THE EMBO JOURNAL

Microtubule poleward flux in human cells is driven by the coordinated action of four kinesins

Yulia STEBLYANKO, Girish Rajendraprasad, Mariana Osswald, Susana Eibes, Ariana Jacome, Stephan Geley, Antonio Pereira, Helder Maiato, and Marin Barisic
DOI: 10.15252/embj.2020105432

Corresponding author(s): Marin Barisic (barisic@cancer.dk)

Review Timeline:

Submission Date:	27th Apr 20
Editorial Decision:	11th Jun 20
Revision Received:	31st Aug 20
Editorial Decision:	21st Sep 20
Revision Received:	24th Sep 20
Accepted:	25th Sep 20

Editor: Hartmut Vodermaier

Transaction Report:

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

1st Editorial Decision 11th Jun 2020

Thank you again for submitting your manuscript on the mechanisms governing microtubule flux in human cells. We have now finally received a complete set of reports from three expert referees, copied below for your information. I am happy to say that all reviewers consider this work of interest and also experimentally solid, and that would therefore like to pursue this work further for EMBO Journal publication. I would therefore invite you to prepare a revised manuscript, incorporating the various comments and suggestions raised by the referees. As you will see, their points mostly pertain to conceptual discussions and clarifications, but there are also a limited number of important control data being requested.

REFEREE REPORTS

Referee #1:

The organization and dynamics of the mitotic spindle are fundamental to the accurate distribution of the genetic material to the daughter cells. It was demonstrated many years ago that microtubules within the spindle undergo a specialized treadmilling like behavior called microtubule flux. During flux, tubulin subunits are added at the microtubule plus ends and removed at the spindle poles, while the microtubules are translocated toward the poles. While there have been several studies that implicated multiple players in this process, there have been relatively few studies that take a detailed look at the molecular mechanisms underlying this process. In the present study the authors carry out a rigorous and thorough analysis of the contributions of a

series of molecular motors and microtubule dynamics regulators to the process of microtubule flux. From these studies they present a new mechanistic model that proposes the coordinated activity of four molecular motors that play distinct roles in coordinating flux. They also provide new insights into the contribution of flux to both spindle size as well as how it is coupled to kinetochore microtubule attachment.

Overall this study is beautifully done with carefully controlled and quantified experiments that are backed up by rigorous controls for knockdown and expression. Each experiment clearly presents the original data from multiple measurements of a robust number of samples. Therefore, the conclusions are well supported by the data. This study provides a new view on the coordination of microtubule flux, and therefore this is an important study that should be of great interest to the readers of EMBO J. I am in favor of eventual publication with a few points below that the authors need to address.

1. In my opinion, the authors present their model too strongly based on the data. One challenge with regards to understanding the mechanisms that control microtubule flux (a challenge that I do not think is easily resolved) is how to determine the direct from the indirect effects. It is clear from this work that kinetochore microtubule attachments, for example, limit the rate of flux. This raises the important point that proper spindle structure and organization are necessary for the coordinated actions of the various motors and dynamics regulators to contribute to flux. The authors assign distinct mechanistic roles to each of the players in the process, but I think that in some cases the motor may be more important as a structural component of the spindle that when disrupted, perturbs the rate of flux. Because there is no easy experimental way to address this question, the authors should carefully go back through the manuscript and edit their conclusions with softer terms- suggests or supports rather than demonstrate or show. In addition, the discussion should be rewritten to make it clear that the authors favor the model that they propose, but they should also include a discussion of the alternative view that some of the effects may be secondary disruptions of spindle structure and organization.
2. Figure 2A is redundant with the videos, which clearly demonstrate the point. Given that the flux measurements are the key to the mechanisms being reported in the paper, and the mitotic timing is secondary- Figure 2 should show the primary data for flux along with quantification, and the mitotic timing can be in the supplement with the videos. Alternatively, the videos themselves are sufficient.
3. I disagree with the conclusions regarding HSET in Figure 4 regarding coupling. It was shown previously that HSET can regulate spindle length independent of K-fibers so it more likely changes spindle organization.
4. In Figure 5D, it would be helpful to switch the order of the graph to match the panels in Figure 5C.
5. In Figure 6, the authors present a new super-resolution localization of Cenp-E to interpolar microtubules and make conclusions about Cenp-E function in flux based on this localization. Given that this localization is distinct from what has been reported previously and is using a different type of microscopy to see the localization, it is important that the authors include the controls of Cenp-E knockdown to show the specificity of antibodies. I am hoping that the authors have this data available and just did not include it in the manuscript, because I am trying hard to not ask authors for new experiments amid the pandemic conditions.

Referee #2:

The mitotic spindle consists of dynamic microtubules (MTs) that continuously flow from the spindle

equator/midzone to the poles. Although the poleward flux of the MTs is conserved in metazoan spindles, its cellular function and the proteins involved in driving its motion remain unclear. In this study, Steblyanko et al. screened for potential candidate proteins based on loss-of-function experiments in human cells. They found that kinesin-4/KIF4A and kinesin-7/CENP-E were the main components that drove flux during late prometaphase/metaphase and early prometaphase, respectively. Furthermore, disruption of kinesin-5/EG5 and kinesin-12/KIF15 showed that KIF4A and CENP-E contribute to the MT flux in a redundant manner with EG5 and KIF15 in both stages. The flux rate correlated with the spindle length, once stable end-on attachments between kinetochores and MTs were established.

While spindle MT flux has been attracting many researchers, the underlying mechanisms are still not fully understood. This study reports a full list of flux-driving motors, and is a breakthrough in spindle research. The presented data are of high quality, implicating novel components such as CENP-E in spindle MT flux. Overall, with a large dataset focused on proteins involved in MT dynamics and various combinations of gene disruption experiments, this study provides novel insights into how MT flux is driven during prometaphase and metaphase. Once the following points are satisfactorily addressed, I recommend the publication of this study.

Major points:
1. Figure 6B: Two control experiments are required to describe this unexpected localization and possible function of CENP-E. 1) CENP-E RNAi followed by immunofluorescence with anti-CENP-E antibody or CENP-E-GFP visualization to validate that the observed signals are not due to cross-reactivity of the antibody with other spindle proteins. 2) CENP-E staining in STLC-treated monopolar spindle to determine whether CENP-E localization is limited to anti-parallel MT overlap.
2. Figure 4: HSET is dispensable in several cell types. The authors should present direct evidence that HSET RNAi alone attenuates spindle MT crosslinking in this particular cell line.
3. I did not understand the authors' logic that flux speed regulates spindle length. Some important discussion points should be addressed:
- What is the spindle length phenotype observed upon MCAK depletion?
- Did the authors consider that MCAK could act exclusively at the centromere, or be involved in general depolymerization in the cytosol?
- I assume that KIF2A depletion has a similar effect to MCAK, namely restoring the spindle length in the absence of flux promoters (KIF15, EG5, and KIF4A). Which data led to the conclusion that MCAK specifically counteracts flux?
- How did the authors exclude the possibility that spindle (MT) length regulates flux speed? With shorter MTs overall in the spindle, the motors may not produce as much force as normal.
- What is the possible mechanism by which MCAK counteracts flux? Does the flux keep the kinetochore MTs away from the inner centromere?
4. The authors explained that MT depolymerization by kinesin-13/KIF2A is a response to flux, rather than the main driving force, and several studies are cited in the introduction and discussion sections. However, KIF2A RNAi significantly reduced the flux rate for both bipolar and monopolar spindles, which is more marked than that observed with KIF4A RNAi (Figure 1E and F). Hence, I do not understand why the authors (and other researchers) eliminated the possibility that KIF2A depolymerase is the main driver of flux. A clear explanation is necessary.

Minor points:
1. In the last paragraph of results section, the bibliography needs to be corrected (e.g. "...in KIF2A depleted cells [11]").
2. In the results section, the authors concluded that "KIF4A's effect on MT-flux is independent of its PRC1-dependent localization at interpolar MTs (Zhu & Jiang, 2005)". Is this an accurate

statement? This manuscript concludes that PRC1 translocation depends on KIF4A transport.
3. "Spindle length" and "spindle size" should be distinguished throughout the manuscript.

Referee #3:

In their manuscript, "Microtubule poleward flux in human cells is driven by the coordinated action of four kinesins," Steblyanko and colleagues use a systematic approach to elucidate the molecular underpinnings of flux in the mitotic spindle, using photoactivation to measure flux while inhibiting potential flux regulators. While previous work has revealed some key proteins involved in flux in other systems, how spindle motors coordinate across time and space in the mammalian spindle is unclear. Here, Steblyanko et al. address this gap by showing a novel role for CENP-E in driving prometaphase flux, establishing different drivers of flux across different stages of mitosis, and elucidating how cross-linkers coordinate and synchronize flux across non-kinetochore microtubules and kinetochore microtubules. They also address the correlation between flux and spindle length, which is a proposed function of flux in spindles.

We appreciate the careful experimental design addressing pertinent open questions, as well as the high quality of the data and presentation. The experimental logic is clear and the data compelling. Overall, these data would be a great asset to the scientific community, though the text could better motivate and clarify these results in the context of previously established models of flux. After addressing major conceptual points regarding the role of KIF2A and the link between flux and spindle length, we would recommend this manuscript for publication.

Major comments:
• The authors define flux as "continuous motion of MTs towards the spindle poles." According to this definition, material does not need to be flowing into and out of microtubules (polymerization and depolymerization, respectively). Thus, microtubules that do not depolymerize at their minus-end can exhibit flux. However, other definitions of flux require both plus- and minus-end dynamics. The implications of the author's definition of flux should be clearly stated, namely 1) whether depolymerization is required for flux and 2) whether mere poleward transport of microtubules with no end dynamics constitutes flux.
• Depending on the author's definition of flux, KIF2A may be a main driver of flux. In Figure 1D, the authors confirm that KIF2A depletion or CLASP depletion strongly inhibit flux as previously shown. This phenotype is much stronger than that of KIF4A inhibition. Thus, the authors should clarify why they conclude that KIF4A, and not KIF2A or CLASP (as other work has proposed), is the main driver of flux during metaphase. Furthermore, the authors propose two models for flux in the introduction. The first relies on kinesin-13 depolymerization at minus-ends coupled to CLASP polymerization at kinetochores. They claim that minus-end depolymerization may be a "reaction" to plus-end dynamics, citing "normal" flux in situations where microtubule minus-ends have been perturbed (Maiato 2004, Matos 2009, Mitchison 2009). They should clarify their definition of flux in these contexts as it seems inconsistent with their results in 1D. If KIF2A's role in minus-end depolymerization is a reaction to plus-end dynamics, then depleting it should not abolish flux.
• The authors propose a model whereby flux is driven by four motors to counteract spindle shortening by MCAK. Thus, flux must be promoting polymerization. Consequently, the authors discuss the interactions and relationship between MCAK and the four motors they identify. How might microtubule transport from these four motors lead to polymerization? Does it result in a bias for plus-end polymerization? Does this require CLASP to polymerize plus-ends? The authors should try to fill this gap in their model (with ideas if not data).

Minor comments:
• Fig 2. Some of the text, figure labels, and figure legends incorrectly refer to the KIF4A ATPase mutant as K64A instead of K94A.
• Fig 2A. The KIF4A mutant that is chromatin non-binding (ΔZip) seems to localize near chromatin better than the ATPase mutant (K94A). Could the authors provide an explanation for this phenotype?
• Fig 2. In the last line of page 7, the authors claim that "KIF4A cannot push [chromosomes] any further due to equivalent forces applied from opposite spindle sides." What is the evidence for this mechanism? It is unclear what is supported by evidence and what is speculation in this paragraph.
• Fig 3. The authors use MUGs to test the contribution of chromatin to flux. However, MUGs have many perturbed features aside from chromatin, including perturbed kinetochores and kinetochore-fibers. These confounding factors should be addressed in the text, and the conclusion that chromatin acts as a locus for KIF4A activity is not clear.
• Fig 4. The authors should rephrase descriptions of the crosslinkers NuMA and PRC1 to accurately reflect the literature.
o On page 8, the authors claim "NuMA is recruited to the spindle poles by the MT minus-end-directed motor Dynein," which may not be an accurate representation given that NuMA has been shown to be recruited to minus-ends without dynein.
o On page 8, the authors should clarify their description of PRC1, "...proposed to bridge KT-MTs with interpolar MTs," to clarify its role as a crosslinker between kinetochore-microtubules and bridge fiber microtubules.
• Fig 4. On page 9, the authors should define "asynchronous flux movements" and be transparent in their methods for measuring it. It would be helpful if the authors could provide an interpretation for why the signal splits into distinct bands, instead of smearing.
• Fig 5. The authors should cite Lecland & Luders 2014, which also found that non-kinetochore microtubules in U2OS cells flux faster than kinetochore-microtubules.
• Fig 8A, 8B, and 8C. Flux rates and spindle lengths are generally quite variable within each experimental condition, so the authors should show the full scatterplot and correlation coefficients from all individual points rather than averages.
• Fig 8D depicts a bipolar spindle after KIF15 depletion and STLC addition. This perturbation has been shown to result in monopoles (i.e. Tanenbaum 2009). The authors should clarify whether these spindles are in the process of collapsing. If so, how and when were they measured for spindle length? Additionally, plotting length over time in the triple inhibitions could show whether these spindles have reached steady-state lengths.
• Discussion. The authors should at least discuss why KIF4A doesn't drive flux during early prometaphase even though it localizes to chromatin at the same time as CENP-E. Why is KIF4A not actively driving flux at this time, and what could be its role at prometaphase? The authors should discuss possible mechanisms by which KIF4A takes over for CENP-E.
• Methods. The authors should describe what photoactivation system and equipment they are using.
• Methods. The authors should clarify the ROIs they drew to define each "kymograph layer.

Referee #1:

The organization and dynamics of the mitotic spindle are fundamental to the accurate distribution of the genetic material to the daughter cells. It was demonstrated many years ago that microtubules within the spindle undergo a specialized treadmilling like behavior called microtubule flux. During flux, tubulin subunits are added at the microtubule plus ends and removed at the spindle poles, while the microtubules are translocated toward the poles. While there have been several studies that implicated multiple players in this process, there have been relatively few studies that take a detailed look at the molecular mechanisms underlying this process. In the present study the authors carry out a rigorous and thorough analysis of the contributions of a series of molecular motors and microtubule dynamics regulators to the process of microtubule flux. From these studies they present a new mechanistic model that proposes the coordinated activity of four molecular motors that play distinct roles in coordinating flux. They also provide new insights into the contribution of flux to both spindle size as well as how it is coupled to kinetochore microtubule attachment.

Overall this study is beautifully done with carefully controlled and quantified experiments that are backed up by rigorous controls for knockdown and expression. Each experiment clearly presents the original data from multiple measurements of a robust number of samples. Therefore, the conclusions are well supported by the data. This study provides a new view on the coordination of microtubule flux, and therefore this is an important study that should be of great interest to the readers of EMBO J. I am in favor of eventual publication with a few points below that the authors need to address.

1. In my opinion, the authors present their model too strongly based on the data. One challenge with regards to understanding the mechanisms that control microtubule flux (a challenge that I do not think is easily resolved) is how to determine the direct from the indirect effects. It is clear from this work that kinetochore microtubule attachments, for example, limit the rate of flux. This raises the important point that proper spindle structure and organization are necessary for the coordinated actions of the various motors and dynamics regulators to contribute to flux. The authors assign distinct mechanistic roles to each of the players in the process, but I think that in some cases the motor may be more important as a structural component of the spindle that when disrupted, perturbs the rate of flux. Because there is no easy experimental way to address this question, the authors should carefully go back through the manuscript and edit their conclusions with softer terms- suggests or supports rather than demonstrate or show. In addition, the discussion should be rewritten to make it clear that the authors favor the model that they propose, but they should also include a discussion of the alternative view that some of the effects may be secondary disruptions of spindle structure and organization.

We thank the reviewer for valuable feedback. We have toned down several of our conclusions throughout the new version of our manuscript by using softer terms. We have also included alternative view to the effect of microtubule crosslinking molecules, discussing that some of the effects that we observed might be due to their impact on the spindle architecture.

2. Figure 2A is redundant with the videos, which clearly demonstrate the point. Given that the flux measurements are the key to the mechanisms being reported in the paper, and the

mitotic timing is secondary- Figure 2 should show the primary data for flux along with quantification, and the mitotic timing can be in the supplement with the videos. Alternatively, the videos themselves are sufficient.

We agree with the reviewer and we now included the primary data for flux into Figure 2 (Fig 2A), while we removed the data depicting KIF4A localization during mitosis to the supplement (Fig EV2C).

3. I disagree with the conclusions regarding HSET in Figure 4 regarding coupling. It was shown previously that HSET can regulate spindle length independent of K-fibers so it more likely changes spindle organization.

We have included alternative view to the effect of HSET, writing that some of the effects that we observed might be due to their impact on the spindle architecture.

4. In Figure 5D, it would be helpful to switch the order of the graph to match the panels in Figure 5C.

We agree that switching the order of the graph to match the panels will be helpful and we changed it accordingly.

5. In Figure 6, the authors present a new super-resolution localization of Cenp-E to interpolar microtubules and make conclusions about Cenp-E function in flux based on this localization. Given that this localization is distinct from what has been reported previously and is using a different type of microscopy to see the localization, it is important that the authors include the controls of Cenp-E knockdown to show the specificity of antibodies. I am hoping that the authors have this data available and just did not include it in the manuscript, because I am trying hard to not ask authors for new experiments amid the pandemic conditions.

We thank the reviewer for drawing our attention to include a control for novel CENP-E localization presented by STED imaging. We now include STED images of fixed HeLa cells stably expressing a bacterial artificial chromosome encoding CENP-E-GFP under control of its own promoter and low copy number (Fig 6C), as well as spinning disk confocal live-cell imaging data of the same cell line (Fig 6 and EV6A). Thus, we show CENP-E localization at the interpolar microtubules throughout mitosis by using two different approaches (GFP-tagging of CENP-E and antibody-based immunostaining of endogenous CENP-E) and two different imaging techniques (STED imaging and spinning disk confocal imaging).

Referee #2:

The mitotic spindle consists of dynamic microtubules (MTs) that continuously flow from the spindle equator/midzone to the poles. Although the poleward flux of the MTs is conserved in metazoan spindles, its cellular function and the proteins involved in driving its motion remain unclear. In this study, Steblyanko et al. screened for potential candidate proteins based on loss-of-function experiments in human cells. They found that kinesin-4/KIF4A and kinesin-7/CENP-E were the main components that drove flux during late prometaphase/metaphase and early prometaphase, respectively. Furthermore, disruption of kinesin-5/EG5 and kinesin-

12/KIF15 showed that KIF4A and CENP-E contribute to the MT flux in a redundant manner with EG5 and KIF15 in both stages. The flux rate correlated with the spindle length, once stable end-on attachments between kinetochores and MTs were established.

We thank the reviewer for the feedback. We would like to briefly comment here that we are not showing that CENP-E and KIF4A, respectively, contribute to the MT flux in a redundant manner with EG5 and KIF15, but that they rather work in synergy with EG5 and KIF15, as their respective triple depletions show strong synergistic effects.

While spindle MT flux has been attracting many researchers, the underlying mechanisms are still not fully understood. This study reports a full list of flux-driving motors, and is a breakthrough in spindle research. The presented data are of high quality, implicating novel components such as CENP-E in spindle MT flux. Overall, with a large dataset focused on proteins involved in MT dynamics and various combinations of gene disruption experiments, this study provides novel insights into how MT flux is driven during prometaphase and metaphase. Once the following points are satisfactorily addressed, I recommend the publication of this study.

Major points:
1. Figure 6B: Two control experiments are required to describe this unexpected localization and possible function of CENP-E. 1) CENP-E RNAi followed by immunofluorescence with anti-CENP-E antibody or CENP-E-GFP visualization to validate that the observed signals are not due to cross-reactivity of the antibody with other spindle proteins. 2) CENP-E staining in STLC-treated monopolar spindle to determine whether CENP-E localization is limited to anti-parallel MT overlap.

We thank the reviewer for highlighting the importance of including a control for novel CENP-E localization presented by STED imaging. We now include STED images of fixed HeLa cells stably expressing a bacterial artificial chromosome encoding CENP-E-GFP under control of its own promotor and low copy number (Fig 6C), as well as spinning disk confocal live-cell imaging data of the same cell line (Fig 6 and EV6A). Thus, we show CENP-E localization at the interpolar microtubules throughout mitosis by using two different approaches (GFP-tagging of CENP-E and antibody-based immunostaining of endogenous CENP-E) and two different imaging techniques (STED imaging and spinning disk confocal imaging). We also included STED images of STLC-treated HeLa-CENP-E-GFP cells and show that CENP-E-GFP associated with bundled microtubules even in monopolar spindles (Fig EV4C). However, whether these bundled MTs are of parallel or antiparallel orientation remains unclear.

2. Figure 4: HSET is dispensable in several cell types. The authors should present direct evidence that HSET RNAi alone attenuates spindle MT crosslinking in this particular cell line.

HSET was shown to be dispensable due to redundancy with NuMA and dynein, but spindle pole defects on bipolar spindles have been reported in absence of functional HSET (Gordon et al, J Cell Biol 2001; Kleylein-Sohn et al, J Cell Sci 2012). We have now also included alternative view to the effect of HSET, discussing that some of the effects that we observed might be due to their impact on the spindle architecture.

3. I did not understand the authors' logic that flux speed regulates spindle length. Some important discussion points should be addressed:

We agree with the reviewer that the previous version of our manuscript lacked detail on how microtubule flux regulates spindle length and we significantly expanded this part in the new version.

- What is the spindle length phenotype observed upon MCAK depletion?

Upon MCAK depletion, spindle length is slightly, but not statistically significantly increased (Fig 8E).

- Did the authors consider that MCAK could act exclusively at the centromere, or be involved in general depolymerization in the cytosol?

Since spindle shortening does not occur in NDC80-depleted cells without end-on kinetochore-microtubule attachments (Fig 8C), we reason that MCAK most likely acts from the centromere, without excluding the possibility of its contribution from the spindle pole.

- I assume that KIF2A depletion has a similar effect to MCAK, namely restoring the spindle length in the absence of flux promoters (KIF15, EG5, and KIF4A). Which data led to the conclusion that MCAK specifically counteracts flux?

We thank the reviewer for the comment and agree that including the KIF2A data will improve our understanding of the process. Using two independent approaches (triple inactivation of KIF15, EG5 and KIF4A vs. depletion of CLASPs), we now show that while MCAK depletion restores spindle length in the absence of flux, depletion of KIF2A does not have any effect on spindle length (Fig 8D and E). This indicates that spindle length under these conditions is specifically regulated by MCAK-driven depolymerization of k-fibers.

- How did the authors exclude the possibility that spindle (MT) length regulates flux speed? With shorter MTs overall in the spindle, the motors may not produce as much force as normal.

Since MCAK depletion was able to rescue the spindle length, but not the microtubule flux rates in cells with depleted flux (triple inactivation of KIF4A, KIF15 and EG5; CLASPs depletion; Fig EV7C), we conclude that microtubule flux was reduced due to the absence of its driving motors, rather than because of the spindle size. We show that the spindle length is specifically dependent on MCAK action on k-fibers, which is counteracted by microtubule flux.

- What is the possible mechanism by which MCAK counteracts flux? Does the flux keep the kinetochore MTs away from the inner centromere?

We have expanded the discussion part on how microtubule flux could counteract MCAK activity. This includes the possibility that kinesins-driven sliding activities apply force to sister kinetochores, which might affect MCAK kinetochore/centromere localization. How this fully works on molecular basis and whether it depends on KT/centromere-localized kinases and phosphatases remains to be solved by future studies.

4. The authors explained that MT depolymerization by kinesin-13/KIF2A is a response to flux, rather than the main driving force, and several studies are cited in the introduction and discussion sections. However, KIF2A RNAi significantly reduced the flux rate for both bipolar and monopolar spindles, which is more marked than that observed with KIF4A RNAi (Figure 1E and F). Hence, I do not understand why the authors (and other researchers) eliminated the possibility that KIF2A depolymerase is the main driver of flux. A clear explanation is necessary.

We have now further highlighted the studies that have shown that microtubule flux can be uncoupled from microtubule minus-end depolymerization, and therefore from KIF2A activity. This, together with our data on CLASPs (Fig 5B), leads us to conclusion that, although KIF2A (together with CLASPs) clearly makes an important component of mitotic microtubule flux, it more likely acts as a microtubule flux governing element than its driving force. We reason that if KIF2A acted as a governor of microtubule flux instead of being its main driving force, one could still expect that its depletion reduce flux, as it is clearly the case. We would also like to highlight here that our model supports that KIF4A synergistically drive microtubule flux with EG5 and KIF15, and therefore we should compare the effects of KIF2A depletion (Fig 1E) to triple inactivation of KIF4A, KIF15 and EG5 (Fig 7C), which results in even stronger flux reduction compared to KIF2A depletion.

Minor points:
1. In the last paragraph of results section, the bibliography needs to be corrected (e.g. "...in KIF2A depleted cells [11]").

We corrected this.

2. In the results section, the authors concluded that "KIF4A's effect on MT-flux is independent of its PRC1-dependent localization at interpolar MTs (Zhu & Jiang, 2005)". Is this an accurate statement? This manuscript concludes that PRC1 translocation depends on KIF4A transport.

We thank the reviewer for drawing our attention to this mistake, which we have corrected by including the accurate references.

3. "Spindle length" and "spindle size" should be distinguished throughout the manuscript.

We agree with the reviewer and we have changed this accordingly.

Referee #3:

In their manuscript, "Microtubule poleward flux in human cells is driven by the coordinated action of four kinesins," Steblyanko and colleagues use a systematic approach to elucidate the molecular underpinnings of flux in the mitotic spindle, using photoactivation to measure flux while inhibiting potential flux regulators. While previous work has revealed some key proteins involved in flux in other systems, how spindle motors coordinate across time and space in the mammalian spindle is unclear. Here, Steblyanko et al. address this gap by showing a novel role for CENP-E in driving prometaphase flux, establishing different drivers of flux across different stages of mitosis, and elucidating how cross-linkers coordinate and synchronize flux across

non-kinetochore microtubules and kinetochore microtubules. They also address the correlation between flux and spindle length, which is a proposed function of flux in spindles.

We appreciate the careful experimental design addressing pertinent open questions, as well as the high quality of the data and presentation. The experimental logic is clear and the data compelling. Overall, these data would be a great asset to the scientific community, though the text could better motivate and clarify these results in the context of previously established models of flux. After addressing major conceptual points regarding the role of KIF2A and the link between flux and spindle length, we would recommend this manuscript for publication.

Major comments:

• The authors define flux as "continuous motion of MTs towards the spindle poles." According to this definition, material does not need to be flowing into and out of microtubules (polymerization and depolymerization, respectively). Thus, microtubules that do not depolymerize at their minus-end can exhibit flux. However, other definitions of flux require both plus- and minus-end dynamics. The implications of the author's definition of flux should be clearly stated, namely 1) whether depolymerization is required for flux and 2) whether mere poleward transport of microtubules with no end dynamics constitutes flux.

We thank the reviewer for the comment and we agree that our microtubule flux definition should have been more clear. In the new version of our manuscript we define microtubule poleward flux as *"a continuous poleward motion of microtubules, typically coordinated with addition of new tubulin subunits at the microtubule plus-ends and their removal at the microtubule minus ends at spindle poles"*. We further discuss the experiments that uncoupled microtubule flux from the minus-end depolymerization, reasoning that transport of these microtubules is driven by the same molecular mechanism and thus should not be excluded from the flux definition.

• Depending on the author's definition of flux, KIF2A may be a main driver of flux. In Figure 1D, the authors confirm that KIF2A depletion or CLASP depletion strongly inhibit flux as previously shown. This phenotype is much stronger than that of KIF4A inhibition. Thus, the authors should clarify why they conclude that KIF4A, and not KIF2A or CLASP (as other work has proposed), is the main driver of flux during metaphase. Furthermore, the authors propose two models for flux in the introduction. The first relies on kinesin-13 depolymerization at minus-ends coupled to CLASP polymerization at kinetochores. They claim that minus-end depolymerization may be a "reaction" to plus-end dynamics, citing "normal" flux in situations where microtubule minus-ends have been perturbed (Maiato 2004, Matos 2009, Mitchison 2009). They should clarify their definition of flux in these contexts as it seems inconsistent with their results in 1D. If KIF2A's role in minus-end depolymerization is a reaction to plus-end dynamics, then depleting it should not abolish flux.

We have now further highlighted the studies that have shown that microtubule flux can be uncoupled from microtubule minus-end depolymerization, and therefore from KIF2A activity. This, together with our data on CLASPs (Fig 5B), leads us to conclusion that although KIF2A (together with CLASPs) clearly makes an important component of mitotic microtubule flux, it more likely acts as a microtubule flux governing element than its driving force. We reason that if KIF2A acted as a governor of microtubule flux instead of being its main driving force, one could still expect that its depletion reduce flux, as it is clearly the case. We would also like to

highlight here that our model supports that KIF4A synergistically drive microtubule flux with EG5 and KIF15, and therefore we should compare the effects of KIF2A depletion (Fig 1E) to triple inactivation of KIF4A, KIF15 and EG5 (Fig 7C), which results in even stronger flux reduction compared to KIF2A depletion.

• The authors propose a model whereby flux is driven by four motors to counteract spindle shortening by MCAK. Thus, flux must be promoting polymerization. Consequently, the authors discuss the interactions and relationship between MCAK and the four motors they identify. How might microtubule transport from these four motors lead to polymerization? Does it result in a bias for plus-end polymerization? Does this require CLASP to polymerize plus-ends? The authors should try to fill this gap in their model (with ideas if not data).

We thank the reviewer for this important comment, which we extensively addressed in the new version of our discussion section. We discuss that microtubule flux, via kinesins-dependent sliding, might exert force to kinetochores/centromere, which counteracts MCAK-dependent depolymerization of k-fibers (perhaps by affecting MCAK localization), eventually providing a bias for their net plus-end polymerization. How this fully works on molecular basis and whether it depends on KT/centromere-localized kinases and phosphatases remains to be addressed by future studies.

Minor comments:
• Fig 2. Some of the text, figure labels, and figure legends incorrectly refer to the KIF4A ATPase mutant as K64A instead of K94A.

We corrected this.

• Fig 2A. The KIF4A mutant that is chromatin non-binding (ΔZip) seems to localize near chromatin better than the ATPase mutant (K94A). Could the authors provide an explanation for this phenotype?

K94A is a rigor mutant that likely binds microtubules stronger, and depending on its expression level it can be better or worse visible on chromatin. In the new version of our manuscript we have included another two examples of the same mutant, where it is clear that this mutant localizes both on the chromatin and spindle (Fig 2A and EV2A; Movie EV3).

• Fig 2. In the last line of page 7, the authors claim that "KIF4A cannot push [chromosomes] any further due to equivalent forces applied from opposite spindle sides." What is the evidence for this mechanism? It is unclear what is supported by evidence and what is speculation in this paragraph.

We corrected the text to clarify that this statement is our speculation.

• Fig 3. The authors use MUGs to test the contribution of chromatin to flux. However, MUGs have many perturbed features aside from chromatin, including perturbed kinetochores and kinetochore-fibers. These confounding factors should be addressed in the text, and the conclusion that chromatin acts as a locus for KIF4A activity is not clear.

We agree with the reviewer's comment and we have added a sentence: *"However, other features that are normally present in MUGs, such as merotelic KT-MT attachments, cannot be excluded to contribute to the observed attenuation of MT-flux."*

• Fig 4. The authors should rephrase descriptions of the crosslinkers NuMA and PRC1 to accurately reflect the literature.
o On page 8, the authors claim "NuMA is recruited to the spindle poles by the MT minus-end-directed motor Dynein," which may not be an accurate representation given that NuMA has been shown to be recruited to minus-ends without dynein.
o On page 8, the authors should clarify their description of PRC1, "...proposed to bridge KT-MTs with interpolar MTs," to clarify its role as a crosslinker between kinetochore-microtubules and bridge fiber microtubules.

We have corrected our descriptions of NuMA and PRC1 and added more recent references.

• Fig 4. On page 9, the authors should define "asynchronous flux movements" and be transparent in their methods for measuring it. It would be helpful if the authors could provide an interpretation for why the signal splits into distinct bands, instead of smearing.

We have expanded our description on "asynchronous flux movements" in the results section by writing: *"…spindles depleted of NuMA and HSET revealed a frequent occurrence of asynchronous flux tracks, as inferred from a multi-stripe pattern on the kymographs of photoactivated spindles (…), suggesting weakened mechanical coupling between non-KT-MTs and KT-MTs."*

We have also expanded the discussion part on it, by writing: *"Instead of the signal smearing, the asynchrony in these kymograph tracks is represented by a few bifurcations. This points to the occurrence of rare, but more severe decoupling incidents, rather than to an increased frequency of small decoupling events. Since k-fibers are more bundled and therefore appear as the brightest signal within a kymograph, the asynchrony likely represents few k-fibers fluxing slower than the others."*

• Fig 5. The authors should cite Lecland & Luders 2014, which also found that non-kinetochore microtubules in U2OS cells flux faster than kinetochore-microtubules.

We thank the reviewer for highlighting this omission, which we have corrected in the new version of our manuscript.

• Fig 8A, 8B, and 8C. Flux rates and spindle lengths are generally quite variable within each experimental condition, so the authors should show the full scatterplot and correlation coefficients from all individual points rather than averages.

We have corrected this and now show the belonging standard deviations and correlation coefficients from all individual points.

• Fig 8D depicts a bipolar spindle after KIF15 depletion and STLC addition. This perturbation has been shown to result in monopoles (i.e. Tanenbaum 2009). The authors should clarify

whether these spindles are in the process of collapsing. If so, how and when were they measured for spindle length? Additionally, plotting length over time in the triple inhibitions could show whether these spindles have reached steady-state lengths.

We clarified this in the methods section by writing: *"Cells with collapsing spindles (e.g. STLC-treated and KIF15-depleted) were discarded from quantifications of MT-flux and spindle length in bipolar spindles and only the ones that maintained the spindle length during the filming were quantified."*

• Discussion. The authors should at least discuss why KIF4A doesn't drive flux during early prometaphase even though it localizes to chromatin at the same time as CENP-E. Why is KIF4A not actively driving flux at this time, and what could be its role at prometaphase? The authors should discuss possible mechanisms by which KIF4A takes over for CENP-E.

We have significantly expanded the discussion on why KIF4A contributes less to microtubule flux in early mitosis than CENP-E and how it could gradually take over during chromosome congression to the metaphase plate.

• Methods. The authors should describe what photoactivation system and equipment they are using.

We have corrected this omission by adding more detail into the methods section.

• Methods. The authors should clarify the ROIs they drew to define each "kymograph layer".

We have corrected this by adding more detail into the methods section.

2nd Editorial Decision 21st Sep 2020

Thank you for submitting your revised manuscript for our editorial consideration. It has now been assessed again by two of the original referees (see below). Given their positive comments, we shall -after incorporation of a few remaining editorial point- be happy to accept the manuscript for EMBO Journal publication!

Please address the following editorial points in a final round of modification.

REFEREE REPORTS

Referee #1:

This is a revised manuscript exploring the molecular mechanisms that drive microtubule flux in the spindle. The initial version of the manuscript was thorough, meticulous and presented important and convincing data providing new insights into the mechanisms that drive flux. The revision has addressed all of my concerns, and the revisions provide increased clarity as well as a more complete discussion of the work in the context of previous studies in this area. It is top-notch cell biology that should be of broad interest, and I highly support publication of this work in EMBO.

Referee #2:

All my concerns have been resolved.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Marin Barisic
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2020-105432

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

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 iustified
- 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.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

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?	The sample size was chosen as a robust number reflecting the common practice from the literature in the field.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	The experiments with inefficient knockdown detected by immunoblotting were not included.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	No.
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Most of the microtubule flux measurements were re-analyzed by another team member to eliminate any bias.
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Yes. The tests were chosen according to how many groups were included and whether the data points were normal distributed or not. Information on statistical tests is included in the figure legends and methods section.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes. The Shapiro–Wilk test of normality was used.
Is there an estimate of variation within each group of data?	Standard deviation was used to indicate the amount of variation within each group of data.

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repor

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tumo

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

Is the variance similar between the groups that are being statistically compared?	F test was used to compare variances and Welch's correction was employed for conditions that did not have equal variances.

C- Reagents

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 have provided the references and/or catalog numbers for antibodies.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	U2OS-PA-GFP-tubulin, U2OS-PA-GFP-tubulin/mCherry-tubulin and U2OS-mEOS-tubulin cell lines were obtained from Duane Compton, Rene Medema and Stephan Geley, respectively, and we have not recenty authenticated them. HeLa-CENPE-GFP cells were obtained from Tony Hyman and we have not recenty authenticated them. To rule out potential mycoplasma contamination, we have been periodically testing all these cell lines by the DAPI-based immunofluorescence or PCR tests.

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

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. 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.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for '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	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No.