Glypican 4 regulates planar cell polarity of endoderm cells by controlling the localization of Cadherin 2

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AUTHORS: Anurag Kakkerla Balaraju, Juan J Rodriguez, Bo Hu, Matthew Murry, and Fang Lin

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

As you will see, the referees express considerable interest in your work, but have some criticisms and suggestions for improvements to your manuscript. If you are able to revise the manuscript along the lines suggested, I will be happy receive a revised version of the manuscript. Please also note that Development will normally permit only one round of major revision.

We are aware that you may be experiencing disruption to the normal running of your lab that make experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please attend to all of the reviewers' comments and 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. 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

In this paper, Balaraju et al. describe at high resolution planar polarized convergent extension (CE) movements of the endodermal cell layer in the zebrafish and demonstrate a role for the glypican Gpc4 in endodermal planar polarization, polarized cell movements, and gut tube formation. Mechanistically, they demonstrate that Cadherin (Cdh2) internalization is disrupted in gpc4 mutants, corresponding with reduced rab5 expression. Importantly, they show that increasing Rab5 activity or reducing Cdh2 both partially rescue the endoderm defects in gpc4 mutants. The resulting model is one in which Gpc4 functions in the endoderm to promote Rab5-dependent Cdh2 internalization and thus changes in cell-cell adhesion that allow cell alignment and polarized movement. The work is compelling and though the conclusions are reminiscent of CE mechanisms in mesoderm and ectoderm, these processes have not previously been described in the endoderm and thus the paper is of high significance. I recommend publication in Development pending the following revisions.

Comments for the author

1) Cell autonomy: The cellular/molecular mechanism that is proposed implies cell-autonomous functions for Gpc4, Rab5c and Cdh2, however the evidence does not rule out a non-cell-autonomous role, or even a non-tissue-autonomous role for any of these components. The chimeric analysis shows that gpc4 functions both cell-autonomously and non-cell autonomously, the Cdh2 knockdown/knockout experiment that rescues the gpc4 mutant phenotype is constitutive, the RN-tre over-expression experiment is also constitutive, and the CA-rab5c-YFP experiment also appears to involve constitutive over-expression. The authors should target RN-tre and CA-Rab5c over-expression specifically to the endoderm with sox32 mRNA and show endoderm-autonomous defects (RN-tre) and rescue (CA-Rab5c). They should also show cell-autonomous rescue of endodermal cell shape by transplanting Cdh2 MO, gpc4-/- cells into the endoderm of wildtype hosts.

2) In Fig. 2 the authors distinguish two classes of effects when donor gpc4-/- cells are transplanted into a wildtype host: sometimes the donor cells have a rounded (mutant) shape, and sometimes they have an elongated (wildtype) shape. These two classes are separated out in Fig. 2H, however no other reason for separating them (their position, their neighbors etc.) is given or even proposed. This arbitrary assignment to one of two “patterns” simply reflects the investigator’s bias. The graph should include all the cell shapes of donor gpc4-/- cells in the same stack, and if the distribution is bimodal, that will be apparent.

3) The classical localization of Vangl2 that represents planar polarization is to the anterior sides of cells however the localization on endoderm in control embryos in Fig. 4 is described as “anterior and posterior”.
Does this manifestation of Vangl2 localization represent a different king of planar polarity? The authors should discuss this.

4) I like that the graphs show both the number of cells and the number of embryos, however the numbers on the graphs are sometimes confusing. For example, pattern #2 in Fig. 2H says that there are 9 cells, but there are 15 dots on the graph. Similarly, Fig. 2B says there are 15 cells but there are 18 dots. The authors should either correct the numbers or better explain what the numbers represent in the figure legends.

Reviewer 2

Advance summary and potential significance to field

The authors provide a very detailed description of endoderm cells migration to the midline in the zebrafish embryo, and the effects of the gpc4 mutant on this process. Their live imaging is beautiful, demonstrating both the active protrusive behavior and junctional rearrangements associated with convergent extension of the cell monolayer. They further dissect the effect of loss of gpc4 showing that it results in a loss of mediolateral cell polarization, loss of polarized localization of Vangl2, enhanced localization of cadherin 2 at the membrane, and decreased expression and activity of Rab5c. Their data support a model in which decreased Rab5c leads to an increase in cdh2 at the membrane, which affects the ability of the cells to polarize and intercalate mediolaterally.

These observations are novel, and substantially advance our knowledge of both endoderm cell behavior during gut morphogenesis, and the cellular effects of loss of glypican-4.

Comments for the author

1. In the legend to Figure S3, panels E and F are marked as XZ images, but on the figure they are labeled as XY.

2. Figure 4 B, C, E, and F. It would be helpful to add measurements of intensity of fluorescence at the AP and ML junctions to confirm the polarized localization of Vangl2, particularly in the sox17:GFP-Vangl2 expressing cells.
Also, it looks in 4B and C as though there might be more Vangl2 in the mutant – is this the case?

3. Figure 5 I,J – Student T-test isn't appropriate for multiple comparisons such as are done in these two panels, a one-way ANOVA and post-hoc test would be better.

4. Figure 6D-D'' – can you include parallel images of the gpc4-/- embryos?
Does Rab5c affect Vangl2 distribution as well as Cdh2?

5. For Movies 1 and 3 – it would be very helpful to have tracking data for the entire movie to show the overall cell intercalation (or lack thereof) in addition to the individual intercalations shown. This could be added to Figure S3.

Reviewer 3

Advance summary and potential significance to field

This manuscript provides a good description of a novel example of convergent extension (CE) by cell intercalation in an under investigated tissue in this regard, the endoderm. Aside from the basic description and analysis, the data suggest a number of interesting questions unique to this system, and details of the CE process in this situation that may may offer new avenues of investigation. The role of Gpc4, the role of Rab5 in cadherin endocytosis, and cadherins in the intercalation process are important findings and all promising issues for going forward in this tissue and organism.

Comments for the author

In regard to the statement in the second paragraph of the results, commenting on Fig. 1A (sticky note 1) "loosely packed without cell-cell contact" – unless the images in hand and the ones appearing in the publication are higher resolution, I would suggest "loosely packed without extensive or apparent contact at this resolution", or some such. As I have learned the hard way in the frog, better resolution may reveal filopodia contacting other cells, and it is not necessary to be absolute, as the point is well-made, the extend of contact is very much different from what follows in B and C. "endoderm cells exhibit different cell behaviors at the late segmentation stage for gut formation."

To be clear, different from what? Early Stages?

I find the correspondences indicated by the arrows in I and J series hard to see.
"Thus, although Gpc4 is not required for apical constriction of endodermal cells at the late segmentation stage, it is necessary for proper endoderm C&E and gut formation."
Under the heading "Gpc4 is required for efficient mediolateral endodermal cell intercalation.", the first sentence distinguishes the two mechanisms, the "cell traction/shuffling (by polarized cell protrusions) and cell-junction shrinkage (by myosin contraction)" by "polarized protrusions", on the one hand, and myosin contractions, on the other, as defining characteristics of the two mechanisms.

However, the "cell-on-cell traction" model involves BOTH mediolateral extension of polarized protrusions AND a following mediolaterally oriented contraction of the cell. These were essential parts of the original polarized protrusive activity/cell traction model and remain so today (see Shih and Keller,1992 Development 116, 901-914; Development 116, 915-930; Keller et al. 1992. In

Gastrulation, Development Supplement 1992, 81-91; Keller et al. 2000 Phil. Trans. R. Soc. Lond. B 355, 897-922;). The actomyosin cytoskeleton, the myosin regulation underlying its contraction, and the relationship of the contraction to C-cadherin cell adhesion and to the protrusive activity have been described in detail (see Skoglund, et al. 2006, Development 135: 2435-2444; Kim and Davidson, 2011 (J. Cell Sci. 124: 635-646; Pfister K. 2016. Development 143, 715-727) (summarized in Keller and Sutherland, 2020, Curr. Top Dev Biol 136m 271-317).

Lately, these models have been confused due to a mistaken version of the polarized protrusive activity/cell traction mechanism that involves largely or only extension of protrusions (see Shindo, 2018, WIREs Dev Biol 2018, 7:e293. doi: 10.1002/wdev.293; Shindo and Wallingford, 2014, Huebner and Wallingford, 2018 cited in this manuscript). This model, or this version of the polarized protrusive activity/cell traction model is actually very different from the cell-on-cell traction mechanism, in which the extension of bilateral protrusions “takes the next step” and then the mediolateral, end-to-end cellular contraction, the “power stroke” generates mediolateral tension, which sums across the tissue to develop high tissue level convergence (Shook et al., 2018) and high tissue level pushing (extension) forces (see Moore, 1994 IEEE Trans. Biomed Eng 4, 45-50); Moore et al.,1995 Development 121, 3131-3140; Zhou et al., Development 137, 2785-2794.

I would very strongly suggest that the authors make it clear whether they are either referring to the original and current mechanism proposed by Keller and colleagues, or this variant, as the two are very different. And if the former, refer to it as the “cell-on-cell traction mechanism” or the “polarized protrusive activity/cell-on-cell traction mechanism” and treat it as involving both protrusive activity and actomyosin contraction in an iterative cycle. And in any case, avoid distinguishing the two by protrusive activity versus contractility.

Under this same heading, I have several suggestions. First, the “junctions” are referred to as “mediolateral” or ML, or anterior-posterior in the text, which is good. But in the legend of Fig. 3, reference to a “vertical” junction appeared, and more may have appeared elsewhere. This refers better to the Drosophila germband situation and is confusing here; I suggest sticking to the appropriate axial references for the fish, ML or AP.

Second, it is unclear whether the various descriptions of movements of the cells and the “junctional” behaviors refers to the apical ends, or the deep ends, or elements of both, as in a z-stack compression or bends of the tissue orthogonal to the imaging axis.

For example: “Some cells remodeled their cell junctions: the mediolateral (ML) junction (Type 3, T3, Fig. 3C1) shrank mediolaterally to allow two cells (#1, #4) to meet to form a center point (Type 2, T2) with two neighboring cells (#2, #3, Fig. 3C2). Following this, a new junction formed along the AP axis (Type 1, T1), which separates these cells (#2, #3, Fig. 3C3)” And there are other references to junction shortening expansion, etc.

I am assuming from their description that this is a continuously apposed tight-junctioned/adherens-junctioned epithelium with continuous apposition of cell boundaries at the apical ends, and a mesenchymal-type organization with cells connected with dynamic focal adhesions at the basal ends. In the movies, I see epithelial apical junctional remodeling and basal crawling between one another at in different regions and at various times, but it is often difficult to tell which is which. In this experimental protocol, the cell membranes are all labeled the same and the resolution is pretty good but not great, and if a basal end of a cell is crawling between two others, it can look like “junctional shortening” as the cell proceeds; even moreso, if two cells are crawling toward one another mediolaterally while separating two others in the A-P axis.

However, if these are the basal ends, this is not a “junction” but a long apposition of two plasma membranes, probably tacked together here and there with very dynamic, “true” junctions of spot welds of cadherin; these junctions are made and broken as the invading cells move in, and the associated membrane surfaces are not “shortening” but being “tractored” on by the invading cells, at least in the cell-on-cell traction mechanism. The invading cells depend on adherence to these apposed surfaces, and on linkage by cell adhesion molecules to the tension bearing/ contractile actomyosin cables of these “substrate” cells. This apparent shortening is a very different thing from the remodeling of a continuous apical junction, which consists of a continuous circumferential array of tight junction/zonula adherens junctional proteins in “belt” form, and all this has to be

dealt with one way or another by a lot of cellular processing, as noted long ago by a true pioneer in the study of cell intercalation, Diane Fristrom (see Fristrom, 1976 Dev. Biol., 54, 163-171; Fristrom, 1988 Tissue and Cell 20, 645-690.

This problem could be lessened with a contrasting membrane label to clearly distinguish protrusive invasion from junctional shortening. In a study of cell intercalation in the mouse neural plate, a single layered epithelium, as here, a random scatter of cells with green membranes on a background of cells with red membranes was generated genetically (Williams et al., 2014 Dev. Cell 29, 34–46). And with live confocal imaging of these contrasting cells, oriented apical junction remodeling and oriented basolateral protrusive activity could be distinguished; both seem to drive mediolateral intercalation and CE, with the latter leading the intercalation most often, and likely the main driver of the process.

I suggest the authors make more complete the description of individual events and their context, as best as they can, on imaging these junctions, in particular, and distinguishing shortening from invasion of other cells, for example. In cases where that is questionable or unclear, acknowledge that this is the case. It won't hurt to acknowledge these caveats and limitations, as I think the movies and the quantitative data solidly make the case that both oriented junctional remodeling/shortening and oriented protrusive activity/cell-on-cell traction are occurring, and that Gpc4 is essential for both. The behavior described is solid and rich enough to build on with more advanced methods.

Going forward, a chimeric two-color labeling, stabilizing movement of the preparation in the Z axis perhaps a shorter interval, and higher resolution may be the approach to take, as this is an incredibly rich system, yet another variant on the diversity of cell intercalation processes. It offers a number of interesting possibilities that could be exploited.

The sections on the effects of Gpc4 on Vangl asymmetric distribution, the effects of over-expression of Cdh2 in gpc4-/-embryos, Gpc4 regulation of Rab5c endocytosis were convincing.

On other matters, again, in the Discussion:
"Our live imaging reveals that endodermal cells employ two modes of intercalation: by cellular protrusions and junction shrinkage—which is consistent with conserved cellular mechanisms used in other cell types (Huebner and Wallingford, 2019)."

Again, I strongly recommend adopting my suggestions above in regard to avoid distinguishing these modes from one another with this misleading and overly simplistic nomenclature. Also, as I note above, "junction shrinkage" may refer to two different phenomena, the first, in an epithelium, bona fide "junction shrinkage" and "boundary shrinkage".

In regard to global tissue interactions:
"Similarly, a mechanical force detected between the mesoderm and endoderm can drive their invagination during Drosophila gastrulation (Mitrossilis et al., 2017)."
another example, posterior midgut invagination is essential for germband extension-
Collinet, C., Rauzi, M., Lenne, P.-F., & Lecuit, T. (2015). Local and tissue-scale forces drive oriented junction growth during tissue extension. Nature Cell Biology, 17, 1247.

Again, in regard to junctional analyses, in the methods, see the section below.

It is not clear that the junctions involved are actually all shrinking, expanding, etc in the sense of an epithelial remodeling, versus, cell invasions driven by oriented protusive activity/cell on cell traction- some are convincing, some are not, but I don't think it essential- it is just that stating that it is one or the other in some cases may make the case less credible. I would nuance this a bit.

"Analyses of cell behaviors underlying endoderm C&E: We analyzed movements of endodermal cells at 7-12s acquired from confocal time-lapse experiments. We manually tracked the changes of junctions over the entire time-lapse at 5-minute time intervals to assess: 1) the number of ML junctions (T3) in every 100 endodermal cells; 2) the rate at which the T3 junction was shrinking (the length of T3 junctions divided by the time required for them contracting to T2; 3) the efficiency of separating one pair of cells (assess if junctions were completed, the changes from T3-

T1 to separate a pair of cells along a perpendicular axis). In addition, we tracked the number of newly formed rosettes and total number of rosettes over the time-lapse period."

Below this, under Cell Protrusion Analysis, some readers may give an argument on whether some of the frames in the movies are really protrusions, as in the movies I downloaded, there are in a few cases of protrusions that are very, very faint and extended and retracted over just a frame or two. Again, having looked at about as many of these images as anybody, I am convinced, overall, but others may be more challenged. Make sure there hasn't been a loss of contrast or resolution somewhere in the handling of these movies.

Overall, this work makes a solid, well-done contribution to understanding CE in challenging context, and in an under investigated system, the endoderm. It raises issues and provides insights, many of which I was excited about and wanting to discuss in the course of writing this review. These will be very productive going forward. I enthusiastically recommend publication with the suggested changes.
Ray Keller

First revision

Author response to reviewers' comments

Responses to Reviewer 1:

Major points:
1. Comment: Cell autonomy: The cellular/molecular mechanism that is proposed implies cell-autonomous functions for Gpc4, Rab5c and Cdh2, however the evidence does not rule out a non-cell-autonomous role, or even a non-tissue-autonomous role for any of these components. The chimeric analysis shows that gpc4 functions both cell-autonomously and non-cell autonomously, the Cdh2 knockdown/knockout experiment that rescues the gpc4 mutant phenotype is constitutive, the RN-tre over-expression experiment is also constitutive, and the CA-rab5c-YFP experiment also appears to involve constitutive over-expression. The authors should target RN-tre and CA-Rab5c over-expression specifically to the endoderm with sox32 mRNA and show endoderm-autonomous defects (RN-tre) and rescue (CA-Rab5c). They should also show cell-autonomous rescue of endodermal cell shape by transplanting Cdh2 MO, gpc4-/- cells into the endoderm of wildtype hosts.
Response: We performed the following experiments suggested by the reviewer to further illustrate the cell-autonomous functions of Gpc4, Rab5c and Cdh2 in the endoderm.
a) We performed the following transplantation. Embryos derived from incrossing gpc4+/- fish served as both donor and host embryos. Donor embryos were injected with cdh2 MO or CA-rab5c mRNA together with sox32 and H2BGFP mRNAs at the one-cell stage. Donor cells were transplanted into host embryos as described in the methods. Donor embryos were genotyped for the gpc4 mutation and host embryos were identified according to their phenotype (short body axis in gpc4-/- embryos).

We found that in wild-type (WT) host embryos, gpc4-/- donor cells injected with either cdh2 MO or CA-rab5c RNA did not display typical gpc4-/- cell morphology, but instead were much elongated. Their LWRs (3.22 for cdh2 MO/gpc4-/- donors; 3.04 for CA-rab5c/gpc4-/- donors) were significantly higher than that in gpc4-/- host endodermal cells (1.49), but were still lower than that in their host WT endodermal cells (3.85). Similar results were found in the gpc4-/- host embryo. Compared with the host gpc4-/- endodermal cells, those cdh2 MO/gpc4-/- donors and CA-rab5c/gpc4-/- donor cells were much more elongated with significantly higher LWRs.

These data demonstrate that reducing Cdh2 expression or overexpressing activating Rab5c in gpc4-/- endodermal cells alleviated their polarity defects, suggesting the effects of the interaction of Gpc4 with Cdh2 or Rab5c take place in endodermal cells.

We presented these data in the new supplementary Fig. S8A-F and described these results in the first paragraph on page 15 and in the last paragraph of page 16.

b) Expression of RN-tre should disrupt Rab5-mediated endocytosis, which could lead to an increase in N-cadherin expression and impaired cell polarity. We also performed additional transplantation to determine the cell-autonomous function of RN-tre in the endoderm. WT embryos injected with RN-tre, sox32 and H2BGFP mRNAs were used as the donors. Donor cells were transplanted into WT embryos. We found that donor endodermal cells expressing RN-tre displayed rounder cell morphology with an LWR that is significantly lower than that of the WT host cells. These data show that targeting Rab5-mediated endocytosis in the endoderm disrupts endodermal cell polarity, suggesting that the effects of Rab5-mediated endocytosis on endoderm are cell autonomous. These data are now presented in the new supplementary Figure 7 and are described in the result session (in the last paragraph of page 16).

2. Comment: In Fig. 2 the authors distinguish two classes of effects when donor gpc4-/- cells are transplanted into a wildtype host: sometimes the donor cells have a rounded (mutant) shape, and sometimes they have an elongated (wildtype) shape. These two classes are separated out in Fig. 2H, however no other reason for separating them (their position, their neighbors etc.) is given or even proposed. This arbitrary assignment to one of two “patterns” simply reflects the investigator’s bias. The graph should include all the cell shapes of donor gpc4-/- cells in the same stack, and if the distribution is bimodal, that will be apparent.

Response: We took the reviewer’s suggestion, and combined data from cells displaying the two patterns of cell morphology. However, we still think it is valuable to show the differences between the two patterns, so that readers can see where those cells come from and we can do statistical analyses between these groups and with other groups. We modified Fig. 2H and the relevant figure legend and text (page 9, line 5-9).

3. Comment: The classical localization of Vangl2 that represents planar polarization is to the anterior sides of cells, however the localization on endoderm in control embryos in Fig. 4 is described as “anterior and posterior”. Does this manifestation of Vangl2 localization represent a different king of planar polarity? The authors should discuss this.

Response: Indeed, our data (Fig 4B, B’) show the anterior enrichment of GFP-Vangl2 in endodermal cells, which is similar with that reported in previous studies. We added a panel (Fig. 4D) to show percentage of endodermal cells with GFP-Vangl2 on the anterior edge of ML boundaries. The relevant text is added in the manuscript (page 12, at the end of the first paragraph).

4. Comment: I like that the graphs show both the number of cells and the number of embryos, however the numbers on the graphs are sometimes confusing. For example, pattern #2 in Fig. 2H says that there are 9 cells, but there are 15 dots on the graph. Similarly, Fig. 2B says there are 15 cells but there are 18 dots. The authors should either correct the numbers or better explain what the numbers represent in the figure legends.

Response: The errors have been corrected.

Responses to Reviewer 2:

1. Comment: In the legend to Figure S3, panels E and F are marked as XZ images, but on the figure they are labeled as XY.
Response: The error has been corrected.
2. Comment: Figure 4 B, C, E, and F. It would be helpful to add measurements of intensity of fluorescence at the AP and ML junctions to confirm the polarized localization of Vangl2, particularly in the sox17:GFP-Vangl2 expressing cells. Also, it looks in 4B and C as though there might be more Vangl2 in the mutant – is this the case?

Response: The intensity of GFP-Vangl2 signal at the AP and ML junctions were measured and the data are presented in a new panel as Fig. 4G. In Fig. 4B and C, we compared the expression pattern of GFP-Vangl2, which is expressed in the entire cell periphery of gpc4-deficient endoderm cells, rather than in the anterior boundary of control cells, thus it is difficult to compared the intensity.

3. Comment: Figure 5 I,J – Student T-test isn't appropriate for multiple comparisons such as are done in these two panels, a one-way ANOVA and post-hoc test would be better.

Response: In Fig. 5 I,J, we performed a Student t-test between two different groups of data, but did not conduct multiple comparisons. The t-test for particular two groups of data are labelled in the new version of figure.

4. Comment: Figure 6D-D'' – can you include parallel images of the gpc4-/- embryos?
Response: We performed additional experiments in gpc4-/- embryos, the results of which are presented in Figure 6 and described in the main text (page 15, at the end of paragraph 2).

5. Comment: Does Rab5c affect Vangl2 distribution as well as Cdh2? Fig 7B, C show overexpressing caRab5c in mutants, reduces cdh2 expression. What about vangl2 distribution? Do experiments in Fig 4, overexpressing caRab5c in gpc4/sox17:GFP-Vangl2 to check the vangl2 distribution (explain).

Response: Unfortunately, we can't perform the experiment suggested as the ca-Rab5c-YFP construct is tagged with YFP, which can't be separated from GFP in our confocal microscopes. Thus, we cannot accurately assess the distribution of Vangl2-GFP in embryos expressing ca-Rab5c-YFP.

6. Comment: For Movies 1 and 3 – it would be very helpful to have tracking data for the entire movie to show the overall cell intercalation (or lack thereof) in addition to the individual intercalations shown. This could be added to Figure S3.

Response: We took the reviewer's suggestion. To demonstrate the overall cell intercalation of the endodermal sheet, the entire image from the movies at the beginning and the end of the time point are shown. Additionally, a few cells are labeled to illustrate their relative positions at the two time points after intercalation. We presented these images in a new supplemental Figure 4 as Figure S3 has already had too many panels.

Responses to Reviewer 3:
We sincerely appreciate Dr. Kelly's insightful comments and discussions, as well constructive suggestions. We performed additional experiments to address the concerns raised. Considering the length of the comments, we listed those critical points here rather than the whole context.

1. Comment: In regard to the statement in the second paragraph of the results, commenting on Fig. 1A (sticky note 1) "loosely packed without cell-cell contact" – unless the images in hand and the ones appearing in the publication are higher resolution, I would suggest "loosely packed without extensive or apparent contact at this resolution", or some such.

Response: We adopted the reviewer's suggestion to rewrite the sentence. (Page 6, paragraph 3, lines 6-7.)

2. Comment:
a) "endoderm cells exhibit different cell behaviors at the late segmentation stage for gut formation." To be clear, different from what? Early Stages?
b) I find the correspondences indicated by the arrows in I and J series hard to see.
c) "Thus, although Gpc4 is not required for apical constriction of endodermal cells at the late segmentation stage, it is necessary for proper endoderm C&E and gut formation.
Response:
a) The specific stages of embryos are stated in the revised manuscript: "Endoderm cells exhibit different cell behaviors during TB-12s and after 12s". (Page 8, paragraph 1, lines 3-4).

b) We cropped and readjusted the images in Fig S3.I-J, so that the phalloidin staining is more evident.

c) The conclusion sentence is also modified as "Thus, Gpc4 is required for polarity and intercalation, but not apical constriction of endodermal cells during endoderm C&E." (Page 8, paragraph 1, lines 6-7).

3. Comment: Under the heading “Gpc4 is required for efficient mediolateral endodermal cell intercalation (Fig. 3).”, the first sentence distinguishes the two mechanisms, the “cell traction/shuffling (by polarized cell protrusions) and cell-junction shrinkage (by myosin contraction)” by “polarized protrusions”, on the one hand, and myosin contractions, on the other, as defining characteristics of the two mechanisms. However, the “cell-on-cell traction” model involves BOTH mediolateral extension of polarized protrusions AND a following mediolaterally oriented contraction of the cell. ....The actomyosin cytoskeleton, the myosin regulation underlying its contraction, and the relationship of the contraction to C-cadherin cell adhesion and to the protrusive activity have been described in detail.

Lately, these models have been confused due to a mistaken version of the polarized protrusive activity/cell traction mechanism that involves largely or only extension of protrusions. This model, or this version of the polarized protrusive activity/cell traction model, is actually very different from the cell-on-cell traction mechanism, in which the extension of bilateral protrusions “takes the next step” and then the mediolateral, end-to-end cellular contraction, the “power stroke” generates mediolateral tension, which sums across the tissue to develop high tissue level convergence

I would very strongly suggest that the authors make it clear whether they are either referring to the original and current mechanism proposed by Keller and colleagues, or this variant, as the two are very different. And if the former, refer to it as the “cell-on-cell traction mechanism” or the “polarized protrusive activity/cell-on-cell traction mechanism” and treat it as involving both protrusive activity and actomyosin contraction in an iterative cycle. And in any case, avoid distinguishing the two by protrusive activity versus contractility.

Response:
We agree with the reviewer’s comments. The myosin activity is necessary for both mechanisms (cell-on-cell traction mediated by polarized protrusive activity and cell-junction shrinking). We rewrote the sentences in the revised manuscript. (Page 9, last paragraph 2).

4. Comment: ….But in the legend of Fig. 3, reference to a “vertical” junction appeared, and more may have appeared elsewhere. This refers better to the Drosophila germband situation and is confusing here; I suggest sticking to the appropriate axial references for the fish, ML or AP.

Response: We have replaced the terminology “vertical junction” with “AP junction” in the revised manuscript. Page 13, paragraph 2, line 4; and Fig. 3 legend text, page 21, line 9.

5. Comment: Second, it is unclear whether the various descriptions of movements of the cells and the “junctional” behaviors refers to the apical ends, or the deep ends, or elements of both, as in a z-stack compression or bends of the tissue orthogonal to the imaging axis..... I am assuming from their description that this is a continuously apposed tight-junctioned/adherens-junctioned epithelium with continuous apposition of cell boundaries at the apical ends, and a mesenchymal-type organization with cells connected with dynamic focal adhesions at the basal ends.
Response: Endodermal cells are very flat (about 5 μm height in XZ view), as shown in Fig. 1F’, thus, it is challenging to distinguish the apical ends and dorsal ends. Additionally, because zebrafish embryos have a huge round-shape yolk in the ventral region and the embryonic tissues are curved, we have to image as much as 100 μm to cover all of the endoderm. Thus, in most our time-lapse experiments, images were taken at 2 μm z intervals, which do not offer high resolution at XZ view to clearly illustrate the exact boundaries of the endodermal cells.

6. Comment: In the movies, I see epithelial apical junctional remodeling and basal crawling between one another at in different regions and at various times, but it is often difficult to tell which is which. In this experimental protocol, the cell membranes are all labeled the same and the resolution is pretty good but not great, and, if a basal end of a cell is crawling between two others, it can look like “junctional shortening” as the cell proceeds; even moreso, if two cells are crawling toward one another mediolaterally while separating two others in the A-P axis.

However, if these are the basal ends, this is not a “junction” but a long apposition of two plasma membranes, probably tacked together here and there with very dynamic, “true” junctions of spot

welds of cadherin; these junctions are made and broken as the invading cells move in, and the associated membrane surfaces are not “shortening” but being “tractored” on by the invading cells, at least in the cell-on-cell traction mechanism. The invading cells depend on adherence to these apposed surfaces, and on linkage by cell adhesion molecules to the tension bearing/ contractile actomyosin cables of these “substrate” cells. This apparent shortening is a very different thing from the remodeling of a continuous apical junction, which consists of a continuous circumferential array of tight junction/zonula adherens junctional proteins in “belt” form, and all this has to be dealt with one way or another by a lot of cellular processing, as noted long ago by a true pioneer in the study of cell intercalation.

This problem could be lessened with a contrasting membrane label to clearly distinguish protrusive invasion from junctional shortening. In a study of cell intercalation in the mouse neural plate, a single layered epithelium, as here, a random scatter of cells with green membranes on a background of cells with red membranes was generated genetically (Williams et al., 2014 Dev. Cell 29, 34–46). And with live confocal imaging of these contrasting cells, oriented apical junction remodeling and oriented basolateral protrusive activity could be distinguished; both seem to drive mediolateral intercalation and CE, with the latter leading the intercalation most often, and likely the main driver of the process.

I suggest the authors make more complete the description of individual events and their context, as best as they can, on imaging these junctions, in particular, and distinguishing shortening from invasion of other cells, for example. In cases where that is questionable or unclear, acknowledge that this is the case. It won’t hurt to acknowledge these caveats and limitations, as I think the movies and the quantitative data solidly make the case that both oriented junctional remodeling/shortening and oriented protrusive activity/cell-on-cell traction are occurring, and that Gpc4 is essential for both. The behavior described is solid and rich enough to build on with more advanced methods.

Going forward, a chimeric two-color labeling, stabilizing movement of the preparation in the Z axis, perhaps a shorter interval, and higher resolution may be the approach to take, as this is an incredibly rich system, yet another variant on the diversity of cell intercalation processes. It offers a number of interesting possibilities that could be exploited. The sections on the effects of Gpc4 on Vangl asymmetric distribution, the effects of over-expression of Cdh2 in gpc4-/-embryos, Gpc4 regulation of Rab5c endocytosis were convincing.
Response: As the reviewer stated, shortening of the cell membrane can result from invading/protruding cells rather than shrinkage of cell-junctions. Thus, the reviewer questioned if the junctional shrinkage we showed is a result of the protrusive invasion or from junctional shortening.

We acknowledge that our current time-lapse images cannot distinguish between these two possibilities. In an effort to address this question, we took the reviewer’s suggestion to image the endoderm with mosaic labeling. We performed endoderm transplantation, so that donor and host endodermal cells are labelled with memGFP and memCherry, respectively. Time-lapse imaging was conducted at higher magnification (40x with zoom 1.5). In this setting, cellular protrusions were evident. Notably, we observed some protrusions extended into the ML junctions of the neighboring cells and some did not. Unfortunately, we did not observe memGFP and memCherry-labeling protrusions meeting each other. However, we found that when protrusions were present near the ML junction from the neighboring cells, they are localized at different Z planes, in which the contracting ML junctions appear to be located dorsally relative to the cellular protrusions. Additionally, we observed that the protrusion extension and ML junction shortening were not synchronized, when the protrusions retracted back to the cell, the junctional shortening continued. Thus, cell protrusions unlikely directly contribute the junctional shortening. Taking together, it is likely that the two different cellular mechanisms (protrusion extension and ML junction shortening) act in concert in endodermal cells during C&E, which is consistent with the “hybrid cell crawling and junction-shrinking model”. However, it is also possible that cellular protrusions have influences on junctional changes. It will be important to identify the molecular mechanisms that regulate these two cellular behaviors in the future.
These new results are presented as a new supplementary Figure 5 and Movie 3. The relevant descriptions and discussion are provided in the revised manuscript (page 10, paragraph 1, line 15)

7. Comment: Below this, under Cell Protrusion Analysis, some readers may give an argument on whether some of the frames in the movies are really protrusions, as in the movies I downloaded, there are in a few cases of protrusions that are very, very faint and extended and retracted over just a frame or two. Again, having looked at about as many of these images as anybody, I am convinced, overall, but others may be more challenged. Make sure there hasn't been a loss of contrast or resolution somewhere in the handling of these movies.
Response: The brightness and contrast in the original time-lapse files were adjusted and new movies were made.

Second decision letter

MS ID#: DEVELOP/2021/199421

MS TITLE: Glypican 4 regulates planar cell polarity of endoderm cells by controlling the localization of Cadherin 2

AUTHORS: Anurag Kakkerla Balaraju, Bo Hu, Juan J Rodriguez, Matthew Murry, and Fang Lin

I have now received all the referees reports on the above manuscript, and am pleased to say that there is just one small issue to address relating to statistical analysis, before we proceed to publication. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

Reviewer 1

Advance summary and potential significance to field

In this paper, Balaraju et al. describe at high resolution planar polarized convergent extension (CE) movements of the endodermal cell layer in the zebrafish and demonstrate a role for the glypican Gpc4 in endodermal planar polarization, polarized cell movements, and gut tube formation. Mechanistically, they demonstrate that Cadherin (Cdh2) internalization is disrupted in gpc4 mutants, corresponding with reduced rab5 expression. Importantly, they show that increasing Rab5 activity or reducing Cdh2 both partially rescue the endoderm defects in gpc4 mutants. The resulting model is one in which Gpc4 functions in the endoderm to promote Rab5-dependent Cdh2 internalization and thus changes in cell-cell adhesion that allow cell alignment and polarized movement. The work is compelling and though the conclusions are reminiscent of CE mechanisms in mesoderm and ectoderm, these processes have not previously been described in the endoderm and thus the paper is of high significance. The authors have addressed the concerns I raised in my review of the previous version of the manuscript and I now recommend publication in Development.

Comments for the author

The authors have adequately addressed the concerns I raised in my review of the previous version of the manuscript. I now recommend publication in Development.

Reviewer 2

Advance summary and potential significance to field

This study provides novel insight into the mechanisms regulating convergence and extension of endoderm cells in the zebrafish embryo.

Comments for the author

The authors have done a commendable job in revising their original manuscript, and have answered most of my concerns. One issue that does still remain, however, is the use of the Student T-test in Figure 5I,J. In these graphs, the authors have made comparisons between the control and cdh2MO,

and between the control and the cdh2-/-, and similar comparisons between the gpc4-/- and gpc4-/- combined with the morpholino and knockout phenotypes. Testing for differences between 3 or more population means requires taking the sample variances into account, thus requiring use of ANOVA instead of a T test.
Apart from this, I find the manuscript to be acceptable for publication.

Reviewer 3

Advance summary and potential significance to field

See the original review.

Comments for the author

I am satisfied with the changes made and with the authors' explanations of the technical limitations in regard to the remaining issues. The other reviewer's comment on the use of the t-test should be followed.
I believe it to be a valuable contribution and I very much enjoyed reading it.

Second revision

Author response to reviewers' comments

Responses to Reviewer 2 and 3:

Comment: One issue that does still remain, however, is the use of the Student T-test in Figure 5I,J. In these graphs, the authors have made comparisons between the control and cdh2MO, and between the control and the cdh2-/-, and similar comparisons between the gpc4-/- and gpc4-/- combined with the morpholino and knockout phenotypes. Testing for differences between 3 or more population means requires taking the sample variances into account, thus requiring use of ANOVA instead of a T test.

Response: As suggested by reviewers #2 and #3, we have conducted the one-way ANOVA analysis followed by Tukey’s multiple comparisons test for the data present in Figure 5 I,J. The new Figure 5 has been presented, and the description of this statistical analysis is provided in the figure legend and the “Materials and Methods” session.

Third decision letter

MS ID#: DEVELOP/2021/199421

MS TITLE: Glypican 4 regulates planar cell polarity of endoderm cells by controlling the localization of Cadherin 2

AUTHORS: Anurag Kakkerla Balaraju, Bo Hu, Juan J Rodriguez, Matthew Murry, and Fang Lin
ARTICLE TYPE: Research Article

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