JCB

Ciliary force responsive striated fibers promote basal body connections and cortical interactions

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Re: JCB manuscript #201904091

Dr. Chad Pearson
University of Colorado - School of Medicine
Department of Cell and Developmental Biology 12801 E. 17th Ave. Room 12104
Aurora, CO 80045

Dear Dr. Pearson,

Thank you for submitting your manuscript entitled "Force responsive length control of basal body-associated striated fibers promotes cilia organization". Your manuscript has been assessed by expert reviewers, whose comments are appended below.

You will see that, although reviewer #2 is not fully convinced that the paper represents enough of a conceptual advance for JCB, the other two reviewers are more enthusiastic about the work. In addition, all three reviewers have raised a number of concerns which must be addressed before the paper would be deemed appropriate for publication, including the need for further evidence to support the main conclusions.

Please let us know if you are able to address the major issues outlined above and wish to submit a revised manuscript to JCB. Note that a substantial amount of additional experimental data likely would be needed to satisfactorily address the concerns of the reviewers. It may be necessary to extend your manuscript to a full Research Article. Our typical timeframe for revisions is three to four months; if submitted within this timeframe, novelty will not be reassessed. We would be open to resubmission at a later date; however, please note that priority and novelty would be reassessed.

In addition, given the scope of the revisions needed, it might be advisable to craft a point-by-point revision plan where you outline how you would propose to address the reviewer concerns so we can give you some feedback prior to you undertaking the time-consuming and laborious efforts involved in revising the study.

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

GENERAL GUIDELINES:
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Please also highlight all changes in the text of the manuscript.

Regardless of how you choose to proceed, we hope that the comments below will prove constructive as your work progresses. You can contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for thinking of JCB as an appropriate place to publish your work.

Sincerely,

Monica Bettencourt-Dias, PhD
Monitoring Editor
JCB

Tim Spencer, PhD
Deputy Editor
Journal of Cell Biology

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

Soh and colleagues explore the function of the striated fiber (SF), a basal body-associated structure, in the ciliate Tetrahymena. The paper contains new observations about the relationships between SFs and the basal bodies (BB). How individual BBs are organized into rows in multiciliated cells is still mostly unknown. The previous elegant study by Pearson lab (Galati et al., JCB 2014), showed that in Tetrahymena, an SF-assemblin protein, DisA, is a part of the SF and is required for proper alignment of BBs into longitudinal rows, likely because of its role in the SF assembly and length regulation. Galati et al also showed that SFs elongate at higher temperature at which cilia beat faster, suggesting that SFs are force-responding organizers of the BB rows. Also, at higher temperature the disA1 mutant phenotype is enhanced. Thus, Galati et al proposed that the increased SF length confers resilience of ciliary rows under higher mechanical stress.

In the current manuscript, Soh et al explore further the above hypothesis. First, they show that the same disA1 mutants can normalize after re-expression of a wild-type DlsA protein. This is a remarkable observation because at the onset of re-expression, the rows of BBs in the disA1 mutant cells are extremely disorganized (as originally described by Jerka-Dziadosz et al Dev Biol 1995). Thus, the disA-1 mutant cells somehow move or reassemble the BBs at the correct positions.

Exactly how this task is accomplished is not clear. The authors show that this phenotypic rescue is accompanied by lengthening of the SFs. They conclude that the elongation of SFs is what drives the recovery of BB organization. However, the data are not entirely convincing. The corresponding images of cells labeled with an anti-SF antibody (Fig. 1C) are of a relatively low resolution (and small size). The original images of SFs in the disA1 mutant published by Jerka-Diadosz et al 1995 are more clear.

Moreover, according to Jerka-Dziadosz et al 1995, in the disA1 mutant, some SFs are shorter but other SFs are actually longer than normal, and the length effects are correlated with the position in the cell (the lengthening was prominent in the left anterior region of the cell). The quantitative data shown in Fig 1C of this manuscript do not indicate an increased range of the SF lengths in the disA1 mutant. Thus, this discrepancy would need to be addressed. To my eyes, the recovering disA1 mutant cell at 8 hrs has some SFs that are longer than those in the wild-type.

There seems to be a weak correlation between the recovery of the BBs organization and the lengthening of the SFs. For example, based on Fig 1C, there is almost a complete recovery of the BB organization at 48 hrs but at the same time the SFs are only about half of the normal length. In addition, the cells imaged at 48 and 336 hrs appear to have BBs that are mostly correctly aligned but have short SFs or perhaps even lack SFs. The extremely long period required for the full recovery of both the SF length and the BB organization to me suggests an alternative model: that an unknown mechanism gradually organizes the BBs into rows and that SFs elongate as the rows reform.

The relatively low resolution of immunofluorescence images with anti-SF is also an issue in other figures including Figs 2,5, S1E-H, S3G. The image shown is Fig S1A (right panel) is excellent; I am assuming that it is an SR-SIM image (the figure legend does not state the type of microscopy used). The authors could address some of my concerns by providing higher resolution images to support their conclusions.

Some mutant images show clusters of very strong SF signals, especially on the left anterior side of the cell (e.g. S1E, S1H). The images are not of sufficient resolution to determine whether these are clusters represent BBs with very long SFs or aggregates of SF material that are not associated with the BBs.

The authors showed previously that SFs elongate at higher temperature and shorten when ciliary motility is inhibited by nickel chloride. It seems that the section of the current manuscript entitled "ciliary forces tune SF length" arrives at the same conclusion. It would helpfull if the authors better anchored these new data to those in the previous paper (Galati et al 2014) so that the reader knows which parts are new and which parts confirm the previous data using different approaches.

The authors used TEM tomography to uncover novel links between the SF originating from the posterior BB and the structures associated with the neighboring anterior BB (the postciliary microtubule bundle and the epiplasm around the ciliary pocket). This is an important observation potentially revealing the structural basis for cohesion of BBs within the row. The authors show that some of these links are only present in the elongated SFs that form under conditions of increased ciliary beating. This is a single observation but if confirmed, this could explain the significance of SF elongation (as the authors have suggested, to establish a more extensive area of linkage between

the adjacent BBs under mechanical stress).

The next part of the paper is a survey of localizations of several additional SF-assemblin proteins of Tetrahymena. It is intriguing that one of them, (related to the original SF-assemblin of Chlamydomonas) localizes to cilia. The remaining proteins show non-identical localization patterns within the SF, some of them are located near the proximal end of SF and others are more uniformly distributed. These data reveal that SFs are complex structures that are polarized at the molecular level. These data open up an avenue for future investigations. One desired outcome would be a mutant of one or more of these SF-assemblins that entirely lacks the SFs.

The authors assess the function of one of the new SF-asemblins, CRO1, which is enriched near the proximal end of the SF. A knockout of CRO1 disorganized the BBs (similarly to the disA1 mutation, in a temperature-sensitive manner). The authors report that 1/3 of the CRO1-KO cells have curved ciliary rows. The authors suggest that these curved rows are a result of inability to elongate SFs at the conditions at which cilia beat more frequently (higher temperature). This part of the manuscript is a bit confusing because it is not clear whether the twisted row phenotype is present predominantly in cells that are grown at higher temperature (I could not find a quantification of the twisted cell phenotype at the two temperatures). Again as stated above the data would be more convincing if higher resolution images were provided. Based on the current images, it is difficult to see where the individual SFs start and end in the crowded rows of cro1 mutants. Also, Jerka-Dziadosz et al reported twisted rows (or whatever was left of them) in the disA1 mutant and thus the phenotype of Cro1-delta is not entirely new. This actually makes some sense because the two proteins have similar localizations in the SFs. A double mutant could reveal an informative interaction between Cro1 and DisA.

Do the changes in the SF length correlate with the corresponding changes in the spacing between adjacent BBs in the wild-type or are the mutants affected in the BB-BB spacing?

Does reduced ciliary motility rescue the cro1-delta phenotypes (short SF and twisted rows)?

This is a rather complex paper and in some parts it is a difficult read. It could help if the authors included a simplified diagram of the Tetrahymena cortex to help the reader to understand the data in figure 3).

The abstract is not well written as it fails to clearly distinguish between the background information (some of which comes from the authors previous work) and the new data and conclusions.

The origin of the name "Cro1" is not stated.

Page 12: I would indicate that the screwy and twisty mutations were described in two different species of ciliates (Paramecium and Tetrahymena).

I would suggest to reduce the usage of terms "elevated force-induced state" and "steady state". The authors may be correct that the high and low temperatures enhance the phenotypes primarily by affecting the ciliary beat rate but there are other explanations that to me have not been entirely excluded. For example, the remaining SF proteins may be able to support the SF organization and dynamics at the lower but not higher temperature. At the least I would indicate the treatment conditions (low or high temperature) in all figure panels on the side of the "elevated FIS" and "steady-state" labels.

Page 25: 5D8 refers to the name of the antibody, not the antigen.

In figures 1, 2, 5, S1, the positions of the higher magnification insets in the cell should be marked.

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

Multiciliated cells, found in both protists and animals, require their numerous basal bodies to be organized into ordered arrays to enable the coordinated beating of cilia and generate productive movement of the cell itself or fluid over the surface of the cell. The mechanisms that underlie the positioning of basal bodies at the cell surface are still poorly understood but are known to involve various basal body accessory structures including the ciliary rootlets, basal feet/subdistal appendages and striated or kinetodesmal fibers. Here, Chad Pearson and colleagues characterize the role of striated fiber proteins in the dynamic organization of basal bodies in the ciliate Tetrahymena. Overall, the work described in this manuscript is technically well executed. However, as detailed below I am not entirely convinced that the conclusions represent enough of a conceptual advance to warrant publication in JCB.

Main point
1. Novelty/significance
Although this is not explicitly stated in the text, this manuscript is clearly a follow-up on a previous publication from the same lab (Galati et al., JCB 2014), which identified a role for the striated fiber protein DisAp in basal body positioning in Tetrahymena. In the original study, DisAp was found to be required for the formation of full length striated fibers. disA mutants displayed defects in basal body orientation, which were exacerbated under conditions of increased cilia-generated stress which in wild-type result in striated fiber elongation. This disorganization was further found to be independent of new basal body assembly, indicating that basal body positioning is dynamically responsive to the cilia-generated forces acting on them.

In the first few figures (Fig 1, 2, S1) the authors essentially retread the ground covered in their earlier study, performing many of the same perturbations (disA mutation, increased temperature, application of NiCl2 to inhibit dynein-dependent cilia motility). While the experiments are not precisely the same (the original study focused on basal body disorientation, the current one primarily on reorientation), the conclusions the authors draw can be found almost verbatim in their earlier study: striated fibers are important for basal body positioning; positioning is independent of cell cycle progression and therefore new basal body assembly; cilia-dependent forces influence striated fiber length and thereby basal body positioning.

The manuscript does break new ground beginning in Figure 3, with an examination of striated fiber contacts with adjacent basal bodies and the cell cortex under different degrees of cilia-generated stress by high-resolution electron tomography, identifying more extensive contacts with the cell cortex under stress conditions which may help to stabilize basal body arrays. The manuscript then proceeds to present an initial characterization of additional striated fiber components, which appear to localize to different striated fiber subdomains. Finally, the authors mutate one of these components, Crop1p, which results in basal body disorganization only under conditions of high stress, which the authors attribute to a function distinct from that of DisAp.

There is clearly more to the manuscript than the first two figures. However, it is a concern that the major conclusions highlighted by the authors in the title and abstract are based almost exclusively

on the first part of the manuscript, not the latter part that is more unequivocally novel.

Other points

2. The authors make much of the evolutionarily conserved role of striated fibers as force-responsive structures that organize multi-ciliary arrays (eg Conclusions, p12). Yet, the proteins they study by their own admission are protist specific. As I understand it there are major differences in the way that the basal body arrays in ciliates and in multiciliated cells in animals are formed and organized (the former are highly ordered and duplicate in situ, while the latter arise in a mass basal body amplification event and dynamically organize at the cell cortex). Non-opisthokonts also generally have a more elaborate cytoskeleton, including post-ciliary microtubules associated with their basal bodies which are missing in animals. Are there then a priori any reasons to expect similarities in how basal bodies are organized/positioned in the two systems?

3. Of the 7 additional striated fiber components described in this study, six (Crop1p, Bbc29p, Bbc39p, Kdf1p, Kdf3p, Kdf4p) were previously localized to striated fibers by the lab of Douglas Chalker (which also named four of them, kinetodesmal fiber protein/Kdf 1, 3 & 4 and Ciliary Row Organizing Protein/Crop1p, Bbc29p and Bbc39p having previously been identified as basal body components by Kilburn et al., JCB 2007). While the supporting data is only found in non-peer reviewed student publications available through the Tetrahymena genome database and SUPRDB, a reference to this prior work would still be in order.

4. 'SF elongation is observed within eight hours after DisAp expression in disA-1 mutants and BBs reorient by 48 hours, suggesting that SF elongation initiates prior to BB re-orientation (Figs. 1C and S1E). Thus, SF elongation is important for promoting BB orientation and re-orientation.' (Results p5)
The logic here appears a little scrambled. If SF elongation is indeed observed before BB reorientation, this is a statement of fact (no need for 'suggests'). However, just because it precedes the latter event, a causal relationship has not been established ('thus,..is important').

5. 'Conversely, SFs of WT cells elongate by 16% when cell swimming and cilia-dependent forces are increased with elevated temperature for four hours (Fig. 2B, C; Elevated force-induced state).' (Results p6)
Changes in temperature are likely to have many different effects on Tetrahymena cells besides altering swimming rate. Can the authors exclude other temperature-induced effects influencing SF length?

6. The authors argue that DisAp and Crop1p and by extension the other 8 SF proteins in Tetrahymena have different specialized functions, with DisAp maintaining steady state SF lengths while Crop1p is required for elevated force-induced SF elongation (Conclusions p12). An alternative view is that that both proteins have similar functions in SF assembly but disA mutation more severely perturbs SFs compared to Crop1. Can the authors exclude that possibility?

Minor comments

7. 'In vertebrates, SFs consist of distinct proteins that form a base (C-Nap1, Centlein, Cep68) that link to proteins that form the striated fiber (Rootletin, Cep68, Lrrc45) (Fang et al., 2014; He et al., 2013; Vlijm et al., 2018).' (Introduction p3)
If Cep68 is in both lists can one really describe the two sets of proteins as distinct?

8. Cro1p is actually Crop1p according to the Tetrahymena genome database (Results p10).

9. The effect of disA mutation on basal body orientation appears much weaker in the present study

compared to Galati et al., 2014 (R value after 24h at 37C 0.8 Fig 1C vs 0.6 Fig 2F, Galati 2014). Is there any reason for this discrepancy?

10. It would be good to describe the effect of NiCl2 in the text, not merely in the Materials and Methods (Results p6)

11. 'Using a semi-automated image analysis routine, we quantified the distance between the peak intensities of SFs and EpiCp and found that SFs do not become detectably closer to the epiplasm upon elongation (Fig. 3C; panel ii). Moreover, SFs extend further along the epiplasm during elevated force-induced state as compared to steady state (Fig. 3C; panel iii). We postulated that elevated force-induced SF elongation promotes resistance against ciliary forces by establishing secondary interaction sites between SFs and the cell cortex, but they were not detectable by fluorescence.' (Results p8)
Having this last sentence as a concluding summary to what is effectively a negative result rather than a lead in to the next paragraph is a little odd as it gives the impression that the authors see the absence of evidence as evidence to support their hypothesis.

12. It is interesting that the number of SFA homologs is significantly higher in the ciliates Paramecium (72) and Tetrahymena (10) with their hundreds of basal bodies than in Chlamydomonas (1) with only two flagella (Results p9). The authors may wish to comment on this.

13. What is the difference between the fluorescence line scans presented in Figs 4D and E?

14. There seems to be something wrong with the datasets being compared by t-test in Fig 5C (WT and mutant rescue clearly are not statistically significantly different).

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

In this study, Soh and coworkers analyze how striated fibers (SFs) orient basal bodies (BBs) in Tetrahymena and how they are dynamically regulated to maintain this orientation in the context of elevated mechanical forces. By monitoring the effect of an acute re-expression of the SF component DisAp in the disA-1 mutant background, they show that SF elongation precedes BB reorientation, suggesting that SF elongation is key to BB orientation. Next, they analyze SF length in conditions that either increase (high temperature) or decrease (oda1 mutant, NiCl) ciliary beating and show that SFs respond dynamically to varying ciliary forces by increasing or decreasing in length, respectively. Using EM-tomography, they analyzed the contact zone between the SF and the post-ciliary microtubule (pcMT) bundle emanating from the anteriorly positioned BB. They show that electron densities connecting the two types of appendages are present both under high and low ciliary forces. They also uncover linkages between the SF distal tip and the epiplasm in cells exposed to high ciliary forces but not at steady state. This suggests that the epiplasm can serve as a second interaction site for SFs upon high ciliary forces, and that these interactions are dynamically regulated. The authors then describe other SFA-related proteins in the Tetrahymena genome belonging to 3 different groups. By fluorescently tagging 8 of them at their endogenous locus, they show that members of groups 2A and 2B localize at specific sites within SFs. Among these, Cro1p localizes the most proximally. The authors generated a cro1Δ mutant strain which displays a strong reduction in motility at high ciliary forces, correlated with BB disorientation and a failure to increase SF length. They conclude that Cro1p is required for force-induced SF elongation, allowing to maintain BB orientation upon high ciliary forces by permitting the formation of additional connections between SFs and the epiplasm.

Overall, the work is very carefully executed and the data presented will be of interest for a broad readership. My only point is the following. In the BB re-orientation assay (P5/Fig. 1C), the R value peaks at 48H post recovery, when SFs are still much shorter than in controls. In the corresponding picture, some of the SFs do not reach the anteriorly positioned BB but the BBs nevertheless form rows. Is this due to the antibody staining or are the SFs indeed not reaching the neighboring BB? In the latter case, this would suggest that BB orientation happens before SFs can make contacts with the next BB. Have the authors performed EM on these cells?

Minor comments:
- P7/Fig. 3C: How to explain the increase in length of the SF-pcMT contacts under high ciliary forces? Do they stretch or are there more MTs in the pcMT bundles?
- Fig. 3, S2: From the different EM presented in the Ms., it appears that the distal tip of the SF is not always at the level of the ciliary pocket, but deeper in the cytoplasm (e.g. Fig. 3A, S2B). Is this connected to BB maturity?
- P8/ Fig. 3E: The frequency (proportion of SFs) at which linkages between the epiplasm and the SF is observed is not indicated.

RESPONSE TO REVIEWERS

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

Soh and colleagues explore the function of the striated fiber (SF), a basal body-associated structure, in the ciliate Tetrahymena. The paper contains new observations about the relationships between SFs and the basal bodies (BB). How individual BBs are organized into rows in multiciliated cells is still mostly unknown. The previous elegant study by Pearson lab (Galati et al., JCB 2014), showed that in Tetrahymena, an SF-assemblin protein, DisA, is a part of the SF and is required for proper alignment of BBs into longitudinal rows, likely because of its role in the SF assembly and length regulation. Galati et al also showed that SFs elongate at higher temperature at which cilia beat faster, suggesting that SFs are force-responding organizers of the BB rows. Also, at higher temperature the disA1 mutant phenotype is enhanced. Thus, Galati et al proposed that the increased SF length confers resilience of ciliary rows under higher mechanical stress.

In the current manuscript, Soh et al explore further the above hypothesis. First, they show that the same disA1 mutants can normalize after re-expression of a wild-type DIsA protein. This is a remarkable observation because at the onset of re-expression, the rows of BBs in the disA1 mutant cells are extremely disorganized (as originally described by Jerka-Dziadosz et al Dev Biol 1995). Thus, the disA-1 mutant cells somehow move or reassemble the BBs at the correct positions. Exactly how this task is accomplished is not clear. The authors show that this phenotypic rescue is accompanied by lengthening of the SFs. They conclude that the elongation of SFs is what drives the recovery of BB organization. However, the data are not entirely convincing. The corresponding images of cells labeled with an anti-SF antibody (Fig. 1C) are of a relatively low resolution (and small size). The original images of SFs in the disA1 mutant published by Jerka-Diadosz et al 1995 are more clear.

We agree that our original statement "Thus, SF elongation is important for promoting BB orientation and re-orientation" is an overstatement. We have revised this statement to "Thus, SF elongation initiates prior to BB re-orientation (Figs. 1C and S1D). This suggests that SF elongation is important for promoting BB orientation and re-orientation".

To understand how BBs re-orient, we tested whether SF elongation in *disA-1* mutants enables disoriented BBs to re-orient by contacting anterior BBs using SF-pcMT interactions. In the revised manuscript, we show that SF elongation during the rescue of the *disA-1* mutant correlates with increased BB coupling via SF-pcMT interactions between posterior and anterior BB neighbors (Fig. 1D). We suggest that SF elongation promotes SF-pcMT interactions for BB re-orientation. As we now discuss, we do not know how these BBs are mechanically reoriented into their polarized orientation to achieve SF-pcMT interactions (Fig. S1G).

In addition to the above quantification of SF-pcMT interaction frequency, we measured the minimal SF length that is required to establish SF-pcMT interactions in WT cells. This is now included in the revised manuscript as follows:

"Based on the relative position of the anterior BB's pcMT distal tip in WT cells, SFs of the posterior BB must attain a minimum length of 0.59±0.25 μm to establish SF-pcMT interactions (Fig S1C). Consistent with this, we observed that disoriented BBs in *disA-1*

mutants begin to regain proper BB orientation once the mean SF length surpassed this minimum length (Fig. 1C, D)."

The poor figure image quality was a software import issue and has been resolved. We have updated our figures with larger images and insets that reveal the high resolution of our imaging.

Moreover, according to Jerka-Dziadosz et al 1995, in the disA1 mutant, some SFs are shorter but other SFs are actually longer than normal, and the length effects are correlated with the position in the cell (the lengthening was prominent in the left anterior region of the cell). The quantitative data shown in Fig 1C of this manuscript do not indicate an increased range of the SF lengths in the disA1 mutant. Thus, this discrepancy would need to be addressed. To my eyes, the recovering disA1 mutant cell at 8 hrs has some SFs that are longer than those in the wild-type.

In this manuscript, all analyses were performed on BBs in the medial region of the cell. SF length in this medial region is less variable compared to the anterior end of the cell. Consistent with Jerka-Dziadosz, we observed both short and long SFs at the anterior end of the cell. To better show the SF length variability, we have included plots that illustrate the SF length distribution for readers to appreciate the variance in SF length. In addition, we have updated our figures with larger images and improved resolution to be able to see the whole cell variability more clearly.

There seems to be a weak correlation between the recovery of the BBs organization and the lengthening of the SFs. For example, based on Fig 1C, there is almost a complete recovery of the BB organization at 48 hrs but at the same time the SFs are only about half of the normal length. In addition, the cells imaged at 48 and 336 hrs appear to have BBs that are mostly correctly aligned but have short SFs or perhaps even lack SFs. The extremely long period required for the full recovery of both the SF length and the BB organization to me suggests an alternative model: that an unknown mechanism gradually organizes the BBs into rows and that SFs elongate as the rows reform.

Based on the long recovery duration, we agree it is plausible that alternative and unknown mechanisms organize BBs into rows and we now include this discussion in the manuscript. It is important to note that when SFs do not elongate, BBs do not re-orient (Fig. S1F, G). We also discovered that single cell *disA-1* isolates undergo BB re-orientation (Fig. S1H). The rate of re-orientation for individual cells was variable within the population. We suggest that this variability is attributed to the degree of BB disorientation individual cells possess at the onset of the experiment. Moreover, when DisAp expression is not induced single cells do not rescue (Fig. S1H; *bottom panel*). These results are reported and further discussed in the revised manuscript.

The relatively low resolution of immunofluorescence images with anti-SF is also an issue in other figures including Figs 2,5, S1E-H, S3G. The image shown is Fig S1A (right panel) is excellent; I am assuming that it is an SR-SIM image (the figure legend does not state the type of microscopy used). The authors could address some of my concerns by providing higher resolution images to support their conclusions.

Fig. S1A is a widefield microscope image. We now indicate this in the revised Figure Legend. The types of microscopy used for all figure images are also indicated in the

Materials and methods. In addition, we have updated our figures with larger images that better represent the resolution of our images. We now include images of SFs and MTs using confocal and structured illumination microscopy (SIM) to show the interactions between SFs and pcMTs (Figs. 1D, S3L).

Some mutant images show clusters of very strong SF signals, especially on the left anterior side of the cell (e.g. S1E, S1H). The images are not of sufficient resolution to determine whether these are clusters represent BBs with very long SFs or aggregates of SF material that are not associated with the BBs.

We now include confocal microscopy and structured illumination microscopy (SIM) super-resolution images to better illustrate regions with strong SF signals (Figs. 1D and S3L). Importantly, our studies focus on the medial region of the cell where the strong SF signals appear less frequently.

The authors showed previously that SFs elongate at higher temperature and shorten when ciliary motility is inhibited by nickel chloride. It seems that the section of the current manuscript entitled "ciliary forces tune SF length" arrives at the same conclusion. It would helpfull if the authors better anchored these new data to those in the previous paper (Galati et al 2014) so that the reader knows which parts are new and which parts confirm the previous data using different approaches.

Galati et al (2014) indeed reported that SFs elongate at high force (high temperature, high viscosity and IBMX) and shorten when at low temperature (25degC; shown in the supplemental data). Galati et al (2014) did not show that $NiCl_2$ shortened SFs but rather that it blocked high temperature induced SF elongation. There were two variables in these experiments (high temperature and $NiCl_2$) and this is why we focus on SF shortening here. In this manuscript, we report that SFs are capable of shortening at low ciliary force using both chemical inhibition ($NiCl_2$) and mutants that allow us to address concerns of non-specific temperature effects on SF length by using the temperature-sensitive *oad1* mutant. The novel contributions and overlap with Galati et al (2014) have now been highlighted as follow:

"SFs were previously shown to shorten at low temperatures (Galati *et al.*, 2014). To establish whether SF length responds to reduced ciliary forces independent of temperature effects, SF length was measured when ciliary beating was inhibited. To inhibit ciliary beating, the temperature sensitive mutant strain, *outer arm deficient 1* (*oad1*; cilia lacking outer arm dynein), was grown at restrictive temperature (37°C for four hours; (Attwell *et al.*, 1992)). Elevated temperature reduces swimming rates of *oad1* cells by 45% and SF length by 11% (Fig. 2B). Similarly, six hours of $NiCl_2$ treatment reduces cell motility and SF length by 67% and 15%, respectively (Fig. S2A). Thus, SF length is dynamically responsive to elevated and reduced ciliary forces (Fig. 2C)."

The authors used TEM tomography to uncover novel links between the SF originating from the posterior BB and the structures associated with the neighboring anterior BB (the postciliary microtubule bundle and the epiplasm around the ciliary pocket). This is an important observation potentially revealing the structural basis for cohesion of BBs within the row. The authors show that some of these links are only present in the elongated SFs that form under conditions of increased ciliary beating. This is a single observation but if

confirmed, this could explain the significance of SF elongation (as the authors have suggested, to establish a more extensive area of linkage between the adjacent BBs under mechanical stress).

The capture of such images is an extremely laborious effort that we have performed over several years. During review of this manuscript, we captured one additional datapoint and this is now included in Fig. S2C (Eg. 2).

The next part of the paper is a survey of localizations of several additional SF-assemblin proteins of Tetrahymena. It is intriguing that one of them, (related to the original SF-assemblin of Chlamydomonas) localizes to cilia. The remaining proteins show non-identical localization patterns within the SF, some of them are located near the proximal end of SF and others are more uniformly distributed. These data reveal that SFs are complex structures that are polarized at the molecular level. These data open up an avenue for future investigations. One desired outcome would be a mutant of one or more of these SF-assemblins that entirely lacks the SFs.

The localization of Cro1p to the SF base prompted us to initially hypothesize that *cro1Δ* knockout would completely inhibit SF formation. However, *cro1Δ* cells have SFs. Our results show that Cro1p minimally contributes to SF length and is required for elevated force-induced elongation. Because of the high sequence similarity between *Tetrahymena* SFA proteins, we predict that they may all contribute to SF length and/or elongation.

The authors assess the function of one of the new SF-asemblins, CRO1, which is enriched near the proximal end of the SF. A knockout of CRO1 disorganized the BBs (similarly to the disA1 mutation, in a temperature-sensitive manner). The authors report that 1/3 of the CRO1-KO cells have curved ciliary rows. The authors suggest that these curved rows are a result of inability to elongate SFs at the conditions at which cilia beat more frequently (higher temperature). This part of the manuscript is a bit confusing because it is not clear whether the twisted row phenotype is present predominantly in cells that are grown at higher temperature (I could not find a quantification of the twisted cell phenotype at the two temperatures).

The incidence of the twisty phenotype increases with elevated force conditions. The phenotype is now quantified and described in the revised text:

"At steady state, the majority of *cro1Δ* cells (76±9%; Mean±SD) exhibit normal BB orientation. The remaining cells either exhibit individual BBs that are locally disoriented and uncoupled from their neighboring BBs in ciliary rows (locally disoriented cells: 20±7%; Mean±SD) or coupled BBs that are positioned within twisted BB rows relative to the cell's anterior-posterior axis (twisted cells: 4±2%; Mean±SD). The elevated force-induced state resulted in a larger proportion of *cro1Δ* cells with BB disorientation (Fig. 5A, C; normal BB orientation: 10±8%; BB disorientation: 90±8% (locally disoriented: 65±8%; twisted cells: 35±8%; Mean±SD; Fig. 5A)."

Again as stated above the data would be more convincing if higher resolution images were provided. Based on the current images, it is difficult to see where the individual SFs start and end in the crowded rows of cro1 mutants. Also, Jerka-Dziadosz et al reported twisted rows (or whatever was left of them) in the disA1 mutant and thus the phenotype of Cro1-delta is not entirely new. This actually makes some sense because the two proteins have similar

localizations in the SFs. A double mutant could reveal an informative interaction between Cro1 and DisA.

We have provided larger images with clear insets of BBs and their SFs. We now reference the *twisty* BB row phenotype observed for *disA-1* (Jerka-Dziadosz et al. (1995)).

We unsuccessfully attempted to generate the suggested double mutant. We believe this is because the double mutant is lethal.

Do the changes in the SF length correlate with the corresponding changes in the spacing between adjacent BBs in the wild-type or are the mutants affected in the BB-BB spacing?

BB spacing remains constant in WT cells but is slightly increased in the *cro1* mutant at both steady state and elevated force-induced state (Fig. S3K). This is now described in the text as follows:

"Moreover, BB spacing is slightly increased in *cro1Δ* cells grown at elevated force-induced state as compared to WT cells, which retain normal BB spacing at both steady state and elevated force-induced state (Fig. S3K)."

Does reduced ciliary motility rescue the cro1-delta phenotypes (short SF and twisted rows)?

***cro1Δ* mutants are sensitive to $NiCl_2$. Therefore, to reduce ciliary forces, *cro1* mutants grown at high temperature were shifted back to 25°C. We then compared the frequency of twisted BB row cells in the population. The proportion of twisted cells decreased with reduced temperature. We conclude that reducing ciliary forces can rescue the twisted BB row phenotype (Fig. S3G).**

This is a rather complex paper and in some parts it is a difficult read. It could help if the authors included a simplified diagram of the Tetrahymena cortex to help the reader to understand the data in figure 3).

We agree. Fig. 3A now includes a model of the *Tetrahymena* cortex to orient readers to the BB, SF, pcMT and epiplasm interfaces shown in the figure. Moreover, we have improved the manuscript text to simplify the manuscript.

The abstract is not well written as it fails to clearly distinguish between the background information (some of which comes from the authors previous work) and the new data and conclusions.

We have significantly edited the title and abstract to highlight the novelties of the entire manuscript and how it differs from our prior publication. Please see below:

Title:

"Dynamic basal body-associated striated fibers promote basal body coupling and cortical interactions"

Abstract:

"Multi-ciliary arrays drive fluid flow and cellular motility using the polarized and coordinated beating of hundreds of motile cilia. Basal bodies (BBs) nucleate and position cilia. BB-associated striated fibers (SFs) promote BB anchorage and orientation at the cell cortex and mutants that shorten SFs cause disoriented BBs. In contrast to the cytotaxis model, we show that disoriented BBs with short SFs can regain normal orientation if SF length is restored. In addition, SFs adopt unique length states (reduced force-induced state, steady state and elevated force-induced state) by their shrinkage and growth to establish BB coupling and cortical interactions via a ciliary force dependent mechanism. *Tetrahymena* SFs comprise at least eight uniquely localizing proteins belonging to the SF-assemblin family. Loss of different SF base proteins disrupts either SF steady state length or ciliary force-induced SF elongation. Thus, the dynamic regulation of SFs promotes BB and cortical interactions to organize ciliary arrays."

The origin of the name "Cro1" is not stated.

The origin of Crop1 and the gene ID in the *Tetrahymena* genome database are now described. This is indicated in the text as follows:

"The localization of the Ciliary Row Organizing-1 Protein to the SF base suggests that it may nucleate SFs, link SFs to BBs and/or influence SF length (Fig. 4C, D; TTHERM_000354599; Cro1p)."

In addition, Dr. Doug Chalker, who identified this gene, is now referenced. Thank you for pointing this out. Because the *CROP1* gene name provided in the genome database does not conform to the *Tetrahymena* gene nomenclature (too many letters), we have renamed it from *CROP1* to *CRO1*.

Page 12: I would indicate that the screwy and twisty mutations were described in two different species of ciliates (Paramecium and Tetrahymena).

This now described in the manuscript as follows:

"The twisted cells are reminiscent of the *twisty* and *screwy* mutants in *Tetrahymena* and *Paramecium*, respectively".

I would suggest to reduce the usage of terms "elevated force-induced state" and "steady state". The authors may be correct that the high and low temperatures enhance the phenotypes primarily by affecting the ciliary beat rate but there are other explanations that to me have not been entirely excluded. For example, the remaining SF proteins may be able to support the SF organization and dynamics at the lower but not higher temperature. At the least I would indicate the treatment conditions (low or high temperature) in all figure panels on the side of the "elevated FIS" and "steady-state" labels.

At the reviewer's request, we indicated the temperature conditions in the figure panels and the figure legends.

Using the temperature-sensitive *oad1* mutant (Fig. 2B), we have addressed the concern of a non-specific temperature effect on SF length in otherwise normal cells. This experiment serves as an important control to show that SF length changes are likely

attributed to ciliary beating. Moreover, our studies using temperature, viscosity and chemical methods (this study and Galati et al. , 2014) all point to the concept that elevated and reduced ciliary force lengthens and shortens SFs ('tunes' SFs).

With regards to mutant effects on SF length control, *disA-1* mutants have short SFs, even at low temperature, and they exhibit a limited lengthening response to the elevated force-induced state. Conversely, *cro1* mutants are nearly normal length but do not respond to the elevated force-induced state. This allowed us to separate the unique function of length generally (in *disA-1*) and elevated force-induced elongation (in *cro1*). Whether other proteins support low temperature functions isn't important for this model. We have revised the manuscript to address this point more generally.

Page 25: 5D8 refers to the name of the antibody, not the antigen.

This has been corrected.

In figures 1, 2, 5, S1, the positions of the higher magnification insets in the cell should be marked.

The positions of higher magnification insets are now indicated.

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

Multiciliated cells, found in both protists and animals, require their numerous basal bodies to be organized into ordered arrays to enable the coordinated beating of cilia and generate productive movement of the cell itself or fluid over the surface of the cell. The mechanisms that underlie the positioning of basal bodies at the cell surface are still poorly understood but are known to involve various basal body accessory structures including the ciliary rootlets, basal feet/subdistal appendages and striated or kinetodesmal fibers. Here, Chad Pearson and colleagues characterize the role of striated fiber proteins in the dynamic organization of basal bodies in the ciliate Tetrahymena. Overall, the work described in this manuscript is technically well executed. However, as detailed below I am not entirely convinced that the conclusions represent enough of a conceptual advance to warrant publication in JCB.

Main point
1. Novelty/significance
Although this is not explicitly stated in the text, this manuscript is clearly a follow-up on a previous publication from the same lab (Galati et al., JCB 2014), which identified a role for the striated fiber protein DisAp in basal body positioning in Tetrahymena. In the original study, DisAp was found to be required for the formation of full length striated fibers. disA mutants displayed defects in basal body orientation, which were exacerbated under conditions of increased cilia-generated stress which in wild-type result in striated fiber elongation. This disorganization was further found to be independent of new basal body assembly, indicating that basal body positioning is dynamically responsive to the cilia-generated forces acting on them.

In the first few figures (Fig 1, 2, S1) the authors essentially retread the ground covered in their earlier study, performing many of the same perturbations (disA mutation, increased temperature, application of NiCl2 to inhibit dynein-dependent cilia motility). While the

experiments are not precisely the same (the original study focused on basal body disorientation, the current one primarily on reorientation), the conclusions the authors draw can be found almost verbatim in their earlier study: striated fibers are important for basal body positioning; positioning is independent of cell cycle progression and therefore new basal body assembly; cilia-dependent forces influence striated fiber length and thereby basal body positioning.

The sections associated with Figs. 1, 2 and S1 were revised to highlight their intellectual and technical advancements. Importantly, these results show that 1) disoriented BBs are capable of re-orientation and this adds an element of plasticity to the cytotaxis model, 2) SF elongation promotes BB coupling by promoting SF-pcMT contacts, 3) severely disoriented BBs can be rescued, and 4) SF length control results from elevated ciliary forces and is not a secondary temperature affect. Please see below for the text included to highlight the revisions:

Concept #1: Disoriented BBs are capable of re-orientation and this introduces the concept of plasticity to the cytotaxis model (Figs. 1 and S1). This is an important and previously unappreciated error correction mechanism for re-positioning disoriented BBs.:
"Cells with severely disoriented BBs in *disA-1* mutants are rescued by the reintroduction of wild-type (WT) *DISA-1* (Galati *et al.*, 2014). Cytotaxis is a nongenetic process whereby pre-existing BBs guide the position and orientation of new BBs such that the existing cortical architecture is propagated to future generations (Beisson and Sonneborn, 1965; Ng and Frankel, 1977; Sonneborn, 1964). However, in contrast to the cytotaxis model, rescue of disoriented BBs in *disA-1* mutants suggests that BBs retain the ability to correct their orientation, even in a landscape of disorganized BBs."

Concept #2: SF elongation promotes BB coupling by supporting SF-pcMT interactions with the pcMT of the anterior BB (Figs. 1 and S1C).:
"Since SF distal ends are juxtaposed to the anterior BB's pcMTs in WT cells, we hypothesized that SF elongation in *disA-1* mutants enables disoriented BBs to establish attachments to the pcMTs of anterior BBs thereby regaining BB orientation. To test this hypothesis, we used fluorescence microscopy to measure the proportion of SF-pcMT interactions during the rescue of *disA-1* mutants. As SFs elongate in *disA-1* mutants, the frequency of BBs with SF-pcMT interactions increases (Fig. 1D). Based on the relative position of the anterior BB's pcMT distal tip in WT cells, SFs of the posterior BB must attain a minimum length of 0.59±0.25 μm to establish SF-pcMT interactions (Fig S1C). Consistent with this, we observed that disoriented BBs in *disA-1* mutants begin to regain proper BB orientation once the mean SF length surpassed this minimum length (Fig. 1C, D)."

Concept #3: Rescue of single cell *disa-1* isolates confirms BB re-orientation occurs in cells with disoriented BBs (Fig. S1H). This is important because it reveals that 1) a single cell with disoriented BBs can recover and 2) rescue occurs even in highly disorganized cells.:
"To test whether BB re-orientation occurs in single cells, we followed the rescue of single cell *disA-1* isolates. Upon the induction of *DISA-1* rescue, *disA-1* isolates undergo BB re-orientation (Fig. S1H; *top panel*). Interestingly, *disA-1* isolates recover at different rates (Fig. S1H; *bottom panel*). This may be attributed to the varying degree of BB disorientation at the onset of the experiment, whereby cells with more severe BB

disorientation require a longer time to recover. Collectively, this suggests that *Tetrahymena* cells possess error correction mechanisms to resolve BB disorientation, which is observed in 5% of WT cells (Fig. S1A). While SF-independent mechanisms that promote BB re-orientation cannot be ruled out, we propose that SF elongation ensures the propagation of orientated BBs to future progeny."

Concept #4: In Fig. 2, SF length responds to both low and high ciliary forces. SFs shorten at low ciliary forces and by using the *oad1* mutant we can also distinguish between non-specific temperature effects that Reviewer 1 was concerned about. This has been highlighted as follows:

"SFs were previously shown to shorten at low temperatures (Galati *et al.*, 2014). To establish whether SF length responds to reduced ciliary forces independent of temperature effects, SF length was measured when ciliary beating was inhibited. To inhibit ciliary beating, the temperature sensitive mutant strain, *outer arm deficient 1* (*oad1*; cilia lacking outer arm dynein), was grown at restrictive temperature (37°C for four hours; (Attwell *et al.*, 1992)). Elevated temperature reduces swimming rates of *oad1* cells by 45% and SF length by 11% (Fig. 2B). Similarly, six hours of $NiCl_2$ treatment reduces cell motility and SF length by 67% and 15%, respectively (Fig. S2A). Thus, SF length is dynamically responsive to elevated and reduced ciliary forces (Fig. 2C)."

The manuscript does break new ground beginning in Figure 3, with an examination of striated fiber contacts with adjacent basal bodies and the cell cortex under different degrees of cilia-generated stress by high-resolution electron tomography, identifying more extensive contacts with the cell cortex under stress conditions which may help to stabilize basal body arrays. The manuscript then proceeds to present an initial characterization of additional striated fiber components, which appear to localize to different striated fiber subdomains. Finally, the authors mutate one of these components, Crop1p, which results in basal body disorganization only under conditions of high stress, which the authors attribute to a function distinct from that of DisAp.

There is clearly more to the manuscript than the first two figures. However, it is a concern that the major conclusions highlighted by the authors in the title and abstract are based almost exclusively on the first part of the manuscript, not the latter part that is more unequivocally novel.

We agree that the title and abstract focused only on Figs. 1 and 2. While we believe that the results in these figures remains important (see above), we appreciate the Reviewer's comment and have revised the title and abstract to highlight the novelties of the entire manuscript.

Title:

"Dynamic basal body-associated striated fibers promote basal body coupling and cortical interactions"

Abstract:

"Multi-ciliary arrays drive fluid flow and cellular motility using the polarized and coordinated beating of hundreds of motile cilia. Basal bodies (BBs) nucleate and position

cilia. BB-associated striated fibers (SFs) promote BB anchorage and orientation at the cell cortex and mutants that shorten SFs cause disoriented BBs. In contrast to the cytotaxis model, we show that disoriented BBs with short SFs can regain normal orientation if SF length is restored. In addition, SFs adopt unique length states (reduced force-induced state, steady state and elevated force-induced state) by their shrinkage and growth to establish BB coupling and cortical interactions via a ciliary force dependent mechanism. *Tetrahymena* SFs comprise at least eight uniquely localizing proteins belonging to the SF-assemblin family. Loss of different SF base proteins disrupts either SF steady state length or ciliary force-induced SF elongation. Thus, the dynamic regulation of SFs promotes BB and cortical interactions to organize ciliary arrays."

Other points

2. The authors make much of the evolutionarily conserved role of striated fibers as force-responsive structures that organize multi-ciliary arrays (eg Conclusions, p12). Yet, the proteins they study by their own admission are protist specific. As I understand it there are major differences in the way that the basal body arrays in ciliates and in multiciliated cells in animals are formed and organized (the former are highly ordered and duplicate in situ, while the latter arise in a mass basal body amplification event and dynamically organize at the cell cortex). Non-opisthokonts also generally have a more elaborate cytoskeleton, including post-ciliary microtubules associated with their basal bodies which are missing in animals. Are there then a priori any reasons to expect similarities in how basal bodies are organized/positioned in the two systems?

There are indeed structural differences in BB biogenesis, BB-associated appendages and organization in multi-ciliary arrays when comparing ciliates to vertebrate multiciliated cells. However, SFs, which are the focus of our manuscript, are structurally conserved. We hypothesize that SF proteins could have arisen from convergent evolution but sequence divergence cannot be ruled out. In our revised manuscript, we reported the unique differences of SFs between systems. This section has been revised as follow:

"Analogous to vertebrate SFs, *Tetrahymena* SFs are composed of a complex and unique network of components that localize to different domains of the SF structure."

3. Of the 7 additional striated fiber components described in this study, six (Crop1p, Bbc29p, Bbc39p, Kdf1p, Kdf3p, Kdf4p) were previously localized to striated fibers by the lab of Douglas Chalker (which also named four of them, kinetodesmal fiber protein/Kdf 1, 3 & 4 and Ciliary Row Organizing Protein/Crop1p, Bbc29p and Bbc39p having previously been identified as basal body components by Kilburn et al., JCB 2007). While the supporting data is only found in non-peer reviewed student publications available through the Tetrahymena genome database and SUPRDB, a reference to this prior work would still be in order.

We now acknowledge Dr. Doug Chalker for the identification of *CRO1* and the localization of other SF proteins. The name *CROP1* was revised to *CRO1* to conform with the *Tetrahymena* gene nomenclature.

4. 'SF elongation is observed within eight hours after DisAp expression in disA-1 mutants and BBs reorient by 48 hours, suggesting that SF elongation initiates prior to BB re-orientation (Figs. 1C and S1C). Thus, SF elongation is important for promoting BB

orientation and re-orientation.' (Results p5)
The logic here appears a little scrambled. If SF elongation is indeed observed before BB reorientation, this is a statement of fact (no need for 'suggests'). However, just because it precedes the latter event, a causal relationship has not been established ('thus,..is important').

We agree, this sentence is now revised to reflect the data that SF elongation occurs before BB re-orientation.

We also agree that the statement "Thus, SF elongation is important for promoting BB orientation and re-orientation" is inaccurate. Both statements have been revised as follow:

"SF elongation begins within eight hours after DisAp expression and BBs reorient by 48 hours. Thus, SF elongation initiates prior to BB re-orientation (Figs. 1C and S1D). This suggests that SF elongation is important for promoting BB orientation and re-orientation."

5. 'Conversely, SFs of WT cells elongate by 16% when cell swimming and cilia-dependent forces are increased with elevated temperature for four hours (Fig. 2B, C; Elevated force-induced state).' (Results p6)
Changes in temperature are likely to have many different effects on Tetrahymena cells besides altering swimming rate. Can the authors exclude other temperature-induced effects influencing SF length?

Fig. 2 addresses this concern of temperature-induced effects. We address this concern using the temperature-sensitive *oad1* mutant (Fig. 2B). Because high temperature in *oad1* mutants that swim slower elicits SF shortening, the observed SF effects are likely specific to differences in ciliary beating. We have now strengthened the text of the manuscript to make the point clearer:

"SFs were previously shown to shorten at low temperatures (Galati *et al.*, 2014). To establish whether SF length responds to reduced ciliary forces independent of temperature effects, SF length was measured when ciliary beating was inhibited. To inhibit ciliary beating, the temperature sensitive mutant strain, *outer arm deficient 1* (*oad1*; cilia lacking outer arm dynein), was grown at restrictive temperature (37°C for four hours; (Attwell *et al.*, 1992)). Elevated temperature reduces swimming rates of *oad1* cells by 45% and SF length by 11% (Fig. 2B). Similarly, six hours of $NiCl_2$ treatment reduces cell motility and SF length by 67% and 15%, respectively (Fig. S2A). Thus, SF length is dynamically responsive to elevated and reduced ciliary forces (Fig. 2C)."

6. The authors argue that DisAp and Crop1p and by extension the other 8 SF proteins in Tetrahymena have different specialized functions, with DisAp maintaining steady state SF lengths while Crop1p is required for elevated force-induced SF elongation (Conclusions p12). An alternative view is that that both proteins have similar functions in SF assembly but disA mutation more severely perturbs SFs compared to Crop1. Can the authors exclude that possibility?

We argue that DisAp and Cro1p have distinct functions because, while *disA-1* mutants are severely short, they elongate slightly from this short state in the presence of elevated ciliary forces. Conversely, *cro1* mutant SFs have longer steady state SFs, but these SFs

do not increase in length with high ciliary forces. These results lead us to conclude that the differences in the two mutants is not simply a change in degree in the cells ability to assemble KFs. Moreover, Cro1p and DisAp have unique localization patterns at the base of the SF.

"SF base proteins possess distinct SF functions whereby DisAp is required for ensuring SF length to achieve SF-pcMT interactions and Cro1p is required for elevated force-induced SF elongation (Figs. 5D and S3L)."

Minor comments

7. 'In vertebrates, SFs consist of distinct proteins that form a base (C-Nap1, Centlein, Cep68) that link to proteins that form the striated fiber (Rootletin, Cep68, Lrrc45) (Fang et al., 2014; He et al., 2013; Vlijm et al., 2018).' (Introduction p3)
If Cep68 is in both lists can one really describe the two sets of proteins as distinct?

We have corrected this in the revised manuscript as follows:

"In vertebrates, SFs consist of proteins proximal to the BB (C-Nap1, Centlein, Cep68) that link to proteins that form the striated fiber (Rootletin, Cep68, Lrrc45)"

8. Cro1p is actually Crop1p according to the Tetrahymena genome database (Results p10).

To remain consistent with the *Tetrahymena* gene nomenclature, we changed the name to *CRO1*. The gene name origin and ID are described in the revised manuscript as follow:

"The localization of the Ciliary Row Organizing-1 Protein to the SF base suggests that it may nucleate SFs, link SFs to BBs and/or influence SF length (Fig. 4C, D; TTHERM_000354599; Cro1p)."

9. The effect of disA mutation on basal body orientation appears much weaker in the present study compared to Galati et al., 2014 (R value after 24h at 37C 0.8 Fig 1C vs 0.6 Fig 2F, Galati 2014). Is there any reason for this discrepancy?

We also noted this discrepancy. At this point, we do not have a reason for these differences. We attribute it to differences in cell and SF sampling. However, because the measurements are consistent through each of the two manuscripts, we do not believe that this influences the conclusions drawn. This has now been described in the Methods section as follows:

"The discrepancy in R values between our prior publication and this manuscript is attributed to slight differences in SF sampling between experimentalists (Galati *et al.*, 2014). Consistency of R value measurements was ensured in this manuscript."

10. It would be good to describe the effect of NiCl2 in the text, not merely in the Materials and Methods (Results p6)

We have included this in the revised manuscript:

“To test whether ciliary beating is required for BB re-orientation, we simultaneously reduced ciliary beating using $NiCl_2$ treatment, which inhibits dynein motors (Larsen and Satir, 1991)”

11. 'Using a semi-automated image analysis routine, we quantified the distance between the peak intensities of SFs and EpiCp and found that SFs do not become detectably closer to the epiplasm upon elongation (Fig. 3C; panel ii). Moreover, SFs extend further along the epiplasm during elevated force-induced state as compared to steady state (Fig. 3C; panel iii). We postulated that elevated force-induced SF elongation promotes resistance against ciliary forces by establishing secondary interaction sites between SFs and the cell cortex, but they were not detectable by fluorescence.' (Results p8)
Having this last sentence as a concluding summary to what is effectively a negative result rather than a lead in to the next paragraph is a little odd as it gives the impression that the authors see the absence of evidence as evidence to support their hypothesis.

We agree. The last sentence has been removed.

12. It is interesting that the number of SFA homologs is significantly higher in the ciliates Paramecium (72) and Tetrahymena (10) with their hundreds of basal bodies than in Chlamydomonas (1) with only two flagella (Results p9). The authors may wish to comment on this.

We think this is a consequence of whole genome duplication and/or gene duplication events in ciliates. We have commented on this as follows:

“Moreover, these organisms possess variable numbers of SFA homologs, possibly resulting from whole genome duplication and/or gene duplication events”

13. What is the difference between the fluorescence line scans presented in Figs 4D and E?

They are the same. The line scans are now combined into Fig. 4D.

14. There seems to be something wrong with the datasets being compared by t-test in Fig 5C (WT and mutant rescue clearly are not statistically significantly different).

The correction has been made.

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

In this study, Soh and coworkers analyze how striated fibers (SFs) orient basal bodies (BBs) in Tetrahymena and how they are dynamically regulated to maintain this orientation in the context of elevated mechanical forces. By monitoring the effect of an acute re-expression of the SF component DisAp in the disA-1 mutant background, they show that SF elongation precedes BB reorientation, suggesting that SF elongation is key to BB orientation. Next, they analyze SF length in conditions that either increase (high temperature) or decrease (oda1 mutant, NiCl) ciliary beating and show that SFs respond dynamically to varying ciliary forces by increasing or decreasing in length, respectively. Using EM-tomography, they analyzed the contact zone between the SF and the post-ciliary microtubule (pcMT) bundle emanating from the anteriorly positioned BB. They show that electron densities connecting the two types of appendages are present both under high and low ciliary forces. They also uncover linkages

between the SF distal tip and the epiplasm in cells exposed to high ciliary forces but not at steady state. This suggests that the epiplasm can serve as a second interaction site for SFs upon high ciliary forces, and that these interactions are dynamically regulated. The authors then describe other SFA-related proteins in the Tetrahymena genome belonging to 3 different groups. By fluorescently tagging 8 of them at their endogenous locus, they show that members of groups 2A and 2B localize at specific sites within SFs. Among these, Cro1p localizes the most proximally. The authors generated a cro1Δ mutant strain which displays a strong reduction in motility at high ciliary forces, correlated with BB disorientation and a failure to increase SF length. They conclude that Cro1p is required for force-induced SF elongation, allowing to maintain BB orientation upon high ciliary forces by permitting the formation of additional connections between SFs and the epiplasm.
Overall, the work is very carefully executed and the data presented will be of interest for a broad readership. My only point is the following. In the BB re-orientation assay (P5/Fig. 1C), the R value peaks at 48H post recovery, when SFs are still much shorter than in controls. In the corresponding picture, some of the SFs do not reach the anteriorly positioned BB but the BBs nevertheless form rows. Is this due to the antibody staining or are the SFs indeed not reaching the neighboring BB? In the latter case, this would suggest that BB orientation happens before SFs can make contacts with the next BB. Have the authors performed EM on these cells?

As Reviewer 1 also pointed out, BBs re-orient without SFs attaining full length or apparently connecting with their anterior BB. We considered using EM to explore what these partially elongated SFs attach to. However, such specific interfaces are difficult to capture with the low number of samples that can be visualized by EM. Thus, to address what the partially extended SFs interact with, we quantified the interaction between SFs and pcMTs using markers for both structures (SFs and MTs) using confocal and SIM super-resolution fluorescence imaging. The partially elongated SFs at 48 hours are capable of attaching to the pcMT of the anterior BB. Similar analyses were included for *cro1* mutants (Fig. S3L). This work is now incorporated into the revised manuscript as follows:

"Since SF distal ends are juxtaposed to the anterior BB's pcMTs in WT cells, we hypothesized that SF elongation in *disA-1* mutants enables disoriented BBs to establish attachments to the pcMTs of anterior BBs thereby regaining BB orientation. To test this hypothesis, we used fluorescence microscopy to measure the proportion of SF-pcMT interactions during the rescue of *disA-1* mutants. As SFs elongate in *disA-1* mutants, the frequency of BBs with SF-pcMT interactions increases (Fig. 1D). Based on the relative position of the anterior BB's pcMT distal tip in WT cells, SFs of the posterior BB must attain a minimum length of 0.59±0.25 μm to establish SF-pcMT interactions (Fig S1C). Consistent with this, we observed that disoriented BBs in *disA-1* mutants begin to regain proper BB orientation once the mean SF length surpassed this minimum length (Fig. 1C, D)."

Minor comments:
- P7/Fig. 3C: How to explain the increase in length of the SF-pcMT contacts under high ciliary forces? Do they stretch or are there more MTs in the pcMT bundles?

We agree, the increase in SF-pcMT contact length could be due to stretching of the linkages or SF or MT subunit addition to either SFs or MTs. At this point, we have not

distinguished between these models. This is a focus of ongoing work in the lab. This point is now discussed in the manuscript:

"Because these linkages were observed under both steady state and elevated force-induced state, they may be constitutive components required for the preservation of BB organization and orientation (Fig. S2B)."

- Fig. 3, S2: From the different EM presented in the Ms., it appears that the distal tip of the SF is not always at the level of the ciliary pocket, but deeper in the cytoplasm (e.g. Fig. 3A, S2B). Is this connected to BB maturity?

Because our EM analyses focused on SFs of mature BBs, we believe that the variability in SF position is unlikely to be a consequence of BB maturity (Fig. S2C). Our fluorescence quantification data also support this. However, it is possible that the variability of the SF distal tip position is representative of SF dynamics such that the distal tip moves toward and away from the epiplasm depending on ciliary beating. In our fluorescence quantification we see larger SEMs at the SF distal tip compared to the SF proximal end. Here the large SEMs are likely because there are fewer data points at the SF distal tip compared to the SF proximal end. Hence, it is still unclear whether the variable position of the SF distal tip is due to ciliary forces and future work is necessary to study this.

- P8/ Fig. 3E: The frequency (proportion of SFs) at which linkages between the epiplasm and the SF is observed is not indicated.

Two out of four EM images depict SF-epiplasm linkages. This is now included in the revised manuscript as follows:

"Unlike SF-pcMT connections, SF-epiplasm linkages were not observed at all SF-epiplasm interfaces around the ciliary pocket (two of four SF-epiplasm interface tomograms exhibited SF-epiplasm linkages) during the elevated force-induced state, potentially due to transient interactions."

Dynamic basal body-associated striated fibers promote basal body coupling and cortical interactions

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Abbreviations: BB, basal body; SF, striated fiber; pcMT, post-ciliary microtubule; tMT, transverse microtubule; WT, wild-type; SFA, striated fiber-assemblin; SIM, structured illumination microscopy.

Keywords: cilia, basal body, striated fiber, ciliary rootlet, *Tetrahymena*

Running Title: Striated fibers organize ciliary arrays

Abstract

Multi-ciliary arrays drive fluid flow and cellular motility using the polarized and coordinated beating of hundreds of motile cilia. Basal bodies (BBs) nucleate and position cilia. BB-associated striated fibers (SFs) promote BB anchorage and orientation at the cell cortex and mutants that shorten SFs cause disoriented BBs. In contrast to the cytotaxis model, we show that disoriented BBs with short SFs can regain normal orientation if SF length is restored. In addition, SFs adopt unique length states (reduced force-induced state, steady state and elevated force-induced state) by their shrinkage and growth to establish BB coupling and cortical interactions via a ciliary force dependent mechanism. *Tetrahymena* SFs comprise at least eight uniquely localizing proteins belonging to the SF-assemblin family. Loss of different SF base proteins disrupts either SF steady state length or ciliary force-induced SF elongation. Thus, the dynamic regulation of SFs promotes BB and cortical interactions to organize ciliary arrays.

Introduction

Multi-ciliary arrays comprise hundreds of hydrodynamically coupled cilia that beat in a coordinated and polarized manner. Basal bodies (BBs) nucleate, position and anchor cilia at the cell cortex. Beating cilia produce both hydrodynamic flow across the cell surface and mechanical forces that are transduced to the BB anchors (Dirksen, 1971; Vernon and Woolley, 2004). Because of the asymmetric nature of ciliary beating, several forces are imposed upon BBs. These include oscillatory, compressive and rotational forces during the power and recovery strokes (Cheung and Jahn, 1976; Narematsu *et al*., 2015; Riedel-Kruse *et al*., 2007). Nevertheless, BBs maintain their position and polar orientation.

Cilia-generated mechanical forces are resisted through BB coupling to neighboring BBs and the cell cortex via BB-appendages. BB-appendage structures can be classified into distal appendages, basal feet and striated fibers (SFs). BB distal appendages promote BB docking to the plasma membrane (Tanos *et al*., 2013). Basal feet and SFs are polarized along the ciliary beat axis but are oriented in opposite directions (Hard and Rieder, 1983; Werner *et al*., 2011). Both structures maintain BB position and orientation by mediating interactions with cortical microtubule, actin and intermediate filament cytoskeletons (Antoniades *et al*., 2014; Kunimoto *et al*., 2012; Lemullois *et al*., 1987; Vladar *et al*., 2012). BB-associated SFs are striated structures that are conserved across ciliated eukaryotes (Holberton *et al*., 1988; Lechtreck and Melkonian, 1991; Yang *et al*., 2002). In vertebrates, SFs consist of proteins proximal to the BB (C-Nap1, Centlein, Cep68) that link to proteins that form the striated fiber (Rootletin, Cep68, Lrrc45) (Fang *et al*., 2014; He *et al*., 2013; Vlijm *et al*., 2018). How BB-associated SFs respond to mechanical forces and interact with the cytoskeleton in multi-ciliated arrays remains poorly understood.

The BBs within *Tetrahymena* multi-ciliary arrays are arranged in longitudinal rows and possess microtubule appendages and SFs. The microtubule appendages consist of both the

post-ciliary microtubule (pcMT) bundles, that project posteriorly from BBs, and the transverse microtubule (tMT) bundles, that extend rightward (when viewed from the outside of the cell) to the adjacent BB row (Allen, 1967; Junker *et al*., 2019). SFs project anteriorly, presumably to connect with anterior BBs, by attaching to the pcMT bundles (Jerka-Dziadosz *et al*., 1995; Pearson and Winey, 2009). Ciliate cortical organization is propagated by cytotaxis, a nongenetic mechanism whereby preexisting BBs and their associated structures guide the organization and orientation of new BBs (Beisson and Sonneborn, 1965; Frankel, 1964; Ng and Frankel, 1977; Sonneborn, 1964). However, it is unclear whether and how the cell's cortical architecture responds when the integrity of the system is compromised.

The BB orientation defective mutant, *disA-1*, revealed that normal SF length is required for proper BB orientation (Galati *et al*., 2014; Jerka-Dziadosz *et al*., 1995). This facilitates BB coupling within BB rows and enables the propagation of metachronal ciliary beating for cellular motility (Narematsu *et al*., 2015; Tamm, 1984; Tamm, 1999). However, the mechanisms by which SFs promote BB organization and orientation and how their lengths are controlled remain unknown.

Here we show that SFs physically link neighboring BBs to each other and to the cell cortex to organize, orient and re-orient BBs. SF lengths respond to changes in ciliary forces such that elevated or reduced cilia-dependent forces cause SFs to elongate and shorten, respectively. Analogous to vertebrate SFs, *Tetrahymena* SFs are composed of a complex and unique network of components that localize to different domains of the SF structure. Components localizing to the SF base ensure both 1) steady state SF length and 2) elevated ciliary force-induced SF elongation. Using mutants of SF base components to separate these functions, we illuminate the important roles that the unique length states of SFs play in organizing and orienting BBs. These findings serve as a foundation for understanding the role of SF dynamics in anchoring BBs and hydrodynamic flow.

Results and discussion

SFs promote BB re-orientation.

Cells with severely disoriented BBs in *disA-1* mutants are rescued by the reintroduction of wild-type (WT) *DISA-1* (Galati *et al*., 2014). Cytotaxis is a nongenetic process whereby pre-existing BBs guide the position and orientation of new BBs such that the existing cortical architecture is propagated to future generations (Beisson and Sonneborn, 1965; Ng and Frankel, 1977; Sonneborn, 1964). However, in contrast to the cytotaxis model, rescue of disoriented BBs in *disA-1* mutants suggests that BBs retain the ability to correct their orientation, even in a landscape of disorganized BBs. To visualize BB re-orientation, we increased BB disorientation by incubating *disA-1* mutants at high ciliary forces (37°C for 24 hours) and then initiated BB re-orientation by acute DisAp expression at low ciliary forces (25°C; Fig. 1A). BB orientation, as defined by the axis of BBs and their associated SFs relative to the cell's anterior-posterior polarity (Fig. 1B; (Galati *et al*., 2014)), was monitored along with SF length relative to time post expression of WT DisAp. SF elongation begins within eight hours after DisAp expression and BBs reorient by 48 hours. Thus, SF elongation initiates prior to BB re-orientation (Figs. 1C and S1D). This suggests that SF elongation is important for promoting BB orientation and re-orientation.

Since SF distal ends are juxtaposed to the anterior BB's pcMTs in WT cells, we hypothesized that SF elongation in *disA-1* mutants enables disoriented BBs to establish attachments to the pcMTs of anterior BBs thereby regaining BB orientation. To test this hypothesis, we used fluorescence microscopy to measure the proportion of SF-pcMT interactions during the rescue of *disA-1* mutants. As SFs elongate in *disA-1* mutants, the frequency of BBs with SF-pcMT interactions increases (Fig. 1D). Based on the relative position of the anterior BB's pcMT distal tip in WT cells, SFs of the posterior BB must attain a minimum length of 0.59±0.25 μm to establish SF-pcMT interactions (Fig S1C). Consistent

with this, we observed that disoriented BBs in *disA-1* mutants begin to regain proper BB orientation once the mean SF length surpassed this minimum length (Fig. 1C, D).

To test whether BB re-orientation occurs in single cells, we followed the rescue of single cell *disA-1* isolates. Upon the induction of *DISA-1* rescue, *disA-1* isolates undergo BB re-orientation (Fig. S1H; *top panel*). Interestingly, *disA-1* isolates recover at different rates (Fig. S1H; *bottom panel*). This may be attributed to the varying degree of BB disorientation at the onset of the experiment, whereby cells with more severe BB disorientation require a longer time to recover. Collectively, this suggests that *Tetrahymena* cells possess error correction mechanisms to resolve BB disorientation, which is observed in 5% of WT cells (Fig. S1A). While SF-independent mechanisms that promote BB re-orientation cannot be ruled out, we propose that SF elongation ensures the propagation of orientated BBs to future progeny.

As BBs assemble during cell division, the spacing between neighboring BBs narrows, and this could promote BB re-orientation upon *DISA-1* rescue (Galati *et al*., 2015). To assess whether cell cycle progression and new BB synthesis are required for BB re-orientation, cells were starved to inhibit both processes and induced for *DISA-1* rescue. Starved *disA-1* cells upon rescue were as efficient as cycling cells in BB re-orientation, indicating that cell cycle progression and new BB synthesis are not required for BB re-orientation (Fig. S1E, F).

Next, we hypothesized that ciliary beating could promote BB re-orientation by translocating BBs to their proper orientation and positioning. To test whether ciliary beating is required for BB re-orientation, we simultaneously reduced ciliary beating using $NiCl_2$ treatment, which inhibits dynein motors (Larsen and Satir, 1991), and induced WT DisAp expression in *disA-1* mutants. Disoriented BBs failed to re-orient with reduced ciliary beating 48 hours post *DISA-1* rescue (Fig. S1G). However, SF length remained short, suggesting that cilia-dependent forces are necessary for SF elongation and for BB re-orientation. Unfortunately,

because SFs did not elongate, we could not determine whether ciliary beating is important for promoting BB re-orientation toward the cell's anterior pole.

Ciliary forces tune SF length.

To investigate the relationship between ciliary forces and SF length, we exposed cells to a range of ciliary forces and quantified SF length. Consistent with our prior work, SFs of WT cells elongate by 16% when cell swimming and cilia-dependent forces are increased with elevated temperature for four hours (Fig. 2A, C; Elevated force-induced state; (Galati *et al.*, 2014)). Because SFs commonly overlap with each other (66%), the number of SFs that could be spatially resolved by conventional fluorescence microscopy was limited. Using structured illumination microscopy (SIM), a larger proportion of SF lengths was resolvable and quantified to show that SFs generally elongate with elevated ciliary forces (Figs. 2A and S1B).

SFs were previously shown to shorten at low temperatures (Galati *et al.*, 2014). To establish whether SF length responds to reduced ciliary forces independent of temperature effects, SF length was measured when ciliary beating was inhibited. To inhibit ciliary beating, the temperature sensitive mutant strain, *outer arm deficient 1* (*oad1*; cilia lacking outer arm dynein), was grown at restrictive temperature (37°C for four hours; (Attwell *et al.*, 1992)). Elevated temperature reduces swimming rates of *oad1* cells by 45% and SF length by 11% (Fig. 2B). Similarly, six hours of $NiCl_2$ treatment reduces cell motility and SF length by 67% and 15%, respectively (Fig. S2A). Thus, SF length is dynamically responsive to elevated and reduced ciliary forces (Fig. 2C).

SFs contact the post-ciliary microtubules and the cell cortex.

Since SF elongation maintains BB orientation in WT cells and SFs promote BB re-orientation in mutant rescue cells ((Galati *et al.*, 2014); Fig. 1), we postulated that SF length above a certain

threshold promotes interactions that facilitate normal BB organization and orientation. Prior work revealed the close proximity and interactions of SFs to BB microtubule appendages and the cell cortex (Iftode *et al*., 1996; Pitelka, 1961). However, it is not known whether SF interactions persist at different ciliary forces. Here, we investigate SF interactions that promote BB positioning and orientation (Fig. 3A).

To determine whether SFs interact with the pcMTs of anterior BBs at varying levels of ciliary force, we measured interactions between these structures using EM-tomography of WT cells grown at normal (25°C; steady state) or high ciliary force (38°C; elevated force-induced state). SFs extend anteriorly, crossing the surface of the anterior BB pcMT bundles in close proximity (Fig. 3A; *panel i*). We observed electron densities that link SFs and pcMTs, suggesting that cortical BBs interact with neighboring BBs by forming bridges between SFs and pcMTs (Fig. 3B and Video 1). These linkages were observed at both normal and high ciliary forces, indicating that, under all conditions measured, SFs physically couple neighboring BBs via pcMTs (Figs. 3B and S2B). The SF-pcMT linkage length is longer in the elevated force-induced state (steady state: 16.0±4.6 nm; elevated force-induced state: 20.0±7.4 nm; Mean±SD; P-value = 0.03). Because these linkages were observed under both steady state and elevated force-induced state, they may be constitutive components required for the preservation of BB organization and orientation (Fig. S2B).

Upon elevated ciliary beating forces SFs elongate beyond the anterior BB's pcMTs, suggesting that the SF distal ends may provide a secondary reinforcing interaction to resist elevated forces from ciliary beating. In addition to their anterior orientation, SFs are also oriented towards the cell cortex, suggesting that SFs may have an interacting partner there (Fig. 3A; *panel ii*; (Allen, 1967; Galati *et al*., 2014)). To quantify the position of SFs relative to the cell cortex, we colocalized SFs with the cortical epiplasm protein, EpiCp, fused to mCherry (Williams *et al*., 1987). Using a semi-automated image analysis routine, the distance between

the peak intensities of SFs and EpiCp was quantified. SFs are not detectably closer to the epiplasm upon elongation (Fig. 3C; *panels ii* and *iv*). However, SFs extend further along the epiplasm during the elevated force-induced state as compared to steady state (Fig. 3C; *panels iii* and *iv*).

To test for SF-cell cortex interactions, we employed EM-tomography to monitor SF's distal end relative to the cortical epiplasm. Consistent with the above fluorescence quantification, SF-epiplasm interactions by longitudinal sections were not observed (Figs. 3D and S2C). However, we observed electron-dense linkages between the elongated SF and the epiplasm surrounding the ciliary pocket in the elevated force-induced state, but not steady state (Fig. 3E and Videos 2 and 3). While such interactions were not observed at steady state, we cannot rule out the possibility that they are transient or not captured. The length of the SF-epiplasm linkage was 22.1±6.0 nm (Mean±SD). Unlike SF-pcMT connections, SF-epiplasm linkages were not observed at all SF-epiplasm interfaces around the ciliary pocket (two of four SF-epiplasm interface tomograms exhibited SF-epiplasm linkages) during the elevated force-induced state, potentially due to transient interactions. This suggests that SF-epiplasm linkages serve as dynamic, secondary interaction sites to reinforce BB organization and orientation when cilia and BBs experience greater mechanical forces.

SFs are composed of a family of uniquely localized SF-assemblin components.

To determine the molecular composition of SFs, we performed phylogenetic analysis on the known SF gene, *DISA-1*, to identify similar genes. As DisAp belongs to the striated fiber-assemblin (SFA) family of proteins, we searched for SFA proteins across a broad set of eukaryotic species and found that SFA homologs are found exclusively amongst protists and Diphoda algae (Fig. 4A and Table S1; (Galati *et al.*, 2014)). Moreover, these organisms possess variable numbers of SFA homologs, possibly resulting from whole genome duplication and/or

gene duplication events (Table S1; Number of SFA homologs: *C. reinhardtii*: 1; *T. thermophila*: 10; *P. tetraurelia*: 72). *Tetrahymena* SFA homologs fall into three orthologous clades, which we designate Group 1, Group 2A and Group 2B. Group 1 and Group 2 SFA proteins can be distinguished by the relative positions of an 18 amino acid consensus sequence (Fig. 4B). This sequence appears near the N-terminus of Group 1 SFA proteins but it is found on the C-terminus in Groups 2A and 2B SFA proteins (Figs. 4B and S2E). In addition, Group 2B SFA proteins possess a single, conserved proline residue that distinguishes them from the Group 2A proteins (Fig. 4B). Hence, we hypothesized that *Tetrahymena* SFA proteins diverged and sub-functionalized.

To investigate whether *Tetrahymena* SFA proteins localize to SFs, we fluorescently tagged these proteins in *Tetrahymena* cells. From our localization analysis, we discovered that the Group 1 SFA homolog (TTHERM_00263290) localizes to cilia (Fig. S2D), which is surprising given that its green algae homolog localizes to SFs (Lechtreck and Melkonian, 1991). Group 2 SFA proteins (Cro1p, Kdf3p, Kdd6p, Bbc39p, Kdf4p, Bbc29p and Kdf1p) localize to SFs (Chalker group; www.suprdb.org). Using endogenously tagged genes under native promoters, we showed with SIM imaging that eight out of ten of the SFA proteins distinctly localize within SFs (Fig. 4C). Thus, *Tetrahymena* SF proteins exhibit unique phylogenetic and localization profiles that may reflect sub-functionalization.

We developed a semi-automated image averaging pipeline to quantify the localization pattern for each *Tetrahymena* SF protein (Fig. 4D). Using BBs and SFs as fiducial marks, fluorescent images of each SF protein were aligned and averaged. To quantify the relative position and distribution of each SF protein along the SF, the fluorescent signal was measured. SF proteins localize to unique domains along the SF length (Figs. 4D; *right panel* and S2F). Among Group 2A proteins, Cro1p and DisAp localize to the SF base while Kdf3p localizes more distally. Group 2B SF proteins (Kdd6p, Bbc39p, Kdf4p, Bbc29p and Kdf1p) localize

along the SF length (Fig. 4D; *right panel*). These localization patterns suggest that there is sub-functionalization of *Tetrahymena* SFA proteins. Moreover, because these components do not localize along the entire SF length, additional proteins likely form the SF distal ends. Coupled with the unique sequence features in each *Tetrahymena* SFA group, it will be interesting to explore how these proteins influence SF assembly, length regulation and function.

Cro1p promotes elevated ciliary force-induced SF elongation.

The localization of the Ciliary Row Organizing-1 Protein to the SF base suggests that it may nucleate SFs, link SFs to BBs and/or influence SF length (Fig. 4C, D; TTHERM_000354599; Cro1p). The latter hypothesis is consistent with the proximal localizing SF component, DisAp, that, when disrupted, causes SF shortening by approximately 60% (Fig. 1; (Galati *et al.*, 2014)). To investigate the effect of Cro1p loss on SFs, *Tetrahymena CRO1* was knocked out in the *Tetrahymena* macronucleus using CoDel (Hayashi and Mochizuki, 2015). Successful *CRO1* knockout was confirmed by the loss of *CRO1* genomic DNA and *CRO1* transcripts (Fig. S3A–C). While exons 1-5 were eliminated, a partial transcript containing exons 6 and 7 was detected (Fig. S3C; white box). Based on an alternate transcriptional start site within exon 7, expression of a truncated 20 amino acid Cro1p cannot be excluded. A *cro1Δ+CRO1* rescue strain was created by reintroducing *CRO1* into the *cro1Δ* cells (Fig. S3D).

At steady state, *cro1Δ* cells swim approximately 11% slower than WT cells (Fig. S3E). At elevated forces, *cro1Δ* cells swim 60% slower than WT cells (Fig. S3E). Thus, *cro1Δ*, like *disA-1* mutants, impede cellular swimming behavior when ciliary beating forces are elevated.

To elucidate the cause of the reduced motility in *cro1Δ* cells, we investigated whether Cro1p loss impacts BB orientation. At steady state, most *cro1Δ* cells exhibit oriented BBs. However, BB disorientation is increased at the elevated force-induced state (Fig. 5A, B). Moreover, BB spacing is slightly increased in *cro1Δ* cells grown at elevated force-induced

state as compared to WT cells, which retain normal BB spacing at both steady state and elevated force-induced state (Fig. S3K). Compared to *disA-1* mutants, *cro1Δ* cells exhibit an intermediate BB disorientation phenotype (Fig. S3L; (Galati *et al*., 2014)). To investigate whether Cro1p loss impacts SF length, we measured SF length (Fig. 5C). *cro1Δ* SFs are 18% shorter than WT SFs during steady state (Fig. 5A, C) and remain short at the elevated ciliary force-induced state (Fig. 5C). Thus, Cro1p loss results in shorter SFs and an inability to lengthen SFs when ciliary forces are increased. Interestingly, the extent of the SF length defect in *disA-1* and *cro1Δ* cells correlates with the extent of BB disorientation (Figs. 5D and S3L; elevated force-induced state; WT: 1.44±0.33 μm; *cro1Δ*: 0.99±0.35 μm; *disA-1*: 0.44±0.22 μm; Mean±SD). Unlike *disA-1* mutants, SFs in *cro1Δ* cells are long enough to establish SF-pcMT interactions and promote normal BB orientation at steady state (Fig. 5B, C). Hence, this further supports the importance of SF length in ensuring proper BB orientation (Fig. 5D).

At steady state, the majority of *cro1Δ* cells (76±9%; Mean±SD) exhibit normal BB orientation. The remaining cells either exhibit individual BBs that are locally disoriented and uncoupled from their neighboring BBs in ciliary rows (locally disoriented cells: 20±7%; Mean±SD) or coupled BBs that are positioned within twisted BB rows relative to the cell's anterior-posterior axis (twisted cells: 4±2%; Mean±SD). The elevated force-induced state resulted in a larger proportion of *cro1Δ* cells with BB disorientation (Fig. 5A, C; normal BB orientation: 10±8%; BB disorientation: 90±8% (locally disoriented: 65±8%; twisted cells: 35±8%; Mean±SD; Fig. 5A). This suggests that SF-pcMT interactions alone are insufficient to maintain BB orientation under the elevated force-induced state. Moreover, it reveals that SF base proteins possess distinct SF functions whereby DisAp is required for ensuring SF length to achieve SF-pcMT interactions and Cro1p is required for elevated force-induced SF elongation (Figs. 5D and S3L).

The twisted cells are reminiscent of the *twisty* and *screwy* mutants in *Tetrahymena* and *Paramecium*, respectively (Fig. S3F; (Frankel, 2008; Jerka-Dziadosz *et al*., 1995; Whittle and Chen-Shan, 1972)). Reduction of ciliary forces by shifting *cro1Δ* cells from 39°C to 25°C for 24 hours rescued twisted BB rows back to WT configuration, indicating that twisted BB rows arise from elevated ciliary forces (Fig. S3G). Because SFs are generally oriented in *cro1Δ* cells with a few examples of local disorientation, we predicted that locally disoriented BBs possess shorter SFs that cause BBs to uncouple from their anterior neighbor. Indeed, locally disoriented BBs possess shorter SFs than BBs in twisted BB rows, which leads to BB uncoupling (Fig. S3H–J, L). Cells with twisted BB rows, on the other hand, possess SFs that are long enough to establish and maintain BB coupling, but they are unable to elongate at the elevated force-induced state (Fig. S3H–J, L). We postulate that this makes BB rows more susceptible to asymmetric ciliary forces and causes BB rows to deviate from the cell's anterior-posterior axis (Figs. 5A and S3F, L; (Cheung and Jahn, 1976)). Collectively, this suggests that Cro1p ensures proper SF lengthening to establish secondary SF-cell cortex interactions during elevated ciliary forces (Fig. 5D).

Conclusions

Here, we show that the evolutionarily conserved, BB-associated SFs are ciliary force-responsive structures that attain unique length states to organize multi-ciliary arrays. To stabilize the positioning and orientation of BBs, SFs establish primary (steady state) and secondary (elevated force-induced state) interactions with the anterior BB's pcMTs and the cell cortex, respectively. This facilitates BB coupling to propagate metachronal ciliary beating for cellular motility. Consistent with the sub-functionalization of SF components, we discovered that one proximally localized SF protein, DisAp, maintains steady state SF lengths while a second proximal SF protein, Cro1p, is required for elevated force-induced SF

elongation. Here, we show that SF length maintenance and elongation promote attachments between neighboring BBs and to the cell cortex to ensure proper BB positioning and orientation. It will be interesting to explore the coordination between BB-associated SFs and microtubule appendages in organizing and orienting BBs.

Figures legends

Figure 1. SFs promote BB re-orientation. (A) The BB re-orientation assay. BB disorientation was exacerbated by shifting *disA-1* mutants from 25°C to 37°C for 24 hours. To promote BB re-orientation, cells were shifted from 37°C to 25°C coincident with WT DisAp protein expression. (B) Schematic of SF length measurements, distance measurements from the posterior BB to the anterior BB's pcMT distal tip to establish the minimal SF length that is required for SF-pcMT contact, and BB orientation analyses. (C) DisAp protein expression leads to SF elongation prior to BB re-orientation. BB re-orientation occurs when SF length surpasses the minimal length that is required for SF-pcMT interactions (arrowhead and dotted line marks the mean distance from the posterior BB to the anterior BB's pcMT distal tip in WT cells). SF length and BB orientation partially recover two weeks post *DISA-1* rescue. BB (red) and SF (green). All small insets show a representative BB and SF. White box marks region of interest (large inset). n ≥ 300 SFs (≥ 30 cells). Mann-Whitney test * denotes P-value < 0.01. Mean±SD. Bars, 10 μm (cell), 1.3 μm (small inset width), 7.8 μm (large inset width). (D) Frequency of SF-pcMT contacts is increased as SF length recovers in *disA-1* mutants. Schematic illustrates the position and orientation of two BBs within a region of interest (white box). BB-associated microtubule appendages (red) and SF (green). n = 240 SFs (24 cells). Bar, 5 μm.

Figure 2. SFs elongate and shorten with ciliary force. (A) Cell motility and SF length of WT cells were elevated by increasing temperature (37°C for four hours). (B) Cell motility and SF length of the temperature sensitive mutant strain, *outer arm deficient 1* (*oad1*), were reduced at restrictive temperature (37°C for four hours; (Attwell *et al*., 1992)). BB (red) and SF (green). SF length quantitation: n = 300 SFs (30 cells); Motility assay: 90 cells. Mann-Whitney test * denotes P-value < 0.01. Mean±SD. Bars, 10 μm (cell), 1.3 μm (inset width), 100 μm (swim

paths). All insets show a representative BB and SF. (C) Schematic illustrating SF length response to varying levels of ciliary force. BB-associated microtubule appendages are not shown.

Figure 3. SFs contact the post-ciliary microtubules and the cell cortex. (A) (i) Schematic of *Tetrahymena* cortical array (cross-sectional interface) illustrating BBs (red) and their associated appendage structures including SFs (green), pcMTs (cyan) and tMT (black). (ii) Schematic depicting the SF-cell cortex longitudinal interface of a single *Tetrahymena* cortical unit. Boxes mark the interfaces of interest. (B) EM tomographic images of the SF-pcMT interface between a pair of BBs at steady state (25°C). Electron-densities (red arrowheads) link SFs to pcMT bundles (EM tomographic slice: i and iv; Model: ii and iii). (C) (i) Fluorescence images illustrating the SF position relative to the cell cortex (epiplasm; EpiC-mCherry (EPI)) at steady state (25°C) and elevated ciliary force-induced state (37°C). (ii and iii) Averaged fluorescence images show that SFs extend towards (y-axis) and along (x-axis) the epiplasm at both steady state and elevated force-induced state. BB (magenta), SF (green) and EPI (red). Dashed line indicates the peak fluorescence intensities of EpiCp-mcherry. (iv) Quantification of SF position relative to the epiplasm. Black dashed line marks the positions where the SF distal ends start to extend along the epiplasm. Steady state: n = 104 SFs (53 cells); Elevated force-induced state: n = 94 SFs (53 cells). Mann-Whitney test * denotes P-value < 0.01. Mean±SEM. (D) EM tomographic images of the longitudinal SF-epiplasm interface. Linkages were not detected between SFs and the epiplasm (white arrowheads) (EM tomographic slice: ii and iv; Model: i and iii). (E) EM tomographic images of the cross-sections of the SF-epiplasm interface at the ciliary pocket at steady state (25°C) and elevated ciliary force-induced state (38°C). Electron-dense linkages (red arrowheads) were observed between SFs and the epiplasm only at the elevated force-induced state (EM tomographic slice: i and iv; Model: ii

and iii). B, D, E: BB (red), SF (green), epiplasm (white), linkages (magenta), pcMTs (cyan) and axoneme (AXO) (dark blue). Bars, 200 nm.

Figure 4. SFs are composed of a family of uniquely localized striated fiber-assemblin (SFA) components. (A) Simplified phylogenetic tree depicting the evolutionary relationships between SFA homologs of selected algae and protists. SFA homologs fall into three evolutionarily distinct groups. *Tt*: *T. thermophila*; *Cr*: *C. reinhardtii*; *Gi*: *G. intestinalis*; *Tg*: *T. gondii*. (B) Domain comparison of previously characterized green algae SFA protein (*C. reinhardtii*; (Lechtreck and Silflow, 1997)) and unique sequence features identified in SFA orthologous groups. (C) SIM imaging shows the unique localization of *Tetrahymena* Group 2 SF proteins. BB (blue), SF (green) and Group 2 SF proteins (white and red). Bar, 500 nm. (D; *left panel*) Averaged fluorescence localization of *Tetrahymena* Group 2 SF proteins. Number of SFs analyzed: Cro1p: 101; DisAp: 107; Kdf3p: 105; Kdd6p: 93; Bbc39p: 107; Kdf4p: 157; Bbc29p: 115; Kdf1p: 109. SFs were gathered from ≥ 60 cells. Bar, 500 nm. (D; *right panel*) Schematic illustrating the relative localization of *Tetrahymena* Group 2 SF proteins along the SF. BB (red), SF (green), Group 2A (orange) and Group 2B (cyan). Green dashed lines mark the BB centroid and SF distal tip. Arrowheads mark the start and end positions of the Group 2 SF proteins as defined by 30% from peak fluorescence intensity. Error bars indicate SEM. Bar, 125 nm.

Figure 5. Cro1p promotes elevated ciliary force-induced SF elongation. (A) Representative images of WT, *cro1Δ* and *cro1Δ* rescue cells during steady (25°C) and elevated force-induced states (39°C for 24 hours). *cro1Δ* cells exhibit more local BB disorientation and twisted BB rows at elevated force-induced state. BB (red) and SF (green). The percentage of each phenotype is calculated based on *cro1Δ* cells that exhibit BB disorientation. All insets show a

representative BB and SF. Bars, 10 μm (cell), 1.3 μm (inset width), 2 μm (BB rows inset). (B) Quantification of BB orientation (R value) of WT, *cro1Δ* and *cro1Δ* rescue cells. c*ro1Δ* cells exhibit BB disorientation at elevated force-induced state. (C) Quantification of SF length of WT, *cro1Δ* and *cro1Δ* rescue cells. SF length of *cro1Δ* cells failed to elongate at the elevated force-induced state. Arrowhead and dotted line mark the minimal SF length that is required for SF-pcMT interactions. n ≥ 350 SFs (≥ 40 cells). Mann-Whitney test * denotes P-value < 0.01. Mean±SD. (D) Model depicting how SF base proteins, DisAp and Cro1p, influence SF length. Loss-of-functions of these proteins either affect primary (SF-pcMT) or secondary (SF-cell cortex) interactions and lead to varying degrees of BB disorganization and disorientation. A, D: BB and epiplasm (red), SF (green), pcMTs (cyan) and linkages (magenta).

Supplemental figure legends

Figure S1. SFs promote BB re-orientation. (A) Widefield image depicting BB disorientation in WT cell. Arrow marks disoriented BBs. Bar, 10 μm (cell), 4.5 μm (inset width). (B) Distribution of SF length measured from SIM-acquired SF-BB images. (C) Distance measurements between posterior BB and anterior BB's pcMT distal tip in WT cells at steady state and elevated force-induced state. Bars, 500 nm. (D) Negative control of BB re-orientation analysis under cycling condition. Recoveries in SF length and BB orientation were attributed to leaky DisAp expression. (E and F) BB re-orientation analysis under non-cycling conditions. (E) Under non-cycling conditions, *DISA-1* rescue promotes SFs lengthening prior to BB re-orientation. (F) Negative control of BB re-orientation analysis under non-cycling condition. SF length and BB orientation recovery does not occur without DisAp expression. (G) BB re-orientation analysis under reduced ciliary force. Under reduced ciliary force, SFs remained short and BBs failed to reorient. BB (red) and SF (green). All small insets show a representative BB and SF. B–G: n = 300 SFs (30 cells). Mann-Whitney test * denotes P-value < 0.01. Mean±SD. D–G: Bars, 10 μm (cell), 1.3 μm (small inset width), 5 μm (BB rows inset). (H) *Top panel*: Single cell *disA-1* isolates are rescued upon DisAp expression. BB (red) and SF (green). Bars, 10 μm (cell), 2 μm (inset). *Bottom panel*: Single cell *disA-1* isolates recovered at different rates.

Figure S2. SF length and position during varying levels of ciliary force, and the molecular composition of SFs. (A) Cell motility and SF length of WT cells were reduced by $NiCl_2$ treatment (25°C for six hours). BB (red) and SF (green). All insets show a representative BB and SF. SF length quantitation: n = 500 SFs (35 cells); Motility assay: 90 cells. Bars, 10 μm (cell), 1.3 μm (inset width), 100 μm (swim paths). (B) EM tomographic images of cross-sectional SF-pcMT interface at steady state (25°C) and elevated ciliary force-induced state

(38°C). Electron-dense linkages exist between SFs and pcMT bundles of the anterior BB (cyan arrowheads: enlarged interface). Bars, 200 nm. (C) EM tomographic images of longitudinal and cross-sectional SF-epiplasm interfaces at steady state and elevated ciliary force-induced state (red arrowheads: enlarged interface; magenta arrowheads: SF-epiplasm linkages). Bars, 200 nm. (D) Localization of non-SF localizing *Tetrahymena* SFA proteins. Multiple labelling strategies indicate that *Tt*Sfap localizes to oral and cortical cilia (white arrowheads). *Tt*Mdlg1p is found in vacuoles. mCh: mCherry. Bars, 10 μm (cell), 6.5 μm (inset width). (E) Sequence logos of group-specific consensus sequences in SFA proteins (X: non-conserved residue). (F) Quantitation of Group 2 SF proteins' distribution along the SF. *Top graph*: Length distribution of Group 2 SF proteins. *Bottom graph*: Start positions of Group 2 SF proteins relative to BB peak intensity. Number of SFs analyzed: Cro1p: 101; DisAp: 107; Kdf3p: 105; Kdd6p: 93; Bbc39p: 107; Kdf4p: 157; Bbc29p: 115; Kdf1p: 109. SFs were obtained from ≥ 60 cells. Mann-Whitney test * denotes P-value < 0.01. Mean±SD.

Figure S3. Cro1p promotes elevated ciliary force-induced SF elongation. (A) Schematic illustrating the genomic locus of *CRO1* and the site that was targeted for DNA elimination (red box). (B) PCR assessment confirmed DNA elimination at the targeted site of *CRO1* genomic locus. (C) RT-PCR assessment confirmed the absence of *CRO1* transcript expression in *cro1Δ* cells (red box). A partial *CRO1* transcript was expressed downstream of the DNA eliminated region (white box). (D) Expression and localization of Cro1p-HA in *cro1Δ* rescue strain. BB (red) and Cro1p-HA (green). Bars, 10 μm (cell), 2 μm (inset). (E) Motility assay of WT, *cro1Δ* and *cro1Δ* rescue cells at steady state and elevated force-induced state. Bar, 100 μm. Motility assay: 90 cells. (F) Mispositioned oral structure in dividing and twisted *cro1Δ* cells. White arrowheads mark matured and developing oral structures. Bar, 10 μm. (G) Twisted cell phenotype is rescued at reduced ciliary forces. To reduce ciliary forces, *cro1Δ* cells were

enriched for the twisted cell phenotype at 39°C for 24 hours before they were temperature shifted to 25°C. Percentage of twisted cell was assessed 24 hours post temperature shift. (H) Schematic analysis to quantify SF length and local SF angle (θ) relative to the anterior BB. (I) Local SF angle is wider for BBs that exhibit local disorientation as compared to BBs within twisted rows in the elevated force-induced state. (J) Locally disoriented BBs possess shorter SFs than BBs in twisted rows. n ≥ 285 SFs (≥ 37 cells). Mann-Whitney test * denotes P-value < 0.01. Mean±SD. (K) BB spacing is marginally increased in *cro1Δ* cells at steady state and elevated force-induced state. n ≥ 120 (≥ 30 cells). (L) SF length correlates with the frequency of SF-pcMT contacts and BB orientation. Schematic illustrates the position and orientation of two BBs within a region of interest (white box). White arrowhead marks BB clusters in *disA-1* mutants. n = 240 SFs (24 cells). Bar, 2 μm.

Supplemental video legends

Video 1. Serial, tomographic slices and model depicting cross-section of SF-pcMT interface at steady state (25°C). Electron-densities link SFs to pcMT bundles. BB (red), SF (green), pcMT (cyan) and linkages (purple). Bar, 200 nm.

Video 2. Serial, tomographic slices and model depicting cross-section of SF-epiplasm interface at steady state (25°C). BB (red), SF (green), epiplasm (white) and linkages are absent. Bar, 200 nm.

Video 3. Serial, tomographic slices and model depicting cross-section of SF-epiplasm interface at elevated force-induced state (38°C). Electron-densities link SF to the epiplasm. BB (red), SF (green), epiplasm (white) and linkages (purple). Bar, 200 nm.

Supplemental tables

Table S1. Phylogenetic analysis of SFA homologs amongst ciliates and algae. Rooted phylogenetic tree of 205 SFA protein sequences gathered from 50 species of protists and algae. Three taxonomically diverse clades are identified and designated as Group 1, Group 2A, and Group 2B.

Table S2. Tabulation of oligo sequences used for fluorescent protein tagging and the functional study of *Tetrahymena* Cro1p.

Supplemental data file

Supplemental data file 1. *SF-assemblin_alignment.fa* - Multiple sequence alignment of 205 SFA protein sequences from 50 protist and algae species.

Materials and methods

Tetrahymena culture

Tetrahymena thermophila cells were grown in 2% SPP media (2% proteose peptone, 0.2% glucose, 0.1% yeast extract, and 0.003% Fe-EDTA). For studies under cycling conditions, cells were analyzed at mid-log phase (4–5 × 10^5 cells/ ml) as determined using a Coulter Counter Z1 (Beckman Coulter). For studies under non-cycling conditions, cells were arrested in the G1 phase of the cell cycle by washing and culturing in 10 mM Tris-HCl (pH 7.4) for 48 hours. Cell concentration was kept at 4–5 × 10^5 cells/ ml. For microscopy experiments, analyses were restricted to nondividing cells as judged by those lacking a developing oral structure. To expose cultures to reduced ciliary forces, *Tetrahymena* cells were propagated in 2% SPP supplemented with 3 mM $NiCl_2$ (Sigma-Aldrich), which was added directly to the culture vessel from a 1 M stock. Dynein-dependent ciliary beating is inhibited by $NiCl_2$, which blocks plasma membrane calcium channels and directly inhibits dynein motors (Larsen and Satir, 1991). For experiments that require an extended duration of $NiCl_2$ treatment, a lower dosage of $NiCl_2$ (2 mM) was used.

Plasmids and *Tetrahymena* strain construction

The BB re-orientation assay was performed using the *disA-1* strain previously described (Galati *et al*., 2014). To initiate rescue in *disA-1* mutants, *DISA-1* expression was induced via a cadmium (II) chloride-driven promoter.

Previously reported *Tetrahymena* strains expressing SFA proteins (Cro1p, Kdf3p, Kdd6p, Bbc39p, Kdf4p, Bbc29p and Kdf1p) localize to the SF (Chalker group; www.suprdb.org). In this study, we created endogenously tagged genes under native promoters. WT *Tetrahymena* cells (B1868) that express mCherry-tagged SF proteins were created using the p4T2-1-mCherryLAP construct (Winey *et al*., 2012). Briefly, the mCherryLAP cassette integrates at the endogenous target gene locus and gene expression remains under the control

of the endogenous promoter. Transformed cells were selected for paromomycin resistance and assorted to at least 2 mg/mL of paromomycin. For information on oligo design, please refer to Supplemental Table S2.

Phylogenetic analysis

We gathered an initial set of SFA related protein sequences from our in-house orthology dataset by running Orthofinder 2.1.2 (Emms and Kelly, 2015) on a curated set of 169 eukaryotic proteomes with Diamond (Buchfink *et al*., 2015). In order to identify sequences that may have been missed, we constructed a custom Hidden Markov Model using the HMMER package (Eddy, 2011) using merged orthologous group sequences that were aligned with MAFFT (v7.271) (Katoh and Standley, 2013). The final alignment was constructed by aligning the inclusive set of sequences with CulstalOmega (v1.2.1) (Sievers *et al*., 2011) and curated by removing variable N- and C- termini sequences. The final set contains 205 SFA sequences (Supplemental data file 1). Phylogenetic trees were constructed using RaxML (v8.2.4) (Stamatakis, 2014) and MrBayes (v3.2.6) (Ronquist *et al*., 2012). Both algorithms generated phylogenetic trees that are in agreement. Raw data is available on request. Since a suitable outgroup could not be determined, the final trees were rooted such that each branch contains eukaryotic wide taxonomic distributions. Coiled-coil regions are predicted during manual curation of SFA protein sequence alignment (Fig. 4B).

Immunocytochemistry

BB and SF labelling

For immuno-cytochemical analyses of BBs and SFs, 7×10^5 cells were pelleted at 600 *g* in a 1.5-ml microcentrifuge tube and fixed for 20 min with 1 ml of 70% ethanol + 0.1% Triton X-100 on ice. Cells were washed with 10 mM phosphate buffered saline (PBS; pH 7.4) and

blocked in 0.5% BSA/ PBS for 1-hour at 4°C. Cells were immunostained by incubating in primary antibody (mouse anti–SF (5D8), 1:500; rabbit anti–centrin (BB), 1:500 (Jerka-Dziadosz *et al*., 1995; Stemm-Wolf *et al*., 2005)) overnight at 4°C followed by secondary antibody (goat anti–mouse Alexa Fluor 488 or 594, 1:2,000; goat anti–rabbit Alexa Fluor 647, 1:2,000; Invitrogen) for a 2-h incubation at 4°C. Cells were mounted in Citifluor mounting media (Citi- fluor LTD) using #1.5 coverslips and sealed with nail polish. All antibodies were diluted in 0.5% BSA/ PBS. Cells were washed (3 × 5 min) with 0.5% BSA/ PBS after primary and secondary antibody incubations.

SF and pcMT labelling

For immuno-cytochemical analyses of SFs and pcMT, 7×10^5 cells were pelleted at 600 *g* in a 1.5-ml microcentrifuge tube and fixed for 5 min with 1 ml of 3.2% PFA/ PHEM + 0.24% Triton X-100 (PHEM; 60 mM 1,4-piperazinediethanesulfonic acid, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 10 mM EGTA, and 2 mM $MgCl_2$, pH 6.9) at 25°C. Next, cells were pelleted and resuspended in 1 ml of ice cold 0.5% Triton X-100 (PHEM) for 10 min on ice at 4°C. Cells were washed with PHEM and blocked in 0.5% BSA/ PHEM for 1-hour at 4°C. Cells were immunostained by incubating in primary antibody (mouse anti–SF (5D8), 1:500; rabbit anti–acetylated alpha tubulin lysine 40 (D20G3; Cell Signaling Technology), 1:100) overnight at 4°C followed by secondary antibody (goat anti–mouse Alexa Fluor 488 or 647, 1:2,000; goat anti–rabbit Alexa Fluor 594, 1:2,000; Invitrogen) for a 2-h incubation at 4°C. Cells were mounted in Citifluor mounting media (Citi- fluor LTD) using #1.5 coverslips and sealed with nail polish. All antibodies were diluted in 0.5% BSA/ PHEM. Cells were washed (3 × 5 min) with 0.5% BSA/ PHEM after primary and secondary antibody incubations.

Light microscopy

Imaging experiments (Figs. 3C, 4D, 5A, S1A, E–H, S2D and S3D, F) were performed with an inverted widefield microscope (Ti Eclipse; Nikon). A 100× Plan-Apochromat (NA 1.4) objective lens (Nikon) was used. Images were captured with a z-step size of 300 nm using a scientific complementary metal-oxide semiconductor camera (Zyla; Andor Technology).

Super-resolution localization experiment of SF-pcMT interactions (Figs. S1C and S3L) and Group 2 SF proteins (Fig. 4C) were performed via structured illumination microscopy (SIM) with the Nikon 3D SIM system. A 100× TIRF objective (NA 1.45) was used. Images were captured with a complementary metal-oxide semiconductor camera (Hamamatsu) with a z-step size of 300 nm. Raw SIM images were reconstructed by the image stack reconstruction algorithm (Nikon Elements).

Confocal microscopy was performed using an inverted microscope (Ti Eclipse) with a 100× Plan-Apochromat (NA 1.43) objective lens (Nikon) and a Swept Field confocal scan head with the 35 μm slit mode (Prairie Technologies). Images were captured with a charge-coupled device (CCD) camera (iXon X3; Andor Technology). Confocal images were also acquired with the A1 Confocal laser microscope (Nikon). All images were acquired with Nikon Elements (Nikon) with a z-step size of 300 nm at room temperature (Figs. 1, 2, S1D and S2A).

Electron tomography

Cells were prepared for electron tomography as previously described (Giddings *et al*., 2010; Meehl *et al*., 2009). Cells were gently spun into 15% dextran (molecular weight 9000–11,000; Sigma-Aldrich) with 5% bovine serum albumin in 2% SPP. A small volume of concentrated cells was transferred to a sample holder and high-pressure frozen using a Wohlwend Compact 02 high pressure freezer (Technotrade International). After low-temperature freeze substitution in 0.25% glutaraldehyde and 0.1% uranyl acetate in acetone, cells were slowly infiltrated with

Lowicryl HM20 resin. Serial thick (250–300 nm) sections were cut using a Leica UCT ultramicrotome. The serial sections were collected on Formvar-coated copper slot grids and poststained with aqueous uranyl acetate followed by Reynold's lead citrate.

Dual-axis tilt series (−60 to +60°) of *Tetrahymena* cells were collected on a FEI Tecnai 300kV FEG-TEM (FEI, Eindhoven, Netherlands). Images were acquired using the SerialEM acquisition program (Mastronarde, 2005) with a Gatan OneView (4k × 4k) camera. Serial section tomograms of *Tetrahymena* cortical structures were generated using the IMOD 4.9 software package (Kremer *et al*., 1996; Mastronarde, 1997). Three tomograms for each interface of interest were analyzed.

***Tetrahymena* motility measurements**

Tetrahymena cell motility was imaged on an inverted widefield microscope using a 20× objective lens (Ti Eclipse; Nikon). Each movie duration is 2.5 seconds (exposure duration: 50 milliseconds; frame rate: 20 frames per second). To quantify swim rates, the relative displacement of the anterior or posterior pole of cells was tracked for 500 ms via the FIJI MTrackJ plugin (Meijering *et al*., 2012). Analyses were restricted to cells that swim along the same xy-plane.

Image analysis: (I) SF length, (II) BB orientation (R value), (III) distance between posterior BB and anterior BB's pcMT distal tip and (IV) frequency of SF-pcMT contact

(I and II) SF length and BB orientation were quantified by a semi-automated strategy previously described (Galati *et al*., 2014). Briefly, all images were first uniformly contrasted prior to quantitation. SF length was measured via the freehand tool (Fig. 1B). To compute BB orientation (R value), the ORIANA circular statistics suite (Kovach Computing Services) was used. Based on the displacement of the SF from its associated BB and the anterior pole of the

cell, an angular measurement is obtained (Fig. 1B). For each *Tetrahymena* cell, at least five angular measurements were gathered from SFs that are positioned within a 10 μm box placed at the cell's medial region. Using the angular measurements, the mean vector and length of the mean vector (R value) were calculated. A R value of 1 indicates that SFs are uniformly oriented towards the anterior pole of the cell. Conversely, a R value of 0 indicates that SFs are randomly oriented. The discrepancy in R values between our prior publication and this manuscript is attributed to slight differences in SF sampling between experimentalists (Galati *et al*., 2014). Consistency of R value measurements was ensured in this manuscript.

(III) To quantitate the position of the anterior BB's pcMT distal tip (relative to the posterior BB), we colocalized SFs with pcMT and measured the distance from the pcMT distal tip to the posterior BB (Fig. 1B). (IV) To measure the frequency of SF-pcMT contacts, we determined the criteria for contact to be an overlap of the SF and pcMT fluorescence signals by at least 2 pixels (220 nm). Both analyses were restricted to SFs and pcMTs at the cell's medial region.

Image analysis: Quantification of SF position relative to the cell cortex (epiplasm)

Fluorescence quantification of SF position relative to the cell cortex was performed by a semi-automated strategy that utilizes the FIJI macro scripting language and plugins (Schindelin *et al*., 2012). Image stacks co-localized for BBs, SFs and the cell cortex (marked by EpiC-mCherry) were pre-processed to generate maximum projections (3 slices; z-step size = 300 nm (FIJI Z Project; Max Intensity plugin)). All analyses were restricted to BBs and their associated SFs that are found in the medial region of the cell. To visualize the longitudinal SF-cell cortex interface, BBs positioned at the side of *Tetrahymena* cells are selected. Each BB unit, which includes a BB, its associated SF and the nearby epiplasm, is cropped as an individual image (5.2 μm × 5.2 μm) for further processing. The average distance between the SF and the

epiplasm is measured based on the peak intensities of the SF (distal 25th percentile of its total length; labelled with mouse anti–SF (5D8)) and the epiplasm marker, EpiC-mCherry (FIJI Plot Profile plugin). The average SF length that spans along the epiplasm is quantified based on SF peak fluorescence intensities that fall within 130 nm from the EpiC-mCherry peak fluorescence intensities.

Image analysis: Fluorescence image averaging

Fluorescence image averaging was performed by a semi-automated strategy that utilizes the FIJI macro scripting language and plugins (Schindelin *et al*., 2012). Image stacks were pre-processed to generate maximum projections of the *Tetrahymena* cell side that is nearer to the cover glass (11 slices; z-step size = 300 nm (FIJI Z Project; Max Intensity plugin)). All analyses were restricted to BBs and their associated SFs that are found in the medial region of the cell. Each selected BB and its associated SF (SF length: 1.1–1.3 μm) is cropped as an individual image (5.2 μm × 5.2 μm) for further processing.

Next, selected BBs and SFs serve as fiducial marks for 2D alignment. To align BBs, the brightest pixel within each BB is identified by gaussian blur (FIJI Gaussian Blur plugin; sigma = 2 pixels). Based on the position of the brightest pixel in each BB, BBs are aligned through the FIJI Translate plugin. Next, a second alignment step based on the SF distal tip was performed. To align SFs along the same axis, SFs are rotated (relative to the BB's brightest pixel) using the FIJI Rotate plugin.

Prior to fluorescence image averaging, the local background fluorescence intensities (defined by the regions directly adjacent to the signal of interest) were measured, averaged and subtracted from each respective channel. Next, all peak intensities for each respective channel were normalized to 1. Finally, fluorescence images of aligned BBs and SFs were averaged using the FIJI Average Intensity plugin.

To measure the average length distribution and relative start positions of Group 2 SF proteins along the SF, we measured the fluorescence distribution of each mCherry-tagged Group 2 SF protein (FIJI Plot Profile plugin). To quantify the length of SF protein-mCherry localization along the SF, a fluorescence-based criterion was used (intensity value ≥ 30% from the peak fluorescence intensity qualifies for length measurement). The start positions of Group 2 SF protein-mCherry along the SF are determined based on their relative positions from the BB centroid. The same fluorescence-based criterion was applied.

Statistical analysis

All datasets were assessed for normal distribution using Shapiro-Wilk normality test. Student's t-test was performed on normally distributed datasets. Mann-Whitney test was performed on datasets that do not conform to a normal distribution. Tests for significance were unpaired and two-tailed. Categorical datasets were analyzed using Chi square test. All error bars indicate SD unless otherwise stated. Statistical significance was set at P-value < 0.01. All analyses were performed on samples obtained from 3 independent experiments.

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August 26, 2019

Re: JCB manuscript #201904091R

Dr. Chad G Pearson
University of Colorado - School of Medicine
Department of Cell and Developmental Biology 12801 E. 17th Ave. Room 12104
Aurora, CO 80045

Dear Chad,

Thank you for submitting your revised manuscript entitled "Dynamic basal body-associated striated fibers promote basal body coupling and cortical interactions". We apologize for the delay in providing you with a decision.

In any case, the manuscript has been seen by the original reviewers whose full comments are appended below. While the reviewers continue to be overall positive about the work in terms of its suitability for JCB, some important issues remain.

You will see that both reviewers have a few lingering concerns that will need to be addressed prior to publication. We hope that you will be able to address these items in a revised version of the manuscript. In particular, reviewer#2 feels that the evidence provided in support of the conclusion that SF elongation enables basal body reorientation remains insufficient. This reviewer has suggested a new plot/analysis approach to address this issue. It should be possible to address the rest of reviewer#2's comments with further explanation and/or additions to the text. Reviewer#1 also questions the generality of these findings and has suggested that you make the model/cell type as clear as possible in the paper. To this end, we suggest that you better illustrate the model type used/cited in the abstract and introduction. However, we do not agree with this reviewer that the term "ciliates" should be added to the title. Finally, we also disagree with this reviewer on the contention that JCB readers may not know what "cytotaxis" means; please be sure to define the term as clearly as you can in the introduction, but we do not feel it is necessary to do so in the abstract.

Our general policy is that papers are considered through only one revision cycle; however, given that the suggested changes are relatively minor we are open to one additional short round of revision. Please note that we will expect to make a final decision without additional reviewer input upon resubmission.

Please submit the final revision within one month, along with a cover letter that includes a point by point response to the remaining reviewer comments.

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

Sincerely,

Monica Bettencourt-Dias, PhD
Monitoring Editor
JCB

Tim Spencer, PhD
Interregnum Executive Editor
Journal of Cell Biology

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

The authors have improved the paper, by providing additional data and in particular by including better images. The most valuable contribution remains the discovery of the SF-PC links, which could be the structural basis of ciliary row formation and maintenance in ciliates.

This and the previous paper (Galati et al 2014) document extensively that the SF proteins are important for the organization of ciliary rows. While the authors do not provide a direct proof, it makes sense to conclude that SF has to have a proper length to connect the neighboring BBs. While it is not a new finding, the paper confirms that the length of the SF changes at different temperatures and under conditions that affect the ciliary beat frequency. In addition, the EM tomography shows that a longer SF has additional contact sites with the BB which is could be important.

However, it is not clear how these findings extend beyond the organization of ciliary rows in ciliates and even how they informs about the function of striated fibers in general. If the authors disagree then they should try to better convey the generality of their findings in the manuscript.

The title is a bit vague and does not convey the main findings well enough. Also, I would consider to include the species (tetrahymena), or at least the phylum name (ciliates) in the title.

In the Introduction multiple sentences state published data without specifying the model species in which the observations were made. Observations made in cell types with vastly different organizations of multiciliated fields and BB accessory structures are lumped together. I would suggest mentioning the model organisms more often so that the reader can judge the generality of statements that the authors make.

The abstract still needs more work. The authors have to make it clear that the research is done in a ciliate model Tetrahymena in the first or second sentence. It is really confusing when the authors go into cytotaxis and SF length changes without first stating that they are describing ciliary rows in a ciliate.

I suspect that very few readers are familiar with the term 'cytotaxis". Thus, the use of this term without definition in the Abstract is awkward. Also, it is not accurate to state that in ciliates cytotaxis is the mechanism that propagates cortical organization. Rather cytotaxis works along with additional cell-wide mechanisms that control the position of organelles in reference to cell ends and around the cell's circumference.

Regarding the new data, it is not clear how the experiments on single cell were made. These observations were made over several days. Did the isolated cells divide during the period of observations?

Also, it is not clear how the observations were made in "non-cycling" (starved) cells. Where these cells first grown at the higher temperature, starved at the higher temperature for 48 hrs and then rescued by induction with cd ions at the lower temperature?

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

In my previous comments on the original manuscript by Soh et al. I had criticized the authors for dwelling in the text on results that appear to largely retread ground covered already in their previous study (Galati et al., JCB 2014). In the revision, they have successfully reworked the text to bring out much better what is actually novel, in particular that basal body reorientation can occur upon rescue of the disA-1 mutant, which argues against the cytotaxis model prevalent in the field. While I appreciate the authors' efforts, there are still significant issues with the experiments as currently presented.

The first is that the correlation between SF elongation and basal body reorientation is not very strong. Presenting the data in scatter plots +/- standard deviation rather than bar graphs with SEM as in the original manuscript, while very welcome, reveals how noisy the data in Fig. 1C and others actually is. Furthermore, the effect of DISA-1 rescue on basal body orientation as opposed to SF length is minimal - comparing Figs. 1C and S1D reveals effectively no difference between +/- cadmium chloride conditions, contrary to what the 48 and 336h recovery images suggest. The authors attribute this to leakiness of the inducible promoter (legend to Fig. S1D), but SF length barely improves without cadmium. The authors make much of the 'magic' distance of 0.59 micron that SFs have to reach before they can establish interactions with pcMTs from neighboring basal bodies and this is indeed plausible. However, the evidence from their quantitations at present is rather weak. One thing that might help to strengthen the correlation is to plot SF length against basal body orientation for individual basal bodies or cells which might better reveal the 0.59 micron inflection point the authors hypothesize exists.

On page 6 the authors state: "As BBs assemble during cell division, the spacing between neighboring BBs narrows, and this could promote BB re-orientation upon DISA-1 rescue. To assess whether cell cycle progression and new BB synthesis are required for BB re-orientation, cells were starved to inhibit both processes and induced for DISA-1 rescue. Starved disA-1 cells upon rescue were as efficient as cycling cells in BB re-orientation, indicating that cell cycle progression and new BB synthesis are not required for BB re-orientation (Fig. S1E, F)." Does this not argue against the authors' own hypothesis? If basal body duplication decreases spacing and proximity is critical for reorientation, duplication may not be required for reorientation but it should aid it. Yet it apparently does not. Why not?

I agree with reviewer 1 that the authors cannot exclude the alternative hypothesis that an unknown mechanism gradually organizes the BBs into rows and that SFs elongate as the rows reform. Further, it bears remarking that cortical organization is not completely lost in disA-1 mutants: Jerka-Dziadosz et al., Dev Biol 1995 reported that longitudinal microtubules are largely unaffected. These microtubules could serve as a guide to realign basal bodies once SFs are

sufficiently elongated. If so, the argument against cytotaxis is severely weakened. Can the authors exclude this possibility?

Minor points
1. Somewhat strangely the graphs between previous and current versions of the manuscript don't always match. For example basal body orientation in Fig. S1E reached an average of >0.85-0.9 at 48h of recovery in the original manuscript, while the same data now presented as a scatter plot reveals an average of nearer 0.8. The same is true for bb orientation in Figs. 1C, S1E, S1G. Why should this be?

2. The revised figures clearly demonstate the large variability of measured parameters like SF length and basal body orientation masked in the original manuscript by the inappropriate use of SEM coupled with the large sample numbers achievable in Tetrahymena. SEM should likewise be replaced by SD in Figs. 3C and 4E.

3. In Fig. S1H, the authors report that there is considerable variability in the time at which basal body orientation is rescued in different disA-1 isolates. This is displayed as percent of rescued single cell isolates. How is 'rescue' assessed (eg R value >0.9 in >50% of cells in isolate)?

4. Why are there comparatively so few measurements for basal body orientation (vs SF length) if both are measured on the same IF signal?

5. In answer to one of reviewer 1's comments the authors report their failure to generate a dCro1/DisA double mutant, which they suspect is due to synthetic lethality. Since this potentially means that a total loss of SFs is lethal in Tetrahymena it may be worth relating this information in the text.

6. Not being an expert on Tetrahymena, I did not question the authors' focus on basal body orientation in the medial region. Is there any reason why SF lengths should be more variable in the anterior region?

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

The authors have addressed the comments satisfactorily and I am therefore pleased to support the publication of this work in JCB.

Minor comment: The y-axis titles in Figure 2A-B are inverted.

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September 10, 2019

RE: JCB Ms. #201904091

Dear Drs. Bettencourt-Dias and Spencer,

We are pleased to submit our revised manuscript entitled, “Ciliary force responsive striated fibers promote basal body connections and cortical interactions” (JCB Ms. #201904091). The revised manuscript is greatly improved by the addition of new analyses and by the comments and clarifications provided by all three reviewers.

To address the concern of Reviewer 2 that we lack evidence to support the conclusion that SF elongation enables BB re-orientation, we performed and included the proposed correlation analysis between SF length and BB orientation during *DISA-1* rescue. Consistent with our prior analyses, BBs with short SFs remain disoriented while BBs with SFs longer than the minimal length for SF-pcMT contacts were oriented (Figs. 1C and S1D). This further supports our hypothesis that SF length is important for proper BB orientation.

Reviewer 1 questioned the generality of our findings. To enable readers to judge the generality of the concepts and models we proposed, we now highlighted the model systems used and referenced in the manuscript. These are now specified in the Abstract and Introduction.

The attached rebuttal describes the included revisions to address all the reviewers’ comments (red).

Please do not hesitate to contact me with questions or comments.

Best Regards,

Chad Pearson

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

The authors have improved the paper, by providing additional data and in particular by including better images. The most valuable contribution remains the discovery of the SF-PC links, which could be the structural basis of ciliary row formation and maintenance in ciliates.

This and the previous paper (Galati et al 2014) document extensively that the SF proteins are important for the organization of ciliary rows. While the authors do not provide a direct proof, it makes sense to conclude that SF has to have a proper length to connect the neighboring BBs. While it is not a new finding, the paper confirms that the length of the SF changes at different temperatures and under conditions that affect the ciliary beat frequency. In addition, the EM tomography shows that a longer SF has additional contact sites with the BB which is could be important.

However, it is not clear how these findings extend beyond the organization of ciliary rows in ciliates and even how they informs about the function of striated fibers in general. If the authors disagree then they should try to better convey the generality of their findings in the manuscript.

We agree with the reviewer that the organization of ciliary rows in ciliates and vertebrates are relatively different. However, the underlying architecture such as basal feet and SFs, and their interactions with cytoskeletal elements (microtubules, actin, intermediate filaments) are hypothesized to perform conserved functions in BB organization in both systems. We have now highlighted this point to help readers appreciate this concept.

"In amphibians, basal feet and SFs are polarized along the ciliary beat axis but are oriented in opposite directions (Hard and Rieder, 1983; Werner *et al.*, 2011). Both structures are generally thought to maintain BB position and orientation by mediating interactions with cortical microtubule, actin and intermediate filament cytoskeletons (Antoniades *et al.*, 2014; Kunimoto *et al.*, 2012; Lemullois *et al.*, 1987; Vladar *et al.*, 2012)."

The title is a bit vague and does not convey the main findings well enough. Also, I would consider to include the species (tetrahymena), or at least the phylum name (ciliates) in the title.

We edited the manuscript title to convey our main findings better. **"Ciliary force responsive striated fibers promote basal body connections and cortical interactions"**

As suggested by the editor, we will not indicate the species or phylum in the title but we emphasized this in the Abstract and Introduction.

In the Introduction multiple sentences state published data without specifying the model species in

which the observations were made. Observations made in cell types with vastly different organizations of multiciliated fields and BB accessory structures are lumped together. I would suggest mentioning the model organisms more often so that the reader can judge the generality of statements that the authors make.

We now specify the broad classification of relevant organisms so that readers can judge the generality of the statements made.

The abstract still needs more work. The authors have to make it clear that the research is done in a ciliate model Tetrahymena in the first or second sentence. It is really confusing when the authors go into cytotaxis and SF length changes without first stating that they are describing ciliary rows in a ciliate.

We specified that we use *Tetrahymena* and that we are referring to ciliary rows in the Abstract.

I suspect that very few readers are familiar with the term 'cytotaxis". Thus, the use of this term without definition in the Abstract is awkward. Also, it is not accurate to state that in ciliates cytotaxis is the mechanism that propagates cortical organization. Rather cytotaxis works along with additional cell-wide mechanisms that control the position of organelles in reference to cell ends and around the cell's circumference.

As suggested by the monitoring editor, we provided a clearer definition of cytotaxis in the Introduction.

We agree with the reviewer that additional mechanisms function with cytotaxis to propagate cortical organization. The manuscript is revised to indicate that local polarity mechanism (cytotaxis), along with global cellular mechanisms, promote cortical organization.

“Ciliate cortical organization is promoted by both global and local polarity cues (Frankel, 1989; Frankel, 2008; Sonneborn, 1964). Cytotaxis is a local and nongenetic polarity mechanism whereby preexisting BBs and their associated structures transmit local polarity information to guide the organization and orientation of new BBs (Beisson and Sonneborn, 1965; Frankel, 1964; Ng and Frankel, 1977; Sonneborn, 1964; Tartar, 1956).”

Regarding the new data, it is not clear how the experiments on single cell were made. These observations were made over several days. Did the isolated cells divide during the period of observations?

DISA-1 rescue cells were grown in media at elevated temperature (37deg) for 24 hours to exacerbate BB disorientation and disorganization. Next, single *DISA-1* rescue cells were isolated and grown in SPP media with and without GFP-DisA expression. The rescue was performed at steady state (room temperature) and cell division was observed during the course of the experiment. We now clarify this in the Results and Methods sections.

Also, it is not clear how the observations were made in "non-cycling" (starved) cells. Where these cells first grown at the higher temperature, starved at the higher temperature for 48 hrs and then rescued by induction with cd ions at the lower temperature?

DISA-1 rescue cells were grown in SPP media at elevated temperature (37deg) for 24 hours to exacerbate BB disorientation and disorganization. Next, the rescue was performed by culturing cells in non-cycling (starved; TRIS buffer) conditions with and without GFP-DisA expression. Like the rescue experiment under cycling condition, GFP-DISA expression was induced at steady state (room temperature). Cells were collected at the respective time points and fixed for immunofluorescence assay and imaged. This is now clarified in the Results and Methods sections.

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

In my previous comments on the original manuscript by Soh et al. I had criticized the authors for dwelling in the text on results that appear to largely retread ground covered already in their previous study (Galati et al., JCB 2014). In the revision, they have successfully reworked the text to bring out much better what is actually novel, in particular that basal body reorientation can occur upon rescue of the disA-1 mutant, which argues against the cytotaxis model prevalent in the field. While I appreciate the authors' efforts, there are still significant issues with the experiments as currently presented.

The first is that the correlation between SF elongation and basal body reorientation is not very strong. Presenting the data in scatter plots +/- standard deviation rather than bar graphs with SEM as in the original manuscript, while very welcome, reveals how noisy the data in Fig. 1C and others actually is. Furthermore, the effect of DISA-1 rescue on basal body orientation as opposed to SF length is minimal - comparing Figs. 1C and S1D reveals effectively no difference between +/- cadmium chloride conditions, contrary to what the 48 and 336h recovery images suggest. The authors attribute this to leakiness of the inducible promoter (legend to Fig. S1D), but SF length barely improves without cadmium. The authors make much of the 'magic' distance of 0.59 micron that SFs have to reach before they can establish interactions with pcMTs from neighboring basal bodies and this is indeed plausible. However, the evidence from their quantitations at present is rather weak. One thing that might help to strengthen the correlation is to plot SF length against basal body orientation for individual basal bodies or cells which might better reveal the 0.59 micron inflection point the authors hypothesize exists.

1. Variance in SF length and BB orientation

As noted by Reviewer 1, there is indeed a relatively high level of noise in the quantified parameters (SF length and BB orientation (R value)). This is consistent with the varying levels of BB disorientation between cells and the variable rates that *DISA-1* rescue isolates undergo BB re-orientation (Figs. 1C; *panel iii*; S1D, I). We postulate that the observed variability was attributed to the degree of BB disorientation individual cells possess at the onset of the experiment and this is now described in the manuscript as follows:

"**Interestingly, *DISA-1* rescue isolates recover at different rates (Fig. S1I; *bottom panel*). Since a subpopulation of cells still retains poor BB orientation even at 336 hours post *DISA-1* rescue induction, we postulate that the difference in recovery rate results from the varying degree of BB disorientation at the onset of the experiment (Figs. 1C; *panel iii*; S1D).**"

2. Discrepancy in the recovery extent of SF length and BB re-orientation during *DISA-1* rescue

The discrepancy in the extent of SF length and BB re-orientation recoveries between the presence and absence of the rescue inducer (cadmium chloride) was confirmed to be due to leaky GFP-DISA expression. At 48 h and 2 weeks post rescue non-induction, low levels of GFP-DISA expression was observed. Consistent with this, the average SF length and BB orientation (R value) increased marginally under the uninduced condition (48 h: Percent of SF length change: +16%, Percent of R value change: +14%; 2 weeks: Percent of SF length change: +9%, Percent of R value change: +9%). However, the majority of these cells possess SFs that fall under the SF-pcMT contact distance (Fig. S1E) and still exhibit BB disorientation as represented in the figure images. The revised manuscript highlights the presence of leaky GFP-DISA expression in the negative control (uninduced condition) in the Results as follows:

"Although mild recoveries in SF length and BB orientation were observed in the negative control (uninduced condition), they were attributed to leaky GFP-DisAp expression (Fig. S1E)."

3. Supplement SF length and BB orientation / raw angle correlation plot

To better support our model that SF lengthening promotes BB re-orientation, we now provide a plot to show the correlation of SF length with BB orientation on a single BB and cell level. We also show that the 0.59 micron inflection point corresponds with the SF length where more BBs show proper orientation. These analyses are included in the revised manuscript (Figs. 1C; *panel iii*; S1D).

On page 6 the authors state: "As BBs assemble during cell division, the spacing between neighboring BBs

narrows, and this could promote BB re-orientation upon DISA-1 rescue. To assess whether cell cycle progression and new BB synthesis are required for BB re-orientation, cells were starved to inhibit both processes and induced for DISA-1 rescue. Starved disA-1 cells upon rescue were as efficient as cycling cells in BB re-orientation, indicating that cell cycle progression and new BB synthesis are not required for BB re-orientation (Fig. S1E, F)." Does this not argue against the authors' own hypothesis? If basal body duplication decreases spacing and proximity is critical for reorientation, duplication may not be required for reorientation but it should aid it. Yet it apparently does not. Why not?

Reviewer 2 correctly points out that our rescue experiments (both cycling and non-cycling conditions) argue that BB duplication is not required for BB re-orientation. This finding rejected our initial hypothesis that BB duplication would promote closer BBs and increased BB re-orientation. We posit that, despite cells having closer BB positioning, BB duplication does not increase the rate of BB reorientation because new BBs are assembling from disoriented BBs, thereby propagating BB disorientation. This point is now discussed in the revised manuscript as follows:

"Non-cycling *DISA-1* rescue cells were equally efficient as cycling cells in BB re-orientation. This suggests that cell cycle progression and new BB synthesis are not required for BB re-orientation (Fig. S1F, G). We postulate this is because new BBs assemble from disoriented BBs, thereby further propagating BB disorientation."

I agree with reviewer 1 that the authors cannot exclude the alternative hypothesis that an unknown mechanism gradually organizes the BBs into rows and that SFs elongate as the rows reform. Further, it bears remarking that cortical organization is not completely lost in disA-1 mutants: Jerka-Dziadosz et al., Dev Biol 1995 reported that longitudinal microtubules are largely unaffected. These microtubules could serve as a guide to realign basal bodies once SFs are sufficiently elongated. If so, the argument against cytotaxis is severely weakened. Can the authors exclude this possibility?

We agree with Reviewers 1 and 2 that there may be alternative SF-independent mechanisms that promote re-orientation and re-organization of BBs into rows. This had been discussed in the earlier revised manuscript and we now highlight this discussion point in the revised manuscript.

We also agree with Reviewer 2 that other cortical structures such as the longitudinal microtubules may serve as a guide to realign BBs once SFs are sufficiently long. However, our EM data suggests that WT SFs are not positioned in close proximity with the longitudinal microtubules. Hence, it is unlikely for the longitudinal microtubules to be involved in this process. This point and the possibility that other undetected cytoskeletal structures may guide BB orientation are now discussed in the revised manuscript as follows:

"While SF-independent mechanisms such as the potential role of neighboring cortical structures that promote BB re-orientation cannot be ruled out, we propose that SF elongation ensures the propagation of orientated BBs to future cell progeny."

Minor points

1. Somewhat strangely the graphs between previous and current versions of the manuscript don't always match. For example basal body orientation in Fig. S1E reached an average of >0.85-0.9 at 48h of recovery in the original manuscript, while the same data now presented as a scatter plot reveals an average of nearer 0.8. The same is true for bb orientation in Figs. 1C, S1E, S1G. Why should this be?

We appreciate the reviewer's discovery of this discrepancy. A scaling error in the BB orientation bar charts shown in the original manuscript was discovered during initial revision and the error was corrected in the scatter plots in the resubmitted manuscript. We have ensured that our data are correctly represented in the revised manuscript.

2. The revised figures clearly demonstate the large variability of measured parameters like SF length and basal body orientation masked in the original manuscript by the inappropriate use of SEM coupled with the large sample numbers achievable in Tetrahymena. SEM should likewise be replaced by SD in Figs. 3C and 4E.

We replaced the SEM with SD in Figs 3C and 4E of the revised manuscript.

3. In Fig. S1H, the authors report that there is considerable variability in the time at which basal body orientation is rescued in different disA-1 isolates. This is displayed as percent of rescued single cell isolates. How is 'rescue' assessed (eg R value >0.9 in >50% of cells in isolate)?

The rescue was assessed as improved cellular morphology and swim path relative to WT cells. We now specify our assessment criteria for 'rescue' in the Methods section as follows:

"The assessment criteria for the rescue of *DISA-1* rescue isolates are normal (i) cell morphology and (ii) swim path."

4. Why are there comparatively so few measurements for basal body orientation (vs SF length) if both are measured on the same IF signal?

BB orientation was quantified as a R value that reflects the variance of the BB orientation relative to the cell's anterior-posterior axis. The BB orientation in each cell is represented by a single R value so that there are fewer R values compared to SF length values. This is now clarified in the Methods section.

5. In answer to one of reviewer 1's comments the authors report their failure to generate a dCro1/DisA double mutant, which they suspect is due to synthetic lethality. Since this potentially means that a total loss of SFs is lethal in Tetrahymena it may be worth relating this information in the text.

We now discuss this in the revised manuscript.

"Based on the distinct functions of DisAp and Cro1p, we hypothesized that the double knockout of *DISA-1* and *CRO1* will lead to cells that do not assemble SFs. However, we failed to obtain clones of the double knockout strain. This suggests that the loss of function of both proteins is synthetically lethal."

6. Not being an expert on Tetrahymena, I did not question the authors' focus on basal body orientation in the medial region. Is there any reason why SF lengths should be more variable in the anterior region?

We focus on BBs in the medial region of the cell because BBs are uniformly spaced as compared to the closely positioned BBs at the cell's anterior end. This serves as a technical advantage as we can sample a large proportion of uniformly positioned BBs in these cells.

Beyond this study, we are interested in understanding whether SF length differs along the length of a *Tetrahymena* cell and how this may be involved in the generation of hydrodynamic forces during ciliary beating. This will be addressed in our future studies.

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

The authors have addressed the comments satisfactorily and I am therefore pleased to support the publication of this work in JCB.

Minor comment: The y-axis titles in Figure 2A-B are inverted.

This is now corrected in the revised manuscript.

Downtown Campus
Denver, Colorado

Anschutz Medical Campus
Aurora, Colorado

September 19, 2019

RE: JCB Manuscript #201904091RR

Dr. Chad G Pearson
University of Colorado - School of Medicine
Department of Cell and Developmental Biology 12801 E. 17th Ave. Room 12104
Aurora, CO 80045

Dear Dr. Pearson:

Thank you for submitting your revised manuscript entitled "Ciliary force responsive striated fibers promote basal body connections and cortical interactions". 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 Reports is < 20,000, 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. You are currently over this limit but we should be able to give you the extra space this time. However, please try to be as concise as possible.

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

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

4) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions (at

least in brief) in the text for readers who may not have access to referenced manuscripts. The text should not refer to methods "...as previously described."

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

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

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

8) Supplemental materials: There are strict limits on the allowable amount of supplemental data. Reports may normally have up to 3 supplemental figures. At the moment, you meet this requirement. However, the layout of STable 1 is not really appropriate for a 'Table' - this seems more appropriate for a 'figure'. Thus, we think that you should rename these diagrams as SFigures 4-6 (this needs to be split into three SFigures since each figure must be able to fit on a single page) - of course, we will allow the extra space for these SFigures in this case. However, please remember to: a) provide individual legends for each figure (they may be brief, if you wish), b) rename STable 2 to STable 1, and c) change all the relevant 'callouts' in the text to reflect these changes.
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 (and please make sure to use the new numbering as well in this summary paragraph).

9) eTOC summary: A ~40-50 word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person.

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) 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.

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Thank you for your attention to these final processing requirements. Please revise and format the manuscript and upload materials within 7 days.

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,

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