JCB

Mutual antagonism between Hippo signaling and cyclin E drives intracellular pattern formation

Yuyang Jiang, Wolfgang Maier, Uzuomaka Chukka, Michael Choromanski, Chinkyu Lee, Ewa Joachimiak, Dorota Wloga, Wayland Yeung, Natarajan Kannan, Joseph Frankel, and Jacek Gaertig

Corresponding Author(s): Jacek Gaertig, University of Georgia

Review Timeline:

Submission Date:	2020-02-17
Editorial Decision:	2020-04-14
Revision Received:	2020-05-11
Editorial Decision:	2020-05-21
Revision Received:	2020-05-27

Monitoring Editor: Daniel Lew

Scientific Editor: Tim Spencer

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

DOI: https://doi.org/10.1083/jcb.202002077

April 14, 2020

Re: JCB manuscript #202002077

Dr. Jacek Gaertig
University of Georgia
724 Biol Sciences
Athens, GA 30602

Dear Dr. Gaertig,

Thank you for submitting your manuscript entitled "Mutual antagonism between Hippo signaling and cyclin E drives intracellular pattern formation". We apologize for the delay in providing you with a decision. The interesting manuscript was positively assessed by expert reviewers, whose comments are appended to this letter.

We invite you to submit a revised manuscript that addresses the reviewers' concerns in full. Please note that while we appreciate reviewer #2's comment regarding the extensive homology modeling data and discussion being moved to the supplementary information, we feel that the phylogenetic/homology data should remain in the main body of the paper.

While you are revising your manuscript, please also attend to the following editorial points to help expedite the publication of your manuscript. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:

Text limits: Character count for an Article 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.

Figures: Articles may have up to 10 main text figures. Figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, http://jcb.rupress.org/site/misc/ifora.xhtml. All figures in accepted manuscripts will be screened prior to publication.

IMPORTANT: It is JCB policy that if requested, original data images must be made available. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original microscopy and blot data images before submitting your revision.

Supplemental information: There are strict limits on the allowable amount of supplemental data. Articles may have up to 5 supplemental figures. Up to 10 supplemental videos or flash animations are allowed. A summary of all supplemental material should appear at the end of the Materials and methods section.

As you may know, the typical timeframe for revisions is three to four months. However, we at JCB

realize that the implementation of social distancing and shelter in place measures that limit spread of COVID-19 also pose challenges to scientific researchers. Lab closures especially are preventing scientists from conducting experiments to further their research.
Therefore, JCB has waived the revision time limit. We recommend that you reach out to the editors once your lab has reopened to decide on an appropriate time frame for resubmission.

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

When submitting the revision, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

We hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised in this letter.

Thank you for this interesting contribution to Journal of Cell Biology. You can contact us at the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Sincerely,

Daniel Lew, PhD
Monitoring Editor
Journal of Cell Biology

Tim Spencer, PhD
Executive Editor
Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

Review of "Mutual antagonism between Hippo signaling and cyclin E drives intracellular pattern formation" by Jiang et al.

In this paper, the authors investigated the role of a cyclin family protein in division plane determination during tandem duplication of the ciliate Tetrahymena thermophila. They characterized previously isolated mutants that show defects in positioning of the cortical subdomain (CS) that are allelic to each other, cdaa-1 and cdaa-4. Using NGS, they identified the causative mutations in a gene previously named as CYC8, which encodes a protein that shows similarity to the Holozoan cyclin E proteins. CdaA-GFP protein was expressed and localized in an interesting pattern in-between the basal bodies in the ciliary rows, specifically in the posterior half of dividing cells. Because the position of the CS correlated with the anterior end of CdaA-GFP localization (in WT and two mutants in which CS positioning is altered), and because CdaA-GFP and CdaI-GFP showed mutually exclusive localization patterns, the authors hypothesized that the CdaA and CdaI (a Hippo homolog) exclude each other from their respective cortical domains to establish the CS. An experiment using a cdaA-1 cdaI-1 double mutant supported this hypothesis. Overall, the experiments in this study were conducted well, and most of the conclusions are

supported by the data. The work will not only be foundational for the future studies of cell division and polarity establishment in ciliates, but also has a broad significance to the larger field on mechanisms of division plane determination and the molecular function of cyclins.

Major comments.
(1) Based on my limited investigation of the literature, this work would be the first report of a "cyclin E" outside of Holozoa and Apusozoa. This notion would have a large implication in the evolution of cycling family proteins, that (a) cyclin E diverged from other cyclins in a common ancestor of most eukaryotes but has since been lost in most lineages, except for Holozoa, Apusozoa, and ciliates, or (b) a horizontal transfer event. It seems that there needs to be a more careful phylogenetic analysis than the one currently presented in this manuscript, i.e., a ML tree of all Holozoan cyclins (based on Cao et al., 2014) + ciliate CdaA homologs. At least all available cyclin sequences (not just CdaA homologs) from ciliates and other alveolates should be included in the tree to test if there is a bipartition between [CdaA+cyclin E] and [the other alveolate+holozoan cyclins].
(2) The major conclusion that CdaA and CdaI inhibits each other for their cortical localization is not experimentally demonstrated, although it is not inconsistent with the presented data, they are correlational. An alternative model would be that the two proteins function in two independent and antagonistic pathways that shift the position of CS, and the CS itself determines where the streaks of CdaA and CdaI localization end. Unless the authors have additional data to demonstrate direct inhibition between the two proteins (for example, sequestering CdaI by artificially targeting CdaA to the anterior half), more caution is recommended in interpretation and discussion.
(3) It is not very clear how the authors concluded that cdaA-1 was a loss-of-function mutation. The "rescue" strain (cdaA-1 + transformed CYC8 sequence) showed a partial phenotype, even though ~50% of the alleles in the macronucleus are WT (Fig. S2A), which does not seem to suggest a simple recessive loss-of-function mutation. Moreover, transformation of the cdaA-1 allele into the macronucleus of WT (which typically does not replace all copies according to the authors and as evidenced in the "rescue" experiment) phenocopied cdaA-1 homozygotes, suggesting a dominant phenotype. On a related note, what is the localization pattern of cdaA-1-GFP? These questions affect the interpretation of many of the data presented in this paper, such as those in Figs. 5-7.
(4) Analysis of many of the imaging data in this manuscript centers around the position of the CS and its correlation with CdaA and CdaI localizations. However, it was not clear how the presumptive cortical subdivision was determined in mutants such as cdaA, elo1, and cdaI. The centrin rows appear continuous in the images (e.g., Fig. 1G-K, Fig. 3D'E', Fig. 4 C'D', Fig. 5 C'D'), and it is difficult for a non-Tetrahymena specialist to predict where the CS will be (or should have been) formed. A more detailed description would be helpful.

Minor points:
(5) Introduction: It would be helpful for readers unfamiliar with ciliates if the authors described how the macro- and micro-nuclei function and are inherited during the vegetative division of Tetrahymena.
(6) Throughout the manuscript, it would be helpful if the panels were labelled with the names of stained proteins with clear indication of colors (as is currently done only in Fig. 7).
(7) Fig. 6 A-C' are supposed to show "pairs of images showing two sides of the same cell." Are they Different Z-sections looked from the same side, or same cells imaged from opposite sides but one of the pairs has been flipped?

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

This paper is on an interesting subject, cell division in Tetrahymena, concerning which I must admit to complete ignorance before reading the paper. The cited Frankel (2008) review was also quite helpful.

Tetrahymena is a very complicated cell with many cortical structures in stereotyped positions. Cell division involves the AP axis effectively duplicating (as per Frankel, if it starts as '12345' it goes to '1234512345') then produces a cleavage furrow between 5 and 1. Mutants were isolated previously that affect the process. The cdaI mutant starts division off fairly normally, then pushes the boundary anteriorly. The new 'oral apparatus' that also formed at a normal posterior location also moves anteriorly; the cell then can divide, producing a minicell (that apparently still has all contents), or can fail complete division. This mutant was previously shown to be a loss of function of a protein kinase related to Hippo; CDA1 largely inhabits the anterior half of the cell, suggesting that it blocks 'sliding' of the division plane to the anterior.

Here, another AP-regulatory gene, cdaA, is identified as a member of the cyclin family, by bulked-segregant analysis of two independent alleles. This is clearly and correctly done. Based on the bioinformatics presented, cdaA is most close to animal cyclin E. It is intriguing and important to know that cdaA is a cyclin, and the mutations are in residues conserved in cyclin E (conservation in other cyclins is not addressed). I can't say that there's anything in the paper beyond this point that makes it at all relevant that cdaA is a cyclin; the paper would read exactly the same if it were a protease or an ion channel, etc. This is fine, of course, but it does suggest to me that the rather extensive discussion of homology modeling, and speculation of which CDK cdaA might interact with, is more appropriate for Supplementary Information. (Different if a kinase assay was carried out; or if another mutation (cdaH, with according to Frankel a similar phenotype??) turned out to be in a CDK, etc).

The paper goes on to show striking symmetry in the behavior of cdaA and the previously published behavior of CdaI, the Hippo-related kinase. CdaI is located anteriorly, cdaA is located posteriorly; removal of either seems to expand the domains of the other. An asymmetry is that cdaA mutants don't divide at all, while cdaI mutants apparently can divide with some frequency although in general at excessively anterior positions. The double cdaA,cdaI mutant is studied, and intriguingly though it is still lethal, many of the cellular phenotypes are reverted; it does divide, I gather usually around the middle. However, interior components of the cortex are apparently disarranged.

The data in the paper are generally clear, though there is an initial investment required (at least there was for me!) to have any idea of what I was looking at.

One aspect that I did wonder about: Tetrahymena has bands of basal body/cilia in lines extending up its surface. It appeared that cdaA and I think also cdaI were localized to spots in the vicinity of the basal bodies, or at least to lines ('streaks' as the paper has it) that also include the BBs, and the localizations cited above (cdaA posterior, cdaI anterior) really apply to these lines/spots. However, it appeared to me looking e.g. at Fig. 3 that there was quite a bit of cdaA-GFP staining in the anterior of the cell, just not localized. There are a number of possibilities here. It should be shown whether this is background staining (i.e. not cdaA-GFP at all, as would be tested by including a cell lacking cdaA-GFP). If it's real, then the total level of cdaA along the AP axis (not just that along the ciliary bands) should be quantified to be sure there really is a gradient. If not, the observations are still interesting but rather different. (For example, maybe cdaA can only find a CDK kinase partner along the bands). In any case this should be addressed.

It is interesting that apparently the cell division cycle overall continues with or without cdaA, at least

this seems to be the implication of the writing here. This is worth a bit more explicit thinking. In animal cells, cyclin E contributes (at least) to activating the complete cyclin B-Cdk1-APC circuit leading to cell division; in Tetrahymena, it could be fundamentally needed just to place a division plane but otherwise not be regulatory of the primary cell cycle oscillator (whatever it is - making no assumptions that it will be cyclin B-CDK1-APC). I gather that at the least, the mitotic division of the micronucleus proceeds with normal timing in the cdaA mutant.

Anyway, overall an interesting story on a remarkable organism.

Authors response to the reviewers comments

We are grateful for the careful analyses of the manuscript, suggestions and constructive criticism. We have attempted to respond to every issue brought by the reviewers and make revisions. The detailed responses to the reviewers comments and the actions taken are below:

Reviewer #1 (Comments to the Authors (Required)):

Review of "Mutual antagonism between Hippo signaling and cyclin E drives intracellular pattern formation" by Jiang et al.

In this paper, the authors investigated the role of a cyclin family protein in division plane determination during tandem duplication of the ciliate Tetrahymena thermophila. They characterized previously isolated mutants that show defects in positioning of the cortical subdomain (CS) that are allelic to each other, cdaa-1 and cdaa-4. Using NGS, they identified the causative mutations in a gene previously named as CYC8, which encodes a protein that shows similarity to the Holozoan cyclin E proteins. CdaA-GFP protein was expressed and localized in an interesting pattern in-between the basal bodies in the ciliary rows, specifically in the posterior half of dividing cells. Because the position of the CS correlated with the anterior end of CdaA-GFP localization (in WT and two mutants in which CS positioning is altered), and because CdaA-GFP and CdaI-GFP showed mutually exclusive localization patterns, the authors hypothesized that the CdaA and CdaI (a Hippo homolog) exclude each other from their respective cortical domains to establish the CS. An experiment using a cdaA-1 cdaI-1 double mutant supported this hypothesis. Overall, the experiments in this study were conducted well, and most of the conclusions are supported by the data. The work will not only be foundational for the future studies of cell division and polarity establishment in ciliates, but also has a broad significance to the larger field on mechanisms of division plane determination and the molecular function of cyclins.

Major comments.
(1) Based on my limited investigation of the literature, this work would be the first report of a "cyclin E" outside of Holozoa and Apusozoa.

Suga et al 2013 (Nature Communications. 4:2325) already reported cyclin E sequences in ciliates (as a side note of their study of Capsaspora). Stover and Rice 2011 (Cell Cycle. 10:1699-1701) published a phylogenetic tree for cyclins of Tetrahymena where CYC8/CdaA groups with the human and Drosophila cyclin E. We have confirmed these observations and functionally linked cyclin E to cell polarity in ciliates.

This notion would have a large implication in the evolution of cycling family proteins, that (a) cyclin E diverged from other cyclins in a common ancestor of most eukaryotes but has since been lost in most lineages, except for Holozoa, Apusozoa, and ciliates, or (b) a horizontal transfer event. It seems that there needs to be a more careful phylogenetic analysis than the one currently presented in this manuscript, i.e., a ML tree of all Holozoan cyclins (based on Cao et al., 2014) + ciliate CdaA homologs. At least all available cyclin sequences (not just CdaA homologs) from ciliates and other alveolates should be included in the tree to test if there is a bipartition between [CdaA+cyclin E] and [the other alveolate+holozoan cyclins].

In the revised manuscript we included a broader phylogenetic analysis by adding all 36 cyclins of Tetrahymena and all cyclins of two non-ciliate alveolates: Toxoplasma gondii (an apicomplexan) and Breviolum minutum (a dinoflagellate). These new data (Fig. S1 B) confirm that CdaA is most closely related to cyclins E. Other Tetrahymena cyclins group with the conserved cyclins of different types including the most conserved cell cycle-associated cyclin B. We also confirmed that among the alveolates, only ciliates have cyclins E.

Clearly more work is needed to reconstruct the evolutionary history of cyclins E. We used phylogenetic analyses to determine the cyclin type that CdaA is most similar to, namely cyclin E. This finding uncovers the remarkable evolutionarily conservation of cyclin E involvement with cell polarity. We suggest that cyclin E had participated in cell polarity in the ancient eukaryotic ancestor. We agree with the reviewer that other evolutionary scenarios are to be considered such as a horizontal transfer or convergence. In the Discussion we now state: "Possibly, the association of cyclin E with cell polarity predates the emergence of Holozoa and ciliates from the common eukaryotic ancestor. However, we can not exclude a possibility that ciliates gained cyclin E during convergent evolution or by horizontal gene transfer."

(2) The major conclusion that CdaA and CdaI inhibits each other for their cortical localization is not experimentally demonstrated, although it is not inconsistent with the presented data, they are correlational.

We believe that the data shown in old Fig. 5 (now Fig. 7 and 8) show convincingly that CdaI and CdaA negatively control the positions of the margins of their respective "streak domains" prior to the cortical subdivision.

An alternative model would be that the two proteins function in two independent and antagonistic pathways that shift the position of CS, and the CS itself determines where the streaks of CdaA and CdaI localization end. Unless the

authors have additional data to demonstrate direct inhibition between the two proteins (for example, sequestering CdaI by artificially targeting CdaA to the anterior half), more caution is recommended in interpretation and discussion.

We think that the "indirect model" suggested by the reviewer, that CdaI and CdaA control their own localizations indirectly by controlling the position of the cortical subdivision, is less likely based on the timing of establishment of the peri-equatorial margins of expression. For both CdaA and CdaI, their peri-equatorial margins are visible before any signs of cortical subdivision. Furthermore, the (preexisting) margin positions correlate with the position of cortical subdivision based on the elo1-1 and cdaI-1 effects. It seems therefore that our model that the topology of CdaA and CdaI guides the position of the CS is more parsimonious.

The experiment that the reviewer suggested, an artificial relocalization, is an excellent idea. However, such an approach can be informative only if such a relocalization affects the entire required activity including upstream regulators and downstream effectors. A considerable effort may be required to find relocalization conditions under which CdaA and CdaI are active ectopically. We used a less perfect tool by overexpressing GFP-CdaA. The data are consistent with inhibition of CdaI by CdaA, based on a close phenocopy of cdaI-1. Interestingly, even overproduced CdaA remains enriched in the posterior cell half, suggesting that other factors anchor CdaA to the posterior cortex.

We agree with the reviewer about the need for careful wording when describing the nature of interactions between CdaI and CdaA. We do not know whether these interactions are direct. For this reason, at the end of the section describing the genetic interactions we state that "CdaI activity (directly or indirectly) inhibits CdaA activity ".

(3) It is not very clear how the authors concluded that cdaA-1 was a loss-of-function mutation. The "rescue" strain (cdaA-1 + transformed CYC8 sequence) showed a partial phenotype, even though ~50% of the alleles in the macronucleus are WT (Fig. S2A), which does not seem to suggest a simple recessive loss-of-function mutation. Moreover, transformation of the cdaA-1 allele into the macronucleus of WT (which typically does not replace all copies according to the authors and as evidenced in the "rescue" experiment) phenocopied cdaA-1 homozygotes, suggesting a dominant phenotype.

cdaA-1 and cdaA-4 behave like classic recessives in standard crosses. By that we mean that a heterozygote cdaA/+ can divide at the restrictive temperature. Also, homology modeling predicted that the two alleles destabilize the cyclin domain. For Sanger sequencing of the rescue clone DNA, we amplified CDAA so the ratio of

transgenic wild-type versus endogenous cdaA-1 base seen on the sequencing chromatograph could be different from the original allelic ratio depending on which alleles by chance were amplified in the first few PCR cycles. The rescue cells, as we noted in the original manuscript, were not entirely wild-type in morphology. For example, many rescue cells were cortical doublets. The phenotype of the rescue cells reflects not only the allelic ratio but also the clonal (cortical) history. The doublet phenotype suggests that the rescue cells originated from mutants that experienced arrests in cell division, likely during the initial phase of selection at the restrictive temperature. Likely, initially the mutant cells that recombined the wild-type DNA fragment, partially recovered their ability to divide and this ability has increased under selective pressure due to gradual increase in the copy number in the polyploid macronucleus.

On a related note, what is the localization pattern of cdaA-1-GFP? These questions affect the interpretation of many of the data presented in this paper, such as those in Figs. 5-7.

We do not have these data yet but our structural modeling suggests that the mutant protein fails to fold at the restrictive temperature. Thus, we would expect the mutant protein to be reduced in quantity and potentially to be mislocalized.

(4) Analysis of many of the imaging data in this manuscript centers around the position of the CS and its correlation with CdaA and CdaI localizations. However, it was not clear how the presumptive cortical subdivision was determined in mutants such as cdaA, elo1, and cdaI. The centrin rows appear continuous in the images (e.g., Fig. 1G-K, Fig. 3D'E', Fig. 4 C'D', Fig. 5 C'D'), and it is difficult for a non-Tetrahymena specialist to predict where the CS will be (or should have been) formed. A more detailed description would be helpful.

In Fig. 1G-K, these are cdaA-1 mutant cells at 39^{o}C most of which do not undergo cortical subdivision. In Fig 3D′ E′, 4C′D′ and 5C′D these cells are in various stages of cortical subdivision. Not all gaps appear at the same time. The identification of cells undergoing the cortical subdivision was based on the presence of at least a few gaps in the rows along the equator.

Minor points:
(5) Introduction: It would be helpful for readers unfamiliar with ciliates if the authors described how the macro- and micro-nuclei function and are inherited during the vegetative division of Tetrahymena.

In the first section of Results where we describe the course of normal cell division, we now state that the micronucleus is the germ-line and the macronucleus is the somatic transcriptionally active nucleus.

(6) Throughout the manuscript, it would be helpful if the panels were labelled with the names of stained proteins with clear indication of colors (as is currently done only in Fig. 7).

We have color coded the labels as suggested.

(7) Fig. 6 A-C' are supposed to show "pairs of images showing two sides of the same cell." Are they Different Z-sections looked from the same side, or same cells imaged from opposite sides but one of the pairs has been flipped?

These are subsets of a single Z stack showing two side of the same cell.

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

This paper is on an interesting subject, cell division in Tetrahymena, concerning which I must admit to complete ignorance before reading the paper. The cited Frankel (2008) review was also quite helpful.

Tetrahymena is a very complicated cell with many cortical structures in stereotyped positions. Cell division involves the AP axis effectively duplicating (as per Frankel, if it starts as '12345' it goes to '1234512345') then produces a cleavage furrow between 5 and 1.

The reviewer has accurately captured the unusual nature of cell division in a ciliate in principle as a developmental process of forming two cells from one by cortical remodeling.

Mutants were isolated previously that affect the process. The cdaI mutant starts division off fairly normally, then pushes the boundary anteriorly. The new 'oral apparatus' that also formed at a normal posterior location also moves anteriorly; the cell then can divide, producing a minicell (that apparently still has all contents), or can fail complete division.

This mutant was previously shown to be a loss of function of a protein kinase related to Hippo; CDA1 largely inhabits the anterior half of the cell, suggesting that it blocks 'sliding' of the division plane to the anterior.

Here, another AP-regulatory gene, cdaA, is identified as a member of the cyclin family, by bulked-segregant analysis of two independent alleles. This is clearly and

correctly done. Based on the bioinformatics presented, cdaA is most close to animal cyclin E. It is intriguing and important to know that cdaA is a cyclin, and the mutations are in residues conserved in cyclin E (conservation in other cyclins is not addressed).

We added the following information the revised manuscript: A1256 is conserved in all human cyclin types except cyclin F. D1366 is conserved in human cyclins E1 and E2 and about half of other human cyclin types have an acidic amino acid at this position.

I can't say that there's anything in the paper beyond this point that makes it at all relevant that cdaA is a cyclin; the paper would read exactly the same if it were a protease or an ion channel, etc. This is fine, of course, but it does suggest to me that the rather extensive discussion of homology modeling, and speculation of which CDK cdaA might interact with, is more appropriate for Supplementary Information. (Different if a kinase assay was carried out; or if another mutation (cdaH, with according to Frankel a similar phenotype??) turned out to be in a CDK, etc).

We believe that it is informative to explore whether CdaA has properties of a genuine cyclin E. One of the surprising observations we made is the remarkable evolutionary conservation of involvement of cyclin E with cell polarity. The modeling data reinforce the idea that CdaA and CdaI interact through (direct or indirect)t inhibitory phosphorylations, a hallmark of polarity networks in animals. We are currently sequencing cdaH-1 and it is very likely that the CDAH gene product works in the same or related pathway with CdaA.

The paper goes on to show striking symmetry in the behavior of cdaA and the previously published behavior of CdaI, the Hippo-related kinase. CdaI is located anteriorly, cdaA is located posteriorly; removal of either seems to expand the domains of the other. An asymmetry is that cdaA mutants don't divide at all, while cdaI mutants apparently can divide with some frequency although in general at excessively anterior positions. The double cdaA, cdaI mutant is studied, and intriguingly though it is still lethal, many of the cellular phenotypes are reverted; it does divide, I gather usually around the middle. However, interior components of the cortex are apparently disarranged.

The data in the paper are generally clear, though there is an initial investment required (at least there was for me!) to have any idea of what I was looking at.

We greatly appreciate that the reviewer took time to read the background literature.

One aspect that I did wonder about: Tetrahymena has bands of basal body/cilia in lines extending up its surface. It appeared that cdaA and I think also cdaI were localized to spots in the vicinity of the basal bodies, or at least to lines ('streaks' as the paper has it) that also include the BBs, and the localizations cited above (cdaA posterior, cdaI anterior) really apply to these lines/spots. However, it appeared to me looking e.g. at Fig. 3 that there was quite a bit of cdaA-GFP staining in the anterior of the cell, just not localized. There are a number of possibilities here. It should be shown whether this is background staining (i.e. not cdaA-GFP at all, as would be tested by including a cell lacking cdaA-GFP). If it's real, then the total level of cdaA along the AP axis (not just that along the ciliary bands) should be quantified to be sure there really is a gradient. If not, the observations are still interesting but rather different. (For example, maybe cdaA can only find a CDK kinase partner along the bands). In any case this should be addressed.

We fully agree with the reviewer that CdaA is also present in the anterior cell half at the stage where CdaA is most polarized (early cortical subdivision). For example, in Fig. 3A, the signal of CdaA-GFP in the anterior half of the single dividing cell is apparent in comparison with the three adjacent non-dividing cells. Thus, CdaA is not excluded from the anterior cell half but rather that it is preferentially retained at the posterior cortex (prior and during early subdivision). This behavior is not surprising in the light of observations made on the properties of cortical polarity proteins in bacteria, yeast and C. elegans. In these diverse models, polarized cortical localizations are to large extent a result of differences in the diffusion rates between cortical and cytoplasmic forms of the same protein. In the revised manuscript we pointed the readers' attention to the presence of an above the background anterior CdaA-GFP at the maximal polarization stage (early cortical subdivision). We also inspected the manuscript to avoid any misleading language that would suggest that the partitioning of CdaA is a consequence of exclusion from the anterior cell half.

It is interesting that apparently the cell division cycle overall continues with or without cdaA, at least this seems to be the implication of the writing here. This is worth a bit more explicit thinking. In animal cells, cyclin E contributes (at least) to activating the complete cyclin B-Cdk1-APC circuit leading to cell division; in Tetrahymena, it could be fundamentally needed just to place a division plane but otherwise not be regulatory of the primary cell cycle oscillator (whatever it is - making no assumptions that it will be cyclin B-CDK1-APC). I gather that at the least, the mitotic division of the micronucleus proceeds with normal timing in the cdaA mutant.

The reviewer has made an important point that cdaA-1 and cdaA-4 alleles do not arrest the cell cycle. Rather these mutations block a discrete step in cortical development that leads to a failure of some downstream events such as cytokinesis and amitosis but despite these failures the cell cycle continues for several generations (which produces the monster phenotype). Thus, it would be accurate to state that in Tetrahymena, CdaA/cyclin E is involved with cell polarity but does not control the cell cycle progression except for activation of the cell division boundary. Interestingly, cyclin E is suspected of having cell cycle-independent roles in animals. We cite Berger et al. who described the role of cyclin E in the asymmetric division of neuroblasts in Drosophila, likely independent of the role of cyclin E in the cell cycle (Berger et al Dev. Biol. 2010, 337:415-424). Ables and Drummond-Barbosa (Development, 2013, 140:530-540) reported an apparent non-cell cycle dependent role for cyclin E in the maintenance of female germ cells in Drosophila. Our paper adds to the emerging picture of cyclin E as a highly conserved regulator of cell polarity, an activity that may be independent of the canonical role of cyclin E during the G1/S transition. Given that there are lineages where the cell cycle functions without cyclin E (fungi and most non-opistokonta), it is even possible that the ancestral role of cyclin E was in cell polarity rather than in the cell cycle progression.

Anyway, overall an interesting story on a remarkable organism.

We thank the reviewers for critical reading and excellent suggestions.

May 21, 2020

RE: JCB Manuscript #202002077R

Dr. Jacek Gaertig
University of Georgia
724 Biol Sciences
Athens, GA 30602

Dear Dr. Gaertig:

Thank you for submitting your revised manuscript entitled "Mutual antagonism between Hippo signaling and cyclin E drives intracellular pattern formation". We have now assessed your revision and we would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

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, http://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 currently below this limit but please bear it in mind when revising.

2) Figures limits: Articles and Tools may have up to 10 main text figures.

3) 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.

4) 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."

5) 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." The materials and methods section should be placed after the Discussion but before the acknowledgements.

6) 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.

7) 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 reconstitutions, surface or volume rendering, gamma adjustments, etc.).

8) 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.
Please note that we do not allow a separate reference section in the supplementary information. Therefore, you will need to remove the supplementary reference section and include any non-duplicate references in the main reference list.

9) Supplemental materials: There are strict limits on the allowable amount of supplemental data. Articles/Tools may have up to 5 supplemental figures. At the moment, you are below this limit but please bear it in mind when revising.
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.

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 video files: See our detailed guidelines for preparing your production-ready images, http://jcb.rupress.org/fig-vid-guidelines.

-- Cover images: If you have any striking images related to this story, we would be happy to consider them for inclusion on the journal cover. Submitted images may also be chosen for highlighting on the journal table of contents or JCB homepage carousel. Images should be uploaded as TIFF or EPS files and must be at least 300 dpi resolution.

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.

The license to publish form must be signed before your manuscript can be sent to production. A link to the electronic license to publish form will be sent to the corresponding author only. Please take a moment to check your funder requirements before choosing the appropriate license.

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 or call (212) 327-8588.

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

Sincerely,

Daniel Lew, PhD
Monitoring Editor
Journal of Cell Biology

Tim Spencer, PhD
Executive Editor
Journal of Cell Biology
