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## Higher-order oligomerization promotes localization of SPOP to liquid nuclear speckles

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*Editor: Hartmut Vodermaier*

### Transaction Report:

(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.)

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Preliminary Editorial Decision

11 November 2015

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Thank you again for submitting your manuscript EMBOJ-2015-88663, "Higher-order oligomerization promotes localization of SPOP to liquid nuclear speckles". After some delay (for which I apologize), we have now received all the reports from three experts, which I am enclosing, copied below. As you will see, the reviewers express some interest in the basic finding of your study, yet they also raise a number of substantive concerns regarding the conclusiveness of the study, including issues regarding the exact identity of the SPOP speckles and their functional significance with regard to ubiquitin ligase assembly and function. Before taking a final decision on this manuscript, I would therefore like to give you an opportunity to consider and respond to the referee reports with a brief point-by-point outline on how the major issues might be addressed/clarified; and to comment on the expected feasibility of such experiments as requested by the reviewers. This tentative response (parts of which we may choose to share and discuss with referees) would be taken into account when making our final decision on this manuscript. I would therefore appreciate if you could send us such a response at your earliest convenience, ideally by early next week. Should you have any further questions in this regard, please do not hesitate to let me know.

Referee #1

(Report for Author)

The manuscript by Mittag et al. contains some very interesting data and I recommend publication in the EMBO journal with minor revision.

The main result here to this reviewers mind is the liquid drop type behaviour from bodies formed from GFP-Gli3(1-455), and that HA-SPOP preferentially co-localises to these bodies. These results are exemplified in figure 2C and 6. This result is novel, timely and builds on an exciting body of work emerging on liquid-drop type membraneless organelles. The significance of these bodies in cellular organisation we are only just starting to see.

The majority of the article is devoted to a biophysical characterisation of the oligomeric assembly of SPOP. I have two major comments on these two main areas, and a number of minor comments. The majority of which I believe can be dealt with textual clarifications and toning down of certain statements. A small number of additional experiments, most notably showing that the bodies formed from GFP-Gli3(1-455) and those that absorb SPOP stain with nuclear speckle markers. This would enhance this story and provide a convincing link that the bodies analysed here can be allied with nuclear speckles.

To illustrate my current reservations, in the abstract:

"Herein, we demonstrate that nuclear speckles have liquid droplet properties and that localization of SPOP to speckles depends upon its ability to form higher-order oligomers through tandem self-association domains. "

As far as I can tell, nuclear speckles are not demonstrated to have liquid droplet properties (bodies formed from GFP-Gli3 have liquid properties, see below) and oligomerisation is not shown to be linked to colocalisation in vivo (perhaps specific interactions are the cause of this lack of localization, and nothing to do with oligomerisation, see below). I do not mean to detract from the great data in this paper; just this reviewer would advocate for qualification in some of the claims, or more data to support them. The core results are outstanding making the overall paper a very worthy article in the view of this reviewer.

#### Major comments

##### 1) Use of the term 'nuclear speckle'.

In Fig 2c, the authors show the results of expressing HA-tagged WT SPOP, and BTB and BACK domain mutants. From the punctate staining pattern of SPOP WT protein, the authors infer that SPOP localises to nuclear speckles. A lot of emphasis is placed on this.

Perhaps the bodies formed by SPOP are formed by SPOP oligomerisation and have nothing to do with nuclear speckles? To link SPOP to nuclear speckles, the authors should simultaneously visualize nuclear speckles using known markers. For example, SC35 antibodies are commercially available and would be one way to easily accomplish this. Using additional nuclear speckle markers would strengthen the case.

The authors conclude nuclear speckles are liquid droplets. What I believe they show is that bodies associated with GFP-Gli3(1-455) have liquid drop properties. Does GFP-Gli3(1-455) co-localise to nuclear speckles, or are these bodies spontaneously formed and are separate to nuclear speckles? At least some HA-SPOP fluorescence is not associated with GFP-Gli3(1-455) fluorescence in the images shown in Fig 6A and E. To evaluate the conclusions drawn from the images, it would be helpful to know how they were generated. For example, were they acquired using confocal or (deconvolved) widefield microscopy? Are the images shown derived from single xy-planes, or were they derived from projections of image stacks (e.g. maximum, average or sum intensity projections)? The argument for HA-SPOP and GFP-Gli3(1-455) co-localisation could be further strengthened by quantifying of the overlap in fluorescence signals. A more direct link to nuclear speckles should be provided to make the claims as stated in the article. In the absence of such a link it's clear that the GFP-Gli3(1-455) bodies have liquid drop properties and that these co-localise to some extent with SPOP, both of which are very neat and compelling findings. Just that without a direct link to nuclear speckles, the precise details of what is seen in the data should be made clearer, and some of the conclusions should be toned down.

##### 2) Page 4 SPOP oligomerization is required for cellular localization

The authors show that the mutations in a truncated SPOP restrict the oligomeric size of SPOP in vitro. No data on the oligomerisation state of SPOP in vivo is shown (unsurprisingly, as to get this is highly challenging). An alternative, arguably simpler possibility is that the specific interaction between Gli3 and SPOP is knocked out by these mutations and is the cause of the lack of colocalisation. Perhaps also the mutations when applied to full length SPOP rather than (28-355), do

not affect oligomerisation of SPOP. I agree with the authors that the causal link between oligomerisation and localization is suggestive. Required is certainly too strong, and no link is 'demonstrated' as the authors claim. So the link here is not as clear as the manuscript implies. The authors should tone this down and qualify the claim. Notable places for this include the abstract, title and conclusion paragraphs.

#### Minor comments

##### 1) Biophysical interrogation of SPOP assembly

The full length protein we are told forms very large oligomers, and the study focuses on truncated construct 28-359.

When the full length protein aggregates, do the authors believe the assembly is a continuation of the assembly that they see in the simplified construct, or something more pathogenic like amyloid assembly? The manuscript would benefit from some data clarifying what is happening with the full length, and a discussion on the subject.

Perhaps the uncontrolled assembly of these molecules occurs when residues 1-28 are present is effectively a continuation of the isodesmic model they propose. Perhaps showing the NTerm in their model will help clarify what it could or could not do.

Moreover, it would be interesting to see whether there is a difference in localisation of HA-full length SPOP and HA-SPOP (residues 28-359). i.e. do the N-terminal 27 residues affect the localisation of the protein in cells?

##### 2) Kinetic modelling

The authors show the results of aggregation simulations, most notably in Fig 3D regarding forming tetramers, and in Fig 5 B and C to justify their assembly model. The precise details should be described clearly in the supplementary information. With any kinetic model, there are assumptions required and these are not clearly defined here. For example, in Fig 3D,

"Figure 3 ... The gray line depicts a fit of the data to a tetramer association model."

There is no single one tetramer association model. A linear model linking monomer to tetramer, but with different rate constants for the formation and destruction rates of each individual step en route to forming a tetramer could be made to fit this curve. On balance of probability, the simplest model with the fewest fitting parameters is the most sensible course, and so I agree with the authors in this. But the authors should state something that is qualified to that effect, that a simple model is consistent with their data, suggesting no need to go to a more complex model, not that the formation of tetramers by mutBTB is not possible. So this statement is too strong:

"Models for oligomerization into discrete tetramers (Figure 3D, gray line) or other discrete oligomers did not fit the data well, ruling out previously proposed tetramers or pentamers (Errington et al, 2012) "

Nothing is ruled out as far as I can see: just that the simpler monomer/dimer model is ruled as being most likely in. Moreover, there are signs of higher order complexes at 5-6 S in both the supplementary information and in fig 3B. Could this be the appearance of tetramers?

Similarly, the agreement of the isodesmic model to data in Fig 5B looks great. But what are the assumptions, what are the fitting parameters, and what precisely are the alternative models?

##### 3) The structural model Fig 3D. and page 6 "According to this model, SPOP oligomers adopt a fibrillar organization with helical propensity."

There are too few details describing the assumptions that went into the model and no validation. The movie shows a helical twist. How sensitive is the twist to the assumptions going into the model? It would be possible for the group to perform, for example, ion-mobility mass spectrometry experiments to estimate the size of the oligomers of various sizes and compare them to their models as a form of cross validation. Alternatively, electron microscopy images could be easily obtained to provide data to support the overall geometry of the proposed models. The model would be more compelling should there be any validation.

##### 4) A note for Figure 5 B

The authors might find it interesting to compare their distributions to the model of 'linear polymersisation'

Oosawa and Kasai, J.Mol.Biol. (1962) 4, 10-21

This model predicts an exponential decay in oligomerisation state with increasing oligomer size at equilibrium, where the rate of decay gives a measurement of the effective Kd of assembly. This model has been used to look at fibrillar assembly including amyloids and actin filaments. It looks

like this will fit this data very well indeed. This finding would further support their isodemic association model where growth comes only from the 'ends' of the oligomer.

5) Significance of aspect ratio

In paragraph 3 the authors describe a difference in the area per cell and aspect ratio of GFP-Gli31-455 bodies in the presence and absence of co-transfection with HA-SPOP. However, it is unclear from Table 2 whether the difference is significant:  $1.0 \pm 0.7$  for HA-SPOP and  $3 \pm 4$  for + GFP-Gli31-455. The distributions appear to have a long tail, and so the mean value might not be the best comparison. The most probable aspect ratio appears to be the same for both, and small differences coming from uncertainties and challenges with performing this analysis.

Not included in this data is the efficiency of co-transfection, which would presumably affect the result. The authors should make it explicit whether they are always measuring the diameter of HA-SPOP bodies in this experiment, or in the case of co-transfected cells, GFP-Gli31-455 bodies. Here, it would be useful to present the actual size (or diameter in the xy plane) distributions of HA-SPOP foci in the presence and absence of GFP-Gli31-455. Perhaps the more extended puncta are the small ones that are inherently difficult to quantify?

6) Figure 2c,

There appears to be a punctate staining of HA-mutBACK, against a background of diffuse material. Only mutBTB (not mutBACK) constructs have a completely diffuse staining pattern. From these data it seems that the BTB-interface is important for oligomerisation and localisation to discrete foci. It would be interesting if the authors could rationalise this result, or at least speculate on it in the manuscript in light of their biophysical data

7) Page 8 "SPOP-fluorescent protein fusion constructs produced mislocalized proteins in the cell, preventing analysis of SPOP dynamics in nuclear speckles."

It would be interesting to know what the authors mean by 'mislocalised'. Maybe a supplemental figure would help.

Minor textual clarifications:

Abstract >"However, higher-order complexes are inherently heterogeneous in size, limiting insight into how size influences function. "

Having heterogeneous complexes does not limit insight, but it does make them experimentally challenging to interrogate. The wording here is unclear.

Page 3 > Protein/protein interactions driving recruitment of components to these bodies are not well understood (Tourrière et al, 2003).

The authors cite amongst other papers the work of Nott et al. In this work the driving forces and specific protein/protein interactions are well-described, linked through mutagenesis work to GF and RG repeats. Moreover this is linked to their ability to uptake nucleic acids. Similar findings are reported in the other cited papers. Similar links have been noted in other organelle forming proteins through low complexity domains. The authors should expand upon this point as the statement implies nothing on this subject is known, which is not the case.

Page 4 >demonstrating that SPOP adopts an ensemble of oligomeric species with a broad size distribution.

The authors argue that a change in elution maximum is due to a change in size distribution. As with sedimentation experiments, a change in smaller oligomer weight with decreasing concentration could also be due to subunit exchange effects (the effective 'on' rate will be  $k^+$  times the free monomer/dimer concentration and so concentration dependent). The authors should tone back the statement. What is clear is that the elution maximum is concentration dependent. There are several biophysical explanations for this.

Page 8 >ranging in size from hundreds of nm

The authors cannot resolve structures of hundreds of nm using light microscopy in this way. This statement should be qualified so that the lower limit is the resolution limit of their apparatus. Bodies might well be smaller than this but it would not be possible to know from these measurements.

Page 8> characteristic time

Do the authors mean half life, or the quantitate  $1/\text{rate}$ ? The authors can turn the rate into a diffusion coefficient, combined with information on the size of the bleaching area, which would enable them to compare the local mobility of their liquid blobs to those of Brangwynne and Nott. That would possibly make for an interesting comparison between the types of liquid drop currently in the literature.

Referee #2

(Report for Author)

SPOP, a MATH-BTB type substrate adaptor of the CUL 3 E3 ubiquitin ligase, regulates cell proliferation and development, and also plays a critical role in suppressing tumor through inhibiting Hh/Gli2 signaling pathway. It may provide an alternative strategy for developing therapeutic agents in the future. Many SPOP mutations were detected in some aggressive cancers. SPOP also has potential use as a novel biomarker of glioma. This study reveals novel molecular events underlying the link between the size distributions of SPOP oligomers to nuclear speckles localization and stimulates CUL3 ubiquitination. The novelty of this study is the linking of the macro-protein complex or oligomers to the localization of SPOP and catalytic efficiency. The idea is interesting as it represents an example of the tandem self-association plays an important role in the localization to nuclear speckles and the protein functions. The authors performed solid experiments supporting their conclusions although the molecular linkage between self-association and the recruitment of SPOP to liquid nuclear speckles is still unclear. The bulk of the data presented are good quality and the manuscript flow is logical.

Major issue

One area of this manuscript can be further improved is to provide *in vivo* functional evidence or further mechanistic insight to support the biophysical studies. For example, there are as many as 200 BTB proteins in human cells. Since one key aspect of the self-association is dependent on the BTB domain, can the authors test how broadly the proposed model applies to other BTB proteins? On the mechanism side, one would like to know how the self-association stimulates the CUL3 polyubiquitylation, facilitating the assembly of multi-subunit of CUL3? Facilitating or enhancing the ub chain elongation like processive factor?

Specific points:

1. In figure 2C, the IF staining may need show the lower magnification pictures not only show the high magnification single cell.
2. In figure 3D, why did not use FDS-AUC assay to analyze the self-association of BACK domain?
3. Complete labeling should be used for the input panels of the following figures to keep the figure formatting consistent throughout: 3A-D, 4B-C, 5C.

Referee #3

(Report for Author)

In the presented manuscript Marzahn and colleagues analyze the higher-order oligomerization of speckle-type POZ protein (SPOP), a component of a cullin E3 ubiquitin-protein ligase complex, which depends on specific tandem self-association domain interaction and is required for localization in nuclear speckles. The authors are trying to demonstrate that nuclear speckles have liquid phase separation which is in agreement with recent findings that many nuclear structures may have liquid properties.

The presented data are interesting but too preliminary for the publication in EMBO J.

They should include the use of an interfering peptide to show that the oligomerization of SPOP *in vitro* is not stochastic aggregation. It is well established that the *in vitro* behavior of an oligomeric complex is completely different to that of an *in vivo* one.

- Fig 2 absolutely needs splicing speckle-specific staining alongside the SPOP visualization. The authors should use the mouse antibody against splicing factor SC-35 (Sigma).

- The *in vivo* oligomeric state should be quantified using the number and brightness assay and shown to be different to the mutants.

- The authors should actually bother to monitor in vivo substrate ubiquitination of each mutant (perhaps in a SPOP-null / CRISPR background to make it interesting)
- I'd like to see the movement of SPOP molecules assessed by single molecule tracking in the wild type and mutant. I'm not satisfied by the statement "SPOP-fluorescent protein fusion constructs produced mislocalized proteins in the cell, preventing analysis of SPOP dynamics in nuclear speckles."
- Finally, I think that <50 cells is far too low for the observations they are claiming re: shape and size. This needs >150 cells at least and should be relatively easy to perform.

Preliminary response

16 November 2015

Thank you for sending the reviewer comments and giving us the chance to lay out a plan to address them. We are planning to send you our response in the next few days and are confident to be able to address many of the reviewer comments.

Some reviewer requests, however, are outside the scope of the work or technically impossible (such as fluorescently labeling SPOP; all fusion constructs we have tried mislocalized in the cell). I would welcome the chance to talk to you on the phone and discuss which requests will actually result in improved insight.

We welcome the chance to respond to the thoughtful comments from the reviewers. We have done so point by point below. Here, we would like to point out the 3 major pieces of data we are prepared to add to the manuscript.

1. We have preliminary data showing co-localization of the speckle marker SC-35 with SPOP.
2. Cross-linking experiments of SPOP WT and mutants in cells recapitulates the in vitro self-association behavior, providing a strong link between self-association and localization in nuclear bodies.
3. Fluorescence anisotropy binding data show that SPOP BTB and BACK domain mutants are able to bind SPOP binding motifs at affinities comparable to the WT.

We think that these data resolve the major open questions left by the scope of our manuscript.

*Referee #1*

*(Report for Author)*

*The manuscript by Mittag et al. contains some very interesting data and I recommend publication in the EMBO journal with minor revision.*

*The main result here to this reviewers mind is the liquid drop type behaviour from bodies formed from GFP-Gli3(1-455), and that HA-SPOP preferentially co-localises to these bodies. These results are exemplified in figure 2C and 6. This result is novel, timely and builds on an exciting body of work emerging on liquid-drop type membraneless organelles. The significance of these bodies in cellular organisation we are only just starting to see.*

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*To illustrate my current reservations, in the abstract:*

*"Herein, we demonstrate that nuclear speckles have liquid droplet properties and that localization of SPOP to speckles depends upon its ability to form higher-order oligomers through tandem self-association domains. "*

*As far as I can tell, nuclear speckles are not demonstrated to have liquid droplet properties (bodies formed from GFP-Gil3 have liquid properties, see below) and oligomerisation is not shown to be*

*linked to colocalisation in vivo (perhaps specific interactions are the cause of this lack of localization, and nothing to do with oligomerisation, see below). I do not mean to detract from the great data in this paper; just this reviewer would advocate for qualification in some of the claims, or more data to support them. The core results are outstanding making the overall paper a very worthy article in the view of this reviewer.*

#### *Major comments*

##### *1) Use of the term 'nuclear speckle'.*

*In Fig 2c, the authors show the results of expressing HA-tagged WT SPOP, and BTB and BACK domain mutants. From the punctate staining pattern of SPOP WT protein, the authors infer that SPOP localises to nuclear speckles. A lot of emphasis is placed on this.*

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*The authors conclude nuclear speckles are liquid droplets. What I believe they show is that bodies associated with GFP-Gli3(1-455) have liquid drop properties. Does GFP-Gli3(1-455) co-localise to nuclear speckles, or are these bodies spontaneously formed and are separate to nuclear speckles? At least some HA-SPOP fluorescence is not associated with GFP-Gli3(1-455) fluorescence in the images shown in Fig 6A and E. To evaluate the conclusions drawn from the images, it would be helpful to know how they were generated. For example, were they acquired using confocal or (deconvolved) widefield microscopy? Are the images shown derived from single xy-planes, or were they derived from projections of image stacks (e.g. maximum, average or sum intensity projections)? The argument for HA-SPOP and GFP-Gli3(1-455) co-localisation could be further strengthened by quantifying of the overlap in fluorescence signals. A more direct link to nuclear speckles should be provided to make the claims as*

*stated in the article. In the absence of such a link it's clear that the GFP-Gli3(1-455) bodies have liquid drop properties and that these co-localise to some extent with SPOP, both of which are very neat and compelling findings. Just that without a direct link to nuclear speckles, the precise details of what is seen in the data should be made clearer, and some of the conclusions should be toned down.*

We agree that it is important to show that the liquid bodies we observe in cells are indeed nuclear speckles; in particular in light of new reports that SPOP can also localize to punctate DNA damage sites (Boysen et al., SPOP mutation leads to genomic instability in prostate cancer. eLife 2015). We have data showing co-localization of SPOP with the nuclear speckle marker SC35 and will also explore whether SPOP co-expressed with Gli3<sup>1-455</sup> localizes to nuclear speckles.

##### *2) Page 4 SPOP oligomerization is required for cellular localization*

*The authors show that the mutations in a truncated SPOP restrict the oligomeric size of SPOP in vitro. No data on the oligomerisation state of SPOP in vivo is shown (unsurprisingly, as to get this is highly challenging). An alternative, arguably simpler possibility is that the specific interaction between Gli3 and SPOP is knocked out by these mutations and is the cause of the lack of colocalisation. Perhaps also the mutations when applied to full length SPOP rather than (28-355), do not affect oligomerisation of SPOP. I agree with the authors that the causal link between oligomerisation and localization is suggestive. Required is certainly too strong, and no link is 'demonstrated' as the authors claim. So the link here is not as clear as the manuscript implies. The authors should tone this down and qualify the claim. Notable places for this include the abstract, title and conclusion paragraphs.*

The reviewer suggests that mutations of the self-association domain interfaces may affect substrate binding, and that the diffuse distribution of the resulting SPOP mutants may be explained by a lack of recruitment to the substrate.

First, we would like to point out that the experiments in Figure X of the manuscript that show punctate localization of SPOP WT and diffuse localization of the SPOP mutants are carried out in the absence of Gli3<sup>1-455</sup>.

Second, we have fluorescence anisotropy binding data that unequivocally demonstrates that these SPOP mutants still bind SPOP binding motifs with normal affinities. We can include these in the manuscript.

Third, we would like to point out that residues 1-26 and 360-374 do not contain oligomerization domains or low-complexity sequences that may lead to self-association. The full-length protein in vitro tends to form amorphous aggregates over time and is likely not going to be useful in further assessing the behavior of SPOP mutants.

Instead, we propose to include new data that demonstrate oligomerization behavior of SPOP WT and mutants in cells similar to that found in vitro.

We performed cross-linking experiments in fresh lysates of NIH 3T3 cells transfected with SPOP WT or one of the self-association deficient mutants. These new data demonstrate that in cells SPOP WT forms large higher-order complexes that are too large to migrate into a SDS PAGE gel. SPOP mutBACK migrates mostly as dimers, while SPOP mutBTB and SPOP mutBTB-BACK migrates mostly as monomers, recapitulating the behavior we see by SEC and MALS in vitro.

#### *Minor comments*

##### *1) Biophysical interrogation of SPOP assembly*

*The full length protein we are told forms very large oligomers, and the study focuses on truncated construct 28-359.*

*When the full length protein aggregates, do the authors believe the assembly is a continuation of the assembly that they see in the simplified construct, or something more pathogenic like amyloid assembly? The manuscript would benefit from some data clarifying what is happening with the full length, and a discussion on the subject.*

*Perhaps the uncontrolled assembly of these molecules occurs when residues 1-28 are present is effectively a continuation of the isodesmic model they propose. Perhaps showing the NTerm in their model will help clarify what it could or could not do.*

*Moreover, it would be interesting to see whether there is a difference in localisation of HA-full length SPOP and HA-SPOP (residues 28-359). i.e. do the N-terminal 27 residues affect the localisation of the protein in cells?*

We do not believe that the aggregation of the full-length protein in vitro is a continuation of the isodesmic self-association of SPOP<sup>28-359</sup>. Indeed, SPOP FL (full-length) forms oligomers of similar size as SPOP<sup>28-359</sup> upon initial purification. On the time-scale of the extensive preparation for CG-MALS analysis, including preparative ultracentrifugation, the protein forms what seem to be amorphous aggregates. SPOP<sup>28-359</sup>, in contrast, forms reversible higher-order oligomers, which adopt a defined size distribution within seconds after diluting the protein from a stock solution. We plan to show SEC data for SPOP FL comparable to SPOP WT, and subsequent irreversible aggregation.

The N-terminus does neither contain a folded oligomerization domain, nor a low-complexity domain of the sort that was recently shown to mediate liquid phase separation. The C-terminus contains a nuclear localization sequence and can therefore not be removed for experiments in cells. We this plan to transiently express HA-SPOP<sup>28-374</sup> in cells to test the necessity of the N-terminus for localization to nuclear speckles.

We will further make a direct comparison of the oligomerization behavior of full-length SPOP WT and mutants in cells and SPOP<sup>28-359</sup> WT and mutants in vitro using cross-linking experiments.

##### *2) Kinetic modelling*

*The authors show the results of aggregation simulations, most notably in Fig 3D regarding forming tetramers, and in Fig 5 B and C to justify their assembly model. The precise details should be described clearly in the supplementary information. With any kinetic model, there are assumptions required and these are not clearly defined here. For example, in Fig 3D,*

*"Figure 3 ... The gray line depicts a fit of the data to a tetramer association model."*

*There is no single one tetramer association model. A linear model linking monomer to tetramer, but with different rate constants for the formation and destruction rates of each individual step en route to forming a tetramer could be made to fit this curve. On balance of probability, the simplest model with the fewest fitting parameters is the most sensible course, and so I agree with the authors in this. But the authors should state something that is qualified to that effect, that a simple model is consistent with their data, suggesting no need to go to a more complex model, not that the formation of tetramers by mutBTB is not possible. So this statement is too strong:*

*"Models for oligomerization into discrete tetramers (Figure 3D, gray line) or other discrete oligomers did not fit the data well, ruling out previously proposed tetramers or pentamers"*



*(Errington et al, 2012) "*

*Nothing is ruled out as far as I can see: just that the simpler monomer/dimer model is ruled as being most likely in. Moreover, there are signs of higher order complexes at 5-6 S in both the supplementary information and in fig 3B. Could this be the appearance of tetramers? Similarly, the agreement of the isodemic model to data in Fig 5B looks great. But what are the assumptions, what are the fitting parameters, and what precisely are the alternative models?*

The reviewer assumes in his or her comments that we are modeling the assembly of SPOP fibrils over time using kinetic models. This is an incorrect assumption, and we apologize if our description has not dissuaded him or her from this assumption. All light scattering measurements were carried out in equilibrium. The data points in Figures 3D and 4 represent individual experiments, in which SPOP protein was diluted from a stock concentration and the LS signal evaluated after equilibration at this new concentration. We can show this in an additional supplementary figure. Since Figure 3D shows the Mw vs concentration in equilibrium, we can unequivocally rule out that the BACK domain tetramerizes.

*3) The structural model Fig 3D. and page 6 "According to this model, SPOP oligomers adopt a fibrillar organization with helical propensity."*

*There are too few details describing the assumptions that went into the model and no validation. The movie shows a helical twist. How sensitive is the twist to the assumptions going into the model? It would be possible for the group to perform, for example, ion-mobility mass spectrometry experiments to estimate the size of the oligomers of various sizes and compare them to their models as a form of cross validation. Alternatively, electron microscopy images could be easily obtained to provide data to support the overall geometry of the proposed models. The model would be more compelling should there be any validation.*

No assumptions go into the model; it relies on crystallography-determined domain-domain interactions only. We included the structural models in Figure 4D and in the supplementary movie to provide intuitive images of the assemblies. In fact, these models could already be made in the absence of our data from the two crystal structures of the BTB dimer and the BACK dimer. The BACK dimer structure is based on only a small fragment of SPOP and was in contrast to biophysical data that was interpreted as tetramerization or pentamerization of SPOP. Our data presented in the manuscript demonstrates that the BACK domain indeed forms dimers that depend on interactions in the crystallographic interface.

The SPOP oligomers are labile. This is one of the premises we make in the introduction. As opposed to stable fibrillar/filamentous assemblies that grow through a nucleation/polymerization mechanism and have been characterized structurally, labile oligomers are largely structurally inaccessible due to their heterogeneous size distribution. We plan to clarify this in the text even more than we do now. Long assemblies that would be visible in EM are always in equilibrium with shorter assemblies; in fact, the population of species of size  $2N-2$  is always larger than that of species  $2N$ . Protein concentrations high enough to result in the preferential incorporation of SPOP monomers in longer filaments (Figure 5C) leads to coating of EM grids. We have tried both EM and AFM and confirmed this reasoning.

*4) A note for Figure 5 B*

*The authors might find it interesting to compare their distributions to the model of 'linear polymerisation'*

*Oosawa and Kasai, J.Mol.Biol. (1962) 4, 10-21*

*This model predicts an exponential decay in oligomerisation state with increasing oligomer size at equilibrium, where the rate of decay gives a measurement of the effective Kd of assembly. This model has been used to look at fibrillar assembly including amyloids and actin filaments. It looks like this will fit this data very well indeed. This finding would further support their isodemic association model where growth comes only from the 'ends' of the oligomer.*

We thank the reviewer for this recommendation and will do so.

*5) Significance of aspect ratio*

*In paragraph 3 the authors describe a difference in the area per cell and aspect ratio of GFP-Gli31-455 bodies in the presence and absence of co-transfection with HA-SPOP. However, it is unclear*

from Table 2 whether the difference is significant: 1.0 +/- 0.7 for HA-SPOP and 3 +/- 4 for + GFP-Gli31-455. The distributions appear to have a long tail, and so the mean value might not be the best comparison. The most probable aspect ratio appears to be the same for both, and small differences coming from uncertainties and challenges with performing this analysis.

We find the aspect ratios of puncta in HA-SPOP-transfected cells and in GFP-Gli3<sup>1-455</sup> + HA-SPOP-transfected cells to be similar within error at 1.1 +/- 0.1 and 1.5 +/- 0.4. We do not claim that there is a difference. We conclude from these data that the bodies are close to spherical, which is in agreement with liquid droplet behavior.

*Not included in this data is the efficiency of co-transfection, which would presumably affect the result. The authors should make it explicit whether they are always measuring the diameter of HA-SPOP bodies in this experiment, or in the case of co-transfected cells, GFP-Gli31-455 bodies. Here, it would be useful to present the actual size (or diameter in the xy plane) distributions of HA-SPOP foci in the presence and absence of GFP-Gli31-455. Perhaps the more extended puncta are the small ones that are inherently difficult to quantify?*

We can include this quantification.

6) Figure 2c,

*There appears to be a punctate staining of HA-mutBACK, against a background of diffuse material. Only mutBTB (not mutBACK) constructs have a completely diffuse staining pattern. From these data it seems that the BTB-interface is important for oligomerisation and localisation to discrete foci. It would be interesting if the authors could rationalise this result, or at least speculate on it in the manuscript in light of their biophysical data*

We agree with the reviewer that, although self-association via both domains mediates localization to nuclear bodies, BTB-mediated self-association (in SPOP mutBACK) plays the larger role of the two domains due to its stronger affinity. This finding is also in agreement with our new cross-linking data.

7) Page 8 "SPOP-fluorescent protein fusion constructs produced mislocalized proteins in the cell, preventing analysis of SPOP dynamics in nuclear speckles."

*It would be interesting to know what the authors mean by 'mislocalised'. Maybe a supplemental figure would help.*

We will include example images in the Supplementary Material showing the incorrect localization of GFP-SPOP and mCherry-SPOP constructs.

*Minor textual clarifications:*

*Abstract > "However, higher-order complexes are inherently heterogeneous in size, limiting insight into how size influences function. "*

*Having heterogeneous complexes does not limit insight, but it does make them experimentally challenging to interrogate. The wording here is unclear.*

In the case of a heterogeneous size distribution, it is often unclear whether the function is mediated preferentially by assemblies of a certain size.

*Page 3 > Protein/protein interactions driving recruitment of components to these bodies are not well understood (Tourrière et al, 2003).*

*The authors cite amongst other papers the work of Nott et al. In this work the driving forces and specific protein/protein interactions are well-described, linked through mutagenesis work to GF and RG repeats. Moreover this is linked to their ability to uptake nucleic acids. Similar findings are reported in the other cited papers. Similar links have been noted in other organelle forming proteins through low complexity domains. The authors should expand upon this point as the statement implies nothing on this subject is known, which is not the case.*

We agree with the reviewer and will endeavor to give a fuller picture. To our knowledge, the role of

interactions of folded domains in the formation of and recruitment to cellular bodies is not well understood.

*Page 4 > demonstrating that SPOP adopts an ensemble of oligomeric species with a broad size distribution.*

*The authors argue that a change in elution maximum is due to a change in size distribution. As with sedimentation experiments, a change in smaller oligomer weight with decreasing concentration could also be due to subunit exchange effects (the effective 'on' rate will be  $k_+$  times the free monomer/dimer concentration and so concentration dependent). The authors should tone back the statement. What is clear is that the elution maximum is concentration dependent. There are several biophysical explanations for this.*

We will take into account this good point.

*Page 8 > ranging in size from hundreds of nm*

*The authors cannot resolve structures of hundreds of nm using light microscopy in this way. This statement should be qualified so that the lower limit is the resolution limit of their apparatus. Bodies might well be smaller than this but it would not be possible to know from these measurements.*

We agree with the reviewer.

*Page 8 > characteristic time*

*Do the authors mean half life, or the quantitate  $1/\text{rate}$ ? The authors can turn the rate into a diffusion coefficient, combined with information on the size of the bleaching area, which would enable them to compare the local mobility of their liquid blobs to those of Brangwynne and Nott. That would possibly make for an interesting comparison between the types of liquid drop currently in the literature.*

We report the inverse of the rate constant. While the arithmetics are possible, we wonder how the diffusion coefficient of Gli3<sup>1-455</sup> in these bodies adds to the message of the paper.

*Referee #2*

*(Report for Author)*

*SPOP, a MATH-BTB type substrate adaptor of the CUL3 E3 ubiquitin ligase, regulates cell proliferation and development, and also plays a critical role in suppressing tumor through inhibiting Hh/Gli2 signaling pathway. It may provide an alternative strategy for developing therapeutic agents in the future. Many SPOP mutations were detected in some aggressive cancers. SPOP also has potential use as a novel biomarker of glioma. This study reveals novel molecular events underlying the link between the size distributions of SPOP oligomers to nuclear speckles localization and stimulates CUL3 ubiquitination. The novelty of this study is the linking of the macro-protein complex or oligomers to the localization of SPOP and catalytic efficiency. The idea is interesting as it represents an example of the tandem self-association plays an important role in the localization to nuclear speckles and the protein functions. The authors performed solid experiments supporting their conclusions although the molecular linkage between self-association and the recruitment of SPOP to liquid nuclear speckles is still unclear. The bulk of the data presented are good quality and the manuscript flow is logical.*

*Major issue*

*One area of this manuscript can be further improved is to provide in vivo functional evidence or further mechanistic insight to support the biophysical studies. For example, there are as many as 200 BTB proteins in human cells. Since one key aspect of the self-association is dependent on the BTB domain, can the authors test how broadly the proposed model applies to other BTB proteins? On the mechanism side, one would like to know how the self-association stimulates the CRL3 polyubiquitylation, facilitating the assembly of multi-subunit of CRL3? Facilitating or enhancing the*

*ub chain elongation like processive factor?*

For SPOP, recruitment to nuclear speckles is mediated by the presence of two functional self-association domains. The BTB domain alone is not enough. In Suppl Figure 5, we address the question whether other substrate adaptors with both BTB and BACK domains may be able to form higher-order oligomers. We find that longer version of the BACK domain, which is present in many substrate adaptors together with KELCH domains, occludes the BACK dimer interface. The only candidates for higher-order self-association we find are a group of proteins specific to rodents.

In this manuscript, we are not attempting to address how SPOP self-association stimulates polyubiquitination. Our structural model as well as data in Errington et al (Structure 2012) suggest that the multi-subunit CRL3 can assemble onto oligomeric SPOP.

We have recently published a manuscript in which we propose that multivalency of SPOP oligomers and multiple SPOP binding motifs in substrates together enhances substrate recruitment, decreases substrate release, and therefore enhances CRL3 processivity.

W.K. Pierce, C.R. Grace, J. Lee, A. Nourse, M.R. Marzahn, E.R. Watson, A.A. High, J. Peng, B.A. Schulman, **T. Mittag**. (2015) Multiple weak linear motifs enhance recruitment and processivity in SPOP-mediated substrate ubiquitination. *J. Mol. Biol.* Epub ahead of print. PMID: 26475525

*Specific points:*

1. *In figure 2C, the IF staining may need show the lower magnification pictures not only show the high magnification single cell.*

Because the transfection efficiency was low and we usually only have a single transfected cell per image with low magnification, we instead offer to include several images per construct in an additional supplementary figure to show the representative nature of the images in Figure 2C.

2. *In figure 3D, why did not use FDS-AUC assay to analyze the self-association of BACK domain?*

We used FDS-AUC to quantify the dissociation constant of the BTB domain, because neither absorbance-detected AUC nor MALS were sensitive enough to detect the protein at concentrations at which it was monomeric. Because the BACK domain dimerizes with a micromolar dissociation constant, sensitivity was not limited and we therefore were able to use absorbance-detected AUC and MALS.

3. *Complete labeling should be used for the input panels of the following figures to keep the figure formatting consistent throughout: 3A-D, 4B-C, 5C.*

We will improve our labels.

*Referee #3*

*(Report for Author)*

*In the presented manuscript Marzahn and colleagues analyze the higher-order oligomerization of speckle-type POZ protein (SPOP), a component of a cullin E3 ubiquitin-protein ligase complex, which depends on specific tandem self-association domain interaction and is required for localization in nuclear speckles. The authors are trying to demonstrate that nuclear speckles have liquid phase separation which is in agreement with recent findings that many nuclear structures may have liquid properties.*

*The presented data are interesting but too preliminary for the publication in EMBO J.*

*They should include the use of an interfering peptide to show that the oligomerization of SPOP in vitro is not stochastic aggregation. It is well established that the in vitro behavior of an oligomeric complex is completely different to that of an in vivo one.*

We demonstrate that higher-order SPOP self-association is mediated by specific, structurally well-defined interfaces. Indeed, higher-order self-association can be nearly completely quantitatively

predicted from the properties of the two independent dimerization domains, further demonstrating the specificity of SPOP self-association.

We thank the reviewer for the suggestion to compete out SPOP self-association. Because the BTB as well as the BACK domains dimerize via complex three-dimensional interfaces, we will use a self-association deficient mutant instead of a peptide. We are already showing limited data to this effect in Suppl. Fig. 7B, in which addition of SPOP mutBACK to SPOP WT at 37 ° C leads to a reduction of the SPOP elution volume. We will expand these experiments.

*- Fig 2 absolutely needs splicing speckle-specific staining alongside the SPOP visualization. The authors should use the mouse antibody against splicing factor SC-35 (Sigma).*

We will include SC-35 staining alongside SPOP visualization.

*- The in vivo oligomeric state should be quantified using the number and brightness assay and shown to be different to the mutants.*

We agree with the reviewer that the number and brightness method would be the optimal method for quantifying the SPOP oligomeric state in a live cell. Unfortunately, this is impossible due to SPOP's mislocalization when fluorescently tagged. We will include example images in the Supplementary Material showing the incorrect localization of GFP-SPOP and mCherry-SPOP constructs.

Instead, we propose to include new data that demonstrate oligomerization behavior of SPOP WT and mutants in cells similar to that found in vitro.

We performed cross-linking experiments in fresh lysates of NIH 3T3 cells transfected with SPOP WT or one of the self-association deficient mutants. These new data demonstrate that in cells SPOP WT forms large higher-order complexes that are too large to migrate into a SDS PAGE gel. SPOP mutBACK migrates mostly as dimers, while SPOP mutBTB and SPOP mutBTB-BACK migrates mostly as monomers, recapitulating the behavior we see by SEC and MALS in vitro.

*- The authors should actually bother to monitor in vivo substrate ubiquitination of each mutant (perhaps in a SPOP-null / CRISPR background to make it interesting)*

We agree with the reviewer that these experiments would be interesting, but they are outside the scope of this manuscript.

*- I'd like to see the movement of SPOP molecules assessed by single molecule tracking in the wild type and mutant. I'm not satisfied by the statement "SPOP-fluorescent protein fusion constructs produced mislocalized proteins in the cell, preventing analysis of SPOP dynamics in nuclear speckles."*

Unfortunately, this is impossible due to SPOP's mislocalization when fluorescently tagged. We will include example images in the Supplementary Material showing the incorrect localization of GFP-SPOP and mCherry-SPOP constructs. Untagged and HA-tagged SPOP were both shown to localize to nuclear speckles. (Nagai Y, Kojima T, Muro Y, Hachiya T, Nishizawa Y, Wakabayashi T & Hagiwara M (1997) Identification of a novel nuclear speckle-type protein, SPOP. *FEBS Lett.* **418**: 23–26

*- Finally, I think that <50 cells is far too low for the observations they are claiming re: shape and size. This needs >150 cells at least and should be relatively easy to perform.*

The conclusion we draw from the analysis of the shape of the cellular bodies is that they are close to spherical on average. If the reviewer thinks that we would be able to draw this conclusion more confidently with >150 cells, we will quantify the sphericity of bodies

1st Editorial Decision

19 November 2015

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Thank you for response letter proposing how you may address the referee comments on your current submission, EMBOJ-2015-93169, during the course of a major revision of the study. I was pleased to read that you appear to be in a good position to answer the majority of concerns raised through further experiments and/or additional clarifications, including the key issues of SPOP colocalization with nuclear speckle markers and of SPOP oligomerization behavior in cells. I would therefore like to invite you to start preparing a revision along the lines suggested. As discussed further by phone, we agree that in-depth follow-up analyses on the functional significance of SPOP oligomerization/localization for ubiquitin ligation would not lie directly within the scope of the present manuscript, but that inclusion of data on the in vivo significance of SPOP oligomerization would clearly further strengthen this work.

Thank you again for the opportunity to consider this work! I look forward to your revision.

1st Revision - authors' response

28 December 2015

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(Begins on next page.)

1st Revision - Author's response

28 December 2015

## **Point-by-point response**

*Referee #1*

*(Report for Author)*

*The manuscript by Mittag et al. contains some very interesting data and I recommend publication in the EMBO journal with minor revision.*

*The main result here to this reviewer's mind is the liquid drop type behaviour from bodies formed from GFP-Gli3(1-455), and that HA-SPOP preferentially co-localises to these bodies. These results are exemplified in figure 2C and 6. This result is novel, timely and builds on an exciting body of work emerging on liquid-drop type membraneless organelles. The significance of these bodies in cellular organisation we are only just starting to see.*

*The majority of the article is devoted to a biophysical characterisation of the oligomeric assembly of SPOP. I have two major comments on these two main areas, and a number of minor comments. The majority of which I believe can be dealt with textual clarifications and toning down of certain*

statements. A small number of additional experiments, most notably showing that the bodies formed from GFP-Gli3(1-455) and those that absorb SPOP stain with nuclear speckle markers. This would enhance this story and provide a convincing link that the bodies analysed here can be allied with nuclear speckles.

To illustrate my current reservations, in the abstract:

"Herein, we demonstrate that nuclear speckles have liquid droplet properties and that localization of SPOP to speckles depends upon its ability to form higher-order oligomers through tandem self-association domains. "

As far as I can tell, nuclear speckles are not demonstrated to have liquid droplet properties (bodies formed from GFP-Gli3 have liquid properties, see below) and oligomerisation is not shown to be linked to colocalisation *in vivo* (perhaps specific interactions are the cause of this lack of localization, and nothing to do with oligomerisation, see below). I do not mean to detract from the great data in this paper; just this reviewer would advocate for qualification in some of the claims, or more data to support them. The core results are outstanding making the overall paper a very worthy article in the view of this reviewer.

We thank the reviewer for these thoughtful comments. In the revised version of the manuscript, we have addressed these two concerns by showing co-localization with the speckle marker SC-35 and by showing that the *in vitro* self-association behavior of WT SPOP and the mutants is recapitulated in cells. See below for more detail. We therefore provide a strong link between the self-association behavior of SPOP *in vitro*, in cells, and its localization to nuclear speckles. We believe we can now conclude "Taken together, all of the data demonstrate that higher-order SPOP oligomers preferentially localize to liquid-like nuclear speckles, whereas self-association deficient SPOP mutants adopt a diffuse distribution in the nucleus."

#### Major comments

1) Use of the term 'nuclear speckle'.

In Fig 2c, the authors show the results of expressing HA-tagged WT SPOP, and BTB and BACK domain mutants. From the punctate staining pattern of SPOP WT protein, the authors infer that SPOP localises to nuclear speckles. A lot of emphasis is placed on this.

Perhaps the bodies formed by SPOP are formed by SPOP oligomerisation and have nothing to do with nuclear speckles? To link SPOP to nuclear speckles, the authors should simultaneously visualize nuclear speckles using known markers. For example, SC35 antibodies are commercially available and would be one way to easily accomplish this. Using additional nuclear speckle markers would strength the case.

The authors conclude nuclear speckles are liquid droplets. What I believe they show is that bodies associated with GFP-Gli3(1-455) have liquid drop properties. Does GFP-Gli3(1-455) co-localise to nuclear speckles, or are these bodies spontaneously formed and are separate to nuclear speckles? At least some HA-SPOP fluorescence is not associated with GFP-Gli3(1-455) fluorescence in the images shown in Fig 6A and E. To evaluate the conclusions drawn from the images, it would be helpful to know how they were generated. For example, were they acquired using confocal or (deconvolved) widefield microscopy? Are the images shown derived from single xy-planes, or were they derived from projections of image stacks (e.g. maximum, average or sum intensity projections)? The argument for HA-SPOP and GFP-Gli3(1-455) co-localisation could be further strengthened by quantifying of the overlap in fluorescence signals. A more direct link to nuclear speckles should be



*provided to make the claims as stated in the article. In the absence of such a link it's clear that the GFP-Gli3(1-455) bodies have liquid drop properties and that these co-localise to some extent with SPOP, both of which are very neat and compelling findings. Just that without a direct link to nuclear speckles, the precise details of what is seen in the data should be made clearer, and some of the conclusions should be toned down.*

We agree that it is important to show that the liquid bodies we observe in cells are indeed nuclear speckles; particularly in light of recent reports that SPOP can also localize to punctate DNA damage sites (Boysen et al., SPOP mutation leads to genomic instability in prostate cancer. eLife 2015). In the new Figure 2, we show that the nuclear speckle marker SC-35 indeed co-localizes with HA-SPOP in single-transfected cells and with HA-SPOP/GFP-Gli3<sup>1-455</sup> in co-transfected cells. We thus demonstrate liquid behavior not only of GFP-Gli3<sup>1-455</sup>-marked bodies, but also of nuclear speckles. We are grateful for the suggestion that has allowed us to strengthen the conclusion that SPOP co-localizes with nuclear speckles.

We have analyzed the overlap of the HA and GFP signals in Figure 2B. The cell has 17 bodies with red/green overlap, and one additional puncta with only red signal. This is also reflected in the fraction of overlapping signal. The overlapped area contains 75% of the punctate HA signal, and 100% of the punctate GFP signal.

The images are single xy-planes acquired with confocal microscopy, as now noted in the figure caption.

## *2) Page 4 SPOP oligomerization is required for cellular localization*

*The authors show that the mutations in a truncated SPOP restrict the oligomeric size of SPOP in vitro. No data on the oligomerisation state of SPOP in vivo is shown (unsurprisingly, as to get this is highly challenging). An alternative, arguably simpler possibility is that the specific interaction between Gli3 and SPOP is knocked out by these mutations and is the cause of the lack of colocalisation. Perhaps also the mutations when applied to full length SPOP rather than (28-355), do not affect oligomerisation of SPOP. I agree with the authors that the causal link between oligomerisation and localization is suggestive. Required is certainly too strong, and no link is 'demonstrated' as the authors claim. So the link here is not as clear as the manuscript implies. The authors should tone this down and qualify the claim. Notable places for this include the abstract, title and conclusion paragraphs.*

To address the question, whether the self-association behavior of SPOP, which we have carefully dissected *in vitro*, is relevant for function in cells, we have added an amide-based crosslinking assay to compare the self-association behavior of SPOP variants *in vitro* and in cell lysates. The pure, recombinant proteins crosslink *in vitro* as expected from our other biophysical measurements: SPOP WT forms higher-order oligomers, SPOP<sup>mutBACK</sup> and SPOP<sup>mutBTB</sup> form mostly dimers, and SPOP<sup>mutBTB-BACK</sup> is mostly monomeric in solution. In lysates of cells expressing HA-tagged SPOP constructs, we observe crosslinking behavior that is in striking agreement; HA-SPOP<sup>1-374</sup> forms large complexes, some too large to enter the gel. HA-SPOP<sup>mutBACK</sup> appears mostly at its dimer molecular weight, HA-SPOP<sup>mutBTB</sup> and HA-SPOP<sup>mutBTB-BACK</sup> appear at their monomer molecular weights. We have qualified the language in the manuscript to say that these results are strongly suggestive of a role for oligomerization in localization to nuclear speckles.

The reviewer suggests an interesting alternative explanation for the diffuse distribution of the SPOP mutants; the mutations of the self-association domain interfaces may affect substrate

binding, and diffuse distribution may be explained by a lack of recruitment to the substrate.

We have now included fluorescence anisotropy binding data that demonstrate the ability of all SPOP mutants studied here to bind a SPOP binding motif containing peptide with affinities comparable to SPOP WT. These data are shown in the current Figure 4A and the new Table 2. A reduction in the SPOP oligomer size may affect binding of multivalent substrates, as we discuss in our recently published manuscript (Pierce et al, Multiple weak linear motifs enhance recruitment and processivity in SPOP-mediated substrate ubiquitination, J. Mol. Biol. 2015). With the ability to bind monovalent substrate intact, this is mechanistically a self-association defect rather than a substrate binding defect.

We would like to point out that residues 1-26 and 360-374 do not contain oligomerization domains or low-complexity sequences that may lead to self-association. The full-length protein tends to form amorphous aggregates *in vitro* (see Supplementary figure 4B) and is not useful in further assessing the behavior of SPOP mutants.

We have carefully dissected the behavior of the individual interfaces and have demonstrated that they are recapitulated in WT SPOP<sup>28-359</sup>. While the full-length protein may add additional complexity, it is impossible for us to dissect the interactions in this protein due to the irreversibility of aggregation.

#### *Minor comments*

##### *1) Biophysical interrogation of SPOP assembly*

*The full length protein we are told forms very large oligomers, and the study focuses on truncated construct 28-359.*

*When the full length protein aggregates, do the authors believe the assembly is a continuation of the assembly that they see in the simplified construct, or something more pathogenic like amyloid assembly? The manuscript would benefit from some data clarifying what is happening with the full length, and a discussion on the subject.*

*Perhaps the uncontrolled assembly of these molecules occurs when residues 1-28 are present is effectively a continuation of the isodesmic model they propose. Perhaps showing the NTerm in their model will help clarify what it could or could not do.*

*Moreover, it would be interesting to see whether there is a difference in localisation of HA-full length SPOP and HA-SPOP (residues 28-359). i.e. do the N-terminal 27 residues affect the localisation of the protein in cells?*

We believe that the isodesmic self-association behavior we have found for SPOP<sup>28-359</sup> also occurs for the full-length protein, but the additional terminal residues lead to behavior that is much less reversible, and hence not amenable to equilibrium analysis. SPOP<sup>28-359</sup> self-associates fully reversibly in all of our experiments (SEC data in Figure 3A, C, light scattering data in Figure 6B and the new Supplementary Figure 8), the full-length protein tends to form amorphous aggregates *in vitro* over time. We now show this difference in behavior in the new Supplementary Figure 4B.

The N-terminus contains neither a folded oligomerization domain, nor a low-complexity domain of the sort that was recently shown to mediate liquid phase separation. The C-terminus contains a nuclear localization sequence and can therefore not be removed for experiments in cells.

##### *2) Kinetic modelling*

*The authors show the results of aggregation simulations, most notably in Fig 3D regarding forming tetramers, and in Fig 5 B and C to justify their assembly model. The precise details should be*

*described clearly in the supplementary information. With any kinetic model, there are assumptions required and these are not clearly defined here. For example, in Fig 3D, "Figure 3 ... The gray line depicts a fit of the data to a tetramer association model." There is no single one tetramer association model. A linear model linking monomer to tetramer, but with different rate constants for the formation and destruction rates of each individual step en route to forming a tetramer could be made to fit this curve. On balance of probability, the simplest model with the fewest fitting parameters is the most sensible course, and so I agree with the authors in this. But the authors should state something that is qualified to that effect, that a simple model is consistent with their data, suggesting no need to go to a more complex model, not that the formation of tetramers by mutBTB is not possible. So this statement is too strong: "Models for oligomerization into discrete tetramers (Figure 3D, gray line) or other discrete oligomers did not fit the data well, ruling out previously proposed tetramers or pentamers (Errington et al, 2012) " Nothing is ruled out as far as I can see: just that the simpler monomer/dimer model is ruled as being most likely in. Moreover, there are signs of higher order complexes at 5-6 S in both the supplementary information and in fig 3B. Could this be the appearance of tetramers? Similarly, the agreement of the isodesmic model to data in Fig 5B looks great. But what are the assumptions, what are the fitting parameters, and what precisely are the alternative models?*

Our analysis of SPOP assembly into higher-order species is a fit of equilibrium species distributions and not a fit of kinetic parameters. All light scattering measurements were carried out in equilibrium. We are now pointing this fact out in more detail in the relevant results section. The data points in Figures 5D and 6 represent individual experiments, in which SPOP protein was diluted from a stock concentration and the LS signal evaluated after equilibration at the new concentration. We have added the new Supplementary Figure 8 to show the rapid re-equilibration within seconds after dilution.

However, the reviewer raises a good point about illustrating the basis for selecting a monomer/dimer equilibrium model versus other equilibrium models such as monomer/trimer, monomer/tetramer etc. We now show the fits for several equilibrium models that clearly show that the monomer/dimer model provides a uniquely compelling fit. This analysis cannot exclude the possibility of trace amounts of larger species that we are unable to detect, but overall the results clearly show that the equilibrium species distribution overwhelmingly consists of monomers and dimers.

The AUC data for which a small peak at 5-6 S is observed is collected on SPOP  $\Delta$ BACK, for which we have observed no sign of tetramers in SEC experiments at millimolar concentrations. This construct was crystallized as a dimer (Zhuang et al., Mol Cell 2009). We think it is more likely that the small peak in  $c(S)$  is attributable to a contaminant.

The only assumption entered into the isodesmic model for fitting SPOP WT self-association was the fact that each addition of a building block happened with the same dissociation constant. The model was broken off at  $n=12$  ((SPOP<sub>2</sub>)<sub>12</sub>, a dodecamer of SPOP dimers) because populations of larger oligomeric species did not contribute to the fit significantly. Fitting parameters were the  $K_D$  and the molecular weight of the building block, which was in agreement with a SPOP dimer. The other models shown in Figure 6B assumed simple dimerization, trimerization, tetramerization etc equilibrium models, in which the building block was again a SPOP dimer, in agreement with the observed light scattering signal.

Of course it is also possible to model a mixture of discrete oligomeric states, although the mechanism through which random populations of different oligomeric states would form is unclear. Such a model revealed that each of the even-numbered oligomerization states was important for the fit of the data. The association constants for their formation were highly correlated, effectively turning the model into an isodesmic model, which is the logical model based on our knowledge of individual domain behavior. We have added detail on the isodesmic model to the Methods section.

*3) The structural model Fig 3D. and page 6 "According to this model, SPOP oligomers adopt a fibrillar organization with helical propensity."*

*There are too few details describing the assumptions that went into the model and no validation. The movie shows a helical twist. How sensitive is the twist to the assumptions going into the model? It would be possible for the group to perform, for example, ion-mobility mass spectrometry experiments to estimate the size of the oligomers of various sizes and compare them to their models as a form of cross validation. Alternatively, electron microscopy images could be easily obtained to provide data to support the overall geometry of the proposed models. The model would be more compelling should there be any validation.*

No assumptions go into the model; it relies on crystallography-determined domain-domain interactions only. We included the structural models in Figure 4D and in the supplementary movie to provide intuitive images of the assemblies. In fact, these models could already be made in the absence of our data from the two crystal structures of the BTB dimer (Zhuang et al., Mol Cell 2009) and the BACK dimer (van Geersdale et al., Act Crystall 2013). The BACK dimer structure is based on only a small fragment of SPOP and was in contrast to biophysical data that was interpreted as tetramerization or pentamerization of SPOP. Our data presented in the manuscript demonstrate that the BACK domain indeed forms dimers, and we thus found it useful to present a combined model of the higher-order oligomeric state. We provide validation by showing that the interface mutants, which are chosen based on the two crystal structures, abrogate the formation of higher-order oligomers.

The SPOP oligomers are labile. It will be especially challenging to characterize them because of the large number of species in rapid equilibrium. The species large enough to detect by EM are generally only present at very low concentrations (see Figure 7B). Figure 6D shows a SPOP octamer to illustrate its architecture. 3% of assemblies would be equal in size or larger in a 27  $\mu$ M SPOP solution. The dodecamer CRL3 assembly in the Supplementary video 2 is populated to an even smaller fraction.

*4) A note for Figure 5 B*

*The authors might find it interesting to compare their distributions to the model of 'linear polymersisation'*

*Oosawa and Kasai, J.Mol.Biol. (1962) 4, 10-21*

*This model predicts an exponential decay in oligomerisation state with increasing oligomer size at equilibrium, where the rate of decay gives a measurement of the effective  $K_d$  of assembly. This model has been used to look at fibrillar assembly including amyloids and actin filaments. It looks like this will fit this data very well indeed. This finding would further support their isodesmic association model where growth comes only from the 'ends' of the oligomer.*

We thank the reviewer for drawing our attention to this model. Indeed, the models are analytically

fully equivalent and therefore result in identical size distributions. We have added a reference to the model.

#### *5) Significance of aspect ratio*

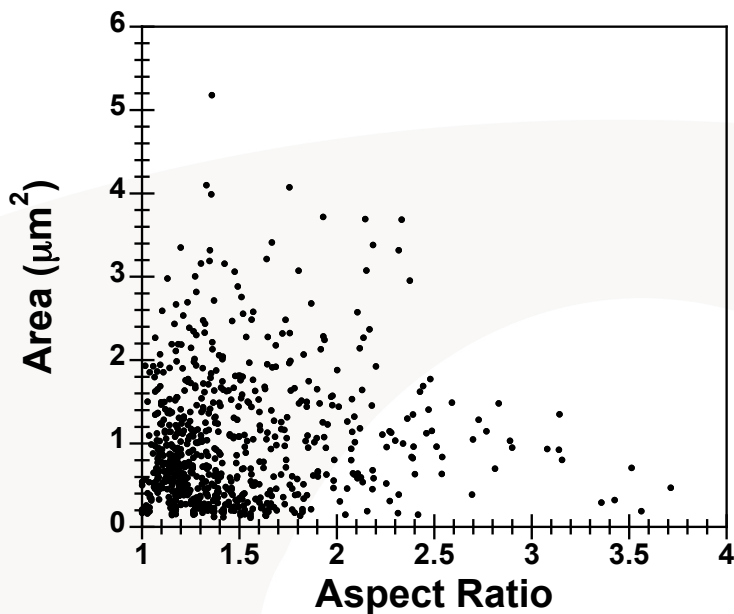
*In paragraph 3 the authors describe a difference in the area per cell and aspect ratio of GFP-Gli31-455 bodies in the presence and absence of co-transfection with HA-SPOP. However, it is unclear from Table 2 whether the difference is significant: 1.0 +/- 0.7 for HA-SPOP and 3 +/- 4 for + GFP-Gli31-455. The distributions appear to have a long tail, and so the mean value might not be the best comparison. The most probable aspect ratio appears to be the same for both, and small differences coming from uncertainties and challenges with performing this analysis.*

We have included data from additional cells (see also the last comment of reviewer 3, 151 and 155, respectively) to be able to make claims about significance of differences. For each cell, the number of speckles, median aspect ratio of the speckles in the cell (intracellular median aspect ratio), and median area of the speckles in the cell (intracellular median area) were determined. Further statistical analyses were performed on these three variables of cell-level data. Confidence intervals for the median of each of these variables under each biological condition (SPOP and SPOP+GLI3) were determined by inversion of the sign test. The Wilcoxon rank-sum test was used to compare the median of each of these three variables across the two biological conditions.

Our results and their interpretation remain the same. A large fraction of speckles have an aspect ratio of  $< 1.3$  (Figure 2F,G), indicative of a high degree of circularity in the horizontal plane. Somewhat surprisingly also to us, the area and the number of speckles observed per cell differed significantly with over-expression of Gli3<sup>1-455</sup> (Table 1 and Supplementary figure 3). This may be due to challenges with the analysis as the reviewer suggests, or may indicate that substrate or the amount of expressed proteins plays a role in regulating the size of nuclear speckles. These possibilities do not interfere with our conclusion that nuclear speckles have liquid-like properties.

*Not included in this data is the efficiency of co-transfection, which would presumably affect the result. The authors should make it explicit whether they are always measuring the diameter of HA-SPOP bodies in this experiment, or in the case of co-transfected cells, GFP-Gli31-455 bodies. Here, it would be useful to present the actual size (or diameter in the xy plane) distributions of HA-SPOP foci in the presence and absence of GFP-Gli31-455. Perhaps the more extended puncta are the small ones that are inherently difficult to quantify?*

We have included this analysis as Supplementary Figure 3. The size distributions are indeed significantly different with and without expression of GFP-Gli3<sup>1-455</sup>, but we think this may be a result of recruitment of more protein to nuclear speckles. The areas and the aspect ratios are not correlated, as shown in the plot below.



6) Figure 2c,

*There appears to be a punctate staining of HA-mutBACK, against a background of diffuse material. Only mutBTB (not mutBACK) constructs have a completely diffuse staining pattern. From these data it seems that the BTB-interface is important for oligomerisation and localisation to discrete foci. It would be interesting if the authors could rationalise this result, or at least speculate on it in the manuscript in light of their biophysical data*

We agree with the reviewer that, although self-association via both domains is correlated with localization to nuclear bodies, BTB-mediated self-association (in SPOP mutBACK) plays the larger role of the two domains due to its stronger affinity. This finding is also in agreement with our new cross-linking data.

7) Page 8 "SPOP-fluorescent protein fusion constructs produced mislocalized proteins in the cell, preventing analysis of SPOP dynamics in nuclear speckles." It would be interesting to know what the authors mean by 'mislocalised'. Maybe a supplemental figure would help.

We have included example images in the new Supplementary Figure 2 showing the incorrect localization of GFP-SPOP, mCherry-SPOP and ReAsH-SPOP.

*Minor textual clarifications:*

*Abstract >"However, higher-order complexes are inherently heterogeneous in size, limiting insight into how size influences function. "*

*Having heterogeneous complexes does not limit insight, but it does make them experimentally challenging to interrogate. The wording here is unclear.*

In the case of a heterogeneous size distribution, it is often unclear whether the function is mediated preferentially by assemblies of a certain size.

Page 3 > *Protein/protein interactions driving recruitment of components to these bodies are not well*

*understood (Tourrière et al, 2003).*

*The authors cite amongst other papers the work of Nott et al. In this work the driving forces and specific protein/protein interactions are well-described, linked through mutagenesis work to GF and RG repeats. Moreover this is linked to their ability to uptake nucleic acids. Similar findings are reported in the other cited papers. Similar links have been noted in other organelle forming proteins through low complexity domains. The authors should expand upon this point as the statement implies nothing on this subject is known, which is not the case.*

We agree with the reviewer that the role of disordered low-complexity sequence domains in driving liquid phase separation has recently emerged as a convincing mechanism for the formation of membrane-less organelles. To our knowledge, the role of folded domain interactions in the recruitment of proteins to cellular bodies is not well understood. We have revised the manuscript to clarify this point.

*Page 4 >demonstrating that SPOP adopts an ensemble of oligomeric species with a broad size distribution.*

*The authors argue that a change in elution maximum is due to a change in size distribution. As with sedimentation experiments, a change in smaller oligomer weight with decreasing concentration could also be due to subunit exchange effects (the effective 'on' rate will be  $k_+$  times the free monomer/dimer concentration and so concentration dependent). The authors should tone back the statement. What is clear is that the elution maximum is concentration dependent. There are several biophysical explanations for this.*

While it is true that the 'effective on-rate' will be concentration dependent, the off-rate constant is not concentration dependent – it simply reflects the life-time of the assembled state. In case of higher-order SPOP oligomerization, two independent exchange processes are operative:

- (a) Slow exchange (on the SEC time scale) in the SPOP BTB dimer, caused by a 1 nM  $K_D$  affinity that we determined by FDS-AUC.
- (b) Fast exchange (on the SEC time scale) in the SPOP oligomer due BACK domain interactions with a micromolar  $K_D$ . We see equilibration of the SPOP oligomer size by MALS after dilution from a stock solution within seconds (see Supplementary Fig 8).

In addition, in SEC we are not looking at the process in equilibrium but under conditions of continuous dilution.

The elution maximum shifts to smaller volumes for increasing protein concentrations, indicating the formation of, on average, larger oligomers. The peak also becomes broader, with a tail to larger elution volumes observed at lower concentrations.

The fast exchange leads to the peak appearing at an average elution volume, but the size distribution changes as a function of concentration, which results in a change of the peak shape in addition to the shift of the peak.

*Page 8 >ranging in size from hundreds of nm*

*The authors cannot resolve structures of hundreds of nm using light microscopy in this way. This statement should be qualified so that the lower limit is the resolution limit of their apparatus. Bodies might well be smaller than this but it would not be possible to know from these measurements.*

We agree with the reviewer and have qualified our statement.

*Page 8> characteristic time*

*Do the authors mean half life, or the quantitate 1/rate? The authors can turn the rate into a diffusion coefficient, combined with information on the size of the bleaching area, which would enable them to compare the local mobility of their liquid blobs to those of Brangwynne and Nott. That would possibly make for an interesting comparison between the types of liquid drop currently in the literature.*

We report the characteristic recovery time, i.e. the inverse of the rate constant.

The diffusion coefficient is calculated as  $D = \frac{a^2}{\tau}$ , where  $a$  is the effective bleaching radius and  $\tau$  is the characteristic recovery time. In our experiments, the bleached spots exceeded the size of the nuclear speckles, making a determination of the bleaching radius unreliable. If we estimate  $a$  to be  $\sim 1 \mu\text{m}$ , the diffusion coefficient  $D$  is  $\sim 0.0526 \mu\text{m}^2 \text{s}^{-1}$ . This is lower than the  $0.24 \mu\text{m}^2 \text{s}^{-1}$  reported for SF2 in the splicing compartment (Phair & Misteli. High mobility of proteins in the mammalian cell nucleus. *Nature* 2000, 404: 604-609), or the  $0.3 \mu\text{m}^2 \text{s}^{-1}$  reported for Ddx4 bodies in cells (Nott et al. Phase transitions of a disordered nuage protein generates environmentally responsive membraneless organelles. *Mol. Cell* 2015, 57: 936-947), but slightly higher than the  $0.01 - 0.025 \mu\text{m}^2 \text{s}^{-1}$  reported for Whi3-containing droplets in vitro (Zhang et al. RNA controls PolyQ protein phase transitions. *Mol. Cell* 2015, 60: 220-230.)

*Referee #2*

*(Report for Author)*

*SPOP, a MATH-BTB type substrate adaptor of the CUL 3 E3 ubiquitin ligase, regulates cell proliferation and development, and also plays a critical role in suppressing tumor through inhibiting Hh/Gli2 signaling pathway. It may provide an alternative strategy for developing therapeutic agents in the future. Many SPOP mutations were detected in some aggressive cancers. SPOP also has potential use as a novel biomarker of glioma. This study reveals novel molecular events underlying the link between the size distributions of SPOP oligomers to nuclear speckles localization and stimulates CUL3 ubiquitination. The novelty of this study is the linking of the macro-protein complex or oligomers to the localization of SPOP and catalytic efficiency. The idea is interesting as it represents an example of the tandem self-association plays an important role in the localization to nuclear speckles and the protein functions. The authors performed solid experiments supporting their conclusions although the molecular linkage between self-association and the recruitment of SPOP to liquid nuclear speckles is still unclear. The bulk of the data presented are good quality and the manuscript flow is logical.*

*Major issue*

*One area of this manuscript can be further improved is to provide in vivo functional evidence or further mechanistic insight to support the biophysical studies. For example, there are as many as 200 BTB proteins in human cells. Since one key aspect of the self-association is dependent on the BTB domain, can the authors test how broadly the proposed model applies to other BTB proteins? On the mechanism side, one would like to know how the self-association stimulates the CRL3 polyubiquitylation, facilitating the assembly of multi-subunit of CRL3? Facilitating or enhancing the*



*ub chain elongation like processive factor?*

We show herein for SPOP that its recruitment to nuclear speckles is mediated by the presence of two functional self-association domains. A functional BTB domain without a functional BACK domain alone is not sufficient (Figure 3D). In Supplementary Figure 10, we address the question whether other substrate adaptors with both BTB and BACK domains may be able to form higher-order oligomers. We find that the longer version of the BACK domain, which is present in many substrate adaptors together with KELCH domains, occludes the BACK dimer interface. The only candidates for higher-order self-association we find are a group of proteins specific to rodents.

In this manuscript, we are not attempting to address how SPOP self-association stimulates polyubiquitination. Our structural model as well as data in Errington *et al.* (Structure 2012) suggest that the multi-subunit CRL3 can assemble in the presence of oligomeric SPOP.

We have recently published a manuscript in which we propose that multivalency of SPOP oligomers and multiple SPOP binding motifs in substrates together enhance substrate recruitment, decrease substrate release, and therefore enhance CRL3 processivity.

W.K. Pierce, C.R. Grace, J. Lee, A. Nourse, M.R. Marzahn, E.R. Watson, A.A. High, J. Peng, B.A. Schulman, T. Mittag. (2015) Multiple weak linear motifs enhance recruitment and processivity in SPOP-mediated substrate ubiquitination. *J. Mol. Biol.* Epub ahead of print. PMID: 26475525

*Specific points:*

1. In figure 2C, the IF staining may need show the lower magnification pictures not only show the high magnification single cell.

Because the transfection efficiency was low and we usually only have a single transfected cell per image with low magnification, we have instead included several images per SPOP mutant in Supplementary figure 6 to show the representative nature of the images in the current Figure 3D.

2. In figure 3D, why did not use FDS-AUC assay to analyze the self-association of BACK domain?

We used FDS-AUC to quantify the dissociation constant of the BTB domain, because neither absorbance-detected AUC nor MALS were sensitive enough to detect the protein at concentrations at which it was monomeric. Because the BACK domain dimerizes with a micromolar dissociation constant, sensitivity was not limited and we were therefore able to use absorbance-detected AUC and MALS.

3. Complete labeling should be used for the input panels of the following figures to keep the figure formatting consistent throughout: 3A-D, 4B-C, 5C.

We have included additional labels.

*Referee #3*

*(Report for Author)*

*In the presented manuscript Marzahn and colleagues analyze the higher-order oligomerization of speckle-type POZ protein (SPOP), a component of a cullin E3 ubiquitin-protein ligase complex, which depends on specific tandem self-association domain interaction and is required for localization in nuclear speckles. The authors are trying to demonstrate that nuclear speckles have liquid phase separation which is in agreement with recent findings that many nuclear structures may have liquid properties.*

*The presented data are interesting but too preliminary for the publication in EMBO J.*

*They should include the use of an interfering peptide to show that the oligomerization of SPOP in vitro is not stochastic aggregation. It is well established that the in vitro behavior of an oligomeric complex is completely different to that of an in vivo one.*

We demonstrate that higher-order SPOP self-association is mediated by specific, structurally well-defined interfaces. Indeed, we have quantitatively dissected SPOP higher-order self-association into the contributions of the two individual dimerization domains. We have substantiated our experiments competing out SPOP self-association that were partially addressed in the current Supplementary Fig. 12. Because the BTB as well as the BACK domains dimerize via complex three-dimensional interfaces, we have used a self-association deficient mutant instead of a peptide. Addition of increasing amounts of SPOP mutBACK, which only forms dimers through the BTB domain, to SPOP WT reduces the size of oligomers formed in a concentration-dependent manner (Figure 3C), showing that the interactions are reversible, can be out-competed, and are not the result of non-native aggregation.

While the cellular environment increases the complexity of the system, we believe that the self-association of SPOP into higher-order complexes occurs essentially in the same manner as we have carefully dissected *in vitro*. Our cross-linking data supports this view.

*- Fig 2 absolutely needs splicing speckle-specific staining alongside the SPOP visualization. The authors should use the mouse antibody against splicing factor SC-35 (Sigma).*

We have included SC-35 staining alongside SPOP visualization as discussed above.

*- The in vivo oligomeric state should be quantified using the number and brightness assay and shown to be different to the mutants.*

*- I'd like to see the movement of SPOP molecules assessed by single molecule tracking in the wild type and mutant. I'm not satisfied by the statement "SPOP-fluorescent protein fusion constructs produced mislocalized proteins in the cell, preventing analysis of SPOP dynamics in nuclear speckles."*

The number and brightness method would be the optimal method for quantifying the SPOP oligomeric state in a live cell. Unfortunately, the mislocalization of fluorescently tagged SPOP in cells prevents single molecule/complex tracking. We have included example images in Supplementary Figure 2 showing the incorrect localization of GFP-SPOP, mCherry-SPOP and ReAsH-SPOP in cells. Untagged and HA-tagged SPOP were both shown to localize to nuclear speckles. (Nagai Y, Kojima T, Muro Y, Hachiya T, Nishizawa Y, Wakabayashi T & Hagiwara M (1997) Identification of a novel nuclear speckle-type protein, SPOP. *FEBS Lett.* **418**: 23–26).

Instead, we have included new data that demonstrate oligomerization behavior of SPOP WT and mutants in cells similar to that observed *in vitro*, as discussed in response to comment 1 of Reviewer 1.

- *The authors should actually bother to monitor in vivo substrate ubiquitination of each mutant (perhaps in a SPOP-null / CRISPR background to make it interesting)*

We agree that the effects on ubiquitination *in vivo* are untested at this stage, but we believe that the role of oligomerization upon cellular localization that we present provides an important framework for understanding how SPOP fulfills its cellular activities. We agree with the reviewer that the suggested experiments are interesting. We have assayed the function of SPOP self-association mutants on Hh pathway signaling by expressing the SPOP variants in the developing wings of *Drosophila melanogaster*. While expression of wild type SPOP leads to a Hh loss-of-function phenotype as expected, SPOP mutBTB leads to a gain-of-function phenotype, suggesting that it can form mixed oligomers with the *Drosophila* SPOP homolog HIB that are inactive.

- *Finally, I think that <50 cells is far too low for the observations they are claiming re: shape and size. This needs >150 cells at least and should be relatively easy to perform.*

We now include the analysis of a larger number of cells to enable greater precision. Our conclusions are unchanged from our previous submission.

Thank you for submitting your revised manuscript for our consideration, and my apologies for the delay in its re-evaluation related still to the turn-of-the-years holiday season and associated backlog. Referee 1 has now looked at the study once more, and I am pleased to say that s/he considers the study significantly improved and is thus in principle supportive of publication. S/he nevertheless still maintains several concerns that would require addressing before acceptance. In particular, s/he is not convinced that part of their original major comment 1 regarding possible effects of Gli1-455 expression itself has been fully addressed. There are suggestions for a few seemingly straightforward control experiments, which I agree would considerably bolster the strength of the major conclusions of the work. At the same time, most other remaining concerns can probably be addressed without further experiments, by additional clarifications and discussions and by tempering of certain statements and conclusions. Please also carefully re-check for inconsistencies between the point-by-point letter and the actual data it refers to, and incorporate certain discussion in the letter also in the main manuscript as suggested by the reviewer.

I am therefore returning the manuscript to you once more for one final round of revision, hoping that this will allow you to satisfactorily deal with the remaining points and clarify them in another point-by-point response letter.

I hope you will be able to make these remaining specific revisions (which I consider justified given the importance of the topic and the potential significance of the conclusions) and resubmit a final version of the manuscript as early as possible. Should you have any further questions in this regard, please do not hesitate to get back to me.

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 REFEREE COMMENT

Referee #1:

The revised manuscript was, in this reviewer's opinion, much improved. Using staining with a fluorescent anti-HA antibody, the authors show that transiently transfected full length HA-SPOP co-localises with nuclear speckle marker SC-35 in the nucleus of NIH-3T3 cells, as previously reported by Nagai et al., 1997 and Figure 2A. The authors then looked at a variety of constructs in which SPOP is conjugated to fluorescent proteins, and found them all to form aggregates in the cytoplasm of cultures cells. Consequently the authors move on to looking at an N-terminally truncated substrate of SPOP, Gli3(1-455) that, when over expressed, localizes into spherical liquid-like droplets in the nucleus.

The work shows that it is highly likely that SPOP recruitment into nuclear speckles requires oligomerisation, a process that receives careful biophysical characterisation.

One major concern remains about one of the key claims, that nuclear speckles, based on data provided here, are liquid-like. Some simple controls and careful rephrasing, described in more detail below, would bolster the claim.

Overall the work is interesting, timely, and provides further information on the new an exploding field of membraneless organelles, and should be published. More care should be made in revision to qualify the claims to match what is actually shown, or to make it clear when induction is being used. The authors frequently state that they 'demonstrate' or 'show' conclusions that has been arrived at through inductive speculation from their data. Changing all instances of these terms to 'suggest' would entirely alleviate this concern.

Major comment:

(1) time-lapse imaging to ask whether nuclear speckles have liquid character;

I raised this as my main concern in the previous review. The authors have added the sp35 antibody binding, which shows the relationship between nuclear speckles and SPOP. The effects of Gli1-455 on the system have not been considered. To my mind, the claim (1) above in its current form does not stand.

The claim about nuclear speckles having liquid like character, in this article, is based on following Gli1-455, the substrate of a protein recruited to nuclear speckles. In previously cited papers describing stress granules and P-bodies having liquid-like character, the protein directly associated

with the body formation was followed. That is not the case here - the liquid nature of the underlying body is being inferred from the substrate of a protein that gets recruited to the body. This distinction should be clearly explained.

This method to study membraneless organelles is neat and novel as far as I am aware but has a potential pitfall. The authors were unable to fluorescently tag SPOP and have it go into a nuclear speckle so they sought other means. HA-SPOP on its own does not appear to affect the morphology of the bodies. But whenever Gli1-455 is present, the number density, size and aspect ratio of the observed bodies change. Their irregular shape characterizes nuclear speckles, yet on addition of Gli1-455, the observed bodies are larger, there are less of them and they are spherical.

This raises a significant issue to my mind that causes problems with this specific claim. Does Gli1-455 form bodies on its own when overexpressed in the nucleus? If you express Gli1-455 at the same time as one of the mutant SPOP do both remain soluble and do they form puncta? I cannot find a control looking at this issue. One should be included to bolster the claim, or the claim should be rephrased to describe what the authors do show.

This control is important as without it, it's possible that bodies formed from overexpressed Gli1-455 are effectively swallowing any nuclear speckles that are present, leading the observed bodies to no longer be representative of nuclear speckles. It would also be compelling to see a reduction in the Gli1-455 concentration, in the over-expression perhaps by observing bodies at earlier times, to show that when relatively dilute it does not perturb the shape, number density and size of the bodies it interacts with.

Alternatively, the authors should tone down their claim to clarify that the bodies that have liquid like character are ones that have been significantly perturbed to the point where they test the definition of a nuclear speckle. In any event, the contrast between previous studies of organelles, and this method, of studying the organelle via the substrate of a recruited protein should be discussed.

I do not believe that making this important aspect clear to readers detracts from the novelty or interest in the article. To restate: saying the Gli1-455 bodies are 'nuclear speckle like' seems reasonable, as sp35 co-localises to them, but calling them nuclear speckles is not.

#### Minor comments

##### 1) Oligomer model:

Although there is not a crystal structure of SPOP that contains all three domains, the two available partial structures from previous studies (PDB IDs: 3HQI (Zhuang et al, 2009) and 4HS2 (van Geersdaele et al, 2013)) can be used to build a model of the oligomeric species of near full-length SPOP without further assumptions (Fig 6D).

The authors should state explicitly which interfaces observed in the crystal structures are used as they describe in their letter, but not the article.

##### 2) SPOP WT

As pointed out in the previous review, care is needed comparing SPOP WT as defined in this article, and the full-length protein. I find naming the 28-359 construct WT misleading. This truncation was worked on because the full length apparently aggregates. When discussing the aggregation model and its consequences, this should be taken into account. This aggregation is either native like, or non-native like, for example, into amyloid. Their model, with higher Kds, would anticipate potentially huge aggregates. One explanation for their data is that the residues removed contribute significantly to the stability.

Only at considerably higher protein concentrations, likely outside of the range that can be biophysically assayed, do we expect that isodesmic association will be dampened due to entropic losses of large oligomers

If that's correct, then results from the full length protein suggests there is no entropic dampening of oligomerisation as it aggregates apparently indefinitely. I would recommend a brief discussion of this in the paper and a clarification of the relationship between full length and WT.

I raised this issue in the previous letter. The authors state in the rebuttal that there is a supplementary figure 4B showing this. I cannot find this figure. This is the closest thing I could find:

Appendix Figure S4. Crosslinking in cells recapitulates the phenotypes observed in vitro.

Which doesn't seem to have the data referred to in the rebuttal.

I would also like to point out to the authors that a high proportion of aggregated material does not mean aggregation is irreversible. It more likely says the equilibrium is shifted firmly towards aggregates. The authors will find a literature on amyloid fibrils where small, but detectable amounts of material are present as soluble when the abundance of material is present as aggregates. Stable aggregates is not the same as irreversible aggregates.

3) Specific comment for second sentence of abstract

However, higher-order complexes are inherently heterogeneous in size, limiting insight into how size influences function.

Higher order complexes are not necessarily heterogeneous. One can find examples of stable 24mers, 48mers, etc. So it's not inherent. I'm not sure what is meant by limited insight. Perhaps it is meant that they are hard to study so we don't know much about them? I would recommend rephrasing this to be clear what is meant.

2nd Revision - authors' response

16 March 2016

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(Begins on next page).

We thank the reviewer for the careful reading of the manuscript and thoughtful comments.

## Point-by-point response

### Referee #1:

*The revised manuscript was, in this reviewer's opinion, much improved.*

*Using staining with a fluorescent anti-HA antibody, the authors show that transiently transfected full length HA-SPOP co-localises with nuclear speckle marker SC-35 in the nucleus of NIH-3T3 cells, as previously reported by Nagai et al., 1997 and Figure 2A. The authors then looked at a variety of constructs in which SPOP is conjugated to fluorescent proteins, and found them all to form aggregates in the cytoplasm of cultures cells. Consequently the authors move on to looking at an N-terminally truncated substrate of SPOP, Gli3(1-455) that, when over expressed, localizes into spherical liquid-like droplets in the nucleus.*

*The work shows that it is highly likely that SPOP recruitment into nuclear speckles requires oligomerisation, a process that receives careful biophysical characterisation.*

*One major concern remains about one of the key claims, that nuclear speckles, based on data provided here, are liquid-like. Some simple controls and careful rephrasing, described in more detail below, would bolster the claim.*

*Overall the work is interesting, timely, and provides further information on the new an exploding field of membraneless organelles, and should be published. More care should be made in revision to qualify the claims to match what is actually shown, or to make it clear when induction is being used. The authors frequently state that they 'demonstrate' or 'show' conclusions that has been arrived at through inductive speculation from their data. Changing all instances of these terms to 'suggest' would entirely alleviate this concern.*

We thank the reviewer for his or her careful reading of the manuscript. The resulting recommendations have enabled us to substantially improve the manuscript. In particular, we are now very careful to state which conclusions we have made through induction.

### Major comment:

*(1) time-lapse imaging to ask whether nuclear speckles have liquid character;*

*I raised this as my main concern in the previous review. The authors have added the sp35 antibody binding, which shows the relationship between nuclear speckles and SPOP. The effects of Gli1-455 on the system have not been considered. To my mind, the claim (1) above in its current form does not stand.*

*The claim about nuclear speckles having liquid like character, in this article, is based on following Gli1-455, the substrate of a protein recruited to nuclear speckles. In previously cited papers describing stress granules and P-bodies having liquid-like character, the protein directly associated with the body formation was followed. That is not the case here - the liquid nature of the underlying body is being inferred from the substrate of a protein that gets recruited to the body. This distinction should be clearly explained.*

The liquid character of membrane-less organelles can be demonstrated through the observation of fusion events (e.g. Brangwynne et al. Science 324:1729-32, 2009 for P granules) and through mapping of the *in cellulo* phase diagram by manipulation of the protein concentration (Weber and Brangwynne Current Biology 25: 641-6, 2015). Fast fluorescence recovery after photobleaching is a hallmark of liquid states but is

not sufficient to show liquid character. Fusion of droplets and their fast coalescence into a spherical state, however, reflects the material properties of the organelles and is hence independent of the manner of visualization. In the past, fusion events of (i) endogenous organelles without fluorescent markers (e.g. Brangwynne et al. Science 324:1729-32, 2009 for P granules), (ii) organelles fluorescently marked with a protein required for their formation (Elbaum-Garfinkle et al. PNAS 112: 7189-94, 2015), and (iii) organelles that were fluorescently marked with proteins localized to them (e.g. Molliex et al. Cell 163: 123-33, 2015 and Patel et al. Cell 162: 1066-76, 2015 for stress granules) have been used to demonstrate liquid properties. In this light, our approach to show liquid behavior is entirely comparable. We have clarified in the text that the molecules we use to probe the material properties of the bodies are not necessarily required for their formation.

*This method to study membraneless organelles is neat and novel as far as I am aware but has a potential pitfall. The authors were unable to fluorescently tag SPOP and have it go into a nuclear speckle so they sought other means. HA-SPOP on its own does not appear to affect the morphology of the bodies. But whenever Gli1-455 is present, the number density, size and aspect ratio of the observed bodies change. Their irregular shape characterizes nuclear speckles, yet on addition of Gli1-455, the observed bodies are larger, there are less of them and they are spherical.*

*This raises a significant issue to my mind that causes problems with this specific claim. Does Gli1-455 form bodies on its own when overexpressed in the nucleus? If you express Gli1-455 at the same time as one of the mutant SPOP do both remain soluble and do they form puncta? I cannot find a control looking at this issue. One should be included to bolster the claim, or the claim should be rephrased to describe what the authors do show.*

*This control is important as without it, it's possible that bodies formed from overexpressed Gli1-455 are effectively swallowing any nuclear speckles that are present, leading the observed bodies to no longer be representative of nuclear speckles. It would also be compelling to see a reduction in the Gli1-455 concentration, in the over-expression perhaps by observing bodies at earlier times, to show that when relatively dilute it does not perturb the shape, number density and size of the bodies it interacts with. Alternatively, the authors should tone down their claim to clarify that the bodies that have liquid like character are ones that have been significantly perturbed to the point where they test the definition of a nuclear speckle. In any event, the contrast between previous studies of organelles, and this method, of studying the organelle via the substrate of a recruited protein should be discussed.*

*I do not believe that making this important aspect clear to readers detracts from the novelty or interest in the article. To restate: saying the Gli1-455 bodies are 'nuclear speckle like' seems reasonable, as sp35 co-localises to them, but calling them nuclear speckles is not.*

We thank the reviewer for these careful comments; they have prompted us to further investigate the nature of the nuclear bodies that SPOP localizes to in the presence of GFP-Gli3<sup>1-455</sup>. They do not stain for SC-35 and therefore are indeed different from nuclear speckles (Figure 2). In the original Figure 2, the weak signal we observed in the SC-35 channel was apparently caused by bleed-through from the strong HA channel. We have now optimized the conditions for SC-35 antibody staining and show that SC-35-positive nuclear speckles are separate from GFP-Gli3<sup>1-455</sup>-positive bodies in single GFP-Gli3<sup>1-455</sup>-transfected and dual GFP-Gli3<sup>1-455</sup>/SPOP-transfected cells. We have added an experiment in which only GFP-Gli3<sup>1-455</sup> was transfected. We note that these bodies are neither nuclear speckles, nor nucleoli, polycomb bodies, PML bodies or Cajal bodies, but have not been able to identify these bodies. We call them Gli3<sup>1-455</sup>- or Gli3<sup>1-455</sup>/SPOP-positive nuclear bodies. They may be generated through overexpression of the exogenous Gli3<sup>1-455</sup>, or a preexisting body that Gli3<sup>1-455</sup> is recruited to.

We therefore observe the localization of SPOP to at least two different punctate nuclear structures. Importantly, both have liquid properties as demonstrated by fusion events. We have confirmed this property for nuclear speckles by time-lapse imaging of SC-35-GFP expressed in NIH 3T3 cells, shown in the new Figure 3. As the reviewer points out, the SC-35-positive nuclear speckles appear more irregular; we have therefore



added several additional snapshots from fusion events to the Fig EV1.

We have drawn the following conclusions: “Together, these results demonstrate that SPOP can localize to nuclear speckles or Gli3<sup>1-455</sup>-positive bodies, which are both membrane-less organelles with liquid droplet character. Our results mirror reports that SPOP can localize to different types of nuclear bodies, specifically to Polycomb bodies and DNA damage foci (Hernández-Muñoz *et al*, 2005), and presumably to PML bodies (Jung *et al*, 2007; Kwon *et al*, 2006). Substrate may play a role in recruiting SPOP to these nuclear bodies, but in contrast, SPOP can also recruit substrate to a nuclear body (Kwon *et al*, 2006). Importantly, all of these compartments are membrane-less organelles, which may have liquid properties. The liquid behavior of DNA damage foci was recently experimentally supported (Patel *et al*, 2015; Altmeyer *et al*, 2015). In conclusion, SPOP localizes to a variety of different nuclear membrane-less organelles, but has not been found diffusely localized.”

*Minor comments*

*1) Oligomer model:*

*Although there is not a crystal structure of SPOP that contains all three domains, the two available partial structures from previous studies (PDB IDs: 3HQI (Zhuang *et al*, 2009) and 4HS2 (van Geersdaele *et al*, 2013)) can be used to build a model of the oligomeric species of near full-length SPOP without further assumptions (Fig 6D).*

*The authors should state explicitly which interfaces observed in the crystal structures are used as they describe in their letter, but not the article.*

We have added this information explicitly to the Figure caption.

*2) SPOP WT*

*As pointed out in the previous review, care is needed comparing SPOP WT as defined in this article, and the full-length protein. I find naming the 28-359 construct WT misleading.*

*This truncation was worked on because the full length apparently aggregates. When discussing the aggregation model and its consequences, this should be taken into account. This aggregation is either native like, or non-native like, for example, into amyloid. Their model, with higher Kds, would anticipate potentially huge aggregates. One explanation for their data is that the residues removed contribute significantly to the stability.*

*Only at considerably higher protein concentrations, likely outside of the range that can be biophysically assayed, do we expect that isodesmic association will be dampened due to entropic losses of large oligomers. If that's correct, then results from the full length protein suggests there is no entropic dampening of oligomerisation as it aggregates apparently indefinitely. I would recommend a brief discussion of this in the paper and a clarification of the relationship between full length and WT.*

*I raised this issue in the previous letter. The authors state in the rebuttal that there is a supplementary figure 4B showing this. I cannot find this figure. This is the closest thing I could find:*

*Appendix Figure S4. Crosslinking in cells recapitulates the phenotypes observed in vitro.*

*Which doesn't seem to have the data referred to in the rebuttal.*

*I would also like to point out to the authors that a high proportion of aggregated material does not mean aggregation is irreversible. It more likely says the equilibrium is shifted firmly towards aggregates. The authors will find a literature on amyloid fibrils where small, but detectable amounts of material are present as soluble when the abundance of material is present as aggregates. Stable aggregates is not the same as irreversible aggregates.*

We agree with the reviewer that the shorter SPOP construct should be renamed, and we now call it SPOP<sup>28-359</sup> throughout the manuscript to clearly distinguish it from full-length WT SPOP used in experiments in cells and flies.

We apologize for mislabeling the location of the Figure, which was in reality Fig EV2B; we think that these previously added data would have resolved the above comments.

We believe that the isodesmic self-association behavior we have found for SPOP<sup>28-359</sup> also occurs for the full-length protein *in vivo*, but the additional terminal residues lead to behavior that is much less reversible *in vitro*, and hence not amenable to equilibrium analysis. While SPOP<sup>28-359</sup> self-associates fully reversibly in all of our experiments (SEC data in Figure 4A, C, light scattering data in Figure 7B and the previously added Supplementary Figure 7), the full-length protein tends to form amorphous aggregates *in vitro* over time. We show this difference in behavior in Fig EV2B. Specifically, protein aggregation was assayed by centrifugation of protein samples, resuspending pelleted material in buffer three times followed by centrifugation, and then resuspending the final insoluble pellet in sample loading dye. The ultracentrifugation conditions are expected to pellet some of the larger SPOP<sup>28-359</sup> oligomeric species. These species are readily soluble in fresh buffer and represent reversibly associated large oligomers. In contrast, the majority of SPOP FL forms insoluble aggregates that do not dissociate even under extensive dilution, but can be resuspended in denaturing gel sample buffer. These results show at least very slow off-rates of SPOP from the aggregates, not only high stability of the aggregates, and are therefore strongly indicative of practical irreversibility of aggregation.

We have included a statement within the caption of the isodesmic self-association model that raises the possibility of the contribution by the N- and C-termini to self-association but indicate that, *in vitro*, they lead to aggregation that is poorly reversible and are therefore not dissected here.

We have no experimental indication for entropic dampening and are simply pointing out this theoretical possibility.

### 3) Specific comment for second sentence of abstract

*However, higher-order complexes are inherently heterogeneous in size, limiting insight into how size influences function.*

*Higher order complexes are not necessarily heterogeneous. One can find examples of stable 24mers, 48mers, etc. So it's not inherent. I'm not sure what is meant by limited insight. Perhaps it is meant that they are hard to study so we don't know much about them? I would recommend rephrasing this to be clear what is meant.*

We are operating under the definition of higher-order oligomers as “oligomers, in which the number of monomers is broadly distributed and can be large”, as defined in the first sentence of the main text. They therefore differ from large oligomers with defined oligomeric state.

Thank you for submitting your re-revised manuscript, including the intriguing new results on distinct types of nuclear bodies formed in the presence of Gli1-455. These clarifying data were highly appreciated by referee 1 (whose comments I am attaching to this message), but they at the same time noticed a potentially serious issue with image data in support of an important control. This concerns Figure 2, where a supposedly empty panel (column 2 "HA", row 2) appears to be a duplication of the control image in column 1 (GFP), row 1. This may well be an oversight during copy-and-paste assembly of the Figure from subpanels, but we nevertheless have to ask you to carefully look into this issue and fully clarify it. This may require provision (for our internal assessment) of original data for this figure, and independent supporting data along the lines suggested by the referee (point 1 A/B/C).

I very much hope that you will be able to swiftly resolve this issue, and please do not hesitate to get back to me for any discussions needed here.

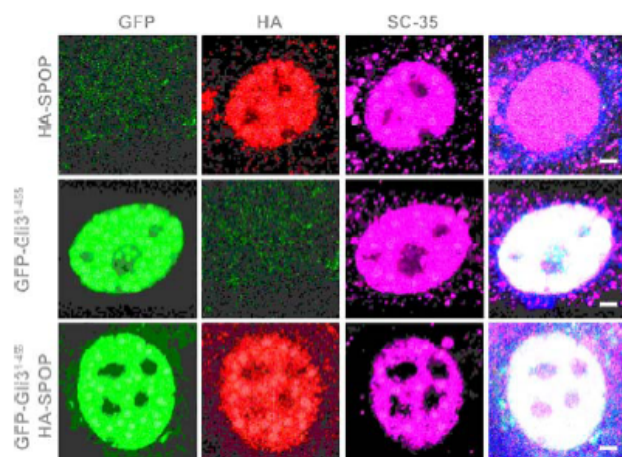
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 REFEREE COMMENTS

Referee #1:

We thank the authors for considering our comments and taking the time to perform the extra control experiments. It's clear that GFP-Gli3(1-455) forms, or is recruited into nuclear bodies that are entirely separate to the nuclear speckles as identified by SC-35. At face value, this invalidates the claim in previous drafts that nuclear speckles are liquid. However, the authors have responded strongly to this finding by adding experiments with SC-35 tagged with GFP. This reagent marks nuclear speckles and allows the authors to follow fluorescence changes in real time. They observe characteristic liquid behaviour in this marker (merging of bodies, and FRAP recovery). This addresses our concerns. This work adds to the rapidly developing field of membraneless organelles as well as providing insights into the requirements of proteins for co-localisation. We recommend publication after fixing 3 further points.

1) The authors note here that the weak signal they observed in the SC-35 channel was bleed through from the strong staining coming from the HA-channel (Fig 2, previous draft). We became concerned that HA-SPOP expression levels might be low or maybe even close to zero, and that fluorescence signal seen in the HA (red) channel was entirely due to bleed through effects. The control to prove that this is not the case is the absence of fluorescence in the HA channel when GFP-Gli-1-455 is expressed (6th panel).

We took a close look at this control. We opened figure 2 in imageJ and altered the pixel intensity to scale rather from 0-255 to 0-5. We obtain the following.



The control panel for the HA channel when GFP-Gli-1-455 is transfected is both false coloured green.

Moreover, it is remarkably similar to the control image in the top right of the figure. This does raise

concerns about this control.

The devil's advocate hypothesis here is that the HA-SPOP fluorescence they see in this experiment is always bleed through from the GFP or SC-35 channels and has nothing to do with HA-SPOP. To address this the authors should:

A) Fix this incongruity. Likely this will mean replacing this image with the one that was intended that is false coloured like the rest in this series.  
B) Show independent verification that HA-SPOP is co-expressed when transfected in these mixtures using a western blot of transfected versus non transfected or similar.  
C) Indicate the transfection efficiencies somewhere in the manuscript. What proportion of cells expresses one component, and what proportion express both when transfecting two proteins? This could be helped by including wider fields of view containing many cells.  
In figure 4D, WT HA-SPOP fluorescence is shown in the absence of stains and GFP. As far as we can tell, this the only data here that conclusively shows that wt-SPOP expresses here. These data do not tell us about expression of HA-SPOP when expressed in combination with Gli1-455.  
This point does not affect the main conclusions of the paper, which centre on nuclear speckles being liquid, and SPOP localisation being dependent on its aggregation state. Both of these points are now well made in our opinion.

2) In the methods section on immunofluorescence, the authors state which primary antibodies they use for staining nuclear body markers and epitopes such as HA or His. But they should also say which fluorescently tagged secondary antibodies were used for imaging, and the filter sets/gating for each channel. For example, they refer to anti-HA antibody (1:250; Clone 3F10, Roche cat# 11867423001) for detection of HA-SPOP. What was the fluorescence secondary antibody was used to detect the primary HA-antibody? Similarly, which secondary antibody was used to stain SC-35 in figure 2 and the other membraneless organelles in the extended view figure.

3) The authors describe a first principles model used to analyse the CG-MALS data. As suggested in previous response letters, they should formulate the mathematical details of this model as supplementary information, together with the alternative models that they use to illustrate poor fits to their data (Fig 7 orange, black, grey). For each model they have a series of coupled equilibria restrained by a mass balance equation. These should be formally stated. These models are not unique, and there are multiple ways to construct them, so the one used by the authors should be made clear.

Additional Correspondence - author

11<sup>th</sup> April 2016

Thank you for the news on the status of our manuscript. We are happy to hear that the reviewer appreciated the new data.

The reviewer is correct that there was an error made while assembling Figure 2. Panel 1 of row 1 was inadvertently duplicated in panel 2 of row 2. We have corrected this and will provide an updated image and original data for your review. We apologize for this mistake.

While we have done extensive Western Blot analyses of V5-SPOP under various conditions (see e.g. Figure 5C for the cross-linking assay in lysates) and some Western Blots of HA-SPOP with other substrates, we will have to blot for HA-SPOP in the presence of GFP-Gli3 1-455. While I do not recognize the basis for the reviewer's specific doubt about HA-SPOP expression, I appreciate that our mistake has contributed to this request and we will be happy to generate these data. It will take us a few days and we plan to send all requested images and additional information to you by the end of the week. We will also include the mathematical self-association models used to fit LS data in Figure 7B. I think this is a reasonable request, but again I do not think that the reviewer has made this request explicitly previously.

Additional Correspondence - editor

11<sup>th</sup> April 2016

Thank you for your swift response. I am happy to hear that you are able to clarify the remaining concerns especially the figure issue, as proposed in your email. My suggestion is you send us the modified and additional files simply via email, so we could just replace them from our side. Please kindly also send along a brief response letter indicating how you addressed the remaining three referee points. I trust that this should resolve all remaining issues before acceptance and publication.

Additional Correspondence - author

15<sup>th</sup> April 2016

Enclosed please find the documents for our manuscript entitled 'High-order oligomerization promotes localization of SPOP to liquid nuclear speckles' that we changed upon the reviewer's request. I have included a revised Figure 2, main manuscript file and Appendix file. For information on specific changes please see the enclosed response letter (begins on next page). I would like to point out here that we have made no changes in the main text, but added information to Figure captions, the Methods section, and into the Appendix file.

We would be happy to provide the raw experimental data for Figure 2 (and any other figure in the manuscript). I could upload the data onto a St. Jude owned server that you could access with a login and password for downloading.

*We thank the authors for considering our comments and taking the time to perform the extra control experiments. It's clear that GFP-Gli3(1-455) forms, or is recruited into nuclear bodies that are entirely separate to the nuclear speckles as identified by SC-35. At face value, this invalidates the claim in previous drafts that nuclear speckles are liquid. However, the authors have responded strongly to this finding by adding experiments with SC-35 tagged with GFP. This reagent marks nuclear speckles and allows the authors to follow fluorescence changes in real time. They observe characteristic liquid behaviour in this marker (merging of bodies, and FRAP recovery).*

*This addresses our concerns. This work adds to the rapidly developing field of membraneless organelles as well as providing insights into the requirements of proteins for co-localisation. We recommend publication after fixing 3 further points.*

We thank the reviewer for the positive evaluation of our work.

*1) The authors note here that the weak signal they observed in the SC-35 channel was bleed through from the strong staining coming from the HA-channel (Fig 2, previous draft). We became concerned that HA-SPOP expression levels might be low or maybe even close to zero, and that fluorescence signal seen in the HA (red) channel was entirely due to bleed through effects. The control to prove that this is not the case is the absence of fluorescence in the HA channel when GFP-Gli-1-455 is expressed (6<sup>th</sup> panel).*

*We took a close look at this control. We opened figure 2 in imageJ and altered the pixel intensity to scale rather from 0-255 to 0-5. We obtain the following.*

*The control panel for the HA channel when GFP-Gli-1-455 is transfected is both false coloured green. Moreover, it is remarkably similar to the control image in the top right of the figure. This does raise concerns about this control.*

*The devil's advocate hypothesis here is that the HA-SPOP fluorescence they see in this experiment is always bleed through from the GFP or SC-35 channels and has nothing to do with HA-SPOP. To address this the authors should:*

*A) Fix this incongruity. Likely this will mean replacing this image with the one that was intended that is false coloured like the rest in this series.*

The reviewer is correct that there was an error made while assembling Figure 2. Panel 1 of row 1 was inadvertently duplicated in panel 2 of row 2. We have corrected this and have provided an updated image and will gladly provide original data for review. We apologize for this mistake.

*B) Show independent verification that HA-SPOP is co-expressed when transfected in these mixtures using a western blot of transfected versus non transfected or similar.*

We have included a Western Blot as Appendix Figure 3, which shows HA-SPOP, GFP-Gli3<sup>1-455</sup> and tubulin for cells transfected with empty vector, pcDNA-HA-SPOP, pcDNA-GFP-Gli3<sup>1-455</sup>, and both constructs. The Western Blot therefore corresponds to the IF images in Figure 2 and shows protein expression for all constructs.

*C) Indicate the transfection efficiencies somewhere in the manuscript. What proportion*

*of cells expresses one component, and what proportion express both when transfecting two proteins? This could be helped by including wider fields of view containing many cells.*

We have quantified the transfection efficiencies of both constructs when transfected individually and together. Transfection efficiencies of pcDNA-GFP-Gli3<sup>1-455</sup> and pcDNA-HA-SPOP are 20-25% and 6-10%, respectively. When both constructs are used, 70-80% of transfected cells express both constructs, as indicated in the caption of Figure 2. Quantification of 5 image fields for all transfection conditions is included in Appendix Tables 1-3.

*In figure 4D, WT HA-SPOP fluorescence is shown in the absence of stains and GFP. As far as we can tell, this the only data here that conclusively shows that wt-SPOP expresses here. These data do not tell us about expression of HA-SPOP when expressed in combination with Gli1-455.*

The bleed through in the signal we falsely interpreted as SC-35 previously resulted from strong HA-SPOP signals, not a weak signal. To show unequivocally that SPOP expresses in combination with Gli3<sup>1-455</sup>, we have included the Western blot in Appendix Figure 3 as indicated above.

*This point does not affect the main conclusions of the paper, which centre on nuclear speckles being liquid, and SPOP localisation being dependent on its aggregation state. Both of these points are now well made in our opinion.*

*2) In the methods section on immunofluorescence, the authors state which primary antibodies they use for staining nuclear body markers and epitopes such as HA or His. But they should also say which fluorescently tagged secondary antibodies were used for imaging, and the filter sets/gating for each channel. For example, they refer to anti-HA antibody (1:250; Clone 3F10, Roche cat# 11867423001) for detection of HA-SPOP. What was the fluorescence secondary antibody was used to detect the primary HA-antibody? Similarly, which secondary antibody was used to stain SC-35 in figure 2 and the other membraneless organelles in the extended view figure.*

We have included the information on secondary antibodies in the Methods section:

Anti mouse-Alexafluor555 (1:1000, Life Technologies), anti rat-Alexafluor647 or Alexafluor488 (1:1000, Life Technologies) and anti rabbit-Alexafluor647 were used as secondary antibodies. For Figure 2, anti-rat Alexafluor 488 was used as secondary antibody when SPOP was transfected alone, and anti-rat Alexafluor647 was used when HA-SPOP was transfected along with GFP-Gli3<sup>1-455</sup>. The images in Figures 2 are pseudocolored. For Figure EV1, anti-rabbit AlexaFluor555 was used as secondary antibody to detect coilin, and anti-mouse AlexaFluor555 was used to detect B23, CBX8 and PML.

*3) The authors describe a first principles model used to analyse the CG-MALS data. As suggested in previous response letters, they should formulate the mathematical details of this model as supplementary information, together with the alternative models that they use to illustrate poor fits to their data (Fig 7 orange, black, grey). For each model they have a series of coupled equilibria restrained by a mass balance equation. These should be formally stated. These models are not unique, and there are multiple ways to*

construct them, so the one used by the authors should be made clear.

We have included the requested information in the Methods section:

Simple monomer-dimer, monomer-trimer, etc. interactions and isodesmic self-association models available in the CALYPSO software (Wyatt Technology Corporation) were used to model the data. In a solution, in which the different scattering species  $X_i$  correspond to different association states of a single protein, the theory of Rayleigh scattering from multicomponent solutions yields the concentration-dependent Rayleigh ratio  $R$

$$\frac{R(0)}{K^*} = \sum_i (iM_x)^2 [X_i],$$

in which  $M_x$  is the molar mass of protein  $X$  and  $[X_i]$  is the concentration of the species  $X_i$ .  $R$  is normalized to an optical constant  $K^*$  defined as

$$K^* = \frac{4\pi^2 n_0^2}{N_A \lambda_0^4} \left( \frac{dn}{dc} \right)^2,$$

where  $n_0$  denotes the refractive index of the solvent,  $\lambda_0$  the vacuum wavelength of incident light (690 nm),  $N_A$ , Avogadro's number, and  $dn/dc$  the specific refractive increment of SPOP.

The concentrations of each species are related to the equilibrium constants and total protein concentration. This results in the following equations for typical monomer-dimer, monomer-trimer, monomer-i-mer interactions:

$$iX \rightleftharpoons X_i; K_A^{(i)} = \frac{[X_i]}{[K]^i}; [X]_{total} = \sum_i i[X_i].$$

In the equations above,  $i=1$  represents the free monomer, the total molar concentration  $[X]_{total}$  is known at each gradient injection, and  $R(0)/K^*$  is measured. Non-linear least square optimization is used to obtain a single  $K_A$  value that fits the data across the entire concentration range of interest.

To describe isodesmic self-association, we used equations previously described by (Attri *et al*, 2010):

$$K_A = \frac{[X_i]}{[X_{i-1}][X_1]}; [X]_{total} = \sum_{i=1}^{\infty} i[X_i] = \frac{[X_1]}{(1-K_A[X_1])^2}.$$

The only assumption entered into the model was the fact that SPOP dimers were treated as self-associating building blocks. The model was broken off at  $n=12$  ( $(SPOP_2)_{12}$ ), a dodecamer of SPOP dimers, because populations of larger oligomeric species did not contribute to the fit significantly. Fitting parameters were the  $K_A$  and the molecular weight of the building block, which was in agreement with a SPOP dimer. For SPOP<sup>28-359</sup>, models that describe the formation of a mixture of discrete oligomeric states revealed that each of these states is important for the fit of the data. The association constants for their formation are highly correlated, effectively turning the model into an isodesmic model.



Thanks for sending all this information and files. I have carefully reviewed them now and don't see the need of returning things once more to the reviewer. We would publish the relevant source data as an online supplement (something we increasingly do and generally encourage for improved data accessibility and credibility).

**YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND** ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Tanja Mittag

Journal Submitted to: EMBO Journal

Manuscript Number: 93169R2

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

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

#### A- Figures

##### 1. Data

The data shown in figures should satisfy the following conditions:

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

##### 2. Captions

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

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/ varied/ perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple  $\chi^2$  tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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

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

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

#### B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	The sample size was not predetermined by statistical power calculations. In response to your question, we used the formula of Noether (1987) to perform post-hoc power calculations for the Wilcoxon (1945) rank-sum test. Noether's formula characterizes the power of Wilcoxon's test in terms of the probability that the value for one randomly selected individual from population A exceeds that of one randomly selected individual from population B. The null hypothesis tested in this study states that this probability is 50%. A sample size of 150 cells per group provides 84.9% power to detect a mild effect size (60% probability that an individual from A has a value that exceeds the value of an individual from B) in a two-sided test with p-value threshold 0.05. Thus, our comparison of 155 cells under the SPOP condition and 151 cells under the SPOP+GLI3 condition had adequate power to detect a biologically meaningful effect.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Methods page 18 and Figure caption 9: For wing analyses, crosses were performed at least twice and ~50 progeny, i.e. 2-3 day old female flies, were analyzed. Representative wings from adult flies were mounted on glass slides using DPX imaging medium.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No samples were excluded from the analysis.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	No randomization was used in assessing wing phenotypes of flies expressing SPOP constructs.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	NA
4.b. For animal studies, include a statement about blinding even if no blinding was done	No blinding of the investigator was used in assessing the wing phenotype of flies expressing SPOP constructs.
5. For every figure, are statistical tests justified as appropriate?	Yes, statistical tests are appropriate for Figures 3 and EV1. No other statistical tests were employed.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	The complete null hypothesis of the Wilcoxon test is that the distribution of values is identical in the two populations; but there is no further assumption regarding the distribution of those values. As such, the rank-sum test is well-known for robustness across many different probability distributions. Therefore, it is not necessary to test whether the data are normally distributed. Furthermore, the quantile-quantile plots shown below indicate that the shape of the distribution of aspect ratios is similar across the two settings. Thus, there is no quantitative evidence suggesting that the statistical test is invalid.
Is there an estimate of variation within each group of data?	Figures 3 and EV1 show groups of data and their variation.
Is the variance similar between the groups that are being statistically compared?	Yes.

#### C- Reagents

#### USEFUL LINKS FOR COMPLETING THIS FORM

<http://www.antibodypedia.com>

<http://1degreebio.org>

<http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo>

<http://grants.nih.gov/grants/olaw/olaw.htm>

<http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm>

<http://ClinicalTrials.gov>

<http://www.consort-statement.org>

<http://www.consort-statement.org/checklists/view/32-consort/66-title>

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

<http://datadryad.org>

<http://figshare.com>

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

<http://www.ebi.ac.uk/ega>

<http://biomodels.net/>

<http://biomodels.net/miriam/>

<http://jij.biochem.sun.ac.za>

[http://oba.od.nih.gov/biosecurity/biosecurity\\_documents.html](http://oba.od.nih.gov/biosecurity/biosecurity_documents.html)

<http://www.selectagents.gov/>

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Methods, p 17.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Methods, p 17. The cell lines are regularly tested for mycoplasma contamination.

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

#### D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Methods, p 18 and caption of Figure 9. Embryo injections to generate transgenic flies were performed by Best Gene, Inc. C765-GAL4 driver flies were obtained from the Bloomington Stock Center. It derives from an Oregon R strain with white mutation. Transgenes carry the mini-white gene to give red eye color. Fly stocks were maintained at 18°C on Jaz agarose (Fisher Scientific) and crosses were performed at 25 °C using standard techniques. Genotypes tested were w;C765-Gal4/P{w,UAS-empty} (control), w;C765-Gal4/P{w,UAS-SPOP}, w;C765-Gal4/P{UAS-SPOP mutBTB}, w;C765-Gal4/P{UAS-SPOP mutBACK}, w;C765-Gal4/P{UAS-SPOP mutBTB-BACK}. For wing analyses, crosses were performed at least twice and ~50 progeny, i.e. 2-3 day old female flies, were analyzed. Representative wings from adult flies were mounted on glass slides using DPX imaging medium.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	confirm compliance.

#### E- Human Subjects

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

#### F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right)).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section.  Examples: <b>Primary Data</b> Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in <i>Shewanella oneidensis</i> MR-1. Gene Expression Omnibus GSE39462 <b>Referenced Data</b> Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CRA/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	NA
22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomedelis (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

#### G- Dual use research of concern

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