Identification of a heterogeneous and dynamic ciliome during embryonic development and cell differentiation

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MS TITLE: Identification of a heterogeneous and dynamic ciliome during embryonic development and cell differentiation

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I have now received all the referees' reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees express considerable interest in your work, but have some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. All three referees have helpful suggestions to improve the manuscript and list a series of points that need clarification and/or changes to the text. The reason for the exclusion of some cilia located proteins from the analysis should be considered. As Referee 1 indicates, including all identified proteins may reveal genes that were previously not linked to cilia function. Including analysis of the NHLBI primary cilia proteome database also appears to be a constructive suggestion. Overall, the referees agree that, suitably revised, the study will provide a timely resource for the field.

If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision. If it would be helpful, you are welcome to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating your plans for addressing the referee's comments, and we will look over this and provide further guidance.

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Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

In their manuscript, "Identification of a heterogeneous and dynamic ciliome during embryonic development and cell differentiation," Elliot et al. investigate cilia heterogeneity in the developing mouse embryo. Several studies have comprehensively defined the ciliome as a singular entity. However, cilia are heterogenous and vary by tissue and developmental stage. This manuscript directly addresses the critical question of cell type specific expression of genes encoding cilia-associated proteins. This work is significant in providing foundational information important for connecting information about gene expression related to cilia to understanding the broad class of ciliopathies and associated spectrum of phenotypes. Moreover, it is novel in providing the community cell type specific datasets for the expression of genes related to cilia. The authors include a user-friendly, web-based interface with which to mine their data, a strength of their work. These datasets sets the stage for closer dissection of the differences in cilia between tissues to better understand the tissue-specific phenotypes that arise from different ciliopathies.
The authors perform transcriptomic analysis on embryos in a tissue and temporal specific manner revealing cilia heterogeneity and show functional significance in CRISPR/Cas9 embryos. The authors show convincing results of tissue-specific cilia based on the DE ciliome among tissues.

Comments for the author

MAJOR ISSUES

1. The authors include several landmark datasets but do not include the NHLBI primary cilia proteome database in the studies that define the ciliome (PMID: 22326026, PMID: 26585297, PMID 33856408). The authors should look to see whether they missed any unique proteins- and, if so, include them or justify why they should be excluded.

2. More rationale should be included as to why the authors chose to curate the analyzed ciliome to only include genes that have a direct role in ciliogenesis and/or cilia function. This would appear to bias a relatively unbiased approach. This seems to introduce more limitations than benefits including:
a. Eliminating genes that are associated with signaling processes like Hh which biases the ciliome.
b. Including genes involved in signal transduction (like Hh) serve as internal validation that these genes are not changing in a tissue dependent manner.
c. Expanding the ciliome to include all genes may reveal genes that were previously not linked to ciliogenesis or cilia function.

3. Figure 1B- The validation experiment is not clearly relevant to the tissue samples used in the transcriptomic approach. The finding of altered ciliary characteristics is unsurprising because it is well known that different cell lines vary in ciliation, cilia length, etc. The authors should directly validate cilia characteristics in the same cell populations used in the transcriptomics (in vivo). As presented, this figure should move to supplemental and the language modified.

4. Figure 1C- In addition to this analysis, the authors should consider the biological relevance of the DE ciliome in human disease by using GnomAD, which provides constraint metrics. This would help provide a link back to human ciliopathies which despite being set up in the intro is currently lacking.

5. Figure 2C- The GO analysis only reveals tissue-specific significant terms for neural compared to face and limb. It is expected that GO terms for DE ciliome in specific tissue

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correspond to known ciliary processes and functions. Therefore, Fig 1D and 1F should be supplemental.

6. Figure 3F- The Venn diagram is not clear on its own that the tissue-type differences are not due to the epithelial versus mesenchymal origin. This figure and accompanying result require reworking so as to be compelling.

7. Figure 3F- The conclusion that the DE ciliome is tissue-specific and not cell-type specific can also be supported by PMID: 27881449. This paper should be included in the discussion.

8. Supplemental Fig.3- An attempt was made to show that changes in the RNA level are maintained at the protein level. However, the western blot was only performed for 3 target genes that showed increases at the transcriptional level (Does this apply for genes that decrease?). While not all genes can be examined, an analysis of the classes of DE genes would better support the idea that DE ciliome contributes to tissue-specific identity at the translational level.

9. Fig 5- The choice of Pcm1 and Tmem107 for the edited F0 embryos needs the rationale explained.
a. A study has already characterized Tmem107 embryo mutants and published cilia and craniofacial phenotypes PMID: 28954202.

b. Pmc1 did not show a ciliary phenotype, leaving only one functional example of a gene from the osteoblastic ciliome. Other examples of differentially expressed genes from the osteogenic ciliome are necessary to conclude that these genes have biological relevance.

c. Why not show that DE ciliome genes are causing ciliary phenotypes specifically in the affected tissue-type in vivo?

10. It would be much easier for the reader to follow if related figures were kept together. Here are two examples:

a. All Pmc1 data (currently supplemental) should be in Fig. 5 with the Tmem107 data.

b. The Venn diagram in supplemental Fig. 2 should accompany the Venn diagram in Fig 1E because both highlight the overlapping v. differential tissue-specific DE ciliome.

MINOR

1. The authors should explain and justify why they switched from tSNE to UMAP plots.

2. While the paper will have a copy editor, the authors should fix the Iimproper capitalization of common nouns throughout manuscript.

Reviewer 2

Advance summary and potential significance to field

Summary: This techniques and resources article investigates the heterogeneity of cilia associated gene expression during mouse head and limb development. The authors identify subsets of ciliome genes that are differentially expressed between these tissues and focus on a category of the ciliome genes that are proposed to be osteogenic-specific. Two of these genes (Pcm1 and Tmem107) are validated for developmental skeletal phenotypes using an F0 CRISPR/Cas9 screen. The RNAseq data are presented as an accessible shiny app for use by the community. This represents an excellent resource and tool for the field.

Comments for the author

We have a few comments for improvement of the manuscript:

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1) Introduction, page 3 last paragraph-top of page 4: the authors describe a number of ciliopathies which have distinct tissue specific phenotypes. This is a vague way to describe and possibly discount a complex series of tissue relationships. It would be better if the authors provided specific examples of their point. What are these ?distinct phenotypes? the authors are referring to, and how do these ?distinct phenotypes? support their argument of a ?degree of heterogeneity beyond the structure of the cilium??

2) They then go on to explain that HH associated genes have been removed from the analysis for this differential expression assessment, however, differential expression of some of these genes (e.g. GLI1/2 vs GLI3) are sure to be contributing to the differential phenotypes observed, especially given that they will follow up later on key osteogenic factors. So, while we don?t particularly object to idea of removal of the Hh signal transduction genes in the analysis, it would be helpful have a more nuanced rationale in the experimental setup. Alternatively, I would favour just including these ten genes in the overall analysis, especially as some of the other genes are surely underappreciated regulators or effectors of Hh signalling (e.g. Tulp3).

3) Page 6 (last paragraph) and fig1c: Please clarify lethal and subviable in terms of the IMPC categories.

Are they lethal prior to organogenesis (e9.5), embryonic (e14), prenatal (e18)?

4) Page 12 and Fig5 e-m ? Panel L, ventral TMEM107 crispant looks to have a fused premaxilla, but hypoplastic basisphenoid and palatine bones. Please provide more detailed description of the phenotypes rather than generalised ?hypoplastic cranial skeleton? statement.

5) Page 20 and 21 ? Mineralisation (alizarin red staining) in supp fig 4. C? looks very non-specific (iPSC, d8 and 12) enough that we are not convinced. Do you have any other means of confirming mineralisation or no beta-glycerophosphate media controls run to check for non-specific mineralisation?

6) Page 21 ? beta glycerol phosphate nomenclature -> (symbol beta) b-glycerophosphate or glycerol 2-phosphate

Reviewer 3

Advance summary and potential significance to field

The manuscript by Elliott et al attempts to demonstrate that there are cilia associated genes (ciliome) that are differentially expressed in different tissues and over developmental time. The documentation of heterogeneity in expression of cilia associated gene is timely. This has important implications for understanding why patient mutations in the ciliopathy class have different phenotypes even though they are considered “cilia” genes.

The strengths of this report are the annotation of cilia related genes that are differentially expressed in different example tissues or developmental stages; the database to allow access to the datasets; some of the confirming studies to show the analysis finds tissue specific expression of cilia associated component.

The weaknesses of this report are that the analysis and interpretation is oversold on a number of points, and some of the experiments said to confirm the relevance of differential expression don’t do so.

Comments for the author

Major points:
1. The title and statements throughout suggest this a much broader study that it is. For example - the sentence in the introduction – “ our data comprehensively reveal that approximately 30% of the ciliome is differentially expressed across tissues….” - the reads as if all tissues were assessed and the study will show a catalog of differential expression in an exhaustive number of tissues and developmental stages. In fact, this is comparing three craniofacial areas to limb and

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two brain areas. These are important, given the impact of ciliopathies on these regions, but it isn't a comprehensive study. Some of this should be toned down to reflect what was actually done in this study.

2. Likewise – the statement at the end of the introduction stating "the ciliome has evolved to account for distinct functions of cell-types in vertebrate species and that it plays an essential role in defining cell/tissue identity and differentiation potential." should be modulated. Cilia peroxisome, mitochondria etc are going to vary depending on the proteins being expressed that go to those locations for their function. And cells vary what they express depending on their origin and developmental trajectories. The cilia aren't defining cell identity but reflecting it. Differential genome expression over time defines it. This all seems overblown.

3. And in the discussion – "our work redefines the primary cilium as a tissue-specific dynamic organelle that changes composition to accommodate cell-type specific function" – some of this work looks at motile cilia, not primary cilia, as part of the basis for claiming there are important differences in different tissues. See point 7 below. And while the work here is interesting I'm not sure it redefines the primary cilium as tissues specific based on comparing brain tissue (motile cilia) with craniofacial tissue (primary cilia).
The changes during osteogenic differentiation are compelling, but not knowing exactly how the ciliome was curated (see point 4) makes it difficult to know what we are analyzing.

4. Results, 1st paragraph – "Manual curation which included the elimination of genes associated with Hh signal transduction…". First, what was done here is important, especially for people wanting to use this as a resource. Explanation of this process and what was removed from the curated ciliome should be provided in supplemental materials.

Second – why remove Smo and Gli given these are proteins that enter into the cilia and are regulated in this location in mouse and humans? Wouldn't this be an obvious candidate for providing cilia associated differences in tissues and in disease phenotypes? It makes me hesitant given what else might have been removed from the ciliome during curation.

5. Results, section 1, paragraph 3, Figure 1 – The fact that cilia in tissue culture cells vary in length and percentage of ciliated cells is well known, so this data isn't surprising, but I don't see how this "validated transcriptomic findings". As to supporting the hypothesis that cilia are not ubiquitous organelles, is that really in question? Cilia in the mouse node are single and motile with a 9+0 axoneme, cilia in the limb mesenchyme are single and non-motile with a 9+0 axoneme, cilia in the brain ventricles are in multiciliate bundles and motile with a 9+2 axoneme. We know cilia are different in different tissues and contexts and the data in Figure 1B aren't compelling in this regard given so many in vivo examples.

6. Results section 1, paragraph 4, Figure 1C – this is an interesting result. Are the genes represented in the non-DE ciliome primarily structural components of the cilia and thus more likely to cause consequence across all tissues? For example centrosomal components that may be involved in spindle assembly as well as cilia formation? Are genes in the DE ciliome more signaling based or genes with multiple orthologs/paralogs in the mouse genome?

7. Results section 2, paragraph 2, Fig 2D – This interpretation seems a bit off. Of course these genes (found in motile cilia) are going to be specific to neural tissue in this analysis, because this is the one tissue assayed that has motile cilia. This is to be expected. It could be used to say the DE ciliome contained these genes as expected, but not as proof that the analysis found something that we didn't already know, which is how it reads to me.

8. Results section2, paragraph 4, Fig 3B – "a subset or genes that was expressed in both tissues" – Is there a better way to display this? It is hard to see low level expression and many of the claims are based on spots that seems to be 0% in size. Doesn't that mean 0% of cells expressed the gene in question?
Given how nicely the RNAscope data is for Rab3il, the data here is underwhelming as presented. I wouldn't predict such nice tissue specific expression from the plot in 3B.

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9. Results section2, paragraph 4 – the data comparing neuroepithelium with MNP mesenchyme is interesting. Do you see something similar comparing MNP mesenchyme with limb mesenchyme? Or is the overlap greater?

10. Results section3m paragraph 2, Figure 5 – are Tmem107 edited MEFS producing longer cilia? Are these cells having issues with cell division by any chance?

11. There is almost no information on the CRISPR F0 mice. How were these genotyped? What sort of mutations were observed? Are these craniofacial defects representative of multiple F0s? Are you sure there are no off-target effects for the CRISPR manipulation?

12. Supp Fig 5 B-G – in my opinion, this is some of the nicest data you have along with the mouse craniofacial defects. The cilia appear to be changing given the accumulation of PCM1 at the base and this is clear from the Ifs. Why not move this to the main text?

Minor points:
1. Figure 1B – I'm not sure what rate of ciliary extension is referring to.
There are no materials and methods explaining what was measured and how. There is note of how cytochalasin was used, but nothing else.

2. Results section 2, paragraph 2 – "essential for the formation and function of 9+0 motile cilia". I assume this is a typo and should be 9+2 motile cilia. The 9+0 motile cilia are primarily found in the mouse node. There is overlap in the ciliome of the motile cilia, but the overlap in neural tissue here would be for ependymal cilia which are 9+2.

3. Results section 2, paragraph 2 – "essential for the formation and function of 9+0 motile cilia [19, 27-30]" – missing Lrrc46, CCDC40 references which are included below:

Yin Y, Mu W, Yu X, Wang Z, Xu K, Wu X, Cai Y, Zhang M, Lu G, Chan WY, Ma J Huang T, Liu H. LRRC46 Accumulates at the Midpiece of Sperm Flagella and Is Essential for Spermiogenesis and Male Fertility in Mouse. Int J Mol Sci. 2022 Jul 31;23(15):8525. doi: 10.3390/ijms23158525. PMID: 35955660; PMCID:
PMC9369233.

Becker-Heck A, Zohn IE, Okabe N, Pollock A, Lenhart KB, Sullivan-Brown J McSheene J, Loges NT, Olbrich H, Haeffner K, Fliegauf M, Horvath J, Reinhardt R Nielsen KG, Marthin JK, Baktai G, Anderson KV, Geisler R, Niswander L, Omran H Burdine RD. The coiled-coil domain containing protein CCDC40 is essential for motile cilia function and left-right axis formation. Nat Genet. 2011 Jan;43(1):79-84. doi: 10.1038/ng.727. Epub 2010 Dec 5. PMID: 21131974; PMCID:
PMC3132183.

4. Results section2, paragraph 4 – Crb3 is noted here, but not in Fig. 3B.
Why call it out here?

5. Materials and methods – cell culture section – "a minimum of 30 cilia were counter per timepoint in triplicate" – this is for lengths and cilogenesis rate, correct? The percentage were the minimum of 400 cells counted in triplicate? Please clarify.

6. Figure 5 legend – the legend says E18.5 skulls but the analysis for PCM1 in Supp Figure 5 says E17.5 mice were phenotyped. Please clarify.

7. Supp Figure 4 – I don't see why cluster Cluster 5 in E11.5 is any more or less similar to E13.5 Cluster 5 based on the data presented in this table.
What were the criteria for deciding these were the two best clusters to compare to each other?

8. Supp Figure 5H – I'm not familiar with the phenotyping method depicted here. What are we looking at? How is the calling done? What is it actually detecting? Loss of probe? How does that get converted to CNV?

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First revision

Author response to reviewers' comments

We thank the Reviewers for their careful and critical analysis. We have addressed all comments, either experimentally or textually. Please see below for a point-by-point rebuttal and find new text in the manuscript document in blue.

Reviewer 1 Comments for the Author:
MAJOR ISSUES

1. The authors include several landmark datasets but do not include the NHLBI primary cilia proteome database in the studies that define the ciliome (PMID: 22326026, PMID: 26585297, PMID 33856408). The authors should look to see whether they missed any unique proteins- and, if so, include them or justify why they should be excluded.

*We thank the Reviewer for this suggestion. We have now cited these three important studies, as well as the database (Mehta et al., 2022). **(See blue text, page 5)** We have also integrated data from these three studies into Supp. File 1 by noting if genes within our ciliome were identified in each study **(Supp. File 1 columns, Z, AA, AB)**.*

Since our goal was to unbiasedly determine the extent of endogenous heterogeneity among cilia across selected tissues in the embryo and all three studies mentioned were performed using cultured cell-lines:

> *22326026- (Ishikawa et al., 2012) isolated primary cilia from a murine kidney cell line, IMCD3 26585297- (Mick et al., 2015) was done with cell lines IMCD3, RPE1-hTERT, MEFs 33856408- (May et al., 2021) was also done in cell lines C2C12, NIH-3T3, HEK293T exposed to Hh. we did not simply add all identified genes to our database.*

Instead, we compared our dataset to with these three proteomic datasets.

- *We first compared the three proteomes to each other. Surprisingly, even between these studies, there was minimal overlap of the ciliary proteome with only 6% (26/426) of proteins being represented in all three data sets.*
- *When we compared proteins present in all three screens (n=26) back to our ciliome, 84% were represented in our ciliome. Of the 4 proteins not represented in our ciliome*
  - *Cemip2- cell surface hyaluronidase reported to be an extracellular matrix regulator*
  - *Kiaa1430 (AKA Cfap97)-cilia- and flagella-associated protein 97*
  - *Pdap1- 28 kDa heat- and acid-stable phosphoprotein with no known*
  - *Ywhaz- 14-3-3 protein zeta/delta reported to be involved in protein binding.*

Together the variation observed between these databases and compared to our ciliome further support our hypothesis of ciliary heterogeneity.

2. More rationale should be included as to why the authors chose to curate the analyzed ciliome to only include genes that have a direct role in ciliogenesis and/or cilia function. This would appear to bias a relatively unbiased approach. This seems to introduce more limitations than benefits including:

a. Eliminating genes that are associated with signaling processes like Hh which biases the ciliome.

b. Including genes involved in signal transduction (like Hh) serve as internal validation that these genes are not changing in a tissue dependent manner.

c. Expanding the ciliome to include all genes may reveal genes that were previously not linked to ciliogenesis or cilia function.

All three Reviewers raised similar concerns regarding our manual curation of the ciliome, thus we have redone our analysis without eliminating any genes.

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3. Figure 1B- The validation experiment is not clearly relevant to the tissue samples used in the transcriptomic approach. The finding of altered ciliary characteristics is unsurprising because it is well known that different cell lines vary in ciliation, cilia length, etc. The authors should directly validate cilia characteristics in the same cell populations used in the transcriptomics (in vivo). As presented, this figure should move to supplemental and the language modified.
We appreciate the Reviewer's comment. We initially performed these experiments in vitro because it allowed us to synchronize cells and better visualize ciliary extension. Performing this type of assay on tissue sections is inherently difficult, especially in mesenchymal tissues that lack epithelial organization, like neural crest and limb, as various of stages of ciliary extension are only captured in a snapshot fashion. Thus, we have moved this data to supplemental Fig. 1 and modified the language in the text. ***(See blue text page. 6)***

4. Figure 1C- In addition to this analysis, the authors should consider the biological relevance of the DE ciliome in human disease by using GnomAD, which provides constraint metrics. This would help provide a link back to human ciliopathies which despite being set up in the intro is currently lacking.
We thank the Reviewer for this thoughtful suggestion. Using gnomAD constraint scores we calculated LOEUF scores for both the non-DE ciliome and the DE ciliome. The DE ciliome genes with a gnomAD constraint score (n = 272, mean = 0.861) were compared to non-DE ciliome genes with a gnomAD constraint score (n = 656, mean = 0.776), and had significantly higher LOEUF scores (t-test p-value = 0.0096), suggesting the DE ciliome genes are under less selection than the non-DE ciliome genes. More specifically, the percentages of DE and non-DE ciliome genes in each LOEUF decile were calculated and compared (New Supp. Table 1). As shown in the table, there is a reduction in the percentage of DE ciliome genes compared to non-DE ciliome genes at low LOEUF deciles (high constraints). As the constraint decreases, the percentage of DE ciliome genes become larger than those of non-DE ciliome genes. This trend is clearly shown in new Figure 1B where the ratio of percentages of DE and non-DE ciliome genes is plotted against LOEUF decile. This data is in alignment with the data from the IMPC shown in Fig. 1C-C'. We have edited the text to include this new and interesting data. ***(See blue text, pages 6-7)***

5. Figure 2C- The GO analysis only reveals tissue-specific significant terms for neural compared to face and limb. It is expected that GO terms for DE ciliome in specific tissue correspond to known ciliary processes and functions. Therefore, Fig 1D and 1F should be supplemental.
We appreciate the Reviewer's suggestion. We believe they were referring to Figs. 2D and 2F (rather than 1D, F) and we have moved these figures to Supp. Fig. 2.

6. Figure 3F- The Venn diagram is not clear on its own that the tissue-type differences are not due to the epithelial versus mesenchymal origin. This figure and accompanying result require reworking so as to be compelling.
We have reworked the visualization of this data using the Rpackage UpSetR. UpSetR is a more scalable alternative to Venn and euler diagrams for visualizing intersecting gene sets that creates visualizations of intersecting gene sets using a novel matrix design, along with visualizations of several common sets, element, and attribute related tasks (Conway et al., 2017). Using UpSetR in ***new Fig. 3F*** *we depict the intersections between genes expressed in the MNP epithelium and neural epithelium, and MNP mesenchyme and neural epithelium. These data show a larger number of gene intersections between neural epithelium and MNP mesenchyme (45) than that in neural epithelium and MNP epithelium (3). Thus, suggesting that cell type (epithelial vs mesenchymal) does not dictate the ciliome.*

7. Figure 3F- The conclusion that the DE ciliome is tissue-specific and not cell-type specific can also be supported by PMID: 27881449. This paper should be included in the discussion.
We thank the Reviewer for this suggestion. This was indeed an oversight on our part, and we have now added this reference to the text. ***(See blue text page 10)***

8. Supplemental Fig.3- An attempt was made to show that changes in the RNA level are maintained at the protein level. However, the western blot was only performed for 3 target genes that showed increases at the transcriptional level (Does this apply for genes that decrease?). While not all genes can be examined, an analysis of the classes of DE genes would better support the idea that DE ciliome contributes to tissue-specific identity at the translational level.

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While we agree with the Reviewer in theory; finding and testing antibodies that worked well for Western Blotting was a costly and time-consuming process. We tested several commercially available antibodies that did not work in our hands. We'd like to reiterate that the purpose of this experiment was to simply confirm that our transcriptional data was conserved on a translational level. We found a representative gene from each group (epithelial, mesenchymal and epithelial and mesenchymal) and a function antibody for each. In addition, we have now compared our transcriptome to the proteomes the Reviewer suggested in point 1.

9. Fig 5- The choice of Pcm1 and Tmem107 for the edited F0 embryos needs the rationale explained.
Pcm1 and Tmem107 were selected for F0 experiments to investigate their role in regulating ostoegenesis during development. Both genes were significantly upregulated during osteogenic differentiation of neural crest cells and represented genes that had other useful reagents (e.g., antibodies, other mouse models) available to validate our data. We were not aware of other groups previously investigating Pcm1 in an osteogenic context, and there was a high-quality antibody available for examining protein expression/localization during osteogenesis. While there was a haploinsufficient mouse model (Zoubovsky et al., 2015), it was only analyzed for its neurological phenotype. Data from the IMPC indicated that mice homozygous for mutations in Pcm1 were subviable and displayed abnormal cranium morphology but no analysis has been done during development. Tmem107 has been previously studied as both an ENU allele (PMID: 22698544) and a knockout (PMID: 28954202) and we wanted to validate whole gene knock-out findings with our rapid CRISPR/Cas9 F0 approach as proof of principle. We have addressed this concern in the text. ***(See blue text, bottom of page 13, top of page 14)***

a. A study has already characterized Tmem107 embryo mutants and published cilia and craniofacial phenotypes PMID: 28954202.
We thank the Reviewer for this call out. We were aware of this study and have now cited this work (Cela et al., 2018).
(See blue text, top of page 14)

b. Pmc1 did not show a ciliary phenotype, leaving only one functional example of a gene from the osteoblastic ciliome. Other examples of differentially expressed genes from the osteogenic ciliome are necessary to conclude that these genes have biological relevance.
We appreciate the Reviewers comment and wish to clarify our rationale and data. We are not suggesting that loss/mutation in genes of the osteogenic ciliome should show a ciliary phenotype. In fact, it is more likely that mutations in these genes do not have a ciliary phenotype, as the embryos are able to survive well into development, unlike most ciliary mutations that result in early embryonic lethality. Our point is that there is a subset of ciliary genes that change expression during skeletogenesis and mutations in these genes result in skeletal phenotypes. PCM1 is a bona fide ciliary gene, previously identified in several ciliary screens. We further provide immunofluorescent data showing PCM1 expression at the centriole (as previously reported). Thus, we do not concur that a lack of an obvious structural phenotype, prevents Pcm1 from serving as an example of a gene within the osteogenic ciliome.

Several other genes that we independently determined to be within the osteogenic ciliome have previously been reported to have skeletal phenotypes when knocked out. We provide summaries of these phenotypes and links to the IMPC pages in Supp File 1. In brief:
Arlbp2 was reported to have significant skeletal phenotypes including abnormal bone structure and abnormal mineral content.
Bbip1 was reported to have significant skeletal phenotypes including kyphosis
Ctnnb1 was reported to have significant skeletal phenotypes including increased bone mineral density, abnormal cranium morphology and abnormal bone mineralization
Pcm1 was reported to have significant skeletal phenotypes including abnormal cranium morphology Trip11 is associated with skeletal disorders including Achondrogenesis Type 1A, Odontochondrodysplasia U2surp was reported to have significant skeletal phenotypes including decreased bone mineral density

Furthermore, we also examined terms for phenotypes associated with genes of the osteogenic ciliome: Ciliary genes exhibiting expression changes during osteoblastic differentiation were associated with ciliopathies and other skeletal pathologies including frontometaphyseal dysplasia,

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spondylometaphyseal dysplasia, osteofibrous dysplasia, and polydactyly (data not shown). ***(See blue text bottom of page 12-top of page 13)***

c. Why not show that DE ciliome genes are causing ciliary phenotypes specifically in the affected tissue-type in vivo?
We thank the Reviewer for this suggestion. We have added this analysis to Fig.2E-E' to show that the DE ciliome represents tissue specific phenotypes. ***(See blue text bottom of page 9-top of page 10)***

10. It would be much easier for the reader to follow if related figures were kept together. Here are two examples:

a. All Pmc1 data (currently supplemental) should be in Fig. 5 with the Tmem107 data.
We have reorganized figures to consolidate all Pcm1 data into one main figure (new Fig. 5). Since the Reviewer has correctly pointed out that Tmem107 null mice have previously been reported, we have moved the Tmem107 data to supplemental figure 5.

b. The Venn diagram in supplemental Fig. 2 should accompany the Venn diagram in Fig 1E because both highlight the overlapping v. differential tissue-specific DE ciliome.
We have moved the Venn diagram from Supp. Fig. 2 to main Fig. 2.

MINOR

1. The authors should explain and justify why they switched from tSNE to UMAP plots.
t-SNE in Supp Fig1 was simply meant to show similarities between replicate tissue sample used for bulk-seq analysis. Essentially, the plot serves as a quality control. UMAP was used to analyze single cell data and show differences in gene expression.

2. While the paper will have a copy editor, the authors should fix the Iimproper capitalization of common nouns throughout manuscript.
We have reviewed the manuscript to try and correct improper capitalization.

Reviewer 2 Advance Summary and Potential Significance to Field:
Summary: This techniques and resources article investigates the heterogeneity of cilia associated gene expression during mouse head and limb development. The authors identify subsets of ciliome genes that are differentially expressed between these tissues and focus on a category of the ciliome genes that are proposed to be osteogenic-specific. Two of these genes (Pcm1 and Tmem107) are validated for developmental skeletal phenotypes using an F0 CRISPR/Cas9 screen. The RNAseq data are presented as an accessible shiny app for use by the community. This represents an excellent resource and tool for the field.

Reviewer 2 Comments for the Author:
We have a few comments for improvement of the manuscript:

1) Introduction, page 3 last paragraph-top of page 4: the authors describe a number of ciliopathies which have distinct tissue specific phenotypes. This is a vague way to describe and possibly discount a complex series of tissue relationships. It would be better if the authors provided specific examples of their point. What are these?distinct phenotypes? The authors are referring to, and how do these ?distinct phenotypes? Support their argument of a ?degree of heterogeneity beyond the structure of the cilium??
We thank the Reviewer for this comment and have addressed it by providing specific ciliopathies that present with tissues-specific phenotypes. ***(See blue text bottom of page 3-top of page 4)***

2) They then go on to explain that HH associated genes have been removed from the analysis for this differential expression assessment, however, differential expression of some of these genes (e.g. GLI1/2 vs GLI3) are sure to be contributing to the differential phenotypes observed, especially given that they will follow up later on key osteogenic factors. So, while we don?t particularly object to idea of removal of the Hh signal transduction genes in the analysis, it would be helpful have a more nuanced rationale in the experimental setup. Alternatively, I would favour just including these ten genes in the overall analysis, especially as some of the other genes are surely

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underappreciated regulators or effectors of Hh signalling (e.g. Tulp3).
We thank the Reviewer for this input. This was a shared critique among Reviewers and as a result, we have added back all the genes that were manually curated out to our initial analysis. Furthermore, we have repeated all analyses with this new ciliome.

3) Page 6 (last paragraph) and fig1c: Please clarify lethal and subviable in terms of the IMPC categories. Are they lethal prior to organogenesis (e9.5), embryonic (e14), prenatal (e18)?
We have adopted the IMPC classifications for our work as described in their primary viability pipeline. In this pipeline, centers breed mice in hetXhet crosses and genotype offspring (n>28) to define lethality as the absence of homozygous mutants at wean and subviability as observing less than half the expected Mendelian ratio (< 12.5%) of homozygous mutants at wean. While some members of IMPC do perform a secondary viability pipeline, the window of lethality is not defined for all lethal lines and thus we opted for the higher-level classification. We have provided references to IMPC and a more expansive definition of terminology in the figure legend of Figure 1. ***(See blue text page 6 and page 30)***

4) Page 12 and Fig5 e-m ? Panel L, ventral TMEM107 crispant looks to have a fused premaxilla, but hypoplastic basisphenoid and palatine bones. Please provide more detailed description of the phenotypes rather than generalised ?hypoplastic cranial skeleton? statement.
We thank the Reviewer for this suggestion and they are indeed correct about the fused premaxilla. We have now labelled the skeletal images in ***Figure 5 and Supplemental Figure 5*** *and provided phenotypic descriptions in the text.* ***(See blue text pages 14, 32, and Supp. Fig. 5 legend)***

5) Page 20 and 21 ? Mineralisation (alizarin red staining) in supp fig 4. C? looks very non-specific (iPSC, d8 and 12) enough that we are not convinced. Do you have any other means of confirming mineralisation or no beta- glycerophosphate media controls run to check for non-specific mineralisation?
We again thank the Reviewer for this comment. We have now analyzed these cells via three distinct assays. In additional to examining expression of RUNX2 (master regulator of osteoblast differentiation) via qPCR and Alizarin Red staining (calcium deposits), we also performed an additional osteogenic assay; the OsteoImage™ Mineralization Assay is a rapid, fluorescent in vitro assay for assessing bone cell mineralization via hydroxyapatite expression and performed this with negative control that lacked beta-glycerophosphate/ascorbic acid. ***(See new Supp. Fig. 4 and new text in Supp. Fig. 4 legend)***

6) Page 21 ? beta glycerol phosphate nomenclature -> (symbol beta) b-glycerophosphate or glycerol 2- phosphate
We thank the Reviewer for bringing this to our attention. ***(See blue text on pages 22, 23, 24, and Supp. Fig. 4 legend)***

Reviewer 3 Advance Summary and Potential Significance to Field: The manuscript by Elliott et al attempts to demonstrate that there are cilia associated genes (ciliome) that are differentially expressed in different tissues and over developmental time. The documentation of heterogeneity in expression of cilia associated gene is timely. This has important implications for understanding why patient mutations in the ciliopathy class have different phenotypes even though they are considered “cilia” genes.

The strengths of this report are the annotation of cilia related genes that are differentially expressed in different example tissues or developmental stages; the database to allow access to the datasets; some of the confirming studies to show the analysis finds tissue specific expression of cilia associated component.

The weaknesses of this report are that the analysis and interpretation is oversold on a number of points, and some of the experiments said to confirm the relevance of differential expression don’t do so.

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Reviewer 3 Comments for the Author:

Major points:
1. The title and statements throughout suggest this a much broader study that it is. For example - the sentence in the introduction – " our data comprehensively reveal that approximately 30% of the ciliome is differentially expressed across tissues…." - the reads as if all tissues were assessed and the study will show a catalog of differential expression in an exhaustive number of tissues and developmental stages. In fact, this is comparing three craniofacial areas to limb and two brain areas. These are important, given the impact of ciliopathies on these regions, but it isn't a comprehensive study. Some of this should be toned down to reflect what was actually done in this study.
*We have edited the sentences in question to state that 30% of the ciliome is differentially expressed across **analyzed** tissues. Furthermore, the first sentence in that paragraph explicitly explains the experimental design in which we compared six distinct embryonic domains that are commonly impacted in ciliopathies.* ***(See blue text pages 4, 15)***

2. Likewise – the statement at the end of the introduction stating "the ciliome has evolved to account for distinct functions of cell-types in vertebrate species and that it plays an essential role in defining cell/tissue identity and differentiation potential." should be modulated. Cilia, peroxisome, mitochondria etc are going to vary depending on the proteins being expressed that go to those locations for their function. And cells vary what they express depending on their origin and developmental trajectories. The cilia aren't defining cell identity but reflecting it. Differential genome expression over time defines it. This all seems overblown.
We have tempered the language and edited the sentence to read "Furthermore, we profiled expression of the ciliome during differentiation of multipotent cranial neural crest cells and observed upregulation of numerous ciliary genes correlating with osteogenic cell fate decisions, suggesting that changes in the ciliome contribute to distinct functions of cell-types in vertebrate species." ***(See blue text page 4)***

3. And in the discussion – "our work redefines the primary cilium as a tissue-specific dynamic organelle that changes composition to accommodate cell- type specific function" – some of this work looks at motile cilia, not primary cilia, as part of the basis for claiming there are important differences in different tissues. See point 7 below. And while the work here is interesting, I'm not sure it redefines the primary cilium as tissues specific based on comparing brain tissue (motile cilia) with craniofacial tissue (primary cilia).
The changes during osteogenic differentiation are compelling, but not knowing exactly how the ciliome was curated (see point 4) makes it difficult to know what we are analyzing.
We have tempered the language in this part of the discussion to read "Thus, our work characterizes the ciliome as both tissue-specific and dynamic and suggests that changes in the ciliome contribute to cell-type specific function." ***(See blue text page 15)***

4. Results, 1st paragraph – "Manual curation which included the elimination of genes associated with Hh signal transduction…". First, what was done here is important, especially for people wanting to use this as a resource. Explanation of this process and what was removed from the curated ciliome should be provided in supplemental materials.

Second – why remove Smo and Gli given these are proteins that enter into the cilia and are regulated in this location in mouse and humans? Wouldn't this be an obvious candidate for providing cilia associated differences in tissues and in disease phenotypes? It makes me hesitant given what else might have been removed from the ciliome during curation.
All three Reviewers had concerns with our manual curation which removed members of the Hh pathway and transcription factors that were parts of other molecular pathways from the ciliome. Thus, we repeated all our analyses without manual curation. Of note, the general trends of our analysis still hold true without curation, validating our original conclusions.

5. Results, section 1, paragraph 3, Figure 1 – The fact that cilia in tissue culture cells vary in length and percentage of ciliated cells is well known, so this data isn't surprising, but I don't see how this "validated transcriptomic findings". As to supporting the hypothesis that cilia are not ubiquitous organelles, is that really in question? Cilia in the mouse node are single and motile with a 9+0 axoneme, cilia in the limb mesenchyme are single and non-motile with a 9+0 axoneme, cilia in the

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brain ventricles are in multiciliate bundles and motile with a 9+2 axoneme. We know cilia are different in different tissues and contexts and the data in Figure 1B aren't compelling in this regard given so many in vivo examples.
*We have moved our in vitro analysis to Supp. Fig. 1 and edited the section in question to read: "While in vivo transcriptomic analysis supported the concept of ciliary heterogeneity within the embryo, in vitro data in cell types that represented organ systems frequently affected in ciliopathies (NIH-3T3-fibroblasts; NE4C-neuroectodermal; and O9- 1-neural crest) [23-25] revealed additional variation in ciliary length, number, and extension rates." **(See blue text page 6)***

6. Results section 1, paragraph 4, Figure 1C – this is an interesting result. Are the genes represented in the non-DE ciliome primarily structural components of the cilia and thus more likely to cause consequence across all tissues? For example centrosomal components that may be involved in spindle assembly as well as cilia formation? Are genes in the DE ciliome more signaling based or genes with multiple orthologs/paralogs in the mouse genome?
*We thank the Reviewer for this comment. We have added **new Supp. File 2** to address Reviewer queries about gene enrichment in the non-DE ciliome. **Supp. File 2** shows GO-term analysis of the non-DE ciliome and reveals that the most significant association are with structural components of the ciliome (e.g., centrosome, axoneme, ciliary tip, ciliary transition zone) compared to the non-DE ciliome. **(See blue text page 7)** This data together with that in Figs. 1B-E supports a conclusion that the non-DE ciliome is more constrained. We also characterized genes in the DE in terms of enrichment within various ciliary compartments, phenotypes and molecular function in **Supp. Fig. 1B-D, Fig. 2C, E, E', and Supp. Fig. 2B, C**.*

7. Results section 2, paragraph 2, Fig 2D – This interpretation seems a bit off. Of course these genes (found in motile cilia) are going to be specific to neural tissue in this analysis, because this is the one tissue assayed that has motile cilia. This is to be expected. It could be used to say the DE ciliome contained these genes as expected, but not as proof that the analysis found something that we didn't already know, which is how it reads to me.
*We again thank the Reviewer for this comment. Our intention was not to portray these results as novel, but to use them as proof of principle for our approach. We have altered the text to ensure that this intention comes across clearly: "Given the fact that the neural tissues were the only tissues to contain motile cilia, these results were expected and served as proof-of-principle and quality control for our approach." **(See blue text page 9)***

8. Results section2, paragraph 4, Fig 3B – "a subset or genes that was expressed in both tissues" – Is there a better way to display this? It is hard to see low level expression and many of the claims are based on spots that seems to be 0% in size. Doesn't that mean 0% of cells expressed the gene in question? Given how nicely the RNAscope data is for Rab3il, the data here is underwhelming as presented. I wouldn't predict such nice tissue specific expression from the plot in 3B.
While we agree that the dot plot isn't striking, it does serve the purpose of comparing groups of genes in epithelial and mesenchymal clusters. We used three additional approaches to validate the conclusion that the ciliome was distinct between epithelial and mesenchymal tissues: feature plots (Fig. 3C-E), RNA scope (Fig. 3C'-E') and Western Blot. Together, these three additional assays supported the conclusions from the dot plot.

9. Results section2, paragraph 4 – the data comparing neuroepithelium with MNP mesenchyme is interesting. Do you see something similar comparing MNP mesenchyme with limb mesenchyme? Or is the overlap greater?
This is an excellent point, unfortunately we were unable to do that comparison for two reasons. First, we did not perform scRNA-seq on limb samples and did not isolate epithelial or mesenchymal tissue from limb samples as it would have been prohibitively time consuming and expensive. Second, the gene set that was exclusively enriched in limb populations only consisted of 10 genes, and as such it is not possible to make any conclusions from the data.

10. Results section3m paragraph 2, Figure 5 – are Tmem107 edited MEFS producing longer cilia? Are these cells having issues with cell division by any chance?
Previous studies have examined cilia in both Tmem107$^{-/-}$ and ENU induced Tmem107 mutants (Cela et al., 2018; Christopher et al., 2012) in vivo on palatal shelf surface (epithelial) and on mesenchymal populations. These groups reported variability in cilia length was higher in

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Tmem107$^{-/-}$ animals than in controls, particularly in the anterior or middle areas of the palate. Furthermore, they reported that mesenchymal cilia appeared to be elongated in Tmem107 $^{-/-}$ animals. We reference these studies within the text. ***(See blue text page 13)***

11. There is almost no information on the CRISPR F0 mice. How were these genotyped? What sort of mutations were observed? Are these craniofacial defects representative of multiple F0s? Are you sure there are no off-target effects for the CRISPR manipulation?
We thank the reviewer for raising these important questions. Yes, all recovered F0 embryos were genotyped first using standard PCR and then quantitatively assessed using droplet digital (ddPCR). Paired guides were designed to generate a deletion eliminating a critical exon (Pcm1) or the entire coding sequence (Tmem107) as depicted in Supplemental Figure 5. Deletions provide a simple means of detection by PCR and are amenable to loss-of-allele (LoA) assays to perform copy number variation (CNV) analysis. The craniofacial defects were detected in several independent embryos; however, given the subviable phenotype of Pcm1, we do expect there to be variability in the Pcm1 phenotype compared to Tmem107. While there is a risk of potential Cas9 off-target activity, all guides were designed to minimize this risk by only accepting guides with 3 or more mismatches and by delivering the gene editing material as Cas9 protein complexed with the guides (RNP) via electroporation which reduces the time Cas9 is active. Additionally, the concurrence in phenotypes between individual embryos is supportive that the phenotype is due to on-target mutation and not off-target which tends to be random. We provide a reference for this methodology in the Methods section on page 26.

12. Supp Fig 5 B-G – in my opinion, this is some of the nicest data you have along with the mouse craniofacial defects. The cilia appear to be changing given the accumulation of PCM1 at the base and this is clear from the Ifs. Why not move this to the main text?
We thank the Reviewer for this suggestion. We have now moved this data to main ***Figure 5****.*

Minor points:
1. Figure 1B – I'm not sure what rate of ciliary extension is referring to.
There are no materials and methods explaining what was measured and how. There is note of how cytochalasin was used, but nothing else.
Cytochalasin D rapidly induces primary cilia formation and over-elongation of cilia in conditions that normally promote ciliary disassembly (Bershteyn et al., 2010; Kim et al., 2015). Thus, we used this approach to determine if the dynamics of ciliogenesis/ciliary extension were different between different cell types. We have now moved this data to ***Supplemental fig. 1*** *and have expanded the methods sections to explain this assay.* ***(See blue text page 20)***

2. Results section 2, paragraph 2 – "essential for the formation and function of 9+0 motile cilia". I assume this is a typo and should be 9+2 motile cilia. The 9+0 motile cilia are primarily found in the mouse node. There is overlap in the ciliome of the motile cilia, but the overlap in neural tissue here would be for ependymal cilia which are 9+2.
We thank the Reviewer for catching this. Since both 9+0 and 9+2 terms came out of the GO-term analysis, we have just removed reference to the microtubule arrangement and simply referred to 'motile cilia'. ***(See blue text page 9)***

3. Results section 2, paragraph 2 – "essential for the formation and function of 9+0 motile cilia [19, 27-30]" – missing Lrrc46, CCDC40 references which are included below:

Yin Y, Mu W, Yu X, Wang Z, Xu K, Wu X, Cai Y, Zhang M, Lu G, Chan WY, Ma J, Huang T, Liu H. LRRC46 Accumulates at the Midpiece of Sperm Flagella and Is Essential for Spermiogenesis and Male Fertility in Mouse. Int J Mol Sci. 2022 Jul 31;23(15):8525. doi: 10.3390/ijms23158525. PMID: 35955660; PMCID: PMC9369233.

Becker-Heck A, Zohn IE, Okabe N, Pollock A, Lenhart KB, Sullivan-Brown J, McSheene J, Loges NT, Olbrich H, Haeffner K, Fliegauf M, Horvath J, Reinhardt R, Nielsen KG, Marthin JK, Baktai G, Anderson KV, Geisler R, Niswander L, Omran H, Burdine RD. The coiled-coil domain containing protein CCDC40 is essential for motile cilia function and left-right axis formation. Nat Genet. 2011 Jan;43(1):79-84. doi: 10.1038/ng.727. Epub 2010 Dec 5. PMID: 21131974; PMCID: PMC3132183.

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This section has been significantly edited based on our new analysis and other Reviewers' comments. Thus, these genes are not referenced in the revision.

4. Results section2, paragraph 4 – Crb3 is noted here, but not in Fig. 3B. Why call it out here?
We thank the Reviewer for catching this. We have removed mention of Crb3 from the text.

5. Materials and methods – cell culture section – "a minimum of 30 cilia were counter per timepoint in triplicate" – this is for lengths and cilogenesis rate, correct? The percentage were the minimum of 400 cells counted in triplicate? Please clarify.
We have edited this section to clarify, and 30 cells were assayed for each cell type to determine cilia extension rate (micron/hour). We have also edited this section to clarify that the percentage of cells extending a cilium was determined by examining 400 cells from each cell type (3T3, NE-4C, O9-1). ***(See blue text page 20)***

6. Figure 5 legend – the legend says E18.5 skulls but the analysis for PCM1 in Supp Figure 5 says E17.5 mice were phenotyped. Please clarify.
We appreciate the reviewer catching this typo. Both Pcm1 and Tmem107 were phenotyped at E17.5. This has been corrected. ***(See blue text page 32)***

7. Supp Figure 4 – I don't see why cluster Cluster 5 in E11.5 is any more or less similar to E13.5 Cluster 5 based on the data presented in this table. What were the criteria for deciding these were the two best clusters to compare to each other?
*We previously published these data sets and their comparisons in Elliott et al., 2020. In brief, Trajectory analysis or 'Pseudotime' analysis was performed using Monocle (*Trapnell et al., 2014*). The integrated E11.5 and E13.5 scRNA- seq dataset was assessed for differential gene expression by original cluster, with the top 2000 being used for ordering. Data dimension reduction was performed using the DDRTree method, and cells were ordered using the orderCells function in Monocle 1. All visualization of the trajectory analysis was performed using functions embedded in Monocle. We have added this reference to the text.*

8. Supp Figure 5H – I'm not familiar with the phenotyping method depicted here. What are we looking at? How is the calling done? What is it actually detecting? Loss of probe? How does that get converted to CNV
We apologize for the confusion on this point. This figure is depicting the genomic organization of each target (Pcm1 and Tmem107) with the position of the guides shown. Additionally, the ddPCR assay is included to indicate the position of the primer probe sets used to perform CNV analysis. The design is a loss-of-allele assay such that a bi- allelic edit would result in a deletion and subsequent loss of probe signal (0 copy), mono-allelic edit (1 copy) or no edit (2 copies). The CNV calculation is performed by normalizing against a reference control, ApoB, which is labeled in separate channel and occurs at normal copy number. All calculations are performed using Bio-rad ddPCR software for CNV determination. The accompanying bar chart is color coded to indicate whether an individual embryo was noted as displaying a gross morphological phenotype highlighting affected embryos are the edited embryos. For CNV reference, a non-edited wild-type control DNA sample is shown in green. The specific details for this experiment are included in the Methods under header "Generation and genotyping of CRISPR/Cas9 edited F0 embryos" on page 26.

Second decision letter

MS ID#: DEVELOP/2022/201237

MS TITLE: Identification of a heterogeneous and dynamic ciliome during embryonic development and cell differentiation

AUTHORS: Kelsey H. Elliott, Sai K. Balchand, Christian Louis Bonatto Paese, Ching-Fang Chang, Yanfen Yang, Kari M. Brown, Daniel Rasicci, Hao He, Konrad Thorner, Praneet Chaturvedi, Stephen A. Murray, Jing Chen, Aleksey Porollo, Kevin A Peterson, and Samantha A Brugmann
ARTICLE TYPE: Techniques and Resources Article

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I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

This manuscript provides the field a user-friendly resource identifying transcripts relevant to cilia in distinct tissue types at distinct times. This is timely as growing evidence shows that cilia have functional tissue specificity.

Comments for the author

The authors have satisfactorily addressed the reviewer comments. The inclusion of the gnomAD analysis strengthens the conclusions.

Reviewer 2

Advance summary and potential significance to field

The authors have created a searchable tool accessible shiny app for searching single cell RNAseq data covering differentially expressed ciliome genes. This is potentially a very useful tool for the field. While curating these datasets, the authors find that approximately 30% of the ciliome is differentially expressed in the embryo, suggesting ciliary heterogeneity and uncovering tissue specificity (in the facial prominences) of ciliary genes. They then use Crispr-Cas9 gene editing to disrupt cilia genes associated with osteogenesis.

Comments for the author

I am satisfied with the authors' response to reviewers.

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