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The oncogenic transcription factor FUS-CHOP can undergo nuclear liquid-liquid phase separation

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MS TITLE: The oncogenic transcription factor FUS-CHOP can undergo nuclear liquid-liquid phase separation

AUTHORS: Izzy Owen, Debra Yee, Hala Wyne, Theodora Myrto Perdikari, Victoria Johnson, Jeremy Smyth, Robert Kortum, Nicolas L Fawzi, and Frank Shewmaker
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers raise a number of substantial criticisms that prevent me from accepting the paper at this stage. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

We are aware that you may be experiencing disruption to the normal running of your lab that makes experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

The authors claim to identify the molecular mechanism underlying FUS-CHOP-induced transcriptional activation. the authors claim that the FUS-CHOP undergoes phase-separation to promote gene transcription.
The authors focus on the co-localization of FUS-CHOP with super-enhancer condensates as a probable mechanism of promoting gene transcription and its resulting oncogenic properties.

The study provides interesting insights into the phase separation properties of FUS-CHOP fusion. The study is of potential interest to the field. I would recommend a few revision points to be addressed before my recommendation for publication in the Journal of Cell Science.

Comments for the author

Revision points essential for publication -

1. Please indicate TEV sites in Fig1 and Fig S1.

2. Please show phase separation of FUS and CHOP alone in cellular experiments in Fig 2C.

3. In Fig 1B- Mention FUS-CHOP type II instead of just Type II.

4. Label fig 1C - FUS-CHOP or CHOP only in the plot.

5.Ectopic expression of proteins often leads to over-expression artifacts leading to condensate formation. Please comment on this and provide appropriate controls in Fig1C to rule out ectopic over-expression artifacts.

6. Please provide quantification (percentage of cells positive for condensates) for Figure 1C and 1D.

7. In Figure 5B- FUS delta 75 seems to have lower expression levels in comparison to other mutants and WT. Please comment.

8. Please provide quantification (percentage of cells positive for condensates) for Figure 5C.

9. In Figure 5D lower panel, please change color scheme as it is very difficult to distinguish the type I and II.

10. Please provide quantification (percentage of cells positive for condensates) for Figure 6C.

11. The authors claim that the loss of a particular number of amino acids from FUS N-term results in abrogated phase separation behavior. Authors should strengthen the claim with additional mutants. like delta aa50-100, delta aa50-125 and delta aa175-250.

12. Why does in Fig 7B, NIH3T3 cells do not express a-CHOP?

13. Please provide quantification (percentage of cells positive for condensates) for Figure 7B.

14. Please provide quantification for co-localization for Figure 7B.

15. Please provide quantification (percentage of cells positive for condensates) for Figure 8A and 8B.

16. Please provide quantification for co-localization for Figure 8B.

17. To causally link phase separation of FUS-CHOP protein with its oncogenic behavior, authors must provide an additional experiment showing - replacement of FUS N-term with other unrelated IDR in CHOP fusion protein can or can't mimic the same properties.

18. A recent publication (33548202) using a similar IDR replacement approach showed FUS IDR can repress transcription when fused with a transcriptional repressor NELF. This finding indicates the contribution of FUS IDR is just to prime for phase separation, whereas the function of phase-separated condensates is attributed by the core protein. Authors should comment on the findings from this publication and compare them with their probable hypothesis of IDR length independent of sequence confers phase separation properties.

19. Authors must show Super enhancer transcriptional output using qPCRs in WT-FUS-CHOP and mutants with loss of phase separation behavior to prove the claims that FUS-CHOP phase separation drives its oncogenic behavior. This experiment is the single most essential experiment to prove the author's claim of FUS-CHOP driving BRD4 phase separation and its role in oncogenic cellular reprogramming.

20. Authors should combine Figs 3 and 4 into one single figure and provide an additional figure with a model summarizing their findings.

21. Overall, the manuscript provides intriguing results and opens the path for possible extensions in the direction of pharmacological interventions but the functional significance from the manuscript remains correlative and undermined.

Reviewer 2

Advance summary and potential significance to field

Overall, this study furthers our understanding of the FUS-CHOP onco-protein localization and biophysical properties in both patient-derived cells and exogenous systems (NIH3T3 plus overexpression). It supports claims of in vivo phase separation with measures of liquidity, sphericity and protein diffusion.
Importantly, FUS-CHOP is also able to form phase-separated droplets in vitro. In both mouse and human cells, the fusion protein forms spherical puncta in the nucleus. Interestingly, these puncta also contain BRD4 in the patient-derived cells, suggesting that they may be sites of highly active transcription like super-enhancers. The functional and mechanistic role of phase separation in living cells has been difficult to determine directly, but its pursuit is a useful and interesting perspective shift for the fields of nuclear organization and cancer progression. This paper seeks to demonstrate that altering phase-separation properties of a transcription factor CHOP, by adding a low-complexity sequence from a known phase-separating protein FUS, can explain the alteration of nuclear localization and function of this fusion protein. Indeed, the fusion protein has liquid-like properties and localizes to structures that are likely phase-separated. This is a novel demonstration that changes in one protein’s phase separation properties can drive the differences in protein function that lead to altered transcription and cancer.

Comments for the author

In this work, Owen et al. investigate the role of the fusion protein FUS-CHOP in nuclear organization and function. They demonstrate that the fusion protein can demix from aqueous solution in vitro and localize to liquid-like clusters in vivo.
Most interestingly, they observe that the change in CHOP protein localization and clustering brought on by fusion with FUS is necessary and sufficient for altering transcription. This work is novel and has implications for the field of chromatin organization and function, and for

understanding of oncogenic progression in specific cancers. With minor edits, I recommend it for publication in JCS.
Please see suggested edits by figure below.
Introduction In the introduction, please add a citation or figure reference for “importantly all MLS-causing FUS-CHOP translocations contain portions of this LC sequence.”
“Likewise, our results suggest FUS-CHOP can undergo a liquid-phase transition in the nucleus, which could provide the mechanism for its emergent gain-of-function oncogenicity. This may be a general mechanism for transcriptional activation by fusion oncoproteins with IDRs.”
Provide more specificity of mechanism here or in the discussion, what would phase separation provide that mis-localization of CHOP would not?

Figure 1.
Please indicate in the figure where the TEV protease site is inserted, I assume between MBP and FUS.
What is the domain structure of CHOP? Does it have a DNA binding domain and any self-interaction domain? Is formation of droplets in vitro concentration-dependent? Were the authors able to make any sort of phase diagram dependent on protein or salt concentration? This would lend understanding to the concentrations required in vivo to create nuclear foci.

Figure 2.
Please provide a scale bar for microscopy images.
Have the authors or previous studies co-stained these nuclei with markers for other known nuclear bodies, i.e. nuclear speckles or PML bodies? Are the fusion proteins partitioning into pre-formed structures or creating novel compartments?
This knowledge would help to interpret whether FUS-CHOP is a driver of phase separation within the nucleus or a ‘client’ of compartments formed by other proteins.
Also, are these FUS-CHOP foci concentration-dependent? It would strengthen the in vivo phase separation mechanism argument to see a saturation concentration, below which the protein is dispersed throughout the nucleus, and above which these foci form.
2B. It is unusual to use a cytoplasmic protein like tubulin as a loading control for nuclear proteins. Have the authors also used a nuclear protein loading control like a histone?

Figure 3.
Please add timescale and scalebar to Figure 3. Did the fusion take similar timeframes for type I and type II fusion proteins? Does this match the FRAP data?

Figure 4.
How were these FRAP data normalized? It seems the fluorescence signal only recovers to 30 percent in the quantification but by eye in the images is brighter than 30% of original by the 60 seconds timepoint. Are these images not shown at the same contrast, or could they be oversaturated?
How does this compare to FRAP of just CHOP without FUS?
The authors may consider combining figures 3 and 4, as these data are both related to the material properties of FUS-CHOP foci and the total number of figures is high.

Figure 5 and 6.
With the FUS-CHOP truncation proteins, both at N-term and internally, it is again interesting to ask whether foci formation is concentration-dependent. If phase separated, we would expect shorter sequences (those with weaker self-self interaction) to need higher concentration to form foci (higher saturation concentration).
5C-D. Please add scale bar to C and report number of cells or bleaches per replicate in D.
6A. Usually internal deletions are denoted with ^, not –

Figure 7.
Do the authors have a way to compare expression levels of the exogenous proteins expressed in NIH3T3 cells with patient derived cells? Are the smaller foci perhaps a result of lower overall protein expression level?

Figure 8.
In this study, the authors did not directly confirm the localization of BRD4 at superenhancers in these cells, I suggest rewording “These data suggest FUS-CHOP and BRD4 occupy the same nuclear condensates at SEs.”, to remove ‘at SEs.’
Pearson correlation coefficient values reported seem very high for the images provided—are these values correct? Are they reproducible in multiple cells?

Discussion
“This suggests FUS-CHOP could be driving phase separation of BRD4 at oncogenic SEs in MLS. These findings provide a mechanism by which oncogenic fusion proteins such as EWS-FLI1 and FUS-CHOP, hijack BRD4 and other bromodomain-containing proteins to induce oncogenic SEs (Chen et al., 2019b; Gollavilli et al., 2018) “
This logic is a bit messy, BRD4 is a protein that phase separates at SEs but the authors claim it is being driven by FUS-CHOP, the data provided in this study show that these proteins colocalize but do not demonstrate which protein is driving the condensation. See suggestions for figure 2 for potential ways to further support claims that FUS-CHOP is indeed driving formation of condensates in vivo.
Also, knowledge of protein-protein interactions would help support these claims as well. Is it known that BRD4 and FUS-CHOP directly interact whereas BRD4 and CHOP do not?

Overall, this study furthers our understanding of the FUS-CHOP onco-protein localization and biophysical properties in both patient-derived cells and exogenous systems (NIH3T3 plus overexpression). It supports claims of in vivo phase separation with measures of liquidity, sphericity and protein diffusion.
Importantly, FUS-CHOP is also able to form phase-separated droplets in vitro. In both mouse and human cells, the fusion protein forms spherical puncta in the nucleus. Interestingly, these puncta also contain BRD4 in the patient-derived cells, suggesting that they may be sites of highly active transcription like super-enhancers. The functional and mechanistic role of phase separation in living cells has been difficult to determine directly, but its pursuit is a useful and interesting perspective shift for the fields of nuclear organization and cancer progression. This paper seeks to demonstrate that altering phase-separation properties of a transcription factor CHOP, by adding a low-complexity sequence from a known phase-separating protein FUS, can explain the alteration of nuclear localization and function of this fusion protein. Indeed, the fusion protein has liquid-like properties and localizes to structures that are likely phase-
separated. This is a novel demonstration that changes in one protein’s phase separation properties can drive the differences in protein function that lead to altered transcription and cancer.

Reviewer 3

Advance summary and potential significance to field

In the manuscript submitted by Owen et al., the authors characterize in detail the ability of the FUS-CHOP protein to undergo phase separation in cells. FUS-CHOP plays a critical role in myxoid liposarcoma, MLS, and other FUS fusions can drive an assortment of sarcomas. The results of this study are important because understanding of phase separation by these cancer-causing fusion proteins is still surprisingly limited, and recent studies offer few answers significant enough for cancer research to build on. The data provided by this study is easy to compare and contrast with previous studies for a new perspective on the questions they’ve raised.

Comments for the author

The novelty of this work has not been fully demonstrated. The fusion of FUS and CHOP is disease relevant, but the authors haven’t completed the story of whether the function of CHOP has any relevance to phase separation. The imaging-based approaches applied here do not yield results identical to observations of FUS-CHOP in the MLS cells, highlighting this question whether the CHOP domain is functional or if these observations are more relatable to LC domain fusions previously reported, not the activity of the fusion in MLS. This needs to be addressed somehow and some options are detailed in major comment #4 below.
Major comments:

1. Recent studies have called into question how to define phase separation by FUS or EWSR1 fusion proteins, particularly for EWS-FLI1, and how the stability or dynamics of assemblies they produce compare to natural condensates associated with transcription (Chong et al., Science 2018; McSwiggen et al., Elife 2019; McSwiggen et al., Genes Dev 2019). The in vitro data in Figure 1B would greatly benefit from FRAP data as performed in vivo, in order to compare with those in vivo results and similar published data for FUS, EWSR1, related proteins, and their fusions.
2. The delta 50-75 truncation of FUS-CHOP does not sufficiently address the question about length vs location of the deletion impacting phase separation. It is noticeable that the first 75 amino acids in FUS are more enriched with tyrosine motifs, which are more tightly spaced than the rest of the LC-domain. A deletion in the latter half of the LC domain (50-100, 75-125, or 50-125) would provide a better indication whether the position and spacing of the motif has a significant influence on FUS-CHOP phase separation in cells. The observation of shortened LC domains such as Types V and IX in Fig S1 may argue they don't impact phase separation, or they do and that's not important to function and MLS. It would also be helpful if the authors acknowledge the number of tyrosine motifs in each deletion, as this is a metric easily compared to a previous publications (Lin et al., JBC 2017, PMID: 28924037; Murthy et al., NSMB 2019, PMID: 31270472; Chong et al., Science 2018, PMID: 29930090).
3. Figures 5 and 6 and their sub-parts are referenced out of order in the results section. This is not aligned with the author guidelines for J Cell Science and also confusing to the reader. Moreover, the purpose of the upper and lower panels in Figure 5D is unclear. Is this the same data presented two ways or different experiments? The description of 5D is vague in the text and the legend, leaving questions about what measurement and experimental protocol is shown. Correcting this may help clarify the section that describes Figures 5 and 6, which was particularly difficult to follow.
4. The primary piece of information that is missing is the role of DNA-binding. The authors reference studies that have already shown phase separation in cells by FUS or LC domain fusions to tags. If the advance achieved here is that the CHOP domain has not precluded the protein to phase separate, this leaves a question whether the protein imaged is functionally competent to bind DNA or just the remainder of protein that isn't bound. Two ways that may address this are: (1) add DNA to the phase separation assay in Fig 1 and infer that DNA binding may or may not also occur in the cell without interfering with phase separation; or, (2) mutate the CHOP domain to test whether the results of figures like 2C draw closer or further away from results in 7B. For the in vitro approach, the more careful analysis offered by the FRAP measurement (see also comment 1 above) and comparing a DNA-binding deficient fusion would increase the value of the result, since it must rely more on inference. For the second option, it would be compelling to see that phase separation in cells by a FUS-CHOP or truncated FUS-CHOP (e.g. delta 25 or 50) mutated to be deficient in DNA-binding is visually distinct from the observations in Fig 2C. However, if there is no engineered solution that can show the CHOP domain or DNA-binding matters, this still needs to be acknowledged by testing it because this is important to the disease models and also central to setting this study apart from previous publications.
5. The authors acknowledge that co-localization of FUS-CHOP and BRD4 on the DNA is previously known (ref Chen et al., 2019b). Since the role of the CHOP domain for the condensates observed in this study is unknown, questions arise whether phase separation by FUS-CHOP is merely in the same euchromatic nuclear compartments as BRD4, or if BRD4 itself can pull FUS-CHOP into a condensate even without a functional CHOP domain. The following would not be so necessary if #4 above was addressed. The results in Fig 8A and 8B do not appear so similar but could be strengthened either by better statistical and quantitative analysis to compare the two, or test whether a DNA-binding deficient FUS-CHOP can colocalize with BRD4, or test if BRD4 in cells still binds the truncations that are unable to phase separate: e.g. delta 75 or delta 125.

First revision

Author response to reviewers' comments

Thank you for giving your attention to our manuscript (*"The oncogenic transcription factor FUS-CHOP can undergo nuclear liquid-liquid phase separation"*). We recognize a thorough critique is a

significant time investment, so we're grateful for the Reviewers' contribution. In response to the Reviewers' comments, we've added new experiments, additional statistical analyses, and updated text. Our Conclusions remain the same, but the Reviewers' suggestions have dramatically enhanced the quality of the manuscript.

In summary of our Conclusions, we characterize novel oncoprotein phase separation in cells and in vitro. We show ectopically expressed FUS-CHOP is undergoing liquid-liquid phase separation (LLPS) by three hallmarks: Puncta are spherical in shape, undergo fusion upon touching, and have dynamic internal rearrangement and external exchange. In myxoid liposarcoma cell lines, we show endogenous FUS- CHOP localized to small nuclear puncta. We observed that the prion-like domain (PrLD) of FUS is necessary for FUS-CHOP phase separation, but no specific region of the domain is essential for the phase transition to occur (only a minimum length of the PrLD low-complexity sequence). Further, we show FUS-CHOP localizes with BRD4 (a marker of super enhancers) within condensates in cancer cell lines, and FUS-CHOP can recruit BRD4 to large phase separated droplets. Lastly, we show DNA-binding deficient FUS-CHOP is still able to undergo phase separation but its colocalization with BRD4 is significantly reduced. Our data show novel phase separation capabilites of the fusion oncogene and suggest a potential mechanism (liquid-liquid phase separation with transcriptional machinery) for FUS- CHOP-induced oncogenesis.

Reviewer 1:
Reviewer 1 Advance Summary and Potential Significance to Field:
The authors claim to identify the molecular mechanism underlying FUS-CHOP-induced transcriptional activation. The authors claim that the FUS-CHOP undergoes phase-separation to promote gene transcription. The authors focus on the co-localization of FUS-CHOP with super-enhancer condensates as a probable mechanism of promoting gene transcription and its resulting oncogenic properties.

The study provides interesting insights into the phase separation properties of FUS-CHOP fusion. The study is of potential interest to the field. I would recommend a few revision points to be addressed before my recommendation for publication in the Journal of Cell Science.

1. *Please indicate TEV sites in Fig1 and Fig S1.*
   We added the TEV cleavage site in figure 1.

2. *Please show phase separation of FUS and CHOP alone in cellular experiments in Fig 2C.*
   We have added fixed cell imaging of ectopic expression for FUS and CHOP alone in Figure 2A. We reference these observations within the Results section.

3. *In Fig 1B- Mention FUS-CHOP type II instead of just Type II.*
   We have changed our labelling, as suggested.

4. *Label fig 1C - FUS-CHOP or CHOP only in the plot.*
   We have changed our labelling, as suggested.

5. *Ectopic expression of proteins often leads to overexpression artifacts leading to condensate formation. Please comment on this and provide appropriate controls in Fig1C to rule out ectopic overexpression artifacts.*
   This is a great point. We previously stated in our discussion: "Ectopic expression is imperfect because it may cause proteins to exceed critical concentrations that would not be normally achieved in vivo (McSwiggen et al., 2019)." As for controls, our western blot in figure 2 shows FUS-CHOP expression both tagged and untagged as compared to endogenous FUS expression. We took the time to ectopically express various concentrations of DNA in our cell model and did not observe differences in the number of cells with nuclear puncta at lower or higher concentrations.

6. *Please provide quantification (percentage of cells positive for condensates) for Figure 1C and 1D.*
   We quantified the number of transfected cells containing nuclear puncta for type I

and II constructs. We have added these data to figure 4D.

7. In Figure 5B- FUS delta 75 seems to have lower expression levels in comparison to other mutants and WT. Please comment.

The truncations had variable expression, but length, not quantity, dictated puncta formation. We characterized ectopically expressed concentrations of type II delta 75 and saw no difference in the number of cells with nuclear puncta. We concluded that concentration effects were not responsible for determining the observed LLPS patterns.

8. Please provide quantification (percentage of cells positive for condensates) for Figure 5C.

We quantified the number of transfected cells containing nuclear puncta for type I and II constructs and added the quantification to figure 4 and S3.

9. In Figure 5D lower panel, please change the color scheme as it is very difficult to distinguish the type I and II.

Previously "Figure 5" is currently "Figure 4" - this panel has been deleted to avoid confusion.

10. Please provide quantification (percentage of cells positive for condensates) for Figure 6C.

We quantified the number of transfected cells containing nuclear puncta for type I and II constructs and added the quantification to supplemental figure 5 and figure S4.

11. The authors claim that the loss of a particular number of amino acids from FUS N-term results in abrogated phase separation behavior. Authors should strengthen the claim with additional mutants. like delta aa50-100, delta aa50- 125 and delta aa175-250.

Additional mutants (delta aa50-125 and delta aa75-125) for type I and type II have been created, tested, and added to figure 5. We would like to highlight that this was a good suggestion and strengthens the conclusion.

12. Why does in Fig 7B, NIH3T3 cells do not express a-CHOP?

CHOP expression is typically repressed. We state in the manuscript: "Normally, CHOP expression is suppressed, but upregulated during differentiation and following cellular stress (Ohoka et al., 2007; Yang et al., 2017)" CHOP expression is suppressed in NIH 3T3s unless under stress conditions.

13. Please provide quantification (percentage of cells positive for condensates) for Figure 7B.

100% of the MLS cancer cell lines show this pattern of FUS-CHOP localization when analyzed by CHOP immunostaining.

14. Please provide quantification for co-localization for Figure 7B.

Co-localization for FUS and CHOP signals would be difficult to interpret since it is a fusion protein.

15. Please provide quantification (percentage of cells positive for condensates) for Figure 8A and 8B.

Quantification of percentage of cells positive for condensates of the full length proteins has been added to figure 4 and S3.

16. Please provide quantification for co-localization for Figure 8B.

We have included the colocalization data, as suggested.

17. To causally link phase separation of FUS-CHOP protein with its oncogenic behavior, authors must provide an additional experiment showing - replacement of FUS N-term with other unrelated IDR in CHOP fusion protein can or can't mimic the same properties. A recent publication (33548202) using a similar IDR replacement approach showed FUS IDR can repress transcription when fused with a transcriptional repressor NELF. This finding indicates the contribution of FUS IDR is just to prime for phase separation, whereas the function of phase-separated condensates is attributed by the core protein. Authors should comment on the findings from this publication and compare them with their probable hypothesis of IDR length independent of sequence confers phase separation properties.

This is an interesting concept. Important to mention, EWS-DDIT3 fusions exist and have been shown to cause myxoid liposarcoma (PMID: 21115923, PMID: 32317857). These references solidify the Reviewer's statement by showing IDR functionality in fusion proteins is to drive phase separation, while the transcriptional landscape is altered by the core protein. We have included this reference to our discussion. In future work we think testing alternative IDRs for the ability cause CHOP oncogenic transformation would be a great approach, but we do not currently have the resources.

19. Authors must show Super enhancer transcriptional output using qPCRs in WT-FUS-CHOP and mutants with loss of phase separation behavior to prove the claims that FUS-CHOP phase separation drives its oncogenic behavior. This experiment is the single most essential experiment to prove the author's claim of FUS-CHOP driving BRD4 phase separation and its role in oncogenic cellular reprogramming.

This is an exciting future direction for this work. While our manuscript was under review, Zuo et al was able to show FUS and EWS fusion proteins can phase separate with DNA and induce transcription. To link phase separation and transcriptional activation, the authors used high salt to disrupt condensate formation and showed a significant decrease of transcriptional output. These data provide a link between fusion protein phase separation and transcriptional regulation in oncogenesis (Zuo et. al, Nat Comm. 2021). PMID: 33674598

20. Authors should combine Figs 3 and 4 into one single figure and provide an additional figure with a model summarizing their findings.

We have combined our figures, as suggested.

21. Overall, the manuscript provides intriguing results and opens the path for possible extensions in the direction of pharmacological interventions but the functional significance from the manuscript remains correlative and undermined.

The work from Zuo et. al, Nat Comm. 2021 complements our findings and strengthens the argument that phase separation is linked to pathogenesis.

Reviewer 2:
Reviewer 2 Comments for the Author:
In this work, Owen et al. investigate the role of the fusion protein FUS-CHOP in nuclear organization and function. They demonstrate that the fusion protein can demix from aqueous solution in vitro and localize to liquid-like clusters in vivo. Most interestingly, they observe that the change in CHOP protein localization and clustering brought on by fusion with FUS is necessary and sufficient for altering transcription. This work is novel and has implications for the field of chromatin organization and function, and for understanding of oncogenic progression in specific cancers. With minor edits, I recommend it for publication in JCS. Please see suggested edits by figure below.

Introduction
In the introduction, please add a citation or figure reference for "importantly, all MLS-causing FUS-CHOP translocations contain portions of this LC sequence."

We have included the appropriate reference for this section.

"Likewise, our results suggest FUS-CHOP can undergo a liquid-phase transition in the nucleus, which could provide the mechanism for its emergent gain-of-function oncogenicity. This may be a general mechanism for transcriptional activation by fusion oncoproteins with IDRs."
Provide more specificity of mechanism here or in the discussion, what would phase separation provide that mis-localization of CHOP would not?

We have elaborated and included more references that show strong evidence for phase separation in transcriptional control.

Figure 1.
Please indicate in the figure where the TEV protease site is inserted, I assume between MBP and FUS.

We indicated the TEV cleavage site in figure 1.

What is the domain structure of CHOP? Does it have a DNA binding domain and any self-

interaction domain?

We have included the structure of CHOP and a better description of CHOP in the text.

Is formation of droplets in vitro concentration-dependent? Were the authors able to make any sort of phase diagram dependent on protein or salt concentration? This would lend understanding to the concentrations required in vivo to create nuclear foci.

We have included data showing the relationship between FUS-CHOP phase separation and salt concentration (Fig 1C). While our manuscript was under review Zuo et al showed FUS and EWS fusion proteins can phase separate with DNA and induce transcription. In this manuscript they were able to create phase diagrams of the fusion proteins with DNA. Their data show DNA lowers the critical concentration necessary for FUS and EWS fusion protein phase separation in vitro, suggesting DNA promotes phase separation. (PMID: 33674598)

Figure 2.
Please provide a scale bar for microscopy images.

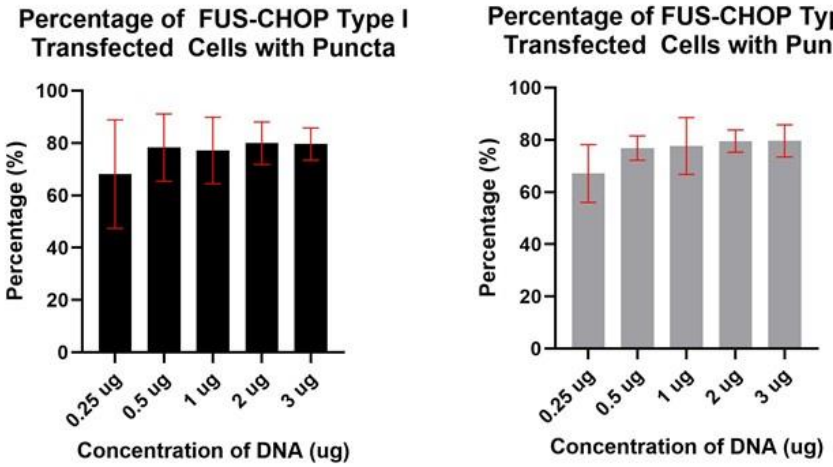
We have included scale bars, as suggested.

Have the authors or previous studies co-stained these nuclei with markers for other known nuclear bodies, i.e. nuclear speckles or PML bodies? Are the fusion proteins partitioning into pre-formed structures or creating novel compartments? This knowledge would help to interpret whether FUS-CHOP is a driver of phase separation within the nucleus or a 'client' of compartments formed by other Proteins.

Previous studies have shown that FUS-CHOP nuclear puncta are distinct from PML bodies (Thelin-Jarnum et al., 2002). Goransson et al. showed Cajal Bodies localize to the periphery of some of the FUS-CHOP nuclear puncta. This finding was recapitulated for other transcriptional condensates in recent data showing Cajal bodies localizing to RNA pol II body periphery (Imada et al, 2021) - (PMID: 33608942). We have mentioned this in the text.

Also, are these FUS-CHOP foci concentration-dependent? It would strengthen the in vivo phase separation mechanism argument to see a saturation concentration, below which the protein is dispersed throughout the nucleus, and above which these foci form.

We used large field of view images to determine if ectopic expression of lower concentrations of FUS-CHOP type I or II resulted in a change in the percentage of cells with nuclear puncta. We counted the number of GFP expressing cells containing puncta and determined at lower concentrations of DNA, we see a slight decrease of cells with puncta, but our transfection efficiency also decreased as a result of lower DNA concentration, but we still see majority of cells with nuclear puncta. We have also included a statement in the text reguarding changes in concentration and nuclear localization. See new data below:



2B. It is unusual to use a cytoplasmic protein like tubulin as a loading control for nuclear proteins. Have the authors also used a nuclear protein loading control like a histone?

We have included endogenous nuclear FUS in all of our blots as a control.

Differences in its signal would indicate loading problems; however, expression comparisons were not used for our experimental goals.

Figure 3.
Please add timescale and scalebar to Figure 3. Did the fusion take similar timeframes for type I and type II fusion proteins? Does this match the FRAP data?

We have included timescales and scale bars on the images. Fusions are occurring in seconds to minutes. The FRAP data shows internal rearrangement and external exchange anywhere from 5 seconds to ~35 seconds.

Figure 4.
How were these FRAP data normalized? It seems the fluorescence signal only recovers to 30 percent in the quantification but by eye in the images is brighter than 30% of original by the 60 seconds timepoint. Are these images not shown at the same contrast, or could they be oversaturated?

The FRAP data was normalized as follows: "The recovery was quantified using the time series analyzer V3 plugin on Fiji. The bleached pixel intensity was subtracted from each data point and then data points were normalized to the pixel intensity before the bleaching occurred." We have reprocessed and updated the images in figure 3B to ensure the contrast was the same and the images were not oversaturated.

How does this compare to FRAP of just CHOP without FUS?

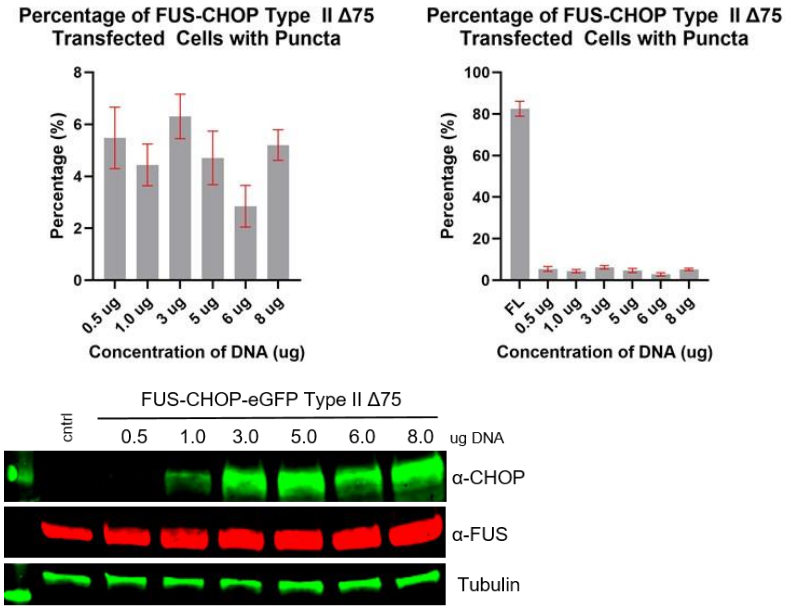
We have included FRAP data for CHOP-eGFP in figure 3D.

The authors may consider combining figures 3 and 4, as these data are both related to the material properties of FUS-CHOP foci and the total number of figures is high.

We have combined our figures, as suggested.

Figure 5 and 6.
With the FUS-CHOP truncation proteins, both at N-term and internally, it is again interesting to ask whether foci formation is concentration-dependent. If phase separated, we would expect shorter sequences (those with weaker self-self interaction) to need higher concentration to form foci (higher saturation concentration).

We quantified the number of transfected cells containing nuclear puncta for type I and II truncation constructs and observed a higher percentage of cells with puncta for those constructs with longer sequences. We ectopically expressed increasing concentrations of the delta-75 type II sequence to determine if a higher concentration of DNA was necessary for formation.
Interestingly, we observed no significant increase in the number of cells with puncta when we transfect cells with 0.5 - 8 ug of DNA. *We have also included a statement in the text reguarding changes in concentration and nuclear localization. See new data below:*



5C-D. Please add scale bar to C and report number of cells or bleaches per replicate in D.

We have included scale bars and numbers, as suggested.

6A. Usually internal deletions are denoted with ^, not -

We have made this adjustment, as suggested.

Figure 7.
Do the authors have a way to compare expression levels of the exogenous proteins expressed in NIH3T3 cells with patient derived cells? Are the smaller foci perhaps a result of lower overall protein expression level?

We noted that our ectopic expression model is imperfect. We hypothesize that ectopic expression may have unknown variables beyond concentration (though expression level is the most prominent variable). When comparing our ectopic expression levels to the expression of FUS-CHOP in the cancer cell line, the ectopic proteins are at greater expression levels. However, for any given ectopic fusion, expression levels did not seem to affect its LLPS. We mention the possibility ectopic expression may alter phase separation in the Discussion. Below is the comparison for your reference:

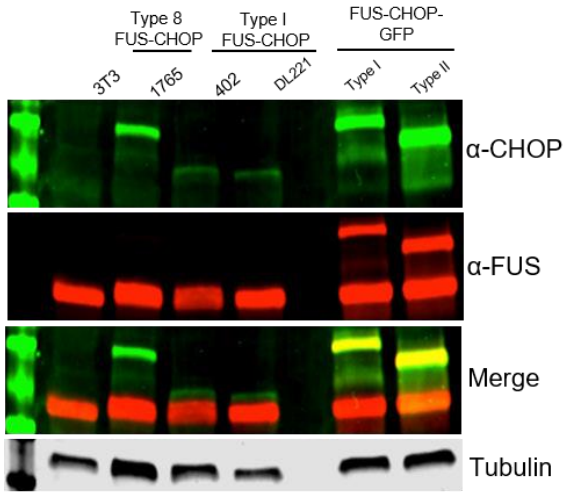


Figure 8.
In this study, the authors did not directly confirm the localization of BRD4 at super enhancers in

these cells, I suggest rewording "These data suggest FUS-CHOP and BRD4 occupy the same nuclear condensates at SEs.", to remove 'at SEs.' Pearson correlation coefficient values reported seem very high for the images provided—are these values correct? Are they reproducible in multiple cells?

We have changed our language to be more precise, as suggested. Pearson's correlation coefficient data was re-evaluated. We have added the data from multiple experiments to the figure to better depict the colocalization values.

Discussion
"This suggests FUS-CHOP could be driving phase separation of BRD4 at oncogenic SEs in MLS. These findings provide a mechanism by which oncogenic fusion proteins, such as EWS-FLI1 and FUS-CHOP, hijack BRD4 and other bromodomain-containing proteins to induce oncogenic SEs (Chen et al., 2019b; Gollavilli et al., 2018) " This logic is a bit messy, BRD4 is a protein that phase separates at SEs but the authors claim it is being driven by FUS-CHOP, the data provided in this study show that these proteins colocalize but do not demonstrate which protein is driving the condensation. See suggestions for figure 2 for potential ways to further support claims that FUS-CHOP is indeed driving formation of condensates in vivo.

We have addressed those suggestions for Figure 2. FUS-CHOP forms distinct compartments from other known and characterized nuclear bodies. Our data demonstrates big droplet accumulation of BRD4 into phase separated condensates following ectopic expression of FUS-CHOP, suggesting FUS-CHOP can recruit BRD4 to these distinct puncta.

Also, knowledge of protein-protein interactions would help support these claims as well. Is it known that BRD4 and FUS-CHOP directly interact whereas BRD4 and CHOP do not?

Previous work showed BRD4 and FUS-CHOP interact in MLS cancer cell lines (Chen et al., 2019). As stated in the manuscript: "Normally, CHOP expression is suppressed, but upregulated during differentiation and following cellular stress (Ohoka et al., 2007; Yang et al., 2017)". Other members of the C/EBP family of transcription factors have been shown to interact with the BET family of proteins, but because CHOP is not expressed under normal conditions, CHOP and BRD4 localization could only be assessed in an ectopic expression model or under a stressed condition (PMID: 30466442).

Overall, this study furthers our understanding of the FUS-CHOP onco-protein localization and biophysical properties in both patient-derived cells and exogenous systems (NIH3T3 plus overexpression). It supports claims of in vivo phase separation with measures of liquidity, sphericity and protein diffusion. Importantly, FUS-CHOP is also able to form phase-separated droplets in vitro. In both mouse and human cells, the fusion protein forms spherical puncta in the nucleus. Interestingly, these puncta also contain BRD4 in the patient-derived cells, suggesting that they may be sites of highly active transcription like super-enhancers. The functional and mechanistic role of phase separation in living cells has been difficult to determine directly, but its pursuit is a useful and interesting perspective shift for the fields of nuclear organization and cancer progression. This paper seeks to demonstrate that altering phase-separation properties of a transcription factor CHOP, by adding a low-complexity sequence from a known phase-separating protein FUS, can explain the alteration of nuclear localization and function of this fusion protein. Indeed, the fusion protein has liquid-like properties and localizes to structures that are likely phase-separated. This is a novel demonstration that changes in one protein's phase separation properties can drive the differences in protein function that lead to altered transcription and cancer.

Reviewer 3:
Reviewer 3 Advance Summary and Potential Significance to Field:
In the manuscript submitted by Owen et al., the authors characterize in detail the ability of the FUS-CHOP protein to undergo phase separation in cells. FUS-CHOP plays a critical role in myxoid liposarcoma, MLS, and other FUS fusions can drive an assortment of sarcomas. The results of this study are important because understanding of phase separation by these cancer-causing fusion proteins is still surprisingly limited, and recent studies offer few answers significant enough for cancer research to build on. The data provided by this study is easy to compare and contrast with previous studies for a new perspective on the questions they've raised.

Reviewer 3 Comments for the Author:
The novelty of this work has not been fully demonstrated. The fusion of FUS and CHOP is disease relevant, but the authors haven't completed the story of whether the function of CHOP has any relevance to phase separation. The imaging-based approaches applied here do not yield results identical to observations of FUS-CHOP in the MLS cells, highlighting this question whether the CHOP domain is functional or if these observations are more relatable to LC domain fusions previously reported, not the activity of the fusion in MLS. This needs to be addressed somehow and some options are detailed in major comment #4 below.

1. Recent studies have called into question how to define phase separation by FUS or EWSR1 fusion proteins, particularly for EWS-FLI1, and how the stability or dynamics of assemblies they produce compare to natural condensates associated with transcription (Chong et al., Science 2018; McSwiggen et al., Elife 2019; McSwiggen et al., Genes Dev 2019). The in vitro data in Figure 1B would greatly benefit from FRAP data as performed in vivo, in order to compare with those in vivo results and similar published data for FUS, EWSR1, related proteins, and their fusions.

While our manuscript was under review Zuo et al was able to show FUS and EWS fusion proteins can phase separate with DNA and induce transcription. In this manuscript they show both FUS and EWS fusion proteins have liquid-like dynamics in vitro when assessed by FRAP. They were also able to show DNA slows the recovery of these puncta, suggesting higher order structures (PMID: 33674598). We have included this reference in our results section.

2. The delta 50-75 truncation of FUS-CHOP does not sufficiently address the question about length vs location of the deletion impacting phase separation. It is noticeable that the first 75 amino acids in FUS are more enriched with tyrosine motifs, which are more tightly spaced than the rest of the LC-domain. A deletion in the latter half of the LC domain (50-100, 75-125, or 50-125) would provide a better indication whether the position and spacing of the motif has a significant influence on FUS-CHOP phase separation in cells. The observation of shortened LC domains such as Types V and IX in Fig S1 may argue they don't impact phase separation, or they do and that's not important to function and MLS. It would also be helpful if the authors acknowledge the number of tyrosine motifs in each deletion, as this is a metric easily compared to a previous publications (Lin et al., JBC 2017, PMID: 28924037; Murthy et al., NSMB 2019, PMID: 31270472; Chong et al., Science 2018, PMID: 29930090).

We designed additional mutants (delta aa50-125 and delta aa75-125) for type I and type II that have been added to the manuscript. The results were similar to the n-terminal truncations, so we believe this suggestion strengthened the conclusions. We also commented in the text and added a table (Supplementary table 1) outlining the number of tyrosine residues removed in each truncation.

3. Figures 5 and 6 and their sub-parts are referenced out of order in the results section. This is not aligned with the author guidelines for J Cell Science and also confusing to the reader. Moreover, the purpose of the upper and lower panels in Figure 5D is unclear. Is this the same data presented two ways or different experiments? The description of 5D is vague in the text and the legend, leaving questions about what measurement and experimental protocol is shown. Correcting this may help clarify the section that describes Figures 5 and 6, which was particularly difficult to follow.

We have removed the confusing data and reorganized the text, as suggested.

4. The primary piece of information that is missing is the role of DNA-binding. The authors reference studies that have already shown phase separation in cells by FUS or LC domain fusions to tags. If the advance achieved here is that the CHOP domain has not precluded the protein to phase separate, this leaves a question whether the protein imaged is functionally competent to bind DNA or just the remainder of protein that isn't bound. Two ways that may address this are: (1) add DNA to the phase separation assay in Fig 1 and infer that DNA binding may or may not also occur in the cell without interfering with phase separation; or,

While our manuscript was under review Zuo et al was able to show FUS and EWS fusion proteins can phase separate with DNA and induce transcription. In this manuscript they show phase diagrams including various concentrations of protein and DNA for both FUS and EWS fusion proteins. They were also able to show DNA lowers the critical concentration necessary

for phase separation (PMID: 33674598). These data infer that DNA promotes phase separation of FUS fusion proteins.

(2) mutate the CHOP domain to test whether the results of figures like 2C draw closer or further away from results in 7B. For the in vitro approach, the more careful analysis offered by the FRAP measurement (see also comment 1 above) and comparing a DNA-binding deficient fusion would increase the value of the result, since it must rely more on inference. For the second option, it would be compelling to see that phase separation in cells by a FUS-CHOP or truncated FUS-CHOP (e.g. delta 25 or 50) mutated to be deficient in DNA-binding is visually distinct from the observations in Fig 2C. However, if there is no engineered solution that can show the CHOP domain or DNA-binding matters, this still needs to be acknowledged by testing it because this is important to the disease models and also central to setting this study apart from previous publications.

We have ectopically expressed a DNA-binding mutant of FUS-CHOP type I and II in cells. Previous work shows deletion of the basic region of CHOP inhibits CHOP DNA binding. Here, we created a previously established DNA binding mutant by removing CHOP amino acids 101-122. (PMID: 8657121). We see both type I and type II FUS-CHOP mutants can still form nuclear puncta, regardless of DNA binding capacity. These data suggest that the N-terminus of FUS is sufficient to drive the interactions necessary for condensate formation without the DNA binding potential from CHOP.

5. The authors acknowledge that co-localization of FUS-CHOP and BRD4 on the DNA is previously known (ref Chen et al., 2019b). Since the role of the CHOP domain for the condensates observed in this study is unknown, questions arise whether phase separation by FUS-CHOP is merely in the same euchromatic nuclear compartments as BRD4, or if BRD4 itself can pull FUS-CHOP into a condensate even without a functional CHOP domain. The following would not be so necessary if #4 above was addressed. The results in Fig 8A and 8B do not appear so similar but could be strengthened either by better statistical and quantitative analysis to compare the two, or test whether a DNA-binding deficient FUS-CHOP can colocalize with BRD4, or test if BRD4 in cells still binds the truncations that are unable to phase separate:
e.g. delta 75 or delta 125.

The Pearson's correlation coefficient data was re-evaluated. We have added the data from multiple experiments to figure 7 to determine colocalization values as accurately as this methodology allows. Additionally, we have ectopically expressed our DNA-binding mutants of FUS-CHOP and probed for BRD4. Interestingly, we see BRD4 localizing with FUS-CHOP nuclear puncta (deficient of DNA binding), but not as readily as observed with the full length constructs. These data suggests that BRD4 and FUS-CHOP interaction is influenced by DNA binding, but is governed by phase separation that is driven by the N-terminus of FUS.

Second decision letter

MS ID#: JOCES/2021/258578

MS TITLE: The oncogenic transcription factor FUS-CHOP can undergo nuclear liquid-liquid phase separation

AUTHORS: Izzy Owen, Debra Yee, Hala Wyne, Theodora Myrto Perdikari, Victoria Johnson, Jeremy Smyth, Robert Kortum, Nicolas L Fawzi, and Frank Shewmaker

ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers recognize that most of their initial criticisms have been addressed in your revised manuscript. However, reviewer #2 still raised issues that will require amendments to your manuscript. I hope that you will be able to carry these out, because I would like to be able to accept your paper.

We are aware that you may be experiencing disruption to the normal running of your lab that makes experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

The manuscript "The oncogenic transcription factor FUS-CHOP can undergo nuclear liquid-liquid phase separation" by Owen et al. presents a well-performed set of experiments and a very intriguing hypothesis linking fusion protein phase separation to transcription of oncogenic genes. The characterization of FUS-CHOP fusion mutants is novel and will provide a base for further investigation in direction of FUS-IDR characterization.
Also, co-localization of FUS-CHOP protein with a super-enhancer marker such as BRD4 is a fundamental finding. The precise characterization of this finding remains a future task to understand the role and crosstalk between super-enhancer proteins and aberrant fusion proteins in cancer progression.

Comments for the author

The manuscript in its current form fulfills the major revision points provided to the authors. The manuscript is suitable for publication in the Journal of Cell Science. The manuscript will be of interest to a broad audience in the fields of transcription, phase separation, and cancer biology. This study provides a starting point for the investigation of transcription factors and their fusion counterparts as a strategy employed by cancer cells to rewire their transcription.

Reviewer 2

Advance summary and potential significance to field

In their manuscript “The oncogenic transcription factor FUS-CHOP can undergo nuclear liquid-liquid phase separation,” Owen et al find that the translocation of FUS onto transcription factor CHOP provides phase separation propensity to the fusion protein in vitro, and changes the nuclear localization pattern of a transfected construct in NIH3T3 cells. The puncta formed in NIH3T3 nuclei fuse and round up, and exhibit rapid FRAP recovery indicative of liquid-like material state. In agreement with previous studies, no specific region of the FUS IDR is required for this behavior; rather the total length of IDR appended to CHOP is important to confer the altered localization and liquid-like properties.
Interestingly, these FUS-CHOP nuclear puncta also include BRD4, a potent transcriptional activator also involved in cancer progression, in both NIH3T3 and MLS patient cells. Together, these data demonstrate that the fusion protein FUS-CHOP has phase-separation tendencies in nuclei of living

cells, and is consistent with an altered transcriptional program due to novel interactions of transcriptional activators like BRD4 with FUS-CHOP's DNA targets.

I hesitate to fully support some of the author's language, as the experiments contained here do not explicitly show phase-separation-dependent functionality in vivo. For example, in the abstract, the authors state that their data "indicate FUS-CHOP forms phase-separated condensates at super enhancer transcriptional sites" and provides "evidence that the FUS-CHOP phase transition is a novel oncogenic mechanism and potential therapeutic target for myxoid liposarcoma."
While colocalization of FUS-CHOP with BRD4 suggests novel interactions, the authors do not investigate whether these are active transcriptional sites or if they map to genomic super-enhancer sites. However, the investigation of the role of FUS in altering CHOP transcription factor's in vitro phase behavior, in vivo nuclear localization and interaction is novel and with careful alteration to wording, I support publication of this manuscript in JCS.

Comments for the author

Owen et al have attempted the requested experiments and made improvements to their original manuscript. I hesitate to fully support some of the author's language, as the experiments contained here do not explicitly show phase-separation-dependent functionality in vivo. For example, in the abstract, the authors state that their data "indicate FUS-CHOP forms phase-separated condensates at super enhancer transcriptional sites." While colocalization of FUS-CHOP with BRD4 suggests novel interactions, the authors do not investigate whether these are active transcriptional sites or if they map to genomic super-enhancer sites. However the investigation of the role of FUS in altering CHOP transcription factor's in vitro phase behavior, in vivo nuclear localization and interaction is novel and with careful alteration to certain wording, I support publication of this manuscript in JCS.

Specific suggestions:
Abstract:
"Our data indicate FUS-CHOP forms phase-separated condensates at super enhancer transcriptional sites."
"Our data indicate FUS-CHOP forms phase-separated condensates that colocalize with BRD4, a marker of super enhancers."
"In an MLS cell line, FUS-CHOP was found to co-localize at SEs with BRD4 – a protein that is proposed to control gene expression through the formation of phase-separated condensates at SEs (Sabari et al., 2018)"
What is the citation for the first half of this sentence? The Sabari paper only covers the second half. Is it data from this paper? Please reword to make more clear.
Page 4. Add figure reference in the sentence, "demonstrating that elements within FUS's LC sequence drive phase separation (Fig. 1B, C).
"Ectopically expressed FUS-CHOP-eGFP is undergoing LLPS in the nucleus."
"Nuclear puncta of ectopically expressed FUS-CHOP-eGFP are liquid-like"
o FRAP and fusion are indicative of liquid-like material state, but do not prove LLPS.

"Imaging the cells over time revealed free movement around the nucleus consistent with Brownian motion"
o Time-lapse imaging reveals diffusive movement of puncta, which occasionally contact each other and subsequently round up (Fig 3A, S2, Movie 1).
"We hypothesize that the RGG repeats present in the longer type I fusion could be driving additional protein-protein or protein-RNA interactions, leading to more mobility throughout the nucleus."
o Having additional interactions should be expected to have slower diffusion, I suggest rewording to "leading to differential diffusive behavior"
Page 5. "Increasing the concentration of FUS-CHOP-eGFP DNA (full length or truncated) in cellular transfections did not yield observable changes in nuclear pattern of expression, indicating LLPS is determined by the length of FUS's PrLD but not expression levels."
o My original suggestion was that if the fusion protein is truly phase separating in vivo, varying the expression level of FUS-CHOP-eGFP protein should lead to a saturation concentration (Csat), below which puncta do not form. It is unclear from this description whether the authors achieved a variety of protein expression levels, especially in the lower expression regime, which

would be essential for measuring the saturation concentration. I suggest removing the indicated sentence, since no conclusion can be made.

Reviewer 3

Advance summary and potential significance to field

From previous review*
In the manuscript submitted by Owen et al., the authors characterize in detail the ability of the FUS-CHOP protein to undergo phase separation in cells. FUS-CHOP plays a critical role in myxoid liposarcoma, MLS, and other FUS fusions can drive an assortment of sarcomas. The results of this study are important because understanding of phase separation by these cancer-causing fusion proteins is still surprisingly limited, and recent studies offer few answers significant enough for cancer research to build on. The data provided by this study is easy to compare and contrast with previous studies for a new perspective on the questions they've raised.

Comments for the author

In the revised manuscript by Owen et al., the authors have done an excellent job addressing my questions. The exciting result that BRD4 can co-localize to the DBD-deficient FUS-CHOP elevates the broad interest of this study as well. This finding matches nicely with findings from a few reports that dominant negative effects of FUS or EWSR1 fusions on some proteins, such as EWSR1, can be reproduced by DBD-deficient mutants (e.g. Gorthi et al., Nature 2018, PMID:29513652). The additional truncations in the LC domain are also a particularly useful addition for the field, which needs more of this study's consistency and completeness for future researchers to build upon. I'm particularly pleased to see this manuscript published in the Journal of Cell Science.

Second revision

Author response to reviewers' comments

We thank the Reviewer for again carefully evaluating our manuscript. The Figures are unchanged, but the conciseness of our text is greatly improved due to the suggestions of the Reviewer. Here are the Reviewer's suggestions paired with our updated text:

Reviewer 2 Advance Summary and Potential Significance to Field:
In their manuscript "The oncogenic transcription factor FUS-CHOP can undergo nuclear liquid-liquid phase separation," Owen et al find that the translocation of FUS onto transcription factor CHOP provides phase separation propensity to the fusion protein in vitro, and changes the nuclear localization pattern of a transfected construct in NIH3T3 cells. The puncta formed in NIH3T3 nuclei fuse and round up, and exhibit rapid FRAP recovery indicative of liquid-like material state. In agreement with previous studies, no specific region of the FUS IDR is required for this behavior; rather the total length of IDR appended to CHOP is important to confer the altered localization and liquid-like properties. Interestingly, these FUS-CHOP nuclear puncta also include BRD4, a potent transcriptional activator also involved in cancer progression, in both NIH3T3 and MLS patient cells. Together, these data demonstrate that the fusion protein FUS-CHOP has phase-separation tendencies in nuclei of living cells, and is consistent with an altered transcriptional program due to novel interactions of transcriptional activators like BRD4 with FUS-CHOP's DNA targets.

I hesitate to fully support some of the author's language, as the experiments contained here do not explicitly show phase-separation-dependent functionality in vivo. For example, in the abstract, the authors state that their data "indicate FUS-CHOP forms phase-separated condensates at super enhancer transcriptional sites" and provides "evidence that the FUS-CHOP phase transition is a novel oncogenic mechanism and potential therapeutic target for myxoid

liposarcoma." While colocalization of FUS-CHOP with BRD4 suggests novel interactions, the authors do not investigate whether these are active transcriptional sites or if they map to genomic super-enhancer sites.
However, the investigation of the role of FUS in altering CHOP transcription factor's in vitro phase behavior, in vivo nuclear localization and interaction is novel and with careful alteration to wording, I support publication of this manuscript in JCS.

Reviewer 2 Comments for the Author:
Owen et al have attempted the requested experiments and made improvements to their original manuscript. I hesitate to fully support some of the author's language, as the experiments contained here do not explicitly show phase-separation-dependent functionality in vivo. For example, in the abstract, the authors state that their data "indicate FUS-CHOP forms phase-separated condensates at super enhancer transcriptional sites." While colocalization of FUS-CHOP with BRD4 suggests novel interactions, the authors do not investigate whether these are active transcriptional sites or if they map to genomic super-enhancer sites. However, the investigation of the role of FUS in altering CHOP transcription factor's in vitro phase behavior, in vivo nuclear localization and interaction is novel and with careful alteration to certain wording, I support publication of this manuscript in JCS.

Specific suggestions:

Abstract:
"Our data indicate FUS-CHOP forms phase-separated condensates at super enhancer transcriptional sites."
"Our data indicate FUS-CHOP forms phase-separated condensates that colocalize with BRD4, a marker of super enhancers."
We have modified our language. The sentence now states: "Our data indicate FUS-CHOP forms phase-separated condensates that colocalize with BRD4, a marker of super enhancer condensates."

"In an MLS cell line, FUS-CHOP was found to co-localize at SEs with BRD4 – a protein that is proposed to control gene expression through the formation of phase-separated condensates at SEs (Sabari et al., 2018)" What is the citation for the first half of this sentence? The Sabari paper only covers the second half. Is it data from this paper?
Please reword to make it more Clear.
We have added the reference (Chen at al 2019b; PMID: 30903020), which shows FUS-CHOP and BRD4 localization to super-enhancer loci in MLS cell lines.

Page 4. Add figure reference in the sentence, "demonstrating that elements within FUS's LC sequence drive phase separation (Fig. 1B, C).
We have added the figure references, as suggested.

"Ectopically expressed FUS-CHOP-eGFP is undergoing LLPS in the nucleus." "Nuclear puncta of ectopically expressed FUS-CHOP-eGFP are liquid-like"
FRAP and fusion are indicative of liquid-like material state, but do not prove LLPS.
We acknowledge the Reviewer's careful language, but we prefer to not make changes in this specific case because we are using the vernacular of the field. Most published studies consider distinct sphere-shaped puncta, puncta that fuse upon touching and puncta that undergo internal rearrangement on the time scale of seconds as undergoing LLPS. Here are some examples:

- **PMID: 31444270**
    18. ***"P granule is a collection of RNA and RNA-binding proteins (RBPs) localized at the posterior cortex of a dividing embryo. P granules appear as spherical droplets with liquid-like properties, and they fuse with one another, deform under shear stress, and flow off the surface of the nucleus. Fluorescence recovery after photobleaching (FRAP) analysis demonstrated rapid turnover rates of constituent proteins, which is indicative of fast molecular rearrangements. These observations together suggested that P granules form through liquid–liquid phase separation (LLPS), distinct from canonical macromolecular assemblies."***

- **PMID: 25288112**
  19. ***"Therefore, over timescales of seconds, P granules have all the key signatures of a liquid state. They fuse, they drip, they are spheres, and they rearrange their contents within seconds (see Figure 3b-d). For any non-membrane-bound compartment in a cell, the turnover properties are sufficient to specify that it is a liquid."***
- **PMID: 29602697**
  20. ***"This is a physical process that occurs when a supersaturated solution of components spontaneously separates into two phases, a dense phase and a dilute phase, that then stably coexist. The proposed liquid-like nature of P granules was evident from their round appearance (the result of minimizing surface tension)*, deformability (fusion and fission events), and dynamic exchange of components. Similar observations were made 2 years later for nucleoli "**

"Imaging the cells over time revealed free movement around the nucleus consistent with Brownian motion" Time-lapse imaging reveals diffusive movement of puncta, which occasionally contact each other and subsequently round up (Fig 3A, S2, Movie 1).
We have modified our language to: "Time-lapse imaging of the cells revealed diffuse movement of the puncta, consistent with Brownian motion. We observed occasional fusion events in which two puncta contact each other and subsequently round up (Fig. 3A, S2, Movie 1)."

"We hypothesize that the RGG repeats present in the longer type I fusion could be driving additional protein-protein or protein-RNA interactions, leading to more mobility throughout the nucleus." Having additional interactions should be expected to have slower diffusion, I suggest rewording to "leading to differential diffusive behavior"
We have modified our language to: "leading to differential mobility throughout the nucleus. However, we did not pursue this observation further."

Page 5. "Increasing the concentration of FUS-CHOP-eGFP DNA (full length or truncated) in cellular transfections did not yield observable changes in nuclear pattern of expression, indicating LLPS is determined by the length of FUS's PrLD, but not expression levels." My original suggestion was that if the fusion protein is truly phase separating in vivo, varying the expression level of FUS-CHOP-eGFP protein should lead to a saturation concentration (Csat), below which puncta do not form. It is unclear from this description whether the authors achieved a variety of protein expression levels, especially in the lower expression regime, which would be essential for measuring the saturation concentration. I suggest removing the indicated sentence, since no conclusion can be made.
To avoid confusion, we have removed the statement regarding concentration and the previous sentence has been modified: "Removing 75 or 125 amino acids resulted in a diffuse pattern of expression, indicating LLPS is determined by the length of FUS's PrLD (Fig. 4C, S3)."

Third decision letter

MS ID#: JOCES/2021/258578

MS TITLE: The oncogenic transcription factor FUS-CHOP can undergo nuclear liquid-liquid phase separation

AUTHORS: Izzy Owen, Debra Yee, Hala Wyne, Theodora Myrto Perdikari, Victoria Johnson, Jeremy Smyth, Robert Kortum, Nicolas L Fawzi, and Frank Shewmaker

ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.