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The IFT81-IFT74 complex acts as an unconventional RabL2 GTPase-activating protein during intraflagellar transport

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

1st Editorial Decision **8th Jun 2022**

Dear Esben,

Thank you for submitting your manuscript "The IFT81-IFT74 complex enhances GTP hydrolysis to inactivate RabL2 during intraflagellar transport" for consideration by The EMBO Journal. I have now read your study carefully and discussed it with my colleagues. I am sorry to say that, based on these discussions, we have decided not to pursue the publication at The EMBO Journal. However, I would like to recommend a transfer to our sister journal EMBO Reports, where the responsible editor would be interested in sending the manuscript for peer review.

We appreciate that your study identifies the IFT-B1 subcomplex, and specifically its IFT-81/74 subunits, as the GAP factor for the RabL2 GTPase, a known regulator of IFT and ciliogenesis. Further findings show that this function of the IFT-B1 subcomplex is conserved in Chlamydomonas reinhardtii. CEP19, another known RabL2-GTP interactor, does not have a GAP activity towards it, thus providing further insight into previously published observations on the interplay between RabL2, CEP19 and IFT-B1 in ciliogenesis. From our side, while we find the identified GAP function of IFT-81/74 towards RabL2 interesting and novel, I am afraid we found that further insights into this new function would be required, either into its role in ciliogenesis in cellulo or into the discussed potential relevance of RabL2 recruitment for IFT complex structural remodelling. Therefore, I am afraid we concluded that the manuscript in its current form is not a sufficiently strong candidate for peer review at The EMBO Journal.

That being said, I appreciate the novelty of these findings, and I have therefore discussed the manuscript with my colleague Deniz Senyilmaz Tiebe at our sister journal EMBO Reports. I am glad to say that she would like to send your manuscript out for peer review. If you are interested in this option, please use the link below to transfer your manuscript to EMBO Reports (no reformatting is required):

Link Not Available

Thank you in any case for giving us the chance to consider your study at our journal. I am sorry that I could not offer better news this time, and I very much hope that you will find the transfer option of interest.

With kind regards,

Ieva

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Dear Ieva,

We are now, after much longer than originally planned, ready to submit our story on RAbL2 to EMBO J. As agreed, we have now carried out an in cellulo analysis of RAbL2 using a structure-guided point mutant in IFT74 that prevents interaction of RABL2 with the IFT complex. Using mammalian RPE1 cells that have one primary cilium, we used CRISPR-Cas9 IFT74 KO cells to rescue with WT or the point mutant preventing IFT association by RabL2. We analyse ciliogenesis, basal-body localization and IFT injection under these conditions. Our data show that the mutant severely hampers with and delays cilium formation, reduces RabL2 localization to the ciliary base and significantly reduces IFT particle injection into cilia.

With this new data, our manuscript now presents a much more complete analysis of RAbL2 functions including: 1) discovery of a novel function of the IFT complex as an activator of GTPase activity of RabL2, 2) comprehensive structural modeling of RabL2 association with CEP19 and the IFT complex and validation of these models using protein biochemistry, site-directed mutagenesis, cross-linking MS, 3) biophysics to measure affinities of RabL2 for CEP19 and the IFT complex and their nucleotide dependencies, and 4) in cellulo analysis of RabL2 using IFT74 CRISPR/CAS KO mammalian cells and rescue experiments with a structure-guided IFT74 mutant.

I hope that you now find the manuscript to be suitable for review with EMBO J.

Best wishes

esben

Appeal - Editorial Decision **10th Mar 2023**

Dear Esben,

Thank you for submitting your manuscript for consideration by the EMBO Journal. We have now received comments from three reviewers, which are included below for your information.

As you will see from the reports, The reviewers find the proposed role of IFT74 as a GAP for RABL2 per se of interest. However, they also indicate several aspects that would have to be improved in the revised manuscript. In particular, they find that a more balanced discussion and interpretation of the data in the context of the previous findings on the role of RABL2 is required. Furthermore, the reviewers find that the basis for exclusion of CEP19 role as a RABL2 effector needs clearer support and explanation. Reviewer #1 also raises several issues regarding the biochemical analysis. Finally, reviewer #3 finds that the specificity of the IFT74 T438R phenotypes to the loss of its interaction of RABL2 remains unclear.

If you find that you are able to address the main issues raised by the reviewers, I would be happy to consider a revised version of the manuscript. I think it would be helpful to discuss the revision in more detail via email or phone/videoconferencing. I should also add that it is The EMBO Journal policy to allow only a single major round of revision and that it is therefore important to resolve the main concerns at this stage.

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, please contact me as soon as possible upon publication of any related work to discuss the appropriate course of action. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess. Please also see the attached instructions for further guidelines on preparation of the revised manuscript.

Please feel free to contact me if you have any further questions regarding the revision. Thank you for the opportunity to consider your work for publication. I look forward to discussing your revision.

With best regards,

Ieva

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Instructions for preparing your revised manuscript:

Please make sure you upload a letter of response to the referees' comments together with the revised manuscript.

Please also check that the title and abstract of the manuscript are brief, yet explicit, even to non-specialists.

When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
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IMPORTANT: When you send the revision we will require
- a point-by-point response to the referees' comments, with a detailed description of the changes made (as a word file).

- a word file of the manuscript text.
- individual production quality figure files (one file per figure)
- a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14602075/authorguide).
- Expanded View files (replacing Supplementary Information)
Please see out instructions to authors
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We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (8th Jun 2023). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions.

Referee #1:

The authors of this manuscript try to biochemically characterise the interaction of RabL2 with CEP19 and the IFT-B1 complex to have a mechanistic insight of IFT initiation and entry. The authors do so by performing several biochemical interactions, GTPase assays, cross linking and using Alphafold to predict the complexes models. They conclude that CEP19 is not a GAP whereas IFTB1 enhances the GTPase activity of RABL2.
It was already reported before that CEP19 and IFTB1 interact with RABL2. However the main finding in my opinion in this paper is the stimulation of RABL2 GTPase activity by IFTB1. Nevertheless this enhancement as reported by the authors is relatively lower than general GAP proteins. . Additionally in the structural model the authors report that there are no residues inserted in the active site and they note that it binds in the effector mode. This finding can be exciting and significant. Nevertheless here are my remarks

1- The authors switch between using 1 mM GTP and 30 uM I guess to move from multiple turnover to single turn over reactions. Nevertheless I don't see the concentrations of RABL2 that was used in the single turnover reactions. Can you please indicate all concentrations in the legend so it can be easily followed

2- A major concern is that the authors mention that they used linear regression to fit the first 500 seconds. If this is a single turn over reaction you have to use single exponential fit not linear fit otherwise you are taking in account the amplitude of the signal which depends on the affinity of RABL2 to the nucleotides (which in turn could be affected by the binding of IFTB1). Please fit the data to a single exponential fit and report those numbers and show the fit. Alternatively do a multiple turnover experiment with saturating amounts of GTP and calculate vmax.

3- In the structure model is there a space for another protein to insert a catalytic residue. In other words can IFTB1 be a kind of co-GAP. This should be mentioned in the discussion section

4- The authors report CEP19 to have a higher affinity to GTP bound RABL2 than GDP bound form (45 times) but they conclude :"This result agrees with the notion that CEP19 senses the nucleotide state of RabL2 but is not a true effector for RabL2". Having a higher affinity toward GTP is actually the definition of an effector. What is that notion that is not a real effector and why do the authors conclude it is not a "true" effector?

5- The authors emphasize that CEP19 is not a GAP and report it in the abstract as a finding. I would only mention or emphasize this finding if it was reported otherwise. If so the authors should cite this.

6- The introduction in my personal opinion is quite spread. I would make it more focused on the question that they want to answer.

Referee #2:

IFT complexes accumulate at the ciliary base before they are transported into cilia. However, how anterograde IFT is initiated is not well understood. Recently, RABL2 has also been implicated in controlling ciliary entry of IFT proteins by its interaction with IFT-B though with distinct mechanisms in Chlamydomonas and mammal. RABL2, a small GTPase interacting with CEP19 at the base of cilia, binds IFT81/74 as a GTP-specific effector to trigger the ciliary entry of IFT complexes as RABL2 mutation does not affect the basal body localization of IFT proteins but typically reduce IFT particles inside cilia (Kanie et al., 2017). The interaction of RabL2 with IFT74/81 was also reported in another study (Mishijima et al., 2017). In contrast, in Chlamydomonas, RABL2 regulates the enrichment of IFT-B at the basal body to control the amounts of IFT-B1 proteins available for assembling the anterograde IFT trains (Zhang et al., 2022 https://doi.org/10.1101/2022.02.13.480273). Thus, it appears that species-specific mechanism may exist. In addition, RABL2 has also been reported to function in regulating the export of BBSome passing through the transition zone to fine-tune ciliary signaling (Duan et al., 2020; Zhang et al., 2022; Zhou et al., 2022). These results indicate that RABL2 is associated with IFT-B and plays critical roles in regulating the anterograde IFT as well as the retrograde transport of BBSome at the ciliary base.

In this paper, Lorentzen and his colleagues have mapped the interactions of RABL2 with CEP19 as well as with IFT81/74 and demonstrated that binding of IFT81/74 enhances GTP hydrolysis of RabL2. They determined the minimal functional region of IFT81/74 to 70 amino acids. AlphaFold modeling and GTPase assays showed that RABL2 interacts with IFT81/74 is evolutionally conserved between Chlamydomonas and human. Moreover, they disrupted the regulation of RABL2 by IFT81/74 via point mutation of IFT74 in RPE1 cells and found severe defects in ciliogenesis and reduced IFT proteins in cilia, which are similar to the RABL2-KO phenotypes. These results are well supported and provide molecular details on the interaction between RABL2 and its partners, which would lead to elucidation of further mechanisms on IFT ciliary entry.

I have only some minor questions and/or comments;
1. Regulation of IFT entry appears to be the major issue to be addressed in this work. To inform the readers well with the related research background, I would suggest mentioning the study from Liang et al., 2014 Dev Cell. This paper is about that phosphorylation functions in regulating the interaction between kinesin-2 and IFT-B to initiate anterograde IFT (Liang et al; 2014 Dev Cell). I bet the regulation of IFT entry by Rabl2 as proposed in this and other papers may be a different layer of regulation.
2. In the abstract, it says that this work provides a rationale for why RabL2 dissociates anterograde IFT trains soon after departure from the ciliary base, which is also depicted in Fig .7. Is there published in vivo evidence in which dissociation of RabL2 from IFT trains occurs when it enters cilia? Duan et al., 2020 (Fig.4) reported that RABL2 could enter cilia and undergo bidirectional IFT in RPE1 cells, which suggests that at least a portion of RABL2 does not dissociate from IFT trains after entering cilia. It will be great to discuss this conflicting data.
3. Just comments. I have a problem to understand how RabL2 mediates IFT entry. Loss of RabL2 or in the presence of the GDP form, IFT-B cannot enter cilia. Why does the GTP form make it possible? Or put it in another way, how does binding of IFT81/74 to RabL2-GTP would triggers IFT entry? What barriers does it break? I would welcome to include a model or hypothesis that the authors might have. Actually I could understand well the explanation in Chlamydomonas, where it says that Rabl2 is involved in recruiting IFT-B to ciliary base. Thus, dysfunction of Rabl2 may lead to limited accumulation of IFT proteins at the ciliary base, which would result in less IFT entry.

Referee #3:

The biochemistry is conducted to high experimental standards throughout. All logical controls are included. The conclusion that a ~70aa coiled coil from IFT74/81 is a GAP for RabL2 is solid and will stand the test of time.

The functional rescue experiments (Fig. 6) are problematic and need to be revised for the paper to be accepted. As detailed below, the prior interpretation that RabL2 is an injector for IFT trains is incorrect and was rigorously rejected by a 2021 EMBO paper from Xueliang Zhu and Xiumin Yan. As already pointed by the Kanie 2017 paper, RabL2 knockout mice are viable, which would not be possible if IFT trains failed to enter cilia.

Major points:

Abstract suggests that the purpose of RABL2 association with IFT is 'to regulate initiation of IFT '. Intro mentions that RabL2 was shown to 'regulate IFT initiation and cilium formation'.
This part of the introduction is unbalanced and reads like a one-sided summary of the authors' past work with Kanie 2017 cited 6 times and Duan 2021 only cited once. (The Kanie 2017 paper is cited 21 times in total in the current manuscript). For the introduction to properly situate the knowns and unknowns of RabL2, the authors need to acknowledge that 1- RabL2 is dispensable for ciliogenesis in mice and 2- a mouse knockin bearing a GTP-locked RabL2 allele displays no alteration of IFT frequency, velocity or processivity. In light of this rigorous study, the suggestion that RabL2-GTP promotes entry of IFT trains into cilia is no longer tenable. Instead, the Duan paper carefully and rigorously articulates a role for RabL2-GTP in antagonizing BBSome binding to IFT-B. An elegant model, which is still largely consistent with past work from the authors, is that the function of RabL2-GTP association with entering IFT trains is to suppress BBSome binding to anterograde IFT so that the BBSome does

not import the cargoes that it exports from cilia.
It is important that the authors state clearly and early in the manuscript that the model of RabL2 injection is not consistent with the viability of the RabL2 knockout mouse, and that the conclusions related to a possible role of RabL2 in IFT injections are limited to RPE cells.
The entire discussion related to RabL2 and IFT train injection on p.23 needs to be re-written to carefully incorporate the results published by Duan et al.

Fig. 6: The major question is whether IFT74 is expressed at the same level at IFT74[T438R] in the rescue lines? A simple FLAG immunoblot would address a trivial interpretation for the observed differences. As pointed by the authors, the quantitation of fluorescence in Fig. 6E 'suggests that IFT74T438R may not be expressed as strongly as WT or the structure of the mutant may be partially disturbed to prevent efficient centriole localization'. The included results suggest that the T438R mutation may affect IFT74 in other ways that by decreasing binding to RabL2, thus invalidating many of the authors' interpretations.

Minor points:
CEP19 discriminates between GTP and GDP RabL2 by a factor of nearly 50. Yet, the authors state that 'CEP19 senses the nucleotide state of RabL2 but is not a true effector for RabL2'. This statement seems at odd with the presented data. Can the authors elaborate on the criteria that make a 'true effector'?

I was initially confused by the authors description of Fig. 1D 'when WT CrRabL2 and GTP were mixed with the IFT-B1 complex and incubated for 3h at room temperature, RabL2 no longer associated with the IFT- B1 complex'. It is because the sizes of IFT27 and RabL2 are extremely close and I believed that there was a RabL2 band in the IFT peak. Could the authors conduct immunoblotting of these samples for RabL2?

Intro reads like an extensive review of IFT train assembly. While the scholarship is outstanding, it could be shortened to better serve the current manuscript and expose the current gaps in knowledge regrading RabL2 function.

Below is the response to the comments by the reviewers for the manuscript by Boegholm et al., with the title 'The IFT81-IFT74 complex enhances GTP hydrolysis to inactivate RabL2 during intraflagellar transport'. We thank the reviewers for their time and constructive comments.

Referee #1:

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1- The authors switch between using 1 mM GTP and 30 uM I guess to move from multiple turnover to single turn over reactions. Nevertheless I don't see the concentrations of RABL2 that was used in the single turnover reactions. Can you please indicate all concentrations in the legend so it can be easily followed

Answer: Yes, we do indeed move from multiple turnover to single turnover reactions. The concentrations of proteins and of GTP in the GTPase experiments are now mentioned in the M&M and in the figure legends.

2- A major concern is that the authors mention that they used linear regression to fit the first 500 seconds. If this is a single turn over reaction you have to use single exponential fit not linear fit otherwise you are taking in account the amplitude of the signal which depends on the affinity of RABL2 to the nucleotides (which in turn could be affected by the binding of IFTB1). Please fit the data to a single exponential fit and report those numbers and show the fit. Alternatively do a multiple turnover experiment with saturating amounts of GTP and calculate vmax.

Answer: thank you for pointing this out. We have now performed a single exponential fit for all our single-turnover experiments. The fitting of GTPase assay data in Figures 2E-H, 3D-E, 5D-E, and 6C has been updated accordingly. Correspondingly, the reaction rate numbers have been revised in these figures and throughout the text of the manuscript. In addition to updated reaction rates we now also provide the quality of fit (R^2) for all experiments in the figures. The single exponential fits give somewhat lower reaction rates than what was obtained using linear regression and the increase in GTP hydrolyses in the IFT-B hexamer compared to RabL2 alone is in the 7-9 fold range. This does, however, not change any of the main conclusions of the manuscript.

3- In the structure model is there a space for another protein to insert a catalytic residue. In other words can IFTB1 be a kind of co-GAP. This should be mentioned in the discussion section

Answer: Thank you for raising this interesting point. We have re-examined the structural model of IFT81/74-RabL2 and indeed, there appears to be sufficient space for another yet unidentified GAP to bind to the complex. This unknown GAP could potentially insert residues into the active site of RabL2, thus accelerating the GTP hydrolysis rate. This possibility is now discussed on page 20 of the revised manuscript.

4- The authors report CEP19 to have a higher affinity to GTP bound RABL2 than GDP bound form (45 times) but they conclude :"This result agrees with the notion that CEP19 senses the nucleotide state of RabL2 but is not a true effector for RabL2". Having a higher affinity toward GTP is actually the definition of an effector. What is that notion that is not a real effector and why do the authors conclude it is not a "true" effector?

Answer: In the case where the switch regions of the small GTPase adopt very different conformations in the GTP- and GDP-bound states, a protein that specifically interacts with these switch regions may, for all practical purposes, only associate with GTP-locked version of the GTPase. This will typically be mirrored by affinities of the effector several orders of magnitude for GTP vs GDP-bound GTPase. Although CEP19 clearly has a preference for GTP-bound RabL2, it does associate with GDP-bond RabL2 and according to our structural modeling uses both switch region-specific as well as unspecific interaction mode.

A typical GTPase effector will associate with the GTP-bound form of the GTPase with an affinity that is at least two orders of magnitude higher than the GDP-bound form. In case of the IFT-B complex, we measure the Kd of the RabL2-GTPgS bound complex as 0.59uM whereas we observe no binding for the GDP-bound form of RabL2 by ITC (at least two orders of magnitude difference). These data suggest that the IFT-B complex is a true effector for RabL2. CEP19 associates with RabL2-GTP in a complex with a Kd that is 45 times lower than that of the CEP19-RAbL2-GDP complex. Whether this is enough to designate CEP19 as a true effector of RabL2 is not so clear to us. From a practical definition, the important issue appears to be if CEP19 can distinguish between RabL2-GTP and RabL2-GDP in a cellular setting. To avoid any confusion, we have re-written the sentences on pg. 8 to now read:

The affinity of CEP19 for RabL2-GDP is thus 45 times lower than for RabL2-GTP. This result aligns with the understanding that CEP19 discerns the nucleotide state of RabL2, demonstrating a clear preference for the GTP-bound state over the GDP-bound state.

5- The authors emphasize that CEP19 is not a GAP and report it in the abstract as a finding. I would only mention or emphasize this finding if it was reported otherwise. If so the authors should cite this.

Answer: We have revised the abstract to exclude the less significant finding that CEP19 is not a GAP for RabL2. This change allows for a clearer focus on the main findings and emphasizes the more important aspects of our research.

6- The introduction in my personal opinion is quite spread. I would make it more focused on the question that they want to answer.

Answer: We have streamlined the introduction by omitting the in-depth information about the IFT-A complex, as it is not central to our current study, and by condensing the description of anterograde and retrograde IFT train ultrastructures. We have retained the sections on IFT complex assembly at the ciliary base and on IFT22/IFT27, as these proteins are integral to the complexes investigated in our manuscript. Additionally, we have heightened the focus on RabL2 in the latter part of the introduction as this is the main protein component under investigation.

Referee #2:

IFT complexes accumulate at the ciliary base before they are transported into cilia. However, how anterograde IFT is initiated is not well understood. Recently, RABL2 has also been implicated in controlling ciliary entry of IFT proteins by its interaction with IFT-B though with distinct mechanisms in Chlamydomonas and mammal. RABL2, a small GTPase interacting with CEP19 at the base of cilia, binds IFT81/74 as a GTP-specific effector to trigger the ciliary entry of IFT complexes as RABL2 mutation does not affect the basal body localization of IFT proteins but typically reduce IFT particles inside cilia (Kanie et al., 2017). The interaction of RabL2 with IFT74/81 was also reported in another study (Mishijima et al., 2017). In contrast, in Chlamydomonas, RABL2 regulates the enrichment of IFT-B at the basal body to control the amounts of IFT-B1 proteins available for assembling the anterograde IFT trains (Zhang et al., 2022 https://doi.org/10.1101/2022.02.13.480273). Thus, it appears that species-specific mechanism may exist. In addition, RABL2 has also been reported to function in regulating the export of BBSome passing through the transition zone to fine-tune ciliary signaling (Duan et al., 2020; Zhang et al., 2022; Zhou et al., 2022). These results indicate that RABL2 is associated with IFT-B and plays critical roles in regulating the anterograde IFT as well as the retrograde transport of BBSome at the ciliary base.

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IFT (Liang et al; 2014 Dev Cell). I bet the regulation of IFT entry by Rabl2 as proposed in this and other papers may be a different layer of regulation.

Answer: We appreciate this important point raised by the reviewer and acknowledge the importance of the work by Liang et al., 2014, that established the role of phosphorylation in modulating the interaction between kinesin-2 and IFT-B, thus facilitating the initiation of anterograde IFT. We agree with the assertion that the regulation of IFT entry by RabL2 and phosphorylation of the kinesin motor at the ciliary tip, could representconsecutive layers of regulation in this complex process. We have modified our discussion section on pg. 23 to emphasize this point and now reference the Liang et al. 2014 study.

2. In the abstract, it says that this work provides a rationale for why RabL2 dissociates anterograde IFT trains soon after departure from the ciliary base, which is also depicted in Fig .7. Is there published in vivo evidence in which dissociation of RabL2 from IFT trains occurs when it enters cilia? Duan et al., 2020 (Fig.4) reported that RABL2 could enter cilia and undergo bidirectional IFT in RPE1 cells, which suggests that at least a portion of RABL2 does not dissociate from IFT trains after entering cilia. It will be great to discuss this conflicting data.

Answer: In the paper published in 2017 [Kanie et al., 2017, Dev Cell, PMID: 28625565], we showed that wild-type RABL2 accumulates at the ciliary base without entering the cilium, whereas GTP-locked form of RABL2 accumulates at both the cilium and the mother centriole (Figure 4A of [Kanie et al., 2017, Dev Cell, PMID: 28625565]). We also confirm that endogenous RABL2 localize to the ciliary base but is barely detectable in the cilium using anti-RABL2 antibody (Figure S2A of [Kanie et al., 2017, Dev Cell, PMID: 28625565]). We further showed using 3D structured illumination microscopy that most of IFT trains (as marked by IFT88) has GTP-locked form of RABL2 in them, whereas very few, if any, of wild-type RABL2 co-localized with ciliary IFT particles (Figure S6A and B). This suggests that wild-type RABL2 undergoes GTP hydrolysis prior to (or soon after) the ciliary entry of IFT, and wild-type RABL2 interacts with IFT complexes mainly at the ciliary base. Duan et al. confirmed that wild-type RABL2 accumulates at the ciliary base and is barely detectable in the cilium, whereas GTP-locked form of RABL2 accumulates in the ciliary shaft (Figure 3B and E of [Duan et al., 2021, EMBO.J, PMID: 33241915]). Duan et al. needed to overexpress 3X mNeonGreen tagged wild-type RABL2 to see its ciliary localization (Fig.4A of [Duan et al., 2021, EMBO.J, PMID: 33241915]). The Chlamydomonas paper that the reviewer mentioned [Zhang et al., 2022, BioRxiv, https://doi.org/10.1101/2022.02.13.480273] also showed that YFP tagged wild-type Rabl2 is undetectable in the cilium, whereas GTP-locked Rabl2 undergoes IFT-like movement. They even expressed 3x mNeonGreen tagged wild-type Rabl2 in Chlamydomonas, and failed to detect ciliary localization of wild-type Rabl2 (Figure S2C of [Zhang et al., 2022, BioRxiv, https://doi.org/10.1101/2022.02.13.480273]).

These data all emphasize that most of, if not all, IFT-associated wild-type RABL2 locates at the ciliary base, and barely enters the cilium. While we cannot exclude the possibility that tiny fraction of RABL2 may localize to the ciliary shaft, it should be safe to say that RABL2 mainly functions at the ciliary base.

3. Just comments. I have a problem to understand how RabL2 mediates IFT entry. Loss of RabL2 or in the presence of the GDP form, IFT-B cannot enter cilia. Why does the GTP form make it possible? Or put it in another way, how does binding of IFT81/74 to RabL2-GTP would triggers IFT entry? What barriers does it break? I would welcome to include a model or hypothesis that the authors might have. Actually I could understand well the explanation in Chlamydomonas, where it says that Rabl2 is involved in recruiting IFT-B to ciliary base. Thus, dysfunction of Rabl2 may lead to limited accumulation of IFT proteins at the ciliary base, which would result in less IFT entry.

Answer: The molecular mechanism of how RabL2-GTP mediates IFT entry is indeed still unknown but could involve a conformational change that allow IFT-B to link to kinesin-II. We now explicitly mention this on pg. 23 of the revised manuscript. The additional sentences on pg. 23 read:

The molecular mechanism through which RabL2 stimulates IFT initiation remains unknown, but it might involve a conformational change within the IFT-B complex that is necessary for its association with the anterograde kinesin-II motor. It is well documented that the phosphorylation of the kinesin-II motor within the cilia of Chlamydomonas disrupts its interaction with IFT-B, an essential step for initiating retrograde IFT from the ciliary tip to the base (Liang et al., Developmental Cell, 2014).

Referee #3:

The biochemistry is conducted to high experimental standards throughout. All logical controls are included. The conclusion that a ~70aa coiled coil from IFT74/81 is a GAP for RabL2 is solid and will stand the test of time.

The functional rescue experiments (Fig. 6) are problematic and need to be revised for the paper to be accepted. As detailed below, the prior interpretation that RabL2 is an injector for IFT trains is incorrect and was rigorously rejected by a 2021 EMBO paper from Xueliang Zhu and Xiumin Yan. As already pointed by the Kanie 2017 paper, RabL2 knockout mice are viable, which would not be possible if IFT trains failed to enter cilia.

*Answer: We appreciate the thorough review and the positive comments on our biochemistry experiments. We acknowledge and appreciate the concerns raised regarding our functional rescue experiments and the interpretation of RabL2's role in IFT initiation. However, we do not acknowledge that a contribution to IFT initiation by RabL2 has been '*rigorously rejected by a 2021 EMBO paper from Xueliang Zhu and Xiumin Yan' for the following reasons:

In the paper published in 2017 [Kanie et al., 2017, Dev Cell, PMID: 28625565], we showed that RABL2 interacts with IFT holocomplex as an effector, and promotes ciliary entry of IFT. Importantly, we showed that small amount of IFT can enter the cilium even in the absence of RABL2A/B (Figure 6C-F of [Kanie et al., 2017, Dev Cell, PMID: 28625565]), strongly suggesting the existence of alternative mechanisms for IFT injection (e.g., intrinsic ability of IFT complex to enter the cilium or other regulator(s)). Consistent with this, both RABL2 and CEP19 knockout RPE cells showed severe kinetic defect of ciliation, but they eventually catch up, albeit not perfectly, on cilium formation (Figure 3C of [Kanie et al., 2017, Dev Cell, PMID:

28625565]). Upon 72 hours serum starvation, 20% of RABL2 knockout cells formed primary cilia, whereas 55-60% of the rescue lines (RABL2 knockouts expressing wild-type RABL2) formed cilia. We therefore highly recommend performing kinetic ciliation assay rather than single time point analysis to better assess cilium formation. [Duan et al., 2021, EMBO.J, PMID: 33241915] performed cilium formation assay after 48 hours of serum starvation in mouse embryonic fibroblasts (MEFs), but in our experiences MEFs complete cilium formation around 24 hours after serum starvation. One may miss the kinetic cilium formation defect if the prolonged serum starvation is induced. Therefore, the absence of cilium formation defect upon 48 hours of serum starvation does not necessarily mean the absence of kinetic cilium formation defect. This issue was pointed out in the peer-review process of the [Duan et al., 2021, EMBO.J, PMID: 33241915], which is open to public, but was not addressed in the revised manuscript. [Duan et al., 2021, EMBO.J, PMID: 33241915] also showed that frequency of cilia was not affected in several cell types in vivo including ependymal cells and kidney epithelia. However, accurately and quantitatively measuring the cilia frequency in tissue samples is a very difficult task, because of the thickness of the sample and difficulty in preservation of cilia in tissue samples. We frequently observe the loss of cilia in kidney epithelia if the sample preparation is not perfect, especially when cardiac perfusion of PFA is not performed. Therefore, we would not be surprised that partial loss of cilia is not detected in tissue samples.

Regarding the phenotype of Rabl2 knockout mice, milder phenotype of Rabl2 knockout mice compared with the severe embryonic lethality caused by complete loss of IFT subunits should not exclude the role of Rabl2 in ciliary entry of IFT. Partial loss of IFT function may not result in complete embryonic lethality and can rather cause much milder ciliopathy phenotypes, such as polydactyly and retinal degeneration. Most well-known and classical example of this is Orpk mice. The IFT88 hypomorph mutant mice are born at Mendelian ratio, and exhibit polydactyly [Moyer et al., 1994, Science, PMID: 8191288] and retinal degeneration [Pazour et al., 2002, JCB, PMID: 11916979]. In the Orpk mice, ciliation is partially, but significantly affected in many tissues [Banizs et al, 2005, Development, PMID: 16284123][Cano et al., 2004, Development, PMID: 15226261][Pazour et al., 2000, JCB, PMID: 11062270]. Therefore, it would not be surprising that Rabl2 knockout mice, which may display partial cilium formation defect as described above, could cause milder ciliopathy phenotypes instead of complete embryonic lethality.

Major points:

Abstract suggests that the purpose of RABL2 association with IFT is 'to regulate initiation of IFT '. Intro mentions that RabL2 was shown to 'regulate IFT initiation and cilium formation'. This part of the introduction is unbalanced and reads like a one-sided summary of the authors' past work with Kanie 2017 cited 6 times and Duan 2021 only cited once. (The Kanie 2017 paper is cited 21 times in total in the current manuscript). For the introduction to properly situate the knowns and unknowns of RabL2, the authors need to acknowledge that 1- RabL2 is dispensable for ciliogenesis in mice and 2- a mouse knockin bearing a GTP-locked RabL2 allele displays no alteration of IFT frequency, velocity or processivity. In light of this rigorous study, the suggestion that RabL2-GTP promotes entry of IFT trains into cilia is no longer tenable. Instead, the Duan paper carefully and rigorously articulates a role for RabL2-

GTP in antagonizing BBSome binding to IFT-B. An elegant model, which is still largely consistent with past work from the authors, is that the function of RabL2-GTP association with entering IFT trains is to suppress BBSome binding to anterograde IFT so that the BBSome does not import the cargoes that it exports from cilia.
It is important that the authors state clearly and early in the manuscript that the model of RabL2 injection is not consistent with the viability of the RabL2 knockout mouse, and that the conclusions related to a possible role of RabL2 in IFT injections are limited to RPE cells. The entire discussion related to RabL2 and IFT train injection on p.23 needs to be re-written to carefully incorporate the results published by Duan et al.

Answer: As we mentioned in the answers to the referee #2 above, we believe that the main function of RABL2 is initiation of intraflagellar transport at the ciliary base. We appreciate the work from Duan et al., and cannot exclude the possibility that a fraction of GTP-bound Rabl2 enters the cilium and may suppress the BBSome exit until Rabl2 hydrolyzes GTP. However, as shown by our previous work as well as the studies from [Duan et al., 2021, EMBO.J, PMID: 33241915] and [Zhang et al., 2022, BioRxiv, https://doi.org/10.1101/2022.02.13.480273], wild-type RABL2 is barely detectable within the cilium whereas the GTP-locked mutant of RABL2 enters the cilium and displays IFT movement. So, it should be safe to say the RABL2 mainly functions at the ciliary base. In the current paper, we showed that IFT74/81 complex has a GAP activity towards RABL2, and RABL2 likely hydrolyzes GTP once it binds to IFT complex (before or soon after IFT trains go into the cilium). This further supports our previous finding that RABL2 mainly functions at the ciliary base.

That said, we do acknowledge that the abstract/introduction in the original submission was unbalanced and have re-written these parts in the revised manuscript. The abstract now states that IFT initiation follows the recruitment of RabL2 and no longer says that RabL2 is absolutely required for said initiation. New abstract on pg. 2 states:

Cilia are important cellular organelles for signaling and motility and are constructed via intraflagellar transport (IFT). RabL2 is a small GTPase that localizes to the basal body of cilia via an interaction with the centriolar protein CEP19 before downstream association with the IFT machinery, which is followed by initiation of IFT…

We have also re-written the introduction on pgs. 6-7 of the revised manuscript to mention both IFT initiation and BBSome regulation as RabL2 functions. The Kanie et al., 2017 paper is now cited 5 times and the Duan et al., 2021 paper 4 times in the new RabL2 introduction section that reads as follows:

More recently, a third GTPase, RabL2, was shown to associate with the IFT-B complex and regulate IFT initiation and cilium formation (Kanie et al., 2017; Nishijima et al., 2017). RabL2 is required for proper ciliogenesis in both Chlamydomonas (Nishijima et al., 2017) and in mammalian cells (Kanie et al., 2017). However, RabL2 is dispensable for ciliogenesis in mice and a mouse knockin bearing a GTP-locked RabL2 allele displays no alteration of IFT frequency, velocity or processivity (Duan et al., 2021). Mutations in RabL2 cause ciliopathies including male infertility because of defects in the assembly of cilia of sperm cells (Ding et al., 2020; Lo et al., 2012). Furthermore, RabL2 controls the ciliary localization

of G-protein coupled receptors (GPCRs) in primary cilia suggesting a conserved role in the assembly/function of both motile and primary cilia (Dateyama et al., 2019). This agrees with the evolutionary conservation of RabL2 in ciliated species and the lack of RabL2 in non-ciliated eukaryotes (Eliáš et al., 2016). RabL2 is recruited to the basal body of cilia via an interaction with the centriolar protein CEP19 (Jakobsen et al., 2011) and subsequently handed over to the IFT-B complex prior to initiation of IFT at the ciliary base (Nishijima et al., 2017; Kanie et al., 2017). Knockout of CEP19 or RabL2 significantly reduces the number of IFT trains in cilia suggesting that RabL2 help control the injection of IFT trains into cilia (Kanie et al., 2017). Wild-type (WT) RabL2 was shown to dissociate from IFT trains shortly after departure from the ciliary base whereas a GTP-locked RabL2 variant (Q80L in human RabL2) stays associated with IFT trains and accumulates in cilia (Kanie et al., 2017; Duan et al., 2021). It was furthermore shown that the S35N RabL2 mutant unable to bind GTP does not rescue ciliogenesis defects of RabL2 knockout cells (Kanie et al., 2017). A recent study in mice suggested that the main function of RabL2 is not IFT initiation but rather the regulation of ciliary export of the BBSome complex and associated cargoes (Duan et al., 2021). This notion was based on observations that BBSome components and cargoes accumulate in RabL2 mutants unable to hydrolyse GTP (Duan et al., 2021). In any case, there is ample evidence that the nucleotide state of RabL2 is important for its ciliary function.

We now mention both the finding that 1) mouse RabL2 is dispensable for ciliogenesis and in mouse and 2) that a GTP-locked variant of RabL2 has the same IFT injection kinetics as WT. Importantly, we have toned down the role of RabL2 function in IFT initiation by changing the sentence' **Knockout of CEP19 or RabL2 significantly reduces the number of IFT trains in cilia suggesting a crucial function for RabL2 in controlling the injection of IFT trains into cilia**' *to* ***'Knockout of CEP19 or RabL2 significantly reduces the number of IFT trains in cilia suggesting that RabL2 help control the injection of IFT trains into cilia'.***

Regarding the discussion section on pages 22-24, we have addressed the reviewer's comments and concerns by revising the text and the RabL2 functional models (Fig. 7) to include both the potential function of RabL2 in IFT injection and in regulating the BBSome. Figure 7 now explicitly shows the function of RabL2 in IFT initiation and in regulation of BBSome trafficking. The text discussing these functions in the discussion part on pgs. 22-24 has been updated accordingly.

Fig. 6: The major question is whether IFT74 is expressed at the same level at IFT74[T438R] in the rescue lines? A simple FLAG immunoblot would address a trivial interpretation for the observed differences. As pointed by the authors, the quantitation of fluorescence in Fig. 6E 'suggests that IFT74T438R may not be expressed as strongly as WT or the structure of the mutant may be partially disturbed to prevent efficient centriole localization'. The included results suggest that the T438R mutation may affect IFT74 in other ways that by decreasing binding to RabL2, thus invalidating many of the authors' interpretations.

Answer: We agree that testing expression level of IFT74 by immunoblotting is important to properly interpret the effect of T438R in IFT74 function. We could not include this in the original manuscript, because Immunoblot experiment had not been setup in Tomoharu Kanie's new lab, where most of the cell biological experiments were performed. We have

now included the immunoblot analysis of the IFT74 mutant. As shown in the revised manuscript, the expression level of the T438R mutant was significantly lower than that of wild-type IFT74 (Fig.6E-F of the revised manuscript). This result at least partially explains why the centrosomal intensity of T438R-IFT74 is lower than that of wild-type (Fig. 6G of the revised manuscript). Although the stability issue of the mutant may partially explain the kinetic ciliation defect of the T438R mutant, the mutant can still fully rescue the stability defect of IFT81 (Fig. S6C of the revised manuscript) and partially rescue the localization defect of IFT81 (Fig. 6H of the revised manuscript), suggesting that the mutant is at least partially functional. Nevertheless, the mutant completely failed to rescue the localization defect of RABL2 (Fig. 6I of the revised manuscript), suggesting that the mutant is unable to bind to RABL2, consistent with the in vitro binding data (Fig. 6B of the revised manuscript). Since RABL2 knockout cells show kinetic defect of ciliation (Fig. 3C of [Kanie et al., 2017, Dev. Cell]) similar to the IFT74 knockout cells expressing T438R mutant of IFT74 (Fig.6D of the revised manuscript), we believe that the ciliation defect of T438R mutant of IFT74 is at least partially attributable to the loss of binding to RABL2. We have updated the text on pgs. 18-19 to reflect the new data.

Minor points:
CEP19 discriminates between GTP and GDP RabL2 by a factor of nearly 50. Yet, the authors state that 'CEP19 senses the nucleotide state of RabL2 but is not a true effector for RabL2'. This statement seems at odd with the presented data. Can the authors elaborate on the criteria that make a 'true effector'?

Answer: We have now rewritten the sentence on pg. 8 to better reflect the 45-fold difference in affinities (for the definition of an effector see also answer to reviewer #1, point 4):

The affinity of CEP19 for RabL2-GDP is thus 45 times lower than for RabL2-GTP. This result aligns with the understanding that CEP19 discerns the nucleotide state of RabL2, demonstrating a clear preference for the GTP-bound state over the GDP-bound state.

I was initially confused by the authors description of Fig. 1D 'when WT CrRabL2 and GTP were mixed with the IFT-B1 complex and incubated for 3h at room temperature, RabL2 no longer associated with the IFT- B1 complex'. It is because the sizes of IFT27 and RabL2 are extremely close and I believed that there was a RabL2 band in the IFT peak. Could the authors conduct immunoblotting of these samples for RabL2?

Answer: Fig. 1D is an ITC experiment titrating RabL2-GDP with CEP19 so we assume that the reviewer refers to Fig. 2D. CrIFT27 and CrRabL2 do indeed have similar Mw and run very close on SDS-gels. However, as seen on the gel displayed in Fig. 1A, IFT27 and RabL2 can indeed be separated with RabL2 migrating somewhat slower than IFT27. We have now repeated the experiment in Fig. 2D with a longer run-time for the SDS-gel, which clearly allows the viewer to tell IFT27 and RabL2 apart.

Intro reads like an extensive review of IFT train assembly. While the scholarship is outstanding, it could be shortened to better serve the current manuscript and expose the current gaps in knowledge regrading RabL2 function.

Answer: we have now shortened the introduction to focus on RabL2 functions more clearly. See also reply to reviewer #1.

Dear Esben,

Thank you for submitting a revised version of your manuscript. Your study has now been seen by all original referees, who find that most of their previous concerns have been addressed. There remain only a few mainly editorial points that have to be addressed before I can extend formal acceptance of the manuscript:

1. Please incorporate the final textual changes as requested by referees #1 and #3.
2. Please check that the funding information is correct and identical both in the manuscript and our online system.
3. Please submit up to five keywords.
4. Our publisher has done their pre-publication check on your manuscript. I have attached the file here. Please take a look at the word file and the comments regarding the figure legends and respond to the issues.
5. Figure panel 5D is not mentioned in the manuscript text.
6. Please remove DOIs from the reference list and update the references according to The EMBO Journal style (up to 10 authors followed by et al.) Further information can be found here: https://www.embopress.org/page/journal/14602075/authorguide#referencesformat.
7. Please rename "Conflict of interest" section into "Disclosure and competing interests statement" (further info: https://www.embopress.org/page/journal/14602075/authorguide#conflictsofinterest).
8. Please add a short table of contents at the beginning of the Appendix.
9. Please rename movies into Movie EV1 etc, remove the legends from the Appendix and zip as a README text file together with the corresponding movie file.
10. Please remove Dataset legends from the Appendix file.
11. Please submit source data as requested by our data editor after invitation to revise the manuscript. I have attached the checklist below - please note that the figure panel numbers refer to the previous version of the manuscript.
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With best wishes,

Ieva

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We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (5th Oct 2023). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions.

Referee #1:

The authors have adequately addressed my concern. They now properly re-fit their data and that resulted in the reduction of their initial calculated fold stimulation of the GTPase activity from 20 to now 7 folds. I have no major concerns. My only remark is that the authors, in the discussion, make comparisons between their quite weak stimulation of GTPases activity and between that of the SAR1 GAP sec23, which have 10 fold stimulation effect. They have to mention in this comparison that binding to the outer coat (sec31) results in further 2-10 stimulation of the sec23 GAP activity.

Referee #2:

My comments/questions have been adequately addressed. I have no further questions.

Referee #3:

The new abstract is better written and more neutral.

The intro of IFT is still too long and includes more material than the reader needs to know to appreciate the work. Most of the section on p.4 from the sentence citing Jordan 2018 to the end of the corresponding paragraph can be condensed into one sentence stating that anterograde trains remodel at the tip to assemble into retrograde trains. The detailed exposé of ciliary GTPases on p.5-6 is also too detailed for the current study. Keeping the intro on a need to know basis will maximize the impact of the paper.

The introduction of RabL2 is significantly improved in its scholarship, although there is sill room for achieving a balanced presentation of the current models. First, the role of RabL2 in regulating BBSome exit from cilia should be stated in the first sentence and on the same level as the author's favorite model to offer a balanced view of RabL2 to the intended audience. The mechanistic model of how RabL2-GTP may antagonize BBSome loading onto anterograde IFT trains needs to be spelled out early and as clearly as the author's favorite model. Ditto for p.24 where the sentence 'This ciliary accumulation of BBSomes and GPCRs let Duan et al., to suggest that RabL2 regulates BBSome mediated ciliary export (Duan et al., 2021).' needs to be changed to 'This ciliary accumulation of the BBSome and its GPCR cargoes in RabL2[Q80L] cells suggests that persistent RabL2-GTP association with IFT-B prevents BBSome association with retrograde IFT trains (Duan 2021). RabL2-GTP highly transient association with entering IFT trains may thus occludes the site on IFT-B used to latch the BBSome onto retrograde trains'.

The authors need to indicate that the defects of RabL2 knockout cells in IFT initiation and number of IFT trains are limited to RPE cells on p.6-7 {RabL2 is required for proper ciliogenesis in both Chlamydomonas (Nishijima et al., 2017) and in mammalian cells (Kanie et al., 2017).}; {Knockout of CEP19 or RabL2 significantly reduces the number of IFT trains in cilia suggesting that RabL2 help control the injection of IFT trains into cilia (Kanie et al., 2017). }; {It was furthermore shown that the S35N RabL2 mutant unable to bind GTP does not rescue ciliogenesis defects of RabL2 knockout cells (Kanie et al., 2017). } . The limitation to RPE cells need to be included in the discussion as well, in particular in the following sentence:
'Interestingly, the effect of CEP19 knockout on ciliogenesis can in part be rescued by over-expressing WT RabL2 and fully rescued by over-expressing RabL2 Q80L (Kanie et al., 2017).'

In the discussion, the following sentence needs to be corrected: 'the continued association of RabL2Q80L-GTP with IFT trains might prevent the recruitment of BBSomes to retrograde IFT trains via IFT27/25 (Eguether et al., 2014).' The reference does not provide any biochemical evidence that the BBSome binds to IFT27/25 inside retrograde IFT trains. The only demonstrated biochemical evidence of the BBSome with and IFT train subunit is with IFT38, although the functional significance of this interaction remains elusive (Nozaki 2019)

Fig. 6E now reveals that the IFT74 mutant is expressed at lower levels than the WT allele.
Fig. 6H finds that the mutant barely increases the centrosomal localization defect of IFT81.
These cautionary findings should lead the authors to further temper their interpretation that the observed defects in ciliogenesis are caused by a decrease in the RabL2-IFT74 interaction. The presented evidence is equally compatible with both interpretations (partial or no effect on RABL2-IFT74 interaction).

DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS
AARHUS UNIVERSITY

Esben Lorentzen, PhD, Associate Professor
Department of Molecular Biology and Genetics
Aarhus University
Denmark

Dear Ieva,

Thank you for the additional comments and edits on our manuscript with the title "**The IFT81-IFT74 complex enhances GTP hydrolysis to inactivate RabL2 during intraflagellar transport**". We have now made the last adjustment of the manuscript according to the comments by reviewers #1 and #3 and carried out the editorial requests. In addition, we noticed that error bars in figure 6D are invisible in many of the samples due to visualization, therefore we modified the figure to make the error bars visible resulting in a new Figure 6.

We also uploaded source data for figures to appropriate databases and link to these in the 'Data Availability' section of the main document. Finally, we have prepared and uploaded a synopsis.

We hope that the manuscript is now ready for publication.

Sincerely,

Esben Lorentzen

Dear Esben,

Thank you for addressing the final points. I sincerely apologise for the delay in communicating the decision due to the high number of submissions we receive at the moment. I am now pleased to inform you that your manuscript has been accepted for publication.

Before we forward your manuscript to our publishers, there are a couple of points that still need to be fixed:
1) In the legend for the Figure 6G-I, K please add the statistical test used, e.g., Student's t-test. I can incorporate this information in the manuscript text for you.
2) I would like to propose a couple of minor edits in the manuscript's title and synopsis. I have also written a short blurb that will accompany the title of your manuscript in our online table of contents. Please take a look and let me know if any changes are needed:

Title:
The IFT81-IFT74 complex acts as an unconventional RabL2 GTPase-activating protein during intraflagellar transport

Blurb:
The IFT-B1 complex inactivates Rab2L, promoting its dissociation from anterograde intraflagellar transport (IFT) trains for correct ciliogenesis

Synopsis
The small GTPase Rab2L regulates assembly and maintenance of cilia by acting on the intraflagellar transport (IFT) complex. Here, the IFT-B1 complex is shown to act as a GTPase-activating protein (GAP) for RabL2, inducing its dissociation from anterograde IFT trains.
• RabL2 interacting protein CEP19 has high affinity for RabL2-GTP but is not a GAP for RabL2.
• The IFT-B1 complex is a GAP for RabL2, but not for IFT27 or IFT22, the small GTPase subunits of IFT-B1.
• 70 residues of IFT81/IFT74 coiled-coil structure constitute a minimal complex that binds RabL2 to activate GTP hydrolysis.
• An IFT74 point-mutant deficient in RabL2 binding disrupts ciliogenesis and injection of IFT particles into cilia of mammalian cells.

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If you have any questions, please do not hesitate to call or email the Editorial Office. Thank you for this contribution to The EMBO Journal and congratulations on a successful publication!

Best regards,

Ieva

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USEFUL LINKS FOR COMPLETING THIS FORM

The EMBO Journal - Author Guidelines
EMBO Reports - Author Guidelines
Molecular Systems Biology - Author Guidelines
EMBO Molecular Medicine - Author Guidelines

Reporting Checklist for Life Science Articles (updated January

This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your manuscript.

Please note that a copy of this checklist will be published alongside your article.

Abridged guidelines for figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:

  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

Materials

Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Yes	Materials and Methods

Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Not Applicable	

DNA and RNA sequences	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Short novel DNA or RNA including primers, probes: provide the sequences.	Not Applicable	

Cell materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Not Applicable	
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Not Applicable	
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Not Applicable	

Experimental animals	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Laboratory animals or Model organisms: Provide species, strain, sex, age, genetic modification status. Provide accession number in repository **OR** supplier name, catalog number, clone number, **OR** RRID.	Not Applicable	
Animal observed in or captured from the field: Provide species, sex, and age where possible**.**	Not Applicable	
Please detail **housing and husbandry conditions**.	Not Applicable	

Plants and microbes	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Plants: provide species and strain, ecotype and cultivar where relevant, unique accession number if available, and source (including location for collected wild specimens).	Not Applicable	
Microbes: provide species and strain, unique accession number if available, and source.	Not Applicable	

Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Not Applicable	

Core facilities	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Not Applicable	

Design

Study protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI.	Not Applicable	
Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

Laboratory protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Not Applicable	

Experimental study design and statistics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about **sample size** estimate even if no statistical methods were used.	Not Applicable	
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Not Applicable	
Include a statement about **blinding** even if no blinding was done.	Not Applicable	
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Not Applicable	
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Not Applicable	

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	Material and Methods
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	

Ethics

Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Not Applicable	
Studies involving **human participants**: Include a statement confirming that **informed consent** was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Not Applicable	
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approva**l (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Not Applicable	
Studies involving **specimen and field samples:** State if relevant **permits** obtained, provide details of authority approving study; if none were required, explain why.	Not Applicable	

Dual Use Research of Concern (DURC)	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Could your study fall under dual use research restrictions? Please check biosecurity documents and list of **select agents and toxins** (CDC): https://www.selectagents.gov/sat/list.htm	Not Applicable	
If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	
If a study is subject to dual use research of concern regulations, is the name of the **authority granting approval and reference number** for the regulatory approval provided in the manuscript?	Not Applicable	

Reporting

The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

Adherence to community standards	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided.	Yes	
For **tumor marker prognostic studies,** we recommend that you follow the **REMARK** reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	Not Applicable	
For **phase II and III randomized controlled trials**, please refer to the **CONSORT** flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	Not Applicable	

Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Yes	MS cross-linking data have been uploaded as EV1 and EV2
Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Yes	3D coordinates (pdb files) of the structural models shown in Figs. 4A-B are uploaded as data sets with the submission. These are also available for download (see data availability section) https://www.modelarchive.org/doi/10.5452/ma-4eif4.
If publicly available data were reused, provide the respective **data citations in the reference list**.	Not Applicable	