JCB

Hemicentin mediated type IV collagen assembly strengthens juxtaposed basement membrane linkage

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February 2, 2022

Re: JCB manuscript #202112096

Dr. David R Sherwood
Duke University
Department of Biology 130 Science Drive Box 90338
Durham, NC 27708

Dear David,

Thank you for submitting your manuscript entitled "Hemicentin mediated type IV collagen assembly strengthens juxtaposed basement membrane linkage" to the Journal of Cell Biology. The manuscript has now been assessed by three expert reviewers, whose reports are appended below. As you can see from these reviews provided by three acknowledged leaders in the various overlapping research areas spanning the elements of this paper, there was considerable potential interest in the conclusions. Although two reviewers were relatively enthusiastic, a third felt that it did not yet provide a sufficiently major advance, at least for JCB. Interestingly, during the initial editorial pre-reviewing process, two evaluators felt that this was a very high-quality paper but was borderline with concerns about its conceptual novelty -- but I felt that it deserved a full peer review. After an assessment of the reviewer feedback, which varied in their level of enthusiasm for this study, our editorial decision is to invite a revision.

Although it may be too difficult to provide highly novel mechanistic information in depth, Reviewer #2 suggests a resolution to this conundrum of conflicting reviews in their second "idea" point. The issues of compensation and especially of the nature of the feedback between hemicentin and some B-LINK components sounds potentially valuable. Please consider carefully whether it will be possible to extend this study in these or some other areas to provide more novelty.

I personally hope that a resubmission will be possible, which would undergo a re-review to evaluate alleviation of the concern about the magnitude of the advance. Regardless of your decision about resubmission, we hope that you will find the reviews informative and useful.

Please let us know if you are able to address the issues outlined above and wish to submit a revised manuscript to JCB. As you may know, the typical timeframe for revisions is three to four months. However, we at JCB realize that the implementation of social distancing and shelter in place measures that limit spread of COVID-19 also pose challenges to scientific researchers. Therefore, JCB has waived the revision time limit. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

If you choose to revise and resubmit your manuscript, please also attend to the editorial points below. Please direct any editorial questions to the journal office.

Thank you very much for your interest in the Journal of Cell Biology.

With kind regards,

Ken

Kenneth Yamada, MD, PhD
Editor, Journal of Cell Biology

Tim Fessenden, PhD
Scientific Editor, Journal of Cell Biology

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If you choose to resubmit, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

Reviewer #1 (Comments to the Authors (Required)):

In this manuscript the authors intends to determine when the utse-beam B-LINK forms in C. elegans and the components involved in the formation and maintenance of this transient BM-BM linkage. They provide evidence that the nascent B-LINK begins to form at the mid-L4 stage and enriches in B-LINK components by the young adult stage, just prior to egg-laying. They also provide evidence that hemicentin and fibulin-initiate utse-seam BM-BM attachment, while type IV collagen is highly stable and functions to maintain the utse-seam B-LINK during the mechanically active time of egg-laying.
The paper is well presented and experiments are performed using state-of-the art techniques and useful reporter c. elegans. There are however several pitfalls. The main one is that all the conclusions drawn are based on in vivo siRNA experiments followed by immunofluorescence analysis. Although, as mentioned above, the images are beautifully presented, no cellular and/or biochemical assays are presented and/or shown do determine whether indeed hemicenitn contributes to collagen IV assembly and how it performs this action. This is also the tile of the paper and one would expect the authors to follow up on their discovery that hemicentin promotes type IV collagen assembly. The data showing that collagen IV is highly stable and functions to maintain the utse-seam B-LINK is again based on quantification of IF images and as written it is felt that the authors performed several perturbations till they were eventually able to test their hypothesis. In conclusion, this is a well performed study that however lacks a clear mechanism of how the stabilization of the B-LINK is established. Finally, given the previous BM-BM and B-LINK discovery from the group, it is felt that this study provides incremental information.

Reviewer #2 (Comments to the Authors (Required)):

The manuscript by Gianakas et al makes a significant contribution to our understanding of how basement membranes are linked together to provide functional support to organs. Specifically, they describe the function, assembly, and maintenance of a C. elegans linkage, a B-LINK, between the utse (hammock-like support for the uterus) and the seam cell that runs along the lateral side of the animal. The authors show that the function of this linkage is to support the uterus during egg-laying, and when the B-Link is compromised, the uterus prolapses and is partially expelled. The authors determine that this structure is first evident in late larval development before it is needed for egg-laying, and the mechanisms for assembling and maintaining the B-LINK are different. They identify many proteins that are required for B-Link function using gene knockdown strategies, and interestingly, they discover that hemicentin acts as a key organizer, secreted locally by the utse; without hemicentin, levels of many matrix proteins are considerably reduced in the B-LINK. In addition to hemicentin, fibulin is required for the initial assembly of the B-LINK, and both fibulin and hemicentin are dynamic, with short-half lives. Unexpectedly, neither hemicentin or fibulin are required later in the adult for B-LINK function, suggesting that hemicentin serves as an organizer but not a functional constituent. In contrast, collagen IV is required later for B-Link function but not initially for its initial assembly.

The data is very strong and clearly presented, and the paper is easy to read, well-organized, well-written. I have no substantive concerns about any of the claims in the manuscript - I am convinced by all of it. I congratulate the authors on their important and interesting study.

I have a few ideas the authors may want to pursue here or another time, and I found a few items that need attention before publication.

An error?
- The last paragraph of the results section closes with a description of perlecan and collagen levels increasing as animals age, but where is this data? Please include it - it makes the loss of hemicentin and fibulin that much more interesting.

Ideas
- The role of hemicentin is really well defined by this study (bravo!). However, the role of fibulin is not so clear. Could fibulin and hemicentin be working to promote early linkage in parallel, partially redundant pathways? You could test this by knocking both down and seeing if the phenotype got worse. This comes up again in the discussion (line 486) when the possibility is raised that fibulin might be compensating for hemicentin in mice. The question of why hemicentin doesn't have this phenotype in mice is a real thorn, and if you could support the compensation-by-fibulin model, that would be helpful to the field.

- The authors don't do much with the super-interesting observation that there is a feedback mechanism between hemicentin and several components of the B-LINK. (Tone down the text, though - you can't say "every component" (line 295) since you didn't test every component - just list the ones you did). They note that when any of several component are lost, the level of hemicentin is increased. It seems likely that the basis for the feedback is mechanical - levels of hemicentin are set by the amount of tension somewhere. Would it be interesting to ask if an infertile worm, without the mechanical tension of egg-laying, also had that feedback?

Small things
- RNAi doesn't work immediately - not like a chemical inhibitor - and a few words (or more) discussing the timing of knockdown and when you expect protein levels to be decreased would be helpful, especially given how stable matrix proteins are usually.
- on line 447, change "residence times" to recovery half-life or something similar. Residence time would need to be calculated from the exponential equation.
-in the methods, there are two mentions of immobilizing worms with polystyrene beads. Either explain this method more or cite a reference.
- in the methods describing FRAP, there is a note in line 622 that the number of repetitions required varied by strain. Please give the range of repetitions and say something about how long this took.
- In Fig. 2, move part D down below part C, it is confusing where it is.
- In all the scatter plots (in all the figures but most obviously true for Fig. 2), it would be good to use gray values to emphasize the data more than the boxes.
- In Fig. 6D, I applaud the authors for showing the data a different way in this spot. But there is no reason to have the error bars. We see the mean from the colored bar, and we see the three data points - anything more is confusing and overly derived with only 3 points.

Reviewer #3 (Comments to the Authors (Required)):

This is an elegant and complete study about the role of hemicentin, fibulin-1, and collagen IV in generating and maintaining the B-LINk between uterine utse and epidermal seam cell basement membranes.

The experiments are well thought, well performed and the results are clear.

My only comment regarding this study is to expand the discussion and speculate why the animals do not have a Rup phenotype when γ-laminin is knocked down [lam-2 (RNAi)) in the L3 stage (Table S1)], since laminins are needed for the cell-extracellular matrices binding and such binding might be needed during mechanical restrains.

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Kenneth Yamada, Ph.D.
Editor, *Journal of Cell Biology*

August 16, 2022

Dear Kenneth,

Thank you again for reviewing our manuscript **"Hemicentin mediated type IV collagen assembly strengthens juxtaposed basement membrane linkage"**. We are very pleased that the reviewers are supportive of our work, and we are grateful for their insightful comments and suggestions. We have now addressed all the requested changes, which has deepened our conclusions and made the study stronger and more thorough with additional novel insights. We believe this work revealing how a basement membrane-basement membrane linkage is formed and maintained between the *C. elegans* utse and seam cells significantly advances our understanding of the mechanisms of assembly, composition and function of the unique matrix components required for these specialized linkages. We expect this study will also help inform our understanding of pathologies affecting basement membrane connections.

We have made the following changes to our manuscript based on the advice of the three external reviewers:

Reviewer comments:

Reviewer #1: In this manuscript the authors intend to determine when the utse-seam B-LINK forms in C. elegans and the components involved in the formation and maintenance of this transient BM-BM linkage. They provide evidence that the nascent B-LINK begins to form at the mid-L4 stage and enriches in B-LINK components by the young adult stage, just prior to egg-laying. They also provide evidence that hemicentin and fibulin-initiate utse-seam BM-BM attachment, while type IV collagen is highly stable and functions to maintain the utse-seam B-LINK during the mechanically active time of egg-laying. The paper is well presented, and experiments are performed using state-of-the art techniques and useful reporter c. elegans.

We thank the reviewer for their appreciation of the techniques and powerful model system used in our study, as well as the overall presentation of the work.

Specific Comments

1) There are however several pitfalls. The main one is that all the conclusions drawn are based on in vivo siRNA experiments followed by immunofluorescence analysis. Although, as mentioned above, the images are beautifully presented, no cellular and/or biochemical assays

are presented and/or shown do determine whether indeed hemicentin contributes to collagen IV assembly and how it performs this action. This is also the tile of the paper and one would expect the authors to follow up on their discovery that hemicentin promotes type IV collagen assembly. The data showing that collagen IV is highly stable and functions to maintain the utse-seam B-LINK is again based on quantification of IF images and as written it is felt that the authors performed several perturbations till they were eventually able to test their hypothesis. In conclusion, this is a well performed study that however lacks a clear mechanism of how the stabilization of the B-LINK is established.

We thank the reviewer for their suggestion to expand upon how hemicentin recruits type IV collagen. To address the concerns of the reviewer in the revised paper we have conducted a focused screen based on genome-wide genetic and RNAi-mediated loss of function of genes and discovered a new and important link between hemicentin and type IV collagen recruitment. As there are no reported direct interactions between hemicentin and type IV collagen, and hemicentin does not recruit type IV collagen in the previously characterized short-term linkage beneath the anchor cell, we hypothesized that an intermediate component might facilitate type IV collagen recruitment. As loss of type IV collagen at the B-LINK leads to a Rup phenotype due to uterine prolapse during egg-laying, we curated all known genes whose loss is reported to cause a Rup phenotype. This led to a list of 403 genes (see new Table S7). Given that recruitment would have to occur in the extracellular space, we then selected genes that encode proteins predicted to be either transmembrane or secreted. This narrowed the list to 52 genes (see Table S7, genes in italics). Within these, we removed genes not likely to be directly involved with collagen recruitment, such as metabolic transporters, cuticular collagens, and gap junction components. This resulted in a list of 11 genes, which we then screened to determine if RNAi mediated knockdown reduced type IV collagen enrichment at the B-LINK. We found that RNAi mediated knockdown of ADAMTS9/20 (*C. elegans gon-1*) led to over a 50% reduction in type IV collagen levels at the B-LINK (see new Fig. 9 D). Upon further characterization, we discovered that an endogenously tagged ADAMTS9/20 localizes to the B-LINK at the late L4 and young adult stage at the time of type IV collagen recruitment (see new Fig. S4 B) and that this ADAMTS9/20 localization is dependent on hemicentin (see new Fig. 9E). Furthermore, we investigated how ADAMTS9/20 affected our other identified components and found that loss of ADAMTS9/20 does not affect hemicentin, perlecan, or fibulin recruitment and that ADAMTS9/20 recruitment is modestly affected by type IV collagen loss (which strengthens the notion of a specific interaction of ADAMTS9/20 with hemicentin and type IV collagen) and not by loss of fibulin-1 or perlecan (see Fig. S4 C). Finally, we used a strain with α1-type IV collagen::mRuby2 and completed fluorescence recovery after photobleaching (FRAP) experiments on control and ADAMTS9/20 RNAi treated animals. We found that in control animals type IV collagen recovered ~20% of its original signal after 2 h, but after ADAMTS9/20 loss it recovered only ~9% (Fig. 10 A-C), providing compelling evidence that ADAMTS9/20 is crucial for normal type IV collagen assembly at the B-LINK. Taken together, this new data strongly places ADAMTS9/20 between hemicentin and type IV collagen buildup and thus provides novel insight into how hemicentin promotes type IV collagen assembly at the B-LINK.

These findings are highlighted in the results and discussion as follows:

Results section Line 390

"Given the critical role of type IV collagen in mediating B-LINK maintenance, we wanted to determine how hemicentin promotes collagen assembly. Hemicentin is not known to bind to type IV collagen (Zhang et al., 2021), so we hypothesized that hemicentin might recruit another protein that mediates collagen assembly. We reasoned that loss of this protein would result in a similar phenotype to type IV collagen loss and thus compiled a list of 403 genes on Wormbase (see Methods) whose knockdown leads to the Rup phenotype (Table S7). We focused on 52 genes encoding secreted or transmembrane proteins and removed genes not likely to be directly involved with collagen recruitment, such as metabolic transporters, cuticular collagens, and gap junction components. RNAi mediated reduction of the remaining 11 genes revealed that loss of ADAMTS9/20 (GON-1) led to the strongest effect on type IV collagen levels—a more than a 50% reduction at the B-LINK at egg-laying onset (Fig. 10 A and B; Table S7). Importantly, ADAMTS9/20::mNG localized to the B-LINK at the time of type IV collagen recruitment, and its localization was dependent on hemicentin, but largely not on other BM components, with only a modest dependence on type IV collagen (Fig. S4 C, Fig. 10 C). Furthermore, loss of ADAMTS9/20 specifically affected collagen recruitment, but did not affect the recruitment of other B-LINK components (Figure S4 C). To examine how ADAMTS9/20 affects type IV collagen assembly, we used a strain with α1-type IV collagen::mRuby2 and performed FRAP experiments on control and ADAMTS9/20 RNAi treated animals. Animals were plated on RNAi at the L2 stage as L1 RNAi made the animals prone to rupturing, which prevented long-term imaging. After photobleaching at the young adult stage (prior to egg-laying), type IV collagen recovered ~20% of its original fluorescence intensity after 2 h. In contrast, in animals where ADAMTS9/20 was reduced, type IV collagen recovered only ~9% (Fig. 10 D). We conclude that ADAMTS9/20 helps mediate hemicentin dependent assembly of type IV collagen at the B-LINK."

Discussion Line 473

"Our data indicates that hemicentin promotes type IV collagen assembly through ADAMTS9/20 (GON-1), as GON-1 B-LINK localization was dependent on hemicentin and its loss reduced type IV collagen assembly. The only identified substrates of ADAMTS9/20 in vertebrates are aggrecan and versican (Kelwick et al., 2015), which are not present in C. elegans. This suggests that GON-1 has another substrate(s) that promotes type IV collagen assembly. Although paradoxical that an ADAMTS protease promotes B-LINK type IV collagen incorporation, the matrix metalloproteinase MMP-1 is required for normal type IV collagen assembly in the BM of Drosophila embryos and larvae (Stevens and Page-McCaw, 2012; Matsubayashi et al., 2020). Proteolysis might facilitate type IV collagen incorporation into extracellular matrices."

2) Finally, given the previous BM-BM and B-LINK discovery from the group, it is felt that this study provides incremental information.

We appreciate the reviewer considering the novelty of our work. A number of new and important discoveries were made in this new study, and we likely did not do an effective job of conveying these. The previous BM-BM B-LINK study published from our group focused on the short-term adhesion system beneath the *C. elegans* anchor cell. Although the long-term utse-seam B-LINK was identified in that paper, hemicentin was the only matrix component found in that work to play a role in this long-term linkage. Notably, even with hemicentin, the regulation of its addition and the function of hemicentin was not elucidated. This study has many new findings that powerfully advance our understanding of BM-BM connection and go well beyond the scope of our prior work.

- First, we have used a strain with optogenetically inducible muscle contraction to show that egg-laying and muscle contractions are directly responsible for the Rup phenotype that occurs after loss of B-LINK components. This experiment directly links the utse-seam BM-BM linkage to resisting the mechanical forces of egg-laying, providing foundational insight into the role of this linkage.

- Additionally, this study not only expands upon the role of hemicentin (see below), but also identifies four other components with roles in this system (type IV collagen, fibulin, perlecan, and ADAMTS9/20).

- Furthermore, we have taken advantage of the strengths of the worm experimental model and developed assays to provide new mechanistic insight into the roles of these matrix molecules. By combining temporal examination of endogenous localization, temporal RNAi knockdown, the ability to endogenously localize BM components, and temporal examination of defects in BM-BM linkage, we have established early roles for hemicentin and fibulin in initiating BM-BM connection and a later role for type IV collagen. Also, we identify hemicentin as a critical organizer for the B-LINK that is secreted by the utse and show with temporal RNAi targeting that hemicentin and fibulin are only required during B-LINK initiation. Offering further support for this result, both endogenous molecules decline dramatically in levels at the B-LINK, once the linkage has been assembled.

- Understanding the temporal roles of matrix components has been challenging, as there are few temporal depletion systems for matrix components in other models. Our ability to temporally control RNAi knockdown and then quantitatively determine endogenous loss and effects on BM-BM linkage represent novel assays that have allowed us to identify previously unknown temporal roles for B-LINK components. Understanding the temporal roles of B-LINK components, and the effects of their loss at particular times in the development of the B-LINK is crucial, as this foundational knowledge will allow us to better inform therapies to restore these components to the proper levels and proper function.

- We also have used fluorescence recovery after photobleaching (FRAP) to examine and compare the dynamics of our identified components, finding that hemicentin and fibulin are the most dynamic, and type IV collagen and perlecan being the most stable.

- Finally, with the new data described above, we have discovered that ADAMTS9/20 links hemicentin to type IV collagen assembly at the B-LINK and conducted FRAP to show that ADAMTS9/20 plays a spatially localized role in promoting type IV collagen addition at the B-LINK. ADAMTS9/20 directed assembly of type IV collagen is a novel finding, which not only has implications for other BM-BM connections, but may also be used in constructing basement membrane with enriched levels of type IV collagen.

These novel findings are highlighted in the Discussion section as follows:

Line 432

"Using LITE-1 optogenetic stimulation of body wall and egg-laying muscle contractions (Edwards et al., 2008) on hemicentin depleted animals (weakened utse-seam B-LINK), we found that egg-laying causes uterine prolapse. These experiments establish that the utse-seam B-LINK forms prior to egg-laying and functions to resist mechanical forces associated with muscle contraction and egg-laying.

Line 439

"Through screening and timed RNAi depletion, we discovered the utse-seam B-LINK has additional matrix components not found at the anchor cell B-LINK including the matricellular protein fibulin-1, and core BM proteins type IV collagen and perlecan.

Line 444

"An important strength of this in vivo model is the ability to perform timed RNAi knockdown through feeding, allowing determination of temporal roles for matrix proteins. Knockdown of the core BM components type IV collagen and laminin at the L1 stage dramatically disrupts the gonadal BM by the young adult stage (Jayadev et al., 2019; Gordon et al., 2019), which leads to the Rup phenotype due to disruption of the anchoring BM. By feeding worms at the L3 stage, we were able to specifically target the later deposition of collagen between the BMs at the B-LINK, and leave the gonadal BM intact, as evidenced by modest collagen reduction in the gonadal BM. This allowed us to establish a functional role for type IV collagen at the B-LINK and determine that laminin was not functionally required at the B-LINK, as L3 RNAi targeting of laminin did not cause uterine prolapse."

Line 486

"Using timed RNAi depletion, photobleaching, and quantitative fluorescence analysis, we also determined distinct properties and temporal requirements for matrix components at the utse-seam B-LINK (summarized in Fig 10 D). We found that hemicentin and fibulin-1 have dynamic associations, with recovery half-lives of less than thirty min, and reach peak levels prior to egg-laying. In contrast, perlecan and type IV collagen reach peak levels later during egg-laying.

Consistent with their early deposition, hemicentin and fibulin-1 loss caused B-LINK disruption and utse-seam splitting at the mid and late L4 stages before type IV collagen levels ramped up."

Line 510

"In contrast to hemicentin and fibulin-1, we found that type IV collagen and perlecan are highly stable at the utse-seam B-LINK and enrich to maximal levels after egg-laying onset. Consistent with this later buildup, type IV collagen depletion only caused utse-seam B-LINK defects at egg-laying onset, suggesting it functions to bear high mechanical loads."

Reviewer #2: The manuscript by Gianakas et al makes a significant contribution to our understanding of how basement membranes are linked together to provide functional support to organs. Specifically, they describe the function, assembly, and maintenance of a C. elegans linkage, a B-LINK, between the utse (hammock-like support for the uterus) and the seam cell that runs along the lateral side of the animal. The authors show that the function of this linkage is to support the uterus during egg-laying, and when the B-Link is compromised, the uterus prolapses and is partially expelled. The authors determine that this structure is first evident in late larval development before it is needed for egg-laying, and the mechanisms for assembling and maintaining the B-LINK are different. They identify many proteins that are required for B-Link function using gene knockdown strategies, and interestingly, they discover that hemicentin acts as a key organizer, secreted locally by the utse; without hemicentin, levels of many matrix proteins are considerably reduced in the B-LINK. In addition to hemicentin, fibulin is required for the initial assembly of the B-LINK, and both fibulin and hemicentin are dynamic, with short-half lives. Unexpectedly, neither hemicentin or fibulin are required later in the adult for B-LINK function, suggesting that hemicentin serves as an organizer but not a functional constituent. In contrast, collagen IV is required later for B-Link function but not initially for its initial assembly.

The data is very strong and clearly presented, and the paper is easy to read, well-organized, well-written. I have no substantive concerns about any of the claims in the manuscript - I am convinced by all of it. I congratulate the authors on their important and interesting study.

I have a few ideas the authors may want to pursue here or another time, and I found a few items that need attention before publication.

We thank the reviewer for their thorough review and appreciation of our study, and for their helpful comments described below. We also are grateful that they highlighted our experimental rigor and clarity of writing.

Experimental comments:

1) The last paragraph of the results section closes with a description of perlecan and collagen levels increasing as animals age, but where is this data? Please include it - it makes the loss of hemicentin and fibulin that much more interesting.

We thank the reviewer for pointing out this omission. It does indeed make the loss of hemicentin and fibulin more interesting and notable. We have now added the referenced data to Fig. 9 B and C.

2) The role of hemicentin is really well defined by this study (bravo!). However, the role of fibulin is not so clear. Could fibulin and hemicentin be working to promote early linkage in parallel, partially redundant pathways? You could test this by knocking both down and seeing if the phenotype got worse. This comes up again in the discussion (line 486) when the possibility is raised that fibulin might be compensating for hemicentin in mice. The question of why hemicentin doesn't have this phenotype in mice is a real thorn, and if you could support the compensation-by-fibulin model, that would be helpful to the field.

We thank the reviewer for their appreciation of how our study defines the role of hemicentin and for highlighting how better understanding the interactions between hemicentin and fibulin could strengthen the study's impact, particularly as loss of hemicentin in vertebrates leads to such mild defects. To address this comment, we knocked down both hemicentin and fibulin together using combined L1 RNAi (see Methods) and imaged the utse and seam cells at the mid-L4, late-L4, and adult onset of egg-laying stages (Table S2). Defects in the B-LINK were indicated by a lack of contact between the utse and seam cells (utse-seam gaps). We found that combined hemicentin and fibulin loss caused a more severe B-LINK defect than either RNAi alone at the late L4 stage. This suggests that hemicentin and fibulin are working in partially redundant pathways at this timepoint and that fibulin could compensate for loss of hemicentin. We have included these data in Fig. 7 B and C and its corresponding figure legend and added the following text to the results section:

Line 319

"Notably, the combined loss of hemicentin and fibulin via double RNAi treatment caused a B-LINK defect at the late L4 more severe than loss of either one alone, suggesting that hemicentin and fibulin have at least partially independent functions (Fig. 7 B and C)."

The following text was also added to the Methods section:

Line 612

"For hemicentin and fibulin double knockdown, cultures of hemicentin and fibulin RNAi were grown normally and mixed in a 1:1 ratio prior to seeding."

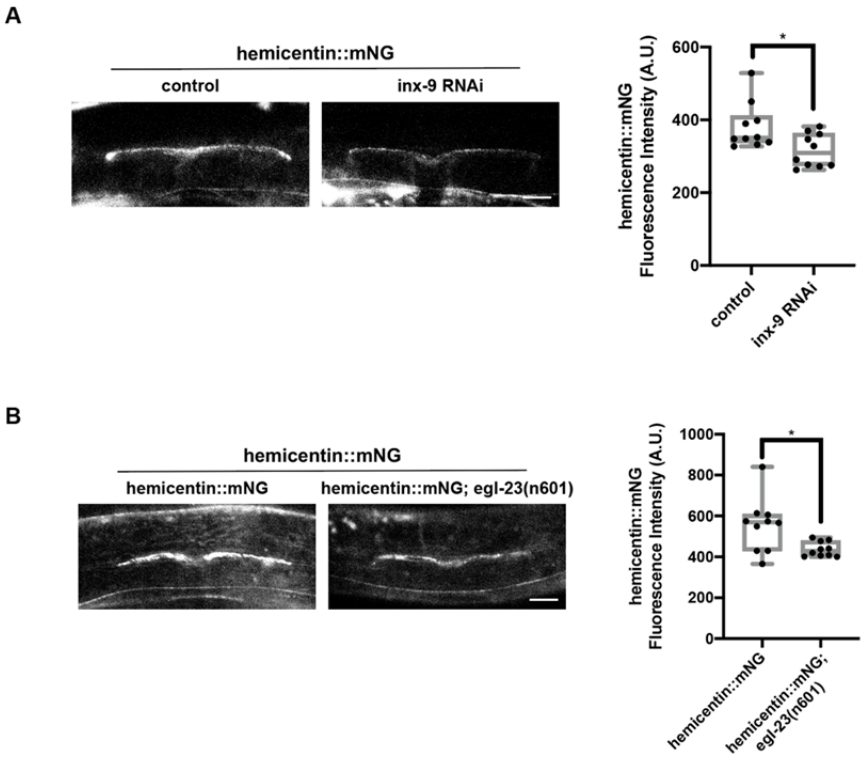
Finally, the following text has been added to the Discussion:

Line 498

"It is likely that hemicentin and fibulin-1 have independent functions in linking the BMs, as loss of fibulin-1 led to defects prior to loss of hemicentin and the combined loss of hemicentin and fibulin-1 led to a greater defect in BM-BM linkage than loss of either alone."

3) The authors don't do much with the super-interesting observation that there is a feedback mechanism between hemicentin and several components of the B-LINK. (Tone down the text, though - you can't say "every component" (line 295) since you didn't test every component - just list the ones you did). They note that when any of several components are lost, the level of hemicentin is increased. It seems likely that the basis for the feedback is mechanical - levels of hemicentin are set by the amount of tension somewhere. Would it be interesting to ask if an infertile worm, without the mechanical tension of egg-laying, also had that feedback?

We thank the reviewer for this very interesting suggestion about mechanical feedback. To address this question, we eliminated egg-laying by treating *C. elegans* with *inx-9* RNAi, which encodes a gap channel protein whose loss causes sterility (Green et al., 2011). We also examined animals harboring a mutation in *egl-23*, which encodes a potassium ion channel protein whose loss abolishes vulval muscle contractions during egg-laying (Trent et al., 1983; Ben Soussia et al., 2019). Both treatments would be expected to reduce mechanical load on the B-LINK and thus hemicentin. Interestingly, in both cases, there was a reduction in hemicentin assembly at the B-LINK (see data below) indicating that after reduction of load on the B-LINK there is increased hemicentin, suggesting that mechanics play a partial role in setting hemicentin levels.



Notably, the effect on hemicentin levels was modest, with only a ~30% reduction in the *egl-23* mutants and a ~15% reduction after treatment with *inx-9* RNAi. As both conditions are expected to result in a significant reduction in mechanical load on the B-LINK, these results suggest that mechanics are most likely not the primary regulator of hemicentin levels. To more thoroughly

understand the role of mechanics, and its impact on hemicentin and other B-LINK components, we would need to pursue further experiments that are outside the scope of this study (especially as we have now discovered in the revised manuscript that ADAMTS9/20 is a critical player in type IV collagen addition). Thus, we have decided to not include this data within the manuscript. We have, however, added the following text to the Discussion to highlight this important question and set up future studies:

Line 466

"It will be interesting in future studies to determine the mechanisms of feedback and if they involve mechanical load."

Additionally, we thank the reviewer for their comment about line 295 and have edited the text as follows:

Line 291

"Consistent with a crucial organizational role at the B-LINK, hemicentin was required for the robust assembly of type IV collagen, fibulin-1, and perlecan (Fig. 6). In addition, loss of each of these components caused a reciprocal increase in hemicentin, suggesting feedback between hemicentin and other B-LINK components (Fig. S2)."

References mentioned above:

Ben Soussia, I., S. El Mouridi, D. Kang, A. Leclercq-Blondel, L. Khoubza, P. Tardy, N. Zariohi, M. Gendrel, F. Lesage, E.-J. Kim, D. Bichet, O. Andrini, and T. Boulin. 2019. Mutation of a single residue promotes gating of vertebrate and invertebrate two-pore domain potassium channels. Nat. Commun. 10:787. doi:10.1038/s41467-019-08710-3.

Green, R.A., H.-L. Kao, A. Audhya, S. Arur, J.R. Mayers, H.N. Fridolfsson, M. Schulman, S. Schloissnig, S. Niessen, K. Laband, S. Wang, D.A. Starr, A.A. Hyman, T. Schedl, A. Desai, F. Piano, K.C. Gunsalus, and K. Oegema. 2011. A high-resolution C. elegans essential gene network based on phenotypic profiling of a complex tissue. Cell. 145:470–482. doi:10.1016/j.cell.2011.03.037.

Trent, C., N. Tsuing, and H.R. Horvitz. 1983. Egg-laying defective mutants of the nematode Caenorhabditis elegans. Genetics. 104:619–647. doi:10.1093/genetics/104.4.619.

Textual changes:

1) - RNAi doesn't work immediately - not like a chemical inhibitor - and a few words (or more) discussing the timing of knockdown and when you expect protein levels to be decreased would be helpful, especially given how stable matrix proteins are usually.

To address this concern, we have added the following text to the Methods section:

Line 625

"Importantly, RNAi does not result in instantaneous protein loss, proteins can have different stabilities, and RNAi has different efficiencies in reducing mRNA. Thus, for RNAi experiments reduction of levels of the targeted protein at the B-LINK were assessed to ensure sufficient knockdown was achieved. Knockdown levels were quantified and are shown in Table S2, S3, S5, and S6. Box plots for all quantified knockdown experiments are in Figure S5."

Furthermore, throughout the study we have ensured that when using RNAi, we examined the endogenous protein levels at the time of imaging to ensure that the protein of interest has been knocked down to a sufficient degree (see Fig. S5, Table S2, S3, S5, S6).

2) on line 447, change "residence times" to recovery half-life or something similar. Residence time would need to be calculated from the exponential equation.

We thank the reviewer for this comment and have adjusted the text as recommended.

3) in the methods, there are two mentions of immobilizing worms with polystyrene beads. Either explain this method more or cite a reference.

We have provided a brief description of this method and included a reference as requested by the reviewer. The following text was added in the Methods:

Line 648

"Worms were mounted on 5% noble agar pads containing 0.01 M sodium azide for imaging for all experiments except for Fig. 5 and 10. For Fig. 5, worms were mounted on 5% noble agar pads with no added anesthetic and added to agar pads in 3 µl undiluted 100nm polystyrene bead solution (Polysciences cat. #64010) (Kim et al., 2013)."

4) in the methods describing FRAP, there is a note in line 622 that the number of repetitions required varied by strain. Please give the range of repetitions and say something about how long this took.

We thank the reviewer for pointing out this omission, and have added the following text to the Methods:

Line 688

"The number of repetitions needed to achieve complete photobleaching varied by strain (number of repetitions varied between 20 and 50 reps, and total beach time ranged between 0.5 and 2.5 s) and was determined experimentally"

5) In Fig. 2, move part D down below part C, it is confusing where it is.

We thank the reviewer for suggesting this change. In Fig. 2 panel D was placed at the top because if we move panel D below panel C, then panel A (experimental timeline) would need to go below panel C as well in order to fit everything into the figure. We purposefully kept the timeline panels as the first panel in each figure for consistency—we are concerned we will make the figure confusing by moving this panel from the top of the figure. Thus, we feel it is best to keep the figure layout as is and we have not moved panel D. If, however, the reviewer feels it is still better to move part D down below part C, we are certainly willing to make that change.

6) In all the scatter plots (in all the figures but most obviously true for Fig. 2), it would be good to use gray values to emphasize the data more than the boxes.

We thank the reviewer for this point, and have modified the following figures with gray values: Fig.2 B, Fig. 6 B, Fig. 7 D, Fig. 8 C and D, Fig. S2 B, Fig. S3 B-D, and Fig. S5 A-D. This recommendation has also been followed for all new figures.

7) In Fig. 6D, I applaud the authors for showing the data a different way in this spot. But there is no reason to have the error bars. We see the mean from the colored bar, and we see the three data points - anything more is confusing and overly derived with only 3 points.

We thank the reviewer for their appreciation of our data presentation in Fig. 6D and for their helpful suggestion. We have removed the error bars as recommended.

Reviewer #3: This is an elegant and complete study about the role of hemicentin, fibulin-1, and collagen IV in generating and maintaining the B-LINk between uterine utse and epidermal seam cell basement membranes.

The experiments are well thought, well performed and the results are clear.

We thank the reviewer for their appreciation of the clarity and completeness of our study.

Specific comments:

1. My only comment regarding this study is to expand the discussion and speculate why the animals do not have a Rup phenotype when γ-laminin is knocked down [lam-2 (RNAi)) in the L3 stage (Table S1)], since laminins are needed for the cell-extracellular matrices binding and such binding might be needed during mechanical restrains.

We thank the reviewer for this suggestion, and we have now updated the discussion with the following text:

Line 444

“An important strength of this in vivo model is the ability to perform timed RNAi knockdown through feeding, allowing determination of temporal roles for matrix proteins. Knockdown of the core BM components type IV collagen and laminin at the L1 stage dramatically disrupts the

gonadal BM by the young adult stage (Jayadev et al., 2019; Gordon et al., 2019), which leads to the Rup phenotype due to disruption of the anchoring BM. By feeding worms at the L3 stage, we were able to specifically target the later deposition of collagen between the BMs at the B-LINK, and leave the gonadal BM intact, as evidenced by modest collagen reduction in the gonadal BM. This allowed us to establish a functional role for type IV collagen at the B-LINK and determine that laminin was not functionally required at the B-LINK, as L3 RNAi targeting of laminin did not cause uterine prolapse."

Manuscript formatting comments: Character count

Due to extensive additions to the results and discussion as requested by the reviewers, we are slightly over the character limit. We have gone through the paper multiple times to shorten it, but at this point will need guidance from the editor as we would need to cut content requested by the reviewers to reduce the character count further.

We would like to sincerely thank all three reviewers for their helpful comments and suggestions. The changes we have made in response to these suggestions have considerably improved the manuscript and broadened the impact of our study. We hope that our revised manuscript will now be suitable for publication in *The Journal of Cell Biology*. Please let me know if there are any additional questions or concerns regarding our submission.

Sincerely,

David R. Sherwood, Ph.D.
Jerry G. and Patricia Crawford Hubbard Professor and Associate Chair of Biology
Co-director, Embryology Course, MBL, Woods Hole, MA
Duke University, Department of Biology
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Bibliography

Ben Soussia, I., S. El Mouridi, D. Kang, A. Leclercq-Blondel, L. Khoubza, P. Tardy, N. Zariohi, M. Gendrel, F. Lesage, E.-J. Kim, D. Bichet, O. Andrini, and T. Boulin. 2019. Mutation of a single residue promotes gating of vertebrate and invertebrate two-pore domain potassium channels. *Nat. Commun.* 10:787. doi:10.1038/s41467-019-08710-3.

Green, R.A., H.-L. Kao, A. Audhya, S. Arur, J.R. Mayers, H.N. Fridolfsson, M. Schulman, S. Schloissnig, S. Niessen, K. Laband, S. Wang, D.A. Starr, A.A. Hyman, T. Schedl, A. Desai, F. Piano, K.C. Gunsalus, and K. Oegema. 2011. A high-resolution C. elegans essential gene network based on phenotypic profiling of a complex tissue. *Cell*. 145:470–482. doi:10.1016/j.cell.2011.03.037.

Kim, E., L. Sun, C.V. Gabel, and C. Fang-Yen. 2013. Long-term imaging of Caenorhabditis elegans using nanoparticle-mediated immobilization. *PLoS One*. 8:e53419. doi:10.1371/journal.pone.0053419.

Trent, C., N. Tsuing, and H.R. Horvitz. 1983. Egg-laying defective mutants of the nematode Caenorhabditis elegans. *Genetics*. 104:619–647. doi:10.1093/genetics/104.4.619.

September 15, 2022

RE: JCB Manuscript #202112096R

Dr. David R Sherwood
Duke University
Department of Biology 130 Science Drive Box 90338
Durham, NC 27708

Dear David,

Thank you for resubmitting your manuscript entitled "Hemicentin mediated type IV collagen assembly strengthens juxtaposed basement membrane linkage". After re-review, it was found that all concerns were resolved in "an outstanding study."

We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

We hope you agree that the rigorous JCB reviewing has helped result in a superb paper about which you and your co-authors can be proud.

With kind regards,

Ken

Kenneth M Yamada, MD, PhD
Editor
Journal of Cell Biology

Tim Fessenden, PhD
Scientific Editor
Journal of Cell Biology

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5) Abstract and title: The abstract should be no longer than 160 words and should communicate the significance of the paper for a general audience. The title should be less than 100 characters including spaces. Make the title concise but accessible to a general readership.

6) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions in the text for readers who may not have access to referenced manuscripts.

7) Please be sure to provide the sequences for all of your primers/oligos and RNAi constructs in the materials and methods. You must also indicate in the methods the source, species, and catalog numbers (where appropriate) for all of your antibodies. Please also indicate the acquisition and quantification methods for immunoblotting/western blots.

8) Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
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b. Type, magnification, and numerical aperture of the objective lenses
c. Temperature
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g. Acquisition software
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9) References: There is no limit to the number of references cited in a manuscript. References should be cited parenthetically in the text by author and year of publication. Abbreviate the names of journals according to PubMed.

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14) A separate author contribution section following the Acknowledgments. All authors should be mentioned and designated by their full names. We encourage use of the CRediT nomenclature.

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Reviewer #1 (Comments to the Authors (Required)):

I would like to thank the authors for taking into account all my major concerns and providing a convincing link between Hemicentrin and collagen assembly. This is truly an outstanding study.