Distinct contributions of ECM proteins to basement membrane mechanical properties in Drosophila

Uwe Töpfer, Karla Yanin Guerra Santillan, Elisabeth Fischer-Friedrich and Christian Dahmann

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Original submission decision letter

MS ID#: DEVELOP/2021/199993
MS TITLE: Distinct contributions of ECM proteins to basement membrane mechanical properties in Drosophila
AUTHORS: Uwe Töpfer, Karla Yanin Guerra Santillan, Elisabeth Fischer-Friedrich, and Christian Dahmann
ARTICLE TYPE: Research Report

Dear Christian,

I apologise for the delay before coming back to you. I have now received all the referees reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see from their reports, the referees recognise the potential of your work, but they also raise significant concerns about it. They all point to the fact that while it is valuable to report quite systematically different tests of the mechanical properties in ECM mutants, a significant number of similar results have been published previously. Given the nature of these concerns, I am afraid I have little choice other than to reject the paper at this stage.

However, having evaluated the paper, I do recognise the potential importance of this work. I would therefore be prepared to consider as a new submission an extension of this study that contains new experiments, data and discussions and that address fully the major concerns of the referees. In particular it would be important to explain the fact that response to osmotic shocks do not correlate straightfowardly with stifness of the ECM as measured by AFM. The work required goes beyond a standard revision of the paper. Please bear in mind that the referees (who may be different from the present reviewers) will assess the novelty of your work in the context of all previous publications, including those published between now and the time of resubmission.

Yours sincerely,

Thomas Lecuit
Handling Editor
Development

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Reviewer 1

Advance Summary and Potential Significance to Field

The manuscript by Töpfer et al. takes a comprehensive approach to dissecting the contributions of the four major basement membrane proteins to the structure and mechanical properties of the basement membrane using the Drosophila egg chamber as a model. Although some of the results have been reported previously, the real strength of this paper lies in having a complete set of carefully performed experiments from one laboratory. This allows comparisons of the different manipulations used and results obtained in a way that is not possible when results are spread across multiple papers. Moreover, there are some surprising findings, such as the observation that egg chamber elongation and resistance to osmotic stress do not necessarily correlate with basement membrane stiffness. In this way, I think this work makes an important contribution to both the matrix and morphogenesis communities and that it is likely to be of interest to the broad readership of Development. The manuscript is also well written and clear in its presentation of both the results and their significance. I do, however, have a few suggestions to improve the impact of the work and to better clarify its relationship to the existing literature that should be addressed prior to publication.

Comments for the author

Major concerns:

The observation that Pcan Levels are reduced under Col IV depletion has been previously shown in the egg chamber by two groups. Please cite Haigo and Bilder, 2011 (Figure S6D) and Isabella and Horne-Badovinac, 2015 (Figure 4) when discussing these results.

The result showing that loss of Laminin does not affect final egg shape conflict with two reports in the literature - Frydman and Spradling, 2001 (Figure 6J) and Andersen and Horne-Badovinac, 2015 (Figure 2G). The laminin antibodies in Drosophila are not great and could be giving an incomplete readout of knock down levels. The authors should consider confirming the strength of their knockdown with another reporter, such as the FlyFos line for either LanA or LanB1. If the discrepancy remains, it should be discussed in the text.

One key piece of data that is missing from the paper is whether the RNAi conditions used affect egg chamber rotation. Rotation affects the structure of the basement membrane and prior work suggests that these changes influence its mechanical properties. Without this information, it is difficult to interpret the results shown in Figures 2-4 fully.

Minor concerns

Lines 59 to 61 - Covalent crosslinks are not required for the collagen IV network to form - the NC1 dimers and 7s tetramers that underlie the interactions between col IV trimers can occur without them. The covalent crosslinking simply strengthens these interactions. Please rephrase.

Line 94 - Using the term "Collagen IV fibers" is confusing for members of the matrix community who do not work on flies. It is hard for them to conceive that a network-forming collagen can have such an organization. It is also not entirely accurate because the fibers also contain other basement membrane proteins (Isabella and Horne-Badovinac, 2016 - Figure 5K). I recommend calling them basement membrane fibers.

Line 122 - The Naba (2012) review is very general. Consider citing Randles et al., Matrix Biology (2017), as it focuses on the proteomics of basement membrane.
Line 137 - Typo (Col IV knockdowns not Col knockdowns)

Line 155 - Typo (Persists not persist)

Line 208 - Typo (were not was)
Line 212 - Typo (Indistinguishably not indistinguishable)
Line 267 - Typo (fibrillar not fibril)

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Line 274 and 280 - It's more specific to say "covalent cross-link". A "molecular cross-link" could include a protein like nidogen that links the collagen IV network to the laminin network

Reviewer 2

Advance Summary and Potential Significance to Field

This brief paper tells about a systematic testing of the four basement membrane molecules that are conserved between animals for their functioning in the Drosophila egg chamber. The fruit fly egg chamber is an established system where shape has been connected to the presence of basement membrane and its mechanical properties. These authors use RNAi against Collagen, Laminin, Nidogen and Perlecan and test the localization of the other molecules, then egg chamber shape, then resistance to osmotic stress and then directly with an AFM.

Comments for the author

A major criticism is that many of the results or conclusions have been published before. The paper acknowledges this by frequently writing "consistent with previous findings." In a rebuttal, the authors could provide a list of which things have been published and which are really novel, in the case that I am overlooking things.

The specific comparison of each mutant in each assay has value, especially with Atomic Force Microscope that is only rarely done. There is also interesting differences between the osmotic stress and AFM assays found. For instance, nidogen mutants are less stiff but they don't burst. Laminin mutants burst a bit faster but also are more stiff. To reconcile the findings, they could do AFM on the poles of the egg chambers, which they talk about are the main position of bursting.

Also they did not see differences in perlecan mutants, while Chlasta et al. 2017 did, which would correlate with the quick bursting. The paper should discuss this more.

They should also discuss why laminin mutants have stiffer basement membranes. This seems very unexpected. The paper says "slight increase" but the figure looks like 50% or more. Also they burst more quickly -why?

The staining in Figure 1 looks quite different from other published stains of Drosophila egg chambers with antibodies to basement membrane components. Those generally do not show cell outlines, or wrinkles. Some of these stains show small fibers, which the paper talks about at the end. Why do these stains look different?

It would be nice to display the data in 1WXYZ also by genotype, so we can see how the levels of each component quantitate in each mutant.

The paper should have a section in the discussion that discusses how the hierarchy of recruitment in the egg chamber compares to the hierarchy in the Drosophila embryo and fat tissue and to C. elegans.

After collagenase treatment, the follicle in S2L seems to change shape by lengthening, while the follicle in S2I does not. Why?

Small points:
Line 149: Fig1J, Z: Col4 does need Ndg (Col4 actually is affected by every other BM mutant)

Line 159: Laminin KD: Col4 upregulated, indicates a compensatory mechanism (rather than independence) between Lamin/Col4 networks?

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Reviewer 3

Advance Summary and Potential Significance to Field

This report from Töpfer et al. is fairly simple - the authors examine four of the major components of extracellular matrix and test interdependencies and a few mechanical properties after individual knockdowns by shRNA. The authors first use shRNA knockdowns of each of the four major ECM proteins (Laminin, perlecan, nidogen, collagen IV) to examine the intensities of each protein after antibody staining of the remaining ECM components. This was, for me, the most interesting part of the paper. Although a fair number of these combinations have been tested previously, the systematic testing is attractive. This does represent one of the major issues for the study - some of the major findings have been previously reported. It seemed that the collagen IV findings would be particularly expected by those familiar with the literature. Additionally, the major mechanical assays that are used have been previously reported (e.g., egg elongation in some of these backgrounds has been well-studied) - for this reason, I do not find the novelty of the study to be particularly high, although perhaps the systematic approach of testing these four components makes up for this to a degree. The systematic conclusions of protein-protein relationships are interesting; however, at times, they should be better qualified given questions about how deep the shRNA disruption is and how long proteins perdure are unclear (more on this below). The authors finish the work by examining how the individual genetic disruptions affect egg chamber elongation (a well-established assay), osmolarity resistance (how long it takes for egg chambers to burst in distilled water), and tissue stiffness (as measured by AFM). The paper is well-written, and statistics appear to be used appropriately.

Comments for the author

1) As mentioned above, the study relies exclusively on shRNA knockdown - the degree of knockdown at the level of protein intensities is usually sizable by stage 8, but it does make some of the negative conclusions tentative. These negative conclusions should be better qualified given that the knockdown is not complete, and how long proteins perdure is not clear (although intensities are assayed at Stage 8, we do not know if they've only reached that level in the last few hours of development, or if the knockdown has been fairly steady). Indeed, what is known about the rates of the individual protein's turnover? It would be nice to know how these protein intensities compare at an earlier stage. Second independent shRNAs are often used to establish specificity and show similarity of effects, though the observed phenotypes do appear to be as expected and the antibody stains suggest specificity.

2) Another issue is that the reported findings do not provide much clarity on how the relative changes in intensity are happening - do the authors think the intensity changes are driven by changes in expression or localization? Is more intracellular retention occurring in the various backgrounds, or extracellular diffusion? It wasn't clear to me how their intensity measurements would respond to the above scenarios.

3) The section of how ECM components affect elongation of the egg chamber seemed particularly repetitive with previous work. Perhaps this helps establish that the shRNA approach is replicating previously observed phenotypes, but it seemed there was little novel to this section.

4) The Introduction seemed a little superficial (perhaps understandable for a Report format), but it would be good to clarify which systems the described literature is from (appeared to be primarily Drosophila). It seemed that some of the ECM knowledge from tissue culture and vertebrate systems would be informative for this study.

5) Why is the y-axis in Fig. 1 from 0-2? It makes it hard to judge the relative differences because of how compressed the graph bars are. Also, what is the second control? The figure legends need more information. It would also be nicer if the bottom graphs were made longer along the y-axis, doesn't seem necessary for the graph to be the exact same size as the image panels. In general, it seemed that data graphs were often too compressed along the y-axis.

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Original submission

First decision letter

MS ID#: DEVELOP/2021/200456

MS TITLE: Distinct contributions of ECM proteins to basement membrane mechanical properties in Drosophila

AUTHORS: Uwe Töpfer, Karla Yanin Guerra Santillan, Elisabeth Fischer-Friedrich, and Christian Dahmann

I sincerely apologize for the long delay before coming back to you. I have now received all the referees reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

The overall evaluation is very positive and we would like to publish your manuscript in Development, provided that the referees' comments can be satisfactorily addressed. Please address the minor comments from Reviewer 3 regarding figures and diagrams to improve clarity of the work. In my opinion no further experiment is required.

Reviewer 1

Advance summary and potential significance to field

This report addresses the contribution of four ECM components to the mechanical properties of the basement membrane of the Drosophila egg chamber. Although the contribution of these ECM components for egg chamber elongation has been reported previously, additional mechanical properties of the basement membrane, including stiffness and strain at distinct regions of the egg chamber have not been described. The systematic comparative analysis of the functional contribution of each of the four components of the ECM to the mechanical properties of the egg chamber is new, important, and enables reliable comparison between the function of each of them.

Comments for the author

The opposite outcome of LanA KD in the pole relative to the central regions is interesting and deserves further analysis.
For example, the authors should address whether laminin distribution differs in the poles relative to the central region? In addition they should find out whether integrin, or actin distribution differs at the pole relative to the central regions in the LanA KD. Other than that, the work is clear, the results are clean and convincing.

Reviewer 2

Advance summary and potential significance to field

The authors have done an impressive job of addressing both my comments and those of the other reviewers. The new data that were added in revision further strengthen the novelty of the finings. This is really a nice piece of work.

Comments for the author

No more suggestions.

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Reviewer 3

Advance summary and potential significance to field

This paper is a revision of an earlier version of the manuscript submitted to Development. The authors have done a very thorough job at addressing the reviewersÂ' comments, providing additional experiments (including new AFM measurements at the posterior pole in addition to previous measurements in the middle of the egg chamber) and corrections in the text. The work is systematic, thoroughly controlled and provides a valuable investigation of the mechanical properties of the basement membrane in an intact organ, which will be of broad interest. As pointed out by the previous reviewers, some of the properties have been tested already by other groups for some of the components of the basement membrane, but I donÂ't see this as a problem: on the contrary, it provides valuable replication in the context of a systematic comparison of different mechanical properties for four ECM components by a single team.

Bénédicte Sanson

Comments for the author

I have only minor suggestions, listed below. These include i) to add a supplementary table to summarise the results of the different essays and to indicate the literature where results have been confirmed or occasionally differ, ii) to provide an additional graph in figure 4 with an anisotropy index to further analyse findings quantitatively.

- Fig 1 panels W-Z: the key below the graph is confusing as the different items are widely spaced out and it is not immediately clear that these are the key for all above graphs rather than labelling specific parts of the histograms. I would suggest grouping the 7 items together to make clear these are in sequence and perhaps add “key” in front of it. Also in panel Z, the data for col IValpha1dsRNA is missing – if intentional, please give a brief explanation of why in legend.
- Diagrams: overall, the diagrams all through the manuscript are very useful and pedagogical. I have one suggestion, however: the diagrams of stage 8 egg chambers are slightly confusing as these are showing a sagittal section rather than a surface view. Perhaps both views should be presented initially and after, a surface view would be more appropriate to highlight the surface follicular cells (the sagittal view shows the nurse cells underneath). For example, see figure S3 A. Also, here, or elsewhere, it would be useful to show in a diagram that the basal side of the follicular cells is facing outside while their apical side is facing the germline.
- Line 154: mislocalisation to lateral membrane: this seems more prevalent at tricellular vertices, which might simply reflect a greater space there compared to lateral bicellular junctions. The authors might want to add a brief comment.
- Line 158: I suggest starting a new paragraph for the conclusions to break up this first section of the results.
- P163: instead of the word “expression”, which suggests intracellular processes such as transcription perhaps the authors should use the mention “presence of” as in previous sentences, as it is not known at which level the interdependency is. Indeed, independency between ECM components could be either at the level of intracellular processes, e.g., transcriptional regulation, mRNA stability, protein trafficking, or extracellular mechanisms, e.g., protein stabilisation, anchorage, or concentration in the extracellular space.

The authors might want to comment on what mechanisms are more likely based on the literature.

- L174: what is the “inner” Laminin network (is there a “outer” one?): define briefly
- L205: nidogen egg chamber at stage 8 in panel G looks a bit pointy too- could there be a similar phenotype as in LanA ds RNA?
- L206: “2 independent lines for perlecan” this statement is ambiguous: do you mean two independent lines used in this work or one line for this work and another line in the Horne-Badovinac paper? If the latter this could be mentioned in the suggested supplementary summary table.
- Fig 3 C: individual data points should be shown on top of histograms as in all other graphs; corresponding legend: is this the total number of burst chambers after 1 hr? This should be specified in the legend, to help understanding the figure independently of the main text.
- L252: “make no or a minor”: add “contribution” for clarity.
- L254: comma missing after “major contribution”.

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- L256: “to more directly” – is it really more direct? I would rephrase mentioning "an alternative method" or perhaps better, "a quantitative measure of stiffness". All methods used in the paper probe the mechanical properties in an indirect manner, as a composite material is sampled rather the isolated matrix. And indeed this is the strength of this paper, to use three distinct methods which probe overlapping mechanical properties in an intact organism.
- L262: the authors might want to write “apparent” basement stiffness since they are indenting not only the matrix but the whole organ, so sampling a composite material. Hence the subsequent tests with collagenase to correlate presence of matrix with stiffness measures.
- L274: mention of “but see Chlasta et al 2017” – the text needs to be self-sufficient, so the authors need to indicate here whether their results are consistent or inconsistent with this citation. To help with these comparisons, I would encourage the authors to publish a table like the table provided for the referees in the rebuttal. This table could be presented in a supplementary figure 6, summarising the conclusions from the different essays in the paper, listing papers where some of these essays had been already performed and whether results are consistent or inconsistent. If inconsistent (I think this is the case for one AFM result), the sup figure legend could be used to indicate briefly why – e.g., use of different AFM parameters (as explained in previous rebuttal).
- L280: stiffness anisotropy: this is interesting and in addition to the graphs E, F in Fig4 presenting the stiffness in the middle versus the posterior pole of the egg chambers, I would encourage the authors to generate an additional graph showing an anisotropy index (for example, a simple ratio between values for the stiffnesses at the pole versus middle), which should demonstrate quantitatively that WT is anisotropic, LanA- even more anisotropic and Ndg and Coll more or less isotropic, which will help discussion and could be cited around L317.

First revision

Author response to reviewers' comments

Response to Reviews

We would like to thank the reviewers for their insightful and constructive comments that have helped us to improve our manuscript.

Summary of the major changes to the manuscript in response to the reviewers’ comments

1. We have added the **new panel B to Figure 3** –showing a stress-strain curve- to explain the physical basis of the bursting of egg chambers in response to osmotic stress: Egg chamber bursting is determined by the failure strain of the basement membrane.
2. We have added the **new panel A to Figure 4** -again showing the same stress-strain curve- to explain how basement membrane stiffness is calculated (based on the linear relationship (modulus) of stress and strain) and how this differs from the physical basis of bursting.
3. We have added the **new panel F to Figure 4** to show our results of measuring basement membrane stiffness at the pole region of egg chambers. We show for the first time that basement membrane stiffness in the pole region of egg chambers depends on Laminin and Collagen IV, but not on Perlecan and Nidogen. These data confirm that basement membrane stiffness does not straightforwardly relate to the response to osmotic shock. Our discussion of the strain-stress curve explains that the bursting of egg chambers (determined by failure strain) and basement membrane stiffness (determined by the linear relationship of stress and strain) reflect two different mechanical properties of the basement membrane.
4. We have added the **new Figure S2A-Z** to show that the knock-down of the four basement membrane components is swift (starting at stage 6) and persists at least through stage 10 of egg chamber development.
5. We have added the **new Figure S3A-U”** to show that the subcellular localizations of Collagen IV and Nidogen are not detectably altered when anyone of the other three basement membrane proteins is knocked-down. Interestingly, however, both Perlecan and Laminin mis-localize to the lateral cell surface in the *Col IV* knock-down. These data reveal a hitherto unknown role of Collagen IV in the trafficking or retention of Perlecan

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and Laminin proteins.

6. We have added the **new Figure S4A-F** to show that egg chamber rotation depends on Collagen IV, but not on Laminin, Nidogen or Perlecan. These data show that the changes in basement membrane composition and mechanical properties observed in the *Lan*, *Ndg*, and *Pcan* knock-downs cannot be accounted for by a lack of proper egg chamber rotation.

Point-by-point response to the reviewers' comments

Reviewer 1 Advance Summary and Potential Significance to Field:
The manuscript by Töpfer et al. takes a comprehensive approach to dissecting the contributions of the four major basement membrane proteins to the structure and mechanical properties of the basement membrane using the Drosophila egg chamber as a model. Although some of the results have been reported previously, the real strength of this paper lies in having a complete set of carefully performed experiments from one laboratory. This allows comparisons of the different manipulations used and results obtained in a way that is not possible when results are spread across multiple papers. Moreover, there are some surprising findings, such as the observation that egg chamber elongation and resistance to osmotic stress do not necessarily correlate with basement membrane stiffness. In this way, I think this work makes an important contribution to both the matrix and morphogenesis communities and that it is likely to be of interest to the broad readership of Development. The manuscript is also well written and clear in its presentation of both the results and their significance. I do, however, have a few suggestions to improve the impact of the work and to better clarify its relationship to the existing literature
that should be addressed prior to publication.

Reviewer 1 Comments for the Author:
Major concerns:

The observation that Pcan Levels are reduced under Col IV depletion has been previously shown in the egg chamber by two groups. Please cite Haigo and Bilder, 2011 (Figure S6D) and Isabella and Horne-Badovinac, 2015 (Figure 4) when discussing these results.
OUR RESPONSE: We have cited the two papers (lines 147,148).

The result showing that loss of Laminin does not affect final egg shape conflict with two reports in the literature - Frydman and Spradling, 2001 (Figure 6J) and Andersen and Horne-Badovinac, 2015 (Figure 2G). The laminin antibodies in Drosophila are not great and could be giving an incomplete readout of knock down levels. The authors should consider confirming the strength of their knockdown with another reporter, such as the FlyFos line for either LanA or LanB1. If the discrepancy remains, it should be discussed in the text.
OUR RESPONSE:
We now show ovarioles of control and *LanA* knock-down animals (Fig. S2A,B). These new data show the specificity of the used Laminin antibody and the strength of the knockdown. Laminin is still detected at early stages before the Gal4 driver used for the knock-down (*GR1-Gal4*) becomes active. Moreover, Laminin is also detected at later stages in stalk cells, in which the *GR1-Gal4* driver is not active.

The studies by Frydman and Spradling (using mosaic *LanA* mutant egg chambers) and Andersen and Horne-Badovinac (using the early active *tj-Gal4* driver to drive *LanA* knock-down) resulted in an early-stage decrease of LanA function (germarium stage onwards). By contrast, the *GR1-Gal4* driver that we have used only leads to *LanA* knock-down starting from stage 5. Thus, the discrepancy might be due to an early function of LanA that is required for the elongation of the egg chamber at a later stage. LanA plays important roles during early ovarian development, for example, during follicle stem cell proliferation (O'Reilly et al., 2008) and anterior-posterior polarity (Diaz de la Loza et al., 2017) that conceivably affect egg chamber elongation. We revised our manuscript as follows: "Previous work showed that reduction of *LanA* activity already during very early egg chamber development (earlier than in our experiments) results in hypo-elongated stage 14 egg chambers (Andersen and Horne- Badovinac, 2016; Frydman and Spradling, 2001), indicating an early function of *LanA* in egg chamber elongation" (lines 201-204).
One key piece of data that is missing from the paper is whether the RNAi conditions used affect egg chamber rotation. Rotation affects the structure of the basement membrane and prior work

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suggests that these changes influence its mechanical properties. Without this information, it is difficult to interpret the results shown in Figures 2-4 fully.
OUR RESPONSE: We have now measured the velocity of egg chamber rotation for controls and the four knock-downs and provide the data in the new Fig. S4. Our data show that velocity is comparable for controls and *Lan, Ndg,* and *Pcan* knock-downs, but significantly reduced in the *Col IV* knockdowns. A reduction of egg chamber rotation in *Col IVα2* mutants was previously reported (Haigo and Bilder, 2011). We conclude that "These data demonstrate that the changes in basement membrane composition and mechanical properties observed in the *Lan, Ndg,* and *Pcan* knock- downs cannot be accounted for by a lack of proper egg chamber rotation" (lines 221- 223).

Minor concerns

Lines 59 to 61 – Covalent crosslinks are not required for the collagen IV network to form – the NC1 dimers and 7s tetramers that underlie the interactions between col IV trimers can occur without them. The covalent crosslinking simply strengthens these interactions. Please rephrase.
OUR RESPONSE: We rephrased the sentence: "Collagen IV is composed of triple- stranded helical structures that form networks by covalent interactions strengthened through their terminal domains."

Line 94 – Using the term "Collagen IV fibers" is confusing for members of the matrix community who do not work on flies. It is hard for them to conceive that a network-forming collagen can have such an organization. It is also not entirely accurate because the fibers also contain other basement membrane proteins (Isabella and Horne-Badovinac, 2016 – Figure 5K). I recommend calling them basement membrane fibers.
OUR RESPONSE: We changed the wording to 'basement membrane fibers'.

Line 122 – The Naba (2012) review is very general. Consider citing Randles et al., Matrix Biology (2017), as it focuses on the proteomics of basement membrane.
OUR RESPONSE: We added the citation.

Line 137 – Typo (Col IV knockdowns not Col knockdowns)
OUR RESPONSE: Thank you. We corrected the typo.

Line 155 – Typo (Persists not persist)
OUR RESPONSE: Thank you. We corrected the typo.

Line 208 – Typo (were not was)
OUR RESPONSE: Thank you. We corrected the typo.

Line 212 – Typo (Indistinguishably not indistinguishable)
OUR RESPONSE: Thank you. We corrected the typo.

Line 267 – Typo (fibrillar not fibril)
OUR RESPONSE: Thank you. We corrected the typo.

Line 274 and 280 – It's more specific to say "covalent cross-link". A "molecular cross-link" could include a protein like nidogen that links the collagen IV network to the laminin network
OUR RESPONSE: We changed the wording to 'covalent cross-link'.

Reviewer 2 Advance Summary and Potential Significance to Field:
This brief paper tells about a systematic testing of the four basement membrane molecules that are conserved between animals for their functioning in the Drosophila egg chamber. The fruit fly egg chamber is an established system where shape has been connected to the presence of basement membrane and its mechanical properties. These authors use RNAi against Collagen, Laminin, Nidogen and Perlecan and test the localization of the other molecules, then egg chamber shape, then resistance to osmotic stress and then directly with an AFM.

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Reviewer 2 Comments for the Author:
A major criticism is that many of the results or conclusions have been published before. The paper acknowledges this by frequently writing “consistent with previous findings.” In a rebuttal, the authors could provide a list of which things have been published and which are really novel, in the case that I am overlooking things.

OUR RESPONSE:

	Dependency on			
	Laminin	Nidogen	Perlecan	Collagen IV
Basement membrane protein level	**This study**	**This study**	**This study**	**This study**
Protein subcellular localization	**This study**	**This study**	**This study**	**This study**
Egg chamber elongation	**This study (stage 1-14)**(Andersen and Horne- Badovinac, 2016) (only stage 14) (Frydman and Spradling, 2001) (only stage 14)	**This study (stage 1- 14) (**Dai et al., 2018) (only stage 14)	**This study (stage 1- 14)** (Isabella and Horne-Badovinac, 2015)	**This study (stage 1- 14)** (Crest et al., 2017; Haigo and Bilder, 2011; Isabella and Horne-Badovinac, 2015)
Resistance of egg chamber to osmotic stress	**This study**	**This study**	**This study**	**This study** (Crest et al., 2017)
Basement membrane stiffness at central region of egg chamber (AFM measurement)	**This study** (Diaz de la Loza et al., 2017) (using *LanA* hypomorphs that lead to small eggs and defects in oocyte positioning)	**This study**	**This study** (Chlasta et al., 2017)	**This study** (Chlasta et al., 2017; Crest et al., 2017)
Basement membrane stiffness at pole of egg chamber (AFM measurement)	**This study**	**This study**	**This study**	**This study**
Egg chamber rotation	**This study** (Diaz de la Loza et al., 2017) (using *LanA* hypomorphs that lead to small eggs and defects in oocyte positioning)	**This study**	**This study**	**This study** (Haigo and Bilder, 2011)

Table: Studies addressing the dependency of basement membrane protein level and localization, elongation, resistance to osmotic stress, basement membrane stiffness and rotation on Laminin, Nidogen, Perlecan or Collagen IV in *Drosophila* egg chambers.

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The specific comparison of each mutant in each assay has value, especially with Atomic Force Microscope that is only rarely done. There is also interesting differences between the osmotic stress and AFM assays found. For instance, nidogen mutants are less stiff but they don't burst. Laminin mutants burst a bit faster but also are more stiff. To reconcile the findings, they could do AFM on the poles of the egg chambers, which they talk about are the main position of bursting.
OUR RESPONSE: We have now performed AFM measurements at the poles of egg chambers from control and *Lan, Ndg, Pcan* and *Col IVα2* knock-down flies and provide this data in the new Fig. 4F. We show that basement membrane stiffness in the pole region is unaffected by knock-down of *Ndg* or *Pcan*. We further show that basement membrane stiffness is decreased by knockdown of *Lan* or *Col IVα2*. Thus, also by measuring stiffness at the poles, the differences between the response to osmotic stress and basement membrane stiffness persists (e.g. *Pcan* knock-down results in an increased percentage of burst egg chambers, but the stiffness at the pole region is unchanged). However, these differences can be easily explained on the ground of the known stress-strain relationship of collagen networks and the failure strain at which the network breaks. We have revised the manuscript as follows: "In general, the bursting of collagen networks is determined by the failure strain of the material, i.e. the amount of deformation at which the network breaks (Fig. 3B) (Roeder et al., 2002; Wang et al., 2002)" (lines 239-242)." And further "For example, the organ-swelling assay interrogates in particular the mechanical properties of the egg chambers at their poles, as suggested by frequent bursting at that location (Crest et al., 2017). The bursting frequency, however, does not correlate with the basement membrane stiffness in the pole region. Knock-down of *Pcan*, for example, resulted in a significantly increased frequency of bursting (Fig. 3C), yet the basement membrane stiffness at the pole region was comparable to controls (Fig. 4F). The bursting assay thus reflects the failure strain of the egg chamber material (see Fig. 3 B) rather than the basement membrane stiffness at the pole region. This is not surprising, as the failure strain and the stiffness often do not correlate positively (Bax et al., 2019; Haut, 1986; Leng et al., 2013)" (lines 305-315).

Also they did not see differences in perlecan mutants, while Chlasta et al. 2017 did, which would correlate with the quick bursting. The paper should discuss this more.
OUR RESPONSE: Chlasta et al. 2017 used a different AFM setup (e.g. max. force of indentation of 3nN versus 0.4 nN in our study; Cantilever stiffness of 0.03 - 0.12 N/m (Chlasta et al. 2017) versus 0.015-0.019 N/m in our study) **compared to our study and studies by (Chen et al., 2019; Crest et al., 2017). The measured basement membrane stiffness of control stage 8 egg chambers by Chlasta et al. 2017 is ~10 times higher compared to the value in our study or the studies by (Chen et al., 2019; Crest et al., 2017). Given these differences, we feel it is difficult to compare our data with the data of Chlasta et al. 2017.**

They should also discuss why laminin mutants have stiffer basement membranes. This seems very unexpected. The paper says "slight increase" but the figure looks like 50% or more. Also they burst more quickly –why?
OUR RESPONSE: We have now also measured basement membrane stiffness in the pole region of control and *LanA* knock-down egg chambers. Interestingly, we find that the stiffness is decreased in *LanA* knock-downs, thus leading to a higher stiffness anisotropy between the pole region and the central region of the egg chamber, which, as we show, relates to the hyper-elongation of the egg chamber. We have revised the manuscript as follows: "Interestingly, knock-down of *LanA* decreased basement membrane stiffness in the pole region (Fig. 4F), whereas in the central region stiffness was increased (see Fig. 4G). This increased stiffness anisotropy of the *LanA* knock- down correlated with the hyper-elongation of *LanA* knock-down egg chambers at stage 8 (Fig. 2F), consistent with the notion that the stiffness anisotropy drives egg chamber elongation (Crest et al., 2017)" (lines 286-291).

It is unclear to us why *LanA* knock-down results in a stiffer membrane (although it is plausible with respect to hyper-elongation, see above) and we therefore would like to refrain from speculation. Similarly, bursting depends on the failure strain (as discussed above), but it is unclear how *LanA* knock-down affects the failure strain.

The staining in Figure 1 looks quite different from other published stains of Drosophila egg chambers with antibodies to basement membrane components. Those generally do not show cell outlines, or wrinkles. Some of these stains show small fibers, which the paper talks about at the end. Why do these stains look different?

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OUR RESPONSE: To our knowledge, antibody stainings revealing Perlecan, Nidogen and Laminin in an entire egg chamber (and not only in a small area) have not been reported. For Collagen IV, most authors detect the GFP fluorescence of the Col IVα2- GFP line and do not fix the sample and perform antibody staining, as we did. Fixation and permeabilization, in our hands, inevitably lead to 'wrinkles' and cell outlines, when viewed at low magnification. We have revised the manuscript: "Note that slight convolutions of the egg chamber surface may be due to the long incubation time of fixation and permeabilization during the antibody staining." (lines 660-661).

It would be nice to display the data in 1WXYZ also by genotype, so we can see how the levels of each component quantitate in each mutant.
OUR RESPONSE: We compare the staining (fluorescence) intensities using one and the same primary antibody. We therefore prefer to plot the fluorescence intensity for a given antibody/ protein for the control and the different knock-downs. As we normalize the fluorescence intensity to the control, we could also, if requested, plot the data by genotype. This would, however, 'duplicate' the display of the data.

The paper should have a section in the discussion that discusses how the hierarchy of recruitment in the egg chamber compares to the hierarchy in the Drosophila embryo and fat tissue and to C. elegans.
OUR RESPONSE: We have mentioned the hierarchy of the recruitment of the ECM components in the Introduction (lines 64-67).

After collagenase treatment, the follicle in S2L seems to change shape by lengthening, while the follicle in S2I does not. Why?
OUR RESPONSE: The time-point after addition of collagenase at which the egg chambers lengthen varies in our hands from egg chamber to egg chamber (independent of the *LanA::GFP* or *Col IVα2::GFP* genotype). Thus, at the indicated incubation time of 60 min, some egg chambers have already elongated, whereas other have not. However, in all egg chambers, collagenase treatment results in a reduction of LanA::GFP or Col IVα2::GFP fluorescence intensity, as shown.

Small points:
Line 149: Fig1J, Z: Col4 does need Ndg (Col4 actually is affected by every other BM mutant) **OUR RESPONSE: We have rephrased the sentence: Second, Laminin expression, and to a lesser extent Collagen IV expression, requires the presence of Nidogen…".**

Line 159: Laminin KD: Col4 upregulated, indicates a compensatory mechanism (rather than independence) between Lamin/Col4 networks?
OUR RESPONSE: We report that *Lan* knock-down results in a slight decrease of Collagen IV (Fig. 1Z) and that *Col IV* knockdown results in Laminin upregulation (Fig. 1 W). We have revised the manuscript to indicate that there might be a compensatory mechanism: "Laminin protein level was decreased (reduction of staining intensity by ~40%) in the *Ndg* knock-down (Fig. 1K,W), was unaffected in the *Pcan* knock-down (Fig. 1O,W) and was slightly increased (by ~20-30%) in the *Col IVα1* or *Col IVα2* knock- downs (Fig. 1S,W), indicating a compensatory increase in Laminin when Collagen IV levels are reduced." (line 137-141).

Reviewer 3 Advance Summary and Potential Significance to Field:
This report from Töpfer et al. is fairly simple – the authors examine four of the major components of extracellular matrix and test interdependencies and a few mechanical properties after individual knockdowns by shRNA. The authors first use shRNA knockdowns of each of the four major ECM proteins (Laminin, perlecan, nidogen, collagen IV) to examine the intensities of each protein after antibody staining of the remaining ECM components. This was, for me, the most interesting part of the paper. Although a fair number of these combinations have been tested previously, the systematic testing is attractive. This does represent one of the major issues for the study – some of the major findings have been previously reported. It seemed that the collagen IV findings would be particularly expected by those familiar with the literature. Additionally, the major mechanical assays that are used have been previously reported (e.g., egg elongation in some of these backgrounds has been well-studied) – for this reason, I do not find the novelty of the study to be particularly high, although perhaps the systematic approach of testing these four components

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makes up for this to a degree. The systematic conclusions of protein-protein relationships are interesting; however, at times, they should be better qualified given questions about how deep the shRNA disruption is and how long proteins perdure are unclear (more on this below). The authors finish the work by examining how the individual genetic disruptions affect egg chamber elongation (a well-established assay), osmolarity resistance (how long it takes for egg chambers to burst in distilled water), and tissue stiffness (as measured by AFM). The paper is well-written, and statistics appear to be used appropriately.

Reviewer 3 Comments for the Author:
1) As mentioned above, the study relies exclusively on shRNA knockdown – the degree of knockdown at the level of protein intensities is usually sizable by stage 8, but it does make some of the negative conclusions tentative. These negative conclusions should be better qualified given that the knockdown is not complete, and how long proteins perdure is not clear (although intensities are assayed at Stage 8, we do not know if they've only reached that level in the last few hours of development, or if the knockdown has been fairly steady). Indeed, what is known about the rates of the individual protein's turnover? It would be nice to know how these protein intensities compare at an earlier stage. Second independent shRNAs are often used to establish specificity and show similarity of effects, though the observed phenotypes do appear to be as expected and the antibody stains suggest specificity.
OUR RESPONSE: First, we have quantified the protein levels at an earlier stage (stage 6) and at a later stage (stage 10) and provide these data in the new Fig. S2. We find that "Decreased protein levels were already detected at stage 6 and persisted at least through stage 10 (Fig. S2A-Z), indicating a swift and steady decrease of the targeted proteins" (lines 132-134). The observation that protein levels are already reduced at stage 6, which is approximately 11-22 hours after the activation of the *GR1-Gal4* line (driving hairpin RNA expression) at stage 3 (Spradling, 1993), indicates a rather fast (hours) basement membrane turnover, consistent with recent observations showing that the half-life of basement membrane components in the *Drosophila* embryo is ~7- 10 h (Matsubayashi et al., 2020).

Second, we had used two different RNAi lines to knock-down Collagen IV and two different RNAi lines to knock-down Laminins. We have now used a second independent RNAi line to knock-down Perlecan to test Perlecan's role during egg chamber elongation. The use of the second RNAi line corroborates our findings that Perlecan is required for egg chamber elongation (see Fig. 1 for reviewers below). We now indicated in the manuscript that two different RNAi lines were used (lines 206 and 348). For Nidogen, we tested two independent RNAi lines. However, only one showed an efficient decrease of Nidogen protein (Fig. 1X, Fig. S2L,X; and data not shown).
This line was also used in previous work (Khadilkar et al., 2020).

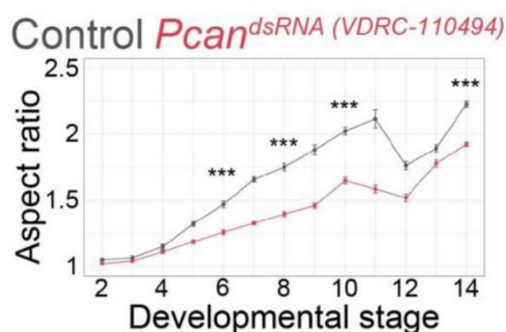

Figure 1 for reviewers. Perlecan is required for egg chamber elongation.
Aspect ratios of egg chambers as a function of developmental stage are shown for the control and knock-down using a second RNAi line for *Pcan*. *n*≥ 5 egg chambers per stage and genotype. Mean±sem are shown. Welsh two-sided t-test was used to compare egg chambers of the two different genotypes at a particular stage. *** p<0.001.

2) Another issue is that the reported findings do not provide much clarity on how the relative changes in intensity are happening – do the authors think the intensity changes are driven by changes in expression or localization? Is more intracellular retention occurring in the various backgrounds, or extracellular diffusion? It wasn't clear to me how their intensity measurements

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would respond to the above scenarios.
OUR RESPONSE: We have now clarified in the Materials and Methods that we measured intensity levels throughout the whole cells (from basal to apical), and not only at the basal surface (=basement membrane). "Image stacks covering the entire apical-basal extent of follicle cells were acquired with the same laser intensity settings and were projected by the maximum projection method. Mean fluorescence intensity of z-projections was measured in corresponding regions of egg chambers using Fiji" (line 366-369). Our intensity measurements are therefore not influenced by changes in the subcellular localization of the basement membrane protein.

In addition, we have now also systematically analyzed the subcellular localization of Laminin, Nidogen, Perlecan and Collagen IV in the four knock-down conditions and display these data in the new Fig. S3. We describe the results as follows: "The subcellular localizations of Collagen IV and Nidogen were not detectably altered when anyone of the other three basement membrane proteins was knocked-down (Fig. S3A, C-S",E-U"). The subcellular localization of Perlecan was also unchanged in the *Lan* or *Ndg* knock-downs (Fig. S3D-P"). Interestingly, however, Perlecan was mislocalized to the lateral cell membranes in the *Col IV* knock-down (Fig. S3T-T"). Similarly, Laminin was mislocalized to the lateral cell membranes in the *Col IV* knockdown (Fig. S3R-R''), but also in the *Pcan* knock-down (Fig. S3N-N"). Laminin localization was unaffected in the *Ndg* knock-down (Fig. 3J-J")" (lines 150-158). And further: "However, the partial mislocalization of Laminin in the *Col IV* or *Pcan* knock-down indicates a role for Collagen IV and Perlecan in Laminin trafficking or retention" (lines 168-170).

3) The section of how ECM components affect elongation of the egg chamber seemed particularly repetitive with previous work. Perhaps this helps establish that the shRNA approach is replicating previously observed phenotypes, but it seemed there was little novel to this section.
OUR RESPONSE: While we agree that our data on egg chamber elongation is, in part, repetitive with previous findings, we would like to point out that egg chamber elongation under conditions of decreased Laminin or Nidogen levels were previously only reported for stage 14 (Andersen and Horne-Badovinac, 2016; Dai et al., 2018; Frydman and Spradling, 2001). We have, on the other hand, analysed egg chamber elongation during the entire egg chamber development (stages 1 -14). Moreover, we have made the, we believe, interesting observation that *LanA* knock-down results in hyperelongated stage 8 egg chamber (Fig. 2F), which correlates with the increased stiffness anisotropy between the pole and central regions of the egg chamber (Fig. 4E,F). This further corroborates previous findings suggesting that stiffness anisotropy promotes egg chamber elongation (Crest et al., 2017).

The Introduction seemed a little superficial (perhaps understandable for a Report format), but it would be good to clarify which systems the described literature is from (appeared to be primarily Drosophila). It seemed that some of the ECM knowledge from tissue culture and vertebrate systems would be informative for this study.
OUR RESPONSE: Lines 48-70 of the Introduction summarize general knowledge of the ECM. The citations refer to work in *C. elegans*, *Drosophila*, mice, human genetic disease and tissue culture experiments. Given the space constraints of the Report format, as the reviewer mentions, we have, for the time being, refrained from extending the Introduction.

4) Why is the y-axis in Fig. 1 from 0-2? It makes it hard to judge the relative differences because of how compressed the graph bars are. Also, what is the second control? The figure legends need more information. It would also be nicer if the bottom graphs were made longer along the y-axis, doesn't seem necessary for the graph to be the exact same size as the image panels. In general, it seemed that data graphs were often too compressed along the y- axis.
OUR RESPONSE: We have revised Fig.1 W-Z. The y-axis is now adjusted to the maximum value of data points (0-1.8). The second control is a control where the staining has been performed in the absence of the primary antibody. This second control allows to measure the background fluorescence of the fluorescently-labelled secondary antibody. This intensity is then subtracted from the mean of fluorescence intensity to calculate the corrected mean fluorescence intensity. We have revised the Materials and Methods: "To calculate the corrected mean fluorescence intensity, the mean fluorescence intensity value was reduced by the mean fluorescence intensity value of 5 egg chambers treated with secondary antibody only" (lines 369-372).

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Second decision letter

MS ID#: DEVELOP/2021/200456

MS TITLE: Distinct contributions of ECM proteins to basement membrane mechanical properties in Drosophila

AUTHORS: Uwe Töpfer, Karla Yanin Guerra Santillan, Elisabeth Fischer-Friedrich, and Christian Dahmann
ARTICLE TYPE: Research Report

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I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.

Reviewer 3

Advance summary and potential significance to field

See my previous review.

Comments for the author

The reviewers have considered all the minor suggestions I had made and I am fully satisfied by their responses. They also have shortened the manuscript and added a diagrammatic summary (Fig. 4H) that is very helpful.

Second revision

Author response to reviewers' comments

We would like to thank the reviewers for their insightful and constructive comments that have helped us to improve our manuscript. We are pleased to see that all three reviewers evaluate our work positively.

On request by the Editor, we have shortened the manuscript to comply with the length limit of *Development*.

Point-by-point response to the comments by Reviewer 3

Reviewer 3 Comments for the Author:

-Fig 1 panels W-Z: the key below the graph is confusing as the different items are widely spaced out and it is not immediately clear that these are the key for all above graphs rather than labelling specific parts of the histograms. I would suggest grouping the 7 items together to make clear these are in sequence and perhaps add “key” in front of it. Also in panel Z, the data for col IValpha1dsRNA is missing – if intentional, please give a brief explanation of why in legend.
OUR RESPONSE: We have revised Fig. 1 as suggested by the reviewer. We have also revised the legend as follows: “(W-Z) Mean fluorescence intensity, normalized to controls, of the indicated antibody stainings of stage 8 egg chambers expressing no transgene (control) or the indicated transgene (key) under *GR1-Gal4*. In (Z), only the *Col IV*α*2*^{dsRNA}, but not the *Col IV*α*1*^{dsRNA} transgene was used.”

-Diagrams: overall, the diagrams all through the manuscript are very useful and pedagogical. I have one suggestion, however: the diagrams of stage 8 egg chambers are slightly confusing as these are showing a sagittal section rather than a surface view. Perhaps both views should be presented initially and after, a surface view would be more appropriate to highlight the surface follicular cells (the sagittal view shows the nurse cells underneath). For example, see figure S3 A. Also, here, or elsewhere, it would be useful to show in a diagram that the basal side of the follicular cells is facing outside while their apical side is facing the germline.
OUR RESPONSE: We now depict surface views of egg chambers in the diagrams throughout the manuscript.
To clarify the location of the basal side of follicle cells, we have enhanced the drawing of the basement membrane in the diagram of Fig. 1B.

-Line 154: mislocalisation to lateral membrane: this seems more prevalent at tricellular vertices, which might simply reflect a greater space there compared to lateral bicellular junctions. The authors might want to add a brief comment.

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OUR RESPONSE: We revised line 154 as follows: "Perlecan mislocalized to lateral cell membranes, in particular at tricellular vertices, in the *Col IV* knock-down (Fig. S3T- T")."

-Line 158: I suggest starting a new paragraph for the conclusions to break up this first section of the results.
OUR RESPONSE: We started a new paragraph.

-P163: instead of the word "expression", which suggests intracellular processes such as transcription, perhaps the authors should use the mention "presence of" as in previous sentences, as it is not known at which level the interdependency is. Indeed, independency between ECM components could be either at the level of intracellular processes, e.g., transcriptional regulation, mRNA stability, protein trafficking, or extracellular mechanisms, e.g., protein stabilisation, anchorage, or concentration in the extracellular space.
The authors might want to comment on what mechanisms are more likely based on the literature.
OUR RESPONSE: We rephrased the sentence as follows: "Second, the presence of Laminin, and to a lesser extent of Collagen IV, requires Nidogen, showing a mutual interdependency between Nidogen and Laminin proteins."

-L174: what is the "inner" Laminin network (is there a "outer" one?): define briefly
OUR RESPONSE: We deleted the word 'inner', as there is no outer laminin network.

-L205: nidogen egg chamber at stage 8 in panel G looks a bit pointy too- could there be a similar phenotype as in LanA ds RNA?
OUR RESPONSE: We do not observe that Nidogen knock-down leads to stage 8 egg chambers with a pointed pole region. These egg chambers have a less elongated shape as compared to controls (Fig. 2G).

-L206: "2 independent lines for perlecan" this statement is ambiguous: do you mean two independent lines used in this work or one line for this work and another line in the Horne-Badovinac paper? If the latter, this could be mentioned in the suggested supplementary summary table
OUR RESPONSE: We revised the sentence as follows: "Knock-down of *Pcan*, using in our work two independent RNAi lines,…"

-Fig 3 C: individual data points should be shown on top of histograms as in all other graphs; corresponding legend: is this the total number of burst chambers after 1 hr? This should be specified in the legend, to help understanding the figure independently of the main text.
OUR RESPONSE: The readout of the organ-swelling assay is either a burst or an intact egg chamber 1h after addition of distilled water. In Fig. 3C, we plot the ratio of burst to intact egg chambers in percentage. We have rephrased the figure legend as follows: "(C) Ratio in percentage of burst to intact stage 8 egg chambers 1 h after addition of distilled water for control and knock-downs as indicated. *n*>19 analyzed egg chambers for each genotype."

-L252: "make no or a minor": add "contribution" for clarity.
OUR RESPONSE: Done. Thank you.

-L254: comma missing after "major contribution".
OUR RESPONSE: Done. Thank you.

-L256: "to more directly" – is it really more direct? I would rephrase mentioning "an alternative method" or perhaps better, "a quantitative measure of stiffness". All methods used in the paper probe the mechanical properties in an indirect manner, as a composite material is sampled rather the isolated matrix. And indeed, this is the strength of this paper, to use three distinct methods which probe overlapping mechanical properties in an intact organism.
OUR RESPONSE: We rephrased the sentence as follows: "We finally analyzed the contribution of Laminin, Nidogen, Perlecan and Collagen IV to apparent basement membrane stiffness…".

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-L262: the authors might want to write “apparent” basement stiffness since they are indenting not only the matrix but the whole organ, so sampling a composite material. Hence the subsequent tests with collagenase to correlate presence of matrix with stiffness measures.
OUR RESPONSE: We added the word ‘apparent’.

-L274: mention of “but see Chlasta et al 2017” – the text needs to be self-sufficient, so the authors needto indicate here whether their results are consistent or inconsistent with this citation. To help with these comparisons, I would encourage the authors to publish a table like the table provided for the referees in the rebuttal. This table could be presented in a supplementary figure 6, summarising the conclusions from the different essays in the paper, listing papers where some of these essays had been already performed and whether results are consistent or inconsistent. If inconsistent (I think this is the case for one AFM result), the sup figure legend could be used to indicate briefly why – e.g., use of different AFM parameters (as explained in previous rebuttal).
OUR RESPONSE: We revised the sentence as follows: “(We note that (Chlasta et al., 2017), using a different AFM setup, reported a decreased basement membrane stiffness in *Pcan* knock-downs.)”
We mention publications showing data related to our findings throughout the text. A table comparing previous and our results would therefore be redundant with the text. We now provide a summary and comparison of the data shown in Figs 2-4 in thenew panel Fig. 4H.

-L280: stiffness anisotropy: this is interesting and in addition to the graphs E, F in Fig4 presenting the stiffness in the middle versus the posterior pole of the egg chambers, I would encourage the authors to generate an additional graph showing an anisotropy index (for example, a simple ratio between values for the stiffnesses at the pole versus middle), which should demonstrate quantitatively that WT is anisotropic, LanA- even more anisotropic and Ndg and Coll more or less isotropic, which will help discussion and could be cited around L317.
OUR RESPONSE: We have added the new panel ‘G’ to Fig. 4 showing the anisotropy index.

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