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Hedgehog pathway modulation by glypican 3-conjugated heparan sulfate

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AUTHORS: Yulu Cherry Liu, Bradley M Wierbowski, and Adrian Salic
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We have now reached a decision on the above manuscript.

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

As you will see, the reviewers raise a number of substantial criticisms that prevent me from accepting the paper at this stage. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

We are aware that you may be experiencing disruption to the normal running of your lab that makes 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 ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

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

Reviewer 1

Advance summary and potential significance to field

This manuscript presents cell biological and biochemical examination of Glypican 3 (GPC3) contributions to Hedgehog (HH) pathway activity. The study suggests that GPC3 promotes the HH response at a level downstream of the pathway receptor Patched (PTCH) and signal transducer Smoothened (SMO). Disruption of GPC3 function prevents GLI transcription factor ciliary enrichment and activation without affecting ciliary trafficking of PTCH or SMO. Using cell lines engineered to specifically disrupt either heparan or chondroitin modifications revealed a specific requirement for heparan modification of GPC3 for its function in the HH pathway. Highlights of the study are that the authors are examining a regulatory step of HH signaling that is not well understood in vertebrates, development of an assay to purify ecto-GPC3 to assess its activity in vitro and development of cell lines providing the ability to manipulate specific sugar modifications of the GPC3 ecto domain. A shortcoming of the manuscript is that it lacks mechanistic insight, so provides only an incremental advance in understanding of how glypicans fit into the vertebrate HH pathway. Specific concerns that need to be addressed prior to publication are detailed below.

Comments for the author

Figure 1B – What is the baseline measurement of Gli1 in the GPC3 KO cells relative to control? Looks like baseline for each cell line is set to 1 since data are shown as fold change following SHH/SAG stimulation. Is baseline Gli1 lower?
Figure 1C-D - Binding assays need statistical analysis. Please provide a brief explanation of how the binding assay was performed when introducing the result. This appears to be a fluorescent cell surface binding assay. Please provide an example fluorescent image for each experimental condition (this would be fine as a supplemental figure). Why is this assay preferred over a biochemical analysis of binding – like an IP/western blot?
Figure 1F-K – Please provide statistical analysis between WT and GPC3KO conditions (not just plus or minus ligand).
Figure 2 – The authors suggest that expression of transfected GPC3 was non-physiological, so they used purified ecto domain to perform assays closer to physiological range. Please provide data showing physiological expression levels. Can cellular images be provided to show that the protein is localizing as expected (i.e. on the cell surface)?
Figure 2C – looks like the arrow might be misaligned.
Figure 2E – Why is the Gli response lower in WT cells exposed to GPC3 ecto and ALFA? This result is confusing. Is it interfering? Shouldn't everything be similar to control in the WT background? The authors mention it is likely a dominant inhibition of endogenous GPC3. This is speculation. It needs to be tested to rule out a nonspecific effect. What happens if you add GPC3 ecto alfa to WT cells expressing Alfa-NB::TM like you do for the GPC3 KO? Does it increase the GLI response?
Figure 3 – Does GAG modification impact cell surface association?
What is the Simpson-Golabi-Behmel syndrome mutation, and is it predicted to impact GAG attachment? Do SGB mutants have similar effects to the GAG mutants examined here?
Figure 3C – Is the difference between SAG for control vs ECTO GAG+ and GAG- significant? Please add statistical analysis. What happens if these experiments are performed in GPC3 knockout cells?
Figure 4B – How was the GPC3 ecto fragment purified? Why so many bands? Are other non-specific proteins co-purifying that may account for the size migration changes plus or minus heparanase and/or chondroitinase? I suspect a western blot would provide clearer/more conclusive results.
Figure 4C – From the data in this panel, the authors conclude that heparan is the essential modification on GPC3 that is important for its effect on HH signaling. Looks like both heparan and chondroitin are important to some extent because the Gli response is reduced to some extent by all Ecto domain proteins regardless of modification. Please provide statistical analysis compared to the control HH response for all Ecto fragments.
Figure 5 – How do these different GPC3 variants affect signaling in GPC3 KO cells? Can you of them rescue?
The authors never clarified how GPC3 loss and over-expression of the ecto-domain yield the same phenotype. What is lost from the ectodomain that is present in the full-length protein? Might this provide information about the mechanism by which GPC3 impacts pathway activity? Alternatively, might the antibody tether be putting GPC3 ecto in an altered conformation that could alter associate with a binding partner that is leading to its inhibitory activity?

The manuscript really needs to include mechanistic investigation of how GPC3 loss (or ecto-fragment addition) blocks GLI activity. Otherwise, it's unclear whether this is a specific effect of GPC3 through the HH pathway or through an accessory/modifying protein that is not a core pathway member, but can impact HH pathway activity. Might GPC3 be affecting GPR161? Since GPC3 is an extracellular protein, reason suggests that it is likely affecting a protein with an extracellular domain. Without finding the specific link between GPC3 and GLI, the study provides only an incremental advance.
Please check to see whether GPR161 exits the PC following pathway activation.
You should also check whether KIF7 shows normal ciliary distribution in GPC3 KO cells, since it is suggested in the text that GPC3 effects might occur through KIF.
Loss of GLI3 R is de-repression and gain of GLI2A is activation. Work from the Robbins lab (in flies, PMC3677211) and Beachy lab (vertebrates, PMC4318143) suggests these activities can be functionally uncoupled. From the GLI3R results, it looks like you are specifically affecting activation. Perhaps testing this would provide mechanistic insight.

Reviewer 2

Advance summary and potential significance to field

In the manuscript entitled, "Hedgehog pathway modulation by glypican 3-conjugated heparan sulfate", Liu and colleagues present evidence that heparan sulfate-decorated GPC3 is required for HH signal transduction through the regulation of GLI ciliary localization, and that unattached, HS-decorated GPC3 can antagonize HH pathway function. This work is certainly thought-provoking, and would be of high interest to a number of researchers. However, as detailed below there are a number of technical flaws that limit enthusiasm for the work, including the major concern that MEFs may not even express GPC3, and that the authors did not examine the requirement for other GPCs that appear to be expressed at much greater levels.
Further, the authors propose a model where extracellular HS, attached only to membrane-untethered GPC3 (and not other GPCs or Syndecans) can alter intracellular GLI ciliary localization. However, the authors do not even attempt to address a mechanism for this highly provocative claim. Given the significant questions regarding the quality of the data presented and the experimental approaches, as well as the lack of evidence to explain their model, this reviewer has significant concerns about the suitability of this work for publication in its current form.

Comments for the author

Major Comments:
1. It is relatively surprising that the authors chose to perform gene editing of Gpc3 in MEFs since previously published RNAseq data in NIH/3T3 cells (an immortalized MEF line) indicates that Gpc3 is not expressed in these cells (https://www.encodeproject.org/experiments/ENCSR000CLW/). Instead, the major Glypicans expressed in these cells are Gpc1 and Gpc4, with relatively lower expression of Gpc6. Do the authors have any evidence that Gpc3 is actually expressed in their MEFs (e.g., either qPCR data, or western blot)? I am very concerned that the authors may have deleted a gene that is not even expressed in this cell line.
2. Along the above lines, have the authors attempted to target any other Glypicans expressed in these cells? Is there any consequence on HH pathway activity? Again, unless the authors provide some evidence that Gpc3 is the major Glypican expressed in their particular MEF line (and is this an immortalized MEF line? I could not find a description of how this line was generated), the question of whether other Glypicans could be equally important in HH signal transduction leaves a large gap in their analysis.
3. In Figure 1L, the authors argue that GLI1 protein induction is increased following SAG treatment in WT MEFs, but not Gpc3-null MEFs. However, inspection of the Tubulin loading control suggests that there is simply more protein loaded in the SAG-treated WT cells than in any of the other wells (cf. lane 2 with the neighboring lanes). Without any quantitation of GLI1 protein normalized to the loading control (and preferably with more than one sample), these data are not convincing.
4. The data in Figure 1F-K are interesting, but I am confused about the baseline levels of GLI in cilia in the Gpc3KO cells– why is there such an apparently significant reduction in GLI ciliary

localization in the absence of Gpc3? And why does the rescue experiment in Figure 2 fail to even bring GLI protein levels back to WT baseline levels in the Gpc3 KO cells? Comparison of GLI ciliary levels in Figure 1K and Figure 2C would suggest a very limited rescue at best.
5. Speaking of the rescue, I am concerned that the authors are using GPC3 purified from HEK293T cells, which likely express a very different set of HS biosynthetic enzymes than MEFs. A better experiment would be to use GPC3 purified from the same cell line where they assess signaling. Given that re-expressing GPC3 does not rescue HH signaling in MEFs (Figure S1B), this becomes an even bigger concern.
6. I am very concerned about the size exclusion chromatography data in Figure 2B (and Figure S2B). Why does the ~65kDa core protein (supposedly lacking GAG modification) migrate at 100kDa? The SDC1 ectodomain (Figure S2B) is also migrating at a larger than expected size (even when you account for SDC1 dimerization, you would expect it to migrate closer to 80kDa).
7. In Figure 5F, the authors perform a key experiment that is almost lost but should be expanded upon. The data indicate that GPC1 and GPC2 are unable to antagonize HH signaling, but can they rescue the HH response in Gpc3 KO cells, as the authors did using GPC3 ecto in Figure 2E? This strikes me as a key experiment that falls well within the authors' expertise.

Minor Comments:
1. In the histograms in Figure 1, the authors should include individual data points (i.e., in Figure 1B, 1E, 1G, 1I, 1K). The authors also need to indicate the type of statistical tests that were performed to assess significance.
2. I believe that there is a typo in the y-axis label in Figure 2E. It should just read mGli1, instead of mGli1R.
3. In Figure 5C, the authors claim that the GPC3 core is inactive, however the data indicate that there is a reduction in Gli1 levels in cells treated with this protein. Again, some indication of the statistical methods used would be helpful here.

First revision

Author response to reviewers' comments

Reviewer #1

Reviewer #1 says: This manuscript presents cell biological and biochemical examination of Glypican 3 (GPC3) contributions to Hedgehog (HH) pathway activity. The study suggests that GPC3 promotes the HH response at a level downstream of the pathway receptor Patched (PTCH) and signal transducer Smoothened (SMO). Disruption of GPC3 function prevents GLI transcription factor ciliary enrichment and activation without affecting ciliary trafficking of PTCH or SMO. Using cell lines engineered to specifically disrupt either heparan or chondroitin modifications revealed a specific requirement for heparan modification of GPC3 for its function in the HH pathway. Highlights of the study are that the authors are examining a regulatory step of HH signaling that is not well understood in vertebrates, development of an assay to purify ecto-GPC3 to assess its activity in vitro and development of cell lines providing the ability to manipulate specific sugar modifications of the GPC3 ecto domain.

Answer: We were happy to see that this reviewer was positive about the level of interest, importance, and the technical approach that we took.

Reviewer #1 says: A shortcoming of the manuscript is that it lacks mechanistic insight, so provides only an incremental advance in understanding of how glypicans fit into the vertebrate HH pathway.

Answer: We thank the reviewer for his/her feedback. GPC3 is an important regulator of the Hh pathway in vertebrates. Our manuscript reports a novel effect of GPC3-associated HS on the Hh pathway. Furthermore, this effect occurs, unexpectedly, downstream of SMO. While we agree that we have not pinpointed the molecular mechanism involved, we feel that our findings represent an important advance in our understanding of the role of glypicans in Hh signaling. We agree that

future mechanistic dissection will be of great interest.

Reviewer #1 says: Figure 1B - What is the baseline measurement of Gli1 in the GPC3 KO cells relative to control? Looks like baseline for each cell line is set to 1 since data are shown as fold change following SHH/SAG stimulation. Is baseline Gli1 lower?

Answer: We apologize for not making this clear. Data was normalized from 0% (untreated) to 100% activation of the Hh pathway (saturating amounts of the SMO agonist, SAG). Baseline Gli1 in WT cells is 0% +/- 0.8, while in GPC3 KO cells is 0.56% +/- 1.31. We better explained how data normalization was performed in the revised manuscript.

Reviewer #1 says: Figure 1C-D - Binding assays need statistical analysis. Please provide a brief explanation of how the binding assay was performed when introducing the result. This appears to be a fluorescent cell surface binding assay. Please provide an example fluorescent image for each experimental condition (this would be fine as a supplemental figure). Why is this assay preferred over a biochemical analysis of binding - like an IP/western blot?

Answer: As requested by the reviewer, in the revised manuscript we incorporate statistical analyses that demonstrate that GPC3 and GPC5 have insignificant binding relative to the negative control (Figure 1C, D), and that GPC3-Ecto does not compete Shh binding to Ptch1 (Figure 1E). We have also incorporated representative fluorescence images for each experiment (Figure S1B,S1C, and S1D). These suggestions improve the strength of our conclusion that GPC3 does not bind Shh or antagonize Shh binding to Ptch1—two claims that have strongly informed the current understanding of GPC3 function in the Hh field.

Regarding the choice of binding assay: We prefer a cell-based assay over IP/Western blotting primarily because the cell-based assay more closely recapitulates how SHH, which is a secreted protein, encounters cell-surface receptors in vivo. Additionally, cell-based binding permits interrogation of interactions with receptors (such as PTCH1) that are too difficult to purify in the amounts required for purely in vitro binding studies. Finally, cell-based binding assays were previously used in the study (Capurro et al., 2008) that originally reported the GPC3-SHH interaction – thus we wanted to use a similar assay to verify this claim.

Reviewer #1 says: Figure 1F-K - Please provide statistical analysis between WT and GPC3KO conditions (not just plus or minus ligand).

Answer: We apologize for the oversight. In the revised manuscript, we are providing the statistical analysis requested by the reviewer (Figure 1 H, J, L of the revised manuscript).

Reviewer #1 says: Figure 2 - The authors suggest that expression of transfected GPC3 was non-physiological, so they used purified ecto domain to perform assays closer to physiological range. Please provide data showing physiological expression levels. Can cellular images be provided to show that the protein is localizing as expected (i.e. on the cell surface)?

Answer: We apologize for the confusion. We meant that we could not establish MEF lines that stably express GPC3, which we clarify in the revised manuscript.
We previously validated cell-surface recruitment of ALFA-tagged EGFP, and we used this system to rescue deletion of another GPI-anchored protein, GAS1 (Wierbowski, B.M, 2020). In the revised manuscript, we added images that demonstrate the recruitment of GPC3-Ecto-ALFA to the cell surface, as requested by the reviewer (Figure S2).

Reviewer #1 says: Figure 2C - looks like the arrow might be misaligned.

Answer: In the revised manuscript, we replaced the arrow with a bracket, to better indicate the GAG modification.

Reviewer #1 says: Figure 2E - Why is the Gli response lower in WT cells exposed to GPC3 ecto and ALFA? This result is confusing. Is it interfering? Shouldn't everything be similar to control in the WT background? The authors mention it is likely a dominant inhibition of endogenous GPC3. This is speculation. It needs to be tested to rule out a nonspecific effect.

Answer: This is important to clarify. WT cells treated with GPC3-Ecto or GPC3-Ecto-ALFA exhibit a blunted response to Hh pathway activation by SAG. In contrast, GPC3-Ecto and GPC3-Ecto-ALFA have no effect on the response of GPC3KO cells to SAG (which response is already reduced). Furthermore, GPC3-Ecto-ALFA, but not GPC3-Ecto, rescues signaling in the GPC3KO cells expressing NbALFA, arguing against a nonspecific inhibitory effect of the two GPC3-Ecto proteins. These data support the idea that GPC3-Ecto has dominant- negative activity towards full-length GPC3.

Reviewer #1 says: What happens if you add GPC3 ecto alfa to WT cells expressing Alfa-NB::TM like you do for the GPC3 KO? Does it increase the GLI response?

Answer: This is a good suggestion. Based on the result we observe when we transiently overexpress GPC3 in wild-type cells (Figure S1E, S1G), we expect that superphysiological levels of GPC3-Ecto recruited to the cell surface will impair the GLI response as well. To test this idea, we performed a titration of GPC3-Ecto-ALFA over a broader range of concentrations as shown in Figure 2C and Figure S2B. We observed a dose dependent rescue effect up to 250nM. However, at 500nM, Hh pathway activation begins to be suppressed again. This result is consistent with tight regulation of GPC3 levels on the cell surface, where either too little or too much suppresses Hh pathway activation. We thank the reviewer for bringing up this issue.

Reviewer #1 says: Figure 3 – Does GAG modification impact cell surface association?

Answer: We have consistently observed low levels of GPC3-Ecto binding to cells, in cell-based binding assays. It is quite likely that glycan modification will affect cell-surface association of unmodified GPC3-Ecto, although it is not clear in which direction. However, our NbALFA recruitment experiments show that enforced GPC3-Ecto recruitment to the cell surface is clearly required for its activity in Hh signaling.

Reviewer #1 says: What is the Simpson-Golabi-Behmel syndrome mutation, and is it predicted to impact GAG attachment? Do SGB mutants have similar effects to the GAG mutants examined here?

Answer: We thank the reviewer for raising this interesting point. Large deletions, frameshifts and truncating nonsense mutations account for 75% of known GPC3 lesions in SGBS. Most such mutations, which terminate in the GPC3 core protein, likely result in misfolded protein. There are a small number of GPC3 missense mutations (~8%), two of which were previously characterized (Vuillaume, ML et.al 2018). One caused protein misfolding, and the other, which occurred in the GPI attachment sequence, resulted in production of HS- modified GPC3-Ecto. These results are consistent with our study, including the dominant negative role we observe for GPC3-Ecto. We cite and discuss this work in the revised manuscript.

Reviewer #1 says: Figure 3C – Is the difference between SAG for control vs ECTO GAG+ and GAG- significant? Please add statistical analysis. What happens if these experiments are performed in GPC3 knockout cells?

Answer: In the revised manuscript, we added the statistical analysis requested by the reviewer. Ciliary recruitment of Smo in response to SAG is not significantly changed under these different conditions.

Reviewer #1 says: Figure 4B – How was the GPC3 ecto fragment purified? Why so many bands? Are other non-specific proteins co-purifying that may account for the size migration changes plus or minus heparanase and/or chondroitinase? I suspect a western blot would provide clearer/more conclusive results.

Answer: We apologize for the confusion caused by not labeling the various species in Figure 4B. GPC3-ecto was purified by affinity followed by gel filtration (illustrated in Figure 2B), so it is of high purity. Some of the additional bands in Figure 4B (~105, 90, 75 for heparinase lanes, ~110 for chondroitinase lanes) represent the GST-tagged enzymes themselves and/or degradation products thereof. Additionally, GPC3 undergoes incomplete furin-mediated proteolytic cleavage, further adding to the number of bands. In the revised manuscript, we updated the labeling of Figure 4B

and added a schematic (Figure S4F), to better clarify the identity of the bands.

Reviewer #1 says: Figure 4C – From the data in this panel, the authors conclude that heparan is the essential modification on GPC3 that is important for its effect on HH signaling. Looks like both heparan and chondroitin are important to some extent because the Gli response is reduced to some extent by all Ecto domain proteins regardless of modification. Please provide statistical analysis compared to the control HH response for all Ecto fragments.

Answer: HS-modified GPC3-Ecto is consistently responsible for most of the Hh inhibitory activity that we observe. At high concentrations, however even unmodified GPC3-Ecto seems to have a contribution. The data in Figure 4C argues strongly against involvement of chondroitin sulfate, since there is no significant difference between GPC3-Ecto preparations from B3GAT3KO and EXT1 KO cells. In response to this request by the reviewer, we have updated the statistical analysis, to compare all GPC3-Ecto versions to the control condition. We also clarify these issues in the text/figure legends.

Reviewer #1 says: Figure 5 – How do these different GPC3 variants affect signaling in GPC3 KO cells? Can you of them rescue?

Answer: The various purified GPC chimeras in Figure 5 were used on WT cells, to pinpoint the modified GPC3 stalk as critical for the inhibitory effect on Hh signaling. These chimeras do not contain an ALFA tag, thus they cannot be used to assay rescue using the NbALFA cell-surface recruitment system. Presumably, the chimeras with inhibitory activity should also rescue loss of GPC3 if artificially recruited to the cell surface.

Reviewer #1 says: The authors never clarified how GPC3 loss and over-expression of the ecto-domain yield the same phenotype. What is lost from the ectodomain that is present in the full-length protein? Might this provide information about the mechanism by which GPC3 impacts pathwayactivity?

Answer: These are important issues, and we apologize for the confusion. We find that loss of GPC3, overexpression of GPC3, and expression/addition of untethered GPC3-Ecto all inhibit Hh pathway activation. This suggests that GPC3 levels at the cell surface must be maintained within a certain window to support proper Hh signaling. The simplest explanation is that GPC3 functions as a scaffold that brings two other factors, Factor X and Factor Y, together on the cell surface to promote a signaling event that, downstream, impacts Gli protein recruitment to cilia. When GPC3 is absent, Factor X and Factor Y cannot be brought together, preventing the downstream effect. In contrast, when GPC3 levels are too high, excess GPC3 may interfere with formation of productive Factor X–GPC3–Factor Y ternary complexes. GPC3-Ecto may function in a similar way, by titrating Factor X and/or Factor Y away from endogenous Factor X–GPC3–Factor Y complexes. We mention this scenario in the Discussion of the revised manuscript, and we thank the reviewer for this question.

Reviewer #1 says: Alternatively, might the antibody tether be putting GPC3 ecto in an altered conformation that could alter associate with a binding partner that is leading to its inhibitory activity?

Answer: We apologize for any confusion. The ALFA-tag is only present on GPC3-Ecto protein, and the NbALFA antibody tether is only expressed, where indicated (Figure 2). The condition in which GPC3-Ecto- ALFA and NbALFA are used together results in rescue of the GPC3 loss phenotype, not inhibition. We also mention that the ALFA peptide is attached to GPC3-Ecto via a flexible linker, with the purpose of preventing any steric clashes. Thus, there should be no reason for concern that the antibody tether might affect GPC3- Ecto conformation in a way that causes inhibitory activity. We thank the reviewer for raising this issue.

Reviewer #1 says: The manuscript really needs to include mechanistic investigation of how GPC3 loss (or ecto-fragment addition) blocks GLI activity. Otherwise, it's unclear whether this is a specific effect of GPC3 through the HH pathway or through an accessory/modifying protein that is not a core pathway member, but can impact HH pathway activity.

Answer: We thank the reviewer for bringing this up. We have adjusted the manuscript to make it clearer that we are not claiming that GPC3 directly impacts a core pathway component. We show that GPC3 affects Gli localization to cilia, but it is difficult to imagine a direct effect on Gli1. We believe it is more likely that GPC3 plays a role in a parallel signaling process, which modifies Gli1 localization to cilia.

Reviewer #1 says: Might GPC3 be affecting GPR161? Since GPC3 is an extracellular protein, reason suggests that it is likely affecting a protein with an extracellular domain.

Answer: This is an interesting idea, and we thank the reviewer for suggesting it. GPR161 is a constitutive repressor of Hh signaling, epistatic to Smo. GPR161 acts at the level of the Gli3 repressor (Gli3R), which is completely lost in the absence of GPR161. In contrast, we see no change in Gli3R levels in GPC3KO cells, or any effect of GPC3-Ecto on the response of Gli3R to Hh pathway activation by SAG. These results suggest that the effect of GPC3 occurs by a distinct mechanism. In the revised manuscript, we mention this difference between the GPR161 and GPC3 phenotypes in Hh signaling.

Reviewer #1 says: You should also check whether KIF7 shows normal ciliary distribution in GPC3 KO cells, since it is suggested in the text that GPC3 effects might occur through KIF.

Answer: We are speculating that GPC3 might act via KIF7. In the revised manuscript, we make clear in the Discussion that this idea requires further testing, in the context of identifying the molecular target of GPC3 in Hh signaling.

Reviewer #1 says: Loss of GLI3 R is de-repression and gain of GLI2A is activation. Work from the Robbins lab (in flies, PMC3677211) and Beachy lab (vertebrates, PMC4318143) suggests these activities can be functionally uncoupled. From the GLI3R results, it looks like you are specifically affecting activation. Perhaps testing this would provide mechanistic insight.

Answer: This is a great suggestion by the reviewer. Testing if and especially how GPC3 affects specifically Gli activation is an important future direction for us.

References:

Capurro MI et al. Glypican-3 inhibits Hedgehog signaling during development by competing with patched for Hedgehog binding. Dev Cell. 2008 May;14(5):700-11.

Wierbowski, B.M et al. Hedgehog pathway activation requires coreceptor-catalyzed, lipid dependent relay of the Sonic Hedgehog ligand. Dev Cell, 2020. 55(4):p.450-467.e8.

Vuillaume ML etl al. A. Mutation update for the GPC3 gene involved in Simpson-Golabi-Behmel syndrome and review of the literature. Hum Mutat. 2018 Jun;39(6):790-805.

Reviewer #2

Reviewer #2 says: In the manuscript entitled, "Hedgehog pathway modulation by glypican 3-conjugated heparan sulfate", Liu and colleagues present evidence that heparan sulfate-decorated GPC3 is required for HH signal transduction through the regulation of GLI ciliary localization, and that unattached, HS-decorated GPC3 can antagonize HH pathway function. This work is certainly thought-provoking, and would be of high interest to a number of researchers.

Answer: We were happy to see that this reviewer was positive about the novelty and level of interest of our work.

Reviewer #2 says: Further, the authors propose a model where extracellular HS, attached only to membrane- untethered GPC3 (and not other GPCs or Syndecans) can alter intracellular GLI ciliary localization. However, the authors do not even attempt to address a mechanism for this highly provocative claim.

Answer: We thank the reviewer for his/her feedback. GPC3 is an important regulator of the Hh pathway in vertebrates. Our manuscript reports a novel effect of GPC3-associated HS on the Hh pathway. Furthermore, this effect occurs, unexpectedly, downstream of SMO. While we agree that we have not pinpointed the molecular mechanism involved, we feel that our findings represent an important advance in our understanding of the role of glypicans in Hh signaling. We agree that future mechanistic dissection will be of great interest.

Reviewer #2 says: It is relatively surprising that the authors chose to perform gene editing of Gpc3 in MEFs since previously published RNAseq data in NIH/3T3 cells (an immortalized MEF line) indicates that Gpc3 is not expressed in these cells (https://www.encodeproject.org/experiments/ENCSR000CLW/). Instead, the major Glypicans expressed in these cells are Gpc1 and Gpc4, with relatively lower expression of Gpc6. Do the authors have any evidence that Gpc3 is actually expressed in their MEFs (e.g., either qPCR data, or western blot)? I am very concerned that the authors may have deleted a gene that is not even expressed in this cell line.

Answer: This is an important issue, and we thank the reviewer for bringing it up. In the revised manuscript, we include data that shows that GPC3 is indeed expressed in MEFs, by qRT-PCR (Figure S1A).

Reviewer #2 says: Along the above lines, have the authors attempted to target any other Glypicans expressed in these cells? Is there any consequence on HH pathway activity? Again, unless the authors provide some evidence that Gpc3 is the major Glypican expressed in their particular MEF line (and is this an immortalized MEF line? I could not find a description of how this line was generated), the question of whether other Glypicans could be equally important in HH signal transduction leaves a large gap in their analysis.

Answer: We thank the reviewer for his/her comments. Regarding the other members of the vertebrate glypican family: we made CRISPR knockouts for GPCs 1, 2, 3 and 5. Unexpectedly, the cells in which we knocked out GPC1, 2 or 5 had abnormal ciliogenesis; as expected, this correlated with impaired Hh signaling, as cilia are essential for Hh pathway activation. GPC3 knockout cells had normal ciliogenesis. Furthermore, between GPC1, 2 and 3 ectodomains (we were unable to purify enough GPC5-Ecto), we only observed dominant- negative effects on Hh signaling with GPC3-Ecto (Figure 5F).

Regarding the MEFs we used: they are an immortalized line that we received as a gift from Dr. James Woodgett (University of Toronto). We mention this in the Methods section of the revised manuscript.

Reviewer #2 says: In Figure 1L, the authors argue that GLI1 protein induction is increased following SAG treatment in WT MEFs, but not Gpc3-null MEFs. However, inspection of the Tubulin loading control suggests that there is simply more protein loaded in the SAG-treated WT cells than in any of the other wells (cf. lane 2 with the neighboring lanes). Without any quantitation of GLI1 protein normalized to the loading control (and preferably with more than one sample), these data are not convincing.

Answer: We thank the reviewer for pointing this out. The qRT-PCR data already demonstrates the increase in Gli1 expression. We also provide a blot for Gli1 that makes this point in Figure 3. In the revised Figure 1, we removed Gli1 panel from Figure 1M.

Reviewer #2 says: The data in Figure 1F-K are interesting, but I am confused about the baseline levels of GLI in cilia in the Gpc3KO cells- why is there such an apparently significant reduction in GLI ciliary localization in the absence of Gpc3?

Answer: This is a good question, and we thank the reviewer for it. We are not sure why basal Gli levels at cilia appear to drop in the GPC3KO cells, though we agree this is potentially an interesting observation. Even at baseline, Gli is trafficked into and out of cilia. If GPC3 plays some role in controlling Gli trafficking or stabilization in cilia, as our evidence suggests, it is possible that GPC3 contributes to basal Gli levels in cilia as well.

Reviewer #2 says: … why does the rescue experiment in Figure 2 fail to even bring GLI protein levels back to WT baseline levels in the Gpc3 KO cells? Comparison of GLI ciliary levels in Figure 1K and Figure 2C would suggest a very limited rescue at best.

Answer: We apologize for the confusion. Figure 1K and Figure 2C show separate experiments, and we display ciliary localization as arbitrary fluorescence units. In general, we compare intensity differences only within the same experiment, since absolute fluorescence values vary between experiments (with cell density, precise staining conditions, exposure time, excitation illumination intensity, etc). In addition, the two experiments were conducted on a different time scale (24-hour treatment with SAG for Figure 1G-L, and 6-hour treatment for Figure 2C). We clarify these issues in the revised manuscript and thank the reviewer or pointing it out.

Reviewer #2 says: Speaking of the rescue, I am concerned that the authors are using GPC3 purified from HEK293T cells, which likely express a very different set of HS biosynthetic enzymes than MEFs. A better experiment would be to use GPC3 purified from the same cell line where they assess signaling.

Answer: We agree that it would be better if it were technically possible to purify recombinant GPC3-Ecto produced by MEFs. However, the yield of protein production by MEFs is insufficient to carry out this experiment. GPC3-Ecto-ALFA produced from HEK293T cells does rescue when recruited to the GPC3KO cells via the NbALFA system - this is strong evidence that HEK293T cells have the required GAG biosynthetic machinery to produce functional GPC3. We thank the reviewer for raising this issue.

Reviewer #2 says: I am very concerned about the size exclusion chromatography data in Figure 2B (and Figure S2B). Why does the ~65kDa core protein (supposedly lacking GAG modification) migrate at 100kDa? The SDC1 ectodomain (Figure S2B) is also migrating at a larger than expected size (even when you account for SDC1 dimerization, you would expect it to migrate closer to 80kDa).

Answer: We apologize for the confusion. Our proteins are tagged with HaloTag, as described in the Methods section - this improves their secretion yield. This tag contributes an additional ~40 kDa. For improved clarity, we mention this fact in the figure legends of the revised manuscript (Figure S3B).

Reviewer #2 says: In Figure 5F, the authors perform a key experiment that is almost lost, but should be expanded upon. The data indicate that GPC1 and GPC2 are unable to antagonize HH signaling, but can they rescue the HH response in Gpc3 KO cells, as the authors did using GPC3 ecto in Figure 2E? This strikes me as a key experiment that falls well within the authors' expertise.

Answer: We apologize for the misunderstanding. This experiment was conducted in WT MEFs, and GPC1- Ecto and GPC2-Ecto are unable to antagonize HH signaling in their wild-type form. However, the chimeras GPC3stalk-GPC1core and GPC3stalk-GPC2core antagonize HH signaling, while the reciprocal chimeras are inactive. This experiment indicates that it is the GPC3 stalk that is responsible for the inhibitory effect.

Reviewer #2 says: In the histograms in Figure 1, the authors should include individual data points (i.e., in Figure 1B, 1E, 1G, 1I, 1K). The authors also need to indicate the type of statistical tests that were performed to assess significance.

Answer: As requested by the reviewer, in the revised manuscript we include individual data points and we provide more detailed explanations of the statistical tests performed.

Reviewer #2 says: I believe that there is a typo in the y-axis label in Figure 2E. It should just read mGli1, instead of mGli1R.

Answer: We apologize for this mistake, which has been corrected in the revised manuscript.

Reviewer #2 says: In Figure 5C, the authors claim that the GPC3 core is inactive, however, the data indicate that there is a reduction in Gli1 levels in cells treated with this protein. Again, some indication of the statistical methods used would be helpful here.

Answer: Indeed, the GPC3 core may have some activity (this could be due to its contribution to the interaction that GPC3 must engage in on the cell surface); however, the effect appears small and is not statistically significant. As requested by the reviewer, we clarify the statistical methods used in the figure legends of the revised manuscript.

Second decision letter

MS ID#: JOCES/2021/259297

MS TITLE: Hedgehog pathway modulation by glypican 3 conjugated heparan sulfate

AUTHORS: Yulu Cherry Liu, Bradley M Wierbowski, and Adrian Salic
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks. I apologize that the Decision of Acceptance took a longer time than usual because one of the reviewers did not return his/her comments on your revision and I had to review the revised manuscript myself.

Reviewer 1

Advance summary and potential significance to field

This manuscript presents cell biological and biochemical examination of Glypican 3 (GPC3) contributions to Hedgehog (HH) pathway activity. The study suggests that GPC3 promotes the HH response at a level downstream of the pathway receptor Patched (PTCH) and signal transducer Smoothened (SMO). Disruption of GPC3 function prevents GLI transcription factor ciliary enrichment and activation without affecting ciliary trafficking of PTCH or SMO. Using cell lines engineered to specifically disrupt either heparan or chondroitin modifications revealed a specific requirement for heparan modification of GPC3 for its function in the HH pathway. Highlights of the study are that the authors are examining a regulatory step of HH signaling that is not well understood in vertebrates, development of an assay to purify ecto-GPC3 to assess its activity in vitro and development of cell lines providing the ability to manipulate specific sugar modifications of the GPC3 ecto domain.

Comments for the author

I am satisfied with the revised submission.