplet fusion and fluorescence recovery after photobleaching, similar to previous observations for FUS and FUS LC at other conditions (Lin et al., 2015, Patel et al., 2015)." The authors should show the FRAP data they are referring to.

We've removed the sentences that discussed FRAP with FUS 12E. We've added a supplementary movie and minimal text to summarize our limited FRAP data with wild-type FUS.

6. "Taken together, these data on isolated FUS LC and full-length FUS demonstrate that the addition of negatively charged phosphate groups or phosphomimetic substitutions impedes FUS self-association and subsequent aggregation." It seems that this statement is not fully warranted; the conditions under which the specific constructs assemble change, and the assemblies do not promote further aggregation.

The new data we present in Figures 2 and S2 better supports our conclusion. Over time, we see unphosphorylated FUS samples evolve from dispersed droplets to large solid aggregates. In control conditions, we don't see phosphorylated FUS (or phosphomimetic FUS 12E) forming solid aggregates.

7. "However, transverse relaxation rate constants, R2, for wild-type is slightly higher than for 12E at both 850 and 500 MHz 1H Larmor, " I believe the missing word is "frequency".

We've added the missing "frequency".

8. "Given that FUS LC is composed of 24 degenerate tyrosine repeats, these changes in intramolecular behavior provide insight into how phosphorylation suppresses both LLPS and aggregation." The authors do not mention anywhere else that the FUS LCD is composed of 24 degenerate tyrosine repeats. It is unclear, why they mention it here, and what it has to do with the suppression of LLPS by Ser/Thr phosphorylation.

We thank the Reviewer for pointing out an unnecessarily confusing sentence. Our intent was merely to emphasize the degenerate, low-complexity sequence of FUS, and how the introduction of charge can have small effects on chain collapse, thus providing a molecular mechanism by which LLPS could be regulated. We've removed the clause regarding the 24 tyrosine repeats. It now reads: "These changes in intramolecular behavior provide insight into how phosphorylation could suppress both LLPS and aggregation."

9. What was the molar ratio of spin-labeled and unlabeled protein in the intermolecular PRE experiments?

We thank the reviewer for pointing out this was not clearly listed, although it was labeled within the figure. We have now updated the Figure 3 legend to state that the concentration was 1:1, 25 μM FUS LC ^{15}N combined with 25 μM spin-labeled.

I am not able to assess whether the coarse-grained simulations that show self-association of the WT LC, but not self-association of the phosphorylated form, are suitable to come to the presented conclusions. Please make sure that a reviewer with expertise in this area can comment on these data.

Referee #3:

In this study, Monahan et al. assess how phosphorylation of the low-complexity domain (LCD) of FUS might impact its phase transition behavior and aggregation propensity, which is of interest for several neurodegenerative disorders (e.g. ALS and FTD) where FUS aggregates accumulate in the cytoplasm of dying neurons. They identify several serines and threonines, including T7, T11, T19, S26, S42, S61, S84 in the FUS LCD that can be phosphorylated by DNA-PK in vitro. In HEK293T cells, upon DNA damage ~17 sites in the FUS LCD become phosphorylated. Pure protein experiments reveal that DNA-PK antagonizes FUS LCD phase transitions and aggregation, as does mutation of 12 putative DNA-PK phosphosites in the FUS LCD to glutamate (FUS12E). In the context of full-length protein, FUS12E undergoes liquid-liquid phase separation like FUS, but fails to fibrillize and displays different sensitivity to salt and RNA. In yeast, FUS12E is less aggregation prone and less toxic. Although several aspects of the study are interesting, I have a number of concerns that reduce my enthusiasm for this study:

1. The detailed opening to this paper tries to resolve which residues in the FUS LCD are actually phosphorylated by DNA-PK in vitro and in cells. However, a clear picture fails to emerge of exactly what phopsho-forms of FUS become populated, and it is not clear whether FUS ever bears multiple DNA-PK phosphorylated serines or threonines. Nonetheless, multiple phosphorylations are subsequently mimicked with the FUS12E variant in the rest of the paper. The lack of clarity here makes it difficult to assess the physiological relevance of the subsequent FUS12E studies. Moreover, what might happen in terms of FUS LCD phosphorylation in a relevant cell type (e.g. motor neurons) or in disease (e.g. ALS/FTD patient samples) is not clear and not addressed in the present study, which is a major weakness.

These are excellent points that we spent quite a bit of time thinking about. Regarding the number of phosphorylations that occur in cells following the additions of DNA-damaging agents, we reasonably conclude that FUS is multiply phosphorylated beyond 6 sites due to the change in migration of FUS in a Western Blot. When we constructed our phosphomimetics, we observed that with each substitution, FUS migrated more slowly, with a greater apparent molecular weight. The population of naturally phosphorylated FUS (following DNA damage) in mammalian cells migrates similarly to FUS with 12 substitutions.

As discussed in the above responses, we have included *in vitro* and in-cell experiments using FUS6E to better capture the behavior predicted by FUS species showing an intermediate degree of phosphorylation. Further, the data presented in yeast with regard to toxicity suggests that amelioration of gain-of-function toxicity is mitigated with as few as 2 phosphorylations, suggesting that fully phosphorylated FUS LCD is not required to result in major changes to FUS's properties.

We have attempted to characterize simultaneous phosphorylation via mass spec – however top down proteomic approaches are far from routine especially for an extremely challenging case such as FUS. Work is ongoing in our groups to address this question, but we believe it is not within reach in the review timeline. The use of human diseased tissue samples, primary neurons and iPSCs are all worth pursuing in future work. However, non-motor neuron cells types are very relevant and worthy of studying since FUS is expressed in many cell types beyond the CNS. Also, FUS's phase separation is regarded as relevant to ribonucleoprotein granule function.

2. DNA-PK is shown to inhibit FUS LCD phase transition and aggregation (Fig. 2). However, it is not clear what concentration of DNA-PK was added. It is possible that DNA-PK is acting as a chaperone and not a kinase in this assay. A control is needed with a kinase-dead DNA-PK mutant or with a DNA-PK small-molecule inhibitor to demonstrate that the effect is due to kinase activity. Likewise, a FUS LCD that cannot be phosphorylated (e.g. S/T to A or even Q/N [to retain the uncharged polar character of the side chain) would be a valuable control. Finally, these DNA-PK experiments should be repeated with full-length FUS as it is not clear whether the full-length FUS would be similarly affected by DNA-PK activity.

We've performed the experiment with full-length FUS and included controls (in our in vitro experiment, we use DNA-PK in the absence of ATP as well as ATP in the absence of DNA-PK) to ensure we weren't getting a chaperone effect with DNA-PK. We thank the Reviewer for challenging this point because our new Figure 2 with these results makes our manuscript stronger.

3. There is a heavy reliance on the phosphomimetic FUS12E variant to ascribe effects of FUS phosphorylation. However, it is not clear whether the glutamate mutations truly mimic phosphoserine or phosphothreonine. Ideally, phosphorylated versions of FUS or FUS LCD would be purified and assessed in terms of phase transition and aggregation behavior. This could be achieved by further fractionation of FUS/FUS-LCD after DNA-PK phosphorylation in vitro, or via introduction of phosphoserine using technology developed by the Chin lab (Rogerson et al. Nat Chem. Biol. 2015. DOI: 10.1038/nchembio.1823), or via semisynthetic approaches. I raise this issue as phosphorylation mimics (S129E/D) do not reproduce the effect of phosphorylation on the structural and aggregation properties of alpha-synuclein in vitro (Paleologou et al. JBC 2008. DOI: 10.1074/jbc.M800747200), and a similar concern applies to FUS. Indeed, phosphorylation extends the ensemble of conformations populated by α-synuclein close to its fully random coil-like dimensions, but this effect is not observed with the S129E/D substitutions. This issue could also apply to FUS, and experiments using phosphorylated FUS are needed to address this issue.

We agree that phosphomimetics do not perfectly recapitulate the phosphorylated form of FUS. The inability to control phosphorylation with DNA-PK and achieve homogenous, uniformly phosphorylated samples is an impediment for many conceivable experiments. We hope our new Figure 2 that shows the effects of phosphorylation on FUS aggregation sufficiently address the Reviewer's concern. Specifically, DNA-PK phosphorylated FUS full-length loses its propensity for solid aggregates.

4. While the observations in yeast (Fig. 4 & 5) are intriguing they need to be validated in more complex model systems, e.g. in primary neurons or in an animal model (e.g. fly), to confirm their relevance to ALS or FTD.

We have extended the results to human cell lines as described above. However, the use of yeast to model neurodegenative-protein aggregation propensity in a crowded eurkaryotic environment is well established. (Khurana V, Lindquist S. Nat Rev Neurosci. 2010; Sun Z, Gitler AD, et al. PLoS Biol. 2011). Our results in the yeast and mammalian models have been strikingly consistent. In the future, we agree the establishment of an animal model would be great, but it is not feasible for the current review period. We hope the Reviewer will find the structural biology / biophysics of phase transition and how it may be altered by post-translational modification is generally relevant to many cellular functions, even in the absence of disease (or animal models of disease).

5. While phosphorylation of the FUS LCD by DNA-PK is proposed to inhibit aggregation and phase transitions it is not clear whether this phosphorylation can promote the reversal of these events. Experiments should be added to determine whether FUS LCD phosphorylation reverses aggregation or phase transitions, as these would provide further support for increasing FUS LCD phosphorylation as a potential therapeutic. For example, do FUS LCD droplets disperse if DNA-PK is added?

This is a really interesting point. The possibility that phosphorylation dissolves droplet compartments into the larger bulk fluid is intriguing, and likely a future direction based on the work presented in this manuscript. Data presented herein suggests that phosphorylated FUS largely retains

the ability to form droplets depending on the RNA concentration, while phosphorylation more profoundly retards the maturation of droplets into pathological aggregates. Thus, the precise role of phosphorylation in dissolving phase separated droplet compartments is ambiguous, and likely to involve extensive additional experimentation to justify its stand-alone publication. Addition of large amount of non-specific DNA needed to stimulate DNA-PK complicate performing the experiments proposed.

6. Finally, FUS that is phosphorylated by DNA-PK appears to accumulate in the cytoplasm (Deng et al. J. Neurosci. 2014. doi: 10.1523/JNEUROSCI.0172-14.2014). Thus, increasing FUS phosphorylation is unlikely to restore nuclear FUS in disease. This issue needs to be discussed.

Our approach in this manuscript is principally to address the biophysical properties of FUS and how these are potentially modified by phosphorylation. Indeed, phosphorylation is unlikely to restore nuclear FUS, however there is considerable evidence that cytoplasmic gain-of-function, in contrast to nuclear loss-of-function, is the major pathway of toxicity (Scekic-Zahirovic 2016), and these data most directly pertain to this mechanism of toxicity.

2nd Editorial Decision 22 June 2017

Thank you for submitting your revised manuscript to The EMBO Journal. Your study has now been seen by referee #1 and 3 and the comments are provided below. As you can see from the comments, both referees appreciate the added data and support publication here. Referee #1 has some remaining suggestion for further improvement of the manuscript. No new experiments are needed. I find the suggestions good and would ask you to take them into consideration. Regarding point 2 (Movie S1) - I think fully OK to keep the movie in. I do think it adds to the manuscript.

REFEREE REPORTS

Referee #1:

The revised manuscript is clearly stronger than the previous version and the major points raised by the three reviewers have been largely addressed, most notably
- extension of in vitro and yeast studies to mammalian cells (HEK293 cells)
- analysis of an „intermediate" phosphorylation-mimicking mutant (6E), demonstrating that also partial phosphorylation affects phase transition of FUS
- validation of turbidity assay results by an alternative method (droplet imaging in DIC microscopy)
- controls demonstrating that DNA-PK and ATP alone do not suppress phase separation of FUS
Although a few points raised by the reviewers were addressed in a rather minimalistic way and could have been addressed more comprehensively, I believe that the study is significant and novel and of high interest to the field of neurodegeneration and cell biology in general. A few minor points should be discussed with the authors, however, before publication in EMBO Journal:

(1) Does the title really appropriately reflect what their data show? They clearly show that in the context of the full length protein, phosphorylation of the FUS-LC domain does NOT prevent or even reduce droplet formation, but what is suppressed is liquid-to-solid state transition, i.e. maturation of liquid droplets into aggregates/solids (see e.g. Fig. 2E and F). I think the current title does not accurately reflect these findings. I also would recommend that the authors explain the concept of liquid-liquid phase separation and liquid-to-solid state transition a bit better (maybe already in the introduction). They mention that "liquid droplets of full-length FUS can convert into fibrillary aggregates over time (Patel et al, 2015)" when they talk about Fig. 2E, it may be helpful to the reader to introduce this concept earlier and clarify that phosphorylation does not disrupt LLPS or phase separation (as stated e.g. on page 10 or in the abstract - this should be corrected) but rather the subsequent aggregation / liquid-to-solid transition.

(2) I would recommend to remove Movie S1 and the paragraph that describes it, as it only shows data on unmodified FUS droplets, but not on phosphorylated / phospho-mimetic FUS droplets, so it does not add any information / insights and it is rather confusing and unclear why they included this

data. Alternatively, they should present FRAP data on unmodified vs. phospho-droplets in comparison.

(3) The image quality shown in Fig. 5A (HEK293 data) is rather poor - it would be nice to include the DAPI channel, so that one can see in the left panel (GFP-FUS-R495X) where nuclei and cytosol are. The cellular data would also be much more convincing if they would add a quantification (e.g. counting the % of cells with cytosolic aggregates or analysis of soluble (S) vs. insoluble (P) material as done in Fig. 5C).

(4) Fig. 2A should be labelled more clearly - were all samples incubated for 1 day at 25{degree sign}C or only the sample shown in the upper right (wild-type FUS LC aggregate)? Text sounds like the latter, but legend sounds like all samples were incubated for 1 day.

(5) More carefully update methods / supplementary methods section, often just WT-FUS and 12E FUS (and not 6E) are mentioned (e.g. turbidity and microscopy, purification of proteins).

Referee #3:

In their revised paper, Fawzi et al. have addresses my previous concerns. In my view, the paper is now suitable for publication.

2nd Revision - authors' response 06 July 2017

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July 6, 2017

Dr. Karin Dumstrei, PhD
Senior Editor | The EMBO Journal
k.dumstrei@embojournal.org

Dear Dr. Dumstrei,

Thank you for communicating the decision to revise our manuscript to improve the manuscript accoroding to the additional constructive feedback from the reviewers. Guided by your editorial remarks, we have now addressed the outstanding concerns in detail. See below.

We are confident that the manuscript is now acceptable for publication and we look forward to your decision.

Nicolas L. Fawzi, Ph.D. and Frank Shewmaker, Ph.D.

Reviewer 1
(1) Does the title really appropriately reflect what their data show? They clearly show that in the context of the full length protein, phosphorylation of the FUS-LC domain does NOT prevent or even reduce droplet formation, but what is suppressed is liquid-to-solid state transition, i.e. maturation of liquid droplets into aggregates/solids (see e.g. Fig. 2E and F). I think the current title does not accurately reflect these findings. I also would recommend that the authors explain the concept of liquid-liquid phase separation and liquid-to-solid state transition a bit better (maybe already in the introduction). They mention that "liquid droplets of full-length FUS can convert into fibrillary aggregates over time (Patel et al, 2015)" when they talk about Fig. 2E, it may be helpful to the reader to introduce this concept earlier and clarify that phosphorylation does not disrupt LLPS or phase separation (as stated e.g. on page 10 or in the abstract - this should be corrected) but rather the subsequent aggregation / liquid-to-solid transition.

We clarify in the main text that we do show that liquid-liquid phase separation is disrupted by phosphorylation of FUS LC (Figure 2A) and phosphomimetic substitution of full length FUS (Figure 2C,D and Figure S2). We have also expanded the description of LLPS in the introduction and of conversion to a solid form.

(2) I would recommend to remove Movie S1 and the paragraph that describes it, as it only shows data on unmodified FUS droplets, but not on phosphorylated / phospho-mimetic FUS droplets, so it does not add any information / insights and it is rather confusing and unclear why they included this data. Alternatively, they should present FRAP data on unmodified vs. phospho-droplets in comparison.

We have clarified this section of the text.

(3) The image quality shown in Fig. 5A (HEK293 data) is rather poor - it would be nice to include the DAPI channel, so that one can see in the left panel (GFP-FUS-R495X) where nuclei and cytosol are. The cellular data would also be much more convincing if they would add a quantification (e.g. counting the % of cells with cytosolic aggregates or analysis of soluble (S) vs. insoluble (P) material as done in Fig. 5C).

We decided on this image processing (identical for all 3 variants) because it highlights how little diffuse (unaggregated) GFP-FUS(R495X) is in the cytoplasm in the absence of phosphomimetic substitutions. However, we have added an inset with altered brightness and Figure S7 that shows the full image with those brightness settings and an additional image with SiR700 nuclear stain overlay. We appreciate that precise quantification would be a nice addition though we suggest that the qualitative picture as presented and reproduced in independent experiments is sufficient.

(4) Fig. 2A should be labelled more clearly - were all samples incubated for 1 day at 25{degree sign}C or only the sample shown in the upper right (wild-type FUS LC aggregate)? Text sounds like the latter, but legend sounds like all samples were incubated for 1 day.

We have updated the labeling to clearly state that all sample were identically incubated.

(5) More carefully update methods / supplementary methods section, often just WT-FUS and 12E FUS (and not 6E) are mentioned (e.g. turbidity and microscopy, purification of proteins).

We have updated the methods and appendix methods carefully. We thank the reviewer for his/her close reading of the manuscript.

3rd Editorial Decision 07 July 2017

Thanks for sending us your revised manuscript. I have now had a chance to take a look at this version and I appreciate the introduced changes. I am therefore very pleased to accept the manuscript for publication here.

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: Nicolas Fawzi
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2016-96394

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

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 χ2 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.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

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?	NA
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
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.	NA
For animal studies, include a statement about randomization even if no randomization was used.	NA
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.	Figure 4B quantification was performed blinded.
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	NA
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	NA
Is there an estimate of variation within each group of data?	NA
Is the variance similar between the groups that are being statistically compared?	NA

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

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

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).	Immunoblotting was performed with rabbit α-FUS monoclonal antibody (Bethyl, #293A) and HRP-conjugated α-rabbit antibody (Southern Biotech).
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Only HEK293T and H4 neuroblastoma cell lines were used.

* 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.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
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.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
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.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
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.	NA
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.	NA

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under '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	BMRB deposition of FUS LC 12E NMR assignment is in process of identifier/record assignment.
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).	NA
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).	NA
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	We have included this section.
22. 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.	NA

G- Dual use research of concern

23. 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.	NA