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Adaptive Pathfinding by Nucleokinesis during Amoeboid Migration

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

1st Editorial Decision **30th Jun 2023**

Dear Jörg,

Thank you for submitting your manuscript for consideration by the EMBO Journal. I apologise for the protracted review process due to the delays in referee report submission. We have now received comments from three reviewers, which are included below for your information.

As you will see from the reports, all reviewers find the study per se of interest, while also pointing out a number of important aspects that would need to be addressed in the final manuscript before they can recommend acceptance of the manuscript. Based on the interest expressed in the reports, I would like to invite you to address the issues raised by the referees in a revised manuscript. I think it would be useful to discuss the revision in more detail via email or phone/videoconferencing - please let me know which option you prefer.

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

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

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

With best wishes,

Ieva

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Instructions for preparing your revised manuscript:

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

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

When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
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IMPORTANT: When you send the revision we will require
- a point-by-point response to the referees' comments, with a detailed description of the changes made (as a word file).
- a word file of the manuscript text.
- individual production quality figure files (one file per figure)
- a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14602075/authorguide).
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Please remember: Digital image enhancement is acceptable practice, as long as it accurately represents the original data and conforms to community standards. If a figure has been subjected to significant electronic manipulation, this must be noted in the figure legend or in the 'Materials and Methods' section. The editors reserve the right to request original versions of figures and the original images that were used to assemble the figure.

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We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (28th Sep 2023). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions.

Referee #1:

In this manuscript Kroll et al., employ microfabrication techniques to study the role played by nuclear movement/positioning on amoeboid cell migration and pathfinding in confined microenvironments. They show that nucleokinesis allows dendritic cells to reorientate as they choose between chemical and mechanical cues. They also show that nucleokinesis requires myosin-II activity. They end the manuscript by showing that nucleokinesis is a conserved mechanism as Dictyostelium also displayed the same behavior. The manuscript is well written, the data is compelling and well controlled. I believe it would be a great addition to the EMBO Journal and I have just a few minor comments:

1-The authors say: "...Indeed, when the two paths were composed of almost equally sized large pores at their path entrance, which are above the threshold of the nuclear pore size sensing mechanism..."
Can the authors point to the data in this manuscript or any other already published paper that characterize the threshold of nuclear pore size sensing mechanism and how this relates to the pore size in figure 2A?
2-Nucleokinesis in figure 3D needs to be quantified.
3-Figures EV4D, E and EV5E, F would benefit from representative images (in addition to the heat maps).
4- The authors say: "...imaging of myosin-IIA localization during amoeboid nucleokinesis using MYH9-GFP encoding dendritic cells revealed an enriched myosin-IIA localization in retracting protrusions closely behind the nucleus...".
This should be contextualized (at least in the discussion) with the work from Matthieu Piel's group (Thiam et al., 2016, Nat. Comms)
5-Authors should try to better explain figure 1E in the main text as I had a hard time understanding it.
6-In figure 1H, what is the difference between gray and red lines?
7- Can the authors show/visualize the actual chemokine gradient/diffusion inside the "distant" versus "close" channel designs?
8-If the authors could perform the micro-channel assay with Dictyostelium (with modified dimensions to account for their smaller size) plus the chemotactic cues (folate) it would make their case stronger. At the very least authors should explain why they chose the environmental maze instead of the micro-channel assay that was used throughout the paper.

Referee #2:

In the Manuscript Adaptive Pathfinding by Nucleokinesis during Amoeboid Migration Kroll et al. describe the phenomenon how the nucleus is moving inside of migrating amoeboid cells and how this movement is needed for the cells to find their path. The authors investigate how amoeboid cells react to chemical versus physical cues and how they can reorient when they are taking a ‚wrong' decision such as a dead end. The authors investigate the role of Microtubules and myosin in this process and conclude that not MTs but myosin is necessary for nucleokinesis. On this particular question we have a major concern which we will address below.
The question how amoeboid cells move in a crowded environment and how their nucleus behaves in such a movement is a long standing and studied question in the biophysical community and while this paper does not explain every detail of the mechanism behind, it offers a piece to the big puzzle and is worth to be published. The experiments are carried out carefully on several cell models and different ways of perturbation and the manuscript is generally written in an excellent scientific English.
In the following we have some specific comments which needs to be addressed:
Major concerns:
1) The use and concentration of nocodazole.
How did the authors decide to use the final concentration of 300nM ? In the titration experiment (EV5B) at those low doses of

nocodazole the migration speed is reduced. In literature, data are published where immune cells migrate faster with depolymerized MTs at higher concentrations e.g. https://doi.org/10.1038/s41467-021-22985-5 or https://doi.org/10.3389/fimmu.2021.729820) . Also, it is known that at low concentrations, nocodazole does STABILIZE MTs instead of depolymerizing them (DOI: 10.3390/cancers13235995 , or DOI: 10.1242/jcs.108.11.3473) . And in fact, a stabilization of MTs via Taxol would indeed result in a reduced migration speed as the authors describe. Another fact which shows that MTs are not depolymerized at 300 nM is the simple fact that the authors analyze the MTOC position with the drug being applied! If nocodazole would depolymerize MTs as the authors claim it would not be possible to find a MTOC ...
Therefore, the conclusions that MTs do not play a role would not hold. Following this, the logic conclusion that an increased contractility happens due to GEF-H1 which gets released by the depolymerized microtubules does not work either.
Is there any way how the authors can convince us that Nocodazole depolymerizes MTs at 300 nM in their cell models?

2) Myosin
The authors claim that inhibiting myosin impairs nukleokinesis which in turns distorts path adaptation and causes arrest in the microenvironment. However, inhibiting myosin has many different effects on the migration of any cells, also amoeboid cells. From the data presented, we do not see why an impaired nucleokinesis could not be the consequence of impaired migration, instead of the cause, as claimed by the authors? Is there a way how the authors can impair specifically nucleokinesis?
3) In the conclusion of the manuscript the authors come to the ,intriguing model that amoeboid nucleokinesis might be solely driven by myosin pushing forces from behind the nucleus, moving the nucleus forward without any requirement of anchorage to the cytoskeleton '. In other words, the nucleus does not play any active role in the migration of amoeboid cell migration and is rather ,dragged along'. In that case, the name ,nucleokinesis' might overstate the role of the nucleus, since it suggests an active role in the mechanism.
4) Its not clear if / in which scenarios the nucleus is pulled and in which it is pushed. Can the authors show the distribution of myosin in channels before, during and after nucleokinesis? Can the authors also provide actin staining during this process?

Minor points

1) The discussion of the position of the nucleus in amoeboid cells is completely missing in the introduction. Here, the focus places on the difference between chemical and physical cues. In contrary, in the discussion of the paper the differences between chemical and physical cues are not mentioned at all anymore, but all focus is on nucleokinesis. A better balance between both ideas will help to keep the readers on board.
2) Statistics: it says in the figures, that every experiment is carried out n=3 and the number of cells. In the M&M it says that all replicates were validated independently and pooled only when all showed similar results. So how many experiments have been done in total/ how many needed to be excluded?
3) The discussion about thresholds when the chemokine cues overrule the pore size cues could be intensified. Can these data on pore sizes be set in relation to in vivo situations (which pore sizes do we expect where in vivo?).
4) Figure 2H: I appreciate trying to add as many information into a figure as possible, and most figures are really done in an excellent way, but that one seems too much. Is there a better way of visualizing these results, perhaps by having more figures? It's quite demanding to the reader to keep track of all the different color codes and data per se ...
5) How close was the chemokine source? How was the source incorporated and when did it start to release ccl19? Is there any way to test the strength of CCL 19 in the channels, e.g. would not cells which are imaged later have less strong gradients then the ones imaged earlier?
6) Figure 3: Did the authors tried a scenario where the second, not blocked channel which is so small that the MTOC cannot overtake the nucleus?
7) 3B: close up not necessary
8) When Figure 3D is discussed: it says it shows a preferential MTOC - forward configuration during DC pathfinding in collagen - I don't see that from this figure/movie. Can it be indicated clearer or another example be shown? Is there more than one cell, so that there is any quantification?
9) Can the authors comment on the differences between the data on myosin IIA knock out and the ones with blebbistatin? I would have expected the contrary (stronger results with KO)

Referee #3:

Kroll and colleagues have submitted a manuscript that seeks to understand how the movement of the nucleus within a rapidly migrating amoeboid cell helps the cell navigate through environments containing competing chemical (gradient) and mechanical (pore-size) cues. The data and movies are a delight. However, as I read through the manuscript, I found I was increasingly confused as to what the authors meant by "nucleokinesis" and that much of the conclusions were based on simple correlations, rather than testing specific mechanistic predictions. My overall impression of the manuscript is that it makes several claims that are not well supported by the data, and that an ambiguous definition of what nucleokinesis is makes it difficult to understand the connections between the results and conclusions made by the authors. My overall enthusiasm for this work is high, and I expect if the authors can address these concerns and the others detailed below, this manuscript is going to provide new insights into how the movement of the nucleus can govern the directional movement of amoeboid cells.

Major concerns:

1. The definition of nucleokinesis is not precise. The definition provided by the authors is quite ambiguous: "Full long-distance nuclear repositioning." I think it would be less confusing/ambiguous if the authors explicitly defined nucleokinesis relative to the motion of the cell body. Obviously, a nucleus is constantly in motion in a moving cell, but I don't think the authors would claim that the nucleus is constantly undergoing nucleokinesis. More likely that nucleokinesis means the nucleus is moving relative to the cell body. Also, the more quantitative they can make their definitions, the better. For example, what distinguishes full nucleokinesis from a partial nucleokinesis, is it velocity relative to the cell body, distance relative to the cell body, or both? What range of these values (or whichever the authors relied on) did the authors use to decide whether the nucleus was undergoing a full or partial repositioning. Do the authors think that the same mechanism applies to both full and partial forms of nucleokinesis? What evidence do they have that it is the same or different mechanisms? Without this level of precision in their definition and discussion of their results, it will be difficult to understand what exactly is being studied in this paper.

2. The ambiguity in the definition of nucleokinesis makes it difficult to understand what the authors are measuring in their experiments. For example, Figure 1D talks about nucleokinesis rate as a percentage, and I am not sure what this represents. Presumably that only 20% of the DC cells imaged use nucleokinesis. Then in Figure 1E, the figure says that DC cells undergo nucleokinesis ~60 times a minute. It is very difficult to reconcile these two pieces of data. 1F and 1G talk about nuclear speed and nuclear distance, but is this during full nuclear positioning or partial nuclear positioning? Also, T-cells only use partial nuclear positioning. Perhaps partial and full repositioning represent two completely different mechanisms? What is the justification that they are the same mechanism?

3. The authors state that two steps of nucleokinesis are correlated to consecutive polarity switches in positioning of the MTOC relative to the nucleus and the direction of cell migration. However, the second switch (or the switch back to the MTOC being behind the nucleus) occurs long after nucleokinesis is complete and the cell and nucleus are moving in tandem again. If that is correct, what is the mechanistic connection between that second switch and nucleokinesis itself? It is possible I am very confused as to what the authors intended to conclude and hope that providing rigorous operational definitions of nucleokinesis could reduce this ambiguity.

4. Many of the conclusions rely on simple correlations, rather than testing specific predictions arising from a strong hypothesis. The best example of this comes from the section of the paper examining where the force driving nucleokinesis is generated. The authors make the very strong conclusion that myosin-based pushing forces drive amoeboid nucleokinesis without any evidence to distinguish pulling versus pushing forces. In fact, in many of their movies the nucleus is able to move forward completely independently of the motion of the trailing edge which is more consistent with a pulling mechanism. It is not clear to me why the authors are convinced that pushing forces are responsible for nucleokinesis, especially for the "full nuclear repositioning" that is so prominent in their representative movies. I think additional experiments are needed to distinguish between pulling and pushing in this model.

Other comments:

1. I'm having a hard time parsing the logic of this sentence. Please say what you mean directly.

"Given that these morphological characteristics are particularly frequent in more shallow gradients (Kay et al, 2008; Andrew & Insall, 2007; Swanson & Taylor, 1982; Gerisch & Keller, 1981) and that deficiency of the Arp2/3 regulator Hem1 causes not only a shape simplification into less ramified shapes but also impaired migration along chemokine gradients (Leithner et al, 2016), it is likely that these alternative protrusions simultaneously measure guidance cues at alternative local sites in the microenvironment".

2. For chemokine versus pore-size, the actual gradients should be measured at the start and end of the assay (possibly by including a soluble fluorophore in the chemokine solution and imaging its distribution). Otherwise, a major conclusion of work is only supported by assumptions.

3. Why do the authors have 2 independent replicates in some of their experiments (Fig. 1) and 3 for others? Three independent replicates are standard in my experience with these types of experiments.

4. I'm not fully convinced that nucleokinesis is required for adaptive pathfinding. Ideally, the authors would design an experiment that would specifically prevent nucleokinesis (repositioning) and then show that the choice between pore-size and gradient is altered in some way. Perhaps testing how blebbistatin treatment affects this choice would be sufficient to strengthen this conclusion. Further, I'm not sure why the authors conclude that nucleokinesis enables adaptive pathfinding when the 8-micron pore is selected over 80% of the time without any nuclear re-positioning (Fig. 2H-J)? Is it only required in the 20% of cases when the cell needs to switch to a second path?

6. Is there any difference in polarity switching when comparing a partial versus a full nucleokinesis event? If there is, it would

suggest they are occurring via different mechanisms.

7. In the discussion the authors state: "...raise the intriguing model that amoeboid nucleokinesis might be solely driven by myosin pushing forces from behind the nucleus, moving the nucleus forward without any requirement of anchorage to the cytoskeleton".

What evidence is this claim (pushing as mentioned above, but also the fact that there is no requirement for cytoskeleton-nucleus connections) based on? This is a bold claim based on the data provided (what evidence is there that it is not connected?).

8. In the results related to figure 3 on page 4 the authors state: "...demonstrating that the nucleus is not only passively dragged by retracting protrusions but actively repositions to its nucleus-forward configuration". I do not understand what they are trying to say and how the data in the figure are connected to this conclusion. I think re-writing to make the logic more plain could help.

9. Perhaps enucleating the cells could be a direct demonstration of the requirement for a nucleus during adaptive pathfinding assuming they are able to migrate at all. I am not saying the authors need to do this experiment since it is risky, but I think their conclusions could be strengthened by a more direct demonstration that nucleokinesis is required for adaptive pathfinding.

Point-by-point reply, *Kroll et al*

We thank the referees for their thoughtful comments and constructive suggestions. We addressed these comments by conducting extensive new experimentation, including confirmation of the experimental setup of competing chemotactic and pore size gradients (e.g., visualization and modelling of the chemokine gradient, new Figures 2B and EV2B; and quantifications of cellular path selections along different pores independent of the chemotactic gradient, new Figures 2A and EV2A), confirmation of the effects of different concentrations of nocodazole on the microtubule cytoskeleton and amoeboid nucleokinesis (new Figures EV6A, EV7C and D), confirmation of the effects of the conditional myosin knockout DCs (new Figures EV9C and D), and visualization of the localization of actin and myosin during amoeboid nucleokinesis (new Figure 4G). Together, these data validate our experimental setup and confirm our findings reported in the initially submitted manuscript. Further, we addressed the particular requirement of myosin forces for nucleokinesis independent of general migration defects (new Figures 4A-D, 5A-C, and 5E). Along this line, we identify Lfc1/GefH1 as a regulator of amoeboid nucleokinesis (new Figures 5F, EV11A-D, and EV11F). These datasets reveal that retractions of 'losing' protrusion containing a nucleus is a particular challenge, requiring myosin forces to reposition the nucleus towards the 'winning' protrusion during the first phase of nucleokinesis and into an intracellular location ahead of the MTOC during the second phase.

Please find the detailed point-by-point responses below, addressing the individual comments and suggestions.

(changes in the text of the revised manuscript are marked in gray)

Referee #1:

In this manuscript Kroll et al., employ microfabrication techniques to study the role played by nuclear movement/positioning on amoeboid cell migration and pathfinding in confined microenvironments. They show that nucleokinesis allows dendritic cells to reorientate as they choose between chemical and mechanical cues. They also show that nucleokinesis requires myosin-II activity. They end the manuscript by showing that nucleokinesis is a conserved mechanism as Dictyostelium also displayed the same behavior. The manuscript is well written, the data is compelling and well controlled. I believe it would be a great addition to the EMBO Journal and I have just a few minor comments.

Thank you for the meaningful comments and experimental suggestions. In the revised manuscript, we addressed these comments, including new experimentation on the visualization of the chemokine gradient (new Figures 2B and EV2B), experimentation on the thresholds of the pore size sensing mechanisms (new Figures 2A and EV2A), and quantification of the MTOC-nucleus axis during migration in collagen matrices (new Figure 3E).

1-The authors say: "...Indeed, when the two paths were composed of almost equally sized large pores at their path entrance, which are above the threshold of the nuclear pore size sensing mechanism..."

Can the authors point to the data in this manuscript or any other already published paper that characterize the threshold of nuclear pore size sensing mechanism and how this relates to the pore size in figure 2A?

In the main text of the revised manuscript, we now directly refer to the paper that described the pore size threshold of the nuclear pore size sensing mechanism (Renkawitz et al, Nature 2019) (main text, lines 122-125). Additionally, to confirm the threshold experimentally in the here described assays, we now performed new experiments in which we generated 2-way path junctions with different pore sizes at the path entrance but equal distances to the chemokine cue (new Figure 2A and EV2A). Specifically, we compared cellular path choices at 8μm vs. 2μm, 8μm vs. 4μm, 8μm vs. 6μm, 8μm vs. 8μm pores, as we have used these pore sizes throughout the manuscript and as they cover the size range of the nuclear pore size sensing mechanism. While the cells did not show any pore size preference when comparing 8μm vs. 6μm and 8μm vs. 8μm pores, they preferred the larger 8μm pore over the smaller 4μm and 2μm pores, confirming the threshold of pore size sensing mechanism when pores are 4μm or smaller. Thus, these data further validate our experimental setup to probe cellular behavior when exposed to competing pore size and chemotactic cues. We added these data to the revised manuscript (new Figure 2A and EV2A).

2-Nucleokinesis in figure 3D needs to be quantified.

We apologize that we missed to quantify the MTOC-nucleus axis configuration during nucleokinesis in collagen matrices in the initial manuscript. We now conducted this quantification, confirming the MTOC-nucleus axis orientation during nucleokinesis in collagen matrices as described in the microchannel experiments. We included this new dataset into the revised manuscript (new Figure 3E).

3-Figures EV4D, E and EV5E, F would benefit from representative images (in addition to the heat maps).

We added these representative images to the revised manuscript: (i) new Figure EV5C shows representative images for figures EV5D and E (former EV4D and E), and (ii) and new Figure EV7F shows representative images for figures EV7E and G (former EV5E and F).

4- The authors say: "…imaging of myosin-IIA localization during amoeboid nucleokinesis using MYH9-GFP encoding dendritic cells revealed an enriched myosin-IIA localization in retracting protrusions closely behind the nucleus"..". This should be contextualized (at least in the discussion) with the work from Matthieu P'el's group (Thiam et al., 2016, Nat. Comms)

Thank you for spotting that we accidently missed to cite this important paper. In the revised manuscript, we now added a careful discussion about the roles of myosin during amoeboid migration, including a reference to the work of Thiam et al, Nat Com 2016 (lines 349-354).

5-Authors should try to better explain figure 1E in the main text as I had a hard time understanding it.

Figure 1E quantifies how often migrating dendritic cells and T cells have multiple simultaneous protrusions during migration, and how often they perform nucleokinesis in a collagen matrix. We apologize that the original explanation in the text was too short. We now provided an additional explanation of Figure 1E in the main text (lines 105-106) and extended the figure legend for better clarity.

6-In figure 1H, what is the difference between gray and red lines?

Thank you for noticing that we missed to describe the color-coding of the lines: the red line shows the mean and the gray lines show the standard error of the mean. We added this information to the figure legend of the revised manuscript.

7- Can the authors show/visualize the actual chemokine gradient/diffusion inside the "distant" versus "close" channel designs?

To visualize the chemokine gradient and assess its diffusion characteristics, we now performed experiments with fluorescent dextran with a molecular weight and hydrodynamic radius comparable to the employed chemokine CCL19. This well-established approach (see e.g., Schwarz et al, Scientific Reports 2016; Frick et al PLoS ONE 2018) revealed higher concentrations at the path junction towards the path closer to the chemokine source and lower concentrations towards the path more distant to the chemokine source (new Figure 2B). Notably, this difference was observable at the beginning of the experiment as well as 2 hours later, reflecting the typical period of time in which we quantified cellular pore choices (new Figure 2B). We complemented this experimental approach with a theoretical simulation approach using the diffusion constant of CCL19, predicting the chemokine gradient with higher chemokine concentrations towards the path closer to the chemokine source (new Figure EV2B). Thus, these approaches together confirm different chemokine concentrations directly at the path junction (this answer is the same as for reviewer 2 “minor point 5”, and reviewer 3 “other comments 2”, suggesting the same controls).

8-If the authors could perform the micro-channel assay with Dictyostelium (with modified dimensions to account for their smaller size) plus the chemotactic cues (folate) it would make their case stronger. At the very least authors should explain why they chose the environmental maze instead of the micro-channel assay that was used throughout the paper.

When we started experimentation with *Dictyostelium* in this project, we initially attempted to load *Dictyostelium* cells into microchannel assays. However, in the microchannel assays, due to technical reasons, cells have to migrate for some hundreds of micrometers through a straight channel until they reach the path decision point in which we can monitor nucleokinesis. When we let *Dictyostelium* cells migrate in the microchannels towards a folate source, we rarely observed cells reaching the path decision point, indicating that they do not migrate for very long distance in a highly directional manner, making it not possible to investigate nucleokinesis. As an experimental alternative, we applied a cAMP gradient as an alternative well-known chemotactic cue for *Dictyostelium*. Yet, this frequently resulted in clustering of the

cells, preventing the analysis of nucleokinesis in single amoebae. We solved these technical issues during the course of the project by loading *Dictyostelium* cells into pillar forests, which offers an environment in which the cells immediately encounter path options once they are loaded, enabling the investigation of nucleokinesis events once the cells start to migrate. We have now provided a detailed explanation of these technical considerations in the methods section of the manuscript.

Referee #2:

In the Manuscript Adaptive Pathfinding by Nucleokinesis during Amoeboid Migration Kroll et al. describe the phenomenon how the nucleus is moving inside of migrating amoeboid cells and how this movement is needed for the cells to find their path. The authors investigate how amoeboid cells react to chemical versus physical cues and how they can reorient when they are taking a ‚wrong' decision such as a dead end. The authors investigate the role of Microtubules and myosin in this process and conclude that not MTs but myosin is necessary for nucleokinesis. On this particular question we have a major concern which we will address below.
The question how amoeboid cells move in a crowded environment and how their nucleus behaves in such a movement is a long standing and studied question in the biophysical community and while this paper does not explain every detail of the mechanism behind, it offers a piece to the big puzzle and is worth to be published.
The experiments are carried out carefully on several cell models and different ways of perturbation and the manuscript is generally written in an excellent scientific English.
In the following we have some specific comments which needs to be addressed:

Thank you for the constructive comments and suggestions. In the revised manuscript, we have addressed these comments as outlined below, including characterization of the effects of different nocodazole concentrations on microtubule depolymerization and amoeboid nucleokinesis (new Figures EV6A, EV7C and D), confirmation of the phenotypes of the conditional myosin knockout DCs (new Figures EV9C and D), visualization of the chemokine gradient (new Figures 2B and EV2B), visualizing the localization of actin and myosin during amoeboid nucleokinesis (new Figure 4G), and quantification of the consequences of myosin inhibition on the general migration behavior versus particular consequences for nucleokinesis (new Figures 4A-D, 5A-C, and 5E), including the identification of Lfc1/GefH1 as a regulator of amoeboid nucleokinesis (new Figures 5F, EV11A-D, and EV11F).

Major concerns:
1) The use and concentration of nocodazole.
How did the authors decide to use the final concentration of 300nM ? In the titration experiment (EV5B) at those low doses of nocodazole the migration speed is reduced. In literature, data are published where immune cells migrate faster with depolymerized MTs at higher concentrations e.g. https://doi.org/10.1038/s41467-021-22985-5 or https://doi.org/10.3389/fimmu.2021.729820) . Also, it is known that at low concentrations, nocodazole does STABILIZE MTs instead of depolymerizing them (DOI: 10.3390/cancers13235995 , or DOI: 10.1242/jcs.108.11.3473) . And in fact, a stabilization of MTs via Taxol would indeed result in a reduced migration speed as the authors describe. Another fact which shows that MTs are not depolymerized at 300 nM is the simple fact that the authors analyze the MTOC position with the drug being applied! If nocodazole would depolymerize MTs as the authors claim it would not be possible to find a MTOC ...
Therefore, the conclusions that MTs do not play a role would not hold. Following this, the logic conclusion that an increased contractility happens due to GEF-H1 which gets released by the depolymerized microtubules does not work either.
Is there any way how the authors can convince us that Nocodazole depolymerizes MTs at 300 nM in their cell models?

To confirm that the concentration of 300nM nocodazole indeed depolymerizes microtubules in our cell model, we performed immunofluorescence staining using the microtubule marker

anti-alpha-tubulin. These experiments revealed in control samples long microtubules nucleating from a single MTOC and spreading through the entire cell (as shown earlier; e.g., Kopf et al, JCB 2020). Yet, already low doses of nocodazole (300nM) caused almost entire depolymerization of the microtubule network, with only short remnants of microtubules at the MTOC and only very occasionally elongated microtubules towards the cellular periphery (new Figure EV6A). This shows that the usage of 300nM of nocodazole largely depolymerizes the microtubule network in our cell system. Further, it also explains why we still are able to detect the MTOC in these cells (despite a much lower signal intensity, as there are only a very few and very short remnant microtubules left at the MTOC). To exclude the possibility that these short and sparse microtubules may still have a functional role during nucleokinesis, we substantially increased the concentration of nocodazole to 10 μM. This resulted in complete depolymerization of the microtubule cytoskeleton, leading to migratory cells without any detectable microtubules (new Figure EV6A). Using this setup, we next measured the efficiency of nucleokinesis. According to our initial findings with low doses of nocodazole (300 nM), also the new experimentation with high doses of nocodazole (10 μM) did not alter the speed of nucleokinesis (new Figures EV7C and D), confirming our conclusions about the not-detectable role of microtubules during amoeboid nucleokinesis.

2) Myosin

The authors claim that inhibiting myosin impairs nukleokinesis which in turns distorts path adaptation and causes arrest in the microenvironment. However, inhibiting myosin has many different effects on the migration of any cells, also amoeboid cells. From the data presented, we do not see why an impaired nucleokinesis could not be the consequence of impaired migration, instead of the cause, as claimed by the authors? Is there a way how the authors can impair specifically nucleokinesis?

Thank you for this important comment. Indeed, inhibition of myosin-II has been reported to cause different phenotypes in amoeboid migration. Yet, our reductionist microchannels enabled us to disentangle the consequences of myosin inhibition on the individual components of amoeboid migration. This revealed that cells migrating in straight channels have the same or even a faster migration velocity upon myosin inhibition (Figure EV8E). Yet, when the cells approached the path decision junction and explored the different paths with simultaneous protrusions, myosin inhibition caused substantially delayed cellular decisions towards the 'winning' path (Figure EV8E). To disentangle whether this phenotype is only a general consequence of slower retraction of the 'losing' protrusion upon myosin inhibition or whether it is caused by affecting nucleokinesis, we quantified in the revised manuscript 'losing' protrusions with and without a nucleus (new Figures 4A-D). Notably, while the retraction of 'losing' protrusions without a nucleus are only mildly slowed down, the retraction of 'losing' protrusions with a nucleus is substantially slowed down upon myosin inhibition (new Figures 5B and E). These data show that myosin is not only generally required to retract protrusions but is also specifically required to retract protrusions with a nucleus that has to be repositioned by nucleokinesis. We further confirmed these data in the revised manuscript, by investigating the consequences of knockout of the RhoA guanine nucleotide exchange factor GEF-H1 (Lfc in mice): while the knockout of Lfc did not impair migration in straight channels (new Figures EV11C and D) and did not impair the orientation of the nucleus-MTOC axis configuration (new Figures EV11A and B), it specifically delayed retractions of 'losing' protrusions but only when they contained a nucleus (new Figure 5F). These findings show that the retraction of

protrusions containing the nucleus is a particular challenge, requiring particular high myosin activity to drive nucleokinesis.

3) In the conclusion of the manuscript the authors come to the ‚intriguing model that amoeboid nucleokinesis might be solely driven by myosin pushing forces from behind the nucleus, moving the nucleus forward without any requirement of anchorage to the cytoskeleton '. In other words, the nucleus does not play any active role in the migration of amoeboid cell migration and is rather ‚dragged along'. In that case, the name ‚nucleokinesis' might overstate the role of the nucleus, since it suggests an active role in the mechanism.

We apologize for the speculative statement regarding an anchorage-independent mechanisms in the discussion of the initial manuscript (see also comment by Reviewer 3). We fully agree that this is not the topic of this manuscript. In the revised manuscript, we excluded this sentence and instead mention in the discussion “It will be interesting to investigate whether the here-described mechanisms of amoeboid nucleokinesis requires anchorage between the actomyosin cytoskeleton and the nucleus, or whether it may be functioning by an anchorage-independent mechanism solely driven by myosin forces located at the rear of the nucleus”. Further, we include now a discussion on the term “nucleokinesis”, which has been defined in neurons as active intracellular repositioning of the nucleus, in line with our data showing that the nucleus is not only dragged along at the rear of the cell but is actively positioned frontward of the MTOC.

4) Its not clear if / in which scenarios the nucleus is pulled and in which it is pushed. Can the authors show the distribution of myosin in channels before, during and after nucleokinesis? Can the authors also provide actin staining during this process?

As suggested, we imaged the localization of myosin and F-actin by using DCs that genetically encode either myosin-GFP (MYH9-GFP) or Lifeact-GFP. To monitor the localization over time specifically during the process of nucleokinesis, we loaded these cells into the microchannel junctions with equally large pore sizes but one blocked path, leading to nucleokinesis events from the blocked to the open path. Thereby we were able to monitor the distribution of myosin and F-actin before, during, and after nucleokinesis, showing relocalisation of myosin from the previous cell rear towards the back of the nucleus-containing ‘losing’ protrusion. Thus, these data confirm the myosin distribution described in the initial manuscript. We added these data to the revised manuscript (new Figure 4G).

Minor points

1) The discussion of the position of the nucleus in amoeboid cells is completely missing in the introduction. Here, the focus places on the difference between chemical and physical cues. In contrary, in the discussion of the paper the differences between chemical and physical cues are not mentioned at all anymore, but all focus is on nucleokinesis. A better balance between both ideas will help to keep the readers on board.

In the revised manuscript, we have improved the balance between the introduction and the discussion sections, ensuring that both concepts – nucleokinesis and adaptive pathfinding

along chemical and physical cues are adequately addressed (new subparagraphs: lines 67-74 and lines 373-386).

2) Statistics: it says in the figures, that every experiment is carried out n=3 and the number of cells. In the M&M it says that all replicates were validated independently and pooled only when all showed similar results. So how many experiments have been done in total/ how many needed to be excluded?

We apologize that our phrasing may have indicated that we excluded particular datasets. We actually did not exclude any datasets. We just wanted to express that we carefully checked the data plots, which pool datapoints from individual cells from different replicates. We now clarified the phrasing in the Materials and Methods section.

3) The discussion about thresholds when the chemokine cues overrule the pore size cues could be intensified. Can these data on pore sizes be set in relation to in vivo situations (which pore sizes do we expect where in vivo?).

We have added an intensified discussion in the revised manuscript regarding the thresholds at which chemokine cues override pore size cues, including a comparison to *in vivo* pore sizes (lines 368-386).

4) Figure 2H: I appreciate trying to add as many information into a figure as possible, and most figures are really done in an excellent way, but that one seems too much. Is there a better way of visualizing these results, perhaps by having more figures? It's quite demanding to the reader to keep track of all the different color codes and data per se ...

We have simplified and extended the former Figure 2H (now Figure 2J) to improve readability and accessibility for the reader.

5) How close was the chemokine source? How was the source incorporated and when did it start to release ccl19? Is there any way to test the strength of CCL 19 in the channels, e.g. would not cells which are imaged later have less strong gradients then the ones imaged earlier?

To visualize the chemokine gradient and assess the diffusion characteristics, we now performed experiments with fluorescent dextran with a molecular weight and hydrodynamic radius comparable to the employed chemokine CCL19. This well-established approach (see e.g., Schwarz et al, Scientific Reports 2016; Frick et al PLoS ONE 2018) revealed higher concentrations at the path junction towards the path closer to the chemokine source and lower concentrations towards the path more distant to the chemokine source (new Figure 2B). Notably, this difference was observable at the beginning of the experiment as well as 2 hours later, reflecting the typical period of time in which we quantified cellular pore choices (new Figure 2B). We complemented this experimental approach with a theoretical simulation approach using the diffusion constant of CCL19, predicting the chemokine gradient with higher chemokine concentrations towards the path closer to the chemokine source (new Figure EV2B). Thus, these approaches together confirm different chemokine concentrations directly

at the path junction (this answer is the same as for reviewer 1 “point 7”, and reviewer 3 “other comments 2”, who suggested the same controls).

6) Figure 3: Did the authors tried a scenario where the second, not blocked channel which is so small that the MTOC cannot overtake the nucleus?

We fully agree that it is very likely that a small channel along the cellular path may result in a delayed repositioning of the MTOC and nucleus during nucleokinesis due to sterical hindrance. Yet, such a small channel would have to have a considerable length, as we typically observe reorientation towards the original nucleus-MTOC axis only some cell lengths after start of nucleokinesis. However, an experimental system with long and extremely narrow channels would expose the cells to considerable mechanical stress, including nuclear envelope rupture, with unpredictable cell responses. Thus, we think that the manuscript would not benefit from this experimental approach that is difficult to interpret.

7) 3B: close up not necessary

We removed the close up in Figure 3B in the revised manuscript.

8) When Figure 3D is discussed: it says it shows a preferential MTOC - forward configuration during DC pathfinding in collagen - I don't see that from this figure/movie. Can it be indicated clearer or another example be shown? Is there more than one cell, so that there is any quantification?

In the initial manuscript we showed an image that included 3 colors, making it difficult to observe the orientation of the nucleus-MTOC axis. We have improved the image representation in the revised manuscript by also showing only the nucleus (Hoechst) and MTOC (EMTB-mCherry) fluorescent channels, so that the nucleus-MTOC axis orientation can better be seen (Figure 3D). In addition, we now also quantified the MTOC-nucleus axis orientation in this dataset, and included it into the revised manuscript (new Figure 3E). (the 2nd part of the answer is similar as for reviewer 1 “point 2”, suggesting the same quantification during migration in collagen networks).

9) Can the authors comment on the differences between the data on myosin IIA knock out and the ones with blebbistatin? I would have expected the contrary (stronger results with KO)

The mild effects of myosin IIA knockout on nucleokinesis are due to the conditional nature of the knockout: the myosin IIA knockout DCs derive from a mouse, in which the deletion of myosin IIA occurs conditionally in DCs during their differentiation with the Cre recombinase being under control of the promoter Cd11c (MyoIIA-Flox*CD11c-Cre), which only gets activated late during differentiation (roughly days 5-7 of differentiation). In our experiments, we used DCs on day 8 of differentiation, suggesting the possibility that the levels of myosin IIA protein may only be reduced but not completely abolished during the experiment. Importantly, this result is in line with a previous manuscript using DCs from the same myosin IIA knockout mouse, also observing milder phenotypes in this experimental system in comparison to myosin inhibition by blebbistatin (Thiam et al, Nat Comm 2016). To further

reduce the levels of myosin IIA in our experiments, we performed new experimentations with DCs that we differentiated longer until day 10, leaving more time for Cre expression under control of CDc11c. We loaded these cells into the microchannels with one blocked path and stained the MTOC with spy-tubulin, and again observed reduced rates of switching in the nucleus-MTOC axis configuration, confirming our findings using the myosin inhibitor blebbistatin (new Figures EV9C and D).

Referee #3:

Kroll and colleagues have submitted a manuscript that seeks to understand how the movement of the nucleus within a rapidly migrating amoeboid cell helps the cell navigate through environments containing competing chemical (gradient) and mechanical (pore-size) cues. The data and movies are a delight. However, as I read through the manuscript, I found I was increasingly confused as to what the authors meant by "nucleokinesis" and that much of the conclusions were based on simple correlations, rather than testing specific mechanistic predictions. My overall impression of the manuscript is that it makes several claims that are not well supported by the data, and that an ambiguous definition of what nucleokinesis is makes it difficult to understand the connections between the results and conclusions made by the authors. My overall enthusiasm for this work is high, and I expect if the authors can address these concerns and the others detailed below, this manuscript is going to provide new insights into how the movement of the nucleus can govern the directional movement of amoeboid cells.

Thank you for the thoughtful comments and suggestions. In the revised manuscript, we clarified the definition of nucleokinesis and also performed a substantial number of new experiments, including visualization and modelling of the chemokine gradient (new Figures 2B and EV2B), visualization of the localization of actin and myosin during amoeboid nucleokinesis (new Figure 4G), and addressing the particular requirement of myosin forces for nucleokinesis (new Figures 4A-D, 5A-C, and 5E), including the identification of Lfc1/GefH1 as a regulator of amoeboid nucleokinesis (new Figures 5F, EV11A-D, and EV11F).

Major concerns:

1. The definition of nucleokinesis is not precise. The definition provided by the authors is quite ambiguous: "Full long-distance nuclear repositioning." I think it would be less confusing/ambiguous if the authors explicitly defined nucleokinesis relative to the motion of the cell body. Obviously, a nucleus is constantly in motion in a moving cell, but I don't think the authors would claim that the nucleus is constantly undergoing nucleokinesis. More likely that nucleokinesis means the nucleus is moving relative to the cell body. Also, the more quantitative they can make their definitions, the better. For example, what distinguishes full nucleokinesis from a partial nucleokinesis, is it velocity relative to the cell body, distance relative to the cell body, or both? What range of these values (or whichever the authors relied on) did the authors use to decide whether the nucleus was undergoing a full or partial repositioning. Do the authors think that the same mechanism applies to both full and partial forms of nucleokinesis? What evidence do they have that it is the same or different mechanisms? Without this level of precision in their definition and discussion of their results, it will be difficult to understand what exactly is being studied in this paper.

We apologize that we have generated this level of confusion by bringing up the terms ‘partial’ and ‘full’ nucleokinesis. Our entire manuscript is about intracellular repositioning of the entire nucleus, which we have called ‘full nucleokinesis’ in the initial version of the manuscript. We defined this phenomenon as intracellular movement of the nucleus at least one nuclear length relative to cell body (see also quantifications of nuclear movement in relation to the cell body in Figure 6B, including the new quantifications in the new Figure EV4A). In the revised

manuscript, we now refer to this behavior simply as ‘nucleokinesis’ (instead of ‘full nucleokinesis'), in line with its definition in the literature on studies in neurons, and better describe its definition in the main text (lines 71-74, 102, and 313-314), figure legend as well as the Material & Method section (lines 543-544).

When we observed these nucleokinesis events in migrating DCs and T cells, we also noted that the nucleus is often strongly deformed, frequently having a shape with two nuclear protrusions reaching towards alternative paths, likely probing for alternative pore sizes (as reported earlier; Renkawitz et al, Nature 2019). In these situations, only parts of the nucleus have to be reorientated towards the finally chosen cell parts. In other words, not the entire but only parts of the nucleus have to be moved from a ‘losing’ protrusion towards the ‘winning’ protrusion. We called the retraction of these short nuclear protrusions towards the cell path ‘partial nucleokinesis’ in the initial manuscript. However, it is important to note that we mention this behavior in the main figures 1 and 2 to most accurately describe the cellular behavior. However, this is only a side aspect of our study, and the entire manuscript (main figures 3, 4, 5, and 6) focusses on the mechanisms of how the entire nucleus repositions from a ‘losing’ towards the ‘winning’ protrusion, as quick and large-distance repositioning of the entire bulky nucleus provides a conceptual challenge for the cell. To prevent any misunderstandings, we now renamed ‘partial’ nucleokinesis’ into ‘repositioning of nuclear protrusions’ and also provide a better definition in the main text (lines 96-101) and figure legends (lines 795-798) of the revised manuscript. We thank the reviewer for bringing up this point to enable us to provide a better definition for the reader.

2. The ambiguity in the definition of nucleokinesis makes it difficult to understand what the authors are measuring in their experiments. For example, Figure 1D talks about nucleokinesis rate as a percentage, and I am not sure what this represents. Presumably that only 20% of the DC cells imaged use nucleokinesis. Then in Figure 1E, the figure says that DC cells undergo nucleokinesis ~60 times a minute. It is very difficult to reconcile these two pieces of data.

Figure 1D quantifies how often the nucleus locates directly into the future ‘winning’ protrusion or to the future ‘losing’ protrusion, the latter necessitating nucleokinesis towards the cell path. Figure 1E quantifies how often migrating dendritic cells and T cells have multiple simultaneous protrusions and how often they perform nucleokinesis in a collagen matrix. We apologize that the original explanation in the text was too short. We now added additional explanations into the figure legend to better explain the data.

1F and 1G talk about nuclear speed and nuclear distance, but is this during full nuclear positioning or partial nuclear positioning? Also, T-cells only use partial nuclear positioning. Perhaps partial and full repositioning represent two completely different mechanisms? What is the justification that they are the same mechanism?

These datasets quantified nucleokinesis (what we have named ‘full nucleokinesis’ in our initial dataset). Please also see our answer to point 1, clarifying the definition of ‘partial’ and ‘full’ nuclear repositioning.

3. The authors state that two steps of nucleokinesis are correlated to consecutive polarity switches in positioning of the MTOC relative to the nucleus and the direction

of cell migration. However, the second switch (or the switch back to the MTOC being behind the nucleus) occurs long after nucleokinesis is complete and the cell and nucleus are moving in tandem again. If that is correct, what is the mechanistic connection between that second switch and nucleokinesis itself? It is possible I am very confused as to what the authors intended to conclude and hope that providing rigorous operational definitions of nucleokinesis could reduce this ambiguity.

When a nucleus locates into a future 'losing' protrusion, it does not only need to be repositioned into the 'winning' protrusion along the cell path, but it also has to be positioned again frontwards of the MTOC. Thus, our data show that nucleokinesis in migrating amoeboid cells is composed of two phases: in the first phase of nucleokinesis, the nucleus travels intracellularly from the 'losing' into the 'winning' protrusion. In the second phase of nucleokinesis, the nucleus travels from the rear of the MTOC to the front of the MTOC. We clarified this aspect in the revised manuscript by extending our description in the main text (lines 185-194). In addition, we included a new quantification of the positioning of the MTOC and nucleus in relation to the cell rear during the second phase of nucleokinesis, underscoring that the nucleus travels intracellularly towards the front of the MTOC (new Figure EV4A).

4. Many of the conclusions rely on simple correlations, rather than testing specific predictions arising from a strong hypothesis. The best example of this comes from the section of the paper examining where the force driving nucleokinesis is generated. The authors make the very strong conclusion that myosin-based pushing forces drive amoeboid nucleokinesis without any evidence to distinguish pulling versus pushing forces. In fact, in many of their movies the nucleus is able to move forward completely independently of the motion of the trailing edge which is more consistent with a pulling mechanism. It is not clear to me why the authors are convinced that pushing forces are responsible for nucleokinesis, especially for the "full nuclear repositioning" that is so prominent in their representative movies. I think additional experiments are needed to distinguish between pulling and pushing in this model.

We apologize that we have raised in our initial manuscript the impression that we would make a definite conclusion that the nucleus is pushed. We checked back on our manuscript and find that we indeed rarely used the phrase 'myosin-based pushing', however only 3 times, yet once in a results subheading. We apologize for that as we did not aim to make a strong conclusion on whether the nucleus is pushed or pulled during amoeboid nucleokinesis. Generally, there is quite of a debate in the literature about pushing and pulling forces, which are often very difficult to discriminate from each other (which has been reviewed for example in Marks, P. C. & Petrie, R. J. Push or pull: how cytoskeletal crosstalk facilitates nuclear movement through 3D environments. *Phys Biol* **19**, 021003 (2022). Thus, we thank the reviewer for spotting the usage of the word 'pushing, and now changed this into 'myosin-driven forces', as we have been describing our findings throughout the rest of the manuscript. Further, we provide a paragraph in the discussions section about whether myosin-based forces may push or pull the nucleus during amoeboid nucleokinesis. Please see also our answer to point 9, describing substantial new experimentation on the specific role of myosin-mediated forces for amoeboid nucleokinesis.

Other comments:

1. I'm having a hard time parsing the logic of this sentence. Please say what you mean directly.

"Given that these morphological characteristics are particularly frequent in more shallow gradients (Kay et al, 2008; Andrew & Insall, 2007; Swanson & Taylor, 1982; Gerisch & Keller, 1981) and that deficiency of the Arp2/3 regulator Hem1 causes not only a shape simplification into less ramified shapes but also impaired migration along chemokine gradients (Leithner et al, 2016), it is likely that these alternative protrusions simultaneously measure guidance cues at alternative local sites in the microenvironment".

In the revised manuscript, we now provide a better readability by rephrasing the content into the following sentences: 'This morphological characteristic of multiple simultaneous protrusions is particularly common in shallow chemotactic gradients (Kay et al, 2008; Andrew & Insall, 2007; Swanson & Taylor, 1982; Gerisch & Keller, 1981), suggesting that cells employ these multiple protrusions to sense chemotactic cues at different micro-locations in their immediate surrounding. This notion is supported by the observation that deficiency of the Arp2/3 regulator Hem1 in motile immune cells not only reduces protrusion formation but also impairs migration along chemotactic gradients (Leithner et al, 2016). Collectively, these findings suggest that cells utilize alternative protrusions to simultaneously explore and measure guidance cues at different local sites in their immediate microenvironment.

2. For chemokine versus pore-size, the actual gradients should be measured at the start and end of the assay (possibly by including a soluble fluorophore in the chemokine solution and imaging its distribution). Otherwise, a major conclusion of work is only supported by assumptions.

To visualize the chemokine gradient and assess the diffusion characteristics, we now performed experiments with fluorescent dextran with a molecular weight and hydrodynamic radius comparable to the employed chemokine CCL19. This well-established approach (see e.g., Schwarz et al, Scientific Reports 2016; Frick et al PLoS ONE 2018) revealed higher concentrations at the path junction towards the path closer to the chemokine source and lower concentrations towards the path more distant to the chemokine source (new Figure 2B). Notably, this difference was observable at the beginning of the experiment as well as 2 hours later, reflecting the typical period of time in which we quantified cellular pore choices (new Figure 2B). We complemented this experimental approach with a theoretical simulation approach using the diffusion constant of CCL19, predicting the chemokine gradient with higher chemokine concentrations towards the path closer to the chemokine source (new Figure EV2B). Thus, these approaches together confirm different chemokine concentrations directly at the path junction (this answer is the same as for reviewer 1 "point 7", and reviewer 2 "minor point 5", suggesting the same controls).

3. Why do the authors have 2 independent replicates in some of their experiments (Fig. 1) and 3 for others? Three independent replicates are standard in my experience with these types of experiments.

We apologize that we missed in the initial manuscript to provide 3 independent replicates of this specific dataset. We added now a third independent replicate to the revised manuscript (Fig 1D and 1E), which is consistent with our initially described results.

4. I'm not fully convinced that nucleokinesis is required for adaptive pathfinding. Ideally, the authors would design an experiment that would specifically prevent nucleokinesis (repositioning) and then show that the choice between pore-size and gradient is altered in some way. Perhaps testing how blebbistatin treatment affects this choice would be sufficient to strengthen this conclusion.

Thank you for this important comment (as also raised by reviewer 2). Indeed, inhibition of myosin has been reported to cause different phenotypes in amoeboid migration. Yet, our reductionist microchannels enabled us to disentangle the consequences of myosin inhibition on the individual components of amoeboid migration. This revealed that cells migrating in straight channels have the same or even a faster migration velocity upon myosin inhibition (Figure EV8E). Yet, when the cells approached the path decision junction and explored the different paths with simultaneous protrusions, myosin inhibition caused substantially delayed cellular decisions towards the 'winning' path (Figure EV8E). To disentangle whether this phenotype is only a general consequence of slower retraction of the 'losing' protrusion upon myosin inhibition or whether it is caused by affecting nucleokinesis, we now quantified in the revised manuscript 'losing' protrusions that either contain a nucleus or do not contain a nucleus. Notably, while the retraction of 'losing' protrusions without a nucleus are only mildly slowed down, 'losing' protrusions that contain a nucleus are substantially slower during retraction upon myosin inhibition (new Figure 5C and E). These data confirm that the activity of myosin is not only generally required to retract protrusions, but are also specifically required to retract protrusions with a nucleus that has to be repositioned by nucleokinesis. Please see also our answer to point 9, in which we show that blebbistatin inhibition causes less frequent adjustments of the path choice by nucleokinesis.

Further, I'm not sure why the authors conclude that nucleokinesis enables adaptive pathfinding when the 8-micron pore is selected over 80% of the time without any nuclear re-positioning (Fig. 2H-J)? Is it only required in the 20% of cases when the cell needs to switch to a second path?

Indeed, as we describe in the main text and Figure 1, the cellular path is mostly identical with the nuclear path. Yet, in 20% of the path decisions, the nucleus is mispositioning into future 'losing' protrusions. This deviation of the nuclear path from the cellular path requires repositioning of the entire nucleus towards the winning protrusion to realign the nuclear with the cellular path. As we show in Figure 2, this path reorientation particularly happens when cells sense a more dominant chemokine cue, enabling them to reorientate their path and their nucleus towards this cue. Considering that fast amoeboid cells constantly generate alternative protrusions and thus constantly have to perform path decision, path adaptions at a frequency of 20% considerably impacts the path of a migrating amoeboid cell (e.g., shown by our finding of stuck cells at path junctions, Fig 5H).

6. Is there any difference in polarity switching when comparing a partial versus a full nucleokinesis event? If there is, it would suggest they are occurring via different mechanisms.

Please see our answer to point 1, clarifying the definition of ‘partial’ and ‘full’ nuclear repositioning.

7. In the discussion the authors state: "...raise the intriguing model that amoeboid nucleokinesis might be solely driven by myosin pushing forces from behind the nucleus, moving the nucleus forward without any requirement of anchorage to the cytoskeleton". What evidence is this claim (pushing as mentioned above, but also the fact that there is no requirement for cytoskeleton-nucleus connections) based on? This is a bold claim based on the data provided (what evidence is there that it is not connected?).

We apologize that we have been tempted to speculate in the discussion of the original manuscript about an anchorage-independent mechanisms (see also comment by Reviewer 2). We fully agree that this is not the topic of this manuscript. In the revised manuscript, we excluded this sentence and instead mention ‘It will be interesting to investigate whether the here-described mechanisms of amoeboid nucleokinesis requires anchorage between the actomyosin cytoskeleton and the nucleus, or whether it may be functioning by an anchorage-independent mechanism solely driven by myosin forces located at the rear of the nucleus’.

8. In the results related to figure 3 on page 4 the authors state: "...demonstrating that the nucleus is not only passively dragged by retracting protrusions but actively repositions to its nucleus-forward configuration". I do not understand what they are trying to say and how the data in the figure are connected to this conclusion. I think re-writing to make the logic more plain could help.

Thank you for the suggestion. In the revised manuscript, we now re-wrote the sentence for better readability: ‘…showing that the nucleus does not passively stay at the cell rear upon retraction of the nucleus-containing ‘losing’ protrusion, but actively repositions to its originally location frontward of the MTOC.

9. Perhaps enucleating the cells could be a direct demonstration of the requirement for a nucleus during adaptive pathfinding assuming they are able to migrate at all. I am not saying the authors need to do this experiment since it is risky, but I think their conclusions could be strengthened by a more direct demonstration that nucleokinesis is required for adaptive pathfinding.

As enucleation protocols typically require adhesive cell types and result in unclear cytoplasmic contents, we now performed alternative demonstrations that nucleokinesis is required for adaptive pathfinding: First, we quantified in the revised manuscript the retraction times of ‘losing’ protrusions with and without a nucleus. Quantification revealed that ‘losing’ protrusions with a nucleus are longer (new Figures 4A and B) and require more time to retract (new Figures 4A, C, and D) than without a nucleus, showing that the retraction of nucleus-containing protrusions is a particular challenge to adapt the cellular path. Second, we tested the consequences of myosin-inhibition for ‘losing’ protrusions with and without a nucleus in environments of competing chemokine and pore size cues. This revealed that nucleus-containing ‘losing’ protrusions are particularly susceptible to myosin inhibition, causing delayed retraction times (new Figure 5B) as well as less frequent path adaptations (new Figure

5C). Third, we confirmed these findings in environments with open and blocked paths, again showing that cells are prone to get stuck in the blocked path when the protrusion in this blocked path contained the nucleus (new Figure 5E). Together, these data support our initial data that repositioning of the nucleus (nucleokinesis) is required to adapt the cellular path in situations in which the nucleus localizes inside future 'losing' protrusions'. We further confirmed these data in the revised manuscript, by investigating the consequences of knockout of the RhoA guanine nucleotide exchange factor GEF-H1 (Lfc in mice): while the knockout of Lfc did not impair migration in straight channels (new Figures EV11C and D) and did not impair the orientation of the nucleus-MTOC axis configuration (new Figures EV11A and B), it specifically delayed retractions of 'losing' protrusions but only when they contained a nucleus (new Figure 5F). These findings show that the retraction of protrusions containing the nucleus is a particular challenge, requiring particular high myosin activity to drive nucleokinesis.

Dear Jörg,

Thank you for submitting a revised version of your manuscript. Your study has now been seen by two of the original referees, who find that their previous concerns have been addressed and now recommend acceptance of the manuscript.

There now remain only a few editorial points that have to be addressed before I can extend acceptance of the manuscript:
1. Please submit up to five keywords.
2. Figure panels 4H-I are not mentioned in the manuscript text, please add the appropriate callouts.
3. CRediT has replaced the traditional author contributions section because it offers a systematic, machine-readable author contributions format that allows for more effective research assessment. Please remove the Authors Contributions from the manuscript and use the free text boxes beneath each contributing author's name in our online submission system to add specific details on the author's contribution. More information is available in our guide to authors.
4. Please rename "Declaration of interests" section into "Disclosure and competing interests statement" (further info: https://www.embopress.org/page/journal/14602075/authorguide#conflictsofinterest).
5. We can accommodate up to 10 main figures and up to five EV figures. Since the manuscript currently contains 12 EV figures, please consider either upgrading some of them to main figures, or assembling seven of the EV figures together with their legends into an Appendix file in the pdf format. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. Please preface the Appendix file with a short table of contents that includes page numbers. See detailed instructions regarding expanded view here: https://www.embopress.org/page/journal/14602075/authorguide#expandedview .
6. Our data editors have flagged the following issues in figure legends that need correcting:
- The information on the number and nature of the replicates (n) is missing in the legend of figures 1f, g; EV1a.
7. In the Data Availability section, please add a resolvable link for the BioStudies (S-BIAD901) dataset. More information about the format of this section can be found here: https://www.embopress.org/page/journal/14602075/authorguide#dataavailability.
8. Papers published in The EMBO Journal are accompanied online by a 'Synopsis' to enhance discoverability of the manuscript. It consists of A) a short (1-2 sentences) summary of the findings and their significance, B) 3-4 bullet points highlighting key results and C) a synopsis image that is 550x300-600 pixels large (width x height, jpeg or png format). You can either show a model or key data in the synopsis image. Please note that the image size is rather small and that text needs to be readable at the final size. Please send us this information together with the revised manuscript.

Please let me know if you have any questions regarding any of these points. You can use the link below to upload the revised files.

Thank you again for giving us the chance to consider your manuscript for The EMBO Journal. I look forward to receiving the final version.

With best wishes,

Ieva

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Further information is available in our Guide For Authors: https://www.embopress.org/page/journal/14602075/authorguide

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (28th Jan 2024). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions.

Referee #1:

The authors' revised manuscript is sound. The new data added not only further supports their claims but also clarifies the role played by Myosin-II during the several steps of nucleokinesis.

I have no further comments. I look forward to seeing this nice work published in the EMBO Journal.

Guilherme Nader

Referee #3:

The authors have fully addressed my comments and concerns in this revised version of their manuscript. I extend my congratulations to the authors for their careful and innovative characterization of the role of nuclear movement during confined amoeboid motility. I look forward to the publication of their work.

The authors addressed the minor editorial issues.

2nd Revision - Editorial Decision **3rd Nov 2023**

Dear Jörg,

Thank you for addressing the final editorial issues. I am now pleased to inform you that your manuscript has been accepted for publication.

Before we forward your manuscript to our publishers, I would like to propose a couple of minor changes in the article abstract and synopsis. I have also written a short blurb that will accompany the title of your manuscript in our online table of contents. Please take a look at the text below and in the attached manuscript text file and let me know if any corrections are necessary. I would need your input on this still today to ensure timely publication of your article due to our publisher transition. I am sorry for this urgency, but I hope that the edits are minor enough to be quickly approved.

Blurb:
Integration of complex chemical and mechanical guidance cues by migrating immune cells and unicellular amoebae requires actomyosin-dependent nuclear repositioning.

Synopsis:
How migrating cells prioritize chemical and mechanical guidance cues during their pathfinding in the local microenvironment largely unknown. This study shows that amoeboid cell migration towards dominant chemotactic and mechanical pore size cues involves long-distance nuclear repositioning that is driven by actomyosin contractility.
• Immune cell migration towards dominant chemotactic guidance cues into narrow microenvironmental pores requires fast and long-distance intracellular repositioning of the nucleus (nucleokinesis) towards the cellular path.
• Nucleokinesis in amoeboid migrating cells requires a two-step polarity switch in the nucleus-MTOC axis configuration,
• Nucleokinesis during amoeboid migration is driven by myosin-II forces and is regulated by the RhoA guanine nucleotide exchange factor GEF-H1/Lfc.
• Major principles of amoeboid nucleokinesis are conserved between mammalian immune cells (dendritic cells, T cells) and the amoeba Dictyostelium discoideum.

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Should you be planning a Press Release on your article, please get in contact with embojournal@wiley.com as early as possible, in order to coordinate publication and release dates.

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

Best regards,

Ieva

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Reporting Checklist for Life Science Articles (updated January
This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your
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1. Data
The data shown in figures should satisfy the following conditions:
- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data

2. Captions
Each figure caption should contain the following information, for each panel where they are relevant:
- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ^2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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

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New materials and reagents need to be available; do any restrictions apply?	Not Applicable	

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Short novel DNA or RNA including primers, probes: provide the sequences.	Not Applicable	

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Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Yes	Materials and Methods

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Include a statement about **sample size** estimate even if no statistical methods were used.	Yes	Materials and Methods
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For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Figure Legends

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