EMBO reports

The two-step cargo recognition mechanism of heterotrimeric kinesin

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Dear Prof. Hirokawa,

Thank you for submitting your research manuscript for consideration by EMBO reports. It has now been seen by three experts in the field, and we have received the full set of their comments, which are included below.

As you will see, referee #1 is very positive and supportive of the work, finds it significant, novel and of high quality, and only has a few minor suggestions for textual improvement. Referee #2 also acknowledges the high technical quality of the study but also identifies limitations and raises the important concern that the main conclusion of the study is not sufficiently supported by the currently available data. Referee #3, on the other hand, finds that this is a very focused study that is suitable only for a specialized readership. However, the conclusion of our editorial assessment, taking also into consideration the reports of referees #1 and #2, is that this study addresses a significant problem in molecular cell biology (i.e. how motor proteins recognize and bind their cargo for transport) and that the advance provided is sufficient for further consideration by EMBO reports.

We would therefore like to invite you to revise your manuscript with the understanding that the concerns of referees #1 and #2 (as detailed in their reports) must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript. If you have any questions or comments, we can also discuss the revisions in a video chat, if you like.

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 usually recommend a revision within 3 months (May 23rd). Please discuss with me the revision progress ahead of this time if you require more time to complete the revisions.

Please note that you can publish the study either as a Report or as an Article. For Reports, the manuscript should not exceed 27,000 characters (including spaces but excluding materials & methods and references) and 5 main plus 5 expanded view figures. The results and discussion sections must further be combined, which will help to shorten the manuscript text by eliminating some redundancy that is inevitable when discussing the same experiments twice. For an Article there are no length limitations, but it should have more than 5 main figures and the results and discussion sections must be separate. In both cases, the entire materials and methods must be included in the main manuscript file.

*****
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6) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here: <https://www.embopress.org/page/journal/14693178/authorguide#expandedview>

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Please remember to provide a reviewer password if the datasets are not yet public.

The accession numbers and database should be listed in a formal "Data availability " section (placed after Materials and Methods) that follows the model below (see also < https://www.embopress.org/page/journal/14693178/authorguide#dataavailability>):

Data availability

The datasets (and computer code) produced in this study are available in the following databases:

- RNA-seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

*** Note: all links should resolve to a page where the data can be accessed. ***

*** Note: the Data Availability Section is restricted to new primary data that are part of this study. ***

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The following points must be specified in each figure legend:

- the name of the statistical test used to generate error bars and P values,
- the number (n) of independent experiments (please specify technical or biological replicates) underlying each data point,
- the nature of the bars and error bars (s.d., s.e.m.)
- If the data are obtained from n {less than or equal to} 2, use scatter plots showing the individual data points.

Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.

See also the guidelines for figure legend preparation:
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- Please also include scale bars in all microscopy images.

10) We now request publication of original source data with the aim of making primary data more accessible and transparent to the reader. Our source data coordinator will contact you to discuss which figure panels we would need source data for and will also provide you with helpful tips on how to upload and organize the files.

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Yours sincerely,

Ioannis Papaioannou, PhD
Editor
EMBO reports

Referee #1:

This excellent paper from Hirokawa and colleagues addresses the general problem of how cytoskeletal motor proteins recognize and bind their intracellular cargo for transport along cytoskeletal tracks. Specifically, they focus on the important MT-based motor protein, heterotrimeric kinesin-2, which has diverse intracellular transport functions in cilia, neuronal processes as well as within the cytoplasm.

Using a variety of structural approaches including small angle X-ray scattering, HS AFM, chemical crosslinking/mass spec and cryo-EM, they uncover a sequential stepwise binding of one of the multiple cargoes of heterotrimeric kinesin-2, namely the RNA-binding tumour suppressor, APC, to the cargo-binding domain of the motor. The work, which seems rigorous and of very high quality, reveals that the cargo interacting domain (ABK comprising the C-terminal regions of KIF3A/3B and KAP) exists in a extended 27nm long conformation containing a presumptive APC-binding cleft formed from the motor subunit tail domains and the accessory KAP subunit (fig. 1E). APC-binding to this cleft induces a conformational change in which the proximal motor subunit coiled-coil stalk collapses onto a portion of APC, forming a compact 19.5 nm motor-cargo complex (fig 2H and fig 3C). The formation of this complex, which is monitored by HS-AFM, is proposed to proceed through D-T-L conformations leads to a "locked" motor-cargo complex that is stabilized by the coiled-coil-cargo interactions (fig 5). The role of cc-cargo binding in the proposed locking mechanism is supported by HS-AFM in which enhanced "tapping" induces the dissociation of cc from APC, which facilitates motor-cargo dissociation.
I think this is a highly significant advance in our understanding of motor-cargo interactions. The work is explained in a clear and

scholarly fashion and, in my opinion, leads to important and plausible conclusions that will lay the foundation for further work on this cutting-edge problem. Therefore, I strongly recommend this work for publication in EMBO reports!

To answer your specific questions:
• 1. Does this manuscript report a single key finding? YES: novel mechanistic pathway for motor-cargo binding.
• 2. Is the reported work of significance, or does it describe a confirmatory finding or one that has already been documented using other methods or in other organisms etc? YES it is significant/NO it is not simply confirmatory.
• 3. Is it of general interest to the molecular biology community?
If YES, please say why, in a single sentence. If NO, please state which more specialized community you feel it is aimed at (or none), in a single word or phrase. YES, because the general problem of how motors recognize, bind and transport their cargo is a poorly understood, cutting-edge topic in cell and molecular biology
• 4. Is the single major finding robustly documented using independent lines of experimental evidence (YES), or is it really just a preliminary report requiring significant further data to become convincing, and thus more suited to a longer¬format article (NO)? YES the work is rigorous and complete.

Minor suggestions for revision: I think that the paper is clearly written and I just have a few minor suggestions for additional comments to be incorporated if the authors feel inclined to do so.
1. In the introduction at the top of page 2 of the introduction where it says ".....and KIF3A/C/KAP3 (Muresan et al, 1998), the authors might also cite the recent paper by Garbouchian et al 2022 Mol Biol Cell which provides convincing evidence that KIF3A/C (like KIF3A/B) forms a heterotrimer with the KAP subunit, something which had previously been disputed by some authors.

2. In the discussion section, it might be useful to discuss the relationship (if any) between the D, T and L conformational states seen in this study with the ionic-strength-dependent conformational changes seen in the purified heterotrimeric kinesin holoenzyme by hydrodynamic and rotary shadow EM studies (cited Wedaman et al, 1996, JCB paper).

3. The heterotrimeric kinesin-cargo interaction is thought to be very flexible and possible lacking the high specificity of e.g. enzyme-substrate interactions. For example, in addition to binding APC and catenin/cadherins, this motor appears bind to IFT-B (in Chlamy and vertebrate cilia) or IFT-A (in C. elegans cilia) or in some cases directly to ciliary tubulins and GLI proteins, as well as to choline acetyl transferase and probably to other cargoes as well. Can the authors discuss any insights their new structural analysis provides into this flexibility of cargo binding?

Referee #2:

Heterotrimeric kinesin KIF3/KAP3 complex binds its cargo through the tail domain. The structures of the trimeric tail domain (ABK) and its complex with a cargo fragment (ABK-APC_ARM) were studied using small-angle X-ray scattering (SAXS), high-speed atomic force microscopy (HS-AFM), and cryogenic electron microscopy (cryo-EM). Ab initio modeling of ABK with SAXS data indicated a globular core with a cleft and an extension attributed to the coiled-coil stalk. On the other hand, the SAXS data of ABK+APC suggested a more compact structure with a maximum dimension (Dmax) of 195 Å reduced from 270 Å of ABK. Cross-linking mass-spectroscopy (XL-MS) revealed that the APC _ARM interacts with both the globular core of the ABK and the coiled-coil. Combining these findings with the dynamic conformational changes observed by HS-AFM and various conformations detected by cryo-EM, the authors proposed a model of stepwise cargo docking. Individual experiments seem to have been performed at high quality. On the other hand, it is not obvious whether the data from different approaches are consistent with each other.

Major points
The HS-AFM and cryo-EM suggest conformational varieties and dynamics both in ABK and ABK-APC. However, the ab initio modeling based on the SAXS data seems to have been done assuming a single conformation for each construct (at least, nothing is mentioned about the conformational heterogeneities and modeling of the conformational ensemble). The models in Fig. 1E and Fig. 2H are averages of 20 runs assuming a uniform structure for each. The rationale for this assumption, which is not supported by HS-AFM and cryo-EM, and the resolution/reliability of the final models (something equivalent to RMSD for the NMR structure,) remain unclear. Multistate modeling or ensemble modeling as well as evaluation of the model resolution (eg. PMID: 27840683) should be considered.

In Fig. 5A, data by AFM, SAXS, and cryo-EM are integrated. Multiple conformations of ABK-APC observed by HS-AFM and cryo-EM are ordered in steps, Form D (docked), Form T (transition), and Form L (locked). However, firm evidence for this temporal order is missing. Moreover, although various 2D configurations of ABK-APC observed by cryo-EM are assigned to the HS-AFM structures, very similar conformational variety is also found for ABK (Fig. EV5B) even in the absence of the APC cargo. These indicate that ABK takes multiple conformations independently of the cargo docking although their probabilities might be affected by the cargo binding. "The stepwise cargo recognition and binding" doesn't seem to be well supported by the current data. A straightforward test of the authors' model is to block the coiled coil-APC interaction by mutation or by swapping the

coiled-coil with one from another protein. This should eliminate the locked conformation if the stepwise model is true.

Minor points
(page 4) What is 'Dmax'?

Fig. 1B What are "M.W.", "L.S." and "R.I."

Fig. 1C What are the colors?
In SEC-MALS (Fig. 1B), the peak elution corresponded to the steady part of the MW curve. Why, in SEC-SAXS, didn't the peak elution (forward scattering) match with the fraction with the steady Rg?
Better reasoning is necessary for choosing the second half of the elution peak. What would be the results if the first half were analyzed?

Fig. 1D How was the theoretical SAXS profile calculated? Was it based on the model in Fig. 1E?

Fig. 1C and Fig. 2F These are the same kind of data (SEC-SAXS). Better in the same style. The x-axis should be in volume (mL) so that we can compare these with Fig. 1B and Fig. 2E (SEC-MALS)

Fig. 2F indicates that Rg of ABK-APC is ~50 Å smaller than that of ABK (~60 Å, Fig. 1C). However, Fig. 1B shows that ABK elutes at 11.5 mL later than 9.75 mL of ABK-APC (Fig. 2E) from the same column (SuperdexTM 200 increase 10/300 column). Are these consistent?

(page 5) "To investigate the transitional conformations through cargo binding between ABK and ABK-APCARM, we continued the observation of ABK-APCARM and intensively induced cargo dissociation by continuous tapping of AFM". This assumes that the association and dissociation take the same intermediate state and it is induced by mechanical stress. This may be true but needs better reasoning.

Fig. 4B and C The main text reads that both of these were done with increasing tapping force. However, this is not clear for B from the legend. Curves for increasing tapping force would be helpful.

Fig. 4D What are the solid and dotted lines? With the current plots, it is not clear whether the variation is due to different molecules or due to the dynamics of individual molecules. The distal-center distance (or length of the protrusion) of each molecule should be plotted against time (3 curves for each construct? Is n=3 enough?).

(Fig. EV5) The circles are too small and the empty gray areas are too large.

Movies of ABK should also be included.

Referee #3:

The manuscript characterizes the structure of the cargo binding tail of KIF3A/B in absence and presence of a fragment of one of its binding partners, APC. The SEC-SAXS and HS-AFM give relatively low resolution structures, but suggest that the coiled-coil is involved in the binding. By enhancing the tapping strength of the AFM, the authors disrupt the complex and make the quite weak argument that the pathway of assembly may be similar to the pathway of AFM-induced disassembly.

The structural information on the cargo binding domain and partner is good information to have in the literature, but this is an exceedingly focused study that is suitable for only a specialized readership. The data from the structures is the content, there are no functional or biological data, and the extrapolations the authors make from their structures are of limited impact.

Referee #1:

This excellent paper from Hirokawa and colleagues addresses the general problem of how cytoskeletal motor proteins recognize and bind their intracellular cargo for transport along cytoskeletal tracks. Specifically, they focus on the important MT-based motor protein, heterotrimeric kinesin-2, which has diverse intracellular transport functions in cilia, neuronal processes as well as within the cytoplasm.

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you feel it is aimed at (or none), in a single word or phrase. YES, because the general problem of how motors recognize, bind and transport their cargo is a poorly understood, cutting-edge topic in cell and molecular biology

• 4. Is the single major finding robustly documented using independent lines of experimental evidence (YES), or is it really just a preliminary report requiring significant further data to become convincing, and thus more suited to a longer format article (NO)? YES the work is rigorous and complete.

Response: We thank the Reviewer for the positive comments.

Minor suggestions for revision: I think that the paper is clearly written and I just have a few minor suggestions for additional comments to be incorporated if the authors feel inclined to do so.

1. In the introduction at the top of page 2 of the introduction where it says ".....and KIF3A/C/KAP3 (Muresan et al, 1998), the authors might also cite the recent paper by Garbouchian et al 2022 Mol Biol Cell which provides convincing evidence that KIF3A/C (like KIF3A/B) forms a heterotrimer with the KAP subunit, something which had previously been disputed by some authors.

Response: We thank the Reviewer for the valuable suggestion. We have added the citation of the mentioned paper (Garbouchian et al., 2022, Mol Biol Cell) at the top of page 3 in the revised manuscript.

2. In the discussion section, it might be useful to discuss the relationship (if any) between the D, T and L conformational states seen in this study with the ionic-strength-dependent conformational changes seen in the purified heterotrimeric kinesin holoenzyme by hydrodynamic and rotary shadow EM studies (cited Wedaman et al, 1996, JCB paper).

Response: Thanks for the constructive suggestion. We have added the discussion on kinesin-2 conformations seen in our study with the relevant findings in the JCB paper (Wedaman et al, 1996) in the revised manuscript.

(p.7, para.1)

"...its stabilizing trend after APC_{ARM} binding (Figs. 4-5). These findings on the conformational heterogeneity of kinesin-2 are in line with the previous rotary shadow EM studies on kinesin-2 that observed both globular and extended conformations (Wedaman ***et al***, 1996)."

(p.8, para.1)

"...This binding mechanism might be implicated in the precise regulation of kinesin-mediated cargo transport by upstream signals such as kinases. In addition, ionic-strength-dependent conformational changes of kinesin-2 were reported that the globular conformer can be converted to the extended conformer with elevated ionic strength, which suggested that ion concentration-mediated conformational change might also be a regulatory factor for cargo loading and unloading of kinesins in cells."

3. The heterotrimeric kinesin-cargo interaction is thought to be very flexible and possible lacking the high specificity of e.g. enzyme-substrate interactions. For example, in addition to binding APC and catenin/cadherins, this motor appears bind to IFT-B (in Chlamy and vertebrate cilia) or IFT-A (in C. elegans cilia) or in some cases directly to ciliary tubulins and GLI proteins, as well as to choline acetyl transferase and probably to other cargoes as well. Can the authors discuss any insights their new structural analysis provides into this flexibility of cargo binding?

Response: Thanks for the valuable comments and suggestion. We have added the discussion on the flexibility of cargo binding of kinesin-2 in the discussion section of the revised manuscript.
(p.8, para.2)

"The heterotrimeric kinesin-cargo interaction is thought to be very flexible as this motor reportedly not only binds ARM domain-containing cargoes including APC and catenin/cadherins, but also interacts with IFT complexes, GLI and many other cargo proteins which possibly lacking the high specificity. Our structural analysis suggests that the extended conformation of KIF3 tail-KAP3 region with a potential binding cavity underlines the specific binding with its high-affinity cargo such as APC, while the flexibility of the binding cavity and the coiled-coil region was also indicated by the intermediate and globular conformations observed, which may confer the additional binding capability with other cargoes on kinesin-2."

Referee #2:

Heterotrimeric kinesin KIF3/KAP3 complex binds its cargo through the tail domain. The structures of the trimeric tail domain (ABK) and its complex with a cargo fragment (ABK-APC_ARM) were studied using small-angle X-ray scattering (SAXS), high-speed atomic force microscopy (HS-AFM), and cryogenic electron microscopy (cryo-EM). Ab initio modeling of ABK with SAXS data indicated a globular core with a cleft and an extension attributed to the coiled-coil stalk. On the other hand, the SAXS data of ABK+APC suggested a more compact structure with a maximum dimension (Dmax) of 195 Å reduced from 270 Å of ABK. Cross-linking mass-spectroscopy (XL-MS) revealed that the APC _ARM interacts with both the globular core of the ABK and the coiled-coil. Combining these findings with the dynamic conformational changes observed by HS-AFM and various conformations detected by cryo-EM, the authors proposed a model of stepwise cargo docking. Individual experiments seem to have been performed at high quality. On the other hand, it is not obvious whether the data from different approaches are consistent with each other.

Major points

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Response: We thank the Reviewer for the valuable comments and suggestions. We have revised the presentation and description of the SAXS results to indicate the conformational varieties observed by SAXS analysis. On the other hand, in the descend side of the peak (regions with a single value of R_g), HS-AFM and Cryo-EM record snapshots of a single molecule, while SAXS shows the average structure of the molecule in solution during the exposure time. In addition, 20 dummy atom models were calculated for the scattering curves, but the approximate structures obtained are explained by a single cluster, suggesting that the average structure over a long period of time is a single conformation. The relative text was revised accordingly.

(p.3, para.4)

“Next, The ABK protein was further investigated in solution by SEC-SAXS analysis. The chromatogram results show that the radius of gyration (R_g) between the ascent and descent sides of the peak are different (Figs. 1C and EV1A). The shape of the scattering profile on the ascent side of the peak differs from that of the descent side of the peak, where the R_g value is stable (Fig. EV1B). In addition, the Guinier plot also shows a rise of the scattering profile on the small angle region (Fig. EV1C). The above results indicated the conformational heterogeneity of ABK protein and a possible existence of oligomeric components in the ascent side of the peak, which made it difficult to obtain a

single structure. Therefore, we calculated the structure using only the descent side of the peak in this paper (Fig. 1C)."

The models in Fig. 1E and Fig. 2H are averages of 20 runs assuming a uniform structure for each. The rationale for this assumption, which is not supported by HS-AFM and cryo-EM, and the resolution/reliability of the final models (something equivalent to RMSD for the NMR structure,) remain unclear. Multistate modeling or ensemble modeling as well as evaluation of the model resolution (eg. PMID: 27840683) should be considered.

Response: We thank the Reviewer for the constructive comments and advices. We have added the results of 20 runs of DAMMIF modeling in the extended figures (Figs. EV1D and EV2C), which indicates that they shared a similar conformation that can be grouped into one cluster. All the structures obtained here exhibited a characteristic formation of a C-shaped upper part and a rod-like lower part for ABK, and an approximately spherical structure for ABK-APC$_{ARM}$. These models also indicate the flexibility of the coiled-coil domain as observed in AFM and cryo-EM analysis consistently.

Evaluations of the model resolution cannot be done since there is no high-resolution structure model of these proteins. Also, we attempted the multistate modeling using also the R_g unstable region of the diffraction data (corresponding to the former part of the ABK peak shown in the Fig. EV1A) but failed to get reliable results because of the conformational heterogeneity, and thus we selected the R_g stable region of low conformational heterogeneity for the reliable modeling of the major conformations as shown by DAMMIF results. We have added the explanations on this issue in the revised manuscript.

(p.4, para.1)

"…20 individual structural models were calculated by the DAMMIF analysis, which were then superimposed and analyzed using cluster analysis. One of the structures was excluded from subsequent calculations based on the normalized spatial discrepancy (NSD) score obtained during the superimposition process. Consequently, the remaining 19 models are presented, and all the structures obtained here exhibited a characteristic formation of a C-shaped upper part and a rod-like lower part (Fig. EV1D). These structures were averaged, and the excess portions of each structure relative to the average structure were filtered out to create an initial structure. This initial structure was used for the final DAMMIN calculation, and the resulting structure from this process was considered as the final model…"

(p.4, para.2)

"…The R_g distribution along the elution peak of ABK-APC$_{ARM}$ is stable compared to ABK (Fig. 2F) and the DAMMIF models exhibit approximately spherical structures (Fig. EV2C)."

In Fig. 5A, data by AFM, SAXS, and cryo-EM are integrated. Multiple conformations of ABK-APC

observed by HS-AFM and cryo-EM are ordered in steps, Form D (docked), Form T (transition), and Form L (locked). However, firm evidence for this temporal order is missing. Moreover, although various 2D configurations of ABK-APC observed by cryo-EM are assigned to the HS-AFM structures, very similar conformational variety is also found for ABK (Fig. EV5B) even in the absence of the APC cargo. These indicate that ABK takes multiple conformations independently of the cargo docking although their probabilities might be affected by the carg binding. "The stepwise cargo recognition and binding" doesn't seem to be well supported by the current data. A straightforward test of the authors' model is to block the coiled coil-APC interaction by mutation or by swapping the coiled-coil with one from another protein. This should eliminate the locked conformation if the stepwise model is true.

Response: Thanks for the valuable comments and suggestions. We have attempted to construct coiled-coil swapping mutations with those from other proteins but failed to obtain soluble fraction for subsequent evaluation as the swapping mutants formed aggregates or highly polymerized complex eluted in the void of SEC. We think that it could result from the susceptibility of the aggregation of coiled coils. Alternatively, we constructed and analyzed a soluble BBK (KIF3B(BCT)-KAP3) complex as an additional group, which shows less conformational flexibility (Fig. EV4 and Movie EV4) and no binding to APC (Fig. EV2B).

Moreover, we agree with the Reviewer that the evidence for the temporal binding model is missing, we thereby revised the “stepwise binding” to be “two-step binding”. Relevant text was revised and discussion was added in the revised manuscript on this issue.

(p.6, para.3)

“In addition, the KIF3-BCT/BCT/KAP3 (BBK) complex that showed no binding with APC (Fig. EV2B) was also purified and evaluated by AFM for comparison (Fig. EV4D-E). The result suggested that BBK exhibits an average length of protrusion similar to ABK but shows less conformational flexibility than ABK and ABK-APC$_{ARM}$ (Fig. EV4F-G).”

(p.8, para.1)

“Moreover, we cannot exclude the possibility that intermediate conformations in cargo association of kinesin-2 are different from what we observed in cargo dissociation by AFM, which needs to be further tested by blocking the coiled coil-cargo interaction in the future.”

Minor points

(page 4) What is 'Dmax'?

Response: We thank the Reviewer for the careful scrutiny. The explanation of D_{max} was added in the text.

“...and the real-space R_g and the maximum particle diameter (D_{max}) were estimated to be ~62.7 Å and 270 Å for ABK (Table EV1).”

Fig. 1B What are "M.W.", "L.S." and "R.I."

Response: We thank the Reviewer for the careful scrutiny. We have added the explanation of these abbreviations (Molecular weight/M.W.; Laser scattering/L.S.; Refractive index/R.I.) in the figure legend of Fig 1B.

Fig. 1C What are the colors?

Response: We thank the Reviewer for the careful scrutiny. We have revised the colors and added the descriptions in the figure legend of Fig. 1C for a clearer presentation.

In SEC-MALS (Fig. 1B), the peak elution corresponded to the steady part of the MW curve. Why, in SEC-SAXS, didn't the peak elution (forward scattering) match with the fraction with the steady Rg? Better reasoning is necessary for choosing the second half of the elution peak. What would be the results if the first half were analyzed?

Response: Thank the Reviewer for the valuable comments. We consider the difference in the ABK peaks between SEC-MALS and SEC-SAXS may result from the different experimental conditions, and a steady MW does not certainly correspond to a steady R_g. We have attempted using the first half of the peak for SAXS analysis and modeling (Fig.EV1). The chromatogram results show that the R_g between the ascent and descent sides of the peak are different (Fig. EV1A). The shape of the scattering profile on the ascent side of the peak differs from that of the descent side of the peak (Fig. EV1B), where the R_g value is stable. In addition, the Guinier plot also shows a rise of the scattering profile on the small angle region (Fig.EV1C). Based on the above results, the ascent side of the peak contains an oligomeric component, making it difficult to obtain a single structure. Therefore, we calculated the structure using only the descent side of the peak in this paper for a reliable analysis. We have added the reasoning and discussion on issue in the revised manuscript.

(p.3, para.4)

“Next, The ABK protein was further investigated in solution by SEC-SAXS analysis. The chromatogram results show that the radius of gyration (R_g) between the ascent and descent sides of the peak are different (Figs. 1C and EV1A). The shape of the scattering profile on the ascent side of the peak differs from that of the descent side of the peak, where the R_g value is stable (Fig. EV1B). In addition, the Guinier plot also shows a rise of the scattering profile on the small angle region (Fig. EV1C). The above results indicated the conformational heterogeneity of ABK protein and a possible existence of oligomeric components in the ascent side of the peak, which made it difficult to obtain a single structure. Therefore, we calculated the structure using only the descent side of the peak in this paper (Fig. 1C).”

Fig. 1D How was the theoretical SAXS profile calculated? Was it based on the model in Fig. 1E?

Response: We thank the Reviewer for the valuable question. The answer to this question is yes. In

Fig. 1D, the theoretical SAXS profile calculated from the final dummy atom model (shown in Fig. 1E) is compared with the experimental SAXS profile. The derivation of the theoretical profile and this comparison are performed by the program DAMMIN.

Fig. 1C and Fig. 2F These are the same kind of data (SEC-SAXS). Better in the same style. The x-axis should be in volume (mL) so that we can compare these with Fig. 1B and Fig. 2E (SEC-MALS)

Response: We thank the Reviewer for these valuable comments. We have revised the figures into the same style for a better comparison.

Fig. 2F (SAXS) indicates that Rg of ABK-APC is ~50 Å smaller than that of ABK (~60 Å, Fig. 1C). However, Fig. 1B (SEC-MALS) shows that ABK elutes at 11.5 mL later than 9.75 mL of ABK-APC (Fig. 2E) from the same column (SuperdexTM 200 increase 10/300 column). Are these consistent?

Response: We thank the Reviewer for this valuable comments. The estimated values from SEC and SAXS result from an integrate effect and balance of stalk radius, molecular volume and molecular shape, etc... The analyzed kinesin-2 complexes exhibit multistate forms as well as conformational flexibility, and ABK is of higher flexibility and heterogeneity than ABK-APC. We think that could be the reason why ABK exhibits a larger R_g than ABK-APC. And in this case, R_g comparison could not normally correspond to comparison of the elution volume in SEC. We have added the discussion on this issue in the revised manuscript.

(page 5) "To investigate the transitional conformations through cargo binding between ABK and ABK-APC$_{ARM}$, we continued the observation of ABK-APC$_{ARM}$ and intensively induced cargo dissociation by continuous tapping of AFM". This assumes that the association and dissociation take the same intermediate state and it is induced by mechanical stress. This may be true but needs better reasoning.

Response: Thanks for the constructive suggestions. The mechanical stress has been used to observed structure changes by AFM previously (e.g., Lin et al., *Nature*, 2019. https://www.nature.com/articles/s41586-019-1499-2). We agree with the Reviewer in that we cannot exclude the possibility because of technical limitations that association and dissociation may take different intermediate states. The relative text was revised to provide clearer description and discussion on this issue.

(p.8, para.1)

“Moreover, we cannot exclude the possibility that intermediate conformations in cargo association of kinesin-2 are different from what we observed in cargo dissociation by AFM, which needs to be further investigated and monitored by advanced techniques in future.”

Fig. 4B and C The main text reads that both of these were done with increasing tapping force.

However, this is not clear for B from the legend. Curves for increasing tapping force would be helpful.

Response: Thanks for the valuable question and suggestions. We have revised the legend of Fig.4B and added the description of "increasing tapping force" of AFM in the method section.

(p.11, para.2)

"We gradually reduced the amplitude setting manually, leading to challenges in determining the exact magnitude of tapping force exerted by the probe during each imaging frame. Nevertheless, we approximated that the tapping force corresponded to approximately 18 pN when the amplitude setting was at 90% of 1 nm free oscillation amplitude, and 22 pN at 70%."

Fig. 4D What are the solid and dotted lines? With the current plots, it is not clear whether the variation is due to different molecules or due to the dynamics of individual molecules. The distal-center distance (or length of the protrusion) of each molecule should be plotted against time (3 curves for each construct? Is n=3 enough?).

Response: We thank the Reviewer for the careful scrutiny. We have revised the figure to distinguish the data plots derived from different group. In addition, explanations of the solid (median) and dotted (quartiles) lines were added in the figure legend. Also, we added extended figure (Fig. EV4D) to show distance plotted against time for three replicates of each construct.

(Fig. EV5) The circles are too small and the empty gray areas are too large.

Response: We thank the Reviewer for the comments. Small circle with large empty areas (i.e. large box size) shown in Fig. EV5 is because we selected small mask diameter for Class3D and Refine3D analysis of better resolution, although high-resolution 3D structure not obtained at last. We presented the raw images to show our reliable refinement process.

Movies of ABK should also be included.

Response: We thank the Reviewer for the suggestion. Movie of ABK was added (Movie EV3).

Dear Prof. Hirokawa,

Thank you for the submission of your revised manuscript to EMBO reports. We have now received the comments of both referees that were asked to re-evaluate your study (their comments are included below).

As you will see, both referees mention that their previous concerns have been successfully addressed, and they now recommend publication. There are only two remaining minor points raised by referee #2, which we would like you to address in a revised manuscript.

From the editorial side, there are also a few things that we need from you before we can proceed with the acceptance of your manuscript:

- Please note that the Abstract should be written in present tense.

- Please update your "Data and materials availability" statement: it should only appear once in the manuscript, at the end of the Materials and Methods; the heading should be "Data availability", and all accession codes and relevant links to external repositories should be provided in a single paragraph. Tokens for confidential access to the data by the reviewers should now be removed from the manuscript, as all datasets should be publicly available at the time of publication.

- Please also change the heading of your competing interests statement to "Disclosure and competing interests statement".

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- We noticed that Figure callouts for Fig. EV4A&B and EV5A&B are missing; please make sure that all panels are called out (in alphabetical order) in your revised manuscript.

- Each movie should be provided in a ZIP folder including its legend in a text file. Please remove movie legends from the main manuscript file.

- We noticed that Fig. 2A is re-used in Fig. EV2A. This should be detailed in the figure legends.

- Similarly, Fig. 4D - D,T,L are reused in Fig. 5A - D,T,L. This should also be mentioned in their figure legends.

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- Your Figure legends have been inspected by our data editors for completeness and accuracy. Please see the required changes in the attached Word file and address all comments in your revised manuscript (with tracked changes and/or with replies to the comments).

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Yours sincerely,

Ioannis Papaioannou, PhD
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EMBO reports

Referee #1:

The authors have addressed my minor questions/suggestions in this revised manuscript. As I said in my review of the initial submission, I think that this is an excellent paper that will be of great interest to scientists who study motor proteins and intracellular transport and to a broad range of cell and molecular biologists who read EMBO reports. Accordingly I strongly recommend publication of this paper in its current form.

Referee #2:

In this revision, the authors addressed all my points. The adjustment of the title with 'two-step' recognition is appreciated. The manuscript is suitable for publication in principle.

(very minor points)
"Caenorbabditis elegans KLP20/KLP11/KAP1 (Signor et al, 1999) " => "Caenorbabditis elegans KLP-20/KLP-11/KAP-1 (Signor et al, 1999)"
"open circles" is more common than "hollow spots" in Figures 1 and 2 legends.

Referee #1:

The authors have addressed my minor questions/suggestions in this revised manuscript. As I said in my review of the initial submission, I think that this is an excellent paper that will be of great interest to scientists who study motor proteins and intracellular transport and to a broad range of cell and molecular biologists who read EMBO reports. Accordingly I strongly recommend publication of this paper in its current form.

Response: We thank the Reviewer for the positive comments and kind recommendation of our paper for publication.

Referee #2:

In this revision, the authors addressed all my points. The adjustment of the title with 'two-step' recognition is appreciated. The manuscript is suitable for publication in principle.

Response: We thank the Reviewer for the favorable consideration and recommendation of our paper.

(very minor points)

"Caenorbabditis elegans KLP20/KLP11/KAP1 (Signor et al, 1999) " => "Caenorbabditis elegans KLP-20/KLP-11/KAP-1 (Signor et al, 1999)"

Response: Thanks for your careful scrutiny. The “KLP20/KLP11/KAP1” has been revised to “KLP-20/KLP-11/KAP-1”

"open circles" is more common than "hollow spots" in Figures 1 and 2 legends.

Response: Thanks for your suggestion. The “hollow spots” has been revised to “open circles”.

Editorial comments

- Please note that the Abstract should be written in present tense.

Response: Thanks for your careful scrutiny. The Abstract has been revised to be in present tense.

- Please update your "Data and materials availability" statement: it should only appear once in the manuscript, at the end of the Materials and Methods; the heading should be "Data availability", and all accession codes and relevant links to external repositories should be provided in a single paragraph. Tokens for confidential access to the data by the reviewers should now be removed from the manuscript, as all datasets should be publicly available at the time of publication.

Response: Thanks. The "Data and materials availability" statement has been updated accordingly.

- Please also change the heading of your competing interests statement to "Disclosure and

competing interests statement".

Response: Thanks. The heading has been changed.

- The author contributions statement should be removed from the manuscript file. Instead, we now use CRediT to specify the contributions of each author in the journal submission system. Please use the free text box to provide more detailed descriptions. See also our guide to authors: <https://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines>;.

Response: Thanks. The author contributions was removed from the manuscript and we used the free text box in submission system with detailed descriptions provided.

- We noticed that Figure callouts for Fig. EV4A&B and EV5A&B are missing; please make sure that all panels are called out (in alphabetical order) in your revised manuscript.

Response: Thanks. The figure callouts have been added and revised.

- Each movie should be provided in a ZIP folder including its legend in a text file. Please remove movie legends from the main manuscript file.

Response: Thanks. The movie file are now submitted as ZIP folders.

- We noticed that Fig. 2A is re-used in Fig. EV2A. This should be detailed in the figure legends.

- Similarly, Fig. 4D - D,T,L are reused in Fig. 5A - D,T,L. This should also be mentioned in their figure legends.

Response: Thanks. We have detailed the re-uses in the figure legends.

- The manuscript sections are in the wrong order. Please follow this order: Title page, Abstract, Keywords, Introduction, Results, Discussion, Materials and Methods, Data availability, Acknowledgements, Disclosure and competing interests statement, References, Figure legends, Expanded View Figure legends.

Response: Thanks. The order of manuscript sections has been corrected.

- Your Figure legends have been inspected by our data editors for completeness and accuracy. Please see the required changes in the attached Word file and address all comments in your revised manuscript (with tracked changes and/or with replies to the comments).

Response: Thanks. We have addressed all comments from data editors.

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Response: Thanks. The synopsis image, short summary and bullet points are submitted.

Prof. Nobutaka Hirokawa
The University of Tokyo
Department of Cell Biology and Anatomy, Graduate School of Medicine
7-3-1 Hongo
Bunkyo-ku, Tokyo 113-0033
Japan

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The data shown in figures should satisfy the following conditions:

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- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
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- a specification of the experimental system investigated (eg cell line, species name).
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- 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.
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  - are tests one-sided or two-sided?
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Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Yes	Materials and Methods
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Yes	Materials and Methods
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Not Applicable	

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Microbes: provide species and strain, unique accession number if available, and source.	Yes	Materials and Methods

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If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Yes	Materials and Methods

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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	
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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	Materials and Methods, Data and materials availability
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?	Not Applicable	
If publicly available data were reused, provide the respective **data citations in the reference list**.	Not Applicable	