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Intraflagellar transport complex proteins 172, 80, 57, 54, 38 and 20 form a stable tubulin-binding IFT-B2 complex

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1st Editorial Decision 05 November 2015

Thank you for the submission of your manuscript entitled "Intraflagellar transport complex proteins 172, 80, 57, 54, 38 and 20 form a stable tubulin-binding IFT-B2 complex" and please accept my apologies for the delay, but we have only now received the comments from the referess, which I copy below.

As you can see from their comments, all three referees are very supportive of your work, but point out to a number of significant concerns that will require your attention before your manuscript can be published in The EMBO Journal. I will not repeat here the referee concerns, which in many cases refer to clarifications and further discussion but may require additional experimental evidence, especially with regards to points raised by referee #3.
Thank you very much again for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE REPORTS
Referee #1:

This interesting paper from the Lorentzen laboratory represents another advance in this group's systematic dissection of the high resolution structure and function of the IFT particles that deliver axoneme subunits, notably tubulin subunits, to their site of assembly for ciliogenesis. This is a cutting edge topic in cell and molecular biology. In the current paper, the authors present evidence that the IFT particles are composed of two subcomplexes, IFTB1 and IFTB2 whose association is mediated by IFT88/52 and IFT57/38. Whereas these investigators previously showed that the IFTB1 subunits IFT74/81 bind tubulin (cited 2013 Science paper), in the current paper they further show

that the IFT54/20 subunits of IFTB2 provide a second tubulin binding site on the IFT particles, consistent with calculations of the required stoichiometry of 2 tubulins per particle required to account for the rate of flagellar growth in Chlamydomonas. This finding, together with the finding that the previously described "peripheral" subunits, IFT172, 80, 57, 54, 38/Qilin, and 20, which many of us assumed were loosely associated with the IFTB core, in fact form a stable IFTB2 subcomplex, represents a significant advance.

The work is, in my opinion, scientifically sound and generally well-written, and I believe that it will be of interest to the EMBO journal readership. Accordingly I recommend publication with only a minor suggestion for revising the introduction.

1. The authors say in the introductory paragraph that "IFT is dependent on the motor protein heterotrimeric kinesin-II to transport....." in a way that sugests that this motor is the sole anterograde IFT motor. This is almost certainly true is Chlamydomonas, but in C. elegans, some vertebrate cilia and possibly in most other cilia, two forms of kinesin-2 participate in transport along the axoneme (Snow et al, 2004, Nat Cell Biol, 6, 1109; Williams et al, 2014, Nature Commun, 5, 5813). I think this should be mentioned in a paper for a broad audience.

2. In relation to the above point, the paper is generally appropriately referenced but the authors might consider adding citations of a couple of ciliogenesis reviews as well, for the more general reader/student who might want to follow up by delving into the background literature on IFT and ciliogenesis e.g. Ishikawa and Marshall, 2011, Nat Rev mol Cell Biol, 12, 222; Nachury et al, 2010, Ann Rev CDB, 26, 59; Pedersen and Rosenbaum, 2008, Curr Topic Dev Biol, 85, 23; Scholey, 2013, Ann Rev CDB, 29, 443; Hou and Witman, 2015, Exp Cell Res, 334, 25).

Referee #2:

Intraflagellar transport is known to play a crucial role in building and maintaining cilia by transporting ciliary cargo from the cell body to the tip of the cilium (anterograde) and from the tip of the cilium to the base (retrograde). Two protein complexes are involved in the IFT, IFTA for the anterograde and IFTB (retrograde). It is known that IFTB is composed of 9 core subunits and 6 peripheral subunits. Here in this paper by Taschner et al titled "Intraflagellar transport complex proteins 172, 80, 57, 54, 38 and 20 form a stable tubulin-binding
IFT-B2 complex", the authors propose the IFTB peripheral subunits to make a stable complex that is linked to the core complex via (IFT88/52) (core)-(IFT57/38) (peripheral). Furthermore they report the crystal structures of CH-domain of CrIFT54 (residues 1-134) CH-domain of MmIFT54 (residues 1-133) CrI and MmIFT52N and show that IFT54 binds tubulin as a potential IFT cargo whereas the CH-domains of IFT38 and IFT57 mediate the interaction with IFT80 and IFT172.
In principle the claims in the paper are important and would represent an advancement in the field and for the general reader if they are true and strongly supported, however I have some major concerns/suggestions regarding this manuscript
The major finding of the paper is that the 6 subunits form a stable complex and the authors show that by gel permeation chromatography. I have several remarks concerning this experiment

1-
a- The peak is broad and covers almost till the end of the column overlapping with elution volumes of the individual proteins and sub-complexes, which would speak of the very weak separation on the column. A major concern is if you have a mixture of different species or a mix of sub-complexes or aggregation of sub-complexes you will not be able to differentiate between this case and the case of having a homogenous population of a stable complex
b- The authors did not estimate the molecular weight of the complex based on the elution volume, can you please provide that
c- On the gel provided the complex does not look like a stoichiometric complex. The right way to do it is to estimate the concentration of each species by first running gels for individual components of known concentrations and then from that you can estimate the concentration of each component (band) on the gel from your peak of the complex
d- If the 6 subunits make a stable complex, the authors should be able to pull down the complex using one component of the complex, this is a very important experiment to show

e- By mixing different components resulting in a complex, the complex peak should be higher (absorption units) than the individual peaks, which is not the case in figure 1b. Did the authors normalize their peaks? Please comment
f- The authors are not showing the full elution profile why is that ? its always good to show the void volume
2- Figure 2 a-c Major concern
a- The authors were running analytical column and then they decided to run a hiload superdex column is there a reason for that?
b- At figure 2-c IFT172 and IFT80 elute as one peak, is this because they have close molecular weight?
c- The peak of the complex of IFT57/IFT38 (around 111kda) is eluting 9 mls earlier than IFT172 (116kda) is the complex oligomerizing ? can the authors comment
d- Most importantly the figure2c there is a shift in the peak of the binary complex, which could be simply a trimeric complex and again the peak of the dimeric complex is overlapping and that would result in 4 bands on the gel. The authors have to show the elution profile of the trimeric complexes to prove there is indeed a tetrameric complex
e- 2e why are the authors using two subunits both are histidine tagged for pull down. It looks like the complex used for pull down is not stoichiometric; if you have a mixture of two different binary complexes how do you differentiate that with this experiment??

3- Figure 5a the complex used for pull down is not stoichiometric is there a reason for that?

In figure 5C it is clear that mixing different components with the complex of IFT88/52 affects the stability of the IFT88/52 complex as it is very obvious from the gel that you are losing the IFT88 band, for example when IFT80 is added
As a general comment running gels of overlapping peaks is not really informative unless the stoichiometry is assessed or there is a clear shift in peaks.
I would have liked to see affinity constants; if the proteins are available one could do ITC for example

Minor comments
Page 6, lacking 787 residues is not right
Page 7 did the authors mean IFT172 and IFT80 do not interact?

Referee #3:

This manuscript reports studies on the composition and function of the 16-subunit IFT-B complex of the larger intraflagellar transport (IFT) complex composed of 22 protein subunits. The IFT-B complex together with the 6-member IFT-A complex and microtubule motor proteins are responsible for ferrying structural components into and out of the cilium. Previously, the IFT-B complex was presumed to be composed of a "core" complex with peripherally associated proteins. This work shows that the "peripheral" proteins of the B complex also form their own separate sub-complex, now named IFT-B2, that associates with the core sub-complex, now re-named IFT-B1, and identifies proteins the mediate the interaction between the 2 sub-complexes. The authors further present evidence that IFT54 in the IFT54/20 heterodimer of the B2 sub-complex binds tubulin in vitro, and thereby becomes the second tubulin binding site in the IFT-B complex, the first being the IFT74/81 module in the IFT-B1 sub-complex. These in vitro reconstitution studies on the structure and composition of the IFT B complex, in particular the reconstitution of almost the entire complex (minus only IFT56) are a tour de force. They are well done and convincing and represent an important advance for the field that will open new avenues for dissecting the structure and function of the IFT complex. That said, the manuscript lacks a solid grounding in in vivo studies, especially with respect to the conclusion drawn that IFT54 is responsible for transporting tubulin as IFT cargo within cilia. The manuscript would be improved if it more purposefully acknowledged and explored the pros and cons of using results from in vitro studies on recombinant proteins to draw conclusions about physiological properties and activities of proteins and protein complexes in the cell. This and other, more minor concerns are outlined below.

1) As is noted in the manuscript, CrIFT38 was not detected in earlier studies of Chlamydomonas IFT complexes. Thus, even though evidence from other organisms suggests that IFT38 is part of the

IFT complex, it would strengthen this manuscript if the authors showed that IFT complexes purified from Chlamydomonas indeed contain IFT38. Recombinant IFT38 was unstable in the studies here unless it was co-expressed with IFT57. Thus, it should be straightforward to identify this heterodimer in Chlamydomonas extracts, even if the full IFT-B2 complex could not be characterized.

2) The authors expressed surprise that the recombinant IFT-B1 component IFT88/52 remains associated with recombinant IFT-B2 complex even at 1 M NaCl, given that in vivo studies with Chlamydomonas showed that B1 and B2 components dissociated at 300 mM NaCl. It is likely that endogenous proteins behave differently than recombinant proteins, and, for example, undergo post-translational modifications that regulate their properties, a possibility that should be discussed in light of this apparent discrepancy.

3) Although the data are solid that IFT54 binds soluble tubulin in these in vitro assays with recombinant IFT proteins, in the absence of in vivo evidence it is premature to conclude that it is the IFT54 protein that binds tubulin subunits as cargo during IFT to assemble a cilium. The authors did raise the idea that IFT54 could carry microtubule protofilaments*, but other functions are also possible. For example, IFT complexes are in close proximity to intact microtubules before and during IFT entry into the cilium, during movement along the axoneme, and during IFT turnaround at the ciliary tip, and it equally likely that the primary function of the tubulin-binding ability of IFT54 could be to mediate these close interactions with microtubules. In support of this idea, mouse IFT54 (MIP-T3) binds soluble tubulin, and the work by Ling and Goeddel in 2000 (as noted in the manuscript), showed that IFT54 also binds strongly to polymerized microtubules as demonstrated by sedimentation assays and by TEM images showing microtubules decorated with MIP-T3. It seems to me that the ability of IFT54 to bind strongly to polymerized microtubules cannot be ignored when attempting to discern its function, and this possibility should me more fully addressed. (*In the discussion where the authors suggest that IFT complexes could transport already pre-polymerized MT protofilaments, they might have meant to say short pieces of polymerized microtubules? I am not aware of any literature that reports the existence of free MT protofiliaments in the cytoplasm.)

4) A second concern about the conclusion that IFT54 carries tubulin as cargo during IFT comes from consideration of the affinity of IFT54/20 for soluble tubulin (Kd = ~3 uM) in light of the report that the concentration of soluble tubulin in the cytoplasm of the Chlamydomonas cell body is ~50 uM and in the cilium is 160-300 uM (Craft et al, 2015 reference). Furthermore, Craft et al showed that tubulin loading onto IFT particles is regulated during ciliary assembly. Thus, many entering IFT complexes lack tubulin in full length cilia, and tubulin is released from IFT complexes at the ciliary tip. These dynamic interactions of the IFT complex with soluble tubulin in vivo seem incompatible with the high affinity in vitro interaction between IFT54/20 and tubulin reported here, and instead argue for another low affinity, dynamically regulated tubulin binding site on the IFT complex.
I should add here that the authors might reasonably conclude that in vivo experiments to demonstrate physiological relevance of these in vitro studies on tubulin binding sites are beyond the scope of the current manuscript. If so, I believe that the field would be better served if the manuscript contained a more full and balanced discussion of the strengths and weakness of the idea that IFT54 is one of the two major sites on the IFT-B complex that transports tubulin into (and out of the cilium). The authors could present other models for the function of the tubulin-binding ability of IFT54, they could indicate that they favor the model that IFT54 indeed transports soluble tubulin, and they could suggest strategies to test their model in vivo. In my opinion, such a more conservative stance would not diminish the exciting results presented in this manuscript, but would make the manuscript even more scholarly, would indicate that the authors recognize the limitations of their own work, and would caution the field that the full story is not yet told.

5) In keeping with the above, the sentence about half-way down the first page of the introduction that begins "Tubulin cargo directly binds . . " should be revised to "In vitro studies raise the possibility that tubulin cargo directly binds . . ."

6) To avoid confusion, the first sentence in the last paragraph of the Introduction should start as follows: "Here we purified recombinant forms of the six peripheral IFT-B complex proteins of [not from] Chlamydomonas to . . ."

7) Figure 7A presents profound, elegant experimental results and should be part of the Results

section, not just briefly described in the Discussion. In addition, Figure 7B needs to be described in the text in Results.

8) The Schou et al 2014 reference reported that IFT 38 contains a CH domain followed by coiled coils. This reference should be cited at the end of the second sentence in the section with the subheading "IFT-B2 binds alpha-beta tubulin . . . "

9) In the middle of that same paragraph, I think the authors meant to text to be "We observed no direct interaction between IFT172 and IFT80 in SEC . . ."

1st Revision - authors' response 15 January 2016

Reviewer #1:
This interesting paper from the Lorentzen laboratory represents another advance in this group's systematic dissection of the high resolution structure and function of the IFT particles that deliver axoneme subunits, notably tubulin subunits, to their site of assembly for ciliogenesis. This is a cutting edge topic in cell and molecular biology. In the current paper, the authors present evidence that the IFT particles are composed of two subcomplexes, IFTB1 and IFTB2 whose association is mediated by IFT88/52 and IFT57/38. Whereas these investigators previously showed that the IFTB1 subunits IFT74/81 bind tubulin (cited 2013 Science paper), in the current paper they further show that the IFT54/20 subunits of IFTB2 provide a second tubulin binding site on the IFT particles, consistent with calculations of the required stoichiometry of 2 tubulins per particle required to account for the rate of flagellar growth in Chlamydomonas. This finding, together with the finding that the previously described "peripheral" subunits, IFT172, 80, 57, 54, 38/Qilin, and 20, which many of us assumed were loosely associated with the IFTB core, in fact form a stable IFTB2 subcomplex, represents a significant advance.

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Answer: We have now included a sentence in the introduction on pg. 2 to describe the 2 IFT kinesin motors of the C.elegans / vertebrate system.

2. In relation to the above point, the paper is generally appropriately referenced but the authors might consider adding citations of a couple of ciliogenesis reviews as well, for the more general reader/student who might want to follow up by delving into the background literature on IFT and ciliogenesis e.g. Ishikawa and Marshall, 2011, Nat Rev mol Cell Biol, 12, 222; Nachury et al, 2010, Ann Rev CDB, 26, 59; Pedersen and Rosenbaum, 2008, Curr Topic Dev Biol, 85, 23; Scholey, 2013, Ann Rev CDB, 29, 443; Hou and Witman, 2015, Exp Cell Res, 334, 25).

Answer: We have now cited three of the above mentioned reviews at the beginning of the introduction on pg. 3.

Reviewer #2
Intraflagellar transport is known to play a crucial role in building and maintaining cilia by transporting ciliary cargo from the cell body to the tip of the cilium (anterograde) and from the tip of the cilium to the base (retrograde). Two protein complexes are involved in the IFT, IFTA for the anterograde and IFTB (retrograde). It is known that IFTB is composed of 9 core subunits and 6 peripheral subunits. Here in this paper by Taschner et al titled "Intraflagellar transport complex proteins 172, 80, 57, 54, 38 and 20 form a stable tubulin-binding IFT-B2 complex", the authors propose the IFTB peripheral subunits to make a stable complex that is linked to the core complex

via (IFT88/52) (core)-(IFT57/38) (peripheral). Furthermore they report the crystal structures of CH-domain of CrIFT54 (residues 1-134) CH-domain of MmIFT54 (residues 1-133) CrI and MmIFT52N and show that IFT54 binds tubulin as a potential IFT cargo whereas the CH-domains of IFT38 and IFT57 mediate the interaction with IFT80 and IFT172.
In principle the claims in the paper are important and would represent an advancement in the field and for the general reader if they are true and strongly supported, however I have some major concerns/suggestions regarding this manuscript.
The major finding of the paper is that the 6 subunits form a stable complex and the authors show that by gel permeation chromatography. I have several remarks concerning this experiment
1-
a- The peak is broad and covers almost till the end of the column overlapping with elution volumes of the individual proteins and sub-complexes, which would speak of the very weak separation on the column. A major concern is if you have a mixture of different species or a mix of sub-complexes or aggregation of sub-complexes you will not be able to differentiate between this case and the case of having a homogenous population of a stable complex

Answer: The broad peak was mainly caused by the presence of excess amounts of individual IFT-B2 complex components but could also be due to multiple conformations of the IFT B2 complex. We now improved our reconstitution by first mixing IFT80, IFT57/38, and IFT54/20 and subjecting this mixture to ion-exchange chromatography. This way we obtained a pentameric complex of higher quality and removed excess individual components. IFT172 could not be added during this step due its salt-labile interaction (as shown in Fig. 5b). This protein was added later to the pre-assembled pentamer, before the entire mixture was subjected to SEC. Using this strategy we were able to obtain a sharper peak in SEC showing less overlap with the elution peak of individual components, and the SDS-PAGE now shows the IFT-B2 complex with improved stoichiometry (new Fig. 1c). The peak is still relatively broad, which can likely be attributed to the inherent conformational flexibility of the complex.

b- The authors did not estimate the molecular weight of the complex based on the elution volume, can you please provide that

Answer: Given that SEC is a shape dependent method for Mw determination and that IFT-B2 contains 4 coiled-coil proteins (predicted to have an elongated shape), the molecular weight estimation from SEC is not particularly informative in this case. For instance, the IFT57/38 complex elutes at 13.1 ml from the Superose6 column (corresponding to a Mw of around 380 kDa for a globular protein), but we show in Fig. S1a using analytical ultracentrifugation that it is hetero-dimeric with a Mw of ~81 kDa. For the hexameric IFT-B2(IFT172DC) complex the Mw estimate from SEC is 1400 kDa (Mw(calculated)=353 kDa) suggesting an elongated shape and/or multimer of IFT-B2. We have now included this information in the manuscript on pg. 6. We are currently carrying out small-angle X-ray scattering experiments to comprehensively address this point (oligomeric state). However, as we are yet unable to make firm conclusions we prefer not to address this point further in the current manuscript.

c- On the gel provided the complex does not look like a stoichiometric complex. The right way to do it is to estimate the concentration of each species by first running gels for individual components of known concentrations and then from that you can estimate the concentration of each component (band) on the gel from your peak of the complex

Answer: We have now improved our reconstitution strategy for the IFT-B2 complex (see answer to question 1a), and the updated gel now shows a complex with improved stoichiometry (Fig. 1c).

d- If the 6 subunits make a stable complex, the authors should be able to pull down the complex using one component of the complex, this is a very important experiment to show

Answer: We agree that this experiment was missing in the initial version of the manuscript, but in Figure 5 we did showed that the hexameric IFT-B2 complex is efficiently pulled down by IFT88/52N-GST, thus implying that the six subunits are stably bound to each other. Although IFT80 was the only subunit of the six IFT-B2 components that contained a His-tag, it could not be used to pull down the entire IFT-B2 complex because IFT172 showed strong non-specific binding to the

Ni2+-NTA resin (as outlined in the Materials and Methods section). To circumvent this problem we have now produced an IFT54/20 complex were IFT20 carries a C-terminal GST tag, and have carried out GST-pulldowns of IFT-B2. The result is shown in a new Fig. 1b, and demonstrates that IFT54/20-GST pulls down the other four IFT-B2 proteins demonstrating that these six IFT subunits form a stable complex. Unfortunately the IFT20-GST protein and the untagged IFT38 protein have nearly identical Mw and cannot be separated on the SDS gel. However, the fact that IFT57 is pulled down implies that its obligate interaction partner IFT38 is also present. This is now indicated in the legend of Figure 1.

e- By mixing different components resulting in a complex, the complex peak should be higher (absorption units) than the individual peaks, which is not the case in figure 1b. Did the authors normalize their peaks? Please comment

Answer: The peaks in Fig. 1b were normalized for better visualization of the result, which is also why there are no numbers indicated on the y-axis. This was necessary because of the significantly different Extinction coefficients of the individual IFT-B2 components. Whereas IFT80 and IFT172 absorb UV quite strongly (Ext.coeff. around 146000 M^{-1} cm^{-1}), this is not the case for IFT57/38 and IFT54/20 (62000 and 25000 M^{-1} cm^{-1}, respectively). Running stoichiometric amounts of all these four IFT-B2 components would thus result in significantly higher peaks (>6x) for IFT80 and IFT172 than for IFT54/20. The main message from the graphs in Fig. 1b is to show that the elution peak of the reconstituted hexameric IFT-B2 complex is clearly separated from the peaks of the individual component, and this is much clearer with normalized peak heights. We have now added a sentence in the figure legend to indicate that the peak heights were normalized for better illustration of the result.

f- The authors are not showing the full elution profile why is that ? its always good to show the void volume

Answer: For space reasons, we only showed the important part of the SEC profile in which the proteins and protein complexes elute. We have now included a larger range starting from 6 ml (with the void volume now indicated at 7.2 ml on that particular column) and still ending at 20 ml (corresponding to a MW of globular proteins of around 20 kDa). Since the elution volumes before 6 ml and after 20 ml are irrelevant for our reconstitution we still prefer not to show these regions in order to better visualize the elution peaks.

2- Figure 2 a-c Major concern a- The authors were running analytical column and then they decided to run a hiload superdex column is there a reason for that?

Answer: We employ a number of different SEC columns depending on amount of and size of the protein(s) and the requirement for resolution at a particular Mw range. If larger amounts (10mg or more) of complex are required for structural studies we typically switch to the hi-load column due to the higher capacity. We believe that the use of a bigger column has no influence on the message conveyed by the figure.

b- At figure 2-c IFT172 and IFT80 elute as one peak, is this because they have close molecular weight?

Answer: Yes, IFT80 and IFT172DC elute at similar volumes presumably due to the similar Mw (85 and 109 kDa) and shapes (both are predicted to contain the same domain architecture and may thus share the same overall shape) and not because of an interaction between the two proteins. We show in Fig. S2a using full-length IFT172 and IFT80 that these two proteins do not bind to each other. We now show in the new Fig. S2b that IFT172DC and IFT80 elute at similar positions on the HiLoad Superdex200 column, and have added a sentence in the figure legend of Fig. 2 which highlights this fact and refers the reader to the new Fig.S2b.

c- The peak of the complex of IFT57/IFT38 (around 111kda) is eluting 9 mls earlier than IFT172 (116kda) is the complex oligomerizing ? can the authors comment

Answer: This is likely due to the shape-dependent nature of SEC. IFT172DC is predicted to contain two N-terminal beta-propellers and may thus be relatively spherical in shape. The IFT57/38

complex is formed via interactions between the long C-terminal predicted coiled-coil domains and thus likely adopts an elongated shape (as does IFT54/20), which leads to a large shift in elution volume compared to a spherically shaped complex of the same size. In Fig. S1a we clearly show using analytical ultracentrifugation that the IFT57/38 complex is a hetero-dimer with a Mw of ~81 kDa, and thus is does not oligomerize under these conditions.

d- Most importantly the figure2c there is a shift in the peak of the binary complex, which could be simply a trimeric complex and again the peak of the dimeric complex is overlapping and that would result in 4 bands on the gel. The authors have to show the elution profile of the trimeric complexes to prove there is indeed a tetrameric complex

Answer: Differences in elution profiles for dimeric (IFT57/38), trimeric (IFT172/57/38 and IFT80/57/38) and tetrameric (IFT172/80/57/38) are relatively small because of the elongated shape of the coiled-coil IFT57/38 complex. Adding a globular (IFT172 or IFT80) to the elongated IFT57/38 complex does not change the longest axis of the complex and thus not the elution volume in SEC much. However, the shifted peak at the elution volume of 56.5ml in Fig. 2c is unlikely to be the result of overlapping smaller complexes eluting at the same volume, rather than a tetrameric complex (IFT57/38/80/172DC). The reason for this is that the case of two overlapping smaller complexes (two trimers or a trimer and a dimer), one would expect the bands for IFT57 and IFT38 to be twice as intense as the bands for IFT80 and/or IFT172DC. This is clearly not the case in Fig. 1c where all four bands are stoichiometric on the gel. The existence of two trimeric (IFT172/57/38 and IFT80/57/38) but not a tetrameric (IFT172/80/57/38) complex would furthermore suggest that the binding of IFT80 and IFT172 to IFT57/38 is mutually exclusive. This is not the case as we show in Fig. 2d that IFT80 and IFT172DC are bound by different parts of the IFT57/38 complex (IFT80 by the CH domain of IFT38, and IFT172DC by the CH-domain of IFT57), and thus there is no reason to believe that the binding to IFT80 and IFT172DC should be mutually exclusive.

e- 2e why are the authors using two subunits both are histidine tagged for pull down. It looks like the complex used for pull down is not stoichiometric; if you have a mixture of two different binary complexes how do you differentiate that with this experiment??

Answer: The pulldown shown in Figure 2e addresses if His-tagged IFT38/57 complex interacts with and pulls down untagged IFT54/20 complex. As both IFT38 and IFT57 have TEV protease cleavable tags we are not able to remove just one of the tags. However, it should not matter if one or both subunits of the bait (IFT38/57) complex are tagged, the important point is that the sub-complex being pulled down (IFT54/20) is untagged. Concerning the stoichiometry of the complex used for pulldown we are sure that IFT57 and IFT38 are stoichiometric, because IFT57 aggregates if not bound to IFT38, and IFT38 is insoluble in the absence of IFT57 (as mentioned in the text). We have purified the IFT57/38 many times (both tagged and untagged), and we have never found excess amounts of any of the two components. The sub-stoichiometric amounts of IFT54/20 pulled-down by tagged IFT57/38 (Fig. 2e) or IFT80 (Fig. 2f) is a result of the relatively weak interactions. As we mention on pg. 7, the complexes between IFT57/38-IFT54/20 and IFT80-IFT54/20 are not strong enough to co-purify. It seems likely that IFT54/20 is stably incorporated into the IFT-B2 complex through simultaneous binding to both IFT57/38 and IFT80 as mentioned on pg. 8.

3- Figure 5a the complex used for pull down is not stoichiometric is there a reason for that?

Answer: Sub-complexes of IFT88 and GST-tagged IFT52 contains excess GST-IFT52 because of the higher expression levels of this subunit and this excess co-purifies at least to some extent with the IFT88/GST52 complex. However, as GST-IFT52 does not interact with IFT-B2 (Fig 5a), the excess of GST-IFT52 does not impact the conclusions drawn from the pull-downs shown in Fig. 5, and it is clear that the interacting bands of the IFT-B2 proteins are stoichiometric with the weaker IFT88 band and not with the stronger IFT52 band.

In figure 5C it is clear that mixing different components with the complex of IFT88/52 affects the stability of the IFT88/52 complex as it is very obvious from the gel that you are losing the IFT88 band, for example when IFT80 is added

Answer: The differences in band intensities were most likely a result of protein loss during the many washing steps as well as the fact that samples stored at -80C were used. We have now repeated this

experiment several times with freshly produced samples and no longer observe this effect. There is no reason to believe that IFT80 actively affects the stability of the IFT88/52 complex. If this were the case, one would expect that the addition of IFT80 would always cause the IFT88 band to be weaker. This is however not the case. Also, we show the reconstitution of a 15-subunit IFT-B complex (containing IFT80) in Fig. 7a, and there is no visible reduction in the intensity of the IFT88 band. The message we want to get across with Fig. 5c is that only IFT57/38, but not IFT54/20 or IFT80, is able to bind directly to IFT88/52. We have now freshly purified protein components to repeat the experiment and have updated Fig. 5c with the new gels, in which the IFT88/52 complex used as bait no longer appear unstable.

As a general comment running gels of overlapping peaks is not really informative unless the stoichiometry is assessed or there is a clear shift in peaks.
I would have liked to see affinity constants; if the proteins are available one could do ITC for example

We observe a clear and reproducible shift for the IFT-B2 complex compared to individual components and (updated Fig. 1c) and again for the 15-subunit IFT-B complex compared to IFT-B1 and B2 (Fig. 7a). We agree that SEC is not optimal for assessing the interaction between elongated proteins and complexes due to the shape-dependent nature of this technique. For this reason our SEC experiments are accompanied by numerous pull-downs using affinity tagged IFT components. The IFT-B complex is stable in vitro and all components stay associated during SEC suggesting dissociation constants in the triple digit nM (or lower) range. We fail to see how the measurement of exact affinities between components within a stable protein complex will add much to our understanding of how the complex works. In the case of tubulin-binding, which represents a potential IFT-cargo interaction we have measured the affinities (1uM for IFT81/74 and 3uM for IFT54), which may help to understand under which conditions tubulin is loaded as a cargo onto IFT trains.

Minor comments
Page 6, lacking 787 residues is not right

Answer: CrIFT172 has 1755 residues and given that IFT172DC used in this study is 1-968, the 'lacking 787' (1755-968=787) seems right.

Page 7 did the authors mean IFT172 and IFT80 do not interact?

Answer: Yes, that is what we meant, the mistake has been corrected.

Referee #3

This manuscript reports studies on the composition and function of the 16-subunit IFT-B complex of the larger intraflagellar transport (IFT) complex composed of 22 protein subunits. The IFT-B complex together with the 6-member IFT-A complex and microtubule motor proteins are responsible for ferrying structural components into and out of the cilium. Previously, the IFT-B complex was presumed to be composed of a "core" complex with peripherally associated proteins. This work shows that the "peripheral" proteins of the B complex also form their own separate sub-complex, now named IFT-B2, that associates with the core sub-complex, now re-named IFT-B1, and identifies proteins the mediate the interaction between the 2 sub-complexes. The authors further present evidence that IFT54 in the IFT54/20 heterodimer of the B2 sub-complex binds tubulin in vitro, and thereby becomes the second tubulin binding site in the IFT-B complex, the first being the IFT74/81 module in the IFT-B1 sub-complex.

These in vitro reconstitution studies on the structure and composition of the IFT B complex, in particular the reconstitution of almost the entire complex (minus only IFT56) are a tour de force. They are well done and convincing and represent an important advance for the field that will open new avenues for dissecting the structure and function of the IFT complex. That said, the manuscript lacks a solid grounding in in vivo studies, especially with respect to the conclusion drawn that IFT54 is responsible for transporting tubulin as IFT cargo within cilia. The manuscript would be improved if it more purposefully acknowledged and explored the pros and cons of using results from in vitro

studies on recombinant proteins to draw conclusions about physiological properties and activities of proteins and protein complexes in the cell. This and other, more minor concerns are outlined below.

1) As is noted in the manuscript, CrIFT38 was not detected in earlier studies of Chlamydomonas IFT complexes. Thus, even though evidence from other organisms suggests that IFT38 is part of the IFT complex, it would strengthen this manuscript if the authors showed that IFT complexes purified from Chlamydomonas indeed contain IFT38. Recombinant IFT38 was unstable in the studies here unless it was co-expressed with IFT57. Thus, it should be straightforward to identify this heterodimer in Chlamydomonas extracts, even if the full IFT-B2 complex could not be characterized.

Answer: To address this point we carried out pull-downs combined with mass-spec of Chlamydomonas whole-cell extracts using GST-tagged IFT20 as bait. This experiment clearly identified IFT38 as an interaction partner suggesting that IFT38 is indeed a bona fide part of the IFT complex in Chlamydomonas. We have included the results of this experiment as a new Fig. S1b.

2) The authors expressed surprise that the recombinant IFT-B1 component IFT88/52 remains associated with recombinant IFT-B2 complex even at 1 M NaCl, given that in vivo studies with Chlamydomonas showed that B1 and B2 components dissociated at 300 mM NaCl. It is likely that endogenous proteins behave differently than recombinant proteins, and, for example, undergo post-translational modifications that regulate their properties, a possibility that should be discussed in light of this apparent discrepancy.

Answer: This is a fair comment and we now mention the possibility that PTMs might affect the stability of the IFTB1-B2 interaction in Chlamydomonas flagellar extracts (pg. 11). However, there is evidence in the literature that the endogenous IFT B1-B2 interaction in mouse cells is stable up to 1 M NaCl (Baker et al, 2003 paper showing that the IFT-B2 components IFT57 and IFT20 co-precipitate with the IFT-B1 component IFT88 after washing with 1 M NaCl), and we have included this reference in the revised version of the manuscript.

3) Although the data are solid that IFT54 binds soluble tubulin in these in vitro assays with recombinant IFT proteins, in the absence of in vivo evidence it is premature to conclude that it is the IFT54 protein that binds tubulin subunits as cargo during IFT to assemble a cilium. The authors did raise the idea that IFT54 could carry microtubule protofilaments*, but other functions are also possible. For example, IFT complexes are in close proximity to intact microtubules before and during IFT entry into the cilium, during movement along the axoneme, and during IFT turnaround at the ciliary tip, and it equally likely that the primary function of the tubulin-binding ability of IFT54 could be to mediate these close interactions with microtubules. In support of this idea, mouse IFT54 (MIP-T3) binds soluble tubulin, and the work by Ling and Goeddel in 2000 (as noted in the manuscript), showed that IFT54 also binds strongly to polymerized microtubules as demonstrated by sedimentation assays and by TEM images showing microtubules decorated with MIP-T3. It seems to me that the ability of IFT54 to bind strongly to polymerized microtubules cannot be ignored when attempting to discern its function, and this possibility should me more fully addressed. (*In the discussion where the authors suggest that IFT complexes could transport already pre-polymerized MT protofilaments, they might have meant to say short pieces of polymerized microtubules? I am not aware of any literature that reports the existence of free MT protofiliaments in the cytoplasm.)

Answer: We agree with the reviewer that the discussion/suggestion of IFT54 function in tubulin-cargo transport was perhaps a bit one-sided in the original submission. We have thus expanded the discussion on pgs. 14-15 and now clearly state that tubulin transport is only one possible function of the CH-domain of IFT54, the other being the regulation of cytoplasmic MT dynamics. To address if CrIFT54 also interacts with MT (as observed for the mammalian counterpart), we carried out a MT-sedimentation assay with CrIFT54/20. The result of this experiment is included in a new Fig S3e and demonstrates that IFT54/20, in addition to soluble tubulin-binding, also interacts with polymerized MT (mentioned on pg. 8 of the revised manuscript). We now clearly state that the functional dissection of the IFT54 CH-domain awaits in vivo experiments (pg. 15). The suggestion that IFT could be transporting a MT-protofilament has been removed from the manuscript.

4) A second concern about the conclusion that IFT54 carries tubulin as cargo during IFT comes from consideration of the affinity of IFT54/20 for soluble tubulin (Kd = ~3 uM) in light of the report

that the concentration of soluble tubulin in the cytoplasm of the Chlamydomonas cell body is ~50 uM and in the cilium is 160-300 uM (Craft et al, 2015 reference). Furthermore, Craft et al showed that tubulin loading onto IFT particles is regulated during ciliary assembly. Thus, many entering IFT complexes lack tubulin in full length cilia, and tubulin is released from IFT complexes at the ciliary tip. These dynamic interactions of the IFT complex with soluble tubulin in vivo seem incompatible with the high affinity in vitro interaction between IFT54/20 and tubulin reported here, and instead argue for another low affinity, dynamically regulated tubulin binding site on the IFT complex.
I should add here that the authors might reasonably conclude that in vivo experiments to demonstrate physiological relevance of these in vitro studies on tubulin binding sites are beyond the scope of the current manuscript. If so, I believe that the field would be better served if the manuscript contained a more full and balanced discussion of the strengths and weakness of the idea that IFT54 is one of the two major sites on the IFT-B complex that transports tubulin into (and out of the cilium). The authors could present other models for the function of the tubulin-binding ability of IFT54, they could indicate that they favor the model that IFT54 indeed transports soluble tubulin, and they could suggest strategies to test their model in vivo. In my opinion, such a more conservative stance would not diminish the exciting results presented in this manuscript, but would make the manuscript even more scholarly, would indicate that the authors recognize the limitations of their own
work, and would caution the field that the full story is not yet told.

Answer: The 50mM of tubulin in the cell body reported by Craft et al., 2015 was not an actual measurement but rather an estimation based on various assumptions. As we do not know with any accuracy the fraction of the tubulin that is polymerized vs the fraction available for IFT, it is hard to make any firm conclusions regarding what the affinity measured in vitro means for the occupancy of tubulin on IFT trains in vivo. However, we agree with the reviewer that observations of fully tubulin-loaded IFT trains in growing flagellar and largely tubulin-empty trains in fully formed steady-state flagella observed by the Lechtreck lab do point to a regulation mechanism beyond mere affinities. These points are now being discussed on pgs. 14-15 of the revised manuscript.

5) In keeping with the above, the sentence about half-way down the first page of the introduction that begins "Tubulin cargo directly binds . . " should be revised to "In vitro studies raise the possibility that tubulin cargo directly binds . . ."

Answer: This has been changed accordingly.

6) To avoid confusion, the first sentence in the last paragraph of the Introduction should start as follows: "Here we purified recombinant forms of the six peripheral IFT-B complex proteins of [not from] Chlamydomonas to . . ."

Answer: We thank the reviewer for pointing this out. The sentence has now been changed accordingly.

7) Figure 7A presents profound, elegant experimental results and should be part of the Results section, not just briefly described in the Discussion. In addition, Figure 7B needs to be described in the text in Results.

Answer: The reconstitution of the 15-subunit IFT-B complex is now covered in a new paragraph of the result section on pg. 13, and Fig. 7B is explicitly referred to in this paragraph.

8) The Schou et al 2014 reference reported that IFT 38 contains a CH domain followed by coiled coils. This reference should be cited at the end of the second sentence in the section with the subheading "IFT-B2 binds alpha-beta tubulin . . . "

Answer: The Schou paper was already cited a few sentences later, where we say that IFT38, 57 and 54 contain additional CH domains. We believe that this is the appropriate place for this citation in this particular paragraph. The second sentence is about the Bhogaraju et al Science paper and deals with the IFT81/74 tubulin-binding module.
We suspect that the reviewer meant the second sentence in the section with the subheading 'Architecture of the IFT-B2 complex'. This sentence deals with the domain architecture of IFT57, IFT54 and IFT38, and we have now included a reference to the Schou paper at this position.

9) In the middle of that same paragraph, I think the authors meant to text to be "We observed no direct interaction between IFT172 and IFT80 in SEC . . ."
subunit identification on pg. 4.
Answer: This mistake has now been corrected.

2nd Editorial Decision 21 January 2016

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Journal Submitted to: EMBO J
Manuscript Number: EMBOJ-2015-93164R

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- 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.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

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.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	N/A
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5. For every figure, are statistical tests justified as appropriate?	Yes, Statistical analysis in the proteomics experiments (Fig. S1B and Fig. S4), were performed by two sample t-test with number of randomization = 250; FDR=0.05 and s0=0.1, The FDR values were used for multiple test correction.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	We estimate the FDR based on 250 randomization, therefore, there is no strict requirement that the data is normally distributed.
Is there an estimate of variation within each group of data?	Yes, it is taken into account in case of two sample t-test
Is the variance similar between the groups that are being statistically compared?	Yes, it is similar between the groups as observed from variance values.

C- Reagents

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6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	We used the : 'mouse monoclonal anti-alpha tubulin, Sigma-Aldrich T9026' antibody to stain for tubulin in western blots
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	N/A

* for all hyperlinks, please see the table at the top right of the document

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F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	Crystal structures reported in the manuscript are deposited to the PDB with codes: 5fmt, 5fmu, 5fmr and 5fms
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