Matrix stiffness regulates Notch signaling activity in endothelial cells

Maibritt Kretschmer, Rose Mamistvalov, David Sprinzak, Angelika M. Vollmar and Stefan Zahler

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Reviewer 1

Evidence, reproducibility and clarity

In this manuscript, Kretschmer and colleagues investigate the role of matrix stiffness in Notch signaling using a series of gain and loss of function experiments (over-expression and inhibitors). As read-outs they use Notch reporter assays, FRAP, transferrin uptake, and immunofluorescence analyses. The authors conclude that softer substrates potentiate Notch signaling. While the questions are interesting and important, I am concerned with the use of inhibitors with off-target or unintended effects, as listed below. There is also some information missing from Materials and Methods which makes it difficult to assess the methodology and resulting conclusions.

Major Concerns

1. A major concern I have is with the use of DAPT to modulate Notch signaling, and investigate the impact on integrins, Yap, cadherins, etc. Gamma-secretase, the target of DAPT, cleaves not only Notch receptors, but also lntegrinB1, Nectins, Cadherins, Ephrins and more. This recent review lists 149 substrates (Guner & Lichtenthaler Seminars in Cell & Developmental Biology 2020). The risk that some of the results reflect DAPT impact on lntegrinB1, Cadherins etc themselves is significant. The authors should validate their findings with more specific modulation of Notch activity, for example with a Notch blocking antibody, with siRNA, or with SAHM1.

2. Furthermore, EGTA was used to "acutely destabilize VE-Cadherin". But EGTA chelates Calcium, which is essential for Notch structure, and EGTA is thus a well-known activator of Notch signaling (see eg Rand MD et al. (2000) Calcium depletion dissociates and activates heterodimeric notch receptors. Mol Cell Biol). The authors rightfully describe and cite this paper, but the use of EGTA nonetheless confounds interpretation. The authors check for NICO levels (at what timepoint?) but the staining is cytoplasmic (also not labelled in the figure per se, but described in the figure legend? - please label the staining in the panel). And in any case, NICO is very short-lived and nuclear staining cannot be taken as a hallmark of signaling activity. In particular if staining is performed at a time point at which the receptor and NICO may have been exhausted/depleted. The authors should validate these observations/conclusions with the Notch reporter to conclusively demonstrate whether EGTA does not activate Notch in their system.

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3. Trans-endocytosis of NECD on different substrates: the authors suggest that trans-endocytosis of NECD by D114 increases on softer substrates. But the authors also show that soft substrates lead to spreading out of cells, which could confound interpretation (is overlapping membranes, not internalization). The authors could validate trans-endocytosis by FAGS: check if red D114+ cells contain more NECD. It is also not clear to me in this experiment whether the authors are looking at green NECD, or Notch1 full length, since they write "overlap of Notch1 and D114", which would not reflect trans-endocytosis but interactions at the cell surface for both cells. Please also define "overlay intensity", or explain further.

4. The authors conclude their introduction with a statement that mechanosensitivity of Notch is linked to endocytosis, but their conclusion from Fig 6C was that Notch stiffness- dependence was independent of endocytosis, using the rhD114.. ?

Minor concerns

1. In the introduction, the authors describe D113 as a Notch ligand that activates Notch signaling in trans. To my knowledge, D113 has only been described as a cis-inhibitor of Notch signaling. {I think this may have arisen during repeated edits of the manuscript!)
2. In the introduction, the authors state that Notch1, D114 and Jag1 control angiogenesis, but then they only describe what Notch1/Dl14 do in the next few sentences. Perhaps one sentence to describe the role of Jag1 would help avoid the feeling of being "left hanging".
3. Data presentation: please show all bar graphs with the individual replicates (dotplots).
4. Data analysis/normalization: many graphs represent normalization of values in multiple steps which are not described in the methods/legends/results. For example, Notch reporter gene activity (Fig 1A) is Firefly divided by Renilla, and presumably nonmalized to the control condition at 1 (or an average of 1 for the three controls?). This is not explained. Also, it is not clear whether the data reported for the Control condition are Huvec on rhD114 compared (normalized) to Huvec on control substrate (and similar for each other condition). What controls are included in this experiment? Please provide the full data to provide insight into the magnitude of activation by D114 itself. Perhaps "Control" is without rhD114? But the bar underneath A/8 implies this rhD114 was used in all conditions.
5. Statistics: data should be presented as means +/- standard deviation, not standard error of the mean (see for example Barde & Barde Perspect Clin Res. 2012): "SEM quantifies uncertainty in estimate of the mean whereas SD indicates dispersion of the data from mean. As readers are generally interested in knowing the variability within sample, descriptive data should be precisely summarized with SD."
6. Statistics:
- a. In the Methods section, the authors state that one-way ANOVA was followed by Dunnett's multiple comparison test, and two-way ANOVA was followed by Tukey's multiple comparison test. Dunnell is used to compare every mean to a control mean, while Tukey is used to compare every mean with every other mean. Fig 1 describes using Dunnell for Fig 1B, but the end of the legend days Tukey was used. However Fig 1A,C show internal pairwise comparisons to plastic. Please be sure to explain which statistics were used where, and why, and if plastic was set as the comparator, please be explicit about this.
- b. Fig 3 uses "Sidak's corrected two-way ANOVA" and "Sidak's multiple comparison test"? I think Sidak is a method to correct alpha or p for multiple comparisons, as stated in the first instance, but it is described why this was used here, and not in other analyses, and whether the authors then applied Tukey's post-hoc test as described in the methods section? Similar comments for Fig 6.
- c. It is counter-intuitive that the plastic -1.SkPa PDMS difference with no error-bar overlap in 1A would be 1-star significance, while the plaslic-70kPa difference with almost overlapping error bars in 1B would be 4-star significance. Please check/show values.
- d. In Fig 1B Figure legend, the authors write "Data is presented in a bar plot and compared with the integrin 131 intensities without DAPT treatment", but this is not the statistical comparison presented.
- e. Fig 38 shows a very minor difference with overlapping error bars as 3-star significance? Is this correct?
7. How much nuclear NICO (NICO intensity) is there in control conditions? (Control missing from Fig 1B, D).
8. A DAPI counterstaining for 1B/D right panels would facilitate evaluation of whether NICO nuclear intensity is increased.The same applies for nuclear YAP assessment in Fig 38. I assume a nuclear

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counter-stain was done for quantification of nuclear NICO intensity, and nuclear YAP intensity, but this is not described in the Materials and Methods, please add a description of how intensity was quantified, and provide nuclear counterstain images. (Also, what is the unit on the y-axis of "intensity" graphs? Arbitrary units (a.u.)?
9. How much "overall" integrin 81 is there in DAPT-treated conditions in Fig 2C? (related to the concept that DAPT could be cleaving integrin B1, it could be depleted at 24 hours..?)
10.More details regarding the analysis procedure need to be added to the Methods Section. Were cells segmented and then mean intensity estimated for the whole cell? Was this done by means of Intensity Ratio Nuclei Cytoplasm Tool plugin for Fiji alone? Were images background corrected, corrected for inhomogeneous illumination, nonmalized? In the case of lntegrin beta 1 active, the expression seems to be patterned, was intensity expressed as mean intensity of every pixel corresponding to cytoplasm? For VE Cadherin staining, how was intensity estimated (only pixels corresponding to membrane were considered or every pixel of the cell)? Many figures are originated from a confocal microscope: were z-stacks acquired and then maximum projections done? Were z-stacks acquired and then fluorescence quantified in 30 images? Was a single plane acquired or analyzed, and if that is the case, how was this plane chosen?
11. In Fig 4A, how is VE-Cadherin intensity quantified? As an average per field of view? Or per cell? And if per cell, how was each cell delineated? And if not per cell, how were equal cell numbers ensured?
12. In FRAP experiment, how was intensity quantified? Was it per cell, per field of view or per region? Was each bleached region analyzed separately, or each cell? The datapoints should be either added to Figure 4C or as supplementary to assess the fitting. How many bleached regions per cell were done and how many cells were analyzed?
13. In FRAP experiment, was bleaching done with an increased pixel dwell time? Was laser intensity increased? Do you have an estimation of laser power (not percentage) or flux?
14. Figure S2 is not referenced in the manuscript - I think a reference to "FigureS3" in the NECD transendocytosis section (no page numbers or line numbering) should be to Fig S2 instead?
15. In Figure 5A NICO nuclear intensity normalized somehow (normalization not explained), and stiffness no longer appears to regulate NICO levels as shown in Figure 1B.
16. Fig 68: From the immuno at right there is a dear stiffness-dependent difference in Transferrin uptake. How were "single cell uptake" and "number of partides" quantified? (How were cell bodies identified?) Uptake could also be verified with FAGS.
17. Fig 6C: there appear to be very different numbers of cells in the brightfield image at right. Are the 70, 1.5, and 0.5 kPa Notch reporter activities different from one another or only different from plastic? Might these results reflect cell density/increased Notch signaling due to more cell-cell contacts?
18. How was the Dl14 coating of the different substrates done?
19. Il would be helpful to describe the composition of Collagen G (Collagen I) in the text (it is a risk to expect vendor information to remain available indefinitely).
20. Please list catalog numbers for all reagents, and dilutions used for antibodies.
21. Instead of using red and green for images, maybe cyan, yellow and/or magenta could be used to help the reader see what is being shown (especially if the reader might be color blind).
22. Packages and tools such as Intensity Ratio Nuclei Cytoplasm Tool plugin for FIJI should be referenced.
https://github.com/MontpellierRessourceslmagerie/imagej_macros_and_scripts/wiki/1ntensity-Ratio-Nuclei-Cytoplasm-Tool#how-to-cite-the-tool

Significance

The concept of how stiffness regulates Notch signaling is of timely interest. While the mechanobiology of Notch has attracted a fair amount of attention (publications), less is known of how stiffness impacts Notch signaling.

The work could be of interest to the Notch field, biomechanics, cell biology/adhesionexperts.
It could be relevant for designing cellular scaffolds for biological or medical applications.

The expertise of this reviewer is Notch and imaging.

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Reviewer 2

Evidence, reproducibility and clarity

Kretchmer et al. investigates the role of substrate stiffness on Notch signalling pathway.
They show increased Notch activity on softer substrates. Transendocytosis of NECD is suggested to be regulated by the substrate stiffness. They also conclude that the softer the substrate the more integrin beta 1 is activated.

Major comments:

Is there difference on a growth rate of cells on softer vrs stiffer gels that could affect cell morphology/signaling pathways?

Nuclear localization of NICO and YAP would be good to validate with western blot.

In Figure 3 and Figure 5, siRNA experiments would strengthen the data. DAPT is not only an inhibitor of Notch but affects to other proteins as well. This should be stated.

How was the mean VE-cadherin branch length determined?This term often refers to angiogenesis assay/sprout formation and maybe another one should be considered here to describe VE-cadherin junction morphology.

Add to all figure texts how many cells were used for the analyses.

In Fig. 6C the cell morphology of HUVECs look abnormal in comparison to other images and should be re-done.

Was all the data normally distributed and thus ANOVA was used? Please add more details on the statistics part. Did you remove outliers?

MTT assay of DAPT would need to be presented as it can be cytotoxic. Cells are not well visible in Fig 2C with DAPT. DAPI and F-actin staining would help to see the cell morphology.

Minor comments:

Please clarify how coating with rhDDL4 is done as this was unclear at least for this reviewer.
HUVECs are known to be hard to transfect. Please provide data on transfection efficiencies of all transiently transfected cells.

Significance

The paper is interesting for the researchers studying angiogenesis and also cancer as the matrix stiffness regulates cancer progression.

My expertise lies in understanding mechanisms of angiogenesis, endothelial cell function and crosstalk with other cell types of the vessel wall. My group also studies Hippo signaling and has vast experience on isolation, culturing and doing experiments on HUVECs and other types of endothelial cells.

Reviewer 3

Evidence, reproducibility and clarity

The authors use different cell culture conditions to alter stiffness (DPMS model) and to measure the effect on Notch signaling and potential upstream and downstream factors. The experiments suggest that softer stiffness leads to higher Notch signaling activity in cultured endothelial cells which had been further stimulated by the Notch ligand DLL4. The data suggest that beta1 integrin activity is

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promoted by Notch which supports previous findings by others. Also, there is a bidirectional interaction with VE-Cadherin also supporting previous findings.
This is a solid study using cultured cells only. The topic is of interest for researches investigating vascular biology, potentially also tumor vascular biology, ECM stiffness and its effect on signaling and Notch signaling per se.

Major comments:

- Are the key conclusions convincing? YES
- Should the authors qualify some of their claims as preliminary or speculative, or remove them altogether? NO
- Would additional experiments be essential to support the claims of the paper? YES, SEE below-
- Are the suggested experiments realistic in terms of time and resources? YES within about a six months' time period.
- Are the data and the methods presented in such a way that they can be reproduced? YES, however, more information is needed about cell density on the plates and the DLL4 expression level on the sender cells.
- Are the experiments adequately replicated and statistical analysis adequate? YES, however showing data points within the bar graphs would improve this study.

1. The authors use recombinant Dl14 or Dl14-expressing ("sender") cells to activate Notch in co-cultured cells. This is per se fine however, one might over-estimate all other observed downstream effects as endogenous Notch activity is lower. It would be important to see how na"ive HUVEC or other primary endothelial cells respond to changes in stiffness. qPCR of Notch target genes such as Hey1, Hey2, Hess, D114 is frequently used as a readout of Notch activity in this context. Also. the Notch transcriptional reporter assay might be a suitable read-out-
2. As the authors mention in the Discussion, cell density could be of utmost importance given the fact that Notch signaling usually is assumed as an in trans signaling event between adjacent cell membranes. However, also other signaling modes (in cis, cis inhibition, JAG1 vs DLL4 ratio) might be important. As such, the authors should carefully document an report on cell density in all experiments. Secondly, the authors should use other conditions such as sparse cell density and thirdly the authors should measure transcriptional effects of stiffness on Notch ligand expression.
3. The authors need to compare stiffness in their model with physiological conditions in developing tissues and ideally also in tumor which often have increased tissue stiffness.
4. Is Notch activation due to changes in stiffness dependent on the presence of ligands or could it be that (unspecific) binding of Notch receptors to ECM could trigger cleavage just by conformational change?

Significance

It was shown that Notch1 acts as a mechanosensor in endothelial cells. However, it is unclear how blood flow activates Notch1. Also, it is clear that stiffness influences blood vessel formation, which is under genetic control of Notch signaling. The importance of this study is to show that stiffness has a strong effect on Notch1 activation (maybe by increasing pulling force of ligands and subsequent endocytosis).

The major limitations of this study are:

1. work was only performed in cell culture, unclear whether there is any relevance in vivo
2. there is an artificial (over)-activation of endothelial Notch signaling by Dl14 expressing cells. Unclear whether this reflects physiological Notch signaling activity.
3. The mechanism how Notch1 gets activated remained elusive.

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Author response to reviewers' comments

1. General Statements

In our study, we demonstrate a mechanical effect on Notch signaling exerted by the stiffness of the extracellular matrix. All Reviewers agree that the manuscript is of interest for cell biologists, researchers from the Notch field, or from biomechanics with special relevance for angiogenesis and tumor biology. In the revised version of the manuscript, we have already addressed many suggestions of the reviewers. The required additional experiments can be performed within approx. six weeks.

2. Description of the planned revisions

Referee #1

1. A major concern I have is with the use of DAPT to modulate Notch signaling, and investigate the impact on integrins, Yap, cadherins, etc. Gamma-secretase, the target of DAPT, cleaves not only Notch receptors, but also IntegrinB1, Nectins, Cadherins, Ephrins and more. This recent review lists 149 substrates (Guner & Lichtenthaler Seminars in Cell & Developmental Biology 2020). The risk that some of the results reflect DAPT impact on IntegrinB1, Cadherins etc themselves is significant. The authors should validate their findings with more specific modulation of Notch activity, for example with a Notch blocking antibody, with siRNA, or with SAHM1.

We agree with the reviewer´s comment and will add additional key experiments using SAHM1 as alternative inhibitor of Notch activity.

2. Furthermore, EGTA was used to "acutely destabilize VE-Cadherin". But EGTA chelates Calcium, which is essential for Notch structure, and EGTA is thus a well-known activator of Notch signaling (see eg Rand MD et al. (2000) Calcium depletion dissociates and activates heterodimeric notch receptors. Mol Cell Biol). The authors rightfully describe and cite this paper, but the use of EGTA nonetheless confounds interpretation. The authors check for NICD levels (at what timepoint?) but the staining is cytoplasmic (also not labelled in the figure per se, but described in the figure legend? - please label the staining in the panel). And in any case, NICD is very short-lived and nuclear staining cannot be taken as a hallmark of signaling activity. In particular if staining is performed at a time point at which the receptor and NICD may have been exhausted/depleted. The authors should validate these observations/conclusions with the Notch reporter to conclusively demonstrate whether EGTA does not activate Notch in their system.

To test whether transient treatment with EGTA causes Notch activation we will repeat this experiment with Notch reporter activity as readout.

3. Trans-endocytosis of NECD on different substrates: the authors suggest that trans-endocytosis of NECD by Dll4 increases on softer substrates. But the authors also show that soft substrates lead to spreading out of cells, which could confound interpretation (is overlapping membranes, not internalization). The authors could validate trans-endocytosis by FACS: check if red Dll4+ cells contain more NECD. It is also not clear to me in this experiment whether the authors are looking at green NECD, or Notch1 full length, since they write "overlap of Notch1 and Dll4", which would not reflect trans-endocytosis but interactions at the cell surface for both cells. Please also define "overlay intensity", or explain further.

We will validate the trans-endocytosis by flow cytometry. In addition, we describe the procedure for microscopic analysis more clearly (methods section, p 4; results section, p 17-19)

Minor Concerns
7. How much nuclear NICD (NICD intensity) is there in control conditions? (Control missing from Fig 1B, D).
We will repeat the experiment and compare the NICD levels with those in non-activated cells on plastic.

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9. How much "overall" integrin B1 is there in DAPT-treated conditions in Fig 2C? (related to the concept that DAPT could be cleaving integrin B1, it could be depleted at 24 hours..?)

We will additionally add this experiment and validate the effect of Notch inhibition on the overall intergrin level by the alternative inhibitor SAHM1

Referee #2

Major comments:
Is there difference on a growth rate of cells on softer vrs stiffer gels that could affect cell morphology/signaling pathways?

This is an important point and we will perform additional respective experiments.

In Figure 3 and Figure 5, siRNA experiments would strengthen the data. DAPT is not only an inhibitor of Notch but affects to other proteins as well. This should be stated.

A similar point was raised by Reviewer#1 with the suggestion to use SAHM1 as an alternative to DAPT. As suggested, we will add these experiments.

MTT assay of DAPT would need to be presented as it can be cytotoxic. Cells are not well visible in Fig 2C with DAPT. DAPI and F-actin staining would help to see the cell morphology.

We will add respective data on cell viability after DAPT (and SAHM1) treatment in a revised version of the manuscript.

Referee #3:
Major comments:

1) The authors use recombinant Dll4 or Dll4-expressing ("sender") cells to activate Notch in co-cultured cells. This is per se fine however, one might over-estimate all other observed downstream effects as endogenous Notch activity is lower. It would be important to see how naïve HUVEC or other primary endothelial cells respond to changes in stiffness. qPCR of Notch target genes such as Hey1, Hey2, Hes5, Dll4 is frequently used as a readout of Notch activity in this context. Also. the Notch transcriptional reporter assay might be a suitable read-out-

In Fig.5A we show data on endogenous Notch activity (- EGTA) on substrates with different stiffness. In this case, NICD levels in the nucleus do not differ. It will definitely be interesting to repeat this experiment based on the reporter gene assay.

2) As the authors mention in the Discussion, cell density could be of utmost importance given the fact that Notch signaling usually is assumed as an in trans signaling event between adjacent cell membranes. However, also other signaling modes (in cis, cis inhibition, JAG1 vs DLL4 ratio) might be important. As such, the authors should carefully document an report on cell density in all experiments. Secondly, the authors should use other conditions such as sparse cell density and thirdly the authors should measure transcriptional effects of stiffness on Notch ligand expression.

In all experiments (with the exception of Fig. 6C) we used confluent cells. With the sparse cells (Fig. 6C) we also observe stiffness dependency. Investigating Notch ligand expression is definitely a good idea and will be investigated in the revised manuscript.

3. Description of the revisions that have already been incorporated in the transferred manuscript

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Referee #1

4. The authors conclude their introduction with a statement that mechanosensitivity of Notch is linked to endocytosis, but their conclusion from Fig 6C was that Notch stiffness-dependence was independent of endocytosis, using the rhDll4..?

We have now rephrased this sentence.

Minor concerns
1. In the introduction, the authors describe Dll3 as a Notch ligand that activates Notch signaling in trans. To my knowledge, Dll3 has only been described as a cis-inhibitor of Notch signaling. (I think this may have arisen during repeated edits of the manuscript!)

This has now been corrected in the current version.

2. In the introduction, the authors state that Notch1, Dll4 and Jag1 control angiogenesis, but then they only describe what Notch1/Dll4 do in the next few sentences. Perhaps one sentence to describe the role of Jag1 would help avoid the feeling of being "left hanging".

This has now been corrected in the current version.

3. Data presentation: please show all bar graphs with the individual replicates (dotplots).

We have now changed all bar graphs into scatter plots.

4. Data analysis/normalization: many graphs represent normalization of values in multiple steps which are not described in the methods/legends/results. For example, Notch reporter gene activity (Fig 1A) is Firefly divided by Renilla, and presumably normalized to the control condition at 1 (or an average of 1 for the three controls?). This is not explained. Also, it is not clear whether the data reported for the Control condition are Huvec on rhDll4 compared (normalized) to Huvec on control substrate (and similar for each other condition). What controls are included in this experiment? Please provide the full data to provide insight into the magnitude of activation by Dll4 itself. Perhaps "Control" is without rhDll4? But the bar underneath A/B implies this rhDll4 was used in all conditions.

We have edited our manuscript accordingly to avoid these ambiguities.

5. Statistics: data should be presented as means +/- standard deviation, not standard error of the mean (see for example Barde & Barde Perspect Clin Res. 2012): "SEM quantifies uncertainty in estimate of the mean whereas SD indicates dispersion of the data from mean. As readers are generally interested in knowing the variability within sample, descriptive data should be precisely summarized with SD."

We now use SD instead of SEM.

6. Statistics:
a. In the Methods section, the authors state that one-way ANOVA was followed by Dunnett's multiple comparison test, and two-way ANOVA was followed by Tukey's multiple comparison test. Dunnett is used to compare every mean to a control mean, while Tukey is used to compare every mean with every other mean. Fig 1 describes using Dunnett for Fig 1B, but the end of the legend days Tukey was used. However Fig 1A,C show internal pairwise comparisons to plastic. Please be sure to explain which statistics were used where, and why, and if plastic was set as the comparator, please be explicit about this.

b. Fig 3 uses "Sidak's corrected two-way ANOVA" and "Sidak's multiple comparison test"? I think Sidak is a method to correct alpha or p for multiple comparisons, as stated in the first instance, but it is described why this was used here, and not in other analyses, and whether the authors then applied Tukey's post-hoc test as described in the methods section? Similar comments for Fig 6.

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c. It is counter-intuitive that the plastic -1.5kPa PDMS difference with no error-bar overlap in 1A would be 1-star significance, while the plastic-70kPa difference with almost overlapping error bars in 1B would be 4-star significance. Please check/show values.

d. In Fig 1B Figure legend, the authors write "Data is presented in a bar plot and compared with the integrin β1 intensities without DAPT treatment", but this is not the statistical comparison presented.

e. Fig 3B shows a very minor difference with overlapping error bars as 3-star significance? Is this correct?

We have checked all statistical issues and corrected where necessary. Since the sample size and variance were homogenous in all comparisons, we now uniformly use ANOVA and Tukey´s multiple comparison test as post hoc to keep things simple.

10. More details regarding the analysis procedure need to be added to the Methods Section. Were cells segmented and then mean intensity estimated for the whole cell? Was this done by means of Intensity Ratio Nuclei Cytoplasm Tool plugin for Fiji alone? Were images background corrected, corrected for inhomogeneous illumination, normalized? In the case of Integrin beta 1 active, the expression seems to be patterned, was intensity expressed as mean intensity of every pixel corresponding to cytoplasm? For VE Cadherin staining, how was intensity estimated (only pixels corresponding to membrane were considered or every pixel of the cell)? Many figures are originated from a confocal microscope: were z-stacks acquired and then maximum projections done? Were z-stacks acquired and then fluorescence quantified in 3D images? Was a single plane acquired or analyzed, and if that is the case, how was this plane chosen?

The requested information has now been inserted in the respective results and method sections.

11. In Fig 4A, how is VE-Cadherin intensity quantified? As an average per field of view? Or per cell? And if per cell, how was each cell delineated? And if not per cell, how were equal cell numbers ensured?

12. In FRAP experiment, how was intensity quantified? Was it per cell, per field of view or per region? Was each bleached region analyzed separately, or each cell? The datapoints should be either added to Figure 4C or as supplementary to assess the fitting. How many bleached regions per cell were done and how many cells were analyzed?

13. In FRAP experiment, was bleaching done with an increased pixel dwell time? Was laser intensity increased? Do you have an estimation of laser power (not percentage) or flux?

These issues are now addressed in more detail in the respective figure legend.

14. Figure S2 is not referenced in the manuscript - I think a reference to "Figure S3" in the NECD transendocytosis section (no page numbers or line numbering) should be to Fig S2 instead?

Sorry for this mistake! We corrected this now.

15. In Figure 5A NICD nuclear intensity normalized somehow (normalization not explained), and stiffness no longer appears to regulate NICD levels as shown in Figure 1B.

We have now described the normalization better in the figure legend. The difference to the results in Fig. 1B is that in Fig. 5A the cells were not activated by Dll4 sender cells or rhDll4 (endogenous Notch activity). This is now stated more clearly.

17. Fig 6C: there appear to be very different numbers of cells in the brightfield image at right. Are the 70, 1.5, and 0.5 kPa Notch reporter activities different from one another or only different from plastic? Might these results reflect cell density/increased Notch signaling due to more cell-cell contacts?

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Unfortunately, with decreasing stiffness the PDMS gels become optically more and more cloudy, giving the false impression of a higher cell number. We tried to circumvent this by changing contrast and brightness of the images, but to no satisfying effect. We now mention this issue in the figure legend.

18. How was the Dll4 coating of the different substrates done?

The coating of the substrates is now described under a specific subheading in the Methods section.

19. It would be helpful to describe the composition of Collagen G (Collagen I) in the text (it is a risk to expect vendor information to remain available indefinitely).

The role and composition of the Collagen G coatings was included in the text (p 7). Further information on the manufacturer of the product used is included in the methods section.

20. Please list catalog numbers for all reagents, and dilutions used for antibodies.

We have added this information wherever possible.

22. Packages and tools such as Intensity Ratio Nuclei Cytoplasm Tool plugin for FIJI should be referenced.

We have now referenced respective tools.

Referee #2

How was the mean VE-cadherin branch length determined? This term often refers to angiogenesis assay/sprout formation and maybe another one should be considered here to describe VE-cadherin junction morphology.
Add to all figure texts how many cells were used for the analyses.

The cell number is now added wherever appropriate.

In Fig. 6C the cell morphology of HUVECs look abnormal in comparison to other images and should be re-done.

In contrast to all other experiments the cells where not confluent in this case. The different morphology is a sign of the lack of neighbours, not of some problem with the cells.

Was all the data normally distributed and thus ANOVA was used? Please add more details on the statistics part. Did you remove outliers?

Like also suggested by Reviewer #1 we have added more information on statistics and streamlined this. The data are normally distributed, outliers were not removed.

Minor comments:
Please clarify how coating with rhDDL4 is done as this was unclear at least for this reviewer.

The coating of the substrates is now described under a specific subheading in the Methods section.

HUVECs are known to be hard to transfect. Please provide data on transfection efficiencies of all transiently transfected cells.

We did not systematically monitor transfection efficiencies in this context, since there was always an internal control (e.g. co-reporter in the reporter gene assay) or the data were obtained on a single cell based quantification. Generally, we yield transfection efficiencies around 30% with HUVECs.

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Referee #3:

3) The authors need to compare stiffness in their model with physiological conditions in developing tissues and ideally also in tumor which often have increased tissue stiffness.

Good point! We have now integrated such comparisons in the Discussion.

4. Description of analyses that authors prefer not to carry out

Referee #1

Minor concerns

8. A DAPI counterstaining for 1B/D right panels would facilitate evaluation of whether NICD nuclear intensity is increased. The same applies for nuclear YAP assessment in Fig 3B. I assume a nuclear counter-stain was done for quantification of nuclear NICD intensity, and nuclear YAP intensity, but this is not described in the Materials and Methods, please add a description of how intensity was quantified, and provide nuclear counterstain images. (Also, what is the unit on the y-axis of "intensity" graphs? Arbitrary units (a.u.)?

The counterstaining method with Hoechst as well as the use of the nuclear staining for quantitative analysis of images are now described in the Methods section and where needed in the figure legends. The y-axis of the intensity graphs now has a dimension (a.u.). We decided against overlay of the nuclear staining with the NICD or YAP images for graphical reasons (visibility of the respective staining).

16. Fig 6B: From the immuno at right there is a clear stiffness-dependent difference in Transferrin uptake. How were "single cell uptake" and "number of particles" quantified? (How were cell bodies identified?) Uptake could also be verified with FACS.

In this point, we disagree with the reviewer: we really do not see a systematic difference in intensities between the different substrates. The process of image analysis is now better described in the figure legend. The result was so clear that we did not use FACS as complementary approach.

21. Instead of using red and green for images, maybe cyan, yellow and/or magenta could be used to help the reader see what is being shown (especially if the reader might be color blind).

We will of course adhere to the respective policy of the publishing journal, once the manuscript is accepted.

Referee #2
Nuclear localization of NICD and YAP would be good to validate with western blot.

Quantification of Western Blots (especially after nuclear isolation) is – at least in our hands – much less sensitive and reliable then quantitative imaging. Furthermore, it needs a lot of cell material we can hardly obtain in our experimental settings on the gels. We do not think that this experiment would significantly strengthen our study.

Referee #3:
4) Is Notch activation due to changes in stiffness dependent on the presence of ligands or could it be that (unspecific) binding of Notch receptors to ECM could trigger cleavage just by conformational change?

Since there is no stiffness dependent response on collagen (Fig. 6C, left panel), an effect of unspecific binding is highly unlikely.

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Original submission

First decision letter

MS ID#: JOCES/2022/260442

MS TITLE: Matrix stiffness regulates Notch signaling activity in endothelial cells

AUTHORS: Maibritt Kretschmer, Rose Mamistvalov, David Sprinzak, Angelika Maria Vollmar, and Stefan Zahler
ARTICLE TYPE: Research Article

Sorry to be slow in reviewing your submission; July is a complex month. I have reviewed your manuscript, the Review Commons transferred reviews, and your revision plan. I think your conclusions are of sufficient interest and your study is within the scope for JCS, though I agree with the reviewers that additional experiments and analyses are necessary. Your revision plan is logical and focuses on the major issues. I agree with your exclusions. I look forward to receiving your revised manuscript and will send it promptly to the original reviewers. Due to the large number of issues they raised, I cannot anticipate the outcome until I see their comments on the revised manuscript. However, I think your plan is good.

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

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

First revision

Author response to reviewers' comments

First, we would like to express our deep gratitude to the reviewers for thoroughly and fairly reviewing our work. All changes in text are highlighted in yellow in the markup copy of the manuscript. The manuscript has been adapted to the style of the Journal of Cell Science (e.g. the Abstract was shortened, and the sequence of sections was changed).

In the following we cite the concerns of the reviewers (bold italics) and respond to them accordingly.

First, we would like to express our deep gratitude to the reviewers for thoroughly and fairly reviewing our work. All changes in text are highlighted in yellow in the markup copy of the manuscript. The manuscript has been adapted to the style of the Journal of Cell Science (e.g. the Abstract was shortened, and the sequence of sections was changed).
In the following we cite the concerns of the reviewers (bold italics) and respond to them accordingly.

Reviewer #1:

Major Concerns

1. A major concern I have is with the use of DAPT to modulate Notch signaling, and investigate the impact on integrins, Yap, cadherins, etc. Gamma-secretase, the target of DAPT, cleaves not only Notch receptors, but also IntegrinB1, Nectins, Cadherins, Ephrins and more. This recent review lists 149 substrates (Guner & Lichtenthaler Seminars in Cell & Developmental Biology 2020). The risk that some of the results reflect DAPT impact on

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IntegrinB1, Cadherins etc themselves is significant. The authors should validate their findings with more specific modulation of Notch activity, for example with a Notch blocking antibody, with siRNA, or with SAHM1.

We agree with the reviewer´s comment. Since HUVECs are very hard to silence by siRNA without impairing overall cellular fitness, we decided to repeat key experiments (integrin activation, Fig. 2B, YAP activity, Fig. 3C, endogenous Notch activity, Fig. 5D) using SAHM1 as alternative inhibitor of Notch activity. SAHM1 in all cases corroborated our previous findings with DAPT, which is quite reassuring in the light of possible unspecific effects of single inhibitiors.

2. Furthermore, EGTA was used to "acutely destabilize VE-Cadherin". But EGTA chelates Calcium, which is essential for Notch structure, and EGTA is thus a well-known activator of Notch signaling (see eg Rand MD et al. (2000) Calcium depletion dissociates and activates heterodimeric notch receptors. Mol Cell Biol). The authors rightfully describe and cite this paper, but the use of EGTA nonetheless confounds interpretation. The authors check for NICD levels (at what timepoint?) but the staining is cytoplasmic (also not labelled in the figure per se, but described in the figure legend?
- please label the staining in the panel). And in any case, NICD is very short-lived and nuclear staining cannot be taken as a hallmark of signaling activity. In particular if staining is performed at a time point at which the receptor and NICD may have been exhausted/depleted. The authors should validate these observations/conclusions with the Notch reporter to conclusively demonstrate whether EGTA does not activate Notch in their system.

We have to admit that the data on blocking cell-cell contacts were presented in a misleading way in the previous version of the manuscript. These data were obtained in a setting where no further stimulation (by sender cells or rhDll4 coating) was induced. Under these conditions, it is quite trivial that cell-cell contacts are crucial for maintaining the basal Notch signalling, and that loosening these by EGTA and/or a blocking antibody reduce Notch signalling irrespective of stiffness. Since this is quite obvious, we have cancelled the respective sub-heading in the Results section and tuned down and clarified the conclusion in the Results and Discussion section.

We would have liked to follow the reviewer´s suggestion to use the reporter gene assay to repeat this experiment. Unfortunately, HUVECs are quite sensitive after transfection, and did not tolerate treatment with EGTA in this case. Since the Notch reporter gene assay and nuclear localization of NICD correlated quite well in our other experiments (Fig. 1B to E), we think that the latter is still sufficient to demonstrate the obvious.
Incubation with EGTA was for 30 min, after removal of EGTA, cells were treated with the VE-cadherin blocking antibody for further 30 min before NICD staining (exhaustion/depletion should not play a role after this short time). The treatment conditions are now stated in the legend to Figure 5.

3. Trans-endocytosis of NECD on different substrates: the authors suggest that trans-endocytosis of NECD by Dll4 increases on softer substrates. But the authors also show that soft substrates lead to spreading out of cells, which could confound interpretation (is overlapping membranes, not internalization). The authors could validate trans-endocytosis by FACS: check if red Dll4+ cells contain more NECD. It is also not clear to me in this experiment whether the authors are looking at green NECD, or Notch1 full length, since they write "overlap of Notch1 and Dll4", which would not reflect trans-endocytosis but interactions at the cell surface for both cells. Please also define "overlay intensity", or explain further.

We have now repeated the trans-endocytosis by flow cytometry. The outcome was comparable to the result from microscopic analysis (new Figure 6B). In addition, we describe the procedure for microscopic analysis more clearly, and state that the plamid encodes for NECD of Notch1 (methods section, p 12 and 13; results section, p 6, legend for Figure 6)

4. The authors conclude their introduction with a statement that mechanosensitivity of Notch is linked to endocytosis, but their conclusion from Fig 6C was that Notch stiffness-dependence was independent of endocytosis, using the rhDll4..?

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We have now rephrased this sentence.

Minor concerns
1. In the introduction, the authors describe Dll3 as a Notch ligand that activates Notch signaling in trans. To my knowledge, Dll3 has only been described as a cis-inhibitor of Notch signaling. (I think this may have arisen during repeated edits of the manuscript!)

This has now been corrected in the current version.

2. In the introduction, the authors state that Notch1, Dll4 and Jag1 control angiogenesis, but then they only describe what Notch1/Dll4 do in the next few sentences. Perhaps one sentence to describe the role of Jag1 would help avoid the feeling of being "left hanging".

This has now been corrected in the current version.

3. Data presentation: please show all bar graphs with the individual replicates (dotplots).
We have now changed all bar graphs into scatter plots.

4. Data analysis/normalization: many graphs represent normalization of values in multiple steps which are not described in the methods/legends/results. For example, Notch reporter gene activity (Fig 1A) is Firefly divided by Renilla, and presumably normalized to the control condition at 1 (or an average of 1 for the three controls?). This is not explained. Also, it is not clear whether the data reported for the Control condition are Huvec on rhDll4 compared (normalized) to Huvec on control substrate (and similar for each other condition). What controls are included in this experiment? Please provide the full data to provide insight into the magnitude of activation by Dll4 itself. Perhaps "Control" is without rhDll4? But the bar underneath A/B implies this rhDll4 was used in all conditions.

We have edited our manuscript accordingly to avoid these ambiguities. The term “control” is now clearly defined in the figure legends.

5. Statistics: data should be presented as means +/- standard deviation, not standard error of the mean (see for example Barde & Barde Perspect Clin Res. 2012): "SEM quantifies uncertainty in estimate of the mean whereas SD indicates dispersion of the data from mean. As readers are generally interested in knowing the variability within sample, descriptive data should be precisely summarized with SD."
We now use SD instead of SEM.

6. Statistics:

a. In the Methods section, the authors state that one-way ANOVA was followed by Dunnett's multiple comparison test, and two-way ANOVA was followed by Tukey's multiple comparison test. Dunnett is used to compare every mean to a control mean, while Tukey is used to compare every mean with every other mean. Fig 1 describes using Dunnett for Fig 1B, but the end of the legend days Tukey was used. However Fig 1A,C show internal pairwise comparisons to plastic. Please be sure to explain which statistics were used where, and why, and if plastic was set as the comparator, please be explicit about this.

b. Fig 3 uses "Sidak's corrected two-way ANOVA" and "Sidak's multiple comparison test"? I think Sidak is a method to correct alpha or p for multiple comparisons, as stated in the first instance, but it is described why this was used here, and not in other analyses, and whether the authors then applied Tukey's post-hoc test as described in the methods section? Similar comments for Fig 6.

c. It is counter-intuitive that the plastic -1.5kPa PDMS difference with no error-bar overlap in 1A would be 1-star significance, while the plastic-70kPa difference with almost overlapping error bars in 1B would be 4-star significance. Please check/show values.

d. In Fig 1B Figure legend, the authors write "Data is presented in a bar plot and compared with the integrin β1 intensities without DAPT treatment", but this is not the statistical

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comparison presented.

e. Fig 3B shows a very minor difference with overlapping error bars as 3-star significance? Is this correct?

We have checked all statistical issues and corrected where necessary. Since the sample size and variance were homogenous in all comparisons we now uniformly use ANOVA and Tukey´s multiple comparison test as post hoc to keep things simple.

7. How much nuclear NICD (NICD intensity) is there in control conditions? (Control missing from Fig 1B, D).

The missing respective controls have now been added to Figure 1D and E.

8. A DAPI counterstaining for 1B/D right panels would facilitate evaluation of whether NICD nuclear intensity is increased. The same applies for nuclear YAP assessment in Fig 3B. I assume a nuclear counter-stain was done for quantification of nuclear NICD intensity, and nuclear YAP intensity, but this is not described in the Materials and Methods, please add a description of how intensity was quantified, and provide nuclear counterstain images. (Also, what is the unit on the y- axis of "intensity" graphs? Arbitrary units (a.u.)?

The counterstaining method with Hoechst as well as the use of the nuclear staining for quantitative analysis of images are now described in the Methods section and where needed in the figure legends. The y-axis of the intensity graphs now has a dimension (a.u.). We decided against overlay of the nuclear staining with the NICD or YAP images for graphical reasons (visibility of the respective staining).

9. How much "overall" integrin B1 is there in DAPT-treated conditions in Fig 2C? (related to the concept that DAPT could be cleaving integrin B1, it could be depleted at 24 hours..?)

We have now validated the absence of an effect of Noch inhibition (DATP or SAHM1) on the overall intergrin level by the alternative inhibitor SAHM1 (new Figure 2A).

10. More details regarding the analysis procedure need to be added to the Methods Section. Were cells segmented and then mean intensity estimated for the whole cell? Was this done by means of Intensity Ratio Nuclei Cytoplasm Tool plugin for Fiji alone? Were images background corrected, corrected for inhomogeneous illumination, normalized? In the case of Integrin beta 1 active, the expression seems to be patterned, was intensity expressed as mean intensity of every pixel corresponding to cytoplasm? For VE Cadherin staining, how was intensity estimated (only pixels corresponding to membrane were considered or every pixel of the cell)? Many figures are originated from a confocal microscope: were z-stacks acquired and then maximum projections done? Were z-stacks acquired and then fluorescence quantified in 3D images? Was a single plane acquired or analyzed, and if that is the case, how was this plane chosen?

The requested information has now been inserted in the respective results and method sections.

11. In Fig 4A, how is VE-Cadherin intensity quantified? As an average per field of view? Or per cell? And if per cell, how was each cell delineated? And if not per cell, how were equal cell numbers ensured?

12. In FRAP experiment, how was intensity quantified? Was it per cell, per field of view or per region? Was each bleached region analyzed separately, or each cell? The datapoints should be either added to Figure 4C or as supplementary to assess the fitting. How many bleached regions per cell were done and how many cells were analyzed?

13. In FRAP experiment, was bleaching done with an increased pixel dwell time? Was laser intensity increased? Do you have an estimation of laser power (not percentage) or flux?

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11 -13: These issues are now described to the best of our knowledge in more detail in the respective figure legend.

14. Figure S2 is not referenced in the manuscript - I think a reference to "Figure S3" in the NECD transendocytosis section (no page numbers or line numbering) should be to Fig S2 instead?

Sorry for this mistake! We corrected this now.

15. In Figure 5A NICD nuclear intensity normalized somehow (normalization not explained), and stiffness no longer appears to regulate NICD levels as shown in Figure 1B.

We have now described the normalization better in the figure legend. The difference to the results in Fig. 1B is that in Fig. 5A the cells were not activated by Dll4 sender cells or rhDll4 (endogenous Notch activity). This is now stated more clearly.

16. Fig 6B: From the immuno at right there is a clear stiffness-dependent difference in Transferrin uptake. How were "single cell uptake" and "number of particles" quantified? (How were cell bodies identified?) Uptake could also be verified with FACS.

In this point, we disagree with the reviewer: we really do not see a systematic difference in intensities between the different substrates. The process of image analysis is now better described in the figure legend to the new supplementary figure S4. We have noe selected representative images better matching with the outcome of quantitative analysis. The result was so clear that we did not use FACS as complementary approach.

17. Fig 6C: there appear to be very different numbers of cells in the brightfield image at right. Are the 70, 1.5, and 0.5 kPa Notch reporter activities different from one another or only different from plastic? Might these results reflect cell density/increased Notch signaling due to more cell-cell contacts?

Unfortunately, with decreasing stiffness the PDMS gels become optically more and more cloudy, giving the false impression of a higher cell number. We tried to circumvent this by changing contrast and brightness of the images, but to no satisfying effect. We now mention this issue in the figure legend.

18. How was the Dll4 coating of the different substrates done?

The coating of the substrates is now described under a specific subheading in the Methods section.

19. It would be helpful to describe the composition of Collagen G (Collagen I) in the text (it is a risk to expect vendor information to remain available indefinitely).

The composition of the Collagen G coatings was included in the text (p 11). Further information on the manufacturer of the product used is included in the methods section.

20. Please list catalog numbers for all reagents, and dilutions used for antibodies.

We have added this information wherever possible.

21. Instead of using red and green for images, maybe cyan, yellow and/or magenta could be used to help the reader see what is being shown (especially if the reader might be color blind).

The Journal of Cell Science requests RGB images.

22. Packages and tools such as Intensity Ratio Nuclei Cytoplasm Tool plugin for FIJI should be referenced.

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We have now referenced respective tools.

Reviewer #2:

Major comments:

Is there difference on a growth rate of cells on softer vrs stiffer gels that could affect cell morphology/signaling pathways?

This is an important point and the respective results have now been added in the new Supplementary Figure S1A.

Nuclear localization of NICD and YAP would be good to validate with western blot.

Quantification of Western Blots (especially after nuclear isolation) is – at least in our hands – much less sensitive and reliable then quantitative imaging. We do not think that this experiment would strengthen our study.
In Figure 3 and Figure 5, siRNA experiments would strengthen the data. DAPT is not only an inhibitor of Notch but affects to other proteins as well. This should be stated.

A similar point was raised by Reviewer#1 with the suggestion to use SAHM1 as an alternative to DAPT. As suggested, we have done these experiments, but see the same outcome like with DAPT (Figures 2, 3 and 5).

How was the mean VE-cadherin branch length determined? This term often refers to angiogenesis assay/sprout formation and maybe another one should be considered here to describe VE-cadherin junction morphology.

Add to all figure texts how many cells were used for the analyses.

The determination of VE cadherin mean junction length is now better described in the legend to Figure 5. The cell number is now added wherever appropriate.

In Fig. 6C the cell morphology of HUVECs look abnormal in comparison to other images and should be re-done.

In contrast to all other experiments the cells where not confluent in this case. The different morphology is a sign of the lack of neighbours, not of some problem with the cells.

Was all the data normally distributed and thus ANOVA was used? Please add more details on the statistics part. Did you remove outliers?

Like also suggested by Reviewer #1 we have added more information on statistics and streamlined this. The data are normally distributed, outliers were not removed.

MTT assay of DAPT would need to be presented as it can be cytotoxic. Cells are not well visible in Fig 2C with DAPT. DAPI and F-actin staining would help to see the cell morphology.

We have now added respective data on cell viability after DAPT (and SAHM1) treatment in Supplementary Figure 1D and E of the revised version of the manuscript.

Minor comments:

Please clarify how coating with rhDDL4 is done as this was unclear at least for this reviewer.

The coating of the substrates is now described under a specific subheading in the Methods section.

HUVECs are known to be hard to transfect. Please provide data on transfection efficiencies of all transiently transfected cells.

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We did not systematically monitor transfection efficiencies in this context, since there was always an internal control (e.g. co-reporter in the reporter gene assay) or the data were obtained on a single cell based quantification. Generally, we yield transfection efficiencies around 30% with HUVECs.

Reviewer #3:

Major comments:

1) The authors use recombinant Dll4 or Dll4-expressing ("sender") cells to activate Notch in co- cultured cells. This is per se fine however, one might over-estimate all other observed downstream effects as endogenous Notch activity is lower. It would be important to see how naïve HUVEC or other primary endothelial cells respond to changes in stiffness. qPCR of Notch target genes such as Hey1, Hey2, Hes5, Dll4 is frequently used as a readout of Notch activity in this context. Also. the Notch transcriptional reporter assay might be a suitable read-out-

In Fig.1A we now show data on endogenous Notch activity (reporter gene assay) on substrates with different stiffness. There is some stiffness dependence, but, like the reviewer suggested, to a lower degree that with additional stimulation.

2) As the authors mention in the Discussion, cell density could be of utmost importance given the fact that Notch signaling usually is assumed as an in trans signaling event between adjacent cell membranes. However, also other signaling modes (in cis, cis inhibition, JAG1 vs DLL4 ratio) might be important. As such, the authors should carefully document an report on cell density in all experiments. Secondly, the authors should use other conditions such as sparse cell density and thirdly the authors should measure transcriptional effects of stiffness on Notch ligand expression.

In all experiments (with the exception of Fig. 6C) we used confluent cells. With the sparse cells (Fig. 6C) we also observe stiffness dependency upon stimulation with Dll4. We now investigated Notch ligand expression (Dll4, Jag 1) as well as Hey1 expression by quantitative PCR (Supplementary Figure S1C).

3) The authors need to compare stiffness in their model with physiological conditions in developing tissues and ideally also in tumor which often have increased tissue stiffness.

Good point! We have now integrated such comparisons in the Discussion.

4) Is Notch activation due to changes in stiffness dependent on the presence of ligands or could it be that (unspecific) binding of Notch receptors to ECM could trigger cleavage just by conformational change?

Since there is no stiffness dependent response on collagen (Fig. 6C, left panel), an effect of unspecific binding is highly unlikely.

Second decision letter

MS ID#: JOCES/2022/260442

MS TITLE: Matrix stiffness regulates Notch signaling activity in endothelial cells

AUTHORS: Maibritt Kretschmer, Rose Mamistvalov, David Sprinzak, Angelika Maria Vollmar, and Stefan Zahler
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

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

As you will see, the reviewers gave favourable reports but raised some critical points that will require amendments to your manuscript. I hope that you will be able to carry these out because I would like to be able to accept your paper, depending on further comments from reviewers.

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

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

Reviewer 1

Advance summary and potential significance to field

I am overall satisfied with the revisions with one exception (see below).

The authors provide important new insights into the impact environmental stiffness (matrix, in this case) has on Notch signaling. This is relative to a wide variety of fields from developmental biology to cancer to therapeutics.

Comments for the author

I had pointed out that figures should be color-blind friendly. The authors replied that JCS requests RGB pictures. The authors may have misunderstood what RGB means.
This is simply a color mode distinction for file types (generally RGB is more suitable for print display and CMYK is more suitable for printing). However figures can be made color-blind friendly in RGB mode.

Most notably, JCS does encourage color-blind friendly formatting:
"We strongly encourage the use of colours that are suitable for colour-blind readers, particularly in the preparation of fluorescent microscopy images. Most notably, we discourage the use of red/green for the display of 2-channel images; authors should consider an alternative colour combination (e.g. magenta/green)."
(https://journals.biologists.com/jcs/pages/manuscript-prep).

Only two figure subpanels are red-green, and could quite easily be reformatted to be accessible to a broader readership.

Reviewer 2

Advance summary and potential significance to field

The paper is interesting for the researchers studying angiogenesis and also cancer as the matrix stiffness regulates cancer progression.

Comments for the author

Major comments:
1. If any type of quantitation is performed, the figure text should include number of wells or cells per group and number of independent experiments performed. This information is missing from some figures.

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2. Validation of nuclear localization of NICD and YAP with another method such as western blot would strengthen the data as it is performed from much higher number of cells than image analysis. Here the data contains only >240 cells. Validation with western blot should be done or clearly state the limitation of the chosen method.

3. Please provide data on cytotoxicity or apoptosis assay on the effect of reporter plasmid transfection in HUVECs (Fig6C). When HUVECs are grown this sparse they start to die.

4. Authors estimated that only 30% of HUVECs are transfected, how does this affect to your data interpretation? Transfection percentage should be added to the manuscript. Also, limitation of using plasmid transfection as a method with HUVECs should be discussed in the manuscript. This is the reason why virus vectors are used with primary endothelial cells and not plasmids.

Second revision

Author response to reviewers' comments

First, we would like to express our deep gratitude to the reviewers for thoroughly and fairly reviewing our work. All changes in text are highlighted in green in the markup copy of the manuscript.

In the following we cite the concerns of the reviewers (bold italics) and respond to them accordingly.

Reviewer 1:

I had pointed out that figures should be color-blind friendly. The authors replied that JCS requests RGB pictures. The authors may have misunderstood what RGB means. This is simply a color mode distinction for file types (generally RGB is more suitable for print display and CMYK is more suitable for printing). However, figures can be made color-blind friendly in RGB mode.
Most notably, JCS does encourage color-blind friendly formatting: "We strongly encourage the use of colours that are suitable for colour-blind readers, particularly in the preparation of fluorescent microscopy images. Most notably, we discourage the use of red/green for the display of 2-channel images; authors should consider an alternative colour combination (e.g. magenta/green)." (https://journals.biologists.com/jcs/pages/manuscript-prep).
Only two figure subpanels are red-green, and could quite easily be reformatted to be accessible to a broader readership.

We are very sorry for our previous misunderstanding! We have now changed Figures 1 and 6, as well as Supplementary Figure S4, accordingly to green/magenta.

Reviewer 2:

Major comments:

1. If any type of quantitation is performed, the figure text should include number of wells or cells per group and number of independent experiments performed. This information is missing from some figures.

We have now completed the missing information in the Figure legends.

2. Validation of nuclear localization of NICD and YAP with another method such as western blot would strengthen the data as it is performed from much higher number of cells than image analysis. Here the data contains only >240 cells. Validation with western blot should be done or clearly state the limitation of the chosen method.

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We think that the optical analysis of over 240 cells has sufficient statistical power to back up our conclusions. The translocation of NICD also correlates very well with the results from the Notch-reporter gene assay and the qPCR for Notch target genes. So we do not think that the use of a further bulk method (especially since it includes cellular fractionation) is necessary to strengthen our findings. We now clearly state the limitation of our methods in the manuscript.

3. Please provide data on cytotoxicity or apoptosis assay on the effect of reporter plasmid transfection in HUVECs (Fig6C). When HUVECs are grown this sparse they start to die.

The reporter gene assay we used is based on the dual luciferase system. This means that the reporter gene (firefly) is transfected along with a co-reporter (renilla). Variations in transfection efficiency from experiment to experiment are thus compensated, as well as putative changes in cell viability or biosynthetic capacity. This is now stated clearly in all respective parts of the manuscript.
Furthermore, transfected cells are detached after 24h and then reseeded. Cells damaged by the transfection procedure will not re-attach, and so only viable cells are left at the beginning of the experiment. The cell density used here is approximately the same we routinely use for proliferation assays with HUVECs. Cell death due to a lack of cell-cell contacts is only observed, when HUVECs are kept as single cells for a prolonged period of time.

4. Authors estimated that only 30% of HUVECs are transfected, how does this affect to your data interpretation? Transfection percentage should be added to the manuscript. Also, limitation of using plasmid transfection as a method with HUVECs should be discussed in the manuscript. This is the reason why virus vectors are used with primary endothelial cells and not plasmids.

As explained above, transfection efficiency is not limiting to our experiments. In the dual luciferase assays the transfection efficiency is normalized, and in the imaging assays a sufficient number of transfected cells was to be identified for quantitative analysis. We would like to point out that while viral vectors are an alternative, transfection of HUVECs with plasmids is still a widely used, accepted and state-of-the-art approach.

Third decision letter

MS ID#: JOCES/2022/260442

MS TITLE: Matrix stiffness regulates Notch signaling activity in endothelial cells

AUTHORS: Maibritt Kretschmer, Rose Mamistvalov, David Sprinzak, Angelika Maria Vollmar, and Stefan Zahler
ARTICLE TYPE: Research Article

Thank you for sending your manuscript to Journal of Cell Science through Review Commons.

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

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