# Periodic subcellular structures undergo long-range synchronized reorganization during *C. elegans* epidermal development

Chunxia Wang, Yuyan Yang, Rong Fu, Yi Zhu and Huimin Zhang
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**Original submission**

First decision letter

MS ID#: JOCES/2020/246793

MS TITLE: Periodic subcellular structures in the C. elegans epidermis undergo long-range synchronized reorganization during epidermal development

AUTHORS: Chunxia Wang, Yuyan Yang, Rong Fu, Yi Zhu, and Huimin Zhang
ARTICLE TYPE: Short Report

We have now reached a decision on the above manuscript.

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

As you will see, the reviewers raise a number of substantial criticisms that prevent me from accepting the paper at this stage. They suggest, however, that a revised version might prove acceptable, if you can address their concerns.

I will highlight here a few things that were discussed in post-review discussion.

There is agreement that it is critical to add statistical analyses and sample size information for all relevant experiments .

There was also a concern about lack of RNAi controls and analysis to confirm efficacy of knock-down in these experiments.

Also, strong conclusions about causality or genetic hierarchies should be avoided without further mechanistic analysis. As stated by one referee, independent evidence to show that these molecules are more directly involved in those processes, rather than playing merely permissive roles, would be needed to support the conclusions as currently stated. Without these, you need to tone down conclusions of the paper, including the strong claim that "the periodic striped patterns in the C.

elegans epidermis is created by the actin-spectrin network, but fixated and maintained by apical membrane attachment structures."

I would also underscore the comments/queries by reviewer 2 regarding actin knockdown (comment #3), molecular epistasis (comment #4), photoconversion (comment #6) and animal bending (comment #7) experiments. While addressing all of these comments would not necessarily require new experiments, you should at least present and explain the data more clearly, and/or interpret the results more cautiously.

If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

*We are aware that you may 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.*

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

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

Reviewer 1

*Advance summary and potential significance to field*

Wang et al. report their observations on the formation and reorganization of the parallel periodic structures in the C. elegans epidermis. In these tissues actin bundles and hemidesmosomes are organized in a periodic manner, but the mechanism of formation and development of these structures was unknown. They found that these periodic structures are assembled in embryonic stages and identified actin and spectrin are the initial detectable components. Interestingly, during larval development, these structures are doubled once at the L3 stage. They also found that a link between the apical side of the epidermal cells and the extracellular matrix is important for this process. These are novel interesting findings and provide implications on the mechanisms of similar repetitive subcellular structures in other cell types. This reviewer has several minor suggestions to improve the manuscript.

*Comments for the author*

1. Line 59. “basically” -> “basally”.

2. Lines 98-100. The body wall muscle defects cause “shortened embryonic body length” due to impaired mechanosensitive cytoskeletal reorganization. This should be clarified. In addition to Zhang et al., 2011, a more recent paper (Lardennois et al., 2019 Nature 573:266-270) describing this mechanism should be added.

3. The hyp7 epidermal cells develop by cell fusion. Please clarify in the text about the timing of cell fusion and initiation/development of the periodic structures.

4. In Fig. 3C, the stripes in bli-1(RNAi) look wider than others. Please clarify in the text whether this is representative of these bli-1(RNAi) worms. Since bli-1 encodes a collagen, is this related to the duplication defects as reported in Fig. 4?

5. Fig. 5. Multiple collagen genes appear to be involved in the stripe duplication. Please add some discussion whether there is any functional redundancy or specificity among the collagen genes. No additional experiments are necessary.

6. Statistic analysis should be performed on quantitative data in Figs 2-4.

Reviewer 2

*Advance summary and potential significance to field*

This manuscript describes an unexpected mechanism by which periodic cellular rings (annuli) on C. elegans skin cells increase in number as animals grow. These rings encompass cytoskeletal elements, junctions, and extracellular proteins. Using transgenic reporters and immunofluorescence, the authors first describe the sequence of events during the initial process of ring formation, identifying spectrin as one of the earliest proteins to adopt a periodic pattern. They then demonstrate that rings double once during development apparently because they are stretched and pulled apart during cellular growth, thus splitting each into two new rings.

This most valuable contribution of this descriptive study is documenting the ring duplication phenomenon, which is unusual and raises exciting mechanistic questions for future work.

*Comments for the author*

Although the data is convincing on the whole, the manuscript would benefit from clarification deeper explanation, and improvements in data presentation. There are also some claims that should either be better justified experimentally, or stated more cautiously.

Specific comments:

1. Throughout the manuscript the authors never use the recognized term for these periodic structures in C. elegans—annuli. The authors should use this term so that readers can search for and connect this study with other literature about them.

2. The RNAi experiments currently do not include controls for efficacy or specificity. For example, RT-PCR or Western blots could assess the magnitude of knock-down. Similarly using multiple RNAi clones for each target may be an easy way to address specificity. However, since this reviewer does not use the C. elegans model, I defer to the expertise of other reviewers for the C. elegans community's current expectations for RNAi controls.

3. Knocking down the actin gene with RNAi is problematic, since the development of all the cells in the animal will be affected, thus making it difficult to interpret effects on annuli as direct. This is also true, perhaps to a lesser extent, for spectrin RNAi. Performing this experiment with spatially or temporally controlled methods (i.e. cell type-specific RNAi, cell type specific RNAi rescue, and/or temporally controlled actin drug inhibitors) could partially alleviate this concern. These experiments are not critical to the main points of the manuscript so if they cannot be addressed experimentally (e.g. due to the Covid crisis), they should at least be interpreted more cautiously.

4. They authors conclude that the periodic spectrin pattern drives formation of the actin pattern, since spc-1 RNAi disrupts the actin pattern. However, to show a genetic hierarchy (rather than interdependence) they should do the reverse experiment: determine if actin disruption affects the spectrin pattern. Perhaps simply backing off the conclusion that the pattern is "initiated by spectrin" would be sufficient to address this concern, since this experiment is not central to the main findings of the manuscript.

5. Related to the point above, the experiments in this study do not justify the statement that "the initial formation of periodic patterns in the C. elegans epidermis is evolutionally conserved". The other periodic spectrin/actin structures with which conservation is implied (in neurites and cilia), are structurally distinct from annuli—for example, they do not contain hemi-desmosomes, intermediate filaments, or extracellular components. It is not surprising that structures comprised

of spectrin and actin depend on spectrin and actin—this observation does not alone indicate that other structures containing spectrin and actin use the same assembly mechanism.

6. The photoconversion experiment (Fig 3E) seems to show that new protein integrates into existing hemi-desmosomes as they divide, but these experiments should be explained better.

In Figure 3E, it is not clear what the boxed region indicates. Was the whole animal photoconverted and the boxed region indicates the region for close up in subsequent panels?
Or was only the boxed region photoconverted? If the latter is correct, the authors cannot conclude that newly synthesized protein was added to existing stripes—it is equally plausible that new Mup4 in the converted region came from exchange with other hemi-desmosomes in unconverted regions. In fact, this explanation is technically possible even if the whole animal was photoconverted, since it appears that photoconversion was far from complete.

Could this photoconversion experiment be repeated at different stages (i.e. each molting cycle). This experiment would determine if Mup-4 synthesis or dynamics increases specifically during the L3 stripe duplication stage.

7. The experiment in Figure S3B is clever and supports the authors' model. The authors should consider moving these panels to a main figure, and should also explain the experiment better. Since the experiment was not well explained and the image in the figure was not completely clear, I did not immediately grasp its rationale or interpretation (When I figured it out though, I thought it was cool!). It would help if the distances within and between doublets in straight and bent animals were quantified. Arrows should also be added to the figure to indicate both fissures and inter-doublet spaces in both straight and bent animals. In the images of the bent animal it is currently impossible to distinguish the doublets from the fissures.

8. One more factor that could be quantified to support the model is the ratio of stripe thickness to animal length at different stages. The model predicts that this ratio should remain relatively steady before and after the L3 stage, but halve during stripe duplication.

Minor/styistic comments:

9. Figure row labels should be used more consistently. For example, in Figure 1, row labels are useful in panel B and E, but are not included in any of the other panels. Row labels are similarly inconsistent in Figure 4. Row labels would help the reader to immediately understand the figure without referring to the legend.

10. The authors should avoid using jargon and precisely define the proteins they are discussing in the main text of the manuscript. For example, the identity of the VAB-10 protein (dystonin, a plakin family member) was never stated. Similarly, in Figure 1B, a reporter is labeled as "IFs", but the specific gene that was tagged is not named. Similarly, the identity of the egl-19 or mup-4 proteins are not described.

11. Auto-correlation analysis (Figure S1) is an interesting way to quantify periodic structures but this analysis is not described well in either the figure legend or the methods section. It would be helpful to add more explanation and justification for this approach. (Incidentally according to the auto-correlation, bli-1 appears to have an interesting higher order pattern does this indicate a subpattern within a pattern?)

12. The manuscript states that, "we tested all the molecules known to be periodically organized in the epidermis and discovered that only a few apically secreted collagens could significantly affect the striped patterning" (line 184). What other molecules were tested?

13. Figure 4D lacks a Y-axis label.

14. Although the manuscript is on the whole clearly written, it should be revised carefully for word choice and grammar. One particular issue (which would understandably not be obvious to a non-native english writer) is that structures should be referred to as "fixed", not "fixated", which has a different common meaning in english.

Reviewer 3

*Advance summary and potential significance to field*

Using C. elegans as a model, the authors reported that the periodic striped patterns in the C. elegans epidermis depend on the actin-spectrin network. They further provide evidence for the importance of extracellular collagens together with extension forces generated from epidermal cell growth in enabling long-range synchronized reorganization of subcellular structures. This beautiful paper is well written, based on a series of rigorous analysis of cellular biological phenotype complemented with elegant genetic studies. The findings provide conceptually new understanding of how periodic subcellular structures are established and maintained in integrity and distribution during cell growth and tissue development and should be of broad interest to the readers in the fields of C. elegans, developmental biology and also cell biology.

*Comments for the author*

One major comment concerns interpretation of the genetic analysis. Although the authors convincingly showed the requirements of actin, spectrin and collagen etc by RNAi or mutants, the data per se only suggest these molecules are genetically required and do not reveal the actual biochemical or cell biological processes by which these molecules might have enabled the establishment of and maintenance of those period subcellular structures. I would suggest the authors seek alternative independent evidence to show that these molecules are more directly involved in those processes, rather than playing merely permissive roles. Without actual mechanistic studies, the authors might want to tone down conclusions of the paper including strong claim that "the periodic striped patterns in the C. elegans epidermis is created by the actin-spectrin network, but fixated and maintained by apical membrane attachment structures."

Another comment concerns lack of quantitative data and statistic analysis for all the images and plots. Please provide numbers of animals imaged with corresponding penetrance of phenotype when applicable, and also statistic significance for all the comparison groups e.g. in Figure 2B, 2C, 3B, 3D, 3F, 4D.

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**First revision**

Author response to reviewers' comments

*Reviewer 1 Advance Summary and Potential Significance to Field:*
*Wang et al. report their observations on the formation and reorganization of the parallel periodic structures in the C. elegans epidermis. In these tissues, actin bundles and hemidesmosomes are organized in a periodic manner, but the mechanism of formation and development of these structures was unknown. They found that these periodic structures are assembled in embryonic stages and identified actin and spectrin are the initial detectable components. Interestingly, during larval development, these structures are doubled once at the L3 stage. They also found that a link between the apical side of the epidermal cells and the extracellular matrix is important for this process. These are novel interesting findings and provide implications on the mechanisms of similar repetitive subcellular structures in other cell types. This reviewer has several minor suggestions to improve the manuscript.*

**We thank the reviewer for his/her positive opinions about our work.**

*Reviewer 1 Comments for the Author:*
*1. Line 59. "basically" -> "basally".*
**This error has been corrected. We thank the reviewer for pointing it out.**

*2. Lines 98-100. The body wall muscle defects cause "shortened embryonic body length" due to*

*impaired mechanosensitive cytoskeletal reorganization. This should be clarified. In addition to Zhang et al., 2011, a more recent paper (Lardennois et al., 2019 Nature 573:266-270) describing this mechanism should be added.*
**We added this recent and most important publication about mechanotransduction and cytoskeletal viscoplasticity in the new version of manuscript. We apologize for the oversight and thank the reviewer for the kind reminder.**

*3. The hyp7 epidermal cells develop by cell fusion. Please clarify in the text about the timing of cell fusion and initiation/development of the periodic structures.*
**The first cell fusion event which initiates the formation of hyp7 syncytium occurs before comma stage, followed by more cell fusions that progress throughout embryogenesis. The hyp 7 syncytium undergoes fusion events with an additional 116 cells continuously during the entire post-embryonic development. Since the initiation of the periodic structures in the epidermal cells happens at about 1.5-fold embryonic stage, and the doubling of the periodic stripes only occurs at L3 stage, the initiation/development of the periodic structures appears not strictly correlated with hyp7 cell fusion. We added the information in the discussion section following the reviewer's suggestion.**

*4. In Fig. 3C, the stripes in bli-1(RNAi) look wider than others. Please clarify in the text whether this is representative of these bli-1(RNAi) worms. Since bli-1 encodes a collagen, is this related to the duplication defects as reported in Fig. 4?*
**No, it is not a representative phenotype. The CeHD stripes in the *bli-1*(RNAi)-treated worm appeared slightly wider than control because that particular individual is slightly older (We used the method of worm synchronization by bleaching, which creates worm populations with an age variation of at least 3 hours). We did not pay attention to the variation of stripe thickness while selecting representative images. The CeHD stripe thickness of bli-1(RNAi)-treated group in general is not significantly different from the control group. We have replaced that particular image with a more representative one from the same set of experiment.**
*5. Fig. 5. Multiple collagen genes appear to be involved in the stripe duplication. Please add some discussion whether there is any functional redundancy or specificity among the collagen genes. No additional experiments are necessary.*
**It was proposed that trimerization of collagen molecules could likely happen between members of cuticle collagens with the same temporal expression patterns, and that the mutation of one molecule could affect the functions of the other two (Johnstone, Trends Genet. 2000). We suspect that this could be the case for the involvement of *dpy-2*, *7* and *10* in the stripe duplication process. We have added some discussion in the text as the reviewer suggested.**

*6. Statistic analysis should be performed on quantitative data in Figs 2-4.*
**Statistic analysis for all the comparison groups has been added in Figure 2-4 as the reviewer requested.**

*Reviewer 2 Advance Summary and Potential Significance to Field:*
*This manuscript describes an unexpected mechanism by which periodic cellular rings (annuli) on C. elegans skin cells increase in number as animals grow. These rings encompass cytoskeletal elements, junctions, and extracellular proteins. Using transgenic reporters and immunofluorescence, the authors first describe the sequence of events during the initial process of ring formation, identifying spectrin as one of the earliest proteins to adopt a periodic pattern. They then demonstrate that rings double once during development, apparently because they are stretched and pulled apart during cellular growth, thus splitting each into two new rings.This most valuable contribution of this descriptive study is documenting the ring duplication phenomenon, which is unusual and raises exciting mechanistic questions for future work.*

**We thank the reviewer for his/her recognition of the potential importance of our work.**

*Reviewer 2 Comments for the Author:*
*Although the data is convincing on the whole, the manuscript would benefit from clarification, deeper explanation, and improvements in data presentation. There are also some claims that should either be better justified experimentally, or stated more cautiously.*

*Specific comments:*

*1. Throughout the manuscript the authors never use the recognized term for these periodic structures in C. elegans—annuli. The authors should use this term so that readers can search for and connect this study with other literature about them.*
**Annuli refer to the pleated-appearing structure of the *C. elegans* cuticle, which is the outer exoskeleton outside of the epidermal cells (www.wormbook.org). Our study mainly focuses on the formation and reorganization of periodic structures WITHIN the epidermal cells, which is the reason why we did not mention the term "annuli" in the text. However, we do believe that the periodic patterns within the epidermal cells may guide the secretion of the cuticle components and the formation of annuli. We added this point in the discussion and thank the reviewer for the suggestion.**

*2. The RNAi experiments currently do not include controls for efficacy or specificity. For example, RT-PCR or Western blots could assess the magnitude of knock-down. Similarly, using multiple RNAi clones for each target may be an easy way to address specificity. However, since this reviewer does not use the C. elegans model, I defer to the expertise of other reviewers for the C. elegans community's current expectations for RNAi controls.* **Following the reviewer's suggestion, controls for RNAi efficacy were added for experiments with results showing that knock-down of certain target genes has no significant effect on stripe reorganization. We feel that the best way to confirm that the function of certain molecule is not required for stripe maintenance is to observe within the same animal the absence of the target molecule and the intact CeHD stripes. By using fluorescent reporters or antibody staining, we confirmed that the periodic stripes formed by apical CeHDs are still present with abolished expression of *let-805*, *vab-10a*, *ifb-1*, *spc-1*, *sma-1*, *bli-1 and unc-52* (Fig. S3). Multiple *mup-4* RNAi clones were used to address RNAi specificity. Our data confirmed that a new RNAi clone targeting 141-1111bp of *mup-4* transcript (Fig. S3A) yielded very similar phenotype as the old RNAi clone targeting 3857-4958bp of *mup-4* transcript (Fig. 3A).**

*3. Knocking down the actin gene with RNAi is problematic, since the development of all the cells in the animal will be affected, thus making it difficult to interpret effects on annuli as direct. This is also true, perhaps to a lesser extent, for spectrin RNAi. Performing this experiment with spatially or temporally controlled methods (i.e.* cell type-specific RNAi, *cell type specific RNAi rescue, and/or temporally controlled actin drug inhibitors) could partially alleviate this concern. These experiments are not critical to the main points of the manuscript, so if they cannot be addressed experimentally (e.g. due to the Covid crisis), they should at least be interpreted more cautiously.*
**Following the reviewer's suggestion, we repeated the experiments of *act-1* and *spc-1* RNAi with an epidermal-specific RNAi strain (Qadota et al., Gene 2007) and replaced the old data obtained using systemic RNAi. The results were shown in new Fig. 1E.**
*4. They authors conclude that the periodic spectrin pattern drives formation of the actin pattern, since spc-1 RNAi disrupts the actin pattern. However, to show a genetic hierarchy (rather than interdependence) they should do the reverse experiment: determine if actin disruption affects the spectrin pattern. Perhaps simply backing off the conclusion that the pattern is "initiated by spectrin" would be sufficient to address this concern, since this experiment is not central to the main findings of the manuscript.*
**We agree that the currently available data cannot fully support our claim. Therefore, we changed the statement "formation of the striped patterns in the *C. elegans* epidermis is initiated by spectrin" into "formation of the striped patterns in the *C. elegans* epidermis start from the alignment of spectrin molecules and actin bundles"**
*5. Related to the point above, the experiments in this study do not justify the statement that "the initial formation of periodic patterns in the C. elegans epidermis is evolutionally conserved". The other periodic spectrin/actin structures with which conservation is implied (in neurites and cilia), are structurally distinct from annuli—for example, they do not contain hemi-desmosomes, intermediate filaments, or extracellular components. It is not surprising that structures comprised of spectrin and actin depend on spectrin and actin—this observation does not alone indicate that other structures containing spectrin and actin use the same assembly mechanism.*
**Following the reviewer's advice, we deleted the statement "the initial formation of periodic patterns in the *C. elegans* epidermis is evolutionally conserved".**
*6. The photoconversion experiment (Fig 3E) seems to show that new protein integrates into existing hemi-desmosomes as they divide, but these experiments should be explained better. In Figure 3E,*

*it is not clear what the boxed region indicates. Was the whole animal photoconverted and the boxed region indicates the region for close up in subsequent panels? Or was only the boxed region photoconverted? If the latter is correct, the authors cannot conclude that newly synthesized protein was added to existing stripes—it is equally plausible that new Mup4 in the converted region came from exchange with other hemi-desmosomes in unconverted regions. In fact, this explanation is technically possible even if the whole animal was photoconverted, since it appears that photoconversion was far from complete. Could this photoconversion experiment be repeated at different stages (i.e. each molting cycle). This experiment would determine if Mup-4 synthesis or dynamics increases specifically during the L3 stripe duplication stage.*
**We agree with the reviewer that the whole-animal photoconversion experiment alone cannot totally rule out the possibility that new MUP-4 in dividing stripes may come from exchange with existing hemidesmosomes in other regions. To clarify this point, we took a different approach and labeled the newly synthesized MUP-4 with heat-shock induced expression system (Fig. S4). The results showed that the GFP-labeled new MUP-4 protein integrates into the dividing CeHD stripes in a random fashion very similar to the one revealed by the photoconversion experiment. We would not presume that MUP-4 synthesis or dynamics increases specifically during the L3 stripe duplication stage, because the CeHDs undergo stripe thickening and circumferential expansion continuously during post-embryonic growth. This experiment is merely to rule out the possibility that new CeHD stripes might form outside of the old stripes. We added diagrams in Fig. S4 to display different hypotheses and clarify our purposes.**

*7. The experiment in Figure S3B is clever and supports the authors' model. The authors should consider moving these panels to a main figure, and should also explain the experiment better. Since the experiment was not well explained and the image in the figure was not completely clear, I did not immediately grasp its rationale or interpretation (When I figured it out though, I thought it was cool!). It would help if the distances within and between doublets in straight and bent animals were quantified. Arrows should also be added to the figure to indicate both fissures and inter-doublet spaces in both straight and bent animals. In the images of the bent animal it is currently impossible to distinguish the doublets from the fissures.*
**Following the reviewer's valuable suggestion, we moved Fig. S3B to the new figure 4, quantified the distances within and between doublets in straight and bent regions and added arrows to indicate fissures and inter-doublet spaces in both straight and bent regions. We also modified the text to better convey the rational and interpretation of this experiment.**

*8. One more factor that could be quantified to support the model is the ratio of stripe thickness to animal length at different stages. The model predicts that this ratio should remain relatively steady before and after the L3 stage, but halve during stripe duplication.*
**Following the reviewer's excellent suggestion, we added dataset showing the ratio of stripe thickness to animal length at different stages in Fig. 4. Consistent with the model's prediction, this ratio indeed remains steady before and after the L3 stage, and halves during stripe duplication.**

*Minor/styistic comments:*

*9. Figure row labels should be used more consistently. For example, in Figure 1, row labels are useful in panel B and E, but are not included in any of the other panels. Row labels are similarly inconsistent in Figure 4. Row labels would help the reader to immediately understand the figure without referring to the legend.*
**Row labels have been added to Fig. 1, Fig 3 and Fig. 4 following the reviewer's advice.**

*10. The authors should avoid using jargon and precisely define the proteins they are discussing in the main text of the manuscript. For example, the identity of the VAB-10 protein (dystonin, a plakin family member) was never stated. Similarly, in Figure 1B, a reporter is labeled as "IFs", but the specific gene that was tagged is not named. Similarly, the identity of the egl-19 or mup-4 proteins are not described.*
**Following the reviewer's suggestion, we added precise descriptions regarding the identities of the proteins discussed in the manuscript.**

*11. Auto-correlation analysis (Figure S1) is an interesting way to quantify periodic structures, but*

*this analysis is not described well in either the figure legend or the methods section. It would be helpful to add more explanation and justification for this approach. (Incidentally, according to the auto-correlation, bli-1 appears to have an interesting higher order pattern, does this indicate a subpattern within a pattern?)*
**We added detailed description of the auto-correlation analysis in the method section following the reviewer's advice. BLI-1 indeed has a subpattern within the common epidermal periodic frame. There are two thin stripes of BLI-1 flanking both sides of every CeHD stripes, plus an additional stripe in the middle of each CeHD stripe.**

*12. The manuscript states that, "we tested all the molecules known to be periodically organized in the epidermis and discovered that only a few apically secreted collagens could significantly affect the striped patterning" (line 184). What other molecules were tested?*
**The molecules known to be periodically organized are *let-805*, *vab-10a*, *ifb-1*, *spc-1*, *sma-1*, *bli-1*, *unc-52*, actin and microtubules, the data related were presented in Fig 3. We also tested all the cuticular collagens that has been reported to have striped organization (dpy-2,4,5,7,10,13,17 and col-19). Data of those that do not affect the stripe patterns were not shown due to space limitation.**

*13. Figure 4D lacks a Y-axis label.*
**The Y-axis label has been added back. We apologize for this oversight and thank the reviewer for spotting the error.**

*14. Although the manuscript is on the whole clearly written, it should be revised carefully for word choice and grammar. One particular issue (which would understandably not be obvious to a non-native english writer) is that structures should be referred to as "fixed", not "fixated", which has a different common meaning in english.*
**We made the following corrections and screened for word and grammar mistakes as the reviewer advised**
**Line 24 "fixated" – "fixed"**
**Line 186 "fixating" – "fixing"**
**Line 203 "fixating" – "fixing"**

*Reviewer 3 Advance Summary and Potential Significance to Field:*
*Using C. elegans as a model, the authors reported that the periodic striped patterns in the C. elegans epidermis depend on the actin-spectrin network. They further provide evidence for the importance of extracellular collagens together with extension forces generated from epidermal cell growth in enabling long-range synchronized reorganization of subcellular structures. This beautiful paper is well written, based on a series of rigorous analysis of cellular biological phenotype complemented with elegant genetic studies. The findings provide conceptually new understanding of how periodic subcellular structures are established and maintained in integrity and distribution during cell growth and tissue development and should be of broad interest to the readers in the fields of C. elegans, developmental biology and also cell biology.*

**We thank the reviewer for his/her acknowledgement of the quality and importance of our work.**

*Reviewer 3 Comments for the Author:*
*One major comment concerns interpretation of the genetic analysis. Although the authors convincingly showed the requirements of actin, spectrin and collagen etc by RNAi or mutants, the data per se only suggest these molecules are genetically required and do not reveal the actual biochemical or cell biological processes by which these molecules might have enabled the establishment of and maintenance of those period subcellular structures. I would suggest the authors seek alternative independent evidence to show that these molecules are more directly involved in those processes, rather than playing merely permissive roles. Without actual mechanistic studies, the authors might want to tone down conclusions of the paper including strong claim that "the periodic striped patterns in the C. elegans epidermis is created by the actin-spectrin network, but fixated and maintained by apical membrane attachment structures."*
**We appreciate the reviewer's concerns and agree that in-depth mechanistic studies will no doubt greatly strengthen our conclusions and improve this manuscript. Unfortunately detailed analysis**

**at the molecular level, particularly biochemical studies are technically challenging, time-consuming and especially difficult during the COVID-19 crisis when both reagent supply and technical support are compromised. Therefore, we modified the statement in question into "the periodic striped patterns in the *C. elegans* epidermis is actin-spectrin dependent, and require the apical membrane attachment structures for maintenance."**
*Another comment concerns lack of quantitative data and statistic analysis for all the images and plots. Please provide numbers of animals imaged with corresponding penetrance of phenotype when applicable, and also statistic significance for all the comparison groups e.g. in Figure 2B, 2C, 3B, 3D, 3F, 4D.*
**The sample sizes and statistical significance for all the comparison groups in Figure 2-4 are added as the reviewer requested.**

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Second decision letter

MS ID#: JOCES/2020/246793

MS TITLE: Periodic subcellular structures undergo long-range synchronized reorganization during C. elegans epidermal development

AUTHORS: Chunxia Wang, Yuyan Yang, Rong Fu, Yi Zhu, and Huimin Zhang
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

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

As you will see, the reviewers gave favourable reports referee #2 raised some critical points that will require amendments to your manuscript. I hope that you will be able to carry these out, because I would like to be able to accept your paper.

*We are aware that you may 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.*

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

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

Reviewer 1

*Advance summary and potential significance to field*

This is a nice report on the formation and reorganization of the parallel periodic structures in the C. elegans epidermis.

*Comments for the author*

The revision clarified all of my previous concerns.

Reviewer 2

*Advance summary and potential significance to field*

This study describes a duplication mechanism for maintaining the periodicity of subcellular structures as an animal grows. This is a new mechanism that will stimulate the field to consider mechanisms for creating and maintaining periodic subcellular patterns.

*Comments for the author*

This manuscript has been substantially improved by numerous changes. The inclusion of statistical analyses for Figure 2-4, the addition of controls for RNAi experiments, and the addition of cell-autonomous actin and spectrin knock-down experiments are particularly appreciated. The inclusion of additional methodological information and the reorganization of the figures are also improvements. A few additional clarifications/minor revisions should still be addressed before the manuscript is ready for publication.

--The response to reviewers and the results section state that spc-1 and act-1 RNAi knockdown were epidermis-specific, but the methods (line 318) states that these experiments were carried out by injecting double stranded RNA into the gonads of mothers. Maybe I lack sufficient C. elegans expertise to understand this method, but it sounds like the dsRNA would be transmitted to all tissues of the progeny with this approach. This issue should be clarified in the methods section.

--The new analysis of stripe thickness/animal length ratio is redundantly described twice in the same paragraph (line 182 and 187). This statement only needs to be made once.

--Some explanations of experiments have been improved, but the observation of stripe distances during movement of the animals could still be explained better.
Specifically, the sentence on line 189 states "…when the epidermis was temporarily stretched, the distance between neighboring CeHD doublets remained constant…", making it sounds like animals were experimentally stretched. I suggest clarifying with something like: "…when the epidermis was temporarily stretched by body bends during normal sinusoidal movement, the distance between neighboring CeHD doublets remained constant…"

--On line 201, the authors state that they "tested all the molecules known to be periodically organized in the epidermis", but do not name the molecules. They clarify in the response to reviewers that these molecules are shown in Figure 3 so Figure 3 should be referenced when this statement is made in the text.

--All the statistical analyses that have been added were done with unpaired T-tests, which suggests that all data were normally distributed. Have the authors verified this? Also, in some cases it seems like a test of multiple comparisons with a post-test would be more appropriate than a T-test (e.g. Figure 2B-C, and Figure 3D).

--The grammar and style in the manuscript should be extensively edited to improve readability.

Reviewer 3

*Advance summary and potential significance to field*

Using C. elegans as a model, the authors reported that the periodic striped patterns in the C. elegans epidermis depend on the actin-spectrin network.
They further provide evidence for the importance of extracellular collagens together with extension forces generated from epidermal cell growth in enabling long-range synchronized reorganization of subcellular structures. This beautiful paper is well written, based on a series of

rigorous analysis of cellular biological phenotype complemented with elegant genetic studies. The findings provide conceptually new understanding of how periodic subcellular structures are established and maintained in integrity and distribution during cell growth and tissue development and should be of broad interest to the readers in the fields of C. elegans, developmental biology and also cell biology.

*Comments for the author*

The authors have satisfactorily addressed my concerns in the revision.

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**Second revision**

Author response to reviewers' comments

*Reviewer 2 Advance Summary and Potential Significance to Field:*
*This study describes a duplication mechanism for maintaining the periodicity of subcellular structures as an animal grows. This is a new mechanism that will stimulate the field to consider mechanisms for creating and maintaining periodic subcellular patterns.*

*Reviewer 2 Comments for the Author:*
*This manuscript has been substantially improved by numerous changes. The inclusion of statistical analyses for Figure 2-4, the addition of controls for RNAi experiments, and the addition of cell-autonomous actin and spectrin knock-down experiments are particularly appreciated. The inclusion of additional methodological information and the reorganization of the figures are also improvements. A few additional clarifications/minor revisions should still be addressed before the manuscript is ready for publication.*

*--The response to reviewers and the results section state that spc-1 and act-1 RNAi knockdown were epidermis-specific, but the methods (line 318) states that these experiments were carried out by injecting double stranded RNA into the gonads of mothers. Maybe I lack sufficient C. elegans expertise to understand this method, but it sounds like the dsRNA would be transmitted to all tissues of the progeny with this approach. This issue should be clarified in the methods section.*

**The principle of *C. elegans* tissue-specific RNAi approach based on *rde-1* mutant was described in Qadota et al., Gene 2007. RDE-1 is a PAZ-PIWI family protein that is essential for processing dsRNA into siRNA (Grishok, FEBS Letters 2005). In our experiments, we used the epidermal-specific RNAi strain NR222. This strain was generated by transgenic expression of wild-type RDE-1 protein in the epidermal cells of the *rde-1(ne219)* loss-of-function mutant. By injecting double stranded RNA into the gonads of mothers, the dsRNA would indeed be transmitted to all tissues of the progeny. However, because the non-epidermal cells are devoid of RDE-1 function, they are unable to process dsRNA into siRNA and knock-down target genes. Only the epidermal cells that express wild-type RDE-1 can carry out the entire RNAi process that ultimately leads to gene silencing. We added the detailed genotype of the epidermal-specific RNAi strain NR222 in the method section to clarify this point.**

*--The new analysis of stripe thickness/animal length ratio is redundantly described twice in the same paragraph (line 182 and 187). This statement only needs to be made once.*
**We deleted the redundant sentence and thank the reviewer for pointing out the mistake.**

*--Some explanations of experiments have been improved, but the observation of stripe distances during movement of the animals could still be explained better. Specifically, the sentence on line 189 states "…when the epidermis was temporarily stretched, the distance between neighboring CeHD doublets remained constant…", making it sounds like animals were experimentally stretched. I suggest clarifying with something like: "…when the epidermis was temporarily stretched by body bends during normal sinusoidal movement, the distance between neighboring CeHD doublets remained constant…"*
**We modified the sentence in question as the reviewer suggested.**

*--On line 201, the authors state that they "tested all the molecules known to be periodically organized in the epidermis", but do not name the molecules. They clarify in the response to reviewers that these molecules are shown in Figure 3, so Figure 3 should be referenced when this statement is made in the text.*
**We added the figure reference and related publications as the reviewer advised.**

*--All the statistical analyses that have been added were done with unpaired T-tests, which suggests that all data were normally distributed. Have the authors verified this? Also, in some cases it seems like a test of multiple comparisons with a post-test would be more appropriate than a T-test (e.g. Figure 2B-C, and Figure 3D).*
**Data normality was verified using the D'Agostino & Pearson normality test and the Shapiro-Wilk normality test (Graphpad Prism 7). We performed Tukey's Multiple Comparison Test on data in Fig. 2B as the reviewer suggested. We do not feel that multiple comparisons test is appropriate for data in Fig. 2C and Fig. 3D, because each mutant or treatment group was only compare with the WT/control group but not other mutant/treatment groups.**

*--The grammar and style in the manuscript should be extensively edited to improve readability.*
**Following the reviewer's advice, we sent the manuscript out for professional English editing and all changes have been highlighted in this new version.**

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Third decision letter

MS ID#: JOCES/2020/246793

MS TITLE: Periodic subcellular structures undergo long-range synchronized reorganization during C. elegans epidermal development

AUTHORS: Chunxia Wang, Yuyan Yang, Rong Fu, Yi Zhu, and Huimin Zhang
ARTICLE TYPE: Research Article

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