JCB

WDR60-mediated dynein-2 loading into cilia powers retrograde IFT and transition zone crossing

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December 11, 2020

Re: JCB manuscript #202010178

Dr. Tiago J. Dantas
Institute for Research and Innovation in Health (i3S)
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Dear Dr. Dantas,

Thank you for submitting your manuscript entitled "WDR60 regulates dynein-2 at multiple stages during retrograde intraflagellar transport." The manuscript has been evaluated by expert reviewers, whose reports are appended below.

You will see that while the reviewers find the premise of your study intriguing they also feel the data presented does not provide sufficient evidence to support the conclusion that transition zone Y-links impede exit of retrograde trains. Unfortunately, after an assessment of these reviews, our editorial decision is against publication in JCB.

Given interest in the topic, we would be open to a re-evaluation of a substantially revised version of the study, but we believe that this would entail a significant amount of additional experimental work. We believe the strategy suggested by reviewer #1 is reasonable and may bring the study to the mechanistic level expected of a JCB paper.

If you would be interested in this possibility, we ask that you submit a revision plan that includes a point-by-point response to all of the reviewer comments, and how you would address them.

If you would like to resubmit this work to JCB, please contact the journal office to discuss an appeal of this decision or you may submit an appeal directly through our manuscript submission system. Please note that priority and novelty would be reassessed at resubmission.

Alternatively, if you wish to expedite publication of the current data you may prefer to consider another journal for this work. Our journal office can transfer your reviewer comments to another journal upon request.

Regardless of how you choose to proceed, we hope that the comments below will prove constructive as your work progresses. We would be happy to discuss the reviewer comments further once you've had a chance to consider the points raised in this letter. You can contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

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

Sincerely,

Maureen Barr, Ph.D.
Monitoring Editor
Journal of Cell Biology

Dan Simon, Ph.D.
Scientific Editor
Journal of Cell Biology

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

This work focuses on the IFT role of the WDR60 intermediate chain of IFT-dynein. Several studies have linked WDR60 to ciliogenesis, dynein-2 function and retrograde IFT, with complete loss of WDR60 in RPE1 cells causing increased ciliary levels of IFT20/40/43/88/57/140 (Hamada 2018; Tsurumi 2018; Vuolo 2019), and depletion of WDR60 in Planaria causing ciliary accumulation of electron dense material presumed to be IFT particles (Patel-King et al 2013). To probe more deeply the IFT role of WDR60 in cilia, the new work performed experiments in C. elegans. The authors formally demonstrate that worm WDR-60 undergoes IFT, and report that WDR60's ciliary targeting and IFT behaviour is not strictly dependent on its B-propellor domain that is known to mediate dynein-2 heavy chain (DHC) binding. Examination of knockout wdr-60 worms showed normal cilia lengths. Using GFP::DHC (knock-in), IFT70/140 and kinesin-11 (KAP1) reporters, wdr-60 worms were found to display ciliary accumulations of all reporters, especially in the middle segment region immediately distal to the transition zone (TZ); they also found that the mutants have reduced frequency and velocity of the reporter-marked retrograde IFT trains. Finally, the work shows that the DHC2 ciliary accumulations in wdr-60 worms depend on two genes involved in TZ Y-link assembly, mksr-2 and nphp-4). The authors interpret this observation to mean that Y-links normally form a block to the ciliary exit of retrograde IFT trains, thus providing one explanation for why the disrupted retrograde IFT trains in wdr-60 mutants back up just before the TZ.
Overall, I found the work to be of high quality. The data appears robust, and most observations are quantified. The data in Figures 1-4 goes a significant distance beyond what has been done before for WDR60, providing a more in-depth knowledge of how retrograde IFT is affect by loss of this dynein-2 intermediate chain. The data in Figures 5 and 6 is arguably the most intriguing, as data supporting the notion that Y-links form a block to retrograde IFT train exit has not, to my knowledge, been reported. However, I have some major comments about this interpretation (see below).

Major comments:

1.The very important conclusion that Y-links prevent the ciliary exit of disrupted retrograde IFT trains in wdr-60 mutants is premature and based on only one correlative experimental observation. Here are some suggestions to consider: (i) The authors really should examine the retrograde IFT accumulation phenotypes in other TZ mutants such as the nphp-4 single mutant as a control for the data in Figure 6. Plus, Y-links are reported to be reduced in nphp-4 single mutants (Lambacher 2016) and it would be interesting therefore to know if that could partially reduce the wdr-60 retrograde IFT defect. Also, since loss of mksr-2 + nphp-4 causes loss of Y-links AND probably also the more proximal transition fibers, it would be very instructive to look at the wdr-60 IFT phenotype in cep-290 background where just the Y-links are affected. (ii) It is possible that the effects they see occur independently of the Y-link model, linked to other functions of mksr-2 and nphp-4. For

example, could these gene regulate the turnover rate of retrograde IFT proteins? Authors could use Western blotting (as in Fig. 2D) to assess this. (iii) Does the retrograde IFT localisation phenotype get progressively worse as wdr-60 mutant worms age (eg. L1 to adult)? Such an observation might help support the proposed model. (iv) Look at the retrograde IFT localisation phenotype in a mutant of another component of IFT-dynein such as XBX-1. Schafer et al 2003 shows ciliary accumulations of IFT proteins that appear somewhat similar, if not more dramatic, to that of wdr-60 mutants. Can those accumulations be cleared by TZ gene loss.

2.Prevo 2015, Oswald 2018 and Jensen 2015 all show that retrograde trains slow down dramatically when they enter the TZ. Furthermore, the Prevo and Jensen papers show that disruption of TZ genes (mksr-1, mks-5) causes IFT trains to increase their speeds through the TZ. From these observations, the authors of those papers conclude that there are roadblocks to IFT trains in the TZ. These important observations must be discussed considering the present paper's conclusions and model.

Minor comments:

1.The second last line of the abstract is not yet fully supported by the data. As it stands, should read ....rescued by disrupting the transition zone genes, mksr-2 and nphp-4.

2. First paragraph of introduction: 'In' the opposition direction, ...

3.First results section; last line of 2nd paragraph - 'Most WDR60 is recruited cilia...'. This does not agree with the images in Fig 1A where you see lots of signal outside cilia in other compartments of the sensory neurons.

4.Page 6; end of 2nd paragraph "... did not significantly affect the ciliary recruitment of WDR-60::GFP..." The data for xbx-1 mutant seems to indicate a loss of WDR-60 signal at the ciliary base.

5.How exactly was the data in Fig. 3G determined, given that the representative kymographs in Fig 3D seem to show that the individual retrograde trains in the mutants seem brighter in the more proximal and distal ciliary regions, compared to more central regions ?

6.The authors repeatedly say that it was 'unexpected' that WRD-60 could have a role in facilitating passage of retrograde IFT trains through the TZ. Why so 'unexpected' ?

7.Authors should probably say something in the discussion about the emerging role of the BBSome cargo adapter in facilitating the crossing of ciliary membrane proteins out of the cilium, across the TZ.

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

WDR60 is a subunit of the IFT dynein/dynein2. Variants in this gene cause short rib polydactyl in human patients. This manuscript reports that mutants in WDR60 in C elegans affects retrograde IFT. Loss results in dynein-2 subunits strongly accumulate near the base of cilia and were reduced along the ciliary axoneme by using tagged locus at the endogenous loci. No change in two transition zone proteins were found in the wdr-60 mutants.

Comments

Previous work in patients and cell lines have suggested that WDR60 affects retrograde IFT and the transition zone (CEP290). This manuscript confirms the first finding, but not the second one. They found that both the number of retrograde particles and the speed were reduced. Unlike the light intermediate chain or the heavy chain mutants, MRKS2 and NPHP4 were not affected. These are not the same proteins as studied in the human patients (RPGRIP1L, TCTN, TMEM67).

They propose some interesting ideas for the role of WDR60 in the discussion, but none of these mechanistic ideas are pursued.

Page1, Line 3 indicates that proteins are descended from a common ancestor. You should think of it as "quantal". Proteins are either orthologs (descended from a common ancestor) or paralogs that arise from a duplication in a particular organism. Thus, one cannot have high homology. I think you mean that the two proteins have high similarity. Protein are either homologous or not homologous.

Page, 1, Line 6. Alike genes encoding for other ... I think the author mean Like not alike

Page 1, Line 10 locus should not be in italics

Page 3 The authors state that structural work suggested that WDR60/WDR34 restrict dynein activity to the tip. I think they mean to say that they may restrict the activation of dynein to the tip as it must be active during retrograde movement.

Is mksr-2 cep290? It would be useful to include the human names of genes.

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

Though the function of WDR60 has been addressed in several studies in mammalian cells regarding ciliary assembly and IFT, its molecular function in IFT is still not clear. To this end, this work addressed this issue in worm by using WDR60 mutants combined with live imaging. The major conclusions that fascinates me are that WDR60 does not affect ciliogenesis but regulate initiation of retrograde IFT and exit of IFT dynein through the transition zone.

However, I found that the evidences presented do not back up those conclusions.
1) Ciliogenesis. As shown in Fig 3B, the length of cilia in the null mutant WDR60 is significantly shorter, which is consistent with the data shown in mammalian cells with WDR60 knockout (Vuolo et al 2018). Thus, WDR60 does affect ciliogenesis though mildly.

2) Initiation of retrograde IFT. Accumulation of IFT dynein in the cilia of the WDR60 mutants is interpreted as defects in the initiation of retrograde IFT. The initiation of retrograde IFT occurs at the ciliary tip. The observed phenotype should be simply due to defects in retrograde IFT, which is expected. If the initiation of retrograde IFT is defective, one should see accumulation of dynein at the ciliary tip, which is not (see Fig 3). Similarly, IFT-A and IFT-B components as shown in Figure 4 also do not accumulate at the ciliary tip. In addition, these data are contradictory to the finding in mammalian cells where both IFT-A and IFT-B components accumulate at the ciliary tip (Vuolo et al 2018). Though the function of WDR60 in different organism may function differently, the data

shown in this paper do not support the author's claim that WDR60 regulates the initiation of retrograde IFT.

3) Exit of IFT dynein. It was shown that IFT dynein accumulates at the TZ area in the WDR60 mutants and disruption of TZ facilitates the return of dynein to the cell body. It was then concluded that WDR60 regulates passage of dynein and/or IFT trains through TZ. However, for the LIC3 mutant, there is also accumulation of dynein at the TZ area (Fig 2B), indicating that IFT dynein also can not pass the TZ in this mutant. Thus, it appears that the integrity of the dynein complex but not WDR60 itself is required for the passage of dynein through the TZ. Secondly, if WDR60 specifically regulates the passage of dynein through the TZ, no possible mechanism is shown.

Minor points
1. P5 "While some signal is detected in the soma and dendrites of these neurons, most WDR-60 is recruited to cilia." From what I see in the images, most signals are in the soma or dendrites.

2. P6. "As endogenous labelling of wdr-60," wrd-60 should be WDR-60? Because the protein is labelled but not the gene.

3. FigS2A, why does the WDR60-FLA-GFP strain have extremely more dye than the control? This is unexpected for a correct knock-in strain.

4. Fig 3A. Dynein in worm is enriched at the TZ region (there is no basal body), which should be around 1 um (see Doroquez et al., 2014 eLife; Snow et al., 2004). If one estimates the length of TZ by dynein fluorescence, it is close to 1 um. Thus I think the second high intensity in the mutants is outside TZ. But in Fig3C, half of the intensity is in the TZ.

JCB manuscript #202010178

Point-by-point Response to all of the Reviewer Comments:

REVIEWER #1

This work focuses on the IFT role of the WDR60 intermediate chain of IFT-dynein. Several studies have linked WDR60 to ciliogenesis, dynein-2 function and retrograde IFT, with complete loss of WDR60 in RPE1 cells causing increased ciliary levels of IFT20/40/43/88/57/140 (Hamada 2018; Tsurumi 2018; Vuolo 2019), and depletion of WDR60 in Planaria causing ciliary accumulation of electron dense material presumed to be IFT particles (Patel-King et al 2013). To probe more deeply the IFT role of WDR60 in cilia, the new work performed experiments in C. elegans. The authors formally demonstrate that worm WDR-60 undergoes IFT, and report that WDR60's ciliary targeting and IFT behaviour is not strictly dependent on its B-propellor domain that is known to mediate dynein-2 heavy chain (DHC) binding. Examination of knockout wdr-60 worms showed normal cilia lengths. Using GFP:HC (knock-in), IFT70/140 and kinesin-11 (KAP1) reporters, wdr-60 worms were found to display ciliary accumulations of all reporters, especially in the middle segment region immediately distal to the transition zone (TZ); they also found that the mutants have reduced frequency and velocity of the reporter-marked retrograde IFT trains. Finally, the work shows that the DHC2 ciliary accumulations in wdr-60 worms depend on two genes involved in TZ Y-link assembly, mksr-2 and nphp-4). The authors interpret this observation to mean that Y-links normally form a block to the ciliary exit of retrograde IFT trains, thus providing one explanation for why the disrupted retrograde IFT trains in wdr-60 mutants back up just before the TZ.

Overall, I found the work to be of high quality. The data appears robust, and most observations are quantified. The data in Figures 1-4 goes a significant distance beyond what has been done before for WDR60, providing a more in-depth knowledge of how retrograde IFT is affect by loss of this dynein-2 intermediate chain. The data in Figures 5 and 6 is arguably the most intriguing, as data supporting the notion that Y-links form a block to retrograde IFT train exit has not, to my knowledge, been reported. However, I have some major comments about this interpretation (see below).

RE: We thank the reviewer for seeing the value of our manuscript and for the time spent reviewing it. We are also grateful for the comments and suggestions, which we tried to address as much as possible.

Major comments:

1.The very important conclusion that Y-links prevent the ciliary exit of disrupted retrograde IFT trains in wdr-60 mutants is premature and based on only one correlative experimental observation. Here are some suggestions to consider: (i) The authors really should examine the retrograde IFT accumulation phenotypes in other TZ mutants such as the nphp-4 single mutant as a control for the data in Figure 6. Plus, Y-links are reported to be reduced in nphp-4 single mutants (Lambacher 2016) and it would be interesting therefore to know if that could partially reduce the wdr-60 retrograde IFT defect.

RE: As suggested by the reviewer, we have analyzed the distribution of the dynein-2 heavy chain (GFP::CHE-3) in the context of a *wdr-60*;*nphp-4* double mutant. We found that disruption of *nphp-4* strongly ameliorates the accumulation of dynein-2 at the distal side of the TZ, providing an almost complete rescue. Due to this remarkable result, we also tested whether NPHP-4 loss improved the

retrograde kinetics of WDR-60-deficient dynein-2 crossing the TZ, which it did, in a manner comparable to the loss of MKS-5 (which we now also include for comparison). These new data advocate that the passage of dynein-2-driven IFT trains through the TZ is mainly restricted by the NPHP module, and they are now included in our new Figures 7 and 9. We also toned down the direct involvement of the Y-links in restricting WDR-60-deficient dynein-2 complex passage through the TZ, given that the exact contribution of each TZ component to the functionality of Y-links is still not fully understood.

Also, since loss of *mksr-2 + nphp-4* causes loss of Y-links AND probably also the more proximal transition fibers, it would be very instructive to look at the wdr-60 IFT phenotype in cep-290 background where just the Y-links are affected.

RE: As suggested, we also tested whether the disruption of *cep-290* is able to reduce the IFT particle accumulation phenotype associated with the loss of WDR-60. In contrast to the other mutations we tested (*mks-5*, *nphp-4*, or *mksr-2;nphp-4*), disruption of *cep-290* does not reduce the accumulation of WDR-60-deficient dynein-2 at the distal side of the TZ. As CEP-290 impairment does not affect the NPHP module (Li et al., 2016; Schouteden et al., 2015), this result further supports that the NPHP module acts as a roadblock for dynein-2 exit from cilia through the TZ. This new data has been added to the manuscript in Figure S5.

(ii) It is possible that the effects they see occur independently of the Y-link model, linked to other functions of mksr-2 and nphp-4. For example, could these gene regulate the turnover rate of retrograde IFT proteins? Authors could use Western blotting (as in Fig. 2D) to assess this.

RE: We thank the reviewer for the suggestion. Unfortunately, we were unable to detect GFP::CHE-3 (>500kDa and expressed only in sensory neurons) as reliably as we detected WDR-60::3xFLAG::GFP. However, we also note that western blots only allow us to look at total protein levels rather than just the ciliary population. Therefore, as an alternative approach to address this concern, we decided to quantify the total GFP::CHE-3 signal ranging from the ciliary base to cilia tip, which has become a good additional readout for our experiments, as it allowed us to uncover that WDR-60 also contributes to the robust recruitment of dynein-2 to cilia (Figures 3C, S3I and S5C,D). We also found that the double *mksr-2;nphp-4* background has the strongest reduction in total levels in both the presence and absence of WDR-60. This is likely because cilia are much shorter in the double mutant background (Figure 7K and Schouteden et al. 2015) and that the ciliary membrane becomes completely detached from the proximal end of the axoneme when mutants of both MKS and NPHP modules are combined (Williams et al., 2011). Given that disruption of MKS-5/RPGRIP1L prevents the assembly of all TZ modules (Jensen et al., 2015; Li et al., 2016) without having such a severe effect on cilium length or on the total levels of ciliary GFP::CHE-3 (Figure 7K and S5C,D), we tested whether the disruption of *mks-5* also facilitates WDR-60-deficient dynein-2 exit from cilia. We obtained an almost complete rescue that is comparable with that of the double *mksr-2;nphp-4* background, which we now include in the manuscript (Figures 7 and 9).

(iii) Does the retrograde IFT localisation phenotype get progressively worse as wdr-60 mutant worms age (eg. L1 to adult)? Such an observation might help support the proposed model.

RE: We thank the reviewer for the nice suggestion. We have repeated our analysis of GFP::CHE-3 distribution in worms at larval stage 2 (L2), at the young adult stage, and at 7 and 18 days post-adulthood to study the progression of the phenotypes associated with loss of WDR-60 (as done in Cornils et al., 2016). We found that, at as early as the L2, the ciliary levels of GFP::CHE-3 were already reduced and

its distribution altered, albeit less than in young adults. This result suggests that these phenotypes arise early on, but get progressively worse as the animals develop (Figure S3). We also found that CHE-3 recruitment and incorporation into cilia continue to increase into adulthood in control worms, while in the *wdr-60(null)* mutants the amount of ciliary CHE-3 only marginally increases. In addition, we found that the abnormal distribution of GFP::CHE-3 does not significantly change with age in *wdr60(null)* animals (7 and 18 days post-adulthood), suggesting that there is no age-dependent suppression of these WDR-60-associated phenotypes, contrasting to what has been observed for some IFT mutants (Cornils et al., 2016). All of this new data is now included in Figure S3.

(iv) Look at the retrograde IFT localisation phenotype in a mutant of another component of IFT-dynein such as XBX-1. Schafer et al 2003 shows ciliary accumulations of IFT proteins that appear somewhat similar, if not more dramatic, to that of wdr-60 mutants. Can those accumulations be cleared by TZ gene loss.

RE: Like the reviewer, we also wondered whether an even stronger reduction in retrograde IFT (with more severe accumulations) could also be rescued by the removal of specific TZ modules or by the complete disruption of the TZ. We liked the suggestion of the reviewer to attempt this experiment with the dynein-2 light intermediate chain (LIC) mutant, *xbx-1(null)*. However, we point out that in these worms the dynein-2 heavy chain CHE-3 (the core subunit of the motor) is not recruited or incorporated into cilia (Yi et al., 2017; our Figure 3A). As an equivalent alternative, we chose to use a strain carrying a specific mutation in dynein-2 HC CHE-3 (K2935Q; Yi et al., 2017) that renders it completely defective for retrograde IFT without preventing its ciliary recruitment. Therefore, this mutant displays a large accumulation of CHE-3 (K2935Q) inside severely shortened cilia (our Figure 8; Yi et al., 2017). Interestingly, combining this CHE-3 mutant with *mks-5*, *nphp-4* or *mksr-2*;*nphp-4* double mutant backgrounds did not decrease dynein-2 accumulation inside cilia. These experiments revealed that even the complete removal of the TZ barrier cannot compensate for the total loss of dynein-2 motility. This important finding also implies that the fewer dynein-2 motors powering retrograde trains in the absence of WDR-60 make an important contribution for the rescue observed upon the disruption of the TZ barrier.

2.Prevo 2015, Oswald 2018 and Jensen 2015 all show that retrograde trains slow down dramatically when they enter the TZ. Furthermore, the Prevo and Jensen papers show that disruption of TZ genes (mksr-1, mks-5) causes IFT trains to increase their speeds through the TZ. From these observations, the authors of those papers conclude that there are roadblocks to IFT trains in the TZ. These important observations must be discussed considering the present paper's conclusions and model.

RE: We thank the reviewer for pointing this out. We now discuss how these earlier studies already hinted that the TZ barrier offers resistance to the passage of IFT trains. The notion that the TZ constitutes a strong gating roadblock is consistent with our observations that the less efficient retrograde trains of *wdr-60* mutants accumulate particularly at the distal side of the TZ.

We also note that by quantifying the average signal of CHE-3 on IFT tracks during our revision experiments, we also found that loss of WDR-60 substantially reduces the amount of dynein-2 loaded onto anterograde trains and, consequently, the availability of dynein-2 motors to drive retrograde trains (Figure 3G). In turn, the reduction in dynein-2 motors powering retrograde trains most likely accounts for both the slower retrograde velocities (Figure 3H), and the tendency of trains to accumulate when they encounter the TZ (Figure 6B,C), as they are less likely to generate enough force to overcome the resistance offered by this barrier. Consistent with this model, and with what was shown by Jensen 2015, we found that removal of MKS-5 substantially increased the velocity of retrograde IFT trains crossing

the TZ in both the presence and absence of WDR-60 (new Figure 9A,C,D). In addition, we found that removal of NPHP-4 also increases the velocities of IFT trains crossing the TZ, particularly in *wdr-60(null)* cilia. These new findings indicate that even though NPHP-4 loss does not impair the TZ to the same extent of MKS-5 disruption, it considerably reduces the resistance offered by the TZ barrier to the underpowered retrograde IFT trains in *wdr-60* mutants. This is also consistent with the almost complete rescue of dynein-2 accumulation at the distal side of the TZ that we observed upon the disruption of NPHP-4 in the *wdr-60*(*null)* background (point 1(i) from the reviewer; new Figure 7G,H).

Minor comments:

1.The second last line of the abstract is not yet fully supported by the data. As it stands, should read ....rescued by disrupting the transition zone genes, mksr-2 and nphp-4.

RE: We agree with the reviewer and we have changed the text also taking into consideration the additional results that we obtained from the revision experiments. The revised text now states that the NPHP module is a key modulator of dynein-2 passage through the TZ, given that disrupting NPHP-4, and consequently, the NPHP module (Blacque and Sanders, 2014; Winkelbauer et al., 2005), almost completely rescues the accumulation of dynein-2 at the distal side of the TZ in the *wdr-60(null)* background and increases dynein-2 velocity across this region.

2. First paragraph of introduction: 'In' the opposition direction, ...

RE: We thank the reviewer for noticing the misspelling and we have corrected it.

3.First results section; last line of 2nd paragraph - 'Most WDR60 is recruited cilia...'. This does not agree with the images in Fig 1A where you see lots of signal outside cilia in other compartments of the sensory neurons.

RE: We agree with the reviewer, and we rephrased the text to reflect that: *"While a large part of the signal is detected in the soma and dendrites of these neurons, WDR-60 is also found inside cilia…"*

4.Page 6; end of 2nd paragraph "... did not significantly affect the ciliary recruitment of WDR-60::GFP..." The data for xbx-1 mutant seems to indicate a loss of WDR-60 signal at the ciliary base.

RE: Loss of the dynein-2 LIC (XBX-1) abolishes dynein-2 HC (GFP::CHE-3) recruitment to cilia (Yi et al., 2017), completely blocking retrograde IFT. While we agree that the WDR-60 signal is indeed very reduced at the base in the *xbx-1(null)* mutant (Figure 2), we believe that this occurs because WDR-60 is not able to exit cilia without retrograde IFT, thus, strongly accumulating inside cilia. In fact, a comparable effect can be observed for CHE-11 and IFT-74 in the *xbx-1(null)* background (now added to Figure 4A,D). Therefore, the results in Figure 2B,C suggest that the recruitment of WDR-60 and its ability to enter cilia remain mostly unaffected by the loss of the dynein-2 LIC. We have nevertheless clarified this in the text of the manuscript: *"Strikingly, the loss of dynein-2 LIC, which is known to destabilize dynein-2 HC (Taylor et al., 2015; Yi et al., 2017), did not prevent the ciliary recruitment of WDR-60::GFP or WDR-60(ΔCT)::GFP (Figure 2B,C). However, it did lead to the accumulation of both forms of WDR-60 inside cilia, likely due to the complete block of retrograde IFT that occurs in the xbx-1 deletion mutant (Schafer et al., 2003; Yi et al., 2017)."* We have also added yellow arrowheads to better label the ciliary base in Figure 2B.

5.How exactly was the data in Fig. 3G determined, given that the representative kymographs in Fig 3D seem to show that the individual retrograde trains in the mutants seem brighter in the more proximal and distal ciliary regions, compared to more central regions?

RE: The average signal intensities of anterogradely or retrogradely moving GFP::CHE-3 particles (Figure 3G) were determined using KymographDirect as in (Mijalkovic et al., 2017). We replaced the GFP::CHE-3 kymographs on Figure 3E for more representative examples with the same acquisition settings. Nevertheless, we agree with the reviewer that the pixel intensities could have some variation along particular IFT tracks in the kymographs. However, we were careful to only analyze tracks that were clearly visible, and we averaged the intensity of all pixels composing each track in order to minimize potential variations. Furthermore, for each GFP::CHE-3 intensity point plotted in Figure 3G, a minimum of 15 tracks from each cilium were analyzed for anterograde or retrograde IFT, and averaged. Then, at least 15 cilia were used to determine the overall GFP::CHE-3 particle intensity undergoing IFT in control and *wdr-60* mutant strains. For clarity, we have added this information to the methods of the manuscript.

We initially analyzed this data as ratios of retrograde/anterograde IFT intensities of each cilium because it better accounted for variations in intensities between data from different cilia. However, we realized that these were less informative than separately plotting the averages of GFP::CHE-3 particle intensities on tracks for each direction (new Figure 3G), as they allow us to directly compare the amount of dynein-2 loaded onto anterograde trains between controls and *wdr-60* mutants. Conversely, this analysis also allowed us to compare the amount of GFP::CHE-3 driving retrograde particles in our strains, and uncover that less dynein-2 motors power retrograde IFT in the absence of WDR-60.

6.The authors repeatedly say that it was 'unexpected' that WRD-60 could have a role in facilitating passage of retrograde IFT trains through the TZ. Why so 'unexpected' ?

RE: We use the term “unexpected” because when we initiated this study we did not anticipate that dynein-2 would particularly accumulate at the distal side of the TZ in *wdr-60* mutants. However, we now have evidence that WDR-60 contributes to efficient transition zone crossing by ensuring the robust loading of dynein-2 into cilia and its availability to power retrograde IFT trains. Therefore, we now removed the term "unexpected" from the text.

7.Authors should probably say something in the discussion about the emerging role of the BBSome cargo adapter in facilitating the crossing of ciliary membrane proteins out of the cilium, across the TZ.

RE: As suggested, we have incorporated into our discussion a paragraph and a revision contemplating the recent literature on transition zone crossing, including the importance of the BBSome in mediating ciliary exit of membrane proteins: *“Emerging evidence points to an important interplay between IFT-A and the BBsome in regulating the traffic of G protein-coupled receptors in and out of cilia across the TZ, in part by coupling the receptors to IFT trains (reviewed in (Nachury and Mick, 2019)).”*

REVIEWER #2

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Loss results in dynein-2 subunits strongly accumulate near the base of cilia and were reduced along the ciliary axoneme by using tagged locus at the endogenous loci. No change in two transition zone proteins were found in the wdr-60 mutants.

Comments

Previous work in patients and cell lines have suggested that WDR60 affects retrograde IFT and the transition zone (CEP290). This manuscript confirms the first finding, but not the second one. They found that both the number of retrograde particles and the speed were reduced. Unlike the light intermediate chain or the heavy chain mutants, MRKS2 and NPHP4 were not affected. These are not the same proteins as studied in the human patients (RPGRIP1L, TCTN, TMEM67).

RE: We would like to first clarify that, in the prior version of our manuscript, we analyzed the recruitment and distribution of MKS-6 (CC2D2A) and TMEM-107 in our mutants, not of MKSR-2 (B9D2) or NPHP-4. Instead, co-disruption of *mksr-2* and *nphp-4* was used to test whether the disruption of the TZ would relieve the accumulation of CHE-3 (dynein-2 heavy chain) inside cilia of *wdr-60* mutants, which it did, as shown in the previous Figure 6 (new Figure 7). This and other results obtained with several TZ mutants clearly provide evidence that the TZ offers resistance to WDR-60-deficient dynein-2 exit from cilia.

To address the concern of the reviewer and provide further evidence of the integrity of the TZ in *wdr-60* mutants, we have now analyzed the localization of two additional TZ components, MKSR-1::tdTomato (B9D1) and GFP::NPHP-4, which we found to also be unaffected in *wdr-60* mutants, but dispersed in the *xbx-1* mutant (shown in new Figure 5). We point out that although we did not analyze the TZ localization of MKS-5 (the *C. elegans* homolog of RPGRIP1L), all four TZ components that we examined rely on this TZ element for their correct localization (Lambacher et al., 2016; Williams et al., 2011). Importantly, we have also analyzed the ability of the TZ to block the entry of RPI-2 (human RP2), a component of the periciliary membrane compartment that is unable to enter cilia when the TZ gating function is intact (Jensen et al., 2018). While loss of XBX-1 resulted in the abnormal entry of RPI-2 into cilia, no RPI-2 signal was detectable inside cilia of *wdr-60* mutants (now included in new Figure 5C,D), attesting that the TZ gating function is maintained in the latter. Together these results strongly advocate for the integrity and functionality of the TZ barrier in the absence of WDR-60 in *C. elegans*. We are quite confident in our analyses given that we readily detected the TZ defects caused by the loss of the dynein-2 LIC XBX-1, which were very similar to what was recently reported for mutations in the dynein-2 HC CHE-3 and other severe retrograde IFT mutants using the same animal model (Jensen et al., 2018; Scheidel and Blacque, 2018). Thus, we think that, in the same way that WDR-60 loss does not preclude axoneme extension (in contrast to *xbx-1*), it is also not severe enough to cause the defects in the localization of TZ components seen in other retrograde IFT mutants (Jensen et al., 2018; Scheidel and Blacque, 2018).

Moreover, as we also discuss in the manuscript, it is conceivable that the differences encountered between the two model systems in which WDR60 has been disrupted might reflect the variations in TZ structure that exist between different types of cells and cilia (Akella et al., 2019; Jana et al., 2018).

They propose some interesting ideas for the role of WDR60 in the discussion, but none of these mechanistic ideas are pursued.

RE: To our knowledge, our manuscript is the first to show kinetics of WDR60 undergoing IFT (in any organism) and to also directly analyze the live dynamics of the endogenous dynein-2 motor subunit in the absence of WDR-60. This clean approach allowed us to uncover that: **(I)** WDR-60 plays a critical

role in both dynein-2 recruitment and in its loading onto anterograde IFT trains for incorporation into cilia (Figure 3C,G); **(II)** WDR-60 is consequently required for enough dynein-2 to become available at the ciliary tip to power normal kinetics of retrograde IFT; (**III**) In the absence of WDR-60, the underpowered retrograde trains (with approximately half of the normal amount of dynein-2 motors; Figure 3G), accumulate at the distal side of TZ (Figure 6), most likely because they are unable to generate enough force to pass through this dense barrier; **(IV)** Complete disruption of the TZ, or just of the NPHP module, substantially rescues dynein-2 accumulation in *wdr-60(null)* cilia (Figure 7), and increases the velocity of retrograde IFT trains crossing the TZ region (Figure 9), further supporting the notion that in the absence of WDR-60, retrograde trains are unable to push their way through the TZ; **(V)** Dynein-2-mediated motility is required for IFT trains to exit cilia, even when no TZ barrier is present (Figure 8).

In addition to these mechanistic insights into how WDR60 contributes to dynein-2 function in retrograde IFT, we also report the following important findings: **(i)** The WDR-60(ΔCT) can be recruited to cilia but the CT β-propeller of WDR-60 nevertheless contributes to its efficient ciliary recruitment (Figure 2); **(ii)** WDR-60 requires retrograde IFT to exit cilia, otherwise it ends up completely accumulated inside cilia **(iii)** The impaired retrograde IFT of WDR-60-deficient cilia is nonetheless sufficient for almost complete axoneme extension (90-95%; Figures 3B and 4B,E), and for partially supporting cilia-mediated sensory functions (Figure 4J,K); **(iv)** Loss of WDR-60 results in increased accumulations of IFT components inside cilia (Figure 4A,C,D,F); **(v)** The dynein-2 LIC (XBX-1) is critical for the integrity and gating capacity of the TZ, while WDR-60 is dispensable for these functions in *C. elegans* (Figure 5);

We believe that the findings in our study greatly improve the understanding of the interplay between WDR-60, retrograde IFT, and ciliary export, which will have an important impact in the field. Finally, we also underline that, to our knowledge, our manuscript is also the first to rescue a ciliary dynein-2 accumulation by targeting the TZ (Figure 7).

Page1, Line 3 indicates that proteins are descended from a common ancestor. You should think of it as "quantal". Proteins are either orthologs (descended from a common ancestor) or paralogs that arise from a duplication in a particular organism. Thus, one cannot have high homology. I think you mean that the two proteins have high similarity. Protein are either homologous or not homologous.

RE: We agree with the reviewer and we have corrected the text accordingly.

Page, 1, Line 6. Alike genes encoding for other ... I think the author mean Like not alike

RE: We thank the reviewer and we have corrected the typo in the text.

Page 1, Line 10 locus should not be in italics

RE: We thank the reviewer and we have corrected it.

Page 3 The authors state that structural work suggested that WDR60/WDR34 restrict dynein activity to the tip. I think they mean to say that they may restrict the activation of dynein to the tip as it must be active during retrograde movement.

RE: We agree with the reviewer and we have rephrased the text accordingly.

Is mksr-2 cep290? It would be useful to include the human names of genes.

RE: We thank the reviewer for the suggestion, and we clarify that MKSR-2 is the homolog of human B9D2. We note that we had already included the human protein homologs in superscript when their names were different, but for simplicity, we have now followed the editor's suggestion to include this information in Table S1, summarizing all the *C. elegans* proteins used in this study and the corresponding human homologs. In addition, we include this information between parenthesis in the first instance that a *C. elegans* protein is mentioned in the text. We hope that this makes our manuscript more accessible to readers that work on other model systems.

REVIEWER #3

Though the function of WDR60 has been addressed in several studies in mammalian cells regarding ciliary assembly and IFT, its molecular function in IFT is still not clear. To this end, this work addressed this issue in worm by using WDR60 mutants combined with live imaging. The major conclusions that fascinates me are that WDR60 does not affect ciliogenesis but regulate initiation of retrograde IFT and exit of IFT dynein through the transition zone.

RE: We thank the reviewer for appreciating the importance of our study.

However, I found that the evidences presented do not back up those conclusions.
1) Ciliogenesis. As shown in Fig 3B, the length of cilia in the null mutant WDR60 is significantly shorter, which is consistent with the data shown in mammalian cells with WDR60 knockout (Vuolo et al 2018). Thus, WDR60 does affect ciliogenesis though mildly.

RE: We agree with the reviewer that based on the measurements made with GFP::CHE-3, there is a small but nevertheless significant effect on cilia length in *wdr-60* null mutants as was reported in Vuolo et al 2018 for WDR60 KO human cells. As the reviewer mentioned, this is a very mild effect when compared to what was observed in the same study for WDR34 KO cilia, which led the authors of Vuolo et al 2018 to propose that these dynein-2 subunits have distinct contributions during dynein-2-mediated retrograde IFT.

To address the reviewer's concern and validate the mild effect in axoneme elongation upon WDR-60 loss that we observed with CHE-3 (Figure 3B), we have now also measured cilia length in *wdr-60* mutants using additional markers such as CHE-11(IFT140; Figure 4B) and IFT-74 (Figure 4E). Our new data confirms that WDR-60 loss does result in a mild defect in axoneme extension, as correctly pointed out by the reviewer. For comparison, we have also measured and included the cilia length in the dynein-2 LIC (XBX-1) mutant, as it severely truncates cilia, mimicking complete loss of dynein-2 function. We have also discussed this aspect further in the context of Vuolo et al 2018 and the remaining literature.

2) Initiation of retrograde IFT. Accumulation of IFT dynein in the cilia of the WDR60 mutants is interpreted as defects in the initiation of retrograde IFT. The initiation of retrograde IFT occurs at the ciliary tip. The observed phenotype should be simply due to defects in retrograde IFT, which is expected. If the initiation of retrograde IFT is defective, one should see accumulation of dynein at the ciliary tip, which is not (see Fig 3).

RE: We understand the reviewer's concern but we point out that this conclusion was not based on the accumulation of dynein-2 near the TZ. The data that supports a decrease in the initiation of retrograde IFT in the absence of WDR-60 comes from our quantifications of the frequency of retrograde IFT events in our *wdr-60* mutants (data shown in Figure 3F). This quantification reflects the number of GFP::CHE-3 particles that start moving in the retrograde direction at the distal segment (measured near the tip of cilia as in (Mijalkovic et al., 2017)). This frequency data shows less dynein-2 HC particles moving in the retrograde direction in the absence of WDR-60. To us this observation indicated that the number of retrograde IFT events initiated in the cilia of these mutants was reduced, hence initially referring to it as a problem in initiation. Nevertheless, we completely agree with the reviewer: Given that dynein-2 HC does not accumulate at the ciliary tip, one can assume that dynein-2 activation still occurs in the absence of WDR-60. To avoid any confusion, we have simply referred to this effect in the text as a reduction in the frequency of retrograde IFT.

To further confirm our results supporting a reduction in the frequency of retrograde IFT in our *wdr-60* mutants, we have now also quantified the rate of CHE-11::mCherry particles in both directions. In agreement with our analysis using labeled CHE-3, we observed a significant decrease in the frequency of CHE-11 particles moving retrogradely, particularly in the *wdr-60* null. This new data is shown in Figure 4I.

Similarly, IFT-A and IFT-B components as shown in Figure 4 also do not accumulate at the ciliary tip. In addition, these data are contradictory to the finding in mammalian cells where both IFT-A and IFT-B components accumulate at the ciliary tip (Vuolo et al 2018). Though the function of WDR60 in different organism may function differently, the data shown in this paper do not support the author's claim that WDR60 regulates the initiation of retrograde IFT.

RE: In Vuolo et al 2018, the authors nicely show how the loss of WDR34 or WDR60 have a differential effect in the distribution profile of IFT components in cilia. In Figure 3A/B of Vuolo et al 2018, the authors analyzed the distribution of IFT140 and IFT43, respectively (two IFT-A components). While they show that these proteins accumulate at the tip of WDR34 KO cilia, it is also evident from their data that both IFT140 and IFT43 particularly accumulate along the axoneme and closer to the base in WDR60 KO cells (Figure 3Aii vs 3Aiii; Figure 3Bii vs 3Biii), similar to what we observe in our WDR-60 mutants with CHE-11(IFT140)::mCherry (our Figure 4A,C).

Regarding IFT-B particles, the authors see various accumulations at the tip and along the axoneme in Figure 2B/C (as we observe with IFT-74 (our Figure 4D,F)). Furthermore, we note that the authors of Vuolo et al., 2018 clearly acknowledge in their discussion section that IFT-B components do not solely accumulate at the ciliary tips of WDR60 KO cilia: *"Loss of either WDR34 or WDR60 leads to IFT particle accumulation at the base of as well as within cilia. We found that loss of WDR60 results in an increase of the IFT-B proteins, IFT20, IFT57, and IFT88, not only at the tip but also close to the base of the cilium. This suggests that IFT-B proteins could be retained at the basal body or around the transition zone. Consistent with these results, mutations in IFT-A or dynein-2 in mice also result in abnormal accumulation of IFT particles near the base of the cilium (Goggolidou et al., 2014;Liem et al., 2012;Ocbina et al., 2011). This has been linked to defects in the export of ciliary cargo across the transition zone (He et al., 2017). Similar defects are seen following disruption of the heavy chain, DHC2/DYNC2H1 (Hou and Witman,2015)*." - This text was directly transcribed from the discussion section of (Vuolo et al., 2018).

Thus, we believe that the IFT distribution profiles that we observed in our *wdr-60* mutant cilia are in agreement with the WDR60 KO data of (Vuolo et al., 2018). All of this information is now debated in the discussion section of our revised manuscript.

3) Exit of IFT dynein. It was shown that IFT dynein accumulates at the TZ area in the WDR60 mutants and disruption of TZ facilitates the return of dynein to the cell body. It was then concluded that WDR60 regulates passage of dynein and/or IFT trains through TZ. However, for the LIC3 mutant, there is also accumulation of dynein at the TZ area (Fig 2B), indicating that IFT dynein also can not pass the TZ in this mutant. Thus, it appears that the integrity of the dynein complex but not WDR60 itself is required for the passage of dynein through the TZ. Secondly, if WDR60 specifically regulates the passage of dynein through the TZ, no possible mechanism is shown.

RE: We would like to first point out that in Figure 2B (same in the revised manuscript), we do not analyze the localization of the dynein-2 HC motor. In that figure, we tested whether the recruitment and distribution of tagged WDR-60 in cilia is dependent of its β-propeller and/or XBX-1(LIC3).

In Figure 3A,C,D, where we do analyze the recruitment and distribution of the dynein-2 HC (CHE-3), the results obtained with *wdr-60* and *xbx-1* mutants are quite different. While we do observe a reduction in the recruitment of dynein-2 to cilia in *wdr-60* mutants, the dynein-2 HC does not enter cilia nor even get recruited to the ciliary base in the *xbx-1* null strain (Figure 3A; Yi et al., 2017). Thus, we believe that the phenotype of a complete block of retrograde IFT observed in the *xbx-1* mutant is distinct from what we observe in our *wdr-60* mutants which still partially recruit the dynein-2 HC and still undergo retrograde IFT, albeit more slowly and less frequently than in wild-type cilia (Figure 3E-H).

Regarding mechanism, the reduction in dynein-2 availability inside cilia and consequent underpowering of retrograde IFT in the absence of WDR-60 is likely to render the IFT trains unable to generate enough force to push through the dense TZ barrier, as we now extensively discuss in our manuscript. Nevertheless, as also mentioned in our discussion, we do not exclude the possibility that WDR-60 might contribute in additional ways to retrograde IFT or for ciliary exit.

Minor points

1. P5, "While some signal is detected in the soma and dendrites of these neurons, most WDR-60 is recruited to cilia." From what I see in the images, most signals are in the soma or dendrites.

RE: We thank the reviewer for highlighting this. We have corrected the text to accurately reflect that a large part of WDR-60 signal is seen in the soma and dendrites of the ciliated sensory neurons.

2. P6. "As endogenous labelling of wdr-60," wrd-60 should be WDR-60? Because the protein is labelled but not the gene.

RE: We thank the reviewer for highlighting this. We changed the text to correctly state the labeling of the WDR-60 protein.

3. FigS2A, why does the WDR60-FLA-GFP strain have extremely more dye than the control? This is unexpected for a correct knock-in strain.

RE: We thank the reviewer for pointing out the seemingly different dye signal in the WDR-60::3xFLAG::GFP strain. However, we note that the dye filling assay is more of a qualitative assay rather than a quantitative technique, as the worms can ingest some dye which results in highly variable signal intensity at their mouth. Therefore, the interpretation of the results from this assay is usually taken as a “yes or no” answer for whether the sensory neurons incorporate the dye through their cilia.

Nevertheless, we have replaced the example in Figure S2A to be more representative of the data and avoid any misinterpretation.

In addition, we are very confident that our knock-in strain is correct. We want to highlight that our 3xFLAG::GFP tag includes a flexible linker to avoid interfering with WDR-60 function and was integrated at the endogenous locus by CRISPR-Cas9 genome editing using gRNAs designed to have no off-targets. Even so, we still outcrossed the knock-in strain with the wild-type strain 6 consecutive times to remove any potential mutations in other regions of the genome, and confirmed the genomic integrity of the *wdr-60::3xflag::gfp* at the locus by sequencing (including outside of the homology arms). Moreover, our results in Figure 1 clearly show that labeled WDR-60 dynamics are identical to the ones of the tagged dynein-2 HC (GFP::CHE-3), which should further advocate that our tagged WDR-60 accurately reports wild-type WDR-60.

4. Fig 3A. Dynein in worm is enriched at the TZ region (there is no basal body), which should be around 1 um (see Doroquez et al., 2014 eLife; Snow et al., 2004). If one estimates the length of TZ by dynein fluorescence, it is close to 1 um. Thus I think the second high intensity in the mutants is outside TZ. But in Fig3C, half of the intensity is in the TZ.

RE: We agree with the reviewer that the TZ is approximately 1 µm. As proposed by the reviewer, we have now standardized the delimitation of this region based in MKS-6::mCherry (Figure 6B,C), a well-established TZ marker (Prevo et al., 2015, Supp. Data; Schouteden et al., 2015; Williams et al., 2011).

Although Snow et al., 2004 was a very nice study, we note that no TZ marker was used to determine the exact region of the TZ in relation to the IFT components imaged. In our study, we carefully imaged CHE-3 relative to MKS-6::mCherry in Figure 6B,C (previous Figure 5). Nevertheless, to better resolve where dynein locates in wild-type and in the absence of WDR-60, we have now also included co-imaging of dynein-2 with the centriolar component, HYLS-1 (HYLS) (Dammermann et al., 2009), that labels the remnants of the degenerated basal body at the ciliary base in the phasmid sensory neurons (Serwas et al., 2017). In addition, we added 3x magnifications of both channels for both marker combinations, to facilitate the analysis.

Several studies show that the intensity of IFT components and dynein-2 itself peak just before entering cilia, adjacent to the proximal side of the TZ (as shown in Scheidel and Blacque, 2018 (Figures 3A, S2B); Williams et al. 2011 (Figure 1D); Prevo et al., 2015 (Supp Figure 1)). Consistent with this, our data in Figure 6B,C (old Figure 5A,D) clearly shows that the largest peak of dynein-2 CHE-3 is present adjacent to the proximal side of the TZ, colocalizing with HYLS-1 at the base of cilia in wild-type controls (new data in Figure 6A). In contrast, in our *wdr-60* mutants, CHE-3 intensity is clearly increased at the distal side of the TZ area in comparison to the pool at the base (new Figure 6B,C). The fact that part of the dynein-2 accumulation overlaps with the TZ further suggest that even when it does manage to start crossing the TZ, it has difficulty in continuing to push through this roadblock. Consistent with this, reducing the resistance offered by the TZ by disrupting key components substantially rescued these accumulations of CHE-3 at the TZ region in the *wdr-60(null)* background.

Finally, as correctly denoted by the reviewer, centrioles degenerate shortly after the initiation of ciliogenesis (Nechipurenko et al., 2017; Nechipurenko and Sengupta, 2017; Serwas et al., 2017), so we were also careful to take that into consideration in our cilia schematics in Figures 1 and 10, to reflect that the outer wall of centriolar microtubules becomes flared (wider at its proximal end) as a consequence of central tube loss, but persists throughout development (Doroquez et al., 2014; Nechipurenko et al., 2017; Nechipurenko and Sengupta, 2017; Serwas et al., 2017).

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Dear Dr. Dantas,

Thank you for submitting your revised manuscript entitled "WDR60-mediated dynein-2 loading into cilia powers retrograde IFT and transition zone crossing." We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines and a couple remaining minor points from reviewers (see details below).

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3) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. Molecular weight or nucleic acid size markers must be included on all gel electrophoresis. Please add a scale bar to Fig. 3A.

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

5) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions (at least in brief) in the text for readers who may not have access to referenced manuscripts. The text should not refer to methods "...as previously described."

6) For all cell lines, vectors, constructs/cDNAs, etc. - all genetic material: please include database / vendor ID (e.g., Addgene, ATCC, etc.) or if unavailable, please briefly describe their basic genetic features, even if described in other published work or gifted to you by other investigators (and provide references where appropriate). Please be sure to provide the sequences for all of your oligos: primers, si/shRNA, RNAi, gRNAs, etc. in the materials and methods. You must also indicate in the methods the source, species, and catalog numbers/vendor identifiers (where appropriate) for all of your antibodies, including secondary. If antibodies are not commercial please add a reference citation if possible.

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

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

9) Supplemental materials: There are strict limits on the allowable amount of supplemental data. Articles may have up to 5 supplemental figures and 10 videos. Please also note that tables, like figures, should be provided as individual, editable files. A summary of all supplemental material should appear at the end of the Materials and methods section. Please include one brief sentence per item.

10) eTOC summary: A ~40-50 word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person. It should begin with "First author name(s) et al..." to match our preferred style.

11) Conflict of interest statement: JCB requires inclusion of a statement in the acknowledgements regarding competing financial interests. If no competing financial interests exist, please include the following statement: "The authors declare no competing financial interests." If competing interests are declared, please follow your statement of these competing interests with the following statement: "The authors declare no further competing financial interests."

12) A separate author contribution section is required following the Acknowledgments in all

research manuscripts. All authors should be mentioned and designated by their first and middle initials and full surnames. We encourage use of the CRediT nomenclature (https://casrai.org/credit/).

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Journal of Cell Biology

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Scientific Editor

Journal of Cell Biology

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

The authors have done a fantastic job in addressing my comments and the new data concerning the NPHP module is a major addition to the study.

The only final comment from me relates to the sentence:
"However, given that the exact contribution of each TZ component to the functionality of Y-links is still not fully understood, it is difficult to directly implicate Y-links in mediating IFT train passage through the TZ."

Because Y-links are very hard to detect or missing entirely in the cep-290 worm mutant (several studies), there is in fact a strong basis for a conclusion that Y-links themselves do not act as roadblocks for the passage of IFT-dynein powered trains (although there is a minor caveat that the TEM data for Y-links was from amphid channel cilia, whereas the data in this paper is from phasmid cilia).

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

The revised manuscript has been significantly improved and the points that were raised have been addressed. I agree that it is suitable for publication.

I have one minor suggestion which should be addressed.
Page 7, Line 6 "Strikingly, the absence of dynein-2 LIC, which is known to destabilize dynein-2 HC (Taylor et al., 2015; Yi et al., 2017)," From my understanding of the two papers that are cited, I did not see the data that loss of LIC destabilizes dynein-2 HC. Interestingly, one recent paper in EMBO J (Zhu X. et al., 2021 40: e105781) showed that LIC directly interacts with IFT54 of the IFT-B complex, which provide explanations why dynein-2 can not enter cilia in the absence of LIC. Thus, the above sentence can be modified as "..., which is known to directly interact with IFT-B for ciliary entry of dynein-2 HC" with the new ref being added.

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Response to the Reviewer Comments:

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RE: While we understand the point made by the reviewer, we would prefer to refrain from taking such strong conclusion considering that there was no TEM imaging carried out in the *cep-290(tm4927)* mutant that we used in our study (Schouteden et al., 2015). Given that this aspect does not affect our conclusion that the NPHP module acts as a roadblock for the passage of IFT-dynein powered trains, we believe that assessing the specific contribution of Y-links should be more thoroughly addressed in future studies.

Accordingly, we have rephrased the sentence pointed out by the reviewer as follows:

"However, additional experimental work will be required to directly determine whether Y-links themselves influence the passage of IFT trains through the TZ."

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

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RE: While we agree with the reviewer that (Yi et al., 2017) does not directly show that loss of dynein-2 LIC destabilizes the HC, the study by (Taylor et al., 2015) clearly shows that mutations that destabilize dynein-2 LIC (DYNC2LI1 in Fig2B/C) strongly reduce the total levels of the dynein-2 HC (DYNC2H1) by western blot in Fig. 3a: *"Mutations in DYNC2LI1 decrease the stability of DYNC2H1"*.

In addition, dynein-2 LIC has been shown to contribute to dynein-2 HC stability in *Chlamydomonas* (Hou et al., 2004 in Fig. 7: *"The level of DHC1b is reduced in d1blic mutant cells and vice versa"*; and Reck et al., 2016 in Fig. 1: *"Knockdown of D1bLIC alters DHC1b stability"*) and in *Trypanosoma* (Blisnick et al., 2014 in Fig. 8: *"DLI1 is required for stability of the dynein heavy chains"*). We have now added these references to our study.

Nevertheless, we agree with the reviewer that the recent paper (Zhu et al., 2021) that shows that dynein-2 LIC directly interacts with IFT54 of the IFT-B complex in *Chlamydomonas* provides important insights for our own work. Therefore, we have rephrased the sentence pointed out by the reviewer as follows:

"Strikingly, although dynein-2 LIC stabilizes the HC (Blisnick et al., 2014; Hou et al., 2004; Reck et al., 2016; Taylor et al., 2015) and contributes to ciliary entry of dynein-2 by directly interacting with IFT-B (Zhu et al., 2021), XBX-1 loss did not significantly affect the ciliary recruitment of WDR-60::GFP or WDR-60(ΔCT)::GFP (Figure 2B,C)".

In addition, we included the important study highlighted by the reviewer in our discussion in page 15, as follows:

"Intriguingly, the observation that a fraction of dynein-2 motors still get incorporated onto anterograde IFT trains indicates that WDR-60 is not the only link that dynein-2 can establish with anterograde trains. This conclusion is further supported by the recent report of a direct interaction between dynein-2 LIC and IFT54 of the IFT-B complex (Zhu et al., 2021) and by Cryo-EM data suggesting that the main contacts between anterograde IFT trains and dynein-2 motors may also involve the HC (Toropova et al., 2019).".

References:

Blisnick, T., J. Buisson, S. Absalon, A. Marie, N. Cayet, and P. Bastin. 2014. The intraflagellar transport dynein complex of trypanosomes is made of a heterodimer of dynein heavy chains and of light and intermediate chains of distinct functions. *Molecular biology of the cell*. 25:2620-2633.

Hou, Y., G.J. Pazour, and G.B. Witman. 2004. A dynein light intermediate chain, D1bLIC, is required for retrograde intraflagellar transport. *Molecular biology of the cell*. 15:4382-4394.

Reck, J., A.M. Schauer, K. VanderWaal Mills, R. Bower, D. Tritschler, C.A. Perrone, and M.E. Porter. 2016. The role of the dynein light intermediate chain in retrograde IFT and flagellar function in Chlamydomonas. *Molecular biology of the cell*. 27:2404-2422.

Schouteden, C., D. Serwas, M. Palfy, and A. Dammermann. 2015. The ciliary transition zone functions in cell adhesion but is dispensable for axoneme assembly in C. elegans. *The Journal of cell biology*. 210:35-44.

Taylor, S.P., T.J. Dantas, I. Duran, S. Wu, R.S. Lachman, C. University of Washington Center for Mendelian Genomics, S.F. Nelson, D.H. Cohn, R.B. Vallee, and D. Krakow. 2015. Mutations in DYNC2LI1 disrupt cilia function and cause short rib polydactyly syndrome. *Nature communications*. 6:7092.

Yi, P., W.J. Li, M.Q. Dong, and G. Ou. 2017. Dynein-Driven Retrograde Intraflagellar Transport Is Triphasic in C. elegans Sensory Cilia. *Curr Biol*. 27:1448-1461 e1447.

Zhu, X., J. Wang, S. Li, K. Lechtreck, and J. Pan. 2021. IFT54 directly interacts with kinesin-II and IFT dynein to regulate anterograde intraflagellar transport. *EMBO J*. 40:e105781.