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A cytoplasmic protein kinase couples engagement of *Chlamydomonas* ciliary receptors to cAMP-dependent cellular responses

Mayanka Awasthi, Peeyush Ranjan, Simon Kelterborn, Peter Hegemann and William J. Snell
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MS ID#: JOCES/2022/259814

MS TITLE: A cytoplasmic protein transduces a ciliary signal into the large increase in cytoplasmic cAMP essential for fertilization in Chlamydomonas

AUTHORS: Mayanka Awasthi, Peeyush Ranjan, Simon Kelterborn, Peter Hegemann, and William J. Snell
ARTICLE TYPE: Short Report

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

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

Awasthi et al., identified a cytoplasmic kinase mediating the flagella adhesion into the increase in cytoplasmic cAMP, which is essential for fertilization in Chlamydomonas. The phosphorylation of this protein is dramatically quick. This work added a critical component in the mating signal transduction pathway in this cilia research model. It will speed the research in the communication between the cilia and cell body and the role of IFT in this process. In general, the data support the conclusion and is very useful to the community of cilia and Chlamydomonas.

Comments for the author

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1.The figure legends are too long and have too much detail; usually, they will not include references.
2.In the Figure 2, the control was Wt(+) X hap(-), why not use the Wt(+) X Wt(-)?
3.In Figure 3B, please add the molecular weight of SAG-1-HA.
4.In lines 205 to lines 207, the authors made such a conclusion" Taken together these results indicated that although cytoplasmic response to ciliary adhesion depended on GSPK, ciliary responses were independent of the protein." But from Figure 3B and Figure 3C, maintaining the level of SAG-1-HA in cilia and the aggregation of gametes at 45 minutes is dependent on GSPK; please clarify this discrepancy.
5.The subtitle between line 234 to line 237 is too long.

Reviewer 2

Advance summary and potential significance to field

Report on Awasthi et al. JOCES-2022-2598814v1

Cilia-based signaling involves the transmission of signals received by the cilium to the cell body to initiate certain downstream responses, such as changes in gene expression. In primary cilium signaling, the signal transmission pathways are often incompletely understood. Here, the authors identify Gamete-Specific Protein Kinase (GSPK) as a first/early responder located within the cell body to cilia adhesion signaling during Chlamydomonas mating. The mating reaction triggered by cilium-to-cilium contacts between gametes of the opposite mating type involves cilium-to-cell body signaling and is the only such pathway known in Chlamydomonas. Mutants in GSPK obtained from the CLiP library or generated by CRISPR, fail to fuse and to initiate other mating-relevant cell body responses whereas the early, cilia-based steps of the signaling cascade are normal. This suggests that GSPK acts at the intersection between the intraciliary and cell body steps of the pathway. The Snell lab has previously characterized other components of this signaling pathway. The experiments shown in this study are carried out with the great expertise testing various aspects and steps of the mating reaction (e.g., cAMP production, cell wall shedding, mating structure development, SAG transport in the cell body and into cilia, cell fusion); the data support the conclusions. The identification of GSPK as an essential player in the mating reaction of Chlamydomonas is, in my opinion, a significant step towards understanding this signaling pathway. To summarize, I believe that this report adds a critical piece to the signaling pathway leading from flagellar adhesion to cell fusion.

Comments for the author

Suggestions for the authors
1. As the authors generated a GSPK-HA rescue strain. I wonder if they have tried to determine the subcellular localization of the protein by immunofluorescence? The results should be reported as readers will be curious whether the protein has a focused subcellular localization, e.g., near the ciliary base?
2. The observation that gspk mutant cells fail to assemble mating structure is important as in nicely illustrtaes the defects of the mutant cell bodies to respond to cilia adhesion. Figure S5, in a condensed form or parts of it, should be moved to the main text as a new panel of Fig. 2.
3. Whereas db-cAMP induces most mating-relevant responses in the gspk mutant, it does not trigger GSPK-HA phosphorylation, which, however, is observed in adhering gametes. As acknowledged by the authors, the study raises a conundrum with respect to the role of cAMP in cilia-to-cell body signaling, since cilia generated cAMP is thought to trigger the down-stream cell body events. While I think the experiments provided in the study are sufficient, the observation raises in my opinion the question if phosphorylation of GSPK is a (necessary) part of the signaling process. Also, it is unclear, if phosphorylation indeed modulates the activity of GSPK. Playing devil's advocate, can the authors exclude that the basic phosphorylation of GSPK-HA is indeed occurring on GSPK and not on the HA moiety? It has been repeatedly observed, that the HA tag itself becomes phosphorylated within the Chlamydomonas ciliary compartment. May be GSPK or some other mating relevant kinase phosphorylates HA tags upon activation. As the specific role of GSPK and its phosphorylation in the signal transduction pathway remain unclear, the discussion could be moderated at times (e.g., "GSPK is present in the cytoplasm and detects the signal within 1 minute after SAG1-SAD1 engagement in the cilia.")
Related to the figure no. 3: Is the x hap2(-) blot in Fig. 4B identical with the 0 and 1 min lanes of the blot shown in panel C?
Additional minor suggestions
- The Main text and supplementary material sections have different titles.
(A cytoplasmic protein (KINASE??) transduces a ciliary signal into the large increase in 3 cytoplasmic cAMP essential for fertilization in Chlamydomonas. And Title: A cytoplasmic protein kinase in Chlamydomonas couples engagement of ciliary receptors to rapid cellular responses (I LIKE THIS ONE BETTER).
- abstract: "spatially distinct cellular compartment". Isn't being spatially distinct part of the definition of a compartment?
"During ciliary adhesion, however, the ciliary barrier is relaxed to allow SAG1 entry and concomitantly SAG1 is also actively recruited from the plasma membrane to become enriched in the cilia as part of a mechanism to support and enhance ciliary adhesion (Cao et al., 2015; Goodenough, 1989; Goodenough and Heuser 1999; Hunnicutt et al., 1990; Ranjan et al., 2019; Saito et al., 1985; Snell 1976; Snell and Moore, 1980; Wiese, 1965)."
On occasion, it would be more convenient for the reader (and referee) to find the relevant original data if only the paper(s) that first made the observations would be cited.
"-Although the phosphorylation state of GSPK is unresponsive to gamete 235 activation induced by db-cAMP, it is responsive to ciliary adhesion, and within 236 1 minute after ciliary adhesion is initiated, the entire cellular complement of 237 GSPK is phosphorylated."
A more condensed subheading should be provided.
-and gametes activated by incubation for 1 hour in db-cAMP buffer (G-A)
The lane in Fig. 1F is labeled differently.
As I had difficulties grasping the key observations and conclusions of the paper on the first reading it, I believe the paper could use some rewording and reorganizing.

First revision

Author response to reviewers' comments

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However, several issues need to be addressed.

1. The figure legends are too long and have too much detail; usually, they will not include references.

We have shortened the figure legends and have removed the references from figure legends wherever possible.

2. In the Figure 2, the control was Wt(+) X hap(-), why not use the Wt(+) X Wt(-)?

In a *Wt*(+) *X Wt*(-) mixtures, the gametes fuse very quickly. Because the *gspk*(-) gametes undergo prolonged adhesion but are strongly reduced in fusion, we felt the appropriate control cells for these experiments would be *hap2*(-) gametes.
hap2(-) gametes lack the conserved gamete fusogen, HAP2, and when mixed with *plus* gametes also continue to undergo prolonged adhesion without fusion. This reasoning is included in the Figure 2 legend.

3. In Figure 3B, please add the molecular weight of SAG-1-HA.

The molecular weight of SAG1-HA is now included in the figure.

4. In lines 205 to lines 207, the authors made such a conclusion" Taken together, these results indicated that although cytoplasmic response to ciliary adhesion depended on GSPK, ciliary responses were independent of the protein." But from Figure 3B and Figure 3C, maintaining the level of SAG-1-HA in cilia and the aggregation of gametes at 45 minutes is dependent on GSPK; please clarify this discrepancy.

We agree that the original sentence was confusing. It has been re-written to indicate that the immediate responses of cilia to ciliary adhesion do not depend on GSPK, but that cell body responses to adhesion do require the protein.

5. The subtitle between line 234 to line 237 is too long.

The subtitle has been shortened.

Reviewer 2 Advance Summary and Potential Significance to Field:
Report on Awasthi et al. JOCES-2022-2598814v1

Cilia-based signaling involves the transmission of signals received by the cilium to the cell body to initiate certain downstream responses, such as changes in gene expression. In primary cilium signaling, the signal transmission pathways are often incompletely understood. Here, the authors identify Gamete-Specific Protein Kinase (GSPK) as a first/early responder located within the cell body to cilia adhesion signaling during Chlamydomonas mating. The mating reaction triggered by cilium-to- cilium contacts between gametes of the opposite mating type involves cilium-to-cell body signaling and is the only such pathway known in Chlamydomonas. Mutants in GSPK obtained from the CLiP library or generated by CRISPR, fail to fuse and to initiate other mating-relevant

cell body responses whereas the early, cilia-based steps of the signaling cascade are normal. This suggests that GSPK acts at the intersection between the intraciliary and cell body steps of the pathway. The Snell lab has previously characterized other components of this signaling pathway. The experiments shown in this study are carried out with the great expertise testing various aspects and steps of the mating reaction (e.g., cAMP production, cell wall shedding, mating structure development, SAG transport in the cell body and into cilia, cell fusion); the data support the conclusions. The identification of GSPK as an essential player in the mating reaction of Chlamydomonas is, in my opinion, a significant step towards understanding this signaling pathway. To summarize, I believe that this report adds a critical piece to the signaling pathway leading from flagellar adhesion to cell fusion.

Reviewer 2 Comments for the Author: Suggestions for the authors.

1. As the authors generated a GSPK-HA rescue strain. I wonder if they have tried to determine the subcellular localization of the protein by immunofluorescence? The results should be reported as readers will be curious whether the protein has a focused subcellular localization, e.g., near the ciliary base?

We feel that our cell fractionation and immunoblotting results are compelling and show that nearly all GSPK is in the cell body. We agree that determining the subcellular localization of GSPK will be exciting and important. Although, our imaging facility is available now, access had been difficult because of COVID restrictions. We feel it will be essential to carry out a thorough analysis of GSPK subcellular localization in resting gametes. But it will also be important to determine whether adhesion-induced phosphorylation of GSPK alters its subcellular localization, and whether its localization changes in gametes of a single mating type activated by incubation in cAMP. Carrying out such extensive analyses will require substantial time and effort, and we hope the reviewer will agree that they are beyond the scope of this current manuscript.

2. The observation that gspk mutant cells fail to assemble mating structure is important as in nicely illustrates the defects of the mutant cell bodies to respond to cilia adhesion. Figure S5, in a condensed form or parts of it, should be moved to the main text as a new panel of Fig. 2.

The images documenting that, unlike WT(+) gametes, *gspk*(+) gametes fail to assemble mating structures are now included in Figure 2.

3. *Whereas db-cAMP induces most mating-relevant responses in the gspk mutant, it does not trigger GSPK-HA phosphorylation, which, however, is observed in adhering gametes. As acknowledged by the authors, the study raises a conundrum with respect to the role of cAMP in cilia-to-cell body signaling, since cilia generated cAMP is thought to trigger the down-stream cell body events. While I think the experiments provided in the study are sufficient, the observation raises in my opinion the question if phosphorylation of GSPK is a (necessary) part of the signaling process. Also, it is unclear, if phosphorylation indeed modulates the activity of GSPK. Playing devil's advocate, can the authors exclude that the basic phosphorylation of GSPK-HA is indeed occurring on GSPK and not on the HA moiety? It has been repeatedly observed, that the HA tag itself becomes phosphorylated within the Chlamydomonas ciliary compartment. May be GSPK or some other mating relevant kinase phosphorylates HA tags upon activation. As the specific role of GSPK and its phosphorylation in the signal transduction pathway remain unclear, the discussion could be moderated at times (e.g., "GSPK is present in the cytoplasm and detects the signal within 1 minute after SAG1-SAD1 engagement in the cilia.").*

We are looking forward to learning much more about the molecular mechanisms of GSPK function in signaling, including determining whether GSPK phosphorylation is a necessary part of the signaling process. On the possibility that phosphorylation might occur on the HA moiety itself within the ciliary compartment: We note that it is highly likely that GSPK phosphorylation occurs in the cytoplasm, since it is unlikely that the entire complement of GSPK could traffic through the cilia within I minute. Also, because we were unaware of experiments showing that the HA tag itself can be phosphorylated in the *Chlamydomonas* ciliary compartment, we searched for examples in the literature, without success. We also did more general PubMed and Google searches for phosphorylation of the HA moiety, again with no success. In addition, we have extensively studied HA-tagged forms of the *plus* gamete mating structure adhesion protein FUS1-HA and the *minus* gamete fusion protein HAP2-HA in gametes undergoing ciliary adhesion and gamete activation and have never detected any evidence that they were phosphorylated. Although these are membrane proteins, the HA tag is in the cytoplasmic domain. Also submission of the sequence of the 3X HA

epitope used for the tag to the NetPhos-3.1 Server indicates that the probability of phosphorylation on the serine is below 0.5 and that a tyrosine is a potential site for an unspecified protein kinase. All in all, we feel it is unlikely that phosphorylation is occurring on the HA-tag itself.

Related to the figure no. 3: Is the x hap2(-) blot in Fig. 4B identical with the 0 and 1 min lanes of the blot shown in panel C?

Yes, they are same. As the figure legend now indicates, those two lanes are reproductions of two of the lanes in Figure 4C and are included to show the shift in migration that occurs during ciliary adhesion. Even though we thought it would be obvious, we appreciate the reminder by the reviewer of the importance of being impeccable in presenting data.

Additional minor suggestions

- *The Main text and supplementary material sections have different titles. (A cytoplasmic protein (KINASE??) transduces a ciliary signal into the large increase in cytoplasmic cAMP essential for fertilization in Chlamydomonas. And Title: A cytoplasmic protein kinase in Chlamydomonas couples engagement of ciliary receptors to rapid cellular responses (I LIKE THIS ONE BETTER).*

We thank the reviewer for the careful reading of the manuscript. The new title is close to the one favored by the reviewer.

- *abstract: "spatially distinct cellular compartment". Isn't being spatially distinct part of the definition of a compartment?*

Agree! We have revised the sentence.

"During ciliary adhesion, however, the ciliary barrier is relaxed to allow SAG1 entry and concomitantly SAG1 is also actively recruited from the plasma membrane to become enriched in the cilia as part of a mechanism to support and enhance ciliary adhesion (Cao et al., 2015; Goodenough, 1989; ~~Goodenough and Heuser, 1999~~; Hunnicutt et al., 1990; Ranjan et al., 2019; Saito et al., 1985; ~~Snell, 1976~~; ~~Snell and Moore, 1980~~; ~~Wiese, 1965~~)." On occasion, it would be more convenient for the reader (and referee) to find the relevant original data if only the paper(s) that first made the observations would be cited.

The references have been revised in keeping with the reviewer's recommendation.

"-Although the phosphorylation state of GSPK is unresponsive to gamete activation induced by db-cAMP, it is responsive to ciliary adhesion, and within 1 minute after ciliary adhesion is initiated, the entire cellular complement of GSPK is phosphorylated." A more condensed subheading should be provided.

The subheading has been shortened.

-and gametes activated by incubation for 1 hour in db-cAMP buffer (G-A). The lane in Fig. 1F is labeled differently.

The label has been corrected.

As I had difficulties grasping the key observations and conclusions of the paper on the first reading it, I believe the paper could use some rewording and reorganizing.

Upon re-reading the paper with "fresh eyes," we saw several places, especially in the Introduction and Conclusion sections, that needed to be clarified and restructured. We feel the revised version is now easier to understand.

Second decision letter

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AUTHORS: Mayanka Awasthi, Peeyush Ranjan, Simon Kelterborn, Peter Hegemann, and William J. Snell
ARTICLE TYPE: Short Report

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

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