Glypican 4 mediates Wnt transport between germ layers via signaling filopodia

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Re: JCB manuscript #202009082

Dr. Fang Lin
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Dear Dr Lin,

We have now received reviews for your manuscript titled "Glypican4 mediates Wnt transport between germ layers via signaling filopodia” from three external experts in the field and their comments are appended. As you will see, they all consider the work to be important, timely and novel. However, they also highlight multiple issues that detract from the solidity of the conclusions and reduce their level of enthusiasm. For these reasons, we are unable to accept your manuscript for publication by JCB in its present form. Responding to the reviewer concerns will require additional experimental work.

Some of the key points raised by Reviewer #1 are that direct evidence is lacking for the conclusion that Wnts are transferred to neighboring cells by filopodia and that this delivery rescues convergence and extension of mesoderm. To validate your conclusion it would be important to show that Wnts are deposited from a producing cell to other cells not expressing the labeled Wnt. Additionally, it is important to show that Wnt5b-mCherry and tagged GPC are functional. This reviewer also notes that all data should be shown in some kind of dot-plot format, to display all the data points, rather than the outdated column-showing-mean format.

Reviewer #2 was more enthusiastic about your study, but suggested that the sample size was underpowered, that the methods to identify the GPI binding site be better described, and that the rigor of the pulldown assays be improved. Reviewer #3 felt that there were some negative controls missing, and that evidence is needed that loss of Gpc4 does not affect local Wnt5b levels. Additionally, this reviewer echoed the concerns of Reviewer #1 that filopodial transfer of Wnts is not entirely convincing.

Given the extensive amount of experimental work involved, you might decide to submit your work elsewhere. However, if you decide to submit a revised manuscript, please note that we will need a point-by-point response to each of the reviewer comments, and that the manuscript will be returned to the original reviewers for their comments.

Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

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

GENERAL GUIDELINES:
Text limits: Character count is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.
Reviewer #1 (Comments to the Authors (Required)):

In the manuscript entitled 'Glypican mediated Wnt transport between germ layers via signaling filopodia' by Hu et al., the authors have identified a mechanism by which Glypican 4 (GPC4) regulates convergence and extension (C&E) of mesoderm and endoderm during zebrafish embryogenesis. The authors note that GPC4 is expressed in posterior endoderm, and the C&E defect in the endoderm and mesoderm caused by gpc4 loss-of-function can be rescued by endoderm-specific expression of GFP-gpc4. The authors show that endodermal cells form filopodia in GPC4-dependent manner, and these filopodia transport Wnt5 and Wnt11f2 to neighboring cells within the endoderm. The formation of endoderm-derived filopodia and the transport of Wnts via filopodia are required for rescue of mesodermal C&E defect caused by gpc4 loss-of-function. Although several studies over the past few years have underscored the importance of filopodia in intercellular communication, whether cell-surface factors determine filopodia formation and function is unknown. Studies in Drosophila have alluded to a role of glypicans in filopodia (cytoneme)
function. Filopodia fail to extend over Dally-like protein (Dlp) mutant cells, compromising Hh signaling. In the Drosophila glioma model, glial-cell derived filopodia hijack Wingless ligand from neurons to sustain aberrant glial growth. The role of glypicans in this process in the glioma model is not known. Additionally, Dlp in flies has been reported to transport Wnt ligand in a bucket-brigade mechanism, shielding the lipid moiety. Whether Dlp regulates Wnt distribution by participating in cytoneme function is unknown. Thus, the study conducted by the authors is both important and novel, identifying the role of cell-surface glypican s in filopodia formation and function and linking glypican function to Wnt distribution.

Although the study is interesting and timely, some of it claims are not well supported. The following experiments and analyses are suggested:

Major concerns:

1) One of the claims that the authors make is that the Wnts from endoderm are delivered to neighboring cells on filopodia. This is an interesting and important claim, but it requires direct evidence. Currently it is shown that Wnts move around on filopodia, not that it is delivered. The authors should strive to label the Wnt producing cells and the Wnt ligand, injecting into an embryo where all cell borders are labeled, to show that Wnts are deposited from a producing cell to other cells not expressing the labeled Wnt.

2) The authors also claim that Wnts are delivered from endodermal cells to mesoderm/ectoderm, resulting in the rescue of the ectoderm/mesoderm C&E phenotype. This claim is depicted in the graphical summary and in result section (para 3). This claim also needs direct evidence. An alternative model would be that Wnt delivery within the endoderm is sufficient to rescue the C&E phenotype. The authors should demonstrate at a minimum that endodermal cells directly contact (labeled) mesoderm, and better, deliver Wnts to the mesoderm.

3) The Wnt5b-mCherry staining is problematic because it appears to be everywhere and looks like noise. Perhaps this can be sorted out with thinner sections, or showing live movies, or showing controls to be clear this isn’t noise, or reducing the amount of RNA injected. At the moment this data is not convincing.

4) Based on controls shown in 6E and 7D, it appears that Wnt5b-mCherry expression does not affect protrusion morphology. However, it is important to show that Wnt5b-mCherry is functional, as tagged proteins can have different sub-cellular localization patterns compared to wild-type protein.

5) Along similar lines, are the tagged Wnts and GPC constructs used in the Co-IP data functional? Are the authors sure that the properties of tagged proteins are comparable to wild-type proteins? The authors should test the functionality of these tagged proteins in vivo.

6) Panel 5D: How often do the authors see the punctae that are far away from the endodermal cells? This (and rest of the panels in the figure) needs quantification.

7) Problems with the analysis of filopodial metrics:
   a. Were the samples scored blinded to identity? If not re-do these analyses in a blinded fashion, and then report it so in the methods.
   b. The interaction experiments with the low levels of DN Cdc42 and LatB do not report the relevant comparisons. In each case, the sox17:GFP-gpc4 embryos to be compared (statistically and in panels) should be those in a gpc4-/- background injected with the RNA/drug compared to those in a wild-type (gpc4+) background *also injected with the RNA/drug*. It is possible that even these low levels of RNA/drug can disrupt the filopodia, regardless of gpc4 status, and if so then the filopodia are not likely to be the site of interaction between gpc4 and the actin cytoskeleton that causes the C&E phenotype.
   c. All data should be shown in some kind of dot-plot format, to display all the data points, rather than the outdated column-showing-mean format.

Minor concerns:

1) It is surprising that the authors say the rescuing GPC4-GFP protein localizes partially to the
cytoplasm (Fig. 5D), as a glypican is expected to localize within membrane-bound compartments and at the cell surface. This suggests that not all the protein is properly folded and may explain why embryos rescued with this construct are more sensitive to disruption by Wnt morpholinos, LatB, and DN Cdc42. The authors should comment on this cellular localization pattern.

2) Fig. 1: It is not common practice to show multiple WISH signal/expression patterns in the same ‘color’ on a single embryo. Could the authors please clarify why they chose this approach? Is it because the dlx and shh expression patterns are well separated and well defined?

3) In Fig. 3:
   a) Because of the GAG chains, all mature glypicans are expected to run as a smear on a gel or blot. In Fig. 3, the glypican band is not a smear. The authors need to comment on what the band represents, as it apparently does not have GAG modifications. Perhaps it is like the fly glypican Dlp, which separates into two subunits under reducing conditions, only one of which is glycosylated (see Wang and Page-McCaw 2014, JCB). Could the authors please clarify how the westerns were performed and if a single band for GPC4 is expected? The lack of glycosylation on this band also explains why the mutant that lacks GAG chains does not run any differently.
   b) For all western and Co-IP data, show the molecular weight ladder and untransfected controls. The latter becomes especially relevant for blots shown in panel B where multiple anti-Myc bands are seen, and the authors points to two specific bands. Are those multiple bands seen because Myc-Wnt11f2 is detected in different sizes because of its biology, or because the anti-Myc recognizes non-specific targets?
   c) Please label (in parenthesis) what proteins the anti-Myc and anti-FLAG bands represent.
   d) Panel B, is it true that Wnt11f2 pulls down more ΔGAG GPC4?

4) Fig. 5:
   a) The authors claim that replacing the GPI cleavage sequence with Sdc-TM domain rescues C&E phenotype arising from GPC LOF, and that this membrane tethered GPC4 that lacks GPI anchor behaves like wild-type GPC4. If this is true (as evidenced by rescue experiment), then would GFP punctae far away from GFP positive endoderm cells be expected in panel G, similar to panel D? Please explain.
   b) Please show split channels in gray scale for better dynamic range.
   c) Panel A: What are the '.' underneath the sequence alignments?
   d) The white asterisks in the panels make it difficult to read the figure. Please take them out.
   e) Panel E: without an mCherry alone panel, it is hard to assess that the arrows are indeed pointing to the extracellular space. Especially because the GFP signal is so blown out. How are the authors identifying/defining extracellular space?
   f) Descriptions for H and I are missing from the text and legend, so it is unclear what the authors want the readers to take away from these panels. Why does ΔCRD or ΔGAG GPC-GFP protein primarily localize to cytosol? Do these deletions/mutations interfere with proper protein folding/localization. If so, then these tools cannot be used to investigate the role of CRD or GAG chains in filopodia formation/function.
   g) The title of this section of text is misleading. Currently it reads, "The ability of endodermal GFP-Gpc4 to rescue body length is not dependent on the GPI anchor". Rather, it should read, "...is not dependent on cleavage of the GPI anchor". Otherwise it reads like the GPI anchor can be deleted with no adverse consequences.

Other comments:
1) In general, the manuscript is hard to read because 1) it does not reference the figures/figure panels in order, 2) sometimes the panels are not referred to in the text at all (Fig. 5 H, I), 3) the overall description is not very clear (for eg. abstract, results para 3) and 4) the sequence in which the data for Figs. 6 and 7 are presented is not intuitive. Following order for data presentation is recommended:
1) Endoderm extends filopodia
2) Filopodia are decorated with GPC4
3) Cells lacking GPC4 fail to form filopodia
4) Filopodia carry Wnt5b
5) Cells that lack GPC4 and filopodia also lack Wnt5.

2) There are several syntax issues and a couple of typos in the text. Results: Para 1, line 5-6. 'Vertebrates have six Gpc isoforms'. Do the authors mean 6 Gpc genes? Isoforms implies splice variants.
4) Intro, first line: Perhaps '...HSPG) family, are anchored to external surface' is more accurate, as 'bound' implies some sort of protein-protein interaction.
5) Please format the gene and protein names as per zebrafish nomenclature rules. They appear varied across the text and incongruent with how the figure panels are labeled.
6) The authors cite McGough, 2020 in the discussion. This citation belongs in the intro as well where the authors summarize different models of Wnt distribution, especially highlighting how this study rules out against lipophorin and exosome model of Wnt transport. The second paragraph in the introduction also needs to be written more clearly: It starts with describing various mechanisms of GPC-mediated regulation of extracellular signaling (not sure what that means either). And then there is another line half way through the paragraph that starts with 'They do so...'. What is 'they'?
7) The legends could be more descriptive. Please spell out the markers that have been used to label different germ layers (dlx3, shh, krox20 etc.)

Please define long-range signaling. The authors use this in the context of communication between endoderm and mesoderm, which are expected to be juxtaposed.

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

In this manuscript, Hu et al. investigate the role of glypican 4 in convergence and extension (C&E) of mesoderm and endoderm in a zebrafish animal model. They established a rescue model for gpc4 loss-of-function by employing an endoderm-specific Tg(sox17:GFP-gpc4) line that restores a shortened body axis phenotype, and endodermal structure morphology in the gpc4-/- fish. The authors further explore the signaling molecules involved in gpc4-related C&E and find that gpc4 interacts with both wnt5b and wnt11f2. First, the authors found that gpc4 physically interact with wnt5b and wnt11f2 via co-immunoprecipitation and showed that the rescue of gpc4 mutants is at least partially mediated by these 2 morphogens. They also showed that the compound wnt5b-/−;wnt11f2-/- mutants have similar phenotypes observed in gpc4 mutants. The authors further showed that gpc4 contributes to the formation of filopodia which mediate the transportation of wnt5b from one cell to another. Additionally, gpc4 mutants was shown to have reduced number as well as shortened filopodia for wnt5b transportation. Collectively, current study reveals the function of gpc4 and its mechanism to work across different germ layers. Lastly, Hu et al. show that gpc4-bound wnt5b is transported in filopodia that interface and communicate between cells. Overall, the data presented is of high quality, and the authors propose a compelling model for transport of wnts through filopodia by means of gpc4. While their endoderm-specific rescue is robust, there are a few comments to be addressed with their pulldown assay to increase the rigor of the data presented. Further, their blastula assay is seen with less enthusiasm compared to the rest of the manuscript, but their conclusions are not reliant on the assay itself, but rather the chimeric constructs generated, which support their hypotheses.

Major Comments
• Figure 1E; Given the variability of physical properties of larvae, the number of larvae measured for length comparison might be a bit too small. Increasing the sample size would increase the scientific
rigor.

• Figure 2 I&J; the sample size between gpc4-/- and gpc4/sox32-/- is very different in both figure 2 I&J (46 vs 10 and 40 vs 14). Extra attention should be paid to make sure the equal variance between the 2 groups, or it might lead to type I error. The authors could also increase the sample size in gpc4/sox32-/- group.

• While the western blot results in figure 3A,B seem compelling, the IP vs. lysate blots seem to be cropped in different ways. This is particularly important for interpreting the presence of the negative control, Flag-JNK in the IP fraction. It would be useful to crop both blots at the same cutoff (possibly using the protein ladder as a reference) and adding an arrowhead where the expected Flag-JNK band would appear in the IP blot. Alternatively, there may be enough space in the figure to place the IP vs lysate panels side-to-side, as opposed to top-down to judge the cropping more accurately in reference to the Flag-gpc4 bands.

• Figure 5A. The authors claim that they find the putative binding site of GPI in a not well-conserved region. The authors should elaborate further on the approach to identify the site. The algorithm used should be cited.

• In Figure 5D, the authors propose a blastula assay to test for the ability for GFP-gpc4 to be delivered to cells beyond the original injection site. However, an alternate explanation is that GFP-gpc4 and H2A-mCherry RNAs can diffuse beyond the original injection site and become expressed in distant cells, albeit at low level. This interpretation is consistent with seeing a large cluster of mCherry nuclei (presumably near the injection site) and some sparse mCherry nuclei a few cells away. In addition, a few puncta can also be seen in 5G,G' where the GPI anchor is replaced by the syndecan TM domain. However, the authors' statement that "GPI cleavage of gpc4 does not drive rescue of the mesoderm" is still supported with their chimeric construct approach and does not require use of the blastula assay.

• While the co-IP results showed that gpc4 could physically bind to wnt5b and wnt11f2, current study only tested the function of gpc4-labeled filopodia in transporting wnt5b. The author should explain their choice of wnt5b.

Minor Comments

• Typo in page 6 of manuscript: "ectorderm" should be ectoderm.

• Figure 2F&H, the genotype labels are truncated.

• In Figure 2, morphant knockdown or genetic knockout of sox32 is used to to suppress the rescue effect of the transgenic GFP-gpc4 construct. Is it possible to perform foxa3 WISH on the sox32-/- or sox32 morphant gpc4-/-/Tg(sox17:GFP-gpc4) fish? If so, it would be compelling to see if the gut, liver, and pancreatic morphology looks like in this fish. Otherwise, a reference to disrupted gut structures in sox32-/- embryos should be enough.

• Figure 2J, statistic test results between Control and sox32-/- should be shown.

• Naming conventions for zebrafish genes and proteins is inconsistent in the manuscript. It is particularly confusing when describing the Western Blot experiment in HEK293 cells. Changing the gene name conventions would make it clear that the zebrafish coding sequences were transfected into human cells, or is that not the case? Several instances describe genes as Wnt11f2, Gpc4, etc. which would refer to mouse proteins, rather than zebrafish.

• While expression of GFP-gpc4 has a significant rescue effect in gpc4-/- embryos, the transgenic lines were generated via a Tol2 transposase system. Using Tol2, it is difficult to control the copy number of transgenic constructs integrated into the genome. In the methods, the authors state that they "utilized lines that express GFP-Gpc4 at a modest level" but it is unclear how this was measured. Presumably this was determined empirically by fluorescence intensity (and phenotypic assessment of embryogenesis), however a qPCR assay comparing gpc4 expression in wild-type vs Tg(sox17:GFP-gpc4) embryos could be provided to give a better sense of relative gene expression to control fish.
Reviewer #3 (Comments to the Authors (Required)):

In this manuscript by Hu et al., the authors provided evidence that Gpc4, essential for convergence and extension (C&E) of both the mesoderm and endoderm, can also act cell non-autonomously in endoderm to regulate C&E in the mesoderm. The authors further showed that Gpc4 is doing so by regulating the formation of signaling filopodia, with which Wnt5b and Wnt11f2 are delivered to cells in distance in another germ layer. The study is interesting and based on rigorously designed genetic experiments, adding new mechanistic information to the roles of Gpc4. The manuscript could be accepted for publication in JCB in principle if the authors can address the following comments:

1. Fig. 3A, B, a negative control with no Myc-Wnt5b or Myc-Wnt11f2 expression in the co-IP experiments should be included.
2. Fig. 6. It is possible that Gpc4 regulates Wnt5b or Wnt11f2 protein levels at the interface of endoderm and mesoderm. With the help of Wnt5b-mCherry, is there any evidence that Gpc4 loss reduced the local Wnt5b protein levels in the mesoderm adjacent to the endoderm?
3. Fig. 8 A and C, and Fig. S5. As filopodia-mediated Wnt protein transport appears to be a major mechanism by which Gpc4 regulates C & E, it is strange why blocking cytoneme formation in the wild type fish by dnCdc42 expression or treatment with Lat B did not lead to C & E defects? According to the model in Fig. 9, reduced filopodia formation should result in reduced Wnt5b and/or Wnt11f2 signaling. Could reduction in Wnt5b and/or Wnt11f2 signaling be observed in a sensitized mutant?
Overall response:

We are very pleased with the reviewers’ positive responses to our manuscript, and greatly appreciate their constructive comments and suggestions. We have carefully considered all of the issues that were raised. To address them, we have gone to great effort to generate new constructs, test new reagents, and perform additional challenging experiments.

Due to the generation of new data, we have added several new figures (7A-B, 8, 10, S2, and S4C-E); to better accommodate these, we have removed Fig. S2. We have also added several new time-lapse movies (Movie 2-7). The revised manuscript includes a total of 10 figures, 5 supplemental figures, and 9 movies.

A detailed, point-by-point response to the reviewers’ comments and a description of changes made to the manuscript are provided below. Due to the extensive nature of the changes, which were needed to make the language sufficiently precise to get our points across, we have not highlighted all of the changes in the manuscript within the text. But for your reference, we have also provided a version in which the changes are marked using the “compare document” tool in the word software.

Responses to Reviewer 1:

Major points

1. **Comment:** One of the claims that the authors make is that the Wnts from endoderm are delivered to neighboring cells on filopodia. This is an interesting and important claim, but it requires direct evidence. Currently it is shown that Wnts move around on filopodia, not that it is delivered. The authors should strive to label the Wnt producing cells and the Wnt ligand, injecting into an embryo where all cell borders are labeled, to show that Wnts are deposited from a producing cell to other cells not expressing the labeled Wnt.

   **Response:** We took the reviewer’s suggestions and performed the following experiments.

   1. **Mosaic labeling of blastula:** Distinct cells from 16-cells stage embryos were injected RNAs encoding *mem-mCherry* and either *wnt11f2-mNeonGreen* or *wnt5b-mNeonGreen*, and RNA encoding *mem-TagBFP* respectively. Thus, a subset of cells in the embryo expressed *mem-mCherry* together with *Wnt11f2-mNeonGreen* or *Wnt5b-mNeonGreen*, and another expressed *mem-TagBFP* only. We performed time-lapse imaging of the injected embryos at 50% epiboly, specifically of the region where one cell population was proximal to the other. We found that cellular protrusions extended from *mem-mCherry*-expressing cells bound *Wnt11f2-mNeonGreen* or *Wnt5b-mNeonGreen*, and transported and deposited these Wnts to the neighboring BFP-expressing cells. These results indicate that *Wnt11f2-mNeonGreen* and *Wnt5b-mNeonGreen* can be deposited by the producing cells to the receiving cells that do not express Wnts. These data are presented in Fig. S4C-E and Movie 5, and the results are described on Page 12, Lines 345-357.

   2. **Endoderm transplantation:** To determine if Wnts can be delivered by endodermal cells, we conducted endoderm transplantation. Donor embryos were injected with
RNAs encoding *mem-mCherry*, *H₂B-GFP*, *wnt5b-mNeonGreen* or *wnt11f2-mNeonGreen*, and *sox32*. Cells from the donor embryos were transplanted into *Tg(sox17:mem-mCherry)* embryos in which endodermal cells were labeled with *mem-mCherry*. In this setting, Wnt5b-mNeonGreen or Wnt11f2-mNeonGreen was expressed by transplanted endodermal cells, whose plasma membrane and nuclei were labeled with mCherry and GFP. As in the case of the blastula assay, we found that donor cells extended cellular protrusions and transported Wnt5b-mNeonGreen or Wnt11f2-mNeonGreen to the neighboring endodermal cells. These data are presented in Fig. 8A-C and Movie 6, and the results are described on Page 13, Lines 359-364.

2. **Comment:**
   
   1. **The authors also claim that Wnts are delivered from endodermal cells to mesoderm/ectoderm, resulting in the rescue of the ectoderm/mesoderm C&E phenotype. This claim is depicted in the graphical summary and in result section (para 3). This claim also needs direct evidence.**
   
   2. **The authors should demonstrate at a minimum that endodermal cells directly contact (labeled) mesoderm, and better, deliver Wnts to the mesoderm.**
   
   3. **An alternative model would be that Wnt delivery within the endoderm is sufficient to rescue the C&E phenotype.**

**Response:**

1. In order to capture Wnts being deposited from endodermal cells to mesodermal and ectodermal cells, it is essential to label Wnts, endodermal cells, and mesodermal and/or ectodermal cells with different fluorescent proteins. In an effort to do this, we created blue fluorescent protein (mem-TagBFP) and far-red fluorescent protein (mem-mCardinal) constructs to label mesodermal cells, and Wnt5b-TagBFP and Wnt5b-mCardinal to label Wnt5b. However, the fluorescent signals from mem-mCardinal and Wnt5b-mCardinal were too dim to be detected. The fluorescent signals from mem-TagBFP and Wnt5b-TagBFP were also faint but could be detected by confocal microscopy using high laser power. Additionally, neither mem-TagBFP nor Wnt5b-TagBFP could be expressed at a high level; the expression of mem-TagBFP caused embryo lethality and that of Wnt5b-TagBFP led to defects in C&E and dorsal-ventral patterning. Thus, these fluorescent signals were bleached quickly with high laser power, making them unsuitable for time-lapse imaging. Therefore, we cannot perform live imaging to monitor Wnts, endodermal cells, and mesodermal/ectodermal cells with three different fluorescence.

2. Our efforts to image endodermal cells that directly contact the mesoderm were more rewarding. We used our newly generated *Tg(β-actin2:mCherry-utrophin)*, which labels actin in all cells with mCherry-Utrophin, and imaged the region where endodermal cells and the notochord are in close proximity. We observed that the protrusions extended from memGFP-labeled endodermal cells towards mCherry-Utrophin-expressing notochord, and that they made contact with notochord cells. These data indicate that endodermal cells do directly contact neighboring mesodermal cells. These data are presented as Fig. 8D and Movie 7, and the results are described in the “Results” section (Page 13, Lines 364-368).
3. To address the question of rescue is resulted from Wnt delivery within the endoderm, it is necessary to manipulate the endoderm without affecting the mesoderm. Because Gpc4 is expressed on cell surface and regulates the distribution of Wnts in the extracellular environment, limiting the impact of Gpc4 to a specific tissue is a challenge. Additionally, it has been shown that mesodermal C&E movements are not influenced by the endoderm, as sox32-mutant embryos, which lack endoderm, have a normal body axis (mesodermal C&E is not disrupted) (Alexander et al., 1999) (Fig. 2). This suggests that mesodermal C&E movements are independent of their endodermal counterparts. Furthermore, we examined endoderm morphology in gpc4\textsuperscript{-}\hspace{1pt}/\hspace{1pt}Tg(sox17:GFP-gpc4) embryos treated with a sub-dose of Latrunculin B (shown in Fig. S5E-H). In the embryos treated with Latrunculin B the mesodermal C&E was disrupted (Latrunculin B suppressed the rescue), although the endoderm in 60% embryos appeared to be normal (rescued by endodermal expression of GFP-Gpc4). We do not have space to present these data in the revised manuscript. These data support to the notion that C&E defects in the mesoderm and endoderm are not necessarily linked. Thus, it is unlikely that Wnt delivery within the endoderm is sufficient to rescue the C&E phenotype of the mesoderm. We have included this point in the “Results” section (Page 6, Lines 158-159).

3. **Comment:** The Wnt5b-mCherry staining is problematic because it appears to be everywhere and looks like noise. Perhaps this can be sorted out with thinner sections, or showing live movies, or showing controls to be clear this isn't noise, or reducing the amount of RNA injected. At the moment this data is not convincing.

**Response:** We agree that the uniform expression of Wnt5b-mCherry secreted by cells injected with the encoding RNA was problematic. We took the reviewer’s suggestion of: presenting fewer Z-sections and cropping the images to focus on the protrusions (Fig. 7A); and providing time-lapse movies that show Wnt5b-mCherry moving along the protrusions (Movies 2, 4).

4. **Comment:** Based on controls shown in 6E and 7D, it appears that Wnt5b-mCherry expression does not affect protrusion morphology. However, it is important to show that Wnt5b-mCherry is functional, as tagged proteins can have different sub-cellular localization patterns compared to wild-type protein.

**Response:**

1. Because the overexpression of Wnt5b can cause severe gastrulation defects, in most of our experiments we can only inject embryos with a small amount of wnt5b RNA. We think that this is why the morphology of the protrusions in these embryos was largely unaffected.

2. To test whether the Wnt5 and Wnt11 constructs used in this study are functional, we first assessed their expression and then conducted functional studies.

   a) The expression patterns of the constructs in zebrafish embryos were assessed following mosaic injection. Specifically, embryos at the 1-cell stage were injected with mem-mCherry RNA so that all cells would be labelled with mem-mCherry; at the 16-cell stage, a single blastula cell was injected with RNAs encoding wnt5b and wnt11, with different tags, together with H\textsubscript{2}B-GFP, so that a subset of cells would express Wnt5b and Wnt11, and their nuclei would be labeled with GFP. Live confocal imaging was performed at 50%
epiboly. Embryos expressing Myc-tagged constructs were fixed for immunostaining using an anti-C-MYC antibody, and then subjected to confocal imaging.

Confocal imaging showed that, in addition to the tagged Wnt protein in the cells with GFP-labeled nuclei, a punctate Wnt signal was present in the extracellular space near those cells, indicating that those Wnts were secreted from the Wnt-expressing cells. These are the predicted sub-cellular and extracellular localization patterns. We present these data as Fig. S2A-D, and the results are described in the “Results” section (Page 8, Lines 202-206; and Page 12, Lines 347-348).

b) Given that wnt11f2 RNA has been shown to rescue the C&E defects in wnt11−/− embryos (Heisenberg et al., 2000), we tested our wnt11f2-tagged constructs for similar abilities. To this end, we injected RNAs encoding different wnt11-tagged proteins into embryos obtained from wnt11 heterozygotes and performed ISH with probes for hgg1, dlx3 and krox20. As shown previously, in wnt11−/− embryos, expression of hgg1 lagged behind that of dlx3 (Heisenberg et al., 2000). We found that this delay in hgg1 localization was almost completely prevented by the expression of either wild-type or tagged Wnt11f2, suggesting that the tagged Wnt11f2 constructs are functional. We present these data in Fig. S2E-I, and describe them in the “Results” section (Page 8, Lines 206-208).

c) Previous studies showed that simply overexpressing Wnt5b is not sufficient to rescue C&E defects in wnt5b−/− embryos; the distribution of Wnt5b is also critical (Kilian et al., 2003; Lin et al., 2010). Moreover, the overexpression of Wnt5b is detrimental, causing a variety of morphogenetic defects, including C&E defects (Kilian et al., 2003). We tested the abilities of different tagged Wnt5b constructs to recapitulate these C&E defects, by injecting wild-type embryos with RNAs encoding the various wnt5b constructs. We found that, expressing various tagged Wnt5b proteins led to C&E defects, suggesting that these Wnt5b-tagged constructs are functional. We present these data in Fig. S2J-L, and describe the results in the “Results” section (Page 8, Lines 206-208).

5. **Comment:** Along similar lines, are the tagged Wnts and GPC constructs used in the Co-IP data functional? Are the authors sure that the properties of tagged proteins are comparable to wild-type proteins? The authors should test the functionality of these tagged proteins in vivo.

**Response:**

a) Regarding the properties of tagged Wnt constructs, please see the response to comment #4.

b) In terms of GFP-Gpc4, our published data showed that injecting gpc4f−/− embryos with a GFP-Gpc4 RNA rescues C&E defects (Hu et al., 2018). Similarly, we found that injecting gpc4f−/− embryos with Flag-gpc4 RNA rescued C&E defects. We present these data in Fig. S3A-B, and describe them in the “Results” section (Page 8, Lines 210-211).
6. **Comment:** Panel 5D: How often do the authors see the punctate that are far away from the endodermal cells? This (and rest of the panels in the figure) needs quantification.

**Response:** Fig. 5D-I show zebrafish embryonic cells in a blastula assay, in which embryos at the one-cell stage were injected with a *mem-mCherry* RNA; when embryos reached the 16-cell stage, a single blastula cell was injected with RNAs encoding one of *GFP-gpc4* constructs and *H2A-mCherry*, resulting in a subset of cells in the embryo that expressed the GFP-Gpc4 construct. Live imaging was performed at 50% epiboly to determine the localizations of the different GFP-Gpc4 proteins. This assay has certain limitations: we cannot control the number of cells expressing the GFP-Gpc4 constructs and where they are located in the embryo. The levels of expression of the constructs are dependent on the amount of RNAs injected, and might differ even when the same amount of RNA is injected in different day. Thus, it is difficult to perform reliable quantification. As such, this assay illustrates only the relative localization of different GFP-Gpc4 constructs.

For the TM construct, we observed that GFP was expressed mainly on the plasma membrane. Cells expressing this construct extended long cellular protrusions and punctate GFP signal was observed in the extracellular space. However, as we explained above, this assay can't perform reliable quantification. Furthermore, our data show that transgenic expression of this construct in the endoderm of *gpc4−/−* embryos rescued mesoderm defects (Fig. S3G-H), suggesting that the ability of endodermal GFP-Gpc4 to rescue body length in *gpc4−/−* embryos is not dependent on cleavage of the GPI anchor. We have incorporated this information in the “Results” section (Page 10, Lines 284-289).

We have also removed the images of the ∆CRD and ∆GAG Gpc4 constructs, as we do not have space to discuss these data in this manuscript.

Finally, we repeated the blastula assay and imaged the embryos at higher magnification to better illustrate the data. New images are presented in Fig. 5D-G, and the text relevant to these findings has been modified (Pages 9-10, Lines 254-289).

7. **Comment:** Problems with measurements of filopodia:

a) *Were the samples scored blinded to identity? If not re-do these analyses in a blinded fashion, and then report it so in the methods.*

b) *The interaction experiments with the low levels of DN Cdc42 and LatB do not report the relevant comparisons. In each case, the *sox17:GFP-gpc4* embryos to be compared (statistically and in panels) should be those in a *gpc4−/−* background injected with the RNA/drug compared to those in a *wild-type (gpc4+)* background also injected with the RNA/drug.*

c) *It is possible that even these low levels of RNA/drug can disrupt the filopodia, regardless of *gpc4* status, and if so then the filopodia are not likely to be the site of interaction between *gpc4* and the actin cytoskeleton that causes the C&E phenotype.*

d) *All data should be shown in some kind of dot-plot format, to display all the data points, rather than the outdated column-showing-mean format.*
Response:

a) All analyses were re-analyzed by different lab members in a blinded fashion, and the averages obtained by different lab members were used. This information was added to the “Microscopy and image analysis” subsection of the methods section (page 30, lines 886-887).

b) In these experiments, gpc4-/- is not a useful control because the formation of filopodia is disrupted, as shown Fig. 6

c) As shown in the Fig. 9A-I, embryos injected with a sub-dose of the dnCdc42 RNA did not have C&E defects, suggesting that this manipulation did not affect cellular protrusions to such an extent that C&E would be disrupted. This is further supported by the analysis of protrusions in wildtype embryos injected with a sub-dose of the dnCdc42 RNA (comparison to uninjected embryos); neither the number nor the length of protrusions generated by endodermal cells differed between the control and RNA-injected embryos. We have added this description to the text (page 13, lines 383-385). Due to space limitations, we cannot present these data as figures there. However, we provide them here for you to review.

In the case of drug treatment, a sub-dose of Lat B did not cause effects on posterior body length in both wildtype and gpc4-/- embryos in no-transgene background (Fig. S5A-D). In the Tg(sox17:GFP-gpc4) background, such treatment resulted in slight shortening of the posterior body in wildtype embryos (Fig. S5G vs E, I) and more significant shortening in gpc4-/- embryos (Fig. S5H vs. F, I). In the latter case, the rescue effect of the transgene was abolished. As expected, this Lat B treatment also inhibited the formation of protrusions in gpc4-/- /Tg(sox17:GFP-gpc4) embryos (Fig. S5J-N, Movie 9). We added this description in the Results section (Page 14, Lines 387-395).

d) All graphs have been changed to the dot-plot format, and some to the super-plot format. The number of embryos and experiments has been added to the figures or figure legends.

Minor concerns

1. **Comment:** It is surprising that the authors say the rescuing GPC4-GFP protein localizes partially to the cytoplasm (Fig. 5D), as a glypican is expected to localize within membrane-bound compartments and at the cell surface. This suggests that
not all the protein is properly folded and may explain why embryos rescued with this construct are more sensitive to disruption by Wnt morpholinos, LatB, and DN Cdc42. The authors should comment on this cellular localization pattern.

Response:

a) Fig. 5D-I show a blastula assay, in which the embryos were injected with RNAs and the proteins were overexpressed. Thus, it is normal that some protein is expressed in the cytoplasm. Also, we imaged the embryos 4-5 hours after injection. Thus, it is possible that some proteins were not completely processed. Additionally, as GPI modification of Gpc is a post-translational process, some GFP-Gpc4 signal in the cytosol is expected. Similarly, a previous study had reported partial cytoplasmic localization of Gpc4-Flag in embryos at 80% epiboly (8.3 hours after injection of gpc4-flag RNA) (Topczewski et al., 2001). Comments on the GFP-Gpc4 expression patterns have been added to the text (Page 9, Lines 255-257).

b) For experiments using Wnt morpholinos, Lat B, and DN Cdc42, the stable transgenic line Tg(sox17:GFP-gpc4) was utilized. In this line, a substantial proportion of GFP-Gpc4 is expressed on the cell membrane, and the endodermal defects are completely rescued (Figs. S1D-F,1A-D). These findings suggest that GFP-Gpc4 expressed in the endoderm is functional.

2. Comments: Fig. 1: It is not common practice to show multiple WISH signal/expression patterns in the same 'color' on a single embryo. Could the authors please clarify why they chose this approach?

Response: These genes have distinct expression patterns, which have been well characterized. Mixed-probe ISH has been widely used in published studies, including those from my and other’s previous work (Heisenberg et al., 2000; Lin et al., 2005). This method can be used to label different tissues simultaneously and to assess the locations of multiple proteins relative to one another.

3. Comments: In Fig. 3:

a) Because of the GAG chains, all mature glypicans are expected to run as a smear on a gel or blot. In Fig. 3, the glypican band is not a smear. The authors need to comment on what the band represents, as it apparently does not have GAG modifications. Perhaps it is like the fly glypican Dlp, which separates into two subunits under reducing conditions, only one of which is glycosylated (see Wang and Page-McCaw 2014, JCB). Could the authors please clarify how the westerns were performed and if a single band for GPC4 is expected? The lack of glycosylation on this band also explains why the mutant that lacks GAG chains does not run any differently.

b) For all western and Co-IP data, show the molecular weight ladder and untransfected controls. The latter becomes especially relevant for blots shown in panel B where multiple anti-Myc bands are seen, and the authors points to two specific bands. Are those multiple bands seen because Myc-Wnt112 is detected in different sizes because of its biology, or because the anti-Myc recognizes non-specific targets?

c) Please label (in parenthesis) what proteins the anti-Myc and anti-FLAG bands represent.
**d) Panel B, is it true that Wnt11f2 pulls down more ΔGAG GPC4?**

**Response:**

a) In the *JCB* paper that the reviewer mentions (Wang and Page-McCaw, 2014), the authors found that Dlp consists of N- and C-terminal domains that are linked by disulfide bonds, and the GAG modifications occur at the C-terminal domain. When the Dlp protein is run on a nonreducing gel, it displays as smear bands because of the GAG modifications. In a reducing gel, in which the disulfide bonds are disrupted, the N- and C-terminal domains run separately. The N-terminal domain was detected as a band at ~50kDa, whereas the C-terminal domain ran as a smear.

In our experiments, we used a reducing gel to run Flag-Gpc4, whose Flag tag is at the N-terminus of the protein. Thus, Flag-Gpc4 was detected as a band at ~60kDa, but not as a smear. This is consistent with the finding in the *JCB* paper, because our Flag antibody did not detect the C-terminal domain of Gpc4. We have added this explanation to the text (Page 8, Lines 215-218).

b) We took the reviewer’s suggestion and repeated the assay with two additional negative control vectors. One is an empty vector that expresses only the Myc tag; the other encodes Myc-tagged Mmp14b. Neither negative control Co-IPs with Flag-Gpc4. These data and the molecular weight ladders have been added to Fig. 4A. We found that the *wt11f2-Myc* construct we used in the previous submission has a nonsense mutation at residue K105 that might produce multiple bands, as observed. We have corrected this mutation and repeated co-IP assay, which revealed a specific band in the precipitate. We present these new data in Figure 4 of the revised manuscript.

c) All proteins in the co-IP assay are now labelled in the Figure.

d) As stated above Since Wnt11f2 used in the previous submission has a mutation as stated above, we can’t make a conclusion if it pulled down more ΔGAG GPC4. We have removed the data for the ΔGAG GPC4 construct, as we do not have space to discuss this construct. Thus, we did not repeat the experiment using ΔGAG GPC4.

4. **Comment: Fig. 5:**

a) The authors claim that replacing the GPI cleavage sequence with Sdc-TM domain rescues C&E phenotype arising from GPC LOF, and that this membrane tethered GPC4 that lacks GPI anchor behaves like wild-type GPC4. If this is true (as evidenced by rescue experiment), then would GFP punctae far away from GFP positive endoderm cells be expected in panel G, similar to panel D? Please explain.

b) Please show split channels in gray scale for better dynamic range.

The white asterisks in the panels make it difficult to read the figure. Please take them out.

Panel E: without an mCherry alone panel, it is hard to assess that the arrows are indeed pointing to the extracellular space. Especially because the GFP signal is so blown out. How are the authors identifying/defining extracellular space?
c) Panel A: What are the '.' underneath the sequence alignments?

d) Descriptions for H and I are missing from the text and legend, so it is unclear what the authors want the readers to take away from these panels. Why does ΔCRD or ΔGAG GPC-GFP protein primarily localize to cytosol? Do these deletions/mutations interfere with proper protein folding/localization. If so, then these tools cannot be used to investigate the role of CRD or GAG chains in filopodia formation/function.

e) The title of this section of text is misleading. Currently it reads, "The ability of endodermal GFP-Gpc4 to rescue body length is not dependent on the GPI anchor". Rather, it should read, "...is not dependent on cleavage of the GPI anchor". Otherwise, it reads like the GPI anchor can be deleted with no adverse consequences.

Response:

a) Like GFP-Gpc4-expressing cells (Fig. 5D), their counterparts expressing GFP-Gpc4Δ517-557-Sdc4TM extended long cellular protrusions (Fig. 5G). Thus, transgenic expression of this construct in the endoderm can rescue C&E defects in gpc4embryos (Fig. S3C-H). Although GFP-Gpc4Δ517-557-Sdc4TM cannot be cleaved at the GPI anchor region, we observed GFP punctae away from the cells expressing this TM construct, similar to what was observed in GFP-Gpc4-expressing cells, which is probably due to two reasons: first, GFP-punctae could be released from the protrusions to the extracellular space; second, GFP-Gpc4Δ517-557-Sdc4TM might be cleaved at other regions rather than the GPI anchor. However, as we responded in the comment #6 (major points), it is difficult to perform reliable qualification on the puncta number in this experiment. Thus, we didn’t compare the number of puncta in cells expressing GFP-Gpc4 or GFP-Gpc4Δ517-557-Sdc4TM. This description has been added to the “Results” section (Page 10, Lines 282-289).

b) We took the reviewer’s suggestions and modified the images in this figure. We performed new experiments using a 40x objective with a 1.5x zoom setting, cropped the images to show fewer cells, show the channels split in grayscale, and replaced the asterisks with smaller dots. These changes have improved the visualization. The extracellular space is defined as the area between cells (yellow arrowheads in Fig. 5E).

c) In panel A, we removed those non-essential symbols and kept only those that are now explained in the figure legend.

d) Due to space limitations, we have opted to remove the data on ΔCRD or ΔGAG GPC-GFP, as these results have no direct relevance to this paper, and we do not use these constructs for further experiments.

e) The title of this section of text has been changed as suggested. "The ability of endodermal GFP-Gpc4 to rescue body length is not dependent on cleavage of the GPI anchor".

Other comments:

1. **Comment**: In general, the manuscript is hard to read. The reviewer suggested to reorganize the introduction and move the discussion of the results of paper
(McGough, 2020) from the discussion to introduction. Please define long-range signaling.

Response: We appreciate reviewer’s suggestions. We rewrote the introduction and re-organized the part of the results section that describes Figs. 7-8, as suggested. We have removed the wording “long-range signaling” as this description is vague.

2. **Comment**: There are several syntax issues and a couple of typos in the text (Results: Para 1, line 5-6.)

Response: Syntax and typos have been fixed.

3. **Comment**: ‘Vertebrates have six Gpc isoforms’. Do the authors mean 6 Gpc genes? Isoforms implies splice variants.

Response: The description has been changed to “Vertebrates have six GPC proteins (GPC1-6)” (Page 3, lines 50-51).

4. **Comment**: Please format the gene and protein names as per zebrafish nomenclature rules. They appear varied across the text and incongruent with how the figure panels are labeled.

Response: We have reviewed the whole manuscript and ensured that the nomenclature rules are used properly throughout the revised manuscript. In zebrafish, gene symbols are italicized, with all letters in lower-case (e.g., brs), protein symbols are not italicized, and the first letter is upper-case (e.g., Brs). In some cases, the details of allele information were provided in the methods section, although for ease of reading, abbreviated versions were used in the remainder of the text.

5. **Comment**: The legends could be more descriptive. Please spell out the markers that have been used to label different germ layers (dlx3, shh, krox20 etc.)

Response: The full names of these genes and what tissues they label have been added in the “Methods” section (Page 27, Lines 785-791).

Responses to Reviewer 2:

Major comments:

1. **Comment**: Figure 1E; Given the variability of physical properties of larvae, the number of larvae measured for length comparison might be a bit too small. Increasing the sample size would increase the scientific rigor.

Response: In zebrafish embryos, the variability of physical properties is remarkably low. Nevertheless, we have performed additional experiments to increase the number of embryos and added the new data to the old. This applies to the experiments shown in Figs. 1, 2, 3, 6.

2. **Comment**: Figure 2 I&J; the sample size between gpc4/- and gpc4/sox32/- is very different in both figure 2 I&J (46 vs 10 and 40 vs 14). Extra attention should be paid to make sure the equal variance between the 2 groups, or it might lead to type I error. The authors could also increase the sample size in gpc4/sox32/- group.

Response: We removed the samples involving three alleles (e.g., gpc4^-/sox32^-) because it led to have much more embryos than double mutant embryos, and
performed additional experiments to increase the sample size. These data are shown in the new graphs in Figure 2.

3. **Comment:** While the western blot results in figure 3A,B seem compelling, the IP vs. lysate blots seem to be cropped in different ways. This is particularly important for interpreting the presence of the negative control, Flag-JNK in the IP fraction (?). It would be useful to crop both blots at the same cutoff (possibly using the protein ladder as a reference) and adding an arrowhead where the expected Flag-JNK band would appear in the IP blot. Alternatively, there may be enough space in the figure to place the IP vs lysate panels side-to-side, as opposed to top-down to judge the cropping more accurately in reference to the Flag-gpc4 bands.

**Response:** As stated in response #3 (minor concerns) to Reviewer #1, we have performed the co-IP assay with two additional negative controls and a corrected wnt11f2-Myc construct (the original contained a mutation we had not been aware of). Additionally, we took the reviewer’s suggestions to crop all blots in the same way, label the protein ladder, add arrowheads indicating the Flag-JNK bands in the IP blot, and rearrange the IP and lysate panels side by side.

4. **Comment:** Figure 5A. The authors claim that they find the putative binding site of GPI in a not well-conserved region. The authors should elaborate further on the approach to identify the site. The algorithm used should be cited.

**Response:** Although the C-terminal region of Gpc4 is not highly conserved, the GPI attachment site (ω) and its adjacent residues (ω+1, ω+2) are statistically favored residues. Among the GPI-anchored proteins in Metazoa, the GPI attachment site (ω) and its adjacent residues (ω+1, ω+2) have been shown to have statistically conserved residues, with: S occupying the ω site 48% of the time; S, A, or G commonly occupying ω+1; and A or G occupying the ω+2 site 70% of the time (Eisenhaber et al., 1998). Using these criteria, we identified putative conserved GPI attachment sites in the C-terminus of Gpc4: SSG in zebrafish, SAG in mammals, and SAA in *Xenopus*. This description has been added in the “Results” section (Page 10, Lines 263-268).

5. **Comment:** In Figure 5D, the authors propose a blastula assay to test for the ability for GFP-gpc4 to be delivered to cells beyond the original injection site. However, an alternate explanation is that GFP-gpc4 and H2A-mCherry RNAs can diffuse beyond the original injection site and become expressed in distant cells, albeit at low levels. This interpretation is consistent with seeing a large cluster of mCherry nuclei (presumably near the injection site) and some sparse mCherry nuclei a few cells away. In addition, a few puncta can also be seen in 5G,G' where the GPI anchor is replaced by the syndecan TM domain. However, the authors’ statement that “GPI cleavage of gpc4 does not drive rescue of the mesoderm” is still supported with their chimeric construct approach and does not require use of the blastula assay.

**Response:** When a single blastula cell is injected with RNA, the RNAs is distributed to the daughter cells during division, and these daughter cells will express the encoded proteins. Although some cells will stay in a large cluster, others migrate away as small clusters or even as single cells. As long as the injected cell received the RNA, its progeny should express mCherry in their nuclei and GFP-Gpc4 on the plasma membrane. In contrast, when cells receive GFP-Gpc4 from a GFP-Gpc4-expressing neighboring cell, the signal is punctate instead of uniform on the plasma
membrane (Fig. 5). Additionally, these cells do not express mCherry in the nuclei (even low expression in the nucleus should be detectable using high laser power). Thus, the expression patterns of the cells that express GFP-Gpc4 differ from those on the cells that receive GFP-Gpc4 from their neighbors.

We recognize that the images are too small to be seen well. We have opted to crop all images for better visualization.

In addition, in embryos expressing the TM construct, we did observe punctate away from the TM-construct expressing cells. Additional comments please see the Response #4 (minor concerns) for the reviewer 1. We have added this explanation to the text (Page 10, Lines 283-289).

6. **Comment:** While the co-IP results showed that gpc4 could physically bind to wnt5b and wnt11f2, current study only tested the function of gpc4-labeled filopodia in transporting wnt5b. The author should explain their choice of wnt5b.

   **Response:** We also generated a wnt11-mNeonGreen construct, and performed similar mosaic and endoderm transplantation. Our results revealed that, like Wnt5b-mNeonGreen, Wnt11-mNeonGreen can be transported to neighboring cells that do not express Wnt11, via filopodia. We also generated a wnt11-mCherry construct, and performed the time-lapse experiment in Tg(sox17:GFP-gpc4) embryos injected with a wnt11-mCherry RNA. Like Wnt5b-mCherry, Wnt11-mCherry was transported by gpc4-labeled filopodia. These data are presented in Fig. 7B and Movie 3 of the revised manuscript.

**Minor Comments**

1. **Comment:** Typo in page 6 of manuscript: "ectorderm" should be ectoderm.

   **Response:** The typo has been corrected.

2. **Comment:** Figure 2F&H, the genotype labels are truncated.

   **Response:** This issue has been corrected.

3. **Comment:** In Figure 2, morphant knockdown or genetic knockout of sox32 is used to suppress the rescue effect of the transgenic GFP-gpc4 construct. Is it possible to perform foxa3 WISH on the sox32−/− or sox32 morphant gpc4−/−/Tg(sox17:GFP-gpc4) fish? If so, it would be compelling to see if the gut, liver, and pancreatic morphology looks like in this fish. Otherwise, a reference to disrupted gut structures in sox32−/− embryos should be enough.

   **Response:** The digestive system is not developed in either the sox32−/− or the sox32 morphants, and thus foxa3 is not expressed in the endoderm in these embryos, as shown previously (Stafford et al., 2006). By performing WISH for foxa3 and using Tg(sox17:EGFP) transgenic line, we did not detect foxa3 expression in endoderm and endodermal GFP signal in both sox32−/− embryos or sox32 morphants (data not shown). Due to the limited space, we are not able to include these data in the revised manuscript. But we have added this description and the reference to the revised manuscript (Page 6, Lines 155-158).

4. **Comment:** Figure 2J, statistic test results between Control and sox32−/− should be shown.
Response: Th statistic test results have been added.

5. Comment: Naming conventions for zebrafish genes and proteins is inconsistent in the manuscript. It is particularly confusing when describing the Western Blot experiment in HEK293 cells. Changing the gene name conventions would make it clear that the zebrafish coding sequences were transfected into human cells, or is that not the case? Several instances describe genes as Wnt11f2, Gpc4, etc. which would refer to mouse proteins, rather than zebrafish.

Response: In most cases, we transfected HEK293 cells with zebrafish genes. In zebrafish, full gene names are italicized (e.g., brass). Gene symbols are also italicized, with all letters in lower-case (e.g., brs). Protein symbols are not italicized, and the first letter is upper-case (e.g., Brs). Thus, gpc4 refers to the gene, whereas Gpc4 refers to the protein.

6. Comment: While expression of GFP-gpc4 has a significant rescue effect in gpc4−/− embryos, the transgenic lines were generated via a Tol2 transposase system. Using Tol2, it is difficult to control the copy number of transgenic constructs integrated into the genome. In the methods, the authors state that they "utilized lines that express GFP-Gpc4 at a modest level" but it is unclear how this was measured. Presumably this was determined empirically by fluorescence intensity (and phenotypic assessment of embryogenesis), however a qPCR assay comparing gpc4 expression in wild-type vs Tg(sox17:GFP-gpc4) embryos could be provided to give a better sense of relative gene expression to control fish.

Response: We did use fluorescence intensity to determine the relative expression levels of GFP-Gpc4. We took the reviewer’s suggestion and examined gpc4 expression, by qPCR of RNAs extracted from sox17:GFP-gpc4 and wild-type (WT) embryos. Our data show that Gpc4 expression levels are twice as high in sox17:GFP-gpc4 vs. wild-type embryos. This result has been added to Fig. S1G and is described in the results section (Page 5, Lines 122-124).

7. Comment: P.13, L1, should be 'Wnt5b-mCherry'.

Response: This has been corrected.

Reviewer #3

1. Comment: Fig. 3A, B, a negative control with no Myc-Wnt5b or Myc-Wnt11f2 expression in the co-IP experiments should be included.

Response: Reviewer #1 and #2 also raised this concern. We now include constructs that expresses Myc tag or Myc-Mmp14b as negative controls. As shown in Fig. 4A, B, Flag-Gpc4 did not precipitate with either Myc or Myc-Mmp14b.

2. Comment: Fig. 6. It is possible that Gpc4 regulates Wnt5b or Wnt11f2 protein levels at the interface of endoderm and mesoderm. With the help of Wnt5b-mCherry, is there any evidence that Gpc4 loss reduced the local Wnt5b protein levels in the mesoderm adjacent to the endoderm?

Response:
Given that the injection of embryos with the \textit{wnt5b-mCherry} RNA results in uniform Wnt5b-mCherry expression, we cannot use this labeling to evaluate the expression pattern in \textit{gpc4}\textsuperscript{-/-} embryos.

To determine whether Gpc4 regulates the levels of Wnt5b or Wnt11f2 protein \textit{in vivo}, it is necessary to assess the expression patterns of endogenous Wnt5b and Wnt11. To this end, we purchased two commercially available antibodies against zebrafish Wnt5b (ORIGene TA34408, AnaSpec AS-55880), as well as one against Wnt11f2 (AnaSpec AS-55661), and tested them for the ability to recognize the proteins.

First, we transfected HEK293 cells with various \textit{wnt5b} and \textit{wnt11f2} constructs (including the native genes and tagged constructs), and conducted Western blotting on those cell lysates. We found that AnaSpec AS-55880 and AnaSpec AS-55661 detected the Wnt5b and Wnt11 proteins, respectively, but that ORIGene TA34408 did not detect Wnt5b.

Second, we performed Western blotting and immunostaining to examine the expression of endogenous Wnt5b and Wnt11 in zebrafish embryos using AnaSpec AS-55880 and AnaSpec AS-5566 antibodies. Western blotting revealed that AnaSpec AS-55880 detected a specific, strong Wnt5b band, but AnaSpec AS-5566 detected only a very faint Wnt11 band. In the case of immunostaining, both antibodies detected signals in nuclei, which is inconsistent with the predicted pattern (Wnt should be expressed in the extracellular space as puncta). We conclude that these antibodies do not work for immunostaining, and only the Wnt5b antibody works for Western blotting. Thus, we are not able to assess the patterns of Wnt5b or Wnt11f2 expression in embryos.

Third, by using Wnt5b antibody (AnaSpec AS-55880), we found that Wnt5b expression levels are not significantly different in the \textit{gpc4}\textsuperscript{-/-} embryos than in their sibling controls. These data are presented in Fig. 10 and described in the results section (Page 14, Lines 404-408) of the revised manuscript.

3. **Comment:**

1) Fig. 8 A and C, and Fig. S5. As filopodia-mediated Wnt protein transport appears to be a major mechanism by which Gpc4 regulates C & E, it is strange why blocking cytoneme formation in the wild type fish by \textit{dnCdc42} expression or treatment with Lat B did not lead to C&E defects?

2) According to the model in Fig. 9, reduced filopodia formation should result in reduced Wnt5b and/or Wnt11f2 signaling. Could...
reduction in Wnt5b and/or Wnt11f2 signaling be observed in a sensitized mutant?

Response:

1) Injection of the dncdc42 RNA at a high dose (250 pg vs 120 pg) or treatment with Lat B at a high dose (0.25 µg/ml vs 0.15 µg/ml) indeed led to C&E defects in embryos. Due to limited space in the manuscript, we present the data only here for you to review (Figure 2 for the reviewer). Thus, it was necessary to use a sub-dose of RNA or Lat B, to avoid significant impacts on C&E from the drug treatment. The fact that such manipulations suppressed the rescue of C&E defects in gpc4-/- embryos by endodermal expression of GFP-Gpc4 suggests that GFP-Gpc4 expressing endodermal cells are sensitized to such manipulations.

2) The expression of phosphorylated JNK (p-JNK) is shown as a readout of activation of the Wnt/PCP signaling pathway (van Amerongen and Nusse, 2009; Yamanaka et al., 2002). By performing western blotting for the expression of p-JNK, we found that levels in gpc4-/- embryos are significantly lower than in control sibling embryos, and that this reduction is reversed in gpc4-/-/Tg(sox17:GFP-gpc4) embryos. These data are consistent with reduced Wnt/PCP signaling in gpc4-/- embryos being responsible for the C&E defects, and with endodermal expression of GFP-Gpc4 helping to restore Wnt/PCP signaling.

Notably, as we described in comment #2, although p-JNK levels are reduced in gpc4-/- embryos, Wnt5b expression is not affected. Thus, Gpc4 likely affects Wnt5b signaling rather than Wnt5b expression levels, and transporting Wnts from the endoderm to the mesoderm helps to restore Wnt5b distribution and signaling. Considering that the distribution of Wnt5b is also necessary for its signaling, we postulate that Gpc4 is important for achieving the Wnt5b distribution that is critical for Wnt/PCP signaling. However, testing this hypothesis directly in vivo will require the ability to examine the Wnt distribution in vivo. We have added our results and this discussion to the revised manuscript (Page 14-15, Lines 405-415, and Page 17-18, Lines 505-515).

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August 23, 2021

RE: JCB Manuscript #202009082R

Dr. Fang Lin
University of Iowa
1-400 BSB, 51 Newton Rd
Iowa city 52242

Dear Dr. Lin:

Thank you for submitting your revised manuscript entitled "Glypican 4 mediates Wnt transport between germ layers via signaling filopodia". The paper has now been seen again by two of the original reviewers who now recommend acceptance and so we would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

**As you will see, reviewer #1 has raised a few remaining minor issues that we would like for you to address in the final revised version of the paper. Please note, though, that while we appreciate the reviewer's position in his/her point #5, we feel that the JNK data should remain in the paper. In addition, while we do not have a formal policy about instances of 'data not shown' (reviewer #1, point #2), we do recommend adding any relevant data to the paper. If the reason that this was not added is due to our 5 supplementary figure limit, just let us know and we can discuss allowing you to have more space. Please also be sure to provide a point-by-point rebuttal detailing how each of the reviewer's final points were addressed in the final revision.**

To avoid unnecessary delays in the acceptance and publication of your paper, please read the following information carefully.

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

Full guidelines are available on our Instructions for Authors page, https://jcb.rupress.org/submission-guidelines#revised. **Submission of a paper that does not conform to JCB guidelines will delay the acceptance of your manuscript.**

1) Text limits: Character count for Articles and Tools is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, and acknowledgments. Count does not include materials and methods, figure legends, references, tables, or supplemental legends. You are below this limit at the moment but please bear it in mind when revising.

2) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. Molecular weight or nucleic acid size markers must be included on all gel electrophoresis.

3) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and
methods. For figures presenting pooled data the statistical measure should be defined in the figure legends. Please also be sure to indicate the statistical tests used in each of your experiments (both in the figure legend itself and in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). Also, since you used parametric tests in your study (e.g. t-tests, ANOVA, etc.), you should have first determined whether the data was normally distributed before selecting that test. In the stats section of the methods, please indicate how you tested for normality. If you did not test for normality, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

4) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions (at least in brief) in the text for readers who may not have access to referenced manuscripts. The text should not refer to methods "...as previously described."

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

6) Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
   a. Make and model of microscope
   b. Type, magnification, and numerical aperture of the objective lenses
   c. Temperature
   d. Imaging medium
   e. Fluorochromes
   f. Camera make and model
   g. Acquisition software
   h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstructions, surface or volume rendering, gamma adjustments, etc.).

7) References: There is no limit to the number of references cited in a manuscript. References should be cited parenthetically in the text by author and year of publication. Abbreviate the names of journals according to PubMed.

8) Supplemental materials: There are normally strict limits on the allowable amount of supplemental data. Articles/Tools may have up to 5 supplemental figures. As mentioned above, though, if you need more space to include the 'data not shown', we should be able to give you some extra space. Please also note that tables, like figures, should be provided as individual, editable files. A summary of all supplemental material should appear at the end of the Materials and methods section.

9) eTOC summary: A ~40-50 word summary that describes the context and significance of the findings for a general readership should be included on the title page. We realize that you have included one already but please note that it should include "First author name(s) et al..." to match our preferred style.

10) Conflict of interest statement: JCB requires inclusion of a statement in the acknowledgements regarding competing financial interests. If no competing financial interests exist, please include the following statement: "The authors declare no competing financial interests." If competing interests...
are declared, please follow your statement of these competing interests with the following statement: "The authors declare no further competing financial interests."

11) A separate author contribution section is required following the Acknowledgments in all research manuscripts. All authors should be mentioned and designated by their first and middle initials and full surnames. We encourage use of the CRediT nomenclature (https://casrai.org/credit/).

12) ORCID IDs: ORCID IDs are unique identifiers allowing researchers to create a record of their various scholarly contributions in a single place. At resubmission of your final files, please consider providing an ORCID ID for as many contributing authors as possible.

B. FINAL FILES:

Please upload the following materials to our online submission system. These items are required prior to acceptance. If you have any questions, contact JCB's Managing Editor, Lindsey Hollander (lhollander@rockefeller.edu).

-- An editable version of the final text (.DOC or .DOCX) is needed for copyediting (no PDFs).

-- High-resolution figure and MP4 video files: See our detailed guidelines for preparing your production-ready images, https://jcb.rupress.org/fig-vid-guidelines.

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**It is JCB policy that if requested, original data images must be made available to the editors. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original data images prior to final submission.**

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Thank you for your attention to these final processing requirements. Please revise and format the manuscript and upload materials within 7 days. If complications arising from measures taken to prevent the spread of COVID-19 will prevent you from meeting this deadline (e.g. if you cannot retrieve necessary files from your laboratory, etc.), please let us know and we can work with you to determine a suitable revision period.

Please contact the journal office with any questions, cellbio@rockefeller.edu.

Thank you for this interesting contribution, we look forward to publishing your paper in Journal of Cell Biology.

Sincerely,

Ian Macara, PhD
Reviewer #1 (Comments to the Authors (Required)):

The authors have sufficiently addressed the major concerns highlighted in the first round of reviews, and the scientific rigor of the experiments has greatly improved.

The authors are urged to address the following minor concerns to improve the readability of the manuscript.
1. Certain data could be described more clearly. For example, in lines 129-132, it would be easier to read if it was made clear that this was a rescue experiment where the gpc4-/- phenotype was rescued by providing GFP-gpc4.
2. The authors should check the journal policy for 'data not shown'. In general, these could be included in supplementary information.
3. Certain figure panels are not described in the text (S2 C,D, S3 C-H, 5 A,B). Along similar lines, some figures and panels are not described in the order of appearance. This makes the manuscript hard to read. The authors should examine the manuscript to identify these instances and edit the manuscript accordingly.
4. This reviewer had a hard time reading graphs in Figure 3. These need to be re-done or need clearer description of how the graphs were generated. In general, the legends could be more descriptive to aid readability.
5. The JNK-related data seem out of place for this manuscript and should be removed. This was also not present in the original submission.
6. The x axes in Fig. 6 C and D are missing labels.

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

The authors did a great job addressing my comments. The manuscript is in a good form for publication in JCB.
Overall response:
We are delighted with the reviewers’ positive responses to our revised manuscript. Below we include a detailed, point-by-point response to comments from Reviewer #1, and a description of related changes made to the manuscript (below). We have highlighted the changes in the manuscript in yellow.

Responses to Reviewer 1:

1. **Comment:** Certain data could be described more clearly. For example, in lines 129-132, it would be easier to read if it was made clear that this was a rescue experiment where the gpc4^- phenotype was rescued by providing GFP-gpc4.
   
   **Response:** We added the following words (in red) to clarify the purpose of the experiment: “To determine whether endodermal defects in gpc4^- embryos are due to gpc4 deficiency specifically in the endoderm, we generated the gpc4^-/Tg(sox17:GFP-gpc4) line and determined whether the phenotypes in gpc4^- embryos can be rescued by expressing GFP-Gpc4 in the endoderm.” (Page 5, Line 130-131).

2. **Comment:** The authors should check the journal policy for ‘data not shown’. In general, these could be included in supplementary information.
   
   **Response:** There are six areas where we used ‘data not shown’ in the main text. We did so in part because the journal allows only five supplementary Figures. We reviewed these descriptions and felt that some of them are unnecessary but that in other cases they represent information valuable to the manuscript. We consulted with the editors on this point and were given permission to include one additional supplementary figure. It shows that injection of a high dose (250 pg) of the cdc42T17N RNA impaired mesodermal C&E in both control siblings and gpc4^- embryos (Fig. S6A-E), whereas injection of such embryos with a sub-dose (120 pg) of this RNA had little impact on the body axes (as shown in Fig. 9A-D,I) and on GFP-Gpc4-labeled cellular protrusions in wild-type embryos (Fig. S6F-H). (Page 14, Line 409-412)

   1) “Compared to control (sibling) embryos, gpc4^- embryos displayed an enlarged gut-tube and malformed digestive organs (duplicated, smaller, or missing; Fig. 1A,B, data not shown).” (Page 5, line 110-112, in the previous submission)

      Given that the detailed information on malformed digestive organs is not the main focus in this manuscript, we have deleted this description. The new sentence is written as follows: “Compared to control (sibling) embryos, gpc4^- embryos displayed an enlarged gut-tube (Fig. 1A,B).” (Page 5, Line 112-113)

   2) “The transcription factor sox32 is required for endoderm development (Alexander et al., 1999); thus, our WISH did not detect foxa3 expression in the endoderm in
sox32\(^{-}\) embryos (not shown), consistent with the previous report (Stafford et al., 2006).” (Page 6, line 155-158, in the previous submission)

The previously published study showed that foxa3 expression is not detectable in the endoderm of sox32 morphants (Stafford et al., 2006); thus, we have changed this sentence to: “The transcription factor sox32 is required for endoderm development (Alexander et al., 1999) and foxa3 expression in the endoderm is not detectable in sox32-deficient embryos (Stafford et al., 2006).” (Page 6, Line 158-160).

3) “We generated a series of C-terminal truncation mutants that lack the potential GPI attachment signal (Fig. 5B, data not shown) and assessed their expression patterns in the blastula assay. We found that Gpc4 lacking AA517-557 (GFP-Gpc4\(\Delta\)517-557) failed to localize to the cell membrane but was present mainly in the extracellular space (Fig. 5E-E\('\)’).” (Page 10, line 268-272, in the previous submission)

We did not include schematics for the series of C-terminal truncation mutants in Fig. 5B. Thus, the “data not shown” wording is not necessary here. The new wording is as follows: “To test if the C-terminal region of zebrafish Gpc4 is responsible for its anchor to the membrane, we generated a series of C-terminal truncation mutants that lack the potential GPI attachment signal and assessed their expression patterns using the mosaic labeling in the blastula. We found that Gpc4 lacking AA517-557 (\(\Delta\)517-557, Fig. 5B) failed to localize to the cell membrane but was present mainly in the extracellular space (Fig. 5E-E\('\)’).” (Page 10-11, Line 289-294)

4) “Although injecting the cdc42T17N RNA impaired C&E (not shown), injection of a low dose had little impact on the gpc4\(^{-}\)/ embryos or their wild-type siblings (Fig. 9A-D,I).” (Page 13, line 377-379, in the previous submission)

In response to a comment from Reviewer #3, we added these data as Fig. 2A-D for the reviewer during the previous submission. We feel that these data are valuable to the manuscript and thus have moved them to Supplementary Figure 6 (Fig. S6A-E) and added the relevant descriptions to the current revision of the manuscript (Page 15, Line 416-419).

5) “Furthermore, confocal live imaging showed that injecting a low dose of the cdc42T17N RNA had little effect on GFP-Gpc4-labeled cellular protrusions in wild-type embryos (not shown), but produced shorter and fewer and filopodia in gpc4\(^{-}\)/Tg(sox17:GFP-Gpc4) embryos than in un-injected counterparts (Fig. 9J-L, Movie 8).” (Page 14, line 383-387, in the previous submission)

In response to a comment from Reviewer #1, we presented these data as Fig. 1 for the reviewer (major comment #7c) during the previous submission. We now present these data in Supplementary Figure 6 (Fig. S6F-H) and include the relevant descriptions in the current revision of the manuscript. (Page 15, Line 423-425)
6) “However, we cannot examine the expression patterns of endogenous Wnt5 and Wnt1112 in embryos because we lack suitable antibodies (not shown)” (Page 14, line 404-405, in the previous submission)

We have deleted the ‘not shown’ because it is not practical to use figure space to show data for antibodies that did not work. (Page 16, Line 445-446)

3. **Comment:** Certain figure panels are not described in the text (S2 C,D, S3 C-H, 5 A,B). Along similar lines, some figures and panels are not described in the order of appearance. This makes the manuscript hard to read. The authors should examine the manuscript to identify these instances and edit the manuscript accordingly.

**Response:** We thank the Reviewer for noticing the missing or unclear descriptions in these panels. All descriptions for the panels in the figures mentioned above and other figures have been updated, and they are highlighted in yellow in this revised manuscript.

1) Fig. S2C,D: We have added a schematic to illustrate the mosaic injection (Fig. S2A), so that Fig. S2C-D’ became to Fig. S2D-E’ in the current version of the manuscript. The descriptions for Fig. S2D-E’ were added: “Similar to wnt-Myc constructs, these mNeonGreen constructs displayed the predicted cellular localizations (Fig. S2C-E’) and were functional (Fig. S2M)” (Page 13, Line 380-382)

2) Fig. S3C-H: The results for these panels were described in the manuscript. (Page 11-12, Line 315-322)

3) Fig. 5A,B: Where possible, we have reorganized panels to reflect the order of their appearance. However, in some cases, the logic of the organization of the figures makes it challenging to do so. In the current version of the manuscript, Fig. 5B,C from the last version is moved to Fig. 5A,B, and the schematic in Fig. 5B is also modified to include all components described in the text. More descriptions have been added to Fig. 5A-C. (Page 9-10, Line 259-263; Page 10-11, Line 273-298)

4) We have made the following additional modifications in the current revision of the manuscript.

   a) We have added new panels or labeling the following figures: Fig. 5B, Fig. S2A.

   b) We have expanded the figure legends to the following figures (Fig. 2,5,6,8, Fig. S1-S3, S5).

   c) New descriptions have been added to the following figures:

      o Fig. S2A (Page 9-10, Line 257-263)
      o Fig. S4A,B (Page 12, Line 344-350)
      o Fig. 7C,D (Page 13, Line 368-371)
4. **Comment:** This reviewer had a hard time reading graphs in Figure 3. These need to be re done or need clearer description of how the graphs were generated. In general, the legends could be more descriptive to aid readability.

**Response:** The Figure 3 has been simplified for easy visualization by removing the symbols for different mutants and adding the labels for the X-axis.

5. **Comment:** The JNK-related data seem out of place for this manuscript and should be removed. This was also not present in the original submission.

**Response:** JNK activation is an additional readout to demonstrate that the endodermal expression of GFP-Gpc4 restored JNK activation in $gpc4^{-/-}$ embryos. Although this was not presented in the original submission, we feel that these data are significant and should be included in the results.

6. **Comment:** The x axes in Fig. 6 C and D are missing labels.

**Response:** The labels for the X-axis in Fig. 6 C and D have been added.