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DLC1 is a direct target of activated YAP/TAZ that drives collective migration and sprouting angiogenesis

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Original submission

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MS ID#: JOCES/2019/239947

MS TITLE: DLC1 is a direct target of activated YAP/TAZ that drives collective migration and sprouting angiogenesis

AUTHORS: Miesje van der Stoel, Lilian Schimmel, Kalim Nawaz, Anne-Marieke van Stalborch, Annett de Haan, Alexandra Klaus-Bergmann, Erik T. Valent, Duco S. Koenis, Geerten P. van Nieuw Amerongen, Carlie J. de Vries, Vivian de Waard, Martijn Gloerich, Jaap D. van Buul, and Stephan Huveneers
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

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

As you will see, the reviewers share enthusiasm for the concept and conclusions of the study. Reviewer 1 is essentially supportive of publication already. Reviewer 2 raises a number of substantial criticisms that prevent me from accepting the paper at this stage. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

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

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

Reviewer 1

Advance summary and potential significance to field

This paper identifies the Rho GAP, DLC1 (ARHGAP7) as a direct transcriptional target of YAP and TAZ, which is required for YAP/TAZ dependent endothelial cell migration and angiogenesis in vitro. Specifically, they identify DLC1 as a regulator of focal adhesion remodeling. This paper contributes a substantial advance to our understanding of collective endothelial cell behavior, and the manuscript is exciting, well-conducted, clearly written, thoroughly controlled, and expansive. This work fills a critical gap in the field, identifying DLC1 as a new effector of YAP/TAZ-mediated feedback control of focal adhesions and cytoskeletal tension.

The authors show direct evidence that DLC1 is a YAP/TAZ-TEAD transcriptional target, that DLC1 is mechanoresponsive, and requires YAP/TAZ-TEAD. They then show that DLC1 depletion prolongs focal adhesion lifetime by reducing FA disassembly, which in turn impairs cell migration and angiogenesis. Finally, and importantly, ectopic DLC1 expression rescues the defects caused by YAP/TAZ depletion.

Comments for the author

It has been many years since I've been able to write this as a review, but this paper should be published as-is without further rounds of review or additional experimentation. The work presented is thorough, and requests for additional experiments would be gratuitous and merely slow the pace of scientific discovery.

some minor misspellings and computer corruptions of greek letters were noted. These can be corrected at the proof stage.

The authors are to be congratulated on a beautiful paper.

Reviewer 2

Advance summary and potential significance to field

The authors demonstrate that depletion of DLC1, whether through RNA interference of YAP or DLC1 itself, negatively affect cells ability to migrate, polarize, and disassemble focal adhesions. Furthermore, they provide convincing evidence that inhibited YAP/TAZ signaling (or depleted DLC1) negatively inhibits angiogenesis in early sprouting stages in spheroids. DLC1 rescue experiments further show recovery of proper collective cell migratory behavior and angiogenic sprouting. The experiments are well-controlled with detailed statistical analysis providing compelling evidence that DLC1 is indeed a modulator of endothelial cell collective cell migration and angiogenesis. The results will be of interest in the fields of Hippo signaling and migration, and these results further provide useful insight in how upstream YAP/TAZ modulate downstream transcriptional targets.

Comments for the author

The manuscript is well-written, concise, and clear, but several issues should be addressed.

1. In Fig. 1d, HEK cells are used to confirm if TEAD binding motif-mutated variants perturbed transcriptional activation of the DLC1 promoter. Given that the majority of this report focuses on HUVEC cells, it would useful to confirm that transfection in HUVECs also shows the same result, in order to eliminate any concern over differences in mechanosensitivity between cell types. Additionally more biologically meaningful ways to express the difference in promoter activity could perhaps be provided, given the small difference in median values of 0.06 and 0.04 relative activity.

2. In Fig. 1g, it would be useful and consistent to show a Western Blot of Yap1 protein level, as shown for Figs 1h,i, as opposed to a decrease in phosphorylated Yap to show activation. Additionally interesting that the DLC1 relative protein level fold change from the YAP-5SA

treatment between the two stiffness conditions remained the same. This should be discussed further, especially in relation to Fig1 a-b. There seems to be no amplification of the YAP signal downstream to DLC1 depending on stiffness

3. In Fig. 2a, there is no quantification of the shRNA used. A figure, such as those shown throughout Fig. 1, would be useful to validate that there is a deep knockdown. Although qualitatively it seems that shDLC1 #1063 and #1064 result in deep knockdown, quantification could be useful for the reader to appreciate the later effects of DLC1 depletion in the scratch assays.

4. The authors report that DLC1 is required for both cell orientation and directional migration shown in Fig. 3. The scratch assays show that DLC1 depletion does prevent "wound" closure in the in vitro model. However, the assays are limited to only pursuing a single phenotype, which makes the question on how important the role of DLC1 in establishing leader cells is. Variations of the scratch assay might add confidence; for example, use a competition assay in which two HUVEC populations are mixed together (i.e., cells with shDLC1 and those with normal DLC1 levels). The authors should confirm that a lack of DLC1 hinders the development of the "leader" cell phenotype. If indeed DLC1 is important, then one should see a cell arrangement (mediated by migration to the frontline) in which the leader alignment consists of DLC1+ cells.

5. In Fig. 3a-c and 5a-c (and further supported by Supplemental Video 2 for shDLC1 #1063), it seems that even with DLC1 and YAP perturbations, there is substantial closure after 12 hours. Could this imply that there are plausible additional compensation mechanisms observed upon DLC1 depletion that may lead to the ~67% wound closing in scratch assays? Fig. 5a also shows decent closure even with shYAP. Is there still nuclear localization of YAP in the leader cells, which could be indicative of a minimal threshold of YAP signaling required to maintain orientation and migration?

In addition, quantification is needed for the differences between the two siDCL1 conditions. There are significant differences between directionality and migration even among the two KD cases in Fig 3e. How much more knockdown does the 3' UTR provided than targeting the gene itself? If not a lot more, is it because a threshold is reached to stop directional migration? Perhaps use a gradient level knockdown to confirm proportionality between knockdown and its effects. Also, discuss further why FA align more in KD.

6. For Fig. 3a-c and 5a-c, given that the Hippo signaling pathway is affected by cell density, are there any phenotypic changes in the leader alignment of cells after the scratch occurs? (e.g. Do they switch from Hippo ON to Hippo OFF?)

7. Minor comments:
- Several of the blot images in the main figures seem too bleached. Specifically, Fig. 1a, 1e 1h, 1i, and 3c (all these crops are mostly on controls).
- The "-" label in Figs. 1g-i is a bit vague, and it would be useful for the authors to specifically state what these conditions are (i.e., starved).
- Heat map has 2 red color values along the scale which makes interpreting it more difficult than needed. The authors should try to have one color per unique frame with no overlapping colors
- Fig 4 caption says that plasmid 1065 is used even though earlier it says 1063 and 1064 pool were used

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First revision

Author response to reviewers' comments

Reviewer 1

Advance Summary and Potential Significance to Field: This paper identifies the Rho GAP, DLC1 (ARHGAP7) as a direct transcriptional target of YAP and TAZ, which is required for YAP/TAZ dependent endothelial cell migration and angiogenesis in vitro. Specifically, they identify DLC1 as a regulator of focal adhesion remodeling. This paper contributes a substantial advance to our

understanding of collective endothelial cell behavior, and the manuscript is exciting, well-conducted, clearly written, thoroughly controlled, and expansive. This work fills a critical gap in the field, identifying DLC1 as a new effector of YAP/TAZ-mediated feedback control of focal adhesions and cytoskeletal tension.

The authors show direct evidence that DLC1 is a YAP/TAZ-TEAD transcriptional target, that DLC1 is mechanoresponsive, and requires YAP/TAZ-TEAD. They then show that DLC1 depletion prolongs focal adhesion lifetime by reducing FA disassembly, which in turn impairs cell migration and angiogenesis. Finally, and importantly, ectopic DLC1 expression rescues the defects caused by YAP/TAZ depletion.

Comments for the Author: It has been many years since I've been able to write this as a review, but this paper should be published as-is without further rounds of review or additional experimentation. The work presented is thorough, and requests for additional experiments would be gratuitous and merely slow the pace of scientific discovery.

Some minor misspellings and computer corruptions of greek letters were noted. These can be corrected at the proof stage. The authors are to be congratulated on a beautiful paper.

We sincerely thank the reviewer for the recommendation to publish our manuscript, and we especially appreciate the very kind supportive words in the evaluation. The manuscript is now corrected for misspellings. We could not find the corrupted greek letters the reviewer refers to, we would be grateful if the reviewer or editors could point out to us where the fonts do not display correctly.

Reviewer 2

Advance Summary and Potential Significance to Field: The authors demonstrate that depletion of DLC1, whether through RNA interference of YAP or DLC1 itself, negatively affect cells' ability to migrate, polarize, and disassemble focal adhesions. Furthermore, they provide convincing evidence that inhibited YAP/TAZ signaling (or depleted DLC1) negatively inhibits angiogenesis in early sprouting stages in spheroids. DLC1 rescue experiments further show recovery of proper collective cell migratory behavior and angiogenic sprouting. The experiments are well-controlled with detailed statistical analysis, providing compelling evidence that DLC1 is indeed a modulator of endothelial cell collective cell migration and angiogenesis. The results will be of interest in the fields of Hippo signaling and migration, and these results further provide useful insight in how upstream YAP/TAZ modulate downstream transcriptional targets.

Comments for the Author: The manuscript is well-written, concise, and clear, but several issues should be addressed.

We sincerely thank the reviewer for the positive evaluation and for providing valuable feedback. We have addressed the remaining issues the reviewer raised as follows:

1. In Fig. 1d, HEK cells are used to confirm if TEAD binding motif-mutated variants perturbed transcriptional activation of the DLC1 promoter. Given that the majority of this report focuses on HUVEC cells, it would useful to confirm that transfection in HUVECs also shows the same result, in order to eliminate any concern over differences in mechanosensitivity between cell types. Additionally, more biologically meaningful ways to express the difference in promoter activity could perhaps be provided, given the small difference in median values of 0.06 and 0.04 relative activity.

Throughout the manuscript we have been working with primary endothelial cells that were genetically modified using lentiviral-based transductions. For the analysis of TEAD binding motif-mutated promoter regions we had to make use of a transient expression plasmid system. Unfortunately, the transfection efficiencies of these plasmids in primary endothelial cells are very low (1000 fold lower than in HEKs). Hence we turned to use HEK cells instead to provide proof-of-principle that the TEAD motif is involved controlling activity of the DLC1 promoter region. We have added a comment in the

corresponding results section of the manuscript to explain our rationale.

In the previous version of our manuscript the promoter activity was plotted as the ratio between firefly and renilla luciferase signal to correct for transfection differences, which is indeed not a direct biological value. In the revised manuscript we related the luciferase signal of the TEAD-mutated promoter region to the wild type promoter activity. DLC1 promoter activity is reduced by ~45% upon mutating the TEAD motif (Fig. 1d).

2. In Fig. 1g, it would be useful and consistent to show a Western Blot of Yap1 protein level, as shown for Figs 1h,i, as opposed to a decrease in phosphorylated Yap to show activation. Additionally, interesting that the DLC1 relative protein level fold change from the YAP-5SA treatment between the two stiffness conditions remained the same. This should be discussed further, especially in relation to Fig1 a-b. There seems to be no amplification of the YAP signal downstream to DLC1 depending on stiffness.
To include the total YAP1 expression levels in the VEGF stimulation experiments we have performed new western blot analysis that are now included in this revised manuscript (Fig. 1g). These data show that VEGF stimulation promotes YAP1 activation and induces DLC1 expression, but does not change total YAP1 levels. Our experiments using constitutive active YAP-5SA shows that increased YAP activation is sufficient to drive DLC1 expression irrespective of the stiffness of the substrate. Indeed the data indicate that there is no amplification of the active YAP driven DLC1 expression once the cells are cultured on soft vs. stiff substrates. This indicates that constitutive activation of YAP overrules the substrate-mediated regulation of DLC1 expression. We added this notion to the discussion of the revised manuscript.

3. In Fig. 2a, there is no quantification of the shRNA used. A figure, such as those shown throughout Fig. 1, would be useful to validate that there is a deep knockdown. Although qualitatively it seems that shDLC1 #1063 and #1064 result in deep knockdown, quantification could be useful for the reader to appreciate the later effects of DLC1 depletion in the scratch assays.
We now quantified the protein expression of the shRNAs used and added the results in Fig. 2a. We observed a significant knockdown of DLC1 using the shRNAs #1063, #1064, and #1065.

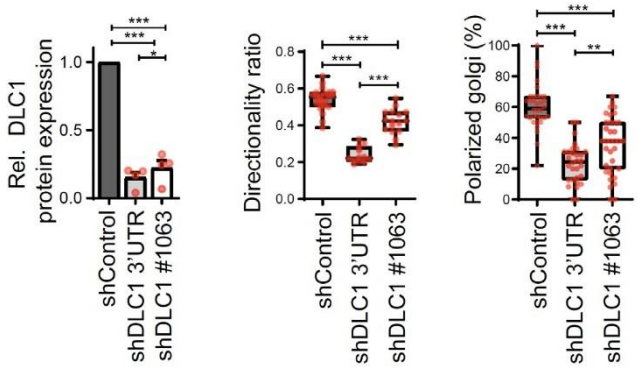
4. The authors report that DLC1 is required for both cell orientation and directional migration, shown in Fig. 3. The scratch assays show that DLC1 depletion does prevent "wound" closure in the in vitro model. However, the assays are limited to only pursuing a single phenotype, which makes the question on how important the role of DLC1 in establishing leader cells is. Variations of the scratch assay might add confidence; for example, use a competition assay in which two HUVEC populations are mixed together (i.e., cells with shDLC1 and those with normal DLC1 levels). The authors should confirm that a lack of DLC1 hinders the development of the "leader" cell phenotype. If indeed DLC1 is important, then one should see a cell arrangement (mediated by migration to the frontline) in which the leader alignment consists of DLC1+ cells.
We thank the reviewer for introducing this very interesting idea. To address this experimentally we generated mosaic endothelial monolayers in which half of the population of cells were expressing shControl (RFP labeled) or shDLC1 (GFP labeled). Next scratch assays were performed and the identity of the leader cells during collective migration was determined. The experiments demonstrated that the leading front is predominantly formed by cells that still express DLC1, whereas DLC1-depleted cells fail to lead during the collective cell migration process (Fig. 3g and Suppl. Movie 3). These results strengthen the conclusion that DLC1 is needed to coordinate collective cell migration.

5. In Fig. 3a-c and 5a-c (and further supported by Supplemental Video 2 for shDLC1 #1063), it seems that even with DLC1 and YAP perturbations, there is substantial closure after 12 hours. Could this imply that there are plausible additional compensation mechanisms observed upon DLC1 depletion that may lead to the ~67% wound closing in scratch assays? Fig. 5a also shows decent closure even with shYAP. Is there still nuclear localization of YAP in the leader cells, which could be indicative of a minimal threshold of YAP signaling required to maintain orientation and migration?

Indeed we think that compensatory mechanisms explain the partial inhibition of scratch wound migration in these experiments. Specifically in endothelial cells the transcriptional co-activator TAZ, the homologue of YAP, is crucial for scratch wound migration, which would compensate the silencing of YAP in scratch assays, as described previously by Neto et al (eLife, 2018). As this is an interesting notion for readers we have now elaborated our discussion to highlight that in addition to YAP, TAZ might serve a strong role in collective endothelial migration as well.

In addition, quantification is needed for the differences between the two siDCL1 conditions. There are significant differences between directionality and migration even among the two KD cases in Fig 3e. How much more knockdown does the 3' UTR provided than targeting the gene itself? If not a lot more, is it because a threshold is reached to stop directional migration? Perhaps use a gradient level knockdown to confirm proportionality between knockdown and its effects. Also, discuss further why FA align more in KD.

To address these comments of the reviewer, we analyzed and quantified the level of knockdown achieved by shDLC1 3'UTR and #1063 in lysates of cells used in the scratch wound migration assays. Even though both shRNA provide a very robust level of knockdown of DLC1 compared to shControl cells, there is a slight significant difference between these two different DLC1 short hairpins. There is a higher knockdown efficiency of the 3'UTR (87.8% knockdown) compared to the #1063 (77.7% knockdown)(Figure for reviewer). Therefore difference in directionality and migration between 3'UTR and #1063 might be caused by a difference in knock down efficiency. Importantly, both knockdown strategies inhibited persistency and migration efficiency compare to shControl cells.



Regarding the finding that focal adhesions align more in the absence of DLC1 we can only speculate. The finding that focal adhesions align more in the absence of DLC1 during collective migration correlates with the observation that DLC1 knockdown resulted in the formation of prominent aligned basal F-actin fibers that terminate at the focal adhesions (Fig. 2b and 3h). The increased focal adhesion alignment fits with the concept that the level of basal F-actin stress fibers is indicative of focal adhesion maturation and force transmission to the ECM (Soiné et al., 2015), which fits with the longer lifetime of the focal adhesions and increased traction forces we detected in DLC1-depleted cells (Fig 2h,i). We added this concept as possible explanation to the discussion section.

6. For Fig. 3a-c and 5a-c, given that the Hippo signaling pathway is affected by cell density, are there any phenotypic changes in the leader alignment of cells after the scratch occurs? (e.g. Do they switch from Hippo ON to Hippo OFF?)

As we show in Suppl. Fig. 3, YAP translocates to the nucleus of endothelial cells at the migration front. This corresponds with a Hippo OFF phenotype (and slightly larger cells). In the follower cells of the dense monolayer, YAP is in the cytoplasm, indeed corresponding with Hippo ON signaling.

7. Minor comments:

-Several of the blot images in the main figures seem too bleached. Specifically, Fig. 1a, 1e, 1h, 1i, and 3c (all these crops are mostly on controls).

Our apologies, the presentation of the blots was adapted accordingly.

-The "-" label in Figs. 1g-i is a bit vague, and it would be useful for the authors to specifically state

what these conditions are (i.e., starved).
We improved the labeling accordingly.

-Heat map has 2 red color values along the scale which makes interpreting it more difficult than needed. The authors should try to have one color per unique frame with no overlapping colors
Thank you for pointing this out. The time frames are now plotted in a unique color spectrum from red (early time points) to blue (late time point).

-Fig 4 caption says that plasmid 1065 is used even though earlier it says 1063 and 1064 pool were used
We apologize for this error. A pool of 1063/1064 shRNAs was used in this experiment. We corrected the caption.

Second decision letter

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AUTHORS: Miesje van der Stoel, Lilian Schimmel, Kalim Nawaz, Anne-Marieke van Stalborch, Annett de Haan, Alexandra Klaus-Bergmann, Erik T. Valent, Duco S. Koenis, Geerten P. van Nieuw Amerongen, Carlie J. de Vries, Vivian de Waard, Martijn Gloerich, Jaap D. van Buul, and Stephan Huveneers
ARTICLE TYPE: Research Article

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

Reviewer 1

Advance summary and potential significance to field

I have no further comments, but reiterate my congratulations to the authors on an excellent paper.

Comments for the author

none

Reviewer 2

Advance summary and potential significance to field

see previous comments...

Comments for the author

The revision is responsive to the concerns.