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Peer Review File

Invasion of glioma cells through confined space requires membrane tension regulation and mechano-electrical coupling via Plexin-B2

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In this study, Junqueira-Alves and colleagues focused on signaling mechanisms that control the plasticity of glioblastoma multiforme (GBM) cells and enable their migration through confined passages, emulating the physical constrains found in the tumor microenvironment. The core message of the paper is that the surface receptor Plexin-B2 is responsible for a mechano-transduction pathway, leading to bio-mechanical and bio-electric changes of GBM cells, promoting their migration through narrow pores of 3D microdevices. Another member of the plexin family, Plexin-D1, has been implicated previously in mechano-transduction. Moreover, the authors of this study (and others) have previously illustrated the links between Plexin-B2 signaling and cell membrane stiffness, due to regulation of actomyosin contractility, as well as cell-cell and cell-matrix adhesion. Although the Authors have corroborated their new working hypothesis in this manuscript with multiple lines of investigation, the underlying mechano-transduction pathway elicited by Plexin-B2 is not well clarified.

Specific comments:
1) The representative Movie S1 comparing GSC cell motility in microchannels with 3 or 8 um restrictions is puzzling, as it seems that the cells entering the two types of channels are already moving at a different speed from the start, before passing through restriction sites. Please clarify.

2) In Figure 1B-C the behavior of cells passing through 3 um restrictions is compared to that of cells passing through 8um pores. However, it should be clarified if the passage through 8 um pores is anyway conditioning cell motility speed or persistence, compared to migration in a non-restricted microchannel? Data in Fig. 1C would in fact suggest that 8 um (but not 3 um) restrictions are sites of significant migration delay. Thus, it is important to understand if motility is actually increased or rather maintained steady upon passage through narrow restrictions.

3) Based on data in Fig. 1D-F the Authors conclude that cell passage through restrictions triggers endocytosis at the leading edge. However, endocytic vesicles are known to traffic across migrating cells towards the leading edge, also involved in integrin and receptor intracellular sorting. Thus, considering the higher speed and directional persistence of cells migrating across 3 um restrictions, the abundance of endosomal vesicles at the leading edge may not necessarily be due to external compression. It would be necessary to track molecules marking the leading edge to confirm localized endocytosis in this site. Moreover, the involvement of endocytosis in cell migration is well established, and the fact that endocytosis inhibitors slow down cell motility (Fig. 1G-H) is not surprising or specifically connected with passage in narrow sites. Indeed, it seems that cells treated with inhibitors migrate basally slowlier from start, even before restrictions. About this point, the wound healing experiments do not provide a comparable control. It would be preferable to assess the impact of endocytosis inhibitors in classical single cell migration assays through a semipermeable membrane (e.g. Boyden chamber/Transwell inserts) or it could be analyzed the migration in 3D channels without restrictions.

4) Plexin-B2 knock-out GBM cells obtained by CRISPR-Cas9 technology were analyzed throughout most of the study. More information should be provided about this model. First, how many independent clones were analyzed and compared to validate the specificity of the observed effects? This is particularly relevant, since in most of the experiments the effect of gene knock-out is not complementary to that of Plexin-B2 overexpression. Moreover, GBM cells of SD2 and SD3 subtype were analyzed and subjected to Plexin-B2 KO. However, in some Figures SD2 cells are shown (e.g. Fig. 2, Fig. 6), while in

others are shown SD3 (e.g. Fig. 4, Fig. 7). Is the functional impact of Plexin-B2 deficiency distinct in the two GBM subtypes?

5) Data included in multiple figures (e.g. Figs. 2D-E-F, S4, and S8) show an inconsistent identical functional impact of Plexin-B2 knock-out and overexpression in the same cells. Although it is conceivable that any disruption of the balanced control of membrane stiffness may lead to similar phenotypes, this explanation severely undermines the hypothesis of a direct regulation by a Plexin-B2 specific signaling pathway.

6) The fact that Plexin-B2 KO cells have a “leaky membrane” (as stated by the Authors), allowing diffuse accumulation of Dextran in the cytosol (Fig. 3 and S6), is puzzling and not sufficiently discussed. Similarly, the posited coincidence of “dextran puncta” with endocytic vesicles is not demonstrated by quantification of co-labeling (and statistics).

7) Data shown in Fig. 2G and Fig. 3C-D should be quantified and statistics provided.

8) The migration of Plexin-B2 KO cells seems to be impaired beyond their capacity to go through 3um restriction points in microchannels. This issue should be carefully analyzed, comparing cell behavior in segments of non-restricted channels and in coincidence of 8um pores.

9) In Fig. 6 and S10, it was analyzed the lipid content and electrical polarity of plasma membrane, concluding that these are altered in Plexin-B2 KO cells (but also equally in OE cells…). It is unclear by which mechanisms the plexin would be supposed to achieve this effect (endocytosis? PLC-gamma regulation?...). Moreover, many observations have been done in quiescent cells, suggesting that the posited regulatory activity of Plexin-B2 is active independently from migration or passage through narrow tunnels. Actually, PIP2 localization at the leading edge of migrating cells is expected, and it should be ruled out that the impairment of this in KO cells is the consequence of their defective migration, rather than a specific sign of defective Plexin-B2 activation. Furthermore, while Annexin-V is commonly associated with the flipped plasma membrane layers typically found in apoptotic cells, here it was used to demonstrate the “internalization of annexin-labeled endosomes at the front of WT GSCs traversing the tunnels, but not in stalled PB2 KO cells” (line 326). The significance of this experiment should be better illustrated. In general, this section of the manuscript is poorly convincing and unable to support the role of Plexin-B2 in cell migration through confined space.

10) Notably, data shown in Fig. 6H could serve to specifically link the findings with Plexin-B2 signaling pathway (and not simply to a role of this receptor controlling migration capacity in general). Thus, a similar approach should be used to dissect the role of Plexin-B2 signaling in the other functional assays in this study. Moreover, should be tackled (or at least discussed) the pathways connecting Rap1-GAP activity with PIP2 and Ca++ regulation (which are generic hallmarks of directional cell migration).

11) A last and major concern is about the complete absence in this study of data concerning ligands known to activate Plexin-B2 (e.g. Sema4C). Although the final section of Results alludes to an alternative mechanism of receptor activation, this remains speculative at the present stage. There is no evidence here that Plexin-B2 is activated by physical force-induced conformational change. Moreover, the fact that extracellular domain flexibility is required for function but not for ligand binding does not rule out that it may be required for ligand-induced conformational changes. Notably, Plexin-B2 ligands are commonly found in the extracellular environment and potentially signaling in autocrine/paracrine manner. It is mandatory to test if the expression of a mutated PlexinB2 unable to bind Sema ligands can still complement the B2-KO phenotype. It is equally recommended to assay the impact of knocking-down (or overexpressing) the major Plexin-B2 ligand, Sema4C, on GBM cell migration through narrow tunnels.

(Remarks on code availability)
I don't understand what this code is. Sorry.

Reviewer #2

(Remarks to the Author)
The authors systematically confirmed the role of Plexin-B2 in glioblastoma cell migration capability using microchannel migration device by combining live-cell imaging techniques, mechanical measurements and statistical analysis. The molecular dynamics simulations by authors revealed the importance of a balance between cortical actin and membrane tension for optimal migration, which is consistent with the molecular mechanism of Plexin-B2. They further investigated the effects of Plexin-B2 on cell membrane internalization, permeability, lipid composition, and membrane charge characteristics, providing crucial insights into the biophysical mechanisms by which Plexin-B2 influences glioblastoma cell migration. The biological validation of this work is quite comprehensive, and the use of microfluidic channels generally confirms the molecular mechanism of Plexin-B2. The description in the Methods section is very detailed, thus enabling other researchers to replicate the experiments. However, we believe some additional experiments or explanations regarding microchannel designs and parameter controls are necessary. Overall, we consider this work to meet the expected standards in the field.

Regarding the microchannel design and experimental methods, we have the following questions:

1.In the "Methods" section, the channel height is designed to be 10 μm. Would this height cause cells to pass through the microchannel in a suspended or near-suspended state while not adhered to the wall surface? It may be necessary to provide existed experimental videos or results of cell migration under slightly lower channel heights (e.g., 5 μm) to address this concern(e.g., Nat Commun 6, 7526 (2015)).

2.In the "Methods" section, the author mentioned ‘cells were seeded into the entry ports’. In the channels containing central chambers and outflow tunnels (Figure 4E), how were the cells injected into each central chamber? It may be necessary for the author to provide clearer explanations.

3.Previous studies indicated that both the local cell density and the geometric structure of microchannels may influence cell migration (Nature Physics 5, 606-612 (2009).). In the supplemental materials' videos (such as Movie S1, Movie S4), we noticed differences in cell density at the inlet among different experimental groups. Could this affect the speed of cell migration? It may require some validation experiments or arguments for explanation.

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)
Manuscript reference NCOMMS-24-12557-T
Journal Nature Communcations
Authors Chrystian Junqueira Alves, Theodore Hannah, Sita Sadia, Christy Kolsteeg, Angela Dixon, Robert Wiener, Ha Nguyen, Murray Tipping, Júlia Silva Ladeira, Paula Fernandes da Costa Franklin, Nathália de Paula Dutra de Nigro, Rodrigo Alves Dias, Priscila V. Zabala Capriles, José Paulo Rodrigues Furtado de Mendonça, Paul Slesinger, Kevin Costa, and Hongyan Zou, Roland Friedel,
Title Invasion of glioma cells through confined space requires membrane tension regulation and mechano-electrical coupling via Plexin-B2

The article by Junqueira Alves et al studies the relationship between Plexin-B2, an axon guidance molecule in glioblastoma (GBM) invasion and their mechanical properties. GBMs are malignant brain tumors that grow uncontrollably and are highly invasive, responsible for the most common and lethal brain cancer. The study suggests that Plexin-B2 regulates cortical actin and membrane tension, enabling GBM cells to adapt to physical constraints and achieve optimal migratory speed and coherence.

1 – Concerning elasticity measurements by AFM, the authors use terminology that is common to many articles in mechanobiology, but which can be misleading. In fact, in the M&M section, the authors talk about cell elasticity, and in the text associated with figure 2, the authors use the terms stiffness or contractility. It would be wise to use a single parameter, such as the apparent Young modulus. A comparison of AFM results with similar cells is necessary. The authors use the term plasticity, which can also be misleading, see for example https://en.wikipedia.org/wiki/Plasticity_(physics). I think the authors are referring to another phenomenon, which it is important to describe correctly. Finally, with regard to Figure 2, the scale of stiffness seems incorrect. There is not a decade between 0.1 and 2.6, or between 2.6 and 5, as the abscissa axis indicates it. The data in the text does not correspond to the figure.

2 - Concerning the simulations, the referee is not convinced that they add any interest to the manuscript. The simulations considerably simplify cellular behavior, and seem to contain assumptions that can only lead to the desired results. For the simulations no quantities (energy, elastic constant) are assigned a unit, which would allow comparison with existing measurements of these quantities, or for the energy with thermal energy. In figure 5C, it is puzzling to see a membrane elastic constant varying from 10 to 5000 (units?), which is not compatible with the variations found in physiological conditions. The referee suggests that these simulations be put in the SI section.

3 - Concerning figure 3, the authors suggest that dextran translocation through a leaky cell membrane. The referee does not share this conclusion. Dextran is an uncharged, water-soluble polymer and interaction with the plasma membrane should be weak. Moreover, as has been seen in cases of non-endocytic internalization of cationic nanoparticles, translocation through a leaky cell membrane is accompanied by toxicity and reduced cell viability. To prove the authors' point, cell viability experiments should be performed.

Minor comments
- The figures as a whole are well structured. Some sub-figures appear rather small (panels in figure 2E, images in figure 2F, boxplot in figure 2A, 3E, boxplots in figure 4 etc.), which will pose a readability problem once published, and others are clearly too busy (e.g. Figure 4). Generally speaking, the figures, especially the fluorescence figures, are too small and difficult to read.
- Page 9, line 211: "...cooling cells to 4 °C, which reduces membrane permeability by increasing..." is not entirely accurate. Lowering the temperature also (and above all) has the effect of greatly slowing down all thermally active processes, and the Maxwell-type probability of event is considerably reduced.

In conclusion, this is an interesting and well-constructed paper, which meets the standards of the journal Nature Communications. I am in favor of its publication in this journal once the requested modifications have been made.

(Remarks on code availability)

Reviewer #4

(Remarks to the Author)
In the article “Invasion of glioma cells through confined space requires membrane tension regulation and mechano-electrical coupling” authors study polarized migration of glioblastoma (GBM) cells through constrictions. Based on biochemical assays and fluorescence imaging they find that, Plexin-B2, a membrane receptor protein controls the polarized migration of GBM cells by balancing the tension of cortical actin and membrane. The authors also report molecular dynamics simulations of a simplistic coarse-grained model of cell which supports their finding that optimal membrane and cortical actin tension are necessary for polarized cell migration.
Please find below some comments and questions on the molecular dynamics study:
1)I do not think the molecular dynamics (MD) simulations provide any new insights to this study. The experimental measurements for PB2 KO and PB2 OE systems have shown PB2 KO system has lower membrane tension (membrane elastic constant) and membrane-cytoskeleton interaction, while PB2 OE system has higher membrane tension and membrane-cytoskeleton interaction. Additionally, experiments also find these two systems do not show polarized migration. Thus, the main conclusion from the MD simulation on the interplay between cortical actin tension and membrane tension in regulating cell migration has already been gleaned from experiments.
2)The MD simulations showing polarized migration for optimal membrane-cytoskeleton interaction and membrane elastic constant (U3=6, κ00=100) do not report polarization of actin filaments to the cell rear and endosomes to the cell front. How then is the migration polarized to one direction?
3)It is difficult to see polarized motility of the cell from video of MD simulations given in SI (movie S11 490809_0_video_4331290_s7gncv.mp4). Why is there a directional preference for motility? This needs to be made clear.
4)The authors write in the discussion that “Future assays can test if GSCs may perform better at constrictions wider than 3μm (a lower limit for nucleus to pass through)”. Given that the MD simulations involve reduced coarse grain systems which are computationally not very expensive for parameter space scanning, can they predict tends of motility under constrictions of different diameters? This would be a great addition to the MD study.

(Remarks on code availability)
I was able to install and run the code.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In this revised manuscript, Junqueira Alves and colleagues have sufficiently addressed most of my concerns and generally improved the work.
However, new Fig. S14 contains important additional results that are not accompanied by adequate controls. The Authors have applied combined CRISPR-based knock out of six Semaphorins, but they show the evidence of successful gene silencing only for two of them (in panel B). One wonders if the KO of Sema4B and Sema4C only may be sufficient to recapitulate the effect of knocking-out the receptor Plexin-B2. This issue should at least be highlighted and discussed in the Results section. Moreover, data shown on the left of panel B indicate that cells subjected to KO of Plexin-B2 suprisingly also completely loose the expression of Sema4B. This could be scientifically relevant or indicate a kind of non-specific impact of gene targeting. The Authors should discuss this issue.

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)
We have seen the supplemented explanation from authors, including the cell seeding method, the density of cells seeded in each experiment, as well as the discussion on how different geometric structures may affect cell migration. These responses basically addressed our questions about the experimental methods.

A minor point that may help support the reliability of the methodology:

Concerning whether cells pass through the 10 μm-height channel in a suspended state (question 2.1), the authors explained that the channel was coated with laminin, thus provided strong adhesion forces. This explanation appears reasonable. however, it would be better to validate this point from existing data or other characterizations. In a suspended state, the cytoskeleton is loosely organized and distinctly different from the adherent state (Nature 463, 485-492, (2010), doi:10.1038/nature08908). We observed the stained cytoskeleton from videos which corresponded to the morphology in adherent state. Therefore, we recommend that the authors briefly discuss this observation or provide higher-resolution images as evidence of cells moving in a non-suspended state.

In summary, we consider the methodology of this work to be reliable, thanks for the efforts from the researchers.

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)
The authors have taken my comments into account, as have those of the other referees. Junqueira Alves' paper is now suitable for publication in Nature Communications.

(Remarks on code availability)

Reviewer #4

(Remarks to the Author)
The questions related to MD simulations have been addressed satisfactorily.

(Remarks on code availability)
The questions related to MD simulations have been addressed satisfactorily.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The Authors have sufficiently addressed my concerns.

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)
All of my questions have been well addressed. This work is suitable for publication in Nature Communications.

(Remarks on code availability)

Response letter NCOMMS-24-12557-T

Junqueira Alves et al., *Invasion of glioma cells through confined space requires membrane tension regulation and mechano-electrical coupling via Plexin-B2*

We thank the reviewers for their constructive critiques. We have performed a series of new experiments and additional analyses to address all points (highlighted in blue in the revised manuscript).

Summary of major new experiments and analyses:

1. New analyses show similar velocity of GBM cells migrating through microchannels with 8 µm constrictions vs. open channels without constrictions. Hence, our observations on confined migration apply to 3 µm constrictions, while 8 µm constrictions do not delay migration.

2. New experiments reveal that endocytosis inhibition also reduced migration in non-confined conditions (i.e., 8 µm constrictions), and also reduced migration persistence.

3. New microchannel migration studies show that Plexin-B2 deletion did not significantly affect velocity or forward movement of GBM cells in non-confined conditions. Hence, Plexin-B2 is mainly responsible for confined migration, while endocytosis is required for GBM cells migration in both confined and non-confined conditions.

4. New experiments show that dominant negative RAP1B phenocopies Plexin-B2 KO in reducing membrane internalization, suggesting Rap1 as a downstream effect of Plexin-B2.

5. New studies show that GBM cells with pan-Sema4 knockout (deleting all six *SEMA4s*) led to reduced migration through micro-tunnels, while ablation of both Plexin-B2 and Sema4s did not increase severity, indicating ligand-receptor communication in the same pathway.

6. Cell viability studies with Calcein Violet dye show that the Plexin-B2 KO cells containing diffuse cytosolic dextran were viable. New quantifications confirm co-labeling of lysotracker signals and with dextran-Alexa488 puncta, supporting endocytic uptake of dextran.

7. New videos with similar cell seeding density and similar distance between entry points and first constrictions confirm enhanced migration of GBM cells in confined condition.

8. Figure layouts were streamlined throughout the manuscript to improve visual clarity and readability.

Point-by-point response:

Reviewer #1

In this study, Junqueira-Alves and colleagues focused on signaling mechanisms that control the plasticity of glioblastoma multiforme (GBM) cells and enable their migration through confined passages, emulating the physical constrains found in the tumor microenvironment. The core message of the paper is that the surface receptor Plexin-B2 is responsible for a mechano-transduction pathway, leading to bio-mechanical and bio-electric changes of GBM cells, promoting their migration through narrow pores of 3D microdevices. Another member of the plexin family, Plexin-D1, has been implicated previously in mechano-transduction. Moreover, the authors of this study (and others) have previously illustrated the links between Plexin-B2 signaling and cell membrane stiffness, due to regulation of actomyosin contractility, as well as cell-cell and cell-matrix adhesion. Although the Authors have corroborated their new working hypothesis in this manuscript with multiple lines of investigation, the underlying mechano-transduction pathway elicited by Plexin-B2 is not well clarified.

Specific comments:

1.1) The representative Movie S1 comparing GSC cell motility in microchannels with 3 or 8 um restrictions is puzzling, as it seems that the cells entering the two types of channels are already moving at a different speed from the start, before passing through restriction sites. Please clarify.

>> We thank the Reviewer for the careful observation. This reason that cells entering the two types of channels appeared to already move at different speeds from the beginning is related to different distances from the entry port to the first constriction, due to the manufacturing process for the microchannel devices. To avoid confusion, we have now provided new videos in which channels have similar distances from entry port to the first constriction (**new Video S1)**.

1.2) In Figure 1B-C the behavior of cells passing through 3 um restrictions is compared to that of cells passing through 8um pores. However, it should be clarified if the passage through 8 um pores is anyway conditioning cell motility speed or persistence, compared to migration in a non-restricted microchannel? Data in Fig. 1C would in fact suggest that 8 um (but not 3 um) restrictions are sites of significant migration delay. Thus, it is important to understand if motility is actually increased or rather maintained steady upon passage through narrow restrictions.

>> We appreciate the thoughtful suggestion. We have conducted additional analysis showing comparable speed of GSCs migrating through channels with 8 µm constriction or "open" channels without constriction (**new Fig. S1f**). Therefore, the 8 µm constrictions do not delay cell migration as compared to open microchannel, whereas 3 µm constrictions result in confined migration with accelerated velocity.

1.3) Based on data in Fig. 1D-F the Authors conclude that cell passage through restrictions triggers endocytosis at the leading edge. However, endocytic vesicles are known to traffic across migrating cells towards the leading edge, also involved in integrin and receptor intracellular sorting. Thus, considering the higher speed and directional persistence of cells migrating across 3 um restrictions, the abundance of endosomal vesicles at the leading edge may not necessarily be due to external compression. It would be necessary to track molecules marking the leading edge to confirm localized endocytosis in this site.

>> We appreciate the suggestion. In constricted GSCs, we also observe smaller number of endocytic vesicles at cell rear (Fig. 1d). We thus avoided using the term 'localized endocytosis', and clarified that we observed increased abundance of endocytic vesicles at the leading edge, which may not necessarily reflect localized endocytosis or external compression at cell front.

Moreover, the involvement of endocytosis in cell migration is well established, and the fact that endocytosis inhibitors slow down cell motility (Fig. 1G-H) is not surprising or specifically connected with passage in narrow sites. Indeed, it seems that cells treated with inhibitors migrate basally slower from start, even before restrictions. About this point, the wound healing experiments do not provide comparable control. It would be preferable to assess the impact of endocytosis inhibitors in classical single cell migration assays through a semipermeable membrane (e.g. Boyden chamber/Transwell inserts) or it could be analyzed the migration in 3D channels without restrictions.

>> We followed the advice and conducted new analysis and tracked migration of GSCs treated with endocytosis inhibitors in micro channels with 8 µm restrictions (effectively similar to open channels, see above) (new **Fig. S2c**). As the Reviewer suspected, endocytosis inhibitors indeed also slowed down velocity through unconfined environment, and migration persistence was also affected, shown by increased ratio of backward vs. forward movement.

1.4) Plexin-B2 knock-out GBM cells obtained by CRISPR-Cas9 technology were analyzed throughout most of the study. More information should be provided about this model. First, how many independent clones were analyzed and compared to validate the specificity of the observed effects? This is particularly relevant, since in most of the experiments the effect of gene knock-out is not complementary to that of Plexin-B2 overexpression. Moreover, GBM cells of SD2 and SD3 subtype were analyzed and subjected to Plexin-B2 KO. However, in some Figures SD2 cells are shown (e.g. Fig. 2, Fig. 6), while in others are shown SD3 (e.g. Fig. 4, Fig. 7). Is the functional impact of Plexin-B2 deficiency distinct in the two GBM subtypes?

>> We have provided further details about Plexin-B2 KO GSCs via CRISPR-Cas9. We avoided picking individual KO clones due to well-known intratumoral heterogeneity of GBM, with different clones from the same parental line behaving differently. We thus chose a population approach by establishing GSC sublines with Cas9 lentivirus with antibiotic selection, and Plexin-B2 ablation was confirmed by WB and IF, shown in Fig. 2 b-c, even though individual cells may harbor a slightly different indel mutation at the cut site, described in detail in our publication Huang et al., 2021 [1]; Suppl. Fig. 3),

Overall, the functional impact of Plexin-B2 KO did not differ between SD2 and SD3 GSC lines. We have prepared a listing of all experiments performed, indicating which GSC line was used (**Table R1** at bottom of this letter). We would like to highlight that the most crucial experiments were repeated and similarly confirmed in both SD2 and SD3 lines. In regard to PB2 overexpression (OE), our results suggest that higher membrane tension and membrane-cytoskeleton interactions in the PB2 OE condition also disrupted confined migration, in line with predictions of the molecular dynamics (MD) simulation (also see **1.5** and Reviewer 4 comment).

1.5) Data included in multiple figures (e.g. Figs. 2D-E-F, S4, and S8) show an inconsistent identical functional impact of Plexin-B2 knock-out and overexpression in the same cells. Although it is conceivable that any disruption of the balanced control of membrane stiffness may lead to similar phenotypes, this explanation severely undermines the hypothesis of a direct regulation by a Plexin-B2 specific signaling pathway.

>> We would like to clarify that our results demonstrate that PB2 KO results in lower membrane tension and membrane-cytoskeleton interaction in GSCs, while the opposite was observed in the PB2 OE condition, i.e. higher membrane tension and membrane-cytoskeleton interactions. In microchannel

migration assays, however, we found that either too high or too low membrane tension appeared similarly detrimental for confined migration, in line with molecular dynamics (MD) simulation predictions.

1.6) The fact that Plexin-B2 KO cells have a "leaky membrane" (as stated by the Authors), allowing diffuse accumulation of Dextran in the cytosol (Fig. 3 and S6), is puzzling and not sufficiently discussed. Similarly, the posited coincidence of "dextran puncta" with endocytic vesicles is not demonstrated by quantification of co-labeling (and statistics).

>> We have added quantifications of co-labeling of dextran puncta and Lysotracker dye, demonstrating that the intracellular puncta corresponded to endocytic vesicles (**Fig. S6a**).

We removed the term "leaky membrane", and added in Discussion that in the literature, several mechanisms of cytosolic uptake of mid-sized and large biomolecules have been described, e.g., disordered lipid packing enabling translocation through membrane (Murayama et al., 2017) [2], transient permeabilization by membrane ruffling induction (Akishiba and Futaki, 2019) [3], or passage through mechanochannels (Ballesteros et al., 2018) [4]. Further studies will be needed to understand the mechanism of the increased cytosolic dextran uptake in Plexin-B2 KO (also see Reviewer 3.3).

1.7) Data shown in Fig. 2G and Fig. 3C-D should be quantified and statistics provided.

>> Done. We included new quantification showing thinner cortical actin in Plexin-B2 KO than control GBM cells (**Fig. 2g**). We also added quantifications demonstrating higher ratio of plasma membrane vs. cytoplasm myr-palm-GFP and -CFP signal in KO cells (new **Fig. 3c, d)**.

1.8) The migration of Plexin-B2 KO cells seems to be impaired beyond their capacity to go through 3um restriction points in microchannels. This issue should be carefully analyzed, comparing cell behavior in segments of non-restricted channels and in coincidence of 8um pores.

>> Done. We conducted new analysis of time-lapse imaging of Plexin-B2 KO vs. control GSCs migrating through microchannels with 3 µm restrictions or 8 µm (non-confined) constrictions (new **Fig. S9**). For the 8 µm restrictions, there was no significant difference of velocity or forward movement between KO and control cells, but backward movement was increased in KO cells, indicating reduced directional consistency. Hence, the effect of Plexin-B2 KO pertains mainly to confined migration through 3 µm

constrictions, whereas migratory consistency was affected in both 3 and 8 µm conditions, suggesting that migratory consistency might be linked to membrane tension status.

1.9) In Fig. 6 and S10, it was analyzed the lipid content and electrical polarity of plasma membrane, concluding that these are altered in Plexin-B2 KO cells (but also equally in OE cells…). It is unclear by which mechanisms the plexin would be supposed to achieve this effect (endocytosis? PLC-gamma regulation?...). Moreover, many observations have been done in quiescent cells, suggesting that the posited regulatory activity of Plexin-B2 is active independently from migration or passage through narrow tunnels. Actually, PIP2 localization at the leading edge of migrating cells is expected, and it should be ruled out that the impairment of this in KO cells is the consequence of their defective migration, rather than a specific sign of defective Plexin-B2 activation. Furthermore, while Annexin-V is commonly associated with the flipped plasma membrane layers typically found in apoptotic cells, here it was used to demonstrate the "internalization of annexin-labeled endosomes at the front of WT GSCs traversing the tunnels, but not in stalled PB2 KO cells" (line 326). The significance of this experiment should be better illustrated. In general, this section of the manuscript is poorly convincing and unable to support the role of Plexin-B2 in cell migration through confined space.

>> We acknowledge that the precise mechanisms of how Plexin-B2 affects lipid content and inner membrane surface charge need further study, but we think the link to regionalized accumulation of endocytic vesicles at the front zone of constricted GSCs is an interesting observation. We added in Discussion that potential effectors of Plexin-RAP signaling may include PLC epsilon [5] and RalGDS [6,7], affecting PIP2 and endocytosis process (illustrated in **Fig. S12a**). In 2D culture ('quiescent' state), PB2 KO and control GSCs showed difference localization (plasma membranes vs. endomembrane) of PIP2 probe and inner membrane charge probe, but in the absence of polarized migration in the 2D culture condition, there was no discernable pattern such as leading edge vs. rear zone. Only in confined migration through 3 µm restrictions, these probes accumulated at cell front (now **Fig. 6b, d**). Whether this is causal or a consequence of confined migration needs further study.

We omitted the Annexin-V section, as it was intended to show phosphoserine at outer membrane leaflet, but we found that Annexin-V was internalized, showing similar results as Memglow.

1.10) Notably, data shown in Fig. 6H could serve to specifically link the findings with Plexin-B2 signaling pathway (and not simply to a role of this receptor controlling migration capacity in general).

Thus, a similar approach should be used to dissect the role of Plexin-B2 signaling in the other functional assays in this study. Moreover, should be tackled (or at least discussed) the pathways connecting Rap1-GAP activity with PIP2 and Ca++ regulation (which are generic hallmarks of directional cell migration).

>> Following the advice, we further dissected Plexin-B2 signaling in controlling plasma membrane internalization/endocytosis. We compared constitutively active (CA) or dominant-negative (DN) isoforms of RAP1B or RAP2A (new **Fig. S7a**). We showed that CA RAP1B, but not RAP 2A, phenocopied Plexin-B2 KO in reducing membrane internalization, shown by membrane-tethered myr-palm GFP. The new data supports engagement of RAP1B, but not RAP2A, in Plexin-B2-mediated membrane turnover in SD2 GSCs.

We also discussed that potential Plexin-RAP effectors may include PLC epsilon [5] and RalGDS [6,7], in connection with PIP2 and calcium signaling, which await future studies in the context of confined migration and GBM invasion.

1.11) A last and major concern is about the complete absence in this study of data concerning ligands known to activate Plexin-B2 (e.g. Sema4C). Although the final section of Results alludes to an alternative mechanism of receptor activation, this remains speculative at the present stage. There is no evidence here that Plexin-B2 is activated by physical force-induced conformational change. Moreover, the fact that extracellular domain flexibility is required for function but not for ligand binding does not rule out that it may be required for ligand-induced conformational changes. Notably, Plexin-B2 ligands are commonly found in the extracellular environment and potentially signaling in autocrine/paracrine manner. It is mandatory to test if the expression of a mutated PlexinB2 unable to bind Sema ligands can still complement the B2-KO phenotype. It is equally recommended to assay the impact of knocking-down (or overexpressing) the major Plexin-B2 ligand, Sema4C, on GBM cell migration through narrow tunnels.

>> We thank the reviewer for the thoughtful suggestions. Following the advice, we tested pan-Sema4 KO in our assays. Plexin-B2 binds to class 4 semaphorins, which are transmembrane proteins (although Sema4D can be cleaved) leading to dimerization of Plexin-B2. Several Sema4s are expressed in GBM cells (Huang et al., 2021 [1]). We thus applied CRISPR/Cas9 to delete all six human Sema4s (*Sema4A, B, C, D, F, G*. In migration assay, pan-Sema4 KO compromised confined migration through 3 μm tunnels and also disrupted regionalized Fluovolt signals at cell rear (new **Fig. S14a-d**). KO of both PB2 and Sema4s did not increase the severity of the phenotype, supporting ligand-receptor communication in the same pathway. Since individual GSC pass in micro tunnels through confinement, Sema4s possibly bind

to Plexin-B2 expressed in the same cell via membrane ruffling (illustrated in model in **Fig. 8d**), in line with previous reports of cell-autonomous signaling semaphorins to co-expressed plexins [8,9].

We agree with the Reviewer that Sema4 may induce conformational changes of the extracellular ring of Plexin-B2. We discussed that future studies will be needed to test if physical force can bend the ring in absence of Sema4. Unfortunately, no mutational strategy is currently known that can abolish the Sema binding surface without disturbing the mechano-sensitivity (mutating the semaphorin binding surface of plexins may disrupt the overall structure). In hESCs, we found that exogenous Sema4C (coated on culture dishes together with laminin, not by adding to culture media) can alter colony organization of control but not PB2 KO cells, which can be rescued by PB2 Lock mutants (now moved to **Fig. S1**5), suggesting that exogenous Sema4 can signal through PB2 without bending the ring in certain contexts.

Reviewer #2

The authors systematically confirmed the role of Plexin-B2 in glioblastoma cell migration capability using microchannel migration device by combining live-cell imaging techniques, mechanical measurements and statistical analysis. The molecular dynamics simulations by authors revealed the importance of a balance between cortical actin and membrane tension for optimal migration, which is consistent with the molecular mechanism of Plexin-B2. They further investigated the effects of Plexin-B2 on cell membrane internalization, permeability, lipid composition, and membrane charge characteristics, providing crucial insights into the biophysical mechanisms by which Plexin-B2 influences glioblastoma cell migration. The biological validation of this work is quite comprehensive, and the use of microfluidic channels generally confirms the molecular mechanism of Plexin-B2. The description in the Methods section is very detailed, thus enabling other researchers to replicate the experiments. However, we believe some additional experiments or explanations regarding microchannel designs and parameter controls are necessary. Overall, we consider this work to meet the expected standards in the field.

>> We appreciate the positive feedback.

Regarding the microchannel design and experimental methods, we have the following questions:

2.1) In the "Methods" section, the channel height is designed to be 10 μm. Would this height cause cells to pass through the microchannel in a suspended or near-suspended state while not adhered to the wall

surface? It may be necessary to provide existed experimental videos or results of cell migration under slightly lower channel heights (e.g., 5 μm) to address this concern (e.g., Nat Commun 6, 7526 (2015)).

>> We appreciate the suggestion. We have clarified in Results and Methods that the microchannel device consists of a glass floor coated with laminin, which provides strong adhesion, while the silicone-based walls and ceiling provide spatial constraints. We thus do not think that GSCs pass through the channel in suspension. We have added to Discussion the consideration of future study designs using 5 μm channel heights according to ref. Chabaud et al., 2015 (*Nat Comm*) [10].

2.2) In the "Methods" section, the author mentioned 'cells were seeded into the entry ports'. In the channels containing central chambers and outflow tunnels (Figure 4E), how were the cells injected into each central chamber? It may be necessary for the author to provide clearer explanations.

>> We have added a new diagram (now **Fig. 5a**) and clarification that cells were seeded into entry ports, from which they then spontaneously migrated through 8 μm wide tunnels into central chambers, connected by outflow tunnels that are 50 μm long and 3 or 8 μm wide.

2.3) Previous studies indicated that both the local cell density and the geometric structure of microchannels may influence cell migration (Nature Physics 5, 606-612 (2009)). In the supplemental materials' videos (such as Movie S1, Movie S4), we noticed differences in cell density at the inlet among different experimental groups. Could this affect the speed of cell migration? It may require some validation experiments or arguments for explanation.

>> We appreciate the careful review. We conducted additional experiments to ensure similar cell density in the inlet at the entrance into the channels, thus minimizing this variation (new **Video S1** and new **Video S7 & S8**). We also added the ref. Mahmud et al., 2009 (*Nat Physics*) [11] and further discussed the complexity of how local cell density and geometric structure of microchannels may influence cell migration (also see reviewer comment 2.1 regarding channel height).

Reviewer #3

The article by Junqueira Alves et al studies the relationship between Plexin-B2, an axon guidance molecule in glioblastoma (GBM) invasion and their mechanical properties. GBMs are malignant brain tumors that grow uncontrollably and are highly invasive, responsible for the most common and lethal brain cancer. The study suggests that Plexin-B2 regulates cortical actin and membrane tension, enabling GBM cells to adapt to physical constraints and achieve optimal migratory speed and coherence.

3.1) Concerning elasticity measurements by AFM, the authors use terminology that is common to many articles in mechanobiology, but which can be misleading. In fact, in the M&M section, the authors talk about cell elasticity, and in the text associated with figure 2, the authors use the terms stiffness or contractility. It would be wise to use a single parameter, such as the apparent Young modulus. A comparison of AFM results with similar cells is necessary. The authors use the term plasticity, which can also be misleading, see for example https://en.wikipedia.org/wiki/Plasticity_(physics). I think the authors are referring to another phenomenon, which it is important to describe correctly. Finally, with regard to Figure 2, the scale of stiffness seems incorrect. There is not a decade between 0.1 and 2.6, or between 2.6 and 5, as the abscissa axis indicates it. The data in the text does not correspond to the figure.

>> We thank for the reviewer's expert advice. In the revision, we avoided using "plasticity" or "elasticity" and changed to "Young's modulus". The scale of stiffness for the x-axis is now corrected (**Fig. 2c**).

We also added in Results: "For comparison, human neural precursor cells (NPCs) have a mean Young's modulus of ~5.9 kPa, while human embryonic stem cells (ESCs) have a mean Young's modulus of ~2.2 kPa, as shown in our previous study [12]."

3.2) Concerning the simulations, the referee is not convinced that they add any interest to the manuscript. The simulations considerably simplify cellular behavior and seem to contain assumptions that can only lead to the desired results. For the simulations no quantities (energy, elastic constant) are assigned a unit, which would allow comparison with existing measurements of these quantities, or for the energy with thermal energy. In figure 5C, it is puzzling to see a membrane elastic constant varying from 10 to 5000 (units?), which is not compatible with the variations found in physiological conditions. The referee suggests that these simulations be put in the SI section.

>> We followed the reviewer's advice and moved the simulations to the SI section (**Fig. S11)**.

3.3) Concerning figure 3, the authors suggest that dextran translocation through a leaky cell membrane. The referee does not share this conclusion. Dextran is an uncharged, water-soluble polymer and interaction with the plasma membrane should be weak. Moreover, as has been seen in cases of non-endocytic internalization of cationic nanoparticles, translocation through a leaky cell membrane is accompanied by toxicity and reduced cell viability. To prove the authors' point, cell viability experiments should be performed.

>>Thank you for the thoughtful comments. We have removed the term “leaky membrane”. We conducted cell viability test using Calcein Violet 450 AM, a membrane-permeable dye that becomes fluorescent after being cleaved by intracellular esterases in live cells. We found that PB2 KO cells with diffuse cytosolic dextran were indeed viable (previously Fig. S13b, now **Fig. S16b**).

We have added to Discussion that several mechanisms of cytosolic uptake of mid-sized and large biomolecules have been described in the literature, e.g., disordered lipid packing enabling translocation through membrane (Murayama et al., 2017) [2], transient permeabilization by membrane ruffling induction (Akishiba and Futaki, 2019) [3], or passage through mechanochannels (Ballesteros et al., 2018) [4]. Future studies are needed to understand the mechanisms of increased dextran uptake in certain Plexin-B2 KO GSCs.

Minor comments

- The figures as a whole are well structured. Some sub-figures appear rather small (panels in figure 2E, images in figure 2F, boxplot in figure 2A, 3E, boxplots in figure 4 etc.), which will pose a readability problem once published, and others are clearly too busy (e.g. Figure 4). Generally speaking, the figures, especially the fluorescence figures, are too small and difficult to read.

>> We have followed the advice by enlarging fluorescence figures and boxplots, and we have also split old Figs. 4 and 6 into two figures each to improve readability.

- Page 9, line 211: "...cooling cells to 4 °C, which reduces membrane permeability by increasing..." is not entirely accurate. Lowering the temperature also (and above all) has the effect of greatly slowing down all thermally active processes, and the Maxwell-type probability of event is considerably reduced.

>> We appreciate the comment. We have now clarified that cooling to 4°C greatly slows down all thermally active processes and particle speed.

In conclusion, this is an interesting and well-constructed paper, which meets the standards of the journal Nature Communications. I am in favor of its publication in this journal once the requested modifications have been made.

>> We appreciate the positive feedback.

Reviewer #4

In the article "Invasion of glioma cells through confined space requires membrane tension regulation and mechano-electrical coupling" authors study polarized migration of glioblastoma (GBM) cells through constrictions. Based on biochemical assays and fluorescence imaging they find that, Plexin-B2, a membrane receptor protein controls the polarized migration of GBM cells by balancing the tension of cortical actin and membrane. The authors also report molecular dynamics simulations of a simplistic coarse-grained model of cell which supports their finding that optimal membrane and cortical actin tension are necessary for polarized cell migration.

Please find below some comments and questions on the molecular dynamics study:

4.1) I do not think the molecular dynamics (MD) simulations provide any new insights to this study. The experimental measurements for PB2 KO and PB2 OE systems have shown PB2 KO system has lower membrane tension (membrane elastic constant) and membrane-cytoskeleton interaction, while PB2 OE system has higher membrane tension and membrane-cytoskeleton interaction. Additionally, experiments also find these two systems do not show polarized migration. Thus, the main conclusion from the MD simulation on the interplay between cortical actin tension and membrane tension in regulating cell migration has already been gleaned from experiments.

>> We appreciate the reviewer's comments. We have moved the MD simulation to SI section (**Fig. S11)**. The computational model that we have developed is based on a coarse-grained approach, aiming to capture the morphology and phenomenology observed in GBM cells. The MD simulation provides a

simplified model to assist understanding of the relationship of membrane tension and membrane-cytoskeletal interaction for polarized migration through confined space, particularly in understanding how PB2 OE also negatively impacts polarized migration in channel migration assay. The MD simulation also allows us to change these properties separately, which is not possible from experiments of PB2 KO or OE, which alters these interconnected processes all at once. Aside from reaffirming experimental observations, the model can predict cell movement and membrane dynamics, thus contributing to our understanding of cell behavior and subcellular components in confined environments.

4.2) The MD simulations showing polarized migration for optimal membrane-cytoskeleton interaction and membrane elastic constant (U3=6, κ00=100) do not report polarization of actin filaments to the cell rear and endosomes to the cell front. How then is the migration polarized to one direction?

>> We thank the reviewer for this question. For a given set of parameters defining a cell, initial velocities were randomly drawn from a Maxwell-Boltzmann distribution at a specific temperature T. For most parameter choices, simulated cell exhibited random movement. However, once a direction was chosen, the movement became progressive, with acceleration tending toward a constant limiting velocity. Of note, in our simulation, the cell is immersed in a thermal reservoir at a constant temperature, thus the energy required to maintain progressive movement comes from this thermal reservoir. In other words, the total energy of the system remains constant.

4.3) It is difficult to see polarized motility of the cell from video of MD simulations given in SI (movie S11 490809_0_video_4331290_s7gncv.mp4). Why is there a directional preference for motility? This needs to be made clear.

>> We have improved the legend of **Video S6** to better indicate the polarized migration. In the MD simulations, there is no clear directional preference for right or left. However, once movement in a specific direction began, it became progressive.

4.4) The authors write in the discussion that "Future assays can test if GSCs may perform better at constrictions wider than 3μm (a lower limit for nucleus to pass through)". Given that the MD simulations involve reduced coarse grain systems which are computationally not very expensive for parameter space

scanning, can they predict tends of motility under constrictions of different diameters? This would be a great addition to the MD study.

>> We thank the reviewer for the suggestion. Our in-silico model, due to its complex network of interactions, still requires considerable computation time. Since each parameter set results in approximately 24 hours of simulation, and we perform 10 simulations to obtain average measurements, the total computational cost is around 240 hours. We are certainly interested in additional simulation for constrictions from 3 µm onward to be compared to wet experiments for future studies.

Reviewer #4 (Remarks on code availability): I was able to install and run the code.

>> Thank you for the confirmation.

Table R1. Overview of studies conducted on the two patient GBM lines SD2 and SD3.

Experiment	Cell Lines		Figure
	SD2	SD3	
Narrow constrictions microchannel	yes	yes	1;4;8;S1;S2;S8;S9;S16
Narrow tunnels microchannel	yes	yes	5;6;7;S10;S13;S14
Endocytosis Inhibitors	yes	no	1;S2;S6;S13
Scratch assay	yes	no	S2
Immunocytochemistry	yes	yes	2;S2;S3;S5;S14;S15;S16
Western blotting	yes	yes	2;8;S5;S14;S15
PLXNB2 knockout or knockdown	yes	yes	2;3;4;5;6;7;8;S3;S4;S5;S6;S8;S9;S10;S12;S13:S14;S15;S16
PLXNB2 overexpression	yes	yes	2;S3;S4;S5;S6;S8;S12
Locked ring mutations	yes	yes	8;S15;S16
Atomic force microscopy	yes	no	2;S3
Membrane tension measurement with Flipper-TR dye	yes	yes	2;S4
Membrane tension measurement with optical tweezer	yes	no	2;S3
Whole-cell patch-clamp recordings	yes	no	S13
Fluorescent protein localization - MyrPalm-CFP, PH-PLCD1-GFP, PH-Btk-GFP, R(+8)-prenyl-GFP	yes	yes	3;6;S7;S12;S16
Dextran-Alexa 488 endocytosis assay	yes	yes	3;S6;S16
Live-cell staining of cytoskeleton, lysosomes, and membrane components (MemGlow, NR12A)	yes	yes	1;2;3;4;5;8;S2;S3;S6;S8;S10;S13
Three-dimensional surface rendering of GSCs	yes	no	S3
FluoVolt live staining	yes	yes	7;S13;S14
Fluo-4 live staining	yes	no	S13
Viscous media assay	yes	no	S10
ERM Ezrin tension	yes	yes	S5;S16
Sema4 KO microchannels	yes	no	S14

References in Response Letter

1. Huang, Y., Tejero, R., Lee, V.K., Brusco, C., Hannah, T., Bertucci, T.B., Junqueira Alves, C., Katsyv, I., Kluge, M., Foty, R., et al. (2021). Plexin-B2 facilitates glioblastoma infiltration by modulating cell biomechanics. Commun Biol *4*, 145. 10.1038/s42003-021-01667-4.

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Response letter NCOMMS-24-12557A

Junqueira Alves et al., *Invasion of glioma cells through confined space requires membrane tension regulation and mechano-electrical coupling via Plexin-B2*

We thank the reviewers for their positive response and comments on our recent resubmission. We have addressed the new comments as follows. Changes in manuscript are in red font.

Reviewer #1

In this revised manuscript, Junqueira Alves and colleagues have sufficiently addressed most of my concerns and generally improved the work.

However, new Fig. S14 contains important additional results that are not accompanied by adequate controls. The Authors have applied combined CRISPR-based knock out of six Semaphorins, but they show the evidence of successful gene silencing only for two of them (in panel B). One wonders if the KO of Sema4B and Sema4C only may be sufficient to recapitulate the effect of knocking-out the receptor Plexin-B2. This issue should at least be highlighted and discussed in the Results section.

>> We agree with the reviewer that the phenotype of the knockout of the six Sema4s (Sema4-KO) may be based already on deletion of Sema4B and Sema4C alone, the two highest expressed Sema4s in our GBM cell lines. **We have modified our Results section to discuss the potential sufficiency of Sema4B/4C KO**.

We would also like to clarify that the "single cut" CRISPR/Cas9 KO approach that we applied creates indel mutations (insertions/deletions) in genes, and in some cases the indels do not create a frameshift, and some protein may still be visible on Western blot (WB). In the case of Sema4 KOs, we assume that also the WB detectable forms are non-functional, as the indels occur in the highly structured Sema domain. We have made similar observations in our previous study Huang et al., 2021 [1], where we describe the details of Cas9 KO of Plexin-B2 (Supp. Fig. 3 therein). **We have expanded these considerations of Sema4 Cas9 KO in the Methods section.**

Moreover, data shown on the left of panel B indicates that cells subjected to KO of Plexin-B2 surprisingly also completely loose the expression of Sema4B. This could be scientifically relevant or indicate a kind of non-specific impact of gene targeting. The Authors should discuss this issue.

>> We appreciate the comment. The fact that the KO of Plexin-B2 reduced the protein levels of Sema4B is intriguing, and one may speculate about a mechanism of cis-interaction and co-regulation of a

Plexin/Sema complex. **We have added a brief discussion of this issue to the Results section.**

We would also like to adjust the Sema4B WB data in S14b for clarity. We apologize that we had applied very high contrast, and we have now adjusted by reducing contrast, revealing low amount of Sema4B protein remaining in PB2 KO and PB2 KO Sema4 KO conditions.

Reviewer #2

We have seen the supplemented explanation from authors, including the cell seeding method, the density of cells seeded in each experiment, as well as the discussion on how different geometric structures may affect cell migration. These responses basically addressed our questions about the experimental methods. A minor point that may help support the reliability of the methodology:

Concerning whether cells pass through the 10 μm-height channel in a suspended state (question 2.1), the authors explained that the channel was coated with laminin, thus provided strong adhesion forces. This explanation appears reasonable. however, it would be better to validate this point from existing data or other characterizations. In a suspended state, the cytoskeleton is loosely organized and distinctly different from the adherent state (Nature 463, 485-492, (2010), doi:10.1038/nature08908). We observed the stained cytoskeleton from videos which corresponded to the morphology in adherent state. Therefore, we recommend that the authors briefly discuss this observation or provide higher-resolution images as evidence of cells moving in a non-suspended state.

In summary, we consider the methodology of this work to be reliable, thanks for the efforts from the researchers.

>> We appreciate the positive feedback and thank the reviewer for the expert comments. **We have added to our Results section now a clarification** that cells in the microchannels, which are laminin coated, migrate in adherent mode (not suspended), similar to a migration mode that has been described for adherence-based migration by others (we added the suggested references, and also work from Friedl lab).

Following this comment of the reviewer, we have also conducted additional immunofluorescence staining for active integrin β1 in GSCs in confined migration, which we would like to share with the

reviewer. We observed that the integrin staining highlighted streaks and dot-like structures where the cells adhere to the laminin bottom, consistent with focal adhesions, further confirming the adherent migration mode (**Fig. A**).

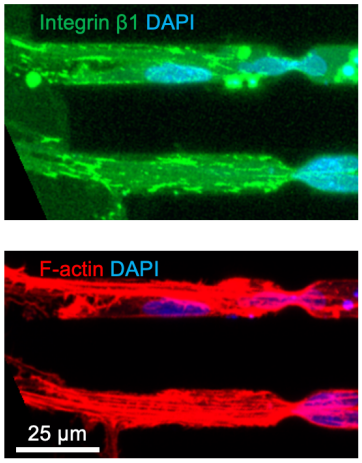

Fig. A. GBM cells in confined migration form focal adhesions in microchannels. IF for integrin β1 and F-actin shows the formation of focal adhesion-like structures at bottom of migrating cells in microchannels, consistent with an adherent migration mode.

We feel that further controls for focal adhesion stainings will be needed and think that it would be outside the scope of this study to include this new data now, but we thank the reviewer again for the comments and we will follow up on the question of adherent migration in future studies.

Reviewer #3

The authors have taken my comments into account, as have those of the other referees. Junqueira Alves' paper is now suitable for publication in Nature Communications.

>> We thank the reviewer very much for the constructive comments in the first round of review.

Reviewer #4

The questions related to MD simulations have been addressed satisfactorily.

>> We thank the reviewer for the expert review of our MD study.

References in Response Letter

1. Huang, Y., Tejero, R., Lee, V.K., Brusco, C., Hannah, T., Bertucci, T.B., Junqueira Alves, C., Katsyv, I., Kluge, M., Foty, R., et al. (2021). Plexin-B2 facilitates glioblastoma infiltration by modulating cell biomechanics. Commun Biol *4*, 145. 10.1038/s42003-021-01667-4.