Trisomy 21 increases microtubules and disrupts centriolar satellite localization

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Reviewer #1 Review (Anna Akhmanova)

Comments to the Authors (Required):
This paper addresses a very interesting question of how the increased gene dosage in cells with trisomy 21, the cause of Down syndrome, leads to defects in ciliation. The authors focus on increased dosage of the large centrosomal scaffolding protein, pericentrin, and propose that increased pericentrin puncta create roadblocks for transport and nucleate non-centrosomal microtubules, which inhibit ciliogenesis by preventing cargoes from reaching the centrosome. As a model system, the authors use RPE1 cells, which were engineered to have 3 or 4 copies of human autosome 21. The model system is a strong feature of this paper; unfortunately, overall, the data are insufficient to support the conclusions, even though there is a lot of quantitative data presented in the manuscript.

Major concerns
1. One of the biggest problems is that the authors for some reason think that all microtubules in RPE1 cells are anchored to centrosomes, while in fact it is firmly established by the work from Kaverina and Rios' labs that approximately half of microtubules in these cells are nucleated and anchored at the Golgi. It is possible that this percentage decreases in serum-starved cells, but this needs to be properly analyzed and discussed. Unfortunately, all microtubule images in the paper are of poor quality, because microtubules look partly disassembled. This is likely because the authors stain microtubules after pre-extraction, and this is apparently performed in a way that leads to partial microtubule depolymerization and leaves the remaining microtubules sparse and curvy. Live imaging, methanol or other fixation method with proper microtubule preservation would be needed to analyze the microtubule network. Dynamic microtubules in RPE1 cells are straight and quite dense - a co-staining of tubulin and EB1 in the same cell would be helpful to illustrate good microtubule preservation. Super-resolution microscopy would be preferable to analyze the details of microtubule organization around the centrosome and determine the actual percentages of centrosome-attached and non-centrosomal microtubules. All microtubule analysis presented in the paper needs to be re-done with proper imaging - the current data simply do not fit with what have been published about RPE1 cells by other labs.
2. The authors extensively discuss changes in trafficking and roadblocks, but the paper doesn't contain a single live imaging experiment, which are now completely standard in the field. Without these, no conclusions can be made about trafficking defects. Live imaging of ciliary cargo would be needed to show that pericentrin puncta indeed disrupt trafficking. It is also not proven at all that non-centrosomal microtubule ends represent "dead-ends", as the authors propose - in fact a lot of cargoes can switch between microtubule tracks, and no evidence is provided that ciliary cargo indeed accumulates at these minus ends. If the authors would like to make any conclusions about transport or trafficking, they would need to do live imaging with appropriate markers and also analyze steady-state distributions of these cargoes.
3. PCM1, a major centriole satellite component, is a binding partner of pericentrin and does not provide a good marker to study cargo transport in this study. It just shows that elevated levels of pericentrin cause expansion of centriolar satellites, but whether this by itself is deleterious for ciliogenesis is unclear. It is, however, important to mention that the most conceptually simple explanation of the phenotype induced by increased levels of pericentrin is sequestration of some ciliogenesis factors into pericentrin puncta, similar to PCM1. This simple possibility needs to be experimentally tested.

4. It is not proven that the elevation of pericentrin levels by 1.5 fold alone is sufficient to inhibit ciliation. It is very nice that the authors show that reducing pericentrin concentration by RNAi rescues ciliation in T21 and Q21 cells, at least to some extent, but it remains possible that additional proteins encoded by autosome 21 contribute to the phenotype caused by pericentrin overexpression.
5. It is not clear how good the quantification of pericentrin levels is. Pericentrin levels, as well as the levels of its major binding partners (CEP215, gamma-tubulin, PCM1) should be analyzed by Western blotting.

Minor concerns.
1. Many quantifications in this paper are shown without illustrating the data that were quantified (see, for example, Figure S3). For every single quantification shown, the data serving as its basis must be provided.
2. 24 hr recovery after cold treatment seems like a very long time - normally, compete recovery should be observed within an hour or so, unless the cells are somehow damaged by this treatment (note that microtubules grow with the rate of 10-20 mkm/min, so it takes a few minutes to regrow complete microtubules, and it shouldn't take longer than an hour before the original microtubule organization is fully restored).
3. Quantification of puncta size - it seems that the authors do not take into account the resolution limit of fluorescence microscopy. Claiming size above or below 50 nm without applying any super-resolution techniques or electron microscopy doesn't seem appropriate. The puncta may appear larger just because they are more bright.

Reviewer #2 Review

Comments to the Authors (Required):
Trisomy 21 is a common congenital disorder that impacts multiple organs but due to the imbalance in expression of hundreds of genes, the molecular pathways involved remain elusive.
In this manuscript Pearson and colleagues investigate the molecular mechanisms by which elevated levels of the centrosomal scaffolding component, Pericentrin (PCNT), impair cilia formation in cells with trisomy 21. This work builds on an excellent report from the same laboratory (Galati et al, 2018) where the authors demonstrated that trisomy 21 cells exhibit a 0.5 fold increase in PCNT levels resulting in cytoplasmic (phase-separated) puncta formation. These puncta disrupt normal trafficking of ciliary assembly and signalling components, ultimately causing reduced cilia formation.

Using isogenic RPE1 cell lines with trisomy or tetrasomy of chr21, the authors here report that depletion or monoallelic deletion of PCNT as well as microtubule depolymerisation can partially restore cilia formation in these cells. Furthermore they find that some PCNT puncta co-localise with PCM1, a core centriolar satellite component. In RPE1 cells PCM1 is vital for cilia formation and its sequestration at these puncta could indeed contribute to reduced ciliogenesis in trisomy 21 cells. Enlarged PCNT puncta do not only associate with microtubules but also nucleate polymers mostly visible in unciliated cells, and thus increased microtubule levels around centrosomes could also have a negative effect on ciliation. Therefore, the authors conclude that trisomy 21 disrupts microtubule-dependent intracellular trafficking hence impairing ciliogenesis.

Data in manuscript is high quality, rigorous and well-presented. The paper is well-written with a good logical flow. Despite these attributes, however, I am not convinced the results represent sufficient conceptual advance over those in the previous publication. Key conclusion from 2018 was that PCNT puncta form roadblocks on microtubules and prevent trafficking of the essential ciliogenesis factor IFT20. Here the authors provide further analysis of PCNT puncta behaviour and their association with and impact on the microtubule network, and demonstrate microtubule-nucleation from these enlarged puncta. Furthermore, they describe a partial co-localisation between puncta and PCM1 in trisomy cells but this is not entirely surprising given that PCNT is a binding partner of PCM1. The data related to PCM1 is also largely correlative; PCM1 co-localisation with PCNT puncta is disrupted at microtubule ends by cold/nocodazole treatment, and cold/nocodazole treatment improves ciliogenesis in trisomy and tetrasomy 21 cells, but whether the latter is truly due to liberation of PCM1 from puncta is not shown. The role of PCM1 in cilia formation is still poorly understood, and while PCM1 is a worthy candidate, the current data is not sufficient to demonstrate a role for PCM1 sequestration in PCNT-driven ciliogenesis failure. As the paper suggests enlarged PCNT puncta are likely to have a dual role in ciliogenesis; i) they could block microtubule-dependent traffic and ii) sequester important components. The authors could have employed a more comprehensive approach to identify the protein content of the puncta whether by a microscopy screen for cilia and centrosome proteins or affinity-based proteomics. Without such information, the story remains fairly narrow providing a relatively small gain in our mechanistic understanding.

Specific points:

1. Depletion of PCNT in cells with 4 pericentrin copies improves cilia formation but only to about 30%, which is less than the 40% seen in cells with 3 copies. This occurs despite centrosomal pericentrin levels reaching diploid levels and no puncta being visible in Fig 2G. How do the authors explain this discrepancy?

2. I am rather puzzled by the cold treatment and nocodazole experiments, where cells are exposed to treatment for 20 or 60 mins, respectively, but assayed only following a 24-hour recovery period. How do authors explain the long-term effects of these treatments especially on microtubule polymer levels? Even in normal disomic cells cold treatment results in decreased

microtubule intensity 24 hours later. Cold treatment clearly has a profound effect on ciliogenesis in trisomy and 21 cells (seemingly stronger effect than nocodazole). Because PCNT puncta persist, I would expect these to associate with/nucleate microtubules as soon as cells are returned to 37C, leaving a relatively short window for initiation of cilia formation. To me these results suggest that as soon as the key factor(s) reaches the centrosome, cilia formation is initiated, and once a cell starts ciliogenesis, the puncta do not matter. Is that so? Cells could be stained for IFT20 and PCM1 0.5-1 hour after start of recovery period following cold or nocodazole treatment to demonstrate increased accumulation of these factors directly brought about by microtubule disruption. Also, it would be interesting to see how soon cilia form following these treatments (i.e. fixing cells 1,2,4 hours into recovery). Timing of cilia formation could provide insight into the molecular players/mechanisms affected by PCNT puncta.

3. I don't doubt that PCM1 and PCNT co-localise in some puncta but it would be useful to know if these correspond to centriolar satellites or if it is only PCM1 that is being taken up. Other satellite markers could be stained for such as CEP131. Moreover, PCM1 staining appears weak and overly scattered in the normal disomy 21 cells in Fig 4G; I would expect to see granules more concentrated around the centrosome region.

4. What about other pericentrin-interacting proteins in the puncta? Is Cdk5rap2 there?

Reviewer #3 Review (Elif Nur Firat Karalar)

Comments to the Authors (Required):
In this manuscript, McCurdy et al. investigated the mechanisms by which elevated PCNT levels interferes with primary cilium assembly and signaling in Trisomy 21. This study was built upon results from their previous study by Galeti et al. 2018. Previously, they showed that PCNT accumulates at centrosomes and assembles into large foci in Trisomy 21 and that this causes cilium assembly and Hedgehog signaling defects via impaired protein trafficking to the centrosomes (i.e. IFT20). Based on their results, they proposed that larger and denser PCNT foci around the centrosome might act as a boundary for trafficking of proteins to the centrosomes and cilia. In the current manuscript, they aimed to the mechanisms that underlie these trafficking defects by focusing on the interplay between PCNT, microtubules and centriolar satellites.

To this end, they first validated that isogenic RPE1 cells engineered with three (T21) or four (Q21) copies of Hsa21 recapitulates the ciliary defects associated with elevated PCNT of T21 patient samples. Using this new system and CRISPR/Cas9 KO of PCNT, they showed that these defects were dependent on PCNT centrosomal levels. In T21 and Q21 cells, PCNT formed large puncta that localized along microtubules and at microtubule ends and enhanced microtubule nucleation. Notably, PCNT puncta co-localized with the centriolar satellite scaffold PCM1 at these sites. Depletion of PCNT and disruption of microtubules rescued PCM1 mislocalization and ciliogenesis defects in T21 and Q21 cells. Collectively, they propose that PCNT foci act as roadblocks along microtubules and dead ends at microtubule ends and disrupt centriolar satellite-mediated protein targeting during ciliogenesis.

The results of the manuscript contributes to our understanding of the microtubule-based transport mechanisms to centrosomes and cilia and how these processes are disrupted in disease states. The presented data are of high quality and general interest, but the authors did not go far enough to dissect the mechanisms by which elevated PCNT levels impairs trafficking and disrupts cilium assembly and function. In its current state, these results are incremental to Galeti et al. 2018 paper except for 1) use of new cell line models that allow dose-dependent investigation of phenotypes, 2) further characterization of microtubule and centriolar satellite-related trafficking defects in T21 and Q21 cells. However, results obtained from these experiments do not provide mechanistic insight into how deregulation of microtubules and centriolar satellites impair centrosomal/ciliary protein targeting in Trisomy 21. For example, which cargoes depend on this trafficking pathway? What is the evidence that larger and denser PCNT foci act as roadblocks and dead ends to the trafficking of these cargoes? How do microtubule and centriolar satellite dynamics differ in their cell line model? Following experiments are required for further investigation of these points:

Fig. 2: Analysis of microtubule dynamics by live imaging in D21, T21 and Q21 is required to demonstrate that PCNT puncta disrupts microtubule dynamics.
Fig. 3: To determine whether PCNT puncta are sites of active MT nucleation, the authors quantified the number of EB3 comets associated with them and also quantified microtubule intensity following cold treatment. Does the intensity of PCNT foci-nucleated microtubules correlate with foci size?
Fig. 3: To investigate the effects of enlarged PCNT puncta and associated microtubule defects on intracellular trafficking, quantitative live imaging of PCNT puncta and associated cargoes are required in D21, T21 and Q21 cells. Such analysis will likely provide further support for their model suggesting that PCNT puncta create roadblocks along microtubules or dead ends at microtubule ends.
Fig. 4: Comparative analysis of centriolar satellite dynamics in D21, T21 and Q21 cells is required for their conclusions on the link between elevated PCNT and centriolar satellite mislocalization and function. Do PCNT-positive and PCNT-negative satellite differ in their dynamic behavior or do PCNT puncta impair trafficking of all centriolar satellite granules independent of the associated proteins?
Fig. 4: Given that PCNT puncta in T21 and Q21 cells co-localized with PCM1 and that PCM1 is the scaffolding protein for

satellites, further co-localization experiments with different satellite proteins will inform whether PCNT puncta selectively sequesters satellite proteins implicated in cilium assembly and function.
Fig. 2-4: A major gap in the study is the lack of characterization of which cargoes are not trafficked properly in T21 and Q21 cells. This information is critical for uncovering the mechanisms that underlie the ciliation defects. In Galeti et al. 2018 paper, they showed a reduction at centrosomal IFT20 levels. Do authors propose that T21 and Q21 cells are impaired in IFT trafficking to the basal body and cilia?
Fig. 4: Given that PCM1 is essential for the assembly and maintenance of centriolar satellites, characterization of T21 and Q21 cells depleted for PCM1 can provide a more direct way to elucidate the interplay between elevated PCNT levels, centriolar satellites and ciliogenesis. Do PCNT puncta still form in these cells and if so, how do their size and number change? What are the consequences of PCM1 depletion on ciliation efficiency of T21 and Q21 cells as compared to D21 cells?

Minor comments:
1- Fig. C and D - Protein levels of PCNT were increased in T21 and Q21 cells: Do authors refer only to centrosomal levels of PCNT? There is no date regarding the total cellular abundance of PCNT.
2- Fig. 2E: What percentage of large PCNT puncta localize to MT ends (staining for MT ends should be included) or along MTs?
3- Fig. S1: CRISPR/Cas9 KO of PCNT in T21 cells should be validated by Western blotting.
4- Fig. 2: Why did the authors choose 0.05 um2 as the threshold for defining large PCNT puncta?
5- The differences in phenotypes reported for cold treatment and nocodazole treatment in T21 and Q21 cells should be clarified and discussed further.
6- Fig. S3: Quantification of microtubule PTMs in different cell lines disrupts the flow of the manuscript. This part could be rewritten to include the reasoning for these experiments. Is there previous literature for a link between PCNT and microtubule PTMs?

RE: Manuscript #E21-10-0517-T
TITLE: Trisomy 21 increases microtubules and disrupts centriolar satellite localization

Dear Dr. Pearson:

Thank you for submitting your work to MboC and for your patience in getting the reviews. As you can see from the reviews below there is disagreement among the two reviewers regarding the advances your manuscript offers. Reviewer 1 feels that the work offers limited new insights beyond your 2018 paper while Reviewer 2 is supportive. Both however bring up several technical points. I would like to invite you to resubmit this work taking the reviewers' comments into account, especially those pertaining to image analysis and rephrasing some of the text to adhere more strictly to the data presented.

Sincerely,

Antonina Roll-Mecak
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Pearson,

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, as described in the Monitoring Editor's decision letter above and the reviewer comments below.

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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
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Reviewer #1 (Remarks to the Author):

This work is continuation of the study published by the authors in 2018 (Galati et al. Dev. Cell, 2018), which demonstrated that Trisomy 21, increases the levels of pericentrin, which accumulates on centrosomes and at cytoplasmic foci surrounding centrosomes. The authors suggested that this disrupts centrosomal trafficking, ciliogenesis, and Shh signaling. They showed that T21 fibroblasts have elevated levels of the centrosomal proteins CDK5RAP2, CEP120, and gamma-tubulin and generate more cytoplasmic microtubules. They proposed that large extra pericentrin-based aggregates increase pericentrosomal crowding and act as roadblocks decreasing trafficking between the cytoplasm, centrosomes, and cilia.
In this work, the authors continue to investigate the mechanisms by which pericentrin imbalance disrupts cilia, using isogenic RPE-1 cells engineered to carry increased copies of chromosome 21 (2, 3, or 4). The authors conclude that pericentrin aggregates increase microtubule density around centrosomes, miss-localize centrosomal satellites, which disrupts ciliogenesis. The strength of the paper is a well-defined model. The weakness of the paper is over-abundance of quantitative data preformed on cells after ciliation (instead of before and during ciliation), most of which provides only circumstantial evidence but does not advance the understanding of the phenomenon mechanistically or conceptually. So, the novelty is minor to Galetti et al., 2018. There are also technical and conceptual concerns.

Specific points:
Imaging resolution is not sufficient for the question in hand.
The authors depict in F4 that the centrosomes in T21 and Q21 cells are larger than in D21. However, the data argues that this is not the case. For instance, Figures 1C and 2A show the same dataset. The difference is that in 2A, the authors use "increased brightens" to illustrate less bright pericentrin aggregates that are found around centrosomes in T21 and Q21. In F2A, T21 and Q21 centrosomes may appear "larger". However, centrosome size measurement (using half-width-at-half-maxima principle) shows that there is no difference in the size of the centrosomes between in D21, T21, and Q21. Similar seems to be the case in other figure panels, for instance F4A and H. This would imply that in T21 and Q21 cells, there is more pericentrin packed within the same centrosome volume. But due to low imaging resolution, it is unclear how is this excess of pericentrin localized. Is it accumulated only to the older mother centrioles that ciliate? Does it obstruct appendages? How many MTs reach centrosomes? These are critical questions that need to be answered before any further conclusions can be made. Especially because there is no live-cell analysis to prove that cargo delivery is indeed perturbed before and during ciliation. Proper centrosome characterization in high resolution will be necessary to reach meaningful conclusions.

The zoning delineated in Figure 2B seems random. Per F2B, the PCM zone extends from 0.5 - 1.2 micrometers from centrosome center, which is approximately four times the size of an average interphase centrosome (two times if the authors consider two adjacent centrosomes as one). Since the PCM is integral to the centrosome, it is unclear what the authors mean by the "PCM region". In addition, it appears that pericentrin and PCM1 aggregates are not always localized symmetrically around centrosomes (F2A, F3H, F4A...) and centrosomes can find themselves outside the major "crowd" of pericentrin and PCM1 aggregates. So, quantification of material within concentric regions cannot really work in cells with asymmetrically distributed aggregates.

I am perplexed by the quantification results for Cep131, Cep290 (S4 A, B, and C) and PCM1 (F4A and D) and accompanying figure panels. The authors measure intensities of these proteins around centrosomes or in whole cells, and there are relatively small differences in their levels between D21, T21, and Q21 cells (less than 2-fold). However, the corresponding image panels, which are supposed to illustrate representative phenotypes, show examples where the differences in the levels of these proteins around centrosomes are 10-20-fold. Why are these two sets of data at odds?

The correlation between pericentrin aggregates and ciliation is inconsistent.
-In Fig. 2D the quantification of "small" and "large" pericentrin puncta shows almost the same values between T21, and Q21. This is inconsistent with major conclusion and incompatible with differences in ciliation between T21 and Q21.
-In FS2B, centrosomal and cytoplasmic pericentrin levels are almost identical in T21 and Q21.
-siRNAPRCNT in T21 and Q21 reduced pericentrin to similar levels, and yet, cilia formation rate remains drastically different. The authors discuss this issue but nevertheless, this inconsistency remains.
-Is F4B and C whole cells PCM1 intensity and PCM intensity across zones seems not significantly different between T21 and Q21. This is inconsistent with a significantly different rate of ciliation between T21 and Q21 cells.
In F2E, the authors indicate that the panel shows "increased brightness". But the panel does not seem to be increased compared to F2A. However, if the brightness were increased, it would reveal numerous pericentrin aggregates in siPCNT Q21, no pericentrin accumulation at the centrosome or pericentrin aggregates in T21, and some in D21. If satellites and pericentrin aggregates are critical to ciliation, how they are entirely absent in D21 in F2B and T21 siPRCNT in F2E? This is not consistent with quantification data, especially in control cells, where standard deviations are minimal.

Pericentrin levels seem to be always higher in unciliated cells, including control D21 cell, which do not suffer from perturbed pericentrin dosage. It would be important to show that all cell lines arrest in the same cell cycle phase after serum starvation. Or, if that is not the case (In my experience, RPE-1 cells do need to be in G0 to form a cilium), to correlate the cell cycle phase with pericentrin/ciliation.

Reviewer #2 (Remarks to the Author):

Trisomy 21 is the cause of Down syndrome, which includes defects in ciliation in patient cells. In this manuscript McCurdy et al. report on a study of increased pericentrin (PCNT) resulting from increased ploidy of Chromosome 21 and its effect on ciliation. In a nice approach the authors use stable cell lines harboring trisomy or tetrasomy HSA21. This condition mimics that of Down Syndrome and gives a more accurate assessment of the effects of all other genes on HSA21. The disomy, trisomy and tetrasomy relationship allows an extended dose response and, in general, the graded effects of increased HSA21, or rescue by selective knockdown or null mutation of PCNT, increases confidence that the observed centrosome and cilia phenotypes are at least in large part due to increased PCNT levels.

The study initial shows that increased HSA21 results in higher PCNT levels at and around the centrosome along with decreased cilia formation. Overall knockdown, or genetic removal of the extra PCNT copy, recue both these phenotypes. The genetic mutation particularly reveals that the increase in other genes on HSA21, sans PCNT, does not disrupt cilia formation. Increased HSA21 ploidy also results in more and larger PCNT puncta surrounding the centrosome, even more so in unciliated fraction of cells, which is recovered following PCNT reduction. The data also suggest that increased PCNT puncta around the centrosome are responsible for increased MT density surrounding centrosomes, particularly in unciliated cells. The authors show that a large fraction of puncta are associated with MTs, and based on the hypothesis that MT trafficking is disrupted, they show that transiently removing some MTs allows more cells to form cilia. To further support the idea of trafficking defects they show that centriolar satellite proteins (PCM1, CEP290 and CEP131) are also increased around the centrosomes and colocalized to large extent with the PCNT puncta. This indicates that trafficking of satellites is indeed impaired by elevated PCNT levels. The authors further explore this by testing PCM1 colocalization with PCNT puncta after reducing PCNT or transiently decreasing MT polymer. Indeed, PCM1 level and colocalization near centrosomes is reduced, showing it is dependent on PCNT. While this suggests trafficking of satellites is impaired, this result would be stronger by including CEP290 and/or CEP131, as PCM1 may associate with PCNT independent of trafficking).

This manuscript addresses an important question that will be interesting to a range of readers. Overall the data presented is high quality and experiments are thorough. The data show evidence of MT nucleation from PCNT puncta and also that centriolar satellites are mislocalized as a result, likely from trafficking defects, providing insight into how elevated PCNT disrupts centriolar trafficking. The use of trisomy and tetrasomy 21 cells also provides a strong method to allow PCNT dosing and control for additional genes in HSA21. The manuscript may leave readers with important mechanistic questions that will need to be elucidate. Overall, this manuscript represents an important advance and is suitable for a brief report format.

Issues:

The authors show data that PCNT and MTs are increased near centrosomes, and that there is no real change near the golgi. Since the golgi and centrosomes are often in close proximity outside of mitosis, this seems like a paradox (for example, Sutterlin & Colanzi, JCB. 2010 Mar 8; 188(5): 621-628. doi: 10.1083/jcb.200910001; Tormanen et al., PLos One. 2019; 14(4): e0215215. doi: 10.1371/journal.pone.0215215). In the reported study, is the golgi comparatively larger such that the increased MTs around the centrosome are inconsequential with respect to the golgi area? Or is centrosomal - golgi proximity disrupted in serum starvation, or in cells with increased HAS21? This should at minimal be addressed, including proposed or known reasons supporting the outcome. If this is not known, ideally the authors may have so-stained cells that would address this apparent paradox.

The authors do not provide any explanation of how they quantified puncta or satellite size even though they report measured sizes well below the resolution of the microscope setup.

In Fig. 4E, the mean and SD appears to not reflect the distribution of data points. This should be carefully verified and checked that the data shown and indicated statistical tests are accurately reported.

The authors propose 2 hypotheses, traffic dead-ends or trafficking disruptions, and use CD and NZ treatments as one approach to test this idea. The results show these treatments reduce the size and number of PCNT puncta, and also the number of MTs around the centrosome. Consistent with this, there is also a reduction in MT-associated puncta. At the bottom of the first section on page 9, the authors state, "PCNT nucleated centrosome-free MTs lead to MT-dependent trafficking dead-ends are disrupted with NZ, and these dead-ends contribute, in part, to primary ciliogenesis defects in T21 and Q21 cells. The remaining PCNT puncta likely create trafficking roadblocks that still reduce primary ciliogenesis." This is stated as a solid conclusion. While the evidence presented would be consistent with the hypothesis, it stops short of demonstrating the actual trafficking defects and that they indeed occur by those two proposed mechanisms. This should be toned down and very clearly described as to what the data does and doesn't show. Even if satellite components will be subsequently tested.

Scale bar sizes appear missing in multiple places (e.g. Fig S2 E and F).

Other concerns:

The manuscript states: "Genetic ablation of a single PCNT allele in T21 cells using CRISPR-Cas9 (leaving cells with 2n PCNT)

also increased the mean cilia frequency to 121% of D21 levels (Fig. 1 E; and Fig. S1, E-I)". This percent seems inaccurate. Please confirm that it is 121% and correct the figure if needed. It currently appears more like 102%.

Additionally, "Genetic ablation of a single PCNT allele in T21 cells using CRISPR-Cas9 (leaving cells with 2n PCNT) also increased the mean cilia frequency to 121% of D21 levels (Fig. 1 E; and Fig. S1, E-I). There is no Fig S1 I...it stops at H.

In the middle of the upper section on page 9 it states, "We attribute this to the negative effects of PCNT puncta on cilia formation that remain after NZ treatment but are dispersed after CD." This statement seems unclear to a reader. Could the authors expand and/or provide 1 or 2 potential examples?

In Fig 2g the PCNT signal is not visible in some panels. Perhaps this could be shown as two insets, one with three channels and one with only the PCNT channel?

The manuscript states "To test whether the elevated levels of centrosomal and pericentrosomal PCNT increase total cellular MTs, we measured the effects of increased HSA21 dosage on MTs". Technically this tests the effects of excess HSA21, not simply PCNT. Perhaps it could be phrased as "we initially measured the effects of..." or otherwise rephrased.

For Fig. S2 E top graph, is the p value for D21 vs T21 also statistically significant?

For Fig. S2 legend: "(I) Left, area of PCNT foci along MTs are larger than 0.05 um (indicated with red line) and increase with Hsa21 ploidy. Bottom, area of PCNT foci at MT minus ends are larger than 0.05 um (indicated with red line). Mean{plus minus} SD. *p < 0.05 (Table 1)". I believe first word in second sentence should say "Right" in place of "Bottom".

I believe the figure callout is wrong for this statement, "In T21 and Q21 cells, an increase in gamma-tubulin and PCNT colocalization is observed at free MT ends (Fig. S3 F)".

This callout is also wrong, "Importantly, whole cell PCNT and PCNT at and surrounding T21 and Q21centrosomes was reduced (Fig. 3, H and I; and Fig. S3 L)". There is no Fig S3L.

In Fig S3 D, presumably the dashed lines represent error but this should be specified clearly in the legend.

In the methods, it sounds like the 10min CD treatment went straight to fixation. Was it given 10 min recovery or simply 10 min depolymerization and fix right away? Please clarify.

In Fig S4 E, the y-axis legend may be missing a space after "of".

McCurdy et al REBUTTAL

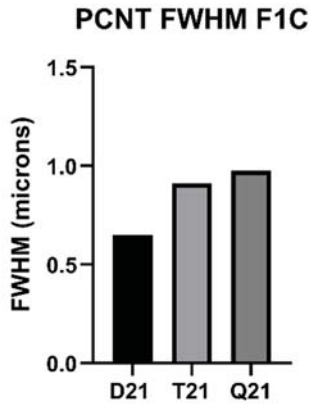
Reviewer #1 (Remarks to the Author):

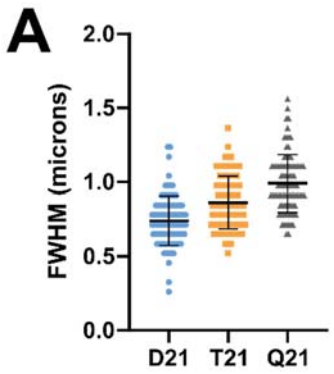
This work is continuation of the study published by the authors in 2018 (Galati et al. Dev. Cell, 2018), which demonstrated that Trisomy 21, increases the levels of pericentrin, which accumulates on centrosomes and at cytoplasmic foci surrounding centrosomes. The authors suggested that this disrupts centrosomal trafficking, ciliogenesis, and Shh signaling. They showed that T21 fibroblasts have elevated levels of the centrosomal proteins CDK5RAP2, CEP120, and gamma-tubulin and generate more cytoplasmic microtubules. They proposed that large extra pericentrin-based aggregates increase pericentrosomal crowding and act as roadblocks decreasing trafficking between the cytoplasm, centrosomes, and cilia.
In this work, the authors continue to investigate the mechanisms by which pericentrin imbalance disrupts cilia, using isogenic RPE-1 cells engineered to carry increased copies of chromosome 21 (2, 3, or 4). The authors conclude that pericentrin aggregates increase microtubule density around centrosomes, miss-localize centrosomal satellites, which disrupts ciliogenesis. **The reviewer is looking to tie a mechanism of centriolar satellites to cilia defects. While centriolar satellites are important for ciliogenesis, we don't directly show the mechanisms by which the centriolar satellite mislocalization observed here directly disrupts ciliation. Nor have any other labs to our knowledge. The revised manuscript makes this point that a direct connection cannot be made at this point but that our results suggest that MT dead end and trafficking roadblock populations of enlarged PCNT puncta contribute to trafficking defects and primary cilia formation defects. The manuscript title has also been changed to reflect this. These insights are critical to understanding the cilia defects found in trisomy 21. (PAGE 11-12)**
The strength of the paper is a well-defined model. The weakness of the paper is over-abundance of quantitative data preformed on cells after ciliation (instead of before and during ciliation) **Our study compares cells with and without cilia at the same timepoint after induction of ciliogenesis with serum starvation (24 hrs). Making comparisons before and after ciliation isn't as simple as this reviewer suggests. Comparing pre and post induction of ciliation would create confounding factors, particular with regard to making conclusions from cells in distinct conditions that affect the cell cycle. Therefore, we will remain focused on our current comparisons. Of note, we have a second manuscript under review that tests the temporal changes associated with serum starvation and induction of cilia. However, this is beyond the scope of this study. We now include a discussion of this point and our future directions to thoroughly address the temporal dynamics of ciliogenesis. (PAGE 11)**, most of which provides only circumstantial evidence but does not advance the understanding of the phenomenon mechanistically or conceptually. So, the novelty is minor to Galetti et al., 2018. There are also technical and conceptual concerns. **In the revised manuscript we outline the novel and mechanistic insights that this manuscript contributes beyond Galati et al. We disagree that this manuscript does not advance our understand of why ciliation is reduced in trisomy 21. Indeed, these studies provide evidence for a model by which changes to PCNT puncta and MT organization in trisomy 21 result in the reduced trafficking and cilia defects initially reported in Galati et al.**

Specific points:

Imaging resolution is not sufficient for the question in hand. **We are unclear what this reviewer means in terms of the resolution required for our experiments. However, we have strived to improve our representative images to more clearly articulate the findings of this study. In particular we now show additional SIM microscopy with image quantification to show the distribution of MT organization (Fig S2).**

The authors depict in F4 that the centrosomes in T21 and Q21 cells are larger than in D21. However, the data argues that this is not the case. For instance, Figures 1C and 2A show the same dataset. **First, F1C and F2A show the same images at different brightness so that the reader can clearly see the pericentrosomal puncta; when brightness is increased the centrosome gets saturated and cannot be quantified – we now make this saturation point clear in the revised manuscript by indicating the amount of increased brightness). Second, the reviewer is referring to our model in F4M showing the centrosome is larger. This is not the focus of this manuscript, and the enlarged centrosome size was already quantified and presented in Galati et al. However, our quantification of the centrosome full width half maximum (FWHM) shows centrosome size increases with HSA21 ploidy as would be expected (see below). These images represent the data acquired throughout this study and the reviewer can see from our biological and technical replicates that we have extensive datasets all around the mean. The top panel below is the FWHM of the representative images present and the lower panel is the FWHM for all centrosomes analyzed in these datasets (n=90 centrosomes).**





The difference is that in 2A, the authors use "increased brightens" to illustrate less bright pericentrin aggregates that are found around centrosomes in T21 and Q21. In F2A, T21 and Q21 centrosomes may appear "larger". However, centrosome size measurement (using half-width-at-half-maxima principle) shows that there is no difference in the size of the centrosomes between in D21, T21, and Q21.

The reason why we indicate "increased brightness" is that we must saturate the centrosome signal to show the pericentrosomal puncta. Because these images are saturated, we would never analyze fluorescence intensities because they would not reveal the dynamic range of our images. As above, if we quantify F2A images without the "increased brightness" and saturation we observe the increased centrosome size consistent with the above averages.

Our revised manuscript now states that centrosome size is increased based on analyses here and Galati et al. (PAGE 20)

Similar seems to be the case in other figure panels, for instance F4A and H. This would imply that in T21 and Q21 cells, there is more pericentrin packed within the same centrosome volume. But due to low imaging resolution, it is unclear how is this excess of pericentrin localized. Is it accumulated only to the older mother centrioles that ciliate?

This is not the focus of this study and it was quantified in Galati et al. The peak intensity of PCNT at the centrosome is greater in T21 and Q21 cells suggesting that more PCNT is packed within the centrosome volume and the increased FWHM would suggest that the centrosome is expanded. PCNT associates with both the mother and daughter centrioles and expands outward. The distribution of PCNT localization at the centrosome is consistent between D21, T21 and Q21 cells. However, it is more expanded with increasing HSA21 dosage. We now include SIM images of PCNT and MTs to show the impact of HSA21 ploidy on PCNT and MT distributions (Fig 3 and S2).

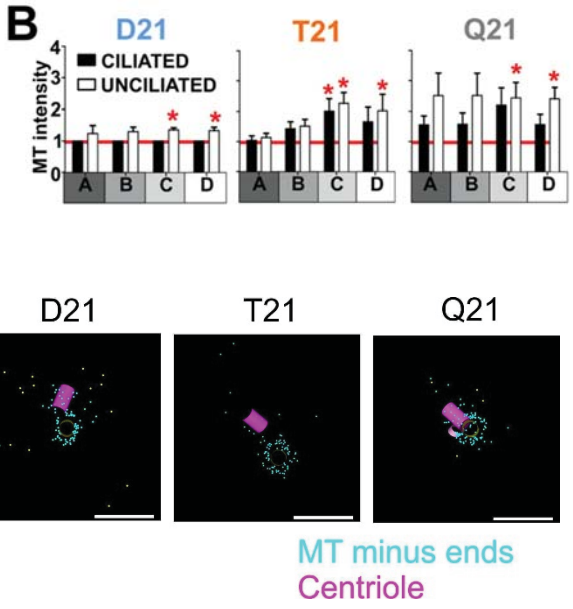
Does it obstruct appendages?

Data from a second manuscript under review indicates that centriolar appendages are unaffected. However, that manuscript provides evidence that that specific trafficking events to the mother centriole appendages are not as efficient in T21 and Q21 compared to D21 cells. Most MTs from the centrosomes in G0 and ciliated RPE-1 cells arise from the centriole

appendages. Our results here suggest that trafficking to the centrosome generally (and therefore likely the centriole appendages) is indeed blocked. The revised manuscript now includes this discussion. (PAGE 12)

How many MTs reach centrosomes?

The plot below (F3B below) and our new and included SIM results suggest that MTs are elevated in the periphery more than at the centrosome in T21 and Q21 compared to D21. This trend does not completely hold up with increasing ploidy and may be a result of our inability to separate these MT populations. We also performed SIM microscopy of MTs and quantified the relative distribution of MTs. The relative number of MTs from the centrosome appears to be similar between the samples but pericentrosomal MTs are increased in T21 and Q21 compared to D21s. These results and further discussions are now included in the revised manuscript. (PAGE 11) Importantly, preliminary data using EM tomography (please see the images below) that is beyond the scope of this manuscript also confirms that approximately the same number of MTs reach or emanate from the centrosome. These results will be published in a second manuscript beyond the scope of this one.



These are critical questions that need to be answered before any further conclusions can be made. Especially because there is no live-cell analysis to prove that cargo delivery is indeed perturbed before and during ciliation. Proper centrosome characterization in high resolution will be necessary to reach meaningful conclusions.

We agree, please see above and our response to Reviewer #2.

The zoning delineated in Figure 2B seems random. Per F2B, the PCM zone extends from 0.5 - 1.2 micrometers from centrosome center, which is approximately four times the size of an average interphase centrosome (two times if the authors consider two adjacent centrosomes as one).

This is a good point and we have now corrected this issue. Our dimensions are now based on where we generally observe fluorescence images to extend for the centrosome (0.0-0.5 μm) and the expanded PCM (0.0-1.2 μm). This junction at 1.2 μm was the precise point where Galati et al identified the peak level of difference in PCNT intensity when comparing D21 to T21 cells (Galati et al 2018). The pericentrosomal region is 1.2-2.0 μm. As one can see in the images these regions encompass the expanded centrosome PCM out to 1.2 μm and the pericentrosomal crowd from 1.2 to 2.0 μm. The revised manuscript now explains the rationale for these chosen dimensions. (PAGE 6)

Since the PCM is integral to the centrosome, it is unclear what the authors mean by the "PCM region".

This is a good point and we have now revised our labeling to be centrosome and expanded centrosome. Because we observe differences between these populations when comparing D21, T21 and Q21, we have opted to keep all four populations.

In addition, it appears that pericentrin and PCM1 aggregates are not always localized symmetrically around centrosomes (F2A, F3H, F4A...) and centrosomes can find themselves outside the major "crowd" of pericentrin and PCM1 aggregates. So, quantification of material within concentric regions cannot really work in cells with asymmetrically distributed aggregates.
We agree and this is a source of noise in our analysis system. Nonetheless, we do not believe that this variance changes our conclusions. Alternatively, we would be required to arbitrarily assign unique regions of centrosome, PCM, and PCNT crowding. We now include a discussion of this source of noise in the Results. (PAGE 6)

I am perplexed by the quantification results for Cep131, Cep290 (S4 A, B, and C) and PCM1 (F4A and D) and accompanying figure panels. The authors measure intensities of these proteins around centrosomes or in whole cells, and there are relatively small differences in their levels between D21, T21, and Q21 cells (less than 2-fold). However, the corresponding image panels, which are supposed to illustrate representative phenotypes, show examples where the differences in the levels of these proteins around centrosomes are 10-20-fold. Why are these two sets of data at odds?

Thank you for this important point. We have replaced these images with images that better represent the mean results presented in the quantification.
The correlation between pericentrin aggregates and ciliation is inconsistent.
-In Fig. 2D the quantification of "small" and "large" pericentrin puncta shows almost the same values between T21, and Q21. This is inconsistent with major conclusion and incompatible with differences in ciliation between T21 and Q21.

If we understand correctly, the reviewer is concerned that we do not see a dose dependent increase in large puncta but that there is a chromosome 21 dose dependent effect on ciliation. We do not agree that this is "incompatible". For unciliated cells, the LP:SP ratio increases with chromosome 21 dosage and this is indeed consistent with the decrease in ciliation with elevated chromosome 21 dosage. We believe that this point is clear in the manuscript.

-In FS2B, centrosomal and cytoplasmic pericentrin levels are almost identical in T21 and Q21.

This is true, the total levels are increased in unciliated Q21, relative to T21, relative to D21. The important result here is that the relative amount of T21 and Q21 are elevated in the cytoplasm compared to the D21. We have now made this point stronger in the revised manuscript.

-siRNAPRCNT in T21 and Q21 reduced pericentrin to similar levels, and yet, cilia formation rate remains drastically different. The authors discuss this issue but nevertheless, this inconsistency remains.

Importantly, we are NOT measuring cilia formation rate. We are measuring the total level of ciliation. We believe we have addressed this with the best models possible. The key point is that the genetic rescue does indeed improve ciliation to the levels found in D21 cells.

-Is F4B and C whole cells PCM1 intensity and PCM intensity across zones seems not significantly different between T21 and Q21. This is inconsistent with a significantly different rate of ciliation between T21 and Q21 cells.

F4B indicates that the total amount of PCM1 in the cell is increased similarly in both T21 and Q21 relative to D21. F4B represents total cellular levels and we do not anticipate that this has a major effect on ciliation. However, F4C shows that there is an increase in PCM1 around the centrosome that is chromosome 21 dose dependent. We suggest that this population has a negative effect on ciliation and this is consistent with the level of ciliation. There is a dose dependent decrease in ciliation with increasing levels of PCM1 crowding at the pericentrosomal region.

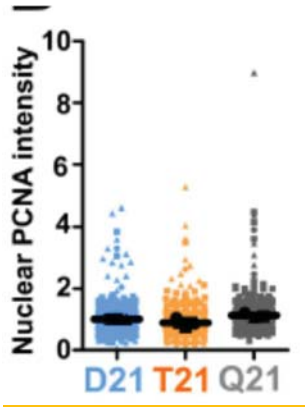
In F2E, the authors indicate that the panel shows "increased brightness". But the panel does not seem to be increased compared to F2A.

As above, "increased brightness" refers to using the identical imaging conditions and scaling to the images in F2A. siRNA reduces total PCNT levels to that comparable to D21 cells in F2A. We now include a description of precisely how much the brightness is increased relative to the unsaturated images.

However, if the brightness were increased, it would reveal numerous pericentrin aggregates in siPCNT Q21, no pericentrin accumulation at the centrosome or pericentrin aggregates in T21, and some in D21. If satellites and pericentrin aggregates are critical to ciliation, how they are entirely absent in D21 in F2B and T21 siPRCNT in F2E? This is not consistent with quantification data, especially in control cells, where standard deviations are minimal.
If we were to increase brightness by scaling with greater brightness compared to F2A then we would indeed observe small aggregates of PCNT. But that is not the point. These are fewer, smaller, and dimmer aggregates in the rescue experiments. We now increase the brightness (while indicating the amount of intensity increased) in a second set of panels to show that such PCNT puncta exist.

Pericentrin levels seem to be always higher in unciliated cells, including control D21 cell, which do not suffer from perturbed pericentrin dosage. It would be important to show that all cell lines arrest in the same cell cycle phase after serum starvation. Or, if that is not the case (In my experience, RPE-1

cells do need to be in G0 to form a cilium), to correlate the cell cycle phase with pericentrin/ciliation. **Our preliminary live cell imaging studies of endogenous PCNT (not shown in this study) suggest that there are variations to PCNT levels upon exit from mitosis and entry into the next cell cycle. Moreover, the variation in PCNT levels of starved D21 cells is greater (%CV: D21=0.59, T21=0.48, and Q21=0.42) than T21 or Q21, suggesting that PCNT levels in this phase of the cell cycle are variable but less so in T21 and Q21. This would suggest that PCNT levels do not strictly reflect the cell cycle state. However, the reviewer's concern that the different levels of ciliation in D21, T21, and Q21 could reflect a cell cycle difference within G0/G1. To test this, we monitored PCNA levels in these cell populations following 24 hours of serum starvation. We find that there are no significant changes to PCNA levels. These results are included below and in Figure S1B (this is the mean of three biological replicates (see three data points (black dots) per condition) of 100 cells per replicate (colored or grey dots)). No statistical difference was observed.**



Reviewer #2 (Remarks to the Author):

Trisomy 21 is the cause of Down syndrome, which includes defects in ciliation in patient cells. In this manuscript McCurdy et al. report on a study of increased pericentrin (PCNT) resulting from increased ploidy of Chromosome 21 and its effect on ciliation. In a nice approach the authors use stable cell lines harboring trisomy or tetrasomy HSA21. This condition mimics that of Down Syndrome and gives a more accurate assessment of the effects of all other genes on HSA21. The disomy, trisomy and tetrasomy relationship allows an extended dose response and, in general, the graded effects of increased HSA21, or rescue by selective knockdown or null mutation of PCNT, increases confidence that the observed centrosome and cilia phenotypes are at least in large part due to increased PCNT levels.

The study initial shows that increased HSA21 results in higher PCNT levels at and around the centrosome along with decreased cilia formation. Overall knockdown, or genetic removal of the extra PCNT copy, recue both these phenotypes. The genetic mutation particularly reveals that the increase in other genes on HSA21, sans PCNT, does not disrupt cilia formation. Increased HSA21 ploidy also results in more and larger PCNT puncta surrounding the centrosome, even more so in unciliated fraction of cells, which is recovered following PCNT reduction. The data also suggest that increased

PCNT puncta around the centrosome are responsible for increased MT density surrounding centrosomes, particularly in unciliated cells. The authors show that a large fraction of puncta are associated with MTs, and based on the hypothesis that MT trafficking is disrupted, they show that transiently removing some MTs allows more cells to form cilia. To further support the idea of trafficking defects they show that centriolar satellite proteins (PCM1, CEP290 and CEP131) are also increased around the centrosomes and colocalized to large extent with the PCNT puncta. This indicates that trafficking of satellites is indeed impaired by elevated PCNT levels. The authors further explore this by testing PCM1 colocalization with PCNT puncta after reducing PCNT or transiently decreasing MT polymer. Indeed, PCM1 level and colocalization near centrosomes is reduced, showing it is dependent on PCNT. While this suggests trafficking of satellites is impaired, this result would be stronger by including CEP290 and/or CEP131, as PCM1 may associate with PCNT independent of trafficking).

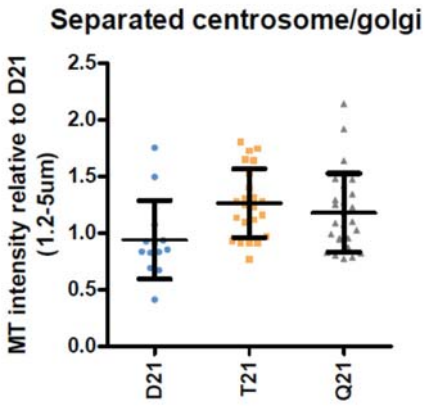
This reviewer had an important insight here and we now include new data showing CEP131 and CEP290 exhibit unique dynamics from PCM1 when exposed to CD and NZ treatment regimes. This suggests that PCM1 which associates with PCNT, is reduced in the pericentrosomal region by CD. CEP131 and CEP290 do not have the same response. We discuss this point in the revised manuscript, favoring the model that all three molecules associate with PCNT puncta and are elevated with HSA21 ploidy, but when PCNT is reduced, PCM1 is more strongly affected in its localization than CEP131 or CEP290. This would further suggest that these molecules can be separated in the satellite localization. In addition, these results suggest that CEP290 further increases in the pericentrosomal region upon MT depolymerization. We thank the reviewer for their insight and these new results are included and discussed in the revised manuscript.

This manuscript addresses an important question that will be interesting to a range of readers. Overall the data presented is high quality and experiments are thorough. The data show evidence of MT nucleation from PCNT puncta and also that centriolar satellites are mislocalized as a result, likely from trafficking defects, providing insight into how elevated PCNT disrupts centriolar trafficking. The use of trisomy and tetrasomy 21 cells also provides a strong method to allow PCNT dosing and control for additional genes in HSA21. The manuscript may leave readers with important mechanistic questions that will need to be elucidate.

We agree and the revised manuscript further elaborates on these important mechanistic questions. (PAGE 11)

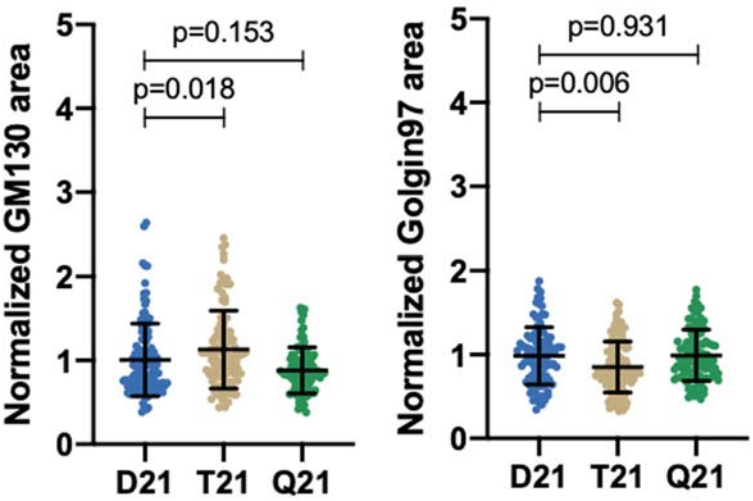
Overall, this manuscript represents an important advance and is suitable for a brief report format.
Issues:
The authors show data that PCNT and MTs are increased near centrosomes, and that there is no real change near the golgi. Since the golgi and centrosomes are often in close proximity outside of mitosis, this seems like a paradox (for example, Sutterlin & Colanzi, JCB. 2010 Mar 8; 188(5): 621-628. doi: 10.1083/jcb.200910001; Tormanen et al., PLos One. 2019; 14(4): e0215215. doi: 10.1371/journal.pone.0215215).

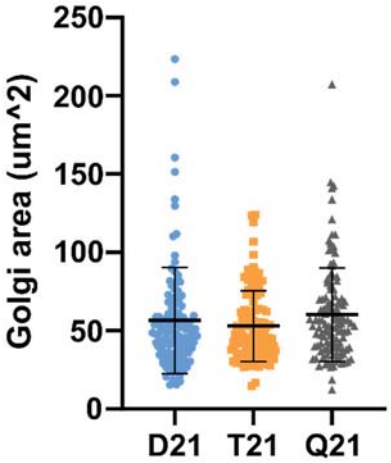
We have revisited this analysis and only measured centrosomal and golgi levels of PCNT and MTs when the centrosome is not overlapping with the golgi apparatus. This allows for accurate measurements of both populations. Importantly, we observe a ploidy dependent increase in MTs associated with the centrosome (see below) but no changes to golgi levels of MTs (if anything there was a reduction). Moreover, our preliminary studies of this using EM tomography found no change in MT nucleation from the golgi apparatus. These results will be presented with other results from the EM tomography and included in a separate manuscript that is now under review.



In the reported study, is the golgi comparatively larger such that the increased MTs around the centrosome are inconsequential with respect to the golgi area?

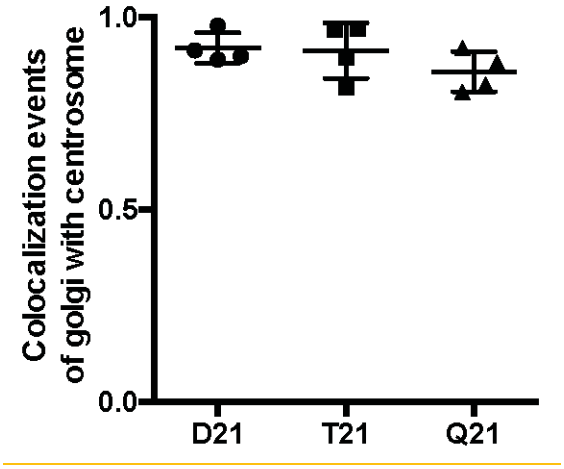
We have measured the golgi size in a second manuscript (https://www.biorxiv.org/content/10.1101/2021.11.10.468107v1). The results are provided below. This lack of a difference in GA size comparing D21, T21, and Q21 is now discussed in the manuscript. Moreover, for each cell analysis of mean MT intensity in this manuscript, we have accounted for the GA area that was measured.





Or is centrosomal - golgi proximity disrupted in serum starvation, or in cells with increased HAS21?

We have tested this question by comparing the frequency of cells with centrosomes separated from the golgi apparatus (not overlapping). While there was a trend of reduced overlap of centrosomes and GA in Q21 cells, we did not observe a significant change (n=4 biological replicates for 30-50 cells per condition per replicate). It is worth noting that there was a large variability between replicates and we are unsure why. To summarize, we do not believe that position of the centrosome relative to the GA has a major impact on ciliation in these studies. We now make this point in the discussion and reference the important work of the Sutterlin lab that the reviewer references above.



This should at minimal be addressed, including proposed or known reasons supporting the outcome. If this is not known, ideally the authors may have so-stained cells that would address this apparent paradox.

To address the reviewer's questions regarding MTs nucleated from the golgi apparatus, we discuss this point and reasons for supporting our model that the MT changes are associated with centrosomal and pericentrosomal MT nucleation and not the golgi apparatus. Further

support for this result was also recently acquired in preliminary EM tomography studies (please see above). We did not observe appreciable differences in the number of MTs emanating from the golgi apparatus. These results are beyond the scope of this manuscript, but this further supports the model that the increase in MTs is from the pericentrosomal and centrosomal regions of the cell.

The authors do not provide any explanation of how they quantified puncta or satellite size even though they report measured sizes well below the resolution of the microscope setup.
The size values provided are a unit area which is not a separation distance reflecting resolution size. A 0.05 μm^2 puncta is the FWHM area that would fill a 3.44 pixel by 3.44 pixel area. Each pixel with our CMOS camera is 0.065 μm in width and therefore 3-4 pixels is approximately the resolution limit of the light microscope (~0.25 μm). Puncta smaller (SP) than this will have a FWHM of fewer than 3.44 pixels while a puncta larger (LP) than this will have more than 3.44 pixels, on average. We now include clear methods for how we quantify all puncta throughout the manuscript

In Fig. 4E, the mean and SD appears to not reflect the distribution of data points. This should be carefully verified and checked that the data shown and indicated statistical tests are accurately reported.

Thank you. We have now revised this calculation.
The authors propose 2 hypotheses, traffic dead-ends or trafficking disruptions, and use CD and NZ treatments as one approach to test this idea. The results show these treatments reduce the size and number of PCNT puncta, and also the number of MTs around the centrosome. Consistent with this, there is also a reduction in MT-associated puncta. At the bottom of the first section on page 9, the authors state, "PCNT nucleated centrosome-free MTs lead to MT-dependent trafficking dead-ends are disrupted with NZ, and these dead-ends contribute, in part, to primary ciliogenesis defects in T21 and Q21 cells. The remaining PCNT puncta likely create trafficking roadblocks that still reduce primary ciliogenesis." This is stated as a solid conclusion. While the evidence presented would be consistent with the hypothesis, it stops short of demonstrating the actual trafficking defects and that they indeed occur by those two proposed mechanisms. This should be toned down and very clearly described as to what the data does and doesn't show. Even if satellite components will be subsequently tested.
We have revised this text to indicate that it supports our model but that additional studies are required to directly show the mechanism. (PAGE 11)

Scale bar sizes appear missing in multiple places (e.g. Fig S2 E and F).
Our versions of Fig S2E and F contain scale bars. All bars were only placed in the bottom right panel for consistent size scaled image panels.

Other concerns:
The manuscript states: "Genetic ablation of a single PCNT allele in T21 cells using CRISPR-Cas9 (leaving cells with 2n PCNT) also increased the mean cilia frequency to 121% of D21 levels (Fig. 1 E; and Fig. S1, E-I)". This percent seems inaccurate. Please confirm that it is 121% and correct the figure if needed. It currently appears more like 102%.

Again, this was an important catch by the reviewer. The correct number is 101% and we have made the appropriate corrections in the text and figure.

Additionally, "Genetic ablation of a single PCNT allele in T21 cells using CRISPR-Cas9 (leaving cells with 2n PCNT) also increased the mean cilia frequency to 121% of D21 levels (Fig. 1 E; and Fig. S1, E-I). There is no Fig S1 I...it stops at H.
This error is now corrected.

In the middle of the upper section on page 9 it states, "We attribute this to the negative effects of PCNT puncta on cilia formation that remain after NZ treatment but are dispersed after CD." This statement seems unclear to a reader. Could the authors expand and/or provide 1 or 2 potential examples?
This is now clarified in the revised manuscript.

In Fig 2g the PCNT signal is not visible in some panels. Perhaps this could be shown as two insets, one with three channels and one with only the PCNT channel?
We now include insets as suggested by the reviewer.

The manuscript states "To test whether the elevated levels of centrosomal and pericentrosomal PCNT increase total cellular MTs, we measured the effects of increased HSA21 dosage on MTs". Technically this tests the effects of excess HSA21, not simply PCNT. Perhaps it could be phrased as "we initially measured the effects of..." or otherwise rephrased.
This is now accurately described in the revised manuscript.

For Fig. S2 E top graph, is the p value for D21 vs T21 also statistically significant?
It is not. We now indicate in the Methods that if a difference is not statistically significant then it is not denoted.

For Fig. S2 legend: "(I) Left, area of PCNT foci along MTs are larger than 0.05 um (indicated with red line) and increase with Hsa21 ploidy. Bottom, area of PCNT foci at MT minus ends are larger than 0.05 um (indicated with red line). Mean{plus minus}
SD. *p < 0.05 (Table 1)". I believe first word in second sentence should say "Right" in place of "Bottom".
This is now corrected.

I believe the figure callout is wrong for this statement, "In T21 and Q21 cells, an increase in gamma-tubulin and PCNT colocalization is observed at free MT ends (Fig. S3 F)".
This is now corrected.

This callout is also wrong, "Importantly, whole cell PCNT and PCNT at and surrounding T21 and Q21centrosomes was reduced (Fig. 3, H and I; and Fig. S3 L)". There is no Fig S3L.
This is now corrected.

In Fig S3 D, presumably the dashed lines represent error but this should be specified clearly in the legend.
This is now corrected.

In the methods, it sounds like the 10min CD treatment went straight to fixation. Was it given 10 min recovery or simply 10 min depolymerization and fix right away? Please clarify.
This is now corrected. Samples were fixed immediately following 10 min depolymerization.

In Fig S4 E, the y-axis legend may be missing a space after "of".
This is now corrected.

RE: Manuscript #E21-10-0517-TR
TITLE: "Trisomy 21 increases microtubules and disrupts centriolar satellite localization"

Dear Dr. Pearson:

Thank you for submitting such a thorough revision of your manuscript. Both reviewers are favorable and I am happy to accept your manuscript as is, but I will leave it up to you whether you want to incorporate any of the suggestions from Reviewer #2.

Please consider these suggestions and then submit your final version.

Thank you for submitting your work to MboC.

Warm regards,
Antonina Roll-Mecak
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Pearson,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript requires minor revisions before it can be published in Molecular Biology of the Cell, as described in the Monitoring Editor's decision letter above and the reviewer comments (if any) below.

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 include a rebuttal letter that details, point-by-point, how the Monitoring Editor's and reviewers' comments have been addressed. (The file type for this letter must be "rebuttal letter"; do not include your response to the Monitoring Editor and reviewers in a "cover letter.") Please bear in mind that your rebuttal letter will be published with your paper if it is accepted, unless you have opted out of publishing the review history.

Authors are allowed 180 days to submit a revision. If this time period is inadequate, please contact us immediately at mboc@ascb.org.

In preparing your revised manuscript, please follow the instruction in the Information for Authors (www.molbiolcell.org/info-for-authors). In particular, to prepare for the possible acceptance of your revised manuscript, submit final, publication-quality figures with your revision as described.

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

Authors of Articles and Brief Communications whose manuscripts have returned for minor revision ("revise only") 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.

Thank you for submitting your manuscript to Molecular Biology of the Cell. Please do not hesitate to contact this office if you have any questions.

Sincerely,

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

Reviewer #1 (Remarks to the Author):

The authors have thoroughly addressed all critical questions and improved/clarified the text. I would be happy to support the publication.

Reviewer #2 (Remarks to the Author):

The authors have done a nice job of addressing the previous reviewers' comments.

- The abstract states, "Cytoplasmic PCNT puncta impact the density, stability and
localization of the MT trafficking network required for primary cilia, as the PCNT puncta sequester cargo peripheral to centrosomes in what we call pericentrosomal crowding." The second half of this sentence seems like an overstatement. More accurately, later in the abstract it is stated "We propose that chromosome 21 aneuploidy disrupts MT-dependent intracellular trafficking required for primary cilia." This later statement seems more accurate given the current level of understanding and I suggest the previous statements be similarly modified.

- I would like to say that the introduction is very well written.

- The text states "Moreover, detyrosinated tubulin was slightly increased in Q21 cells and acetylation and glutamylation remained unchanged under the conditions tested (Fig. S2 H)." I would suggest the word polyglutamylation in place of glutamylation, as detyrosination results in what is called glutamylated tubulin.

- The text states "Thus, PCNT-associated free MTs contribute to primary cilia defects." Perhaps the phrasing "likely contribute to" would be more accurate.

This reviewer finds the following logic/statements to be unclear: "First, PCNT puncta localized along MTs act as trafficking roadblocks. In this model, enlarged PCNT puncta either sterically block the movement of other cargoes or bind and sequester cargos as they encounter PCNT puncta." The next statement is abrupt and seems to unequivocally discount the previous statement. "Cargo can translocate around objects on MTs (Can et al., 2014; Conway et al., 2012)." So then, roadblocks are out of the question or not? Perhaps elaborate more. "However, our prior work suggests that the trafficking of enlarged PCNT puncta is reduced along MTs (Galati et al., 2018), and PCNT-associated cargos no longer traffic efficiently." It would help new readers if the authors could more clearly delineate the two proposed models and the evidence for/against each.

The text states "For Q21 cells, this could suggest an already achieved saturation of enlarged PCNT puncta along MTs, limiting space along MTs for redistribution of puncta affected by NZ (Fig. 4 L)." Based on the images of enlarged PCNT, at least in the periphery areas, it is unclear why one would consider total MT binding along lattices to be saturated and/or limited. This concept would benefit from explanation.

RE: Manuscript #E21-10-0517-TRR
TITLE: "Trisomy 21 increases microtubules and disrupts centriolar satellite localization"

Dear Dr. Pearson:

Thank you for submitting this work.I am pleased to accept your manuscript for publication in Molecular Biology of the Cell. I hope that you found the review process constructive and fair. I also encourage you to take advantage of the video abstract feature we have. It can be a fun project for your trainees and makes the article more accessible. And we can all use some fun, team projects after the last 2 years!

Sincerely,
Antonina Roll-Mecak
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Pearson:

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.

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
