Whole-genome screen identifies diverse pathways that negatively regulate ciliogenesis

Marion Failler, Ariadna Giro-Perafita, Mikito Owa, Shalini Srivastava, Chi Yun, David Kahler, Derya Unutmaz, Francisco Esteva, Irma Sanchez, and Brian Dynlacht

Corresponding author(s): Brian Dynlacht, NYU School of Medicine

Review Timeline:

|                   |               |
|-------------------|---------------|
| Submission Date:  | 2020-02-12    |
| Editorial Decision| 2020-02-13    |
| Revision Received | 2020-11-06    |
| Accepted:         | 2020-11-09    |

Editor-in-Chief: Matthew Welch

Transaction Report:

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

Thank you for submitting your interesting paper to Mol.Biol.Cell and for including the reviews from Dev. Cell. Under the assumption that you have NOT transferred your paper to another Cell journal, we are happy to consider your work.

There are two issues I feel you should consider:

1) All of your manipulations are based on siRNAs or shRNAs. You should confirm the phenotype for the most critical hits in your screen by either creating CRISPR-based knockout cell lines or rescuing the effects of knockdowns via reintroduction of siRNA- or shRNA-resistant expression constructs.

2) I believe that the well known association of integrin adhesion with actin remodeling and YAP/TAZ nuclear localization and the following paper provide a mechanistic explanation for your results:

Kim J, Jo H, Hong H, Kim MH, Kim JM, Lee JK, Heo WD, Kim J. Actin remodelling factors control ciliogenesis by regulating YAP/TAZ activity and vesicle trafficking. Nat Commun. 2015 Apr 7;6:6781. doi: 10.1038/ncomms7781.

I would urge you to consider the possibility of a simple experiment e.g. reversing the effect of one of your siRNA knockdowns with activated YAP might add to the impact of your work.

I would leave it up to you as to which of the many other comments from Dev. Cell you choose to address. Many of the minor comments re: citing references, etc. should require little effort on your part.

Prof. Mark Ginsberg

Dear Dr. Dynlacht:

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript is not acceptable for publication at this time, but may be deemed acceptable after specific revisions are made.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

When submitting your revision online please use the link below, and include a cover letter that details, point-by-point, how the Monitoring Editor's and reviewers comments have been addressed.
When entering the author names online, enter them exactly as they appear on the manuscript title page. Please send only the latest revised manuscript. DO NOT resend any previous versions. Authors are allowed 180 days to submit a revision. If this time period is inadequate, please contact us immediately at mboc@ascb.org.

Revised manuscripts are assigned to the original Monitoring Editor whenever possible. However, special circumstances may preclude this. Also, revised manuscripts are often sent out for re-review, usually to the original reviewers, when possible. The Monitoring Editor may solicit additional reviews if it is deemed necessary to render a completely informed decision.

To prepare for the possible acceptance of your revised manuscript, submit final, publication-quality figures with your revision.

**MBoC PRODUCTION FILE REQUIREMENTS:**

**MANUSCRIPT and TABLE FILES** must be submitted in either .doc or .rtf format.

Because the quality of artwork reproduction is important, MBoC requires that all artwork be prepared using professional graphic art software. Word processing and presentation software packages (such as Word and Powerpoint) are inadequate for preparing high-quality digital artwork.

Figure File Types. For revised manuscripts, figure files should be in .tif, .eps, or .pdf format. Files in .eps or .pdf formats must have their fonts embedded, and the images in them must meet the resolution requirements below.

**Figure Size.** Prepare figures at the size they are to be published.

- 1 column wide: Figure width should be 4.23-8.47 cm
- 1 to 1.5 columns wide: Figure width should be 10.16-13.3 cm
- 2 columns wide: Figure width should be 14.4-17.57 cm

The figure height must be less than 22.5 cm

Resolution and Color Mode.
All images should be submitted at a minimum of 300dpi.
Save all color figures in RGB mode at 8 bits/channel.
Save all black and white images in Grayscale.

File Size. Final figures should be <10 MB in size. Figures larger than 10 MB are likely to be returned for modification. Tips for managing file sizes:
1. crop out all extraneous white space
2. RGB color mode for color images, Grayscale for images not containing color
3. avoid excessive use of imbedded color
4. select the LZW compression option when saving tif files in Photoshop, this is a lossless compression mechanism

Locants and Labels. Locants and labels can be between 1.5 and 2 mm high. Wherever possible, place locants and labels within the figures.

Line Images. Prepare line drawings at one-column width (less than 8.47 cm) or less if the graph or
histogram is relatively simple. Symbols should be at least 1 mm high and large enough to be distinguishable from the lines connecting them.

To submit the cover letter, revised version, and figures, please use this link (please enable cookies, or cut and paste URL): Link Not Available

Please contact us with any questions at mboc@ascb.org.

Thank you for submitting your manuscript to Molecular Biology of the Cell. We look forward to receiving your revised paper.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org

------------------------------------------------------------------------
November 6, 2020

RE: E20-02-0111
Title: Whole genome screen identifies diverse pathways that negatively regulate ciliogenesis

Dear Dr. Ginsberg,

We thank you for a thoughtful and careful review of our manuscript. We apologize for the lengthy revision time, necessitated by (1) a three month shutdown owing to the fact that New York City was an epicenter of the pandemic and (2) the need to replace the first two authors who left the lab with another fellow who was likewise delayed in her ability to enter the US because of a travel ban. Although we are still faced with numerous restrictions, we have made considerable progress and believe that we have fully addressed the concerns raised in your decision letter. Herewith, we present a revision for your examination. Below, we first address both of the issues that you raised previously, and secondly, we have addressed the comments that were raised in the initial Developmental Cell critique.

We believe that your comments were exceptionally helpful and right on target, and as detailed below, we are happy to report that the suggested rescue experiments were successful. With respect to the concerns raised at Developmental Cell, we feel that many of them were clearly beyond the scope of a first report, and to address each and every one of them would minimally require an additional two years. As you have suggested, a large number of concerns could be addressed through editorial changes, and where appropriate, we have made these revisions.

We believe that the above modifications have led to a substantially improved manuscript. We hope you agree that it has been significantly improved and that our paper is now acceptable for publication.

Sincerely,

Brian D. Dynlacht
Mol Bio Cell comments

Please see our responses to comments from Editor/Reviewers in bold.

1. " All of your manipulations are based on siRNAs or shRNAs. You should confirm the phenotype for the most critical hits in your screen by either creating CRISPR-based knockout cell lines or rescuing the effects of knockdowns via re-introduction of siRNA or shRNA-resistant expression constructs.

2. " I believe that the well known association of integrin adhesion with actin remodeling and YAP/TAZ nuclear localization and the following paper provide a mechanistic explanation for your results: Kim J, Jo H, Hong H, Kim MH, Kim JM, Lee JK, Heo WD, Kim J. Actin remodelling factors control ciliogenesis by regulating YAP/TAZ activity and vesicle trafficking. Nat Commun. 2015 Apr 7;6:6781 doi:10.1038/ncomms7781. I would urge you to consider the possibility of a simple experiment eg. reversing the effect of one on your siRNA knockdowns with activated YAP might add to the impact of your work."

We thank the Editor for suggesting these experiments, and we appreciate the need for additional confirmatory experiments. We did not attempt to knock out genes of interest using CRISPR because a knock-out would result in a permanently ciliated state that would cause growth arrest. In that case, such a cell line would not proliferate and would be unobtainable. We wish to make several points regarding this concern and any off-target or non-specific effects. First, our initial screen used a pool of siRNAs, and to follow up these candidates, we have used a set of shRNAs and siRNAs distinct from the ones employed in the initial screen. Second, we have supplied a protein (Vitronectin, VTN) to the extracellular matrix (ECM) to rescue enhanced ciliation induced by VTN depletion.

We have also further directly addressed both of the above issues by performing two additional rescue experiments suggested by the Editor. For these experiments, we selected a representative candidate gene from our screen (Fermt2) that is essential for integrin activation and focal adhesion assembly-mediated stabilization of the actin cytoskeleton. This protein is thus a pivotal link connecting the ECM to integrin activation and the actin cytoskeleton. In the first experiment, we have tested whether expression of RNAi-resistant, recombinant GFP-Fermt2 can rescue depletion of Fermt2. Second, as described below, we have asked whether expression of activated YAP can suppress enhanced ciliation in cells depleted of Fermt2. The results of these experiments are shown in new Fig. 7.

In these experiments, we showed that activated YAP can block ciliation induced through FERMT2 knock-down. This experiment was performed in RPE1 cell lines stably expressing GFP-YAP55A (activated YAP), GFP control, or FERMT2-GFP. Each of these cell lines was then infected with a lentivirus expressing a FERMT2 shRNA which targets a region within the 3' UTR of the FERMT2 gene. Virally transduced cells were selected and then fixed, and ciliation was scored by immuno-staining cilia with the anti-polyglutamylation antibody GT355. As shown in Fig. 7A/B, FERMT2 knock-down in control GFP cells induced robust ciliation to levels comparable to what was observed in the parental RPE1 cell line (compare to Fig. 3D). In contrast, FERMT2-GFP cells have higher FERMT2 expression than GFP control cells, FERMT2
shRNA expression in these cells was unable to induce ciliation to the same degree as in the control, indicating rescue of this phenotype. Finally, we observed substantially reduced levels of ciliation in cells expressing constitutively active GFP-YAP. Importantly, a requirement for integrin activation is that two proteins identified in our screen (TLN1 and Fermt2) must bind to the cytoplasmic tail of β integrins. Since integrin activation promotes and stabilizes the assembly of the actin cytoskeleton, our screen suggests that stabilization of the actin cytoskeleton through activation of integrins via intracellular or outside-in signaling inhibits ciliogenesis.

The above observations are important for two reasons. First, we can now conclusively rule out any off-target or non-specific effects as a basis for our findings. Second, the experiment using activated YAP has allowed us to build upon our previous findings to provide a mechanistic explanation for our results linking the ECM to focal adhesions, the actin pathway, and ciliation. We have now incorporated these findings into a new model (Fig. 7).

We thank the Editor for suggesting a critical experiment which has enabled us to provide an elegant model to explain our results.

DEVELOPMENTAL-CELL-D-19-01076 Review

"Whole-genome screen identifies diverse pathways that negatively regulate ciliogenesis"

Please see our responses in bold corresponding to each Reviewer comment.

Reviewer #1:

The results presented by Failler et al are novel and surprising, as cell-ECM interactions have not to my knowledge been previously linked to ciliogenesis. Furthermore, their experiments are in general quite well-controlled (see below). My main issue is that while these observations hint at biology that is clearly interesting and important, the underlying mechanisms remain incompletely characterized. Thus, I feel that the paper is not suitable for publication in Dev Cell in its current form. Below I summarize specific issues that the authors could address to strengthen their manuscript:

MAJOR ISSUES:
1. The authors show that disruption of cell-ECM interactions blocks ciliogenesis, but they do not offer any molecular links between these processes. What are the signaling events that connect cell-ECM interactions to primary cilium formation? Does "outside-in" integrin signaling, for example, affect any of the myriad factors (such as centrosomal or basal body proteins) known to participate in building a cillum?

In response to this comment and the Editor at MBoC, we have performed additional experiments that shed light on mechanisms underlying the connection between ECM interactions and ciliogenesis. As shown in new Fig. 7, we now show that loss of one
representative candidate gene (Fermt2), which is essential for integrin activation and focal adhesion assembly-mediated stabilization of the actin cytoskeleton, can be rescued by expression of constitutively active YAP, suggesting that cytoplasmic retention/inactivation of YAP could explain the increased ciliogenesis associated with Fermt2 depletion.

2. The authors demonstrate that the presence of a cilium inhibits cell migration, but again they do not offer a mechanistic link that would explain this effect. Such a link is critical because in other cell types cilia appear to promote cell migration, raising the question of why cancer cells display the opposite effect.

As we state in the Discussion, it is not clear why epithelial cells differ from fibroblasts with respect to the impact of ciliation on migration. This will require additional experimentation beyond the scope of this initial report.

3. The authors argue that "restoring cytoskeletal organization and proper interactions with the ECM might . . . [prevent] the metastatic progression of primary epithelial cancers in aggressive solid tumors such as TNBC" but this is a highly speculative statement as it is based entirely on changes in in vitro cell migration assays. If the authors were able to offer any additional evidence that restoration of cilia blocks tumorigenesis (such as in vivo transplantation-based assays), it would help to strengthen their case significantly.

(Note: I don't expect the authors to fully address all of these issues in depth, as that is beyond the scope of a single manuscript. But the present manuscript will be much stronger and more complete if they can either provide at least some additional experimental evidence in each of these three areas, or go into significantly more depth for one of them.)

The Reviewer raises a valid point, and we have relegated this speculation to the Discussion section. We agree that it would be useful in the future to test this hypothesis using in vitro (additional migration and invasion assays) and in vivo (xenograft) experiments.

4. All of their manipulations are based on siRNAs or shRNAs. Given the issues with nonspecificity and off-target effects that have plagued previous RNA interference-based screens, the authors should confirm the phenotype for the most critical hits in their screen by either creating CRISPR-based knockout cell lines (preferred), or, at minimum, rescuing the effects of their knockdowns via reintroduction of siRNA- or shRNA-resistant expression constructs.

We agree that off-target effects can be an issue, and we have addressed this concern as follows using four different strategies. First, our initial screen used a pool of siRNAs, and to follow up these candidates, we have used a set of shRNAs and siRNAs distinct from the ones employed in the initial screen. Importantly, we did not attempt to knock out genes of interest using CRISPR because a knock-out would result in a permanently ciliated state that would cause growth arrest. In that case, such a cell line would not proliferate and would be unobtainable. Second, in one case, we used a protein (Vitronectin, VTN) to rescue
enhanced ciliation induced by VTN depletion. Third, we have used expression of RNAi-resistant, recombinant GFP-Fermt2 to rescue depletion of Fermt2. Fourth, as described above, we showed that expression of activated YAP suppressed enhanced ciliation in cells depleted of Fermt2. Based on all of these considerations, we can effectively rule out off-target or non-specific effects as a basis for our findings.

MINOR ISSUES:
1. The authors somewhat oversell the concept that primary cilia restrict tumorigenesis. Work from the Reiter, Alvarez-Buylla, and Scott labs has clearly demonstrated that in some tumors, notably those driven by Hedgehog pathway activation, cilia are pro-tumorigenic. Thus, the relationship between primary cilia and tumorigenesis is not as straightforward as the authors' introductory statement implies. The authors should tone down this point in their introduction and discussion statements to provide a more even-handed account of the prior literature.

We have included a balanced discussion in the Introduction to address this point.

2. The authors do not provide any information about the magnitude of effects from their primary screen. Presumably some siRNAs strongly enhance ciliogenesis while others do so more weakly? This information should be incorporated into a supplementary table and/or explanatory diagrams (i.e., Fig 2) for the readers' benefit.

We can certainly include the full Excel sheets with the raw results from the screen in a supplemental table. However, this would represent a massive amount of data that would be difficult to appreciate without great effort. Therefore, we have included a Supplemental Table (new Supplemental Table 1) that summarizes the average ciliation frequency for all positive candidates/hits in our screen.

3. The overall rate of ciliation in Hs578T cells is still considerably lower than RPE cells, even after the siRNA knockdown treatments. Do any of the siRNAs (or classes of siRNAs) produce synergistic effects when combined, such that ciliogenesis is further enhanced?

We thank the Reviewer for this excellent suggestion. We have indeed attempted to test this using multiple shRNAs and combinations of drugs. We performed combined depletion of several candidates, including VTN+FERMT2, RAC1+FERMT2, RAC1+VTN, and FERMT2+LAMB4 using shRNAs, counted cilia, and measured RNA. We did not see a synergistic response, most likely because the drugs and shRNAs inhibit common/overlapping pathways. Since no clear synergy was observed, and the number of possible combinations that can be tested is quite large, we did not pursue this line of experimentation. It will be interesting to expand the number of permutations targeting distinct pathways in future experiments.

Reviewer #2: Dev Cell
Failler … Sanchez & Dynlatch
The authors have carried out an elegant screen with a breast cancer cell line with low ciliation. With this, they have carried out a whole genome siRNA screen and identified several potential negative regulators of ciliogenesis, as their downregulation leads to an increase in ciliogenesis in these cancer cells. They repeated some of these experiments in RPE-1 cells, which are somewhat more "normal" cells. They conclude that some of the identified genes inhibit ciliation in both normal and cancer cells.

They find a cohort of genes encoding ECM proteins. Adding an integrin-ligand, vitronectin rescued the enhanced ciliation observed upon silencing this gene.

They claim a reciprocal relationship between ciliation and cellular adhesion to the ECM.

This is an elegant screen. However, I am not convinced about the mechanistical relationship between cilia and integrins. I am not convinced that removing integrins directly affects ciliogenesis. They also fail to mention Guen et al, Lees and Weimberg (2017) showing that expression of EMT promoters twist and snail promote ciliogenesis in breast cancer models.

Some assumptions in the introduction are not accurate and they need revision.

The Introduction has been examined, and there are no inaccurate statements.

Have they measured hedgehog pathway activation in these cells? Are these cilia functional?

We have not measured Hh responsiveness in Hs578T cilia. However, we have stained Rab8 and AC3 at the centrosome and IFT88 and INPP5A within the cilium in these same cells, and their patterns appeared normal. This suggests that the assembly pathway is intact and that at least partially functional cilia are assembled.

Figure 1 is very straightforward. Very big effort and thorough approach.

However, I have some concerns about this work expressed below:

1- Are these cancer cells less ciliated? Or are the cilia shorter? Have they examined IFT88 localization? Are these cilia functional?

We refer the Reviewer to Fig. 3. Ciliation is reduced in serum starved Hs578T as compared to RPE1, where ~20% of Hs578T cells are ciliated as compared to >80% of RPE1. Indeed, this was one consideration for our screen, as it provided a wider dynamic range in which to examine restoration of ciliation. The cilia are not appreciably shorter in these cancer cells. We have observed proper IFT88 localization in these cells suggesting that cilia are likely to be normal.

2- It has been shown that integrins localize to primary cilia in MDCK cells (PMID:15226154).
Did they check this localization in their case? In fact it has been shown that integrins regulate calcium sensing at the cilium.

Indeed, we have cited this paper in our manuscript and are only aware of this single publication documenting integrin localization in cilia. In fact, the Reviewer has overstated the conclusions from this prior study, as the function of integrins at cilia was not specifically investigated. We have not observed integrin staining at primary cilia, but we have not rigorously pursued this area. Further, the presence or absence of integrins in the organelle itself has no bearing on our model since it is more likely the case that ciliation is triggered through a signaling pathway from the ECM and focal adhesions to the basal body.

3- They mention "assembly of a cilium could abolish the ability of cells to form a mitotic spindle" For this statement, they are citing a review from 2008, when antibodies such as Arl13B were not widely used. While it is clear that cilia are lost in certain cancer situations, cilia are also important for tumorigenesis and for drug resistance and are also present in cells after the G1/S transition (PMID: 30458140). They are also present during rapid divisions in embryogenesis. Thus, this is a dangerous and poor informed statement to make.

The presence of a cilium would indeed preclude assembly of a mitotic spindle. Indeed, we are not aware of a single study documenting mitotic spindle assembly in a ciliated mammalian cell. Nevertheless, the reviewer is correct that some tumors assemble cilia and they are present during rapid divisions during embryogenesis. These cilia are likely to be transiently assembled, and our statement was intended to suggest that a persistently ciliated state would abrogate mitotic spindle assembly. Therefore, we have slightly modified this sentence to indicate that persistence of a primary cilium throughout the cell cycle would be expected to abolish the ability of cells to form a mitotic spindle.

4- They cite a paper from Yuan, 2010, showing that primary cilia is absent in breast cancer cell lines. This paper is of poor quality and the presence of cilia was assessed by merely staining for acetylated a-tubulin, which is not always sufficient to detect primary cilia in highly tubulin-acetylated cancer cells.

Numerous studies from our lab and others have documented the absence of cilia, or severely decreased ciliation, in breast cancer cell lines. These studies were performed using a combination of markers, including Arl13b, which is the standard marker for cilium assembly.

5- I am not convinced that restoring cilia would prevent proliferation, as embryonic cells are highly proliferative and at the same time are ciliated with a functional hedgehog signaling pathway.

Please see Point #3 above. Persistent ciliation would be expected to prevent proliferation.

6- Kim et al, 2010, carried out a functional screen for modulators of ciliogenesis. It would have
been important to use some of these a control. Why are the hits different? Why is this not thoroughly addressed?

We disagree with the reviewer that this represents a problem because the screens were carried out using quite different methods. For example, Kim et al. used different cell lines, a different, smaller siRNA library, and cells were serum starved. In addition, the Kim et al. screen was optimized to identify proteins that promote rather than inhibit ciliogenesis (cells were serum starved prior to scoring), and the primary screen was performed in RPE-1 cells instead of breast cancer cells. Indeed, whereas the Kim screen uncovered many positive regulators, many fewer ciliogenesis inhibitors were identified. Thus, there are many technical differences that can explain the diverse outcomes.

As stated above, the majority of hits from the Kim et al. screen were positive regulators of ciliogenesis, and identifying such hits was not our goal. Our goal was to identify ciliation inhibitors. Thus, their hits would not represent good controls. We did, however, recover multiple hits in the actin assembly pathway, and this pathway was identified in the Kim et al. screen among the few ciliogenesis inhibitors they uncovered. Thus, there was overlap at the pathway level. We have now added additional explanatory notes on this point in the Discussion.

7- They mention the paper from Yuan et al, 2010, but fail to cite the PNAS 2017 paper by Guen et al, Lees and Weimberg, showing that expression of EMT promoters twist and snail promote ciliogenesis in breast cancer models. How do they reconcile this with their work? Why is this paper not cited? It is confusing that this paper is not cited.

We have not explored EMT in the context of ciliation in our cell lines. Hence, we cannot comment on the Guen et al. study as it pertains to our findings.

8- It is not clear how the Z-score is calculated. Can you explain?

The z-score is the number of standard deviations from the mean. 
\[ Z\text{-score} = \frac{\text{measurement} - \text{mean}}{\text{SD}} \]

The robust (Rob) z-score substitutes the outlier-insensitive median and median absolute deviation (MAD) for mean and standard deviation in the z-score calculation: 
\[ \text{Rob } Z\text{-score} = \frac{\text{measurement} - \text{Med}}{\text{MAD}} \]

The Z score is frequently used to normalize data in a way that provides information about the strength of each siRNA relative to the rest of the sample distribution. However, because it is less sensitive to outliers, the Rob z-score is generally considered preferable for RNAi screens.

We have added this explanatory text to the Methods section.

9- I imagine it is difficult to test this, but for some of the important hits it would be important to examine how their role in ciliogenesis correlates with their expression levels.

While this is a worthwhile suggestion for future experiments, this would indeed be difficult to test. It would be even more challenging to extrapolate useful information since it makes
the assumption that expression of a single gene can explain the ciliation phenotype. This is unlikely to prove correct, especially given the many gene expression differences between breast cancer cell lines.

10- The difference between Basal A and Basal B breast cancer cell lines needs to be better explained. Why ciliary regulators have a different effect between the two?

As stated in the Introduction, these cell lines were distinguished initially based on expression profiling, wherein basal B cells have a mesenchymal-like expression profile. We have included brief explanatory statements in the Introduction to explain the differences between Basal A and B breast cancer cell lines, but the underlying differences as they pertain to ciliogenesis are unknown.

11- It has been shown that cilia are required for differentiation (PMID:19190184). Thus, it is misleading to cite differentiation as the mechanism behind a lack of cilia. It has not been proven that undifferentiated cells have less cilia. Stem cells have cilia. Undifferentiated cells also have cilia. So, it has not been proven that cells that are de-differentiated have less cilia. In fact, highly dividing embryos have plenty of cilia. They also fail to cite the paper by Guen et al, Lees and Weimberg, showing that expression of EMT promoters twist and snail promote ciliogenesis in breast cancer models.

We are aware that cilia are required in the context of adipocyte differentiation and other systems, and nowhere do we state that differentiation is a mechanism underlying loss of cilia. Please see point #7 above regarding the reference to Guen et al.

12- It is a problem that the hits did not overlap with the Kim et al 2010 paper. The screen was done in RPE-1 cells. Could have they incorporated some of the hits from Kim et al as a control when doing the validation in RPE-1? Why did only a single hit overlap?

Please see point #6 above. We disagree with the reviewer that this represents a problem because the screens were carried out using vastly different methods (including, most importantly, different cell lines and siRNA libraries) with different goals and optimization.

13- It is worrisome that the expression levels change amongst cell lines, but this is unfortunately very common. Could they level the expression of some of these hits with lentiviral transduction. Perhaps knocking down the endogenous protein and re-expressing the gene of interests at similar levels. Could the effects in ciliogenesis now be similar?

We are not sure as to what point is being raised here, and it is unclear why it is worrisome that protein expression varies among cell lines. While interesting, the suggested experiments would be quite labor-intensive and time-consuming, and in our opinion, they would not add new information to our study.

14- Figure 2 is very confusing, 2A is OK, with the concerns I expressed above about why the cells are different and whether the differences could be overcome by restoring expression levels
15- It is not clear how figure 2C contributes to the understanding of the paper, it is very busy and convoluted. We know from table 1 which genes are associated with the different functions. How does this contribute to further understanding the paper?

Figure 2C is a summary that integrates the diverse candidate genes while schematically showing how the pathways intersect. We agree that the figure can be simplified. We have therefore retained it in the revised manuscript as a supplemental figure (Sup Fig. 2B) after enlarging and simplifying the image to make it less “busy and convoluted.”

16- Similarly, the string analysis figure is not clear.

We have improved the presentation of the STRING analysis by enlarging the figure.

17- they mention: "To examine the relationship between these proteins and ciliation, we blocked the activity of RACK1 and PDE4D using either individual shRNAs (targeting sequences distinct from those used in the screen) or a small molecule inhibitor of PDE4D (GEBR-7B). Interestingly, suppression of RACK1 or PDE4D protein expression or activity markedly enhanced ciliation of Hs578T cells (Supplemental Fig. 1), independently confirming that these proteins inhibit ciliogenesis”

For the regulators of the actin cytoskeleton, and the molecules involved in cell adhesion and migration, what is the abundance of those in these cells, does it correlate with their effect in ciliogenesis? They mention VAV2 but they do not knock down VAV2, what is the effect of knocking down VAV2 in this context?

As detailed here and elsewhere in this rebuttal, the issue of abundance is complex and not easy to address, especially because the answer would depend on whether the given protein is a limiting component and whether there are other genes that encode functionally related proteins (e.g., integrins), among other considerations. As such, it is beyond the scope of the current report. Owing to the large number of candidates, we have not methodically explored VAV2 knock-down.

18- The information from STRING analysis is not sufficient to draw the conclusions stated.

The utility of STRING analysis has been established in many publications. It is based on clearly stated criteria provided with the open source software. While STRING and any other proteome-wide analytical tools have certain limitations, we clearly state that the conclusions in this figure are based on modeling using STRING.

19- In Figure 3A, how do they explain the differences in the ciliogenesis increase? For instance, the linker protein between pathways they identify in STRING, ITGB1, has the lowest mRNA levels upon knockdown but the smallest effect in ciliogenesis increase.
It is worth re-stating that ITGB1 is one of many integrin subunits expressed in cells. There are 8 β and 18 α subunits expressed in any given cell that can assemble into 24 different receptors.

20- when they say: "we knocked down each of these candidates using short hairpin RNAs (shRNAs) distinct from those used in our screen.", could they use both hairpins in side-by-side experiments?

We have not used both hairpins in side-by-side experiments. Experiments with distinct shRNAs were performed to rule out any possible off-target effects observed with the siRNA pools in the initial screen.

21- when they say: "we found that in nearly all cases, the number of proliferative, Ki67-positive RPE1 cells markedly decreased, concomitant with gene suppression and enhanced ciliation, suggesting that cilium assembly was linked with cell cycle exit in normal epithelial cells (Fig. 3G)."

This is an over assumption, in Figure 3G, 3 out of 6 genes downregulated have no striking effect in the Ki67 positive cells, this half of the genes. Thus, this statement has to be modified accordingly. Also, it is not discussed whether these effects have to do at all with their effect in ciliogenesis or whether this is just an unrelated effect.

We have modified this statement to reflect the Reviewer’s points.

22- can some of these effects in ciliogenesis be rescued with re-expression of the genes of interest?

We have addressed this concern as follows using three different strategies. First, in one case, we used a protein (Vitronectin, VTN) to rescue enhanced ciliation induced by VTN depletion. Second, we have used expression of RNAi resistant, recombinant GFP-Fermt2 to rescue depletion of Fermt2. Third, we have used expression of activated YAP to suppress enhanced ciliation in cells depleted of Fermt2. Based on all of these considerations, we can effectively rule out off-target or non-specific effects as a basis for our findings.

23- Figure 5A, the figure has no error bars. If this is the case, then increasing contractility in normal, RPE1 cells should decrease ciliogenesis. Could they test this? Could they re-express these genes, examine the cell's contractility and quantify ciliogenesis? Many migrating cell and invasive cells have perfectly functional cilia.

Error bars are shown in Fig. 5A. The experiments to address contractility are interesting, but they are beyond the scope of the current report.

24- Figure 5C, although the effects of restoring vitronectin are significant in decreasing ciliogenesis, these values are different that other figures where the percent of ciliogenesis is Hs578T. Why is this? The difference between BSA treated cells and vitronectin treated cells in
10% to 20%. In other figures in these cells the increase in cilia is up to 50% or 90% in serum starved cells.

That is not correct in Hs578T, where it increases to 30% (Fig. 3). There is no difference after BSA treatment in starved cells. For Hs578T, percent ciliation in control (CTR) conditions is below 10% in all figures. The values mentioned by the Reviewer (50-90%) are for RPE1 cells. Regarding the levels for VTN KD, the range is 20-30%.

25- could the effect of the knockdown of these proteins be rescued?

Please see point #22 above. We have addressed this concern as follows using several different strategies. First, in one case, we used a protein (Vitronectin, VTN) to rescue enhanced ciliation induced by VTN depletion. Second, we have used expression of RNAI-resistant, recombinant GFP-Fermt2 to rescue depletion of Fermt2. Third, we have used expression of activated YAP to suppress enhanced ciliation in cells depleted of Fermt2. Based on all of these considerations, we can effectively rule out off-target or non-specific effects as a basis for our findings.

26- Figure 6, it is not clear that the decrease in invasion is related to the increase in cilia. In addition to CEP83 could they downregulate other proteins required for ciliogenesis such as IFT88 and Odf2? These results are contradictory with many results in the literature. Did they examine CEP83 levels by western blot and immunostaining? The half life of centriolar proteins is so long that the mRNA levels might not be informative in the short timeframe of the siRNA experiments.

We disagree with the reviewer that our findings contradict “many results in the literature,” and we are not aware of the findings he/she is referencing. We have tried multiple times to silence IFT88, but we found that the silencing was not effective. Therefore, we tested Cep83 ablation and succeeded in depleting this protein, as determined by western blotting and immunofluorescence. However, since ablation of Cep83 is sufficient to abolish ciliation, our data indicate that cilium assembly is indeed responsible for the effect on migration.

Did they do these experiments in RPE1 cells? Did they test this in fibroblasts? Did they test this with other types of invasion assays such as serum-dependent invasion or migration towards a growth factor.

Figure 6 shows the results for Hs578T using shRNAs and RPE1 using CEP83 knockdown with siRNAs. We have not tested the ablation in other cell lines besides those shown in the manuscript, nor have we performed other types of invasion or migration assays since this was not the primary focus of our study. These are all worthwhile experiments that will be performed in the future.

Reviewer #3: This manuscript reports the results of a whole-genome siRNA screen for factors
that promote the disassembly (or prevent the maintenance) of primary cilia. They perform the primary screen in Hs578T breast cancer cells, motivated in part by evidence in the literature to suggest that cilia are downregulated in breast cancer. As usual, this yielded a substantial number of hits, the majority of which were confirmed in a secondary screen that also encompassed other breast cancer cell lines. Bioinformatic analysis suggested that the hits include genes associated with the cytoskeleton, mitochondria and ion transport.

In particular, the authors identify a number of genes that are associated with integrin adhesion, the extracellular matrix and potentially the adhesion-associated cytoskeleton. They accordingly choose to explore these further to test the potential for these to provide new insight into cilial homeostasis. This is the focused experimental work that would enhance the relevance of the screen for the general audience of Developmental Cell. They choose to test genes such as talin, Rac1 and vitronectin. As per their abstract, they conclude that their findings suggest "an unanticipated reciprocal relationship between ciliation and cellular adhesion onto the extracellular matrix…"

However, I feel that this material is too preliminary for them to make a convincing case at the present time.

1. Fig 3 is foundational data, as it shows that depleting a number of their candidates appears to increase ciliation. The shRNAs used in these experiments were different from the siRNA screen. However, I think more effort is needed to exclude off-target effects, if this is to be the foundation of their focused analysis. The addition of vitronectin to VN-depleted cells helps for one case, but not the others. (Nor is it clear to me that the vitronectin addition serves as more than a control for the VN RNAi.)

We agree that off-target effects can be an issue, and we have addressed this concern as follows using four different strategies. First, our initial screen used a pool of siRNAs, and to follow up these candidates, we have used a set of shRNAs and siRNAs distinct from the ones employed in the initial screen. Second, in one case, we used a protein (Vitronectin, VTN) to rescue enhanced ciliation induced by VTN depletion. This experiment also shows that VTN can rescue ciliation after it is supplied to the extracellular matrix. Third, we have used expression of RNAi resistant, recombinant GFP-Fermt2 to rescue depletion of Fermt2. Fourth, as described above, we have used expression of activated YAP to suppress enhanced ciliation in cells depleted of Fermt2. Based on all of these considerations, we can effectively rule out off-target or non-specific effects as a basis for our findings.

2. Fig 4 does not seem to provide any new insights. It is well established that depleting talin or Rac1 affects focal adhesion formation and the cytoskeleton.

We disagree with the Reviewer’s assessment. This figure provides the necessary evidence to show the impact of protein depletion on the cytoskeleton.

3. Fig 5. Using Y27632 is a very indirect approach to investigate the link between stress fibers and enhanced ciliogenesis. In contrast to controls, Y27632 did not further enhance cilial recovery
in cells where genes such as laminin and Rac1 had been depleted. Based on this the author conclude that "our candidate genes most likely suppress cilium assembly by inducing changes in cell contractility. But this observation is also compatible with an alternative scenario, where e.g. laminin depletion maximally enhances ciliary recovery in a contraction-independent fashion, so that Y27632 cannot have any additional effect.

The Reviewer makes an excellent point. We have incorporated this alternative conclusion into our discussion.

4. Fig 6 seeks to link changes in cilia to cell migration. As expected, depleting genes such as talin and Rac1 compromises cell migration, as well as increasing cilia. It is therefore interesting that depleting cilia with CEP83 RNAi can correct the migration phenotype, at least in some cases. However, I think that this should be pursued in greater depth. For example, why is it that CEP83 RNAi "corrects" wound healing in only a subset of proteins, but seems to restore transwell migration for all the candidates tested? How have the authors performed their statistical analyses and has this been performed for the transwell migration assays? Can the authors correct for possible changes in cell proliferation? If the links pertain for only a subset of proteins, why is this so (e.g. why vitronectin but not laminin - does it reflect different adhesion complexes)?

While the differences were significant (using t-test) for certain genes, we have elected to remove the transwell data because we believe that additional experimentation will be required to optimize this assay to gauge the full dynamic range of responses across multiple genes. Since this will necessitate considerable optimization (timing, growth conditions, depletion kinetics), we have chosen to focus our conclusions on the related wound-healing assay, for which we present extensive and reproducible data.
Dear Dr. Dynlacht:

I am pleased to accept your manuscript for publication in Molecular Biology of the Cell.

Thanks for your efforts to address the most outstanding issues in these difficult times.

Sincerely,
Mark Ginsberg
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Dynlacht:

Congratulations on the acceptance of your manuscript.

A PDF of your manuscript will be published on MBoC in Press, an early release version of the journal, within 10 days. The date your manuscript appears at www.molbiolcell.org/toc/mboc/0/0 is the official publication date. Your manuscript will also be scheduled for publication in the next available issue of MBoC.

Within approximately four weeks you will receive a PDF page proof of your article.

Your paper is among those chosen by the Editorial Board for Highlights from MBoC. Highlights from MBoC appears in the ASCB Newsletter and highlights the important articles from the most recent issue of MBoC.

All Highlights papers are also considered for the MBoC Paper of the Year. In order to be eligible for this award, however, the first author of the paper must be a student or postdoc. Please email me to indicate if this paper is eligible for Paper of the Year.

Would you like to see an image related to your accepted manuscript on the cover of MBoC? Please contact the MBoC Editorial Office at mboc@ascb.org to learn how to submit an image.

Authors of Articles and Brief Communications are encouraged to create a short video abstract to accompany their article when it is published. These video abstracts, known as Science Sketches, are up to 2 minutes long and will be published on YouTube and then embedded in the article abstract. Science Sketch Editors on the MBoC Editorial Board will provide guidance as you prepare your video. Information about how to prepare and submit a video abstract is available at www.molbiolcell.org/science-sketches. Please contact mboc@ascb.org if you are interested in creating a Science Sketch.

We are pleased that you chose to publish your work in MBoC.
Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org