The intrinsically disordered protein SPE-18 promotes localized assembly of MSP in *Caenorhabditis elegans* spermatocytes

Kari L. Price, Marc Presler, Christopher Uyehara and Diane Shakes
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MS TITLE: The intrinsically disordered protein SPE-18 promotes localized assembly of the major sperm protein in C. elegans spermatocytes

AUTHORS: Kari L Price, Marc Presler, Christopher Uyehara, and Diane Shakes

Thank you submitting your manuscript to Development. I have received all three reviewer comments, and as you will the overall evaluation is positive and we would like to publish a revised manuscript in Development, provided that the referees' comments can be satisfactorily addressed. Each reviewer offers numerous suggestions, largely textual to greatly improve the clarity of the study and enable more effective data presentation. In addition, and if possible to perform, reviewer 1 suggests analysis of *spe-18* mutant germlines with anti-MSP and anti-tubulin antibodies to assess the localization of these proteins. Reviewer 2 makes similar recommendations, if possible, on localization analysis between SPE-18 with 1C4 and SPE-18 and MSP.

Please attend to all of the reviewers' comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so.

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

Reviewer 1

Advance summary and potential significance to field

The manuscript focuses on the role of a protein called SPE-18 that had previously been identified through mutagenesis screens to be important for spermatogenesis in C. elegans. The paper

determines the molecular identity of SPE-18 as an intrinsically disordered protein that is required to regulate the dynamics of the sperm-specific cytoskeletal protein Major Sperm Protein (MSP). MSP is an interesting cytoskeletal protein that functions in sperm motility by forming polymers; however, the regulation and dynamics of MSP polymer formation during sperm formation has many unknown aspects. SPE-18 is shown to be important to sequester MSP into paracrystalline structures in Fibrous Bodies (FBs) as sperm form. Without SPE-18, cells do not progress through meiosis and fail to form functional sperm.

The manuscript is well written. The most exciting advance in this paper is the characterization of MSP dynamics relative to FB formation and SPE-18 localization during sperm formation. The dynamic localization of SPE-18 relative to this process and the phenotypes observed when SPE-18 is mutated or absent showing altered MSP dynamics are evidence that this intrinsically disordered protein modulates the forms that MSP can have during spermatogenesis. Overall, the execution of the experiments and quality of the data are excellent. They allow one of the first clear observation of these dynamics and how this cytoskeletal system can be regulated, which has so far been missing in the literature. Fertility researchers and those that study cytoskeletal dynamics will find this paper interesting. It is particularly important to not base all 'rules' of cytoskeletal dynamics on tubulin or actin and to take into account different systems that allow us to learn new aspects of cytoskeletal biology. The topic and overall novelty are appropriate for Development.

Comments for the author

There are some questions about the manuscript in the current form that are detailed below. They are not intended to require more new experiments but instead to improve the clarity and interpretation of the experiments.

-One aspect that could be strengthened is the link between SPE-18 and the MSP dynamics. The most striking data is found in Figure 3E, which beautifully shows SPE-18 localization relative to MSP recruitment and storage in FBs. Is it possible to show more images of MSP localization at different stages of spermatogenesis (as in Figure 3E, at least early stages) when SPE-18 is either mutated or absent? This could strengthen the role of SPE-18 in these dynamics. There is only one image, Figure 2A, that shows one time point of MSP localization when SPE-18 is mutated. It is difficult to appreciate what happens when SPE-18 is absent in terms of where MSP is localized.

-The authors state the spe-18 mutants arrest in meiosis and do not complete cytokinesis (abstract, Page 8 Ln 6). They also find cells often have 1-3 small chromatin bodies. Can the authors cite previous studies or show the cells do not undergo chromosome segregation? Lack of typical tubulin structures that mark divisions during sperm formation? From the description it is not clear how the authors envision how 1, 2, or 3-4 chromatin structure might arise. In general, can the authors better address why they think meiosis fails without SPE-18?

-The authors mention that packing MSP may prevent interferences with actin and tubulin-mediated events – do they see any types of disruption in these events?

-Can the authors better address why the MSP need to be stored in FBs in the discussion? This addresses a sentence in their abstract "Our findings reveal an alternative strategy for sequestering cytoskeletal elements, not as monomers but in localized, bundled polymers." Concentration of MSP in FBs allows focused segregation of the mass of MSP into spermatids, not the residual body like the other cytoskeletal components actin and tubulin. Noting this could better highlight different strategies in use of cytoskeletal components to carry out different aspects of development in this case.

-It would be good to see how the findings of SPE-18 dynamics fits in with original schematics of FBMO dynamics. The dot-like localization pattern on the sides of the rods are particularly striking. How does what is in Figure 7 that tie in with the FBMO structure shown in Figure 1?

Reviewer 2

Advance summary and potential significance to field

The manuscript by Price, et al identifies a regulatory protein for coordination of major sperm protein (MSP) use in C elegans sperm, both as an organizer for storage, and to promote release for ultimate sperm motility. The protein SPE-18 shows sequence and physical properties consistent with a mostly intrinsically disordered protein (IDP). The authors argue that its IDP nature and predicted phosphorylation sites are the reason for its developmental functions, but there is little molecular evidence presented. Rather, the authors present a much more compelling genetic and cell biological argument for the function of SPE-18 using functional links to associated spe- mutation phenotypes and very elegant immunofluorescence across all stages of spermatogenesis. The latter findings really sell the them of this paper as a newly discovered mechanism that corrals MSP in meiotic and differentiation stages of sperm production. This has tangible value for others interested in non-actin/myosin-mediated modes of cell motility.
The results presented are clear and robust. They generally support the conclusions drawn by the authors, though in a few instances other interpretations should be considered. These are noted in the suggestions below.

Comments for the author

Specific Comments:
1. The layout of some figures, and their organization within the manuscript are inefficient or confusing. The middle portion of the Results section is choppy, composed of many paragraphs of 2 or 3 sentences; this leads to a loss of continuity in thought.
2. P. 4 line 22. The phrase “amenable for” should be replaced by “amenable to”.
3. Fig 2; p.7, lines 13-24. The description of the molecular nature of the spe-18 allele in Fig 3 would be better introduced as Fig 2, prior to the description of the phenotype in the current Fig 2. The major emphasis in the subsequent figures involves imaging/immunofluorescence that follows direction from the current Fig 2.
4. Fig 2, p. 8, lines 9-19. Fig 2C shows a spe-18 phenotype with multinucleated secondary spermatocytes of 2 types: MI arrest and AI arrest. It would be helpful to know the percentage of each, particularly because it appears that the MI decreases and the AI decreases. Some comment on that change is also warranted.
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6. p. 9 lines 1-10. The nature of the mutation is a late nonsense mutation (last exon). The phenotype is similar to a spe-18(RNAi) phenotype. It is suggested that hc133 is a loss of function allele, which is likely. However, the authors may want to address whether the truncated SPE-18 is perhaps stable and represents a dominant negative protein. For instance, it would be helpful to know if the heterozygote shows no spermatocyte phenotype.
7. p. 10, lines 7-12. The heat denaturation experiment with recombinant SPE-18 demonstrates properties of solubility, but does not “…predict that SPE-18 functions as an intrinsically disordered protein.”
8. p. 11, lines 8-13, Fig 4A. SPE-18 is observed as a 39 kDa protein in the western blot. However, the antibody cross-reacts prominently with several other proteins; only p100 is addressed in the legend. Others (e.g. 52 kDa) should be acknowledged. Is there any knowledge of related somatic proteins, and could they account for residual immunostaining in Fig 4C?
9. Labeling within the figures and legends should be consistent throughout the manuscript. In some cases, “anaphase I” is abbreviated “AI”, and in others it is “Ana I”; the same is true for pachytene, etc. One consisted labeling system would make figures clearer.
10. Several figures have image panels that are too small to see detail, relative to other panels in the same figure. This is seen most clearly in Fig 4, where the examples “P”, “K”, etc in panel D are very small relative to those in panel B and C. Also, where appropriate, it would be helpful show regions of primary spermatocytes and secondary spermatocytes (e.g. Fig 1 and Fig 4)

11. Fig 5, Fig 7 legends. The legend has very little information and could be more informative. There is no text in the legend for Fig 7. The diagram needs substantial explanation for the non-expert in nematode spermatogenesis. In Fig 7, the position or role of SPE-18 should be included in the yellow "organelle" after step 2.
12. Fig S4 could use color coded staining identities (e.g. red SPE-18) as in other figures.
13. p. 15-16, lines 27-3. The final statement is unnecessarily vague: "…coupled to some property of the invidualized spermatids that is distinct…." It is apparent that SPE-18 becomes destabilized (ubiquitinated?) in spermatids after it becomes actively segregated away from RBs. State clearly the model most consistent with observations. Also, "SPE-10" in line 29 should apparently be SPE-18.
14. p. 17, lines 5-6. It seems there is a second interpretation of the elongated morphology of late SPE-18::MSP complexes that is passive rather than active. It is possible that MSP polymerization actively elongates the SPE-18 structure and causes the "bar bell" shape.

Reviewer 3

Advance summary and potential significance to field

Price and colleagues show that the spermatocyte-arrest mutant, *spe-18 (hc133)*, is required for Major Sperm Protein (MSP) localization to a subcellular spermatocyte-specific organelle, the Fibrous Body (FB). MSP sequestration to the FB is likely required to ensure proper chromosome segregation during the meiotic divisions, as well as proper partitioning of MSP to nascent spermatids. Local polymerization of MSP is required for directional, ameboid movement of nematode sperm in the absence of actin, tubulin, and molecular motors (e.g., myosin).
Little is known about the cellular components of the FB, or the underlying genetic and cell biological requirements for FB assembly.

This study posits the identification of an early assembly factor for the FB, the intrinsically disordered protein SPE-18, and documents requirements for *spe-18* in MSP localization within the FB. SPE-18 subcellular localization in spermatocytes is undoubtedly dynamic, and its juxtaposition with MSP in FBs suggests that it is a likely candidate for an FB-associated protein and putative assembly factor. However, the key claims and findings in the manuscript hinge upon interpretation of immunofluorescence, in particular colocalization experiments that lack sufficient magnification and spatial resolution to substantiate the stated conclusions. Although the study hints at an unappreciated complexity to creation of MSP inclusion bodies, and hints that FB assembly and MSP polymerization may utilize liquid phase condensation much like other cytoskeletal proteins, the results are too preliminary to corroborate the proposed model of action presented by the authors.

Comments for the author

The manuscript is well-written and easy to follow. My reservations about the main conclusions hinge on the presentation and interpretation of immunofluorescence data, particularly experiments that co-stain with two antibodies. Using available microscopy technologies at your institution, are you able to obtain higher magnification, higher resolution images (for example, generating Z-stacks using a confocal microscope, using a 60x or 100x objective?). This would be particularly helpful to bolster the claims of subcellular colocalization between SPE-18 and 1C4 (Figure 4D, P and K stages) and SPE-18 and MSP (Figure 4E, esp. diplotene stage).

Additional comments:
Figure 2 B-D, and associated text (Results, p.8, paragraph 2): Please consider scoring and quantifying the spermatocyte arrest and chromosome segregation phenotypes described in the text and shown in the DAPI-stained images for the control, *spe-18* mutants and *F32A11.3* RNAi knock-downs. (# DAPI-positive foci per cell for each stage, karyosome – spermatid). A graphical representation of the quantified data would be more convincing than the representative images alone.

Figure 4D: I am having a difficult time appreciating the anti-1C4 (MO marker) signal, even in the grayscale images, particularly for the P and K stages. Perhaps showing the grayscale single-channel images for the larger panel, along with arrows that are color-coded differently and/or labeled

according to stage, would better illustrate the potential association of the MO and SPE-18 early in FB formation.

Results, p.13 Line 31 – p. 14, Line 1: Please refer to the data presented in Figure 2A in the text.

Results, p. 14, Paragraph 1/Figure 5: Could anti-1C4/anti-SPE-18 costaining be performed in *spe-6(hc49)* mutants to lend credence to your claim that the SPE-18-positive foci in karyosome-stage and later spermatocytes are "pre-FB" structures? What do you think accounts for the delay in appearance of these puncta in the *spe-6(hc49)* mutants, and how do you know that they are analogous to "pre-FB" structures in wildtype pachytene-stage spermatocytes? Have you performed anti-SPE-18/anti-MSP costaining in <I>*spe-6(hc49)*</I> mutants?

In the future, and beyond the scope of this study, have you considered generating transgenic lines carrying fluorescently-tagged SPE-18 and MSP to visualize FB assembly dynamics via time-lapse microscopy? Alternatively, have efforts been made to do subcellular fractionation to enrich for FBs, for downstream biochemical analysis of components (e.g., by mass-spectrometry)?

First revision

Author response to reviewers' comments

Responses to the reviewer comments

Reviewer 1 Advance Summary and Potential Significance to Field:
The manuscript focuses on the role of a protein called SPE-18 that had previously been identified through mutagenesis screens to be important for spermatogenesis in C. elegans. The paper determines the molecular identity of SPE-18 as an intrinsically disordered protein that is required to regulate the dynamics of the sperm-specific cytoskeletal protein Major Sperm Protein (MSP). MSP is an interesting cytoskeletal protein that functions in sperm motility by forming polymers; however, the regulation and dynamics of MSP polymer formation during sperm formation has many unknown aspects. SPE-18 is shown to be important to sequester MSP into paracrystalline structures in Fibrous Bodies (FBs) as sperm form. Without SPE-18, cells do not progress through meiosis and fail to form functional sperm.

The manuscript is well written. The most exciting advance in this paper is the characterization of MSP dynamics relative to FB formation and SPE-18 localization during sperm formation. The dynamic localization of SPE-18 relative to this process and the phenotypes observed when SPE-18 is mutated or absent showing altered MSP dynamics are evidence that this intrinsically disordered protein modulates the forms that MSP can have during spermatogenesis. Overall, the execution of the experiments and quality of the data are excellent. They allow one of the first clear observation of these dynamics and how this cytoskeletal system can be regulated, which has so far been missing in the literature. Fertility researchers and those that study cytoskeletal dynamics will find this paper interesting. It is particularly important to not base all 'rules' of cytoskeletal dynamics on tubulin or actin and to take into account different systems that allow us to learn new aspects of cytoskeletal biology. The topic and overall novelty are appropriate for Development.

We thank reviewer 1 for their overall enthusiasm for this manuscript and recognition that this paper provides important new contributions to our understanding of MSP dynamics.

Reviewer 1 Comments for the Author:
There are some questions about the manuscript in the current form that are detailed below. They are not intended to require more new experiments but instead to improve the clarity and interpretation of the experiments.

-One aspect that could be strengthened is the link between SPE-18 and the MSP dynamics. The most

striking data is found in Figure 3E, which beautifully shows SPE-18 localization relative to MSP recruitment and storage in FBs. Is it possible to show more images of MSP localization at different stages of spermatogenesis (as in Figure 3E, at least early stages) when SPE-18 is either mutated or absent? This could strengthen the role of SPE-18 in these dynamics. There is only one image, Figure 2A, that shows one time point of MSP localization when SPE-18 is mutated. It is difficult to appreciate what happens when SPE-18 is absent in terms of where MSP is localized.

The revised version includes a new figure 3 in which proximal gonad regions are shown. This new figure highlights the distinction between the diffuse unpolymerized MSP pattern in *spe- 6(hc49)* mutants and the distinct MSP patterns in *spe-18*. Importantly, we found that 25°C *spe- 18* spermatocytes contain cortical MSP fibers. These new experiments clarify that SPE-6 is essential for MSP polymerization whereas SPE-18 is required for localized assembly and organization of MSP into FBs.

-The authors state the *spe-18* mutants arrest in meiosis and do not complete cytokinesis (abstract, Page 8 Ln 6). They also find cells often have 1-3 small chromatin bodies. Can the authors cite previous studies or show the cells do not undergo chromosome segregation? Lack of typical tubulin structures that mark divisions during sperm formation? From the description it is not clear how the authors envision how 1, 2, or 3-4 chromatin structure might arise. In general, can the authors better address why they think meiosis fails without SPE-18?

This is the first paper to characterize the *spe-18* phenotype, so there are no previous studies to cite. As per the reviewers suggestion, we have expanded our analysis to include DAPI/tubulin staining (Figure 2 F-H). This expanded analysis suggests that meiotic prophase is largely unaffected. The mutant spermatocytes enter M-phase but have defects in meiotic chromosome segregation. Undivided spermatocytes with 3-4 chromatin structures are associated with multipolar spindles with 4 microtubule asters. In terminally arrested spermatocytes, the microtubules reorganize into a unique, polymerized but disorganized pattern. The localization pattern of SPE-18 does not immediately suggest why meiosis fails without SPE-18, but we speculate in the discussion that either cytosolic SPE-18 has additional functions beyond the scope of this paper or in the absence of SPE-18, the components normally sequestered by SPE-18 interfere with chromosome segregation.

-The authors mention that packing MSP may prevent interferences with actin and tubulin- mediated events – do they see any types of disruption in these events?

As shown in the expanded figure 2, the mutant spermatocytes exhibit a progression of metaphase I and multipolar spindles that fail to support proper chromosome segregation. Likewise (in new Figure 2 images), actin within the mutant spermatocytes remains uniform at the cortex and neither concentrates in contractile rings nor partitions to support residual body formation in budding figures. MSP at the cortex could interfere with both astral microtubules and actin during anaphase.

-Can the authors better address why the MSP need to be stored in FBs in the discussion? This addresses a sentence in their abstract “Our findings reveal an alternative strategy for sequestering cytoskeletal elements, not as monomers but in localized, bundled polymers.” Concentration of MSP in FBs allows focused segregation of the mass of MSP into spermatids, not the residual body like the other cytoskeletal components actin and tubulin. Noting this could better highlight different strategies in use of cytoskeletal components to carry out different aspects of development in this case.

This point is address in the discussion – and bolstered by new data showing both unregulated MSP polymers as well as defects in the microtubule spindles and actin rearrangements.

-It would be good to see how the findings of SPE-18 dynamics fits in with original schematics of FBMO dynamics. The dot-like localization pattern on the sides of the rods are particularly striking. How does what is in Figure 7 that tie in with the FBMO structure shown in Figure 1?

In the revised manuscript, we not only revised the Figure 7 schematics but also added schematics to earlier figures to show how details of developing MO structures that are known from published electron micrographs (Roberts et al., 1986) may relate to the immunofluorescence images.

Reviewer 2 Advance Summary and Potential Significance to Field:
The manuscript by Price, et al identifies a regulatory protein for coordination of major sperm protein (MSP) use in C elegans sperm, both as an organizer for storage, and to promote release for ultimate sperm motility. The protein SPE-18 shows sequence and physical properties consistent with a mostly intrinsically disordered protein (IDP). The authors argue that its IDP nature and predicted phosphorylation sites are the reason for its developmental functions, but there is little molecular evidence presented. Rather, the authors present a much more compelling genetic and cell biological argument for the function of SPE-18 using functional links to associated spe- mutation phenotypes and very elegant immunofluorescence across all stages of spermatogenesis. The latter findings really sell the them of this paper as a newly discovered mechanism that corrals MSP in meiotic and differentiation stages of sperm production. This has tangible value for others interested in non-actin/myosin-mediated modes of cell motility.
The results presented are clear and robust. They generally support the conclusions drawn by the authors, though in a few instances other interpretations should be considered. These are noted in the suggestions below.

We want to thank reviewer 2 for their overall enthusiasm of the work.

Reviewer 2 Comments for the Author:
Specific Comments:

1. The layout of some figures, and their organization within the manuscript are inefficient or confusing. The middle portion of the Results section is choppy, composed of many paragraphs of 2 or 3 sentences; this leads to a loss of continuity in thought.
In response to these comments and the addition of the new tubulin and expanded MSP studies, both the figures and the results text have been significantly reorganized. The final sections of the results still contain a couple of short sections, but hopefully the other changes address this reviewer's comment.

2. P. 4 line 22. The phrase "amenable for" should be replaced by "amenable to". Sentence has been revised

3. Fig 2; p.7, lines 13-24. The description of the molecular nature of the spe-18 allele in Fig 3 would be better introduced as Fig 2, prior to the description of the phenotype in the current Fig 2. The major emphasis in the subsequent figures involves imaging/immunofluorescence that follows direction from the current Fig 2.
We appreciate this suggestion but opted to keep the now expanded phenotypic analysis of the *spe-18* spermatocytes before the molecular analysis.

4. Fig 2, p. 8, lines 9-19. Fig 2C shows a spe-18 phenotype with multinucleated secondary spermatocytes of 2 types: MI arrest and AI arrest. It would be helpful to know the percentage of each, particularly because it appears that the MI decreases and the AI decreases. Some comment on that change is also warranted.
Quantitation of chromosome segregation patterns has been added to the main text.

5. Fig 3, p. 9 lines 12-19. The initial description of C elegans SPE-18 deals with its homology to other nematode and non-nematode species. Yet that is not depicted in Fig 3A, even though the briggsae and remanei sequences are shown; the purpose of the latter are not clear. The alignment of all the species is shown in supplemental Fig S1, but would be better included as Fig 3A. This would also remove the need for Fig 3B. Instead, the authors could show the disordered regions, helices and phosphorylation sites in a linear, colored box diagram in B.

We appreciate the reviewer's excellent suggestion for improving the presentation of Figure 3 (now Figure 4). We have moved the alignment of all species that was in Fig S1 to panel A of the new figure and in it have highlighted the region of high homology that was previously in Fig 3B. In panel B, we now present the SPE-18 protein structure as a linear, colored box diagram as suggested. In the diagram, we show the disordered regions (in magenta), predicted helices/sheets (in blue, orange, or green bars), as well as predicted phosphorylation and ubiquitination sites. Predicted iTasser structures are shown in panel C.

6. p. 9 lines 1-10. The nature of the mutation is a late nonsense mutation (last exon). The phenotype is similar to a *spe-18*(RNAi) phenotype. It is suggested that hc133 is a loss of function allele, which is likely. However, the authors may want to address whether the truncated SPE-18 is perhaps stable and represents a dominant negative protein. For instance, it would be helpful to know if the heterozygote shows no spermatocyte phenotype.

Examples of *spe-18* heterozygotes have been added to figure 2, and there is no spermatocyte phenotype in the heterozygotes. In addition, our western blot (Fig. 5A) and immunofluorescence (Fig. 5C) data indicates that *spe-18* mutants do not produce detectable levels of SPE-18.

7. p. 10, lines 7-12. The heat denaturation experiment with recombinant SPE-18 demonstrates properties of solubility, but does not "…predict that SPE-18 functions as an intrinsically disordered protein."

We revised the wording to emphasize that the heat resistant property of SPE-18 is that it remains soluble in heated solutions. This solubility experiment tests one common property of intrinsically disordered proteins – that their normal structure lacks extensive internal hydrophobic residues that contribute to their structure under normal conditions and to their aggregation when these residues become surface exposed at elevated temperatures.

8. p. 11, lines 8-13, Fig 4A. SPE-18 is observed as a 39 kDa protein in the western blot. However, the antibody cross-reacts prominently with several other proteins; only p100 is addressed in the legend. Others (e.g. 52 kDa) should be acknowledged. Is there any knowledge of related somatic proteins, and could they account for residual immunostaining in Fig 4C?

Based on pBLAST analysis and the YenZym's methods for selecting the peptide antigen, we don't think that there are related somatic proteins cross-reacting. The non-specific bands on the western would indeed be problematic if this was the main focus of our experiments. However in the revised results text, we note that "Despite the non-specific bands in the westerns, antibody binding was not above background in either *spe-18(hc133)* (Fig. 5C, Fig. S2C, D) or *spe-44* (Fig. S2E, F) gonads, confirming the specificity of the antibody for immunocytology."

9. Labeling within the figures and legends should be consistent throughout the manuscript. In some cases, "anaphase I" is abbreviated "AI", and in others it is "Ana I"; the same is true for pachytene, etc. One consisted labeling system would make figures clearer.

With the exception of the longer labels retained in figure 1, this change has been made.

10. Several figures have image panels that are too small to see detail, relative to other panels in the same figure. This is seen most clearly in Fig 4, where the examples "P", "K", etc in panel D are very small relative to those in panel B and C. Also, where appropriate, it would be helpful show regions of primary spermatocytes and secondary spermatocytes (e.g. Fig 1 and Fig 4).

Adjustments have been made to many of the figures in order to address this point.

11. Fig 5, Fig 7 legends. The legend has very little information and could be more informative. There is no text in the legend for Fig 7. The diagram needs substantial explanation for the non-expert in nematode spermatogenesis. In Fig 7, the position or role of SPE-18 should be included in the yellow "organelle" after step 2.
A revised figure 5 is now Figure 6. Figure 7 has been extensively revised and now contains a descriptive legend.

12. Fig S4 could use color coded staining identities (e.g. red SPE-18) as in other figures.
Done. (This is now S3)

13. p. 15-16, lines 27-3. The final statement is unnecessarily vague: "…coupled to some property of the individualized spermatids that is distinct…." It is apparent that SPE-18 becomes destabilized (ubiquitinated?) in spermatids after it becomes actively segregated away from RBs. State clearly the model most consistent with observations. Also, "SPE-10" in line 29 should apparently be SPE-18

(Fixed). Revised

14. p. 17, lines 5-6. It seems there is a second interpretation of the elongated morphology of late SPE-18::MSP complexes that is passive rather than active. It is possible that MSP polymerization actively elongates the SPE-18 structure and causes the "bar bell" shape.
Good point. The alternative explanation of "who shapes who" has been added to the discussion.

Reviewer 3 Advance Summary and Potential Significance to Field:
Price and colleagues show that the spermatocyte-arrest mutant, *spe-18 (hc133)*, is required for Major Sperm Protein (MSP) localization to a subcellular, spermatocyte-specific organelle, the Fibrous Body (FB). MSP sequestration to the FB is likely required to ensure proper chromosome segregation during the meiotic divisions, as well as proper partitioning of MSP to nascent spermatids. Local polymerization of MSP is required for directional, ameboid movement of nematode sperm in the absence of actin, tubulin, and molecular motors (e.g., myosin). Little is known about the cellular components of the FB, or the underlying genetic and cell biological requirements for FB assembly.

This study posits the identification of an early assembly factor for the FB, the intrinsically disordered protein SPE-18, and documents requirements for *spe-18* in MSP localization within the FB. SPE-18 subcellular localization in spermatocytes is undoubtedly dynamic, and its juxtaposition with MSP in FBs suggests that it is a likely candidate for an FB-associated protein and putative assembly factor. However, the key claims and findings in the manuscript hinge upon interpretation of immunofluorescence, in particular colocalization experiments, that lack sufficient magnification and spatial resolution to substantiate the stated conclusions. Although the study hints at an unappreciated complexity to creation of MSP inclusion bodies, and hints that FB assembly and MSP polymerization may utilize liquid phase condensation much like other cytoskeletal proteins, the results are too preliminary to corroborate the proposed model of action presented by the authors.
The key structures are indeed quite small, and some details will require super-resolution microscopy and/or immuno-EM. However in the revised manuscript, we've both tempered the language in places and provided better images in others. In addition, the suggested *spe-6* experiments have added significantly to the results.

Reviewer 3 Comments for the Author:
The manuscript is well-written and easy to follow. My reservations about the main conclusions hinge on the presentation and interpretation of immunofluorescence data, particularly experiments that co-stain with two antibodies. Using available microscopy technologies at your institution, are you able to obtain higher magnification, higher resolution images (for example, generating Z-stacks using a confocal microscope, using a 60x or 100x objective?). This would be particularly helpful to bolster the claims of subcellular colocalization between SPE-18 and 1CB4 (Figure 4D, P and K stages) and SPE-18 and MSP (Figure 4E, esp. diplotene stage).

While Covid related restrictions prevented me from collaborating with other faculty to undertake new confocal studies, the revised version of the manuscript includes
a) enlarged, deconvolution images of MSP-SPE-18 colocalization (Fig. 5E – formerly Fig. 4). In parallel, the wording and graphical images have been updated.
b) New images of 1CB4-SPE-18 colocalization (New Fig. 6A – formerly Figure 4D)
Note, all images were taken using a 60X objective – and then the gonad images were downsized while some of the spermatocyte images were further enlarged or contrast-enhanced (as noted in the figure legend). However these structures are very small.

Additional comments:
Figure 2 B-D, and associated text (Results, p.8, paragraph 2): Please consider scoring and quantifying the spermatocyte arrest and chromosome segregation phenotypes described in the text and shown in the DAPI-stained images for the control, *spe-18* mutants and *F32A11.3* RNAi knock-downs. (# DAPI-positive foci per cell for each stage, karyosome – spermatid). A graphical representation of the quantified data would be more convincing than the representative images alone.
We added quantitative data related to the chromosome segregation defects to the main text. We did not quantify the RNAi phenotype as it was highly variable and RNAi mediated depletion within

the sperm producing gonad is problematic. However the RNAi phenotype was sufficient similar enough to the mutant phenotype to convince us that we were looking at the right gene.

Figure 4D: I am having a difficult time appreciating the anti-1C4 (MO marker) signal, even in the grayscale images, particularly for the P and K stages. Perhaps showing the grayscale single- channel images for the larger panel, along with arrows that are color-coded differently and/or labeled according to stage, would better illustrate the potential association of the MO and SPE- 18 early in FB formation.
In new Figure 6, we've added enlargements and contrast enhanced inverted images to aid in the visualization of these very small structures. We've also added schematics that use information from previously published transmission electron micrograph to show how the immunofluorescence images may relate to known ultrastructure of developing FB-MO complexes.

Results, p.13 Line 31 – p. 14, Line 1: Please refer to the data presented in Figure 2A in the text.
In the revised text, we refer to the even more relevant Figure 3B.

Results, p. 14, Paragraph 1/Figure 5: Could anti-1C4/anti-SPE-18 costaining be performed in *spe-6(hc49)* mutants to lend credence to your claim that the SPE-18-positive foci in karyosome-stage and later spermatocytes are "pre-FB" structures?
Great idea – we included these experiments in figure 6. This experiment adds significantly to the model.

What do you think accounts for the delay in appearance of these puncta in the *spe-6(hc49)* mutants, and how do you know that they are analogous to "pre-FB" structures in wildtype pachytene-stage spermatocytes?
We've added a sentence to this section of the results noting that the apparent delay might be a problem of detecting very small foci or alternatively that there is some synergy between SPE-18 and either another FB component or SPE-6 itself.

Have you performed anti-SPE-18/anti-MSP co-staining in *spe-6(hc49)* mutants?

We did not do the double-labelling of SPE-18 and MSP because expanded studies of MSP staining in *spe-6(hc49)* mutants at multiple temperatures convinced us that MSP is always diffuse (unpolymerized) in *spe-6* mutants. On the other hand, our new experiments and revised figure show that SPE-18 eventually co-localizes with the 1CB4 (MO) marker in *spe-6(hc49)* spermatocytes.

In the future, and beyond the scope of this study, have you considered generating transgenic lines carrying fluorescently-tagged SPE-18 and MSP to visualize FB assembly dynamics via time-lapse microscopy?
Although we do not current have such constructs, we do hope to eventually examine SPE-18 and MSP patterns through time either in transgenic worms or *in vitro*.

Alternatively, have efforts been made to do subcellular fractionation to enrich for FBs, for downstream biochemical analysis of components (e.g., by mass-spectrometry)?
Historically, fractionation of nematode spermatids has proven tricky as the organelles tend to stick together. However it's possible that it will be easier to fractionate FBs from spermatocytes.

Second decision letter

MS ID#: DEVELOP/2020/195875

MS TITLE: The intrinsically disordered protein SPE-18 promotes localized assembly of the major sperm protein in C. elegans spermatocytes

AUTHORS: Kari L Price, Marc Presler, Christopher Uyehara, and Diane Shakes

ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks. The referee reports on this version are available below.

Reviewer 1

Advance summary and potential significance to field

The manuscript characterizes the role of a protein called SPE-18 for regulating cytoskeletal protein dynamics during spermatogenesis in C. elegans. The paper determines the molecular identity of SPE-18 as an intrinsically disordered protein that is required to regulate the dynamics of the sperm-specific cytoskeletal protein Major Sperm Protein (MSP. In the revision, the authors bolster the evidence that shows that SPE-18 is important to sequester MSP into bundles as sperm form. This advances our knowledge of how MSP dynamics are regulated relative to FB formation. The quality of the data are excellent and allow a clear observation how this cytoskeletal system can be regulated, which has so far been missing in the literature. Fertility researchers and those that study cytoskeletal dynamics will find this paper interesting. As noted in a previous review, it is important to not base all 'rules' of cytoskeletal dynamics on tubulin or actin and to take into account different systems that allow us to learn new aspects of cytoskeletal biology. The topic and overall novelty are appropriate for Development.

Comments for the author

The authors have addressed all concerns satisfactorily.

Reviewer 3

Advance summary and potential significance to field

Price and colleagues show that the intrinsically disordered protein SPE-18 is required for Major Sperm Protein (MSP) localization and arrayed assembly within a subcellular, spermatocyte-specific organelle, the Fibrous Body (FB). Analysis of the spe-18 null mutant phenotype revealed that SPE-18-mediated sequestration of MSP within FB is necessary to ensure proper chromosome segregation during the meiotic divisions, and proper partitioning of MSP to nascent spermatids. Loss of SPE-18 causes meiotic arrest and male sterility. Local polymerization of MSP is required for directional, ameboid movement of nematode sperm in the absence of actin, tubulin, and molecular motors (e.g., myosin). This study is the first to reveal that MSP fiber organization within FBs requires accessory proteins, like SPE-18. FB growth reveals previously unknown and unanticipated dynamics between SPE-18 and MSP. Finally, degradation of SPE-18 was necessary, but not sufficient, for FB disassembly, revealing that much is left to be discovered about the cell biological processes underlying this developmental transition. This work will appeal to a broad audience, including members of the readership interested in cytoskeletal regulation, liquid phase condensation, gametogenesis, and cell motility.

Comments for the author

The revised manuscript by Price and colleagues (DEVELOP-2020_195875v2-Shakes) is significantly improved and attended to the most pressing reviewer concerns and questions. I appreciated the careful phenotypic characterization that now appears in Figure 2, including the addition of the anti-tubulin and actin stains on WT and spe-18 mutant spermatocytes at two temperatures, which clarifies the types of chromosome segregation defects seen in arrested spermatocytes. In Figure 5, the visualization of the FB structures is much improved with the addition of the deconvolution images in Figure 5E. I feel that the models for FB-MO assembly put forward are plausible given the available data, and that this study will no doubt seed efforts to obtain a closer view of the process once external collaborations are possible. I agree that this model begs to be interrogated in vitro, and that super-resolution light microscopy or advanced electron microscopy methods would greatly help to resolve these tiny sub-organelle structures. It is clear that this study opens the door to the

unappreciated complexity of FB-MO components that likely exist, as well as the future potential to uncover novel regulatory mechanisms impacting MSP fiber assembly, SPE-18-dependent FB-MO nucleation and maturation, and the regulated disassembly of these structures in nascent spermatocytes. Given the substantive improvements and the strength of the genetic analysis and phenotypic characterization, I recommend the manuscript for publication in Development. Thank you for the opportunity to review your work.