Nuclear body phase separation drives telomere clustering in ALT cancer cells

Huaiying Zhang, Rongwei Zhao, Jason Tones, Michel Liu, Robert Dilley, David Chenoweth, Roger Greenberg, and Michael Lampson

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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.)

RE: E19-10-0589
TITLE: Nuclear body phase separation drives telomere clustering in ALT cancer cells

Dear Dr. Zhang,

As you will see, while the reviewers find our observations interesting, they also raise major issues which need to be addressed before we can further consider the manuscript. Specifically, both referees would like to see a more in-depth characterization of the condensates that you observe. Secondly, and more importantly, the referees are both requesting stronger evidence for a functional role of the condensates in ALT. As indicated by both referees, your system offers a unique opportunity to test the functional relevance of a condensate. I refer you to the referees' specific comments for details on these and additional technical points that would need to be addressed before further consideration of the manuscript.

Dr. Tom Misteli

Dear Dr. Zhang:

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript is not acceptable for publication at this time, but may be deemed acceptable after specific revisions are made. Any specific areas to be addressed are outlined in the reviewer comments included below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

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Please contact us with any questions at mboc@ascb.org.

Thank you for submitting your manuscript to Molecular Biology of the Cell. We look forward to receiving your revised paper.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org

Reviewer #1 (Remarks to the Author):

Comments on Zhang et. al. Molecular Biology of the Cell E19-10-0589.

In this study by Zhang et al., the authors characterize the role of liquid-liquid phase separation in the formation of ALT-associated promyelocytic leukemia (PML) nuclear bodies (APBs). have a long lasting experience in the field and previously Previous studies showed that introducing DNA damage at telomeres leads to APB formation, telomere clustering within the induced APBs, and telomere elongation via HR repair (Cho et al., 2014). These studies also showed a role for Rad51-driven homology search in promoting telomere clustering (Cho et al., 2014). Nevertheless the mechanism governing APB assembly and function are still unclear, also due to limitations in how to study this biological process. Here, the authors show that APBs have liquid-like behavior in vivo, and are enriched for SUMOylated proteins. Expression of a chemical dimerizer that links the telomeric protein Trf1 and a SUMO-binding motif (SIM) induces a liquid-like behavior at telomeres and telomere clustering, even in the absence of telomeric damage and repair proteins. This supports a role for SUMOylation in inducing the liquid-like behavior of APBs, consistent with recent studies (Min et al., 2019). Expression of a chemical dimerizer that links the telomeric protein Trf1 to a RGG domain of Laf-1, which is known to induce non-APB liquid-like behavior, is sufficient to induce telomere clustering even in the absence of damage or damage-induced SUMOylation, supporting a model where the liquid-like behavior promotes telomere clustering. Together, these data support a role for telomeric-damage induced SUMOylation in telomeric clustering via phase separation.

One of the main strengths of this paper is the generation and validation of new tools to induce a reversible SUMOylation-driven phase separation at telomeres. The role of SUMOylation in phase separation and telomere clustering has been recently shown (Min et al., 2019), and this study provides a strong independent validation of this response. However, a major missing point in the study by Zhang et al. is the characterization of the role of damage-induce phase separation in telomere repair, which is also important to validate the newly generated tools. Statistical validations and a higher number of cells/replicates should also be provided for most experiments.

Major points:

1) The damage-induced (Fok1) and chemical dimerizer-induced telomere clusters display liquid-like behavior, including coalescence after collision and dynamic exchange of components by FRAP. It would be important to provide an independent validation that these structures are, indeed, phase separated. For example, are these clusters disrupted by 1,6-hexanediol, a compound known to disrupt liquid-like condensates?

2) The authors propose a model where telomere clustering depends on the liquid properties of APBs and leads to telomere elongation in ALT cells. However, repair is never investigated throughout the paper. This also questions the extent to which the tools generated (dimerizer-induced telomere clustering with SIM or Laf-1) mimic the endogenous phase separated state for telomeres in ALT cells. The consequences of impairing the liquid properties of ABPs on HR repair of telomeres should be shown by measuring telomere length (such as by terminal restriction fragment

analysis and/or Q-FISH) or Rad51 recruitment at telomeres.

3) A limited number of cells and experiments are shown throughout the paper. Experiments should be provided in triplicates and with a quantification of several cells/exp to account for variability across individual cells and experimental variations (Fig 1B,C; Fig. 2C,D,E; Fig. S2, S3, S4, etc).

4) How do the authors reconcile their model where phase separation drives telomere clustering in ALT cells with previous models where directed motions characterize homology search in ALT cells (which in turn drives clustering)? This should be added to the discussion.

5) The authors state in the discussion that their 'findings provide an opportunity to target physical-chemical properties of APBs for cancer therapy in ALT without affecting the function of their DNA repair components'.

Liquid-liquid phase separation is critical to organize nuclear activities spatially and temporally in membraneless domains. To what extent other chromatin domains rely on phase separation for their spatial-temporal confinement and for their biological functions is a matter of intense investigation, but tools that disrupt phase separation described here (such as by interfering with SUMOylation) are likely to affect other nuclear functions. How the authors are suggesting to target APBs without affecting other nuclear functions requires a more extensive discussion.

Minor points:

1. Introduction - lines 70-73. While APBs are proposed to be sites of telomere recombination during ALT, the precise functions of these specialized PML nuclear bodies are poorly understood and whether their formation requires a DNA damage response. The sentence needs to be completed.

2. Fig. 1B,D: it is unclear how many clustering events are quantified here. A quantification of the frequency of clustering over several cells and 3+ experiments should be provided. SD/SEM values should also be provided.

3. table in supplementary information showing that APB components contains SIM domain or are SUMOylated (or both) including references, could be provided for the readers.Figures 1D, 2B-D, S1, S2: It would be clearer if the authors specified in the figures and graphs the mutant to which they refer (i.e. 'Fok1 mutant' or 'SIM mutant' instead of 'mutant').

4. Figure S1: should be shown with the same color scheme as Fig. 1D, for clarity. Quantifications of the percent of telomeres with SUMO 2/3 foci and Integrated intensity of SUMO 2/3 foci on telomeres/cell should also be provided (similar to Fig. 1D).

5. Fig. 2E appears to show defective recruitment of telomeres of the SIM mutant. A quantification should be provided to test whether this is the case. If not, a better set of images should be provided. If the SIM mutant is, instead, defective in its recruitment to telomeres, that would provide an alternative explanation to the lower level of SUMOylated proteins at telomeres.

6. Figure S2: should be shown with the same color scheme as figure 2B, for clarity.
7.
8. 'Decoupling of APB functions' - page 7. Literature references should be added in the first paragraph.

9. Figure S3 - text in the figure: Clutering> clustering

10. Figure S4 - insets indicating FokI, SIM colocalization with 53BP1 and POLD3 should be provided inside the images. Quantifications are also missing for this figure.

11. Discussion - line 284. 'Coalescence of APB liquid droplets drives telomere clustering (Figure 3A-E) may provide'... this sentence is missing 'that'.

12. Line 139. ABPs > APBs

Reviewer #2 (Remarks to the Author):

Alternative lengthening of telomeres (ALT) is used by around 10-15% of cancers to prevent telomere shortening and senescence, and targeting ALT is therefore a promising anti-cancer strategy. Several mechanistic aspects of ALT remain poorly understood however, including the precise role of ALT-associated promyelocytic leukemia (PML) nuclear bodies (APB) for telomere maintenance and stability. In their manuscript, Zhang et al. employ a chemically-induced protein dimerization approach to trigger telomere-associated SIM-SUMO interactions, which they report drives APB formation by liquid-liquid phase separation (LLPS) and promotes telomere clustering. 53BP1, PCNA and POLD3 were not prominently enriched in these chemically-induced APBs, and inducing liquid condensation at telomeres by a different means, i.e. exploiting the LLPS properties of the LAF-1 RGG domain tethered to telomeres, also resulted in telomere fusions. Based on these findings, the authors propose that LLPS drives telomere clustering in ALT cells.
The approach taken by Zhang et al. to form APB-like structures through chemically-induced TRF1-SIM dimerization and SIM-SUMO interactions is interesting, the quality of the data is overall convincing, and the manuscript is well written. The presented paper is a rather brief report, with some similarity to a recently published study (Min et al. Genes Dev. 2019 Jul 1;33(13-14):814-827). Nevertheless, the report by Zhang et al. would make a nice and useful addition to the field, in particular if the following points could be addressed:

1. In the abstract the authors outline "two potential mechanisms to promote telomere elongation", which they want to test: "condensation to enrich DNA repair factors for telomere synthesis and coalescence to cluster telomeres to provide repair templates". As specified further in the following two points, the manuscript in its current form does not seem to go far enough to address these two models, and additional experiments are required to more thoroughly evaluate the contribution of condensation for repair factor retention and the contribution of coalescence for telomere maintenance.

2. The authors argue that they have decoupled APB functions and that LLPS drives telomere clustering independent of specific protein components of the liquid condensates. In terms of functional relevance, how does such clustering affect ALT and telomere maintenance? Would enforced clustering using the dimerization system enhance ALT and promote telomere recombination and lengthening? Would this be reversed by TMP addition? In the context of the model put forward by the authors, it might even be informative to test whether the LAF-1 RGG domain can functionally substitute for the SIM. And what happens if the TRF1-SIM dimerization system is expressed in non-ALT cells, would this lead to telomere clustering?

3. On a somewhat related note, the authors seem to suggest that LLPS at telomeres has no effect on the recruitment of 53BP1, PCNA and POLD3 (BLM and RAD52 would be two other interesting

candidates to test in this context). Wouldn´t it be possible to use the dimerization system (WT and SIM mutant) in the TRF1-FokI cells (WT and nuclease-dead) and test whether the chemically enforced SIM-SUMO-driven LLPS impacts protein composition around telomeres and perhaps facilitates repair factor retention? Along these lines, the relative enrichment of 53BP1, PCNA and POLD3 upon FokI induction, SIM tethering, and combined should be quantified.

4. It would be good to test whether the chemical dimerization-induced liquid condensates share common features with other phase-separated compartments, e.g. are they sensitive to 1,6-hexanediol and increasing salt concentration?

5. For the analyses in Figure 1B,C and 3B-E, please clarify in the legends how many foci from how many cells were analyzed and, if possible, provide the data for both the GFP and the mCherry signal (Figure 3).

6. With reference to the final sentences of the discussion (page 11, lines 316-320), composition control and clustering my not be as strictly separatable and may rather represent two sides of the same coin. The experiments performed in this study, as interesting as they are, in my opinion do not exclude that APB-like condensates induced by the TRF1-SIM dimerization affect composition control (not observing a pronounced enrichment of three selected proteins by IF is not sufficient for such a conclusion), and they also do not provide sufficient evidence that the only functional role of clustering is to promote proximity to repair templates. Composition control and clustering could still be interconnected, and I would therefore recommend to tone down or rephrase such statements.

Minor points

1. In light of the similarities between telomeres and DNA double-strand breaks and their relevance for genome stability, the authors may want to mention or discuss recent findings related to LLPS at DNA strand breaks (Pessina et al. Nat Cell Biol. 2019 Sep 30. doi: 10.1038/s41556-019-0392-4; Kilic et al. EMBO J. 2019 Aug 15;38(16):e101379).

2. Likewise, the authors may want to consider to refer to the "SUMO glue" model put forward by Stefan Jentsch (Cell. 2012 Nov 9;151(4):807-820).

3. On page 9, line 247, the authors may want to briefly discuss that in addition to sumoylation/desumoylation other PTMs implicated in regulating LLPS may also contribute to the regulation or fine-tuning of APBs.

4. On page 8, line 221, it should probably read "low complexity protein" instead of "no complexity protein".

Response to Review Comments

Reviewer #1 (Remarks to the Author):

Comments on Zhang et. al. Molecular Biology of the Cell E19-10-0589.

In this study by Zhang et al., the authors characterize the role of liquid-liquid phase separation in the formation of ALT-associated promyelocytic leukemia (PML) nuclear bodies (APBs). have a long lasting experience in the field and previously Previous studies showed that introducing DNA damage at telomeres leads to APB formation, telomere clustering within the induced APBs, and telomere elongation via HR repair (Cho et al., 2014). These studies also showed a role for Rad51-driven homology search in promoting telomere clustering (Cho et al., 2014). Nevertheless the mechanism governing APB assembly and function are still unclear, also due to limitations in how to study this biological process. Here, the authors show that APBs have liquid-like behavior in vivo, and are enriched for SUMOylated proteins. Expression of a chemical dimerizer that links the telomeric protein Trf1 and a SUMO-binding motif (SIM) induces a liquid-like behavior at telomeres and telomere clustering, even in the absence of telomeric damage and repair proteins. This supports a role for SUMOylation in inducing the liquid-like behavior of APBs, consistent with recent studies (Min et al., 2019). Expression of a chemical dimerizer that links the telomeric protein Trf1 to a RGG domain of Laf-1, which is known to induce non-APB liquid-like behavior, is sufficient to induce telomere clustering even in the absence of damage or damage-induced SUMOylation, supporting a model where the liquid-like behavior promotes telomere clustering. Together, these data support a role for telomeric-damage induced SUMOylation in telomeric clustering via phase separation.

One of the main strengths of this paper is the generation and validation of new tools to induce a reversible SUMOylation-driven phase separation at telomeres. The role of SUMOylation in phase separation and telomere clustering has been recently shown (Min et al., 2019), and this study provides a strong independent validation of this response. However, a major missing point in the study by Zhang et al. is the characterization of the role of damage-induce phase separation in telomere repair, which is also important to validate the newly generated tools. Statistical validations and a higher number of cells/replicates should also be provided for most experiments.

Major points:

1) The damage-induced (Fok1) and chemical dimerizer-induced telomere clusters display liquid-like behavior, including coalescence after collision and dynamic exchange of components by FRAP. It would be important to provide an independent validation that these structures are, indeed, phase separated. For example, are these clusters disrupted by 1,6-hexanediol, a compound known to disrupt liquid-like condensates?

We added 1,6-hexanediol or salt to dimerization-induced droplets and observed dissolution. The result is now added to Figure 3-S1. The lab closed due to COVID-19 before we had a chance to test these treatments on FokI-induced droplets. Given the similarity in properties shown with fusion and FRAP between droplets induced by FokI or dimerizer, we believe the evidence presented here is sufficient for demonstrating APB liquid behavior.

2) The authors propose a model where telomere clustering depends on the liquid properties of APBs and leads to telomere elongation in ALT cells. However, repair is never investigated throughout the paper. This also questions the extent to which the tools generated (dimerizer-induced telomere clustering with SIM or Laf-1) mimic the endogenous phase separated state for telomeres in ALT cells. The consequences of impairing the liquid properties of ABPs on HR repair of telomeres should be shown by measuring telomere length (such as by terminal restriction fragment analysis and/or Q-FISH) or Rad51 recruitment at telomeres.

We agree that impairing the liquid properties of APBs (or any intracellular condensates) would be an ideal experiment to demonstrate the functional significance of condensate material properties. However, the difficulty is that there is no existing method that can disrupt the liquid properties of condensates without dissolving the condensates completely or changing their composition. That is the reason we use a synthetic approach to show that de novo APBs cluster telomeres and that such clustering does not depend on APB chemistry, as LAF-1 droplets can cluster telomeres as well. Developing an elegant method to disrupt APB liquid properties without affecting other aspects of APB condensate is a future goal but beyond the scope of this manuscript.

We also agree that effects of telomere clustering on telomere elongation should be investigated. Using EdU labeling to monitor nascent DNA synthesis, we find that telomere clustering does not induce more telomere DNA synthesis. This result, shown in Figure 4-S3, agrees with a previous publication (Min et al. 2019, *Genes Dev*) and with our prior findings (Dilley et al. 3026, *Nature*) showing that telomere clustering is required but not sufficient for telomere DNA synthesis in ALT. Unfortunately, we were not able to finish the Q-FISH experiment suggested by the reviewer before the lab closed.

3) A limited number of cells and experiments are shown throughout the paper. Experiments should be provided in triplicates and with a quantification of several cells/exp to account for variability across individual cells and experimental variations (Fig 1B,C; Fig. 2C,D,E; Fig. S2, S3, S4, etc).

We increased the sample size in figures mentioned by the reviewer and others (Figure 3B,C,G,H, Figure 4B,C, and Figure 5F). We note that averaging is not appropriate in Figures 1B and Figure 3D because the decay curve depends on the size of the droplets. For those two figures, we plotted the dependence of fusion time on fusion length for multiple fusion events in Figure 1-S1A and Figure 3-S1A.

4) How do the authors reconcile their model where phase separation drives telomere clustering in ALT cells with previous models where directed motions characterize homology search in ALT cells (which in turn drives clustering)? This should be added to the discussion.

We added this to the Discussion: “We previously showed that DNA damage increases telomere mobility of chromosomally attached telomeres (Cho et al., 2014), indicating that DNA damage not only nucleates APB condensates to enable telomere clustering through droplet coalescence, but also actively modulates clustering efficiency by increasing the chance of APB collision. Nuclear actin polymerization increases the mobility of DNA damage sites to cluster DNA damage foci for homology-directed DNA repair (Schrank et al., 2018). It remains to be determined whether actin polymerization increases telomere mobility in response to DNA

damage in ALT cells, and whether and how it depends on telomere protein sumoylation or APB condensation.”

5) The authors state in the discussion that their 'findings provide an opportunity to target physical-chemical properties of APBs for cancer therapy in At affecting the function of their DNA repair components'.

Liquid-liquid phase separation is critical to organize nuclear activities spatially and temporally in membrane-less domains. To what extent other chromatin domains rely on phase separation for their spatial-temporal confinement and for their biological functions is a matter of intense investigation, but tools that disrupt phase separation described here (such as by interfering with SUMOylation) are likely to affect other nuclear functions. How the authors are suggesting to target APBs without affecting other nuclear functions requires a more extensive discussion.

We added this in the Discussion: Since sumoylation is involved in many cellular functions, globally targeting sumoylation to prevent APB condensation would have many side effects. Instead, approaches to disrupt APB liquid properties or recruitment of important factors to APBs would be more attractive. For example, pushing APB condensates into gel or solid phase (Shin et al., 2017) by increasing molecule density or interaction strength within APBs would prevent reversible telomere clustering, inhibit dynamic retention of DNA repair factors within APBs and thus prevent telomere elongation.

Minor points:

1. Introduction - lines 70-73. While APBs are proposed to be sites of telomere recombination during ALT, the precise functions of these specialized PML nuclear bodies are poorly understood and whether their formation requires a DNA damage response. The sentence needs to be completed.

This error is corrected.

2. Fig. 1B,D: it is unclear how many clustering events are quantified here. A quantification of the frequency of clustering over several cells and 3+ experiments should be provided. SD/SEM values should also be provided.

The number of events analyzed in Figure 1B and Figure 3D are now shown as the dependence of decay time on fusion length (Figure 1-S1A and Figure 3-S1A). Instead of quantifying the clustering frequency which depends on time interval and droplet size, our quantification of droplet number over time (Figure 3C) reflects the degree of clustering.

3. table in supplementary information showing that APB components contains SIM domain or are SUMOylated (or both) including references, could be provided for the readers. Figures 1D, 2B-D, S1, S2: It would be clearer if the authors specified in the figures and graphs the mutant to which they refer (i.e. 'Fok1 mutant' or 'SIM mutant' instead of 'mutant').

Please see supplemental table 1 for APB components containing sumoylation sites and SIM. We made changes to the figures to clarify the different mutants.

4. Figure S1: should be shown with the same color scheme as Fig. 1D, for clarity.

Quantifications of the percent of telomeres with SUMO 2/3 foci and Integrated intensity of SUMO 2/3 foci on telomeres/cell should also be provided (similar to Fig. 1D).

The changes are made.

5. Fig. 2E appears to show defective recruitment of telomeres of the SIM mutant. A quantification should be provided to test whether this is the case. If not, a better set of images should be provided. If the SIM mutant is, instead, defective in its recruitment to telomeres, that would provide an alternative explanation to the lower level of SUMOylated proteins at telomeres.

A better set of images is now presented. The loss of fluorescence during the FISH assay is more obvious for weak SIM mutant foci, making quantification less accurate. We planned to use an mCherry antibody for better imaging but could not finish before the lab closed. Based on the high colocalization in bright cells and our previous results (Cho et al. 2014, *Cell*) showing FokI-TRF1 and telomere DNA colocalization, we believe our conclusion is valid that both SIM and SIM mutant are recruited to telomeres by dimerization.

6. Figure S2: should be shown with the same color scheme as figure 2B, for clarity.

The color for each channel is the same. Different composites are shown in Figure 2B and Figure 2-S1A to indicate co-localization.

7.
8. 'Decoupling of APB functions' - page 7. Literature references should be added in the first paragraph.

This paragraph states our model; it is not from the literature.

9. Figure S3 - text in the figure: Clutering> clustering

The change is made.

10. Figure S4 - insets indicating FokI, SIM colocalization with 53BP1 and POLD3 should be provided inside the images. Quantifications are also missing for this figure.

Inserts and quantifications are added (now Figure 4-S1).

11. Discussion - line 284. 'Coalescence of APB liquid droplets drives telomere clustering (Figure 3A-E) may provide'... this sentence is missing 'that'.

The change is made.

12. Line 139. ABPs > APBs

Corrected.

Reviewer #2 (Remarks to the Author):

Alternative lengthening of telomeres (ALT) is used by around 10-15% of cancers to prevent telomere shortening and senescence, and targeting ALT is therefore a promising anti-cancer strategy. Several mechanistic aspects of ALT remain poorly understood however, including the precise role of ALT-associated promyelocytic leukemia (PML) nuclear bodies (APB) for telomere maintenance and stability. In their manuscript, Zhang et al. employ a chemically-induced protein dimerization approach to trigger telomere-associated SIM-SUMO interactions, which they report drives APB formation by liquid-liquid phase separation (LLPS) and promotes telomere clustering. 53BP1, PCNA and POLD3 were not prominently enriched in these chemically-induced APBs, and inducing liquid condensation at telomeres by a different means, i.e. exploiting the LLPS properties of the LAF-1 RGG domain tethered to telomeres, also resulted in telomere fusions. Based on these findings, the authors propose that LLPS drives telomere clustering in ALT cells.
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1. In the abstract the authors outline "two potential mechanisms to promote telomere elongation", which they want to test: "condensation to enrich DNA repair factors for telomere synthesis and coalescence to cluster telomeres to provide repair templates". As specified further in the following two points, the manuscript in its current form does not seem to go far enough to address these two models, and additional experiments are required to more thoroughly evaluate the contribution of condensation for repair factor retention and the contribution of coalescence for telomere maintenance.

To address the contribution of condensation to repair factor retention, we fused PCNA with SUMO1 and observed enrichment in SIM dimerization induced condensates, highlighting that APB as a condensate can recruit DNA repair factors through SUMO-SIM interactions. The result is in Figure 4-S2. To determine whether telomere clustering is sufficient for telomere synthesis, we monitored nascent DNA synthesis with EdU labeling and found that clustering alone is not sufficient to induce telomere DNA synthesis, agreeing with published results (Min et al. 2019, *Genes Dev; Dilley et al 2016; Nature*). The result is in Figure 4-S3.

2. The authors argue that they have decoupled APB functions and that LLPS drives telomere clustering independent of specific protein components of the liquid condensates. In terms of functional relevance, how does such clustering affect ALT and telomere maintenance? Would enforced clustering using the dimerization system enhance ALT and promote telomere recombination and lengthening? Would this be reversed by TMP addition? In the context of the model put forward by the authors, it might even be informative to test whether the LAF-1 RGG domain can functionally substitute for the SIM. And what happens if the TRF1-SIM dimerization system is expressed in non-ALT cells, would this lead to telomere clustering?

We thank the reviewer for these great suggestions. Our results suggest that both APB functions, recruiting DNA repair factors and telomere clustering, are required for telomere synthesis. This is supported by the added experiment in Figure 4-S3 showing that telomere clustering is not sufficient to induce telomere DNA synthesis, agreeing with previous results (Min et al. 2019,

Genes Dev). It is also in agreement with recent work that came out while our work was in revision showing APBs promote BLM recruitment to telomeres and telomere length maintenance during ALT (Loe et al. GenesDev 2020). In terms of non-ALT cells, in which DNA damage on telomeres does not cluster telomeres (Cho et al. 2014, *Cell*), we did observe telomere clustering by recruiting SIM to telomeres (data not shown). We are still working on understanding the differences between these cases, which we believe are beyond the scope of this paper.

3. On a somewhat related note, the authors seem to suggest that LLPS at telomeres has no effect on the recruitment of 53BP1, PCNA and POLD3 (BLM and RAD52 would be two other interesting candidates to test in this context). Wouldn´t it be possible to use the dimerization system (WT and SIM mutant) in the TRF1-FokI cells (WT and nuclease-dead) and test whether the chemically enforced SIM-SUMO-driven LLPS impacts protein composition around telomeres and perhaps facilitates repair factor retention? Along these lines, the relative enrichment of 53BP1, PCNA and POLD3 upon FokI induction, SIM tethering, and combined should be quantified.

Our model is that APB condensates act as scaffolds to recruit or retain factors such as 53BP1, PCNA and others through SUMO-SIM interaction directly or indirectly. Our data indicate that SIM dimerization-induced LLPS is not sufficient for this function, possibly because SIM dimerization mimics telomere protein sumoylation-induced LLPS but not other aspects of the DNA damage response such as sumoylation/phosphorylation of DNA repair proteins. We show that FokI can induce telomere protein sumoylation, APB LLPS and recruitment of DNA repair factors, and therefore we believe that recruiting SIM on top of FokI tethering will not further facilitate repair factor retention. Our goal of using chemical dimerization is to mimic APB LLPS induced by DNA damage and ask how APB condensates retain repair factors. For example, Figure 4 and the newly added Figure 4-S2 show that SIM dimerization-induced condensates cannot enrich PCNA but can enrich a PCNA-SUMO1 fusion protein.

4. It would be good to test whether the chemical dimerization-induced liquid condensates share common features with other phase-separated compartments, e.g. are they sensitive to 1,6-hexanediol and increasing salt concentration?

Following this suggestion, we did observe droplet disruption by hexanediol and salt. The result is in Figure 3-S1.

5. For the analyses in Figure 1B,C and 3B-E, please clarify in the legends how many foci from how many cells were analyzed and, if possible, provide the data for both the GFP and the mCherry signal (Figure 3).

These changes are made. For 1B and 3D, average across fusion events are not appropriate because the decay curve depends on the size of the droplets. For those two figures, we plotted the dependence of fusion time on fusion length in Figure 1-S1A and Figure 3-S1A.

6. With reference to the final sentences of the discussion (page 11, lines 316-320), composition control and clustering my not be as strictly separatable and may rather represent two sides of the same coin. The experiments performed in this study, as interesting as they are, in my opinion do not exclude that APB-like condensates induced by the TRF1-SIM dimerization affect composition control (not observing a pronounced enrichment of three selected proteins by IF is

not sufficient for such a conclusion), and they also do not provide sufficient evidence that the only functional role of clustering is to promote proximity to repair templates. Composition control and clustering could still be interconnected, and I would therefore recommend to tone down or rephrase such statements.

Totally agree! We added this in our discussion: In addition, DNA repair factors required for telomere DNA synthesis may be selectively retained in APBs by regulating chemical properties of APB condensate scaffold and client molecules.

Minor points

1. In light of the similarities between telomeres and DNA double-strand breaks and their relevance for genome stability, the authors may want to mention or discuss recent findings related to LLPS at DNA strand breaks (Pessina et al. Nat Cell Biol. 2019 Sep 30. doi: 10.1038/s41556-019-0392-4; Kilic et al. EMBO J. 2019 Aug 15;38(16):e101379).

2. Likewise, the authors may want to consider to refer to the "SUMO glue" model put forward by Stefan Jentsch (Cell. 2012 Nov 9;151(4):807-820).

We thank the reviewer for these insightful suggestions. We added this in the discussion: 'Indeed, sumoylation is proposed to generate a glue that holds DNA repair factors together (Psakhye and Jentsch, 2012), which may form through SUMO-SIM driven phase separation as observed here. In addition, PARylation and transcription can drive phase separation of DNA repair factors at damage sites (Altmeyer et al., 2015; Kilic et al., 2019; Pessina et al., 2019; Singatulina et al., 2019). It remains to be determined how sumoylation coordinates with PARylation, transcription and other DNA damage signaling to facilitate DNA repair through phase separation. As PARylation is one of the earliest events during DNA damage recognition, it is possible that a temporal order of signals beginning with PARP activity and culminating in SUMO-SIM interactions is responsible for phase separation of DNA damage foci. Furthermore, PML bodies associate with genomic loci other than telomeres in non-ALT cells to regulate multiple functions including DNA repair, transcription, viral genome replication and heterochromatin domain formation (Dellaire and Bazett-Jones, 2004; Eskiw et al., 2004; Ching et al., 2005; Luciani et al., 2006; Shastrula et al., 2019). Our work demonstrates local sumoylation as a mechanism for generating telomere association of PML bodies, by either directly nucleating PML bodies or enabling sumoylated telomeres to fuse with existing PML bodies to form APBs. Similarly, protein sumoylation at other genomic loci may trigger PML association. Supporting this notion, a recent study finds that viral protein sumoylation is required for association of PML bodies with viral replication centers (Stubbe et al., 2020).'

3. On page 9, line 247, the authors may want to briefly discuss that in addition to sumoylation/desumoylation other PTMs implicated in regulating LLPS may also contribute to the regulation or fine-tuning of APBs.

We added this: 'Other posttranslational modifications known to regulate phase separation, such as phosphorylation (Snead and Gladfelter, 2019), may play a role in APB condensation or dissolution as well, either by directly controlling de/sumoylation or modulating SUMO-SIM interaction strength (Chang et al., 2011; Hendriks et al., 2017).'

4. On page 8, line 221, it should probably read "low complexity protein" instead of "no complexity protein".

Yes indeed. This error is now corrected.

RE: Manuscript #E19-10-0589R
TITLE: "Nuclear body phase separation drives telomere clustering in ALT cancer cells"

Dear Dr. Zhang:

As you will see from the comments, referee 2 is satisfied with your revisions, but referee 1 raises a number of point which still need to be addressed. In particular, since not directly addressed by experimentation it will be important to tone down some the claims regarding the functional roles of phase-separation in the repair process as outlined by the referee. Maybe more importantly, additional experimentation or explanation needs to be provided for the loss of SIM in the 1,6-hexanediol experiment and what that behavior means for the interpretation of the recruitment approach used in the study. I ask you to include these clarifications in a revised manuscript. Note that this will be an exception to the usual MBoC policy of only considering a single round of revision and will be the final revision.

Sincerely,
Tom Misteli
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Zhang,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript requires minor revisions before it can be published in Molecular Biology of the Cell, as described in the Monitoring Editor's decision letter above and the reviewer comments (if any) below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

When submitting your revision include a rebuttal letter that details, point-by-point, how the Monitoring Editor's and reviewers' comments have been addressed. (The file type for this letter must be "rebuttal letter"; do not include your response to the Monitoring Editor and reviewers in a "cover letter.") Please bear in mind that your rebuttal letter will be published with your paper if it is accepted, unless you have opted out of publishing the review history.

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In preparing your revised manuscript, please follow the instruction in the Information for Authors (www.molbiolcell.org/info-for-authors). In particular, to prepare for the possible acceptance of your revised manuscript, submit final, publication-quality figures with your revision as described.

To submit the rebuttal letter, revised version, and figures, please use this link (please enable

cookies, or cut and paste URL): Link Not Available

Authors of Articles and Brief Communications whose manuscripts have returned for minor revision ("revise only") are encouraged to create a short video abstract to accompany their article when it is published. These video abstracts, known as Science Sketches, are up to 2 minutes long and will be published on YouTube and then embedded in the article abstract. Science Sketch Editors on the MBoC Editorial Board will provide guidance as you prepare your video. Information about how to prepare and submit a video abstract is available at www.molbiolcell.org/science-sketches. Please contact mboc@ascb.org if you are interested in creating a Science Sketch.

Thank you for submitting your manuscript to Molecular Biology of the Cell. Please do not hesitate to contact this office if you have any questions.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org

Reviewer #1 (Remarks to the Author):

This revised version of the manuscript by Zhang et al provides a more convincing description of a new tool to induce a reversible SUMOylation-driven phase separation at telomeres. The role of SUMOylation in phase separation and telomere clustering has been recently shown (Min et al., 2019), and this study provides an independent validation of this response.

Several points rained on the previous version of the manuscript have been addressed in a satisfactory manner. Specifically, the authors extended they quantifications to at least two independent datasets for most experiments. They also provided a more integrated discussion with previous data, and a better evaluation of potential therapeutic approaches, greatly improving the manuscript.

However, I am still concerned that an independent validation that these structures are phase separated is lacking (see comment 1). Statistical validations, additional replicates, and information on the number of experiments quantified is still lacking in many instances. Specific points that should be addressed before publication are:

1) The authors performed treatments with 1,6-hexanediol or NaCl to provide independent validation that the structures they see have the properties of condensates. However, these treatments result in reduction of signal for both SIM and Trf1. I expected Trf1 signals to remain and separating into smaller and less rounded signals, similar to TMP addition in figure 3F. What is observed suggests photobleaching effects rather than disassembly of condensates. Perhaps the treatment with these chemicals need to be better titrated for providing meaningful results.

2) It is unclear what is the population size and the number of replicates for several experiments (3B,C,G,H; 3S1B; 3S2B,C). P values for significant comparisons are also missing (3B,C,G,H; 5B,C; 3S2)

Further, it is unclear how representative quantifications shown in 1B, 3D, and 5D are, as only one event is shown. Maybe rather quantify the number of fusion event per cell over time, rather than the quantifying the aspect ratio of an individual fusion event, which is not very informative.

The meaning of error bars in 1C; 3C,G,H; 5C needs to be specified.

3) The authors provide a reasonable explanation to justify why the role of phase separation-induced clustering in repair cannot be directly tested. In this case, statements implying a role for phase separation in ALT repair should be significantly toned down.

For example:

Abstract:
"We find that APBs behave as liquid condensates in response to telomere DNA damage, suggesting two potential mechanisms to promote telomere elongation: condensation to enrich DNA repair factors for telomere synthesis and coalescence to cluster telomeres to provide repair templates".
> telomere elongation does not occur in the system tested, and telomere elongation in damaged telomeres has not been tested, so this statement should be rephrased.

(Page 9 line 261) "These findings indicate that APB condensates are nucleated on telomeres via sumoylation and can be dissolved via desumoylation, implicating sumoylation dynamics in the initiation and resolution of telomere recombination".
> telomere recombination has not been tested, so this is an overstatement.

(Page 10, line 269) "Our observation that sumoylation nucleates APB condensates as a mechanism for ALT telomere elongation..."
> again, the effects of condensates on telomere elongation in conditions of damaged telomeres was not investigates. Also, in undamaged telomeres, there is no effect on telomere elongation, so this statement is incorrect.

Reviewer #2 (Remarks to the Author):

The authors have addressed all my comments in an adequate and satisfactory manner. I endorse publication of this study.

Minor corrections:

Line 312 should be singular "promote"
Line 342 should be "due to"

E19-10-0589 Rebuttal Letter

Reviewer #1 (Remarks to the Author):

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Several points rained on the previous version of the manuscript have been addressed in a satisfactory manner. Specifically, the authors extended they quantifications to at least two independent datasets for most experiments. They also provided a more integrated discussion with previous data, and a better evaluation of potential therapeutic approaches, greatly improving the manuscript.

However, I am still concerned that an independent validation that these structures are phase separated is lacking (see comment 1). Statistical validations, additional replicates, and information on the number of experiments quantified is still lacking in many instances. Specific points that should be addressed before publication are:

1) The authors performed treatments with 1,6-hexanediol or NaCl to provide independent validation that the structures they see have the properties of condensates. However, these treatments result in reduction of signal for both SIM and Trf1. I expected Trf1 signals to remain and separating into smaller and less rounded signals, similar to TMP addition in figure 3F. What is observed suggests photobleaching effects rather than disassembly of condensates. Perhaps the treatment with these chemicals need to be better titrated for providing meaningful results.

SIM phase separation leads to TRF1 enrichment on telomeres because TRF1 is linked to SIM through dimerizers (see TRF1 intensity increase after dimerization in Figure 3B). Therefore, there are two populations of TRF1 on the telomere: one population that directly binds to DNA and one population that is enriched because of SIM phase separation. When we use 1,6-hexanediol or NaCl to dissolve SIM condensates, the TRF1 population that is enriched through SIM condensation is expected to be lost, therefore resulting in dimmer TRF1 foci. The difference with Figure 3F is that reversing dimerization acts on the TRF1-SIM linkage (i.e., eliminates the seed for phase separation) while 1,6-hexanediol and NaCl act on the SUMO-SIM interaction (i.e., weaken the driving force for phase separation). To confirm that the loss of intensity in the foci is not due to photobleaching, we took an image before adding NaCl, and then one image after adding 0, 0.2, 0.4, or 0.6 M NaCl to avoid imaging cells many times. For the 0 M NaCl control, we did not observe intensity loss while for other concentrations we saw intensity loss both in TRF1 and SIM in a NaCl concentration dependent manner, indicating that the intensity loss is not due to photobleaching. The new results are shown in Figure 3-S1 F, G.

2) It is unclear what is the population size and the number of replicates for several experiments (3B,C,G,H; 3S1B; 3S2B,C). P values for significant comparisons are also missing (3B,C,G,H; 5B,C; 3S2)

Further, it is unclear how representative quantifications shown in 1B, 3D, and 5D are, as only one event is shown. Maybe rather quantify the number of fusion event per cell over time, rather than the quantifying the aspect ratio of an individual fusion event, which is not very informative.

The meaning of error bars in 1C; 3C,G,H; 5C needs to be specified.

We added the population size and the number of replicates in 3B,C,G,H; 3S1B,C,D, 3S2B,C, and 5B,C.
P values between the first time point and last time point are added to the captions of Figure 3BCGH, 5BC and 3S2.

The number of fusion events per cell over time is reflected in the change in telomere number over time. Quantifying the aspect ratio is informative because the aspect ratio needs to approach 1 for a liquid. This is the most direct evidence for the liquid property of condensates. 1,6-hexanediol or NaCl targets interactions that are important for phase separation but not the liquid property itself.

The meaning of error bars is specified in 1C, 3C,G,H, and 5C.

3) The authors provide a reasonable explanation to justify why the role of phase separation-induced clustering in repair cannot be directly tested. In this case, statements implying a role for phase separation in ALT repair should be significantly toned down.

For example:

Abstract:
"We find that APBs behave as liquid condensates in response to telomere DNA damage, suggesting two potential mechanisms to promote telomere elongation: condensation to enrich DNA repair factors for telomere synthesis and coalescence to cluster telomeres to provide repair templates".
> telomere elongation does not occur in the system tested, and telomere elongation in damaged telomeres has not been tested, so this statement should be rephrased.

This sentence is changed to: “We find that APBs behave as liquid condensates in response to telomere DNA damage, suggesting two potential functions: condensation to enrich DNA repair factors and coalescence to cluster telomeres.”

(Page 9 line 261) "These findings indicate that APB condensates are nucleated on telomeres via sumoylation and can be dissolved via desumoylation, implicating sumoylation dynamics in the initiation and resolution of telomere recombination".
> telomere recombination has not been tested, so this is an overstatement.

This is changed to: “These findings indicate that APB condensates are nucleated on telomeres via sumoylation and can be dissolved via desumoylation.”

(Page 10, line 269) "Our observation that sumoylation nucleates APB condensates as a mechanism for ALT telomere elongation..."
> again, the effects of condensates on telomere elongation in conditions of damaged telomeres was not investigates. Also, in undamaged telomeres, there is no effect on telomere elongation, so this statement is incorrect.

This is changed to: “Our observation that sumoylation nucleates APB condensates as a mechanism for ALT telomere clustering ...”

Reviewer #2 (Remarks to the Author):

The authors have addressed all my comments in an adequate and satisfactory manner. I endorse publication of this study.

Minor corrections:

Line 312 should be singular "promote"
Line 342 should be "due to

These errors are corrected.

RE: Manuscript #E19-10-0589RR
TITLE: "Nuclear body phase separation drives telomere clustering in ALT cancer cells"

Dear Dr. Zhang:

I am pleased to accept your manuscript for publication in Molecular Biology of the Cell.

Sincerely,
Tom Misteli
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Zhang:

Congratulations on the acceptance of your manuscript.

A PDF of your manuscript will be published on MBoC in Press, an early release version of the journal, within 10 days. The date your manuscript appears at www.molbiolcell.org/toc/mboc/0/0 is the official publication date. Your manuscript will also be scheduled for publication in the next available issue of MBoC.

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