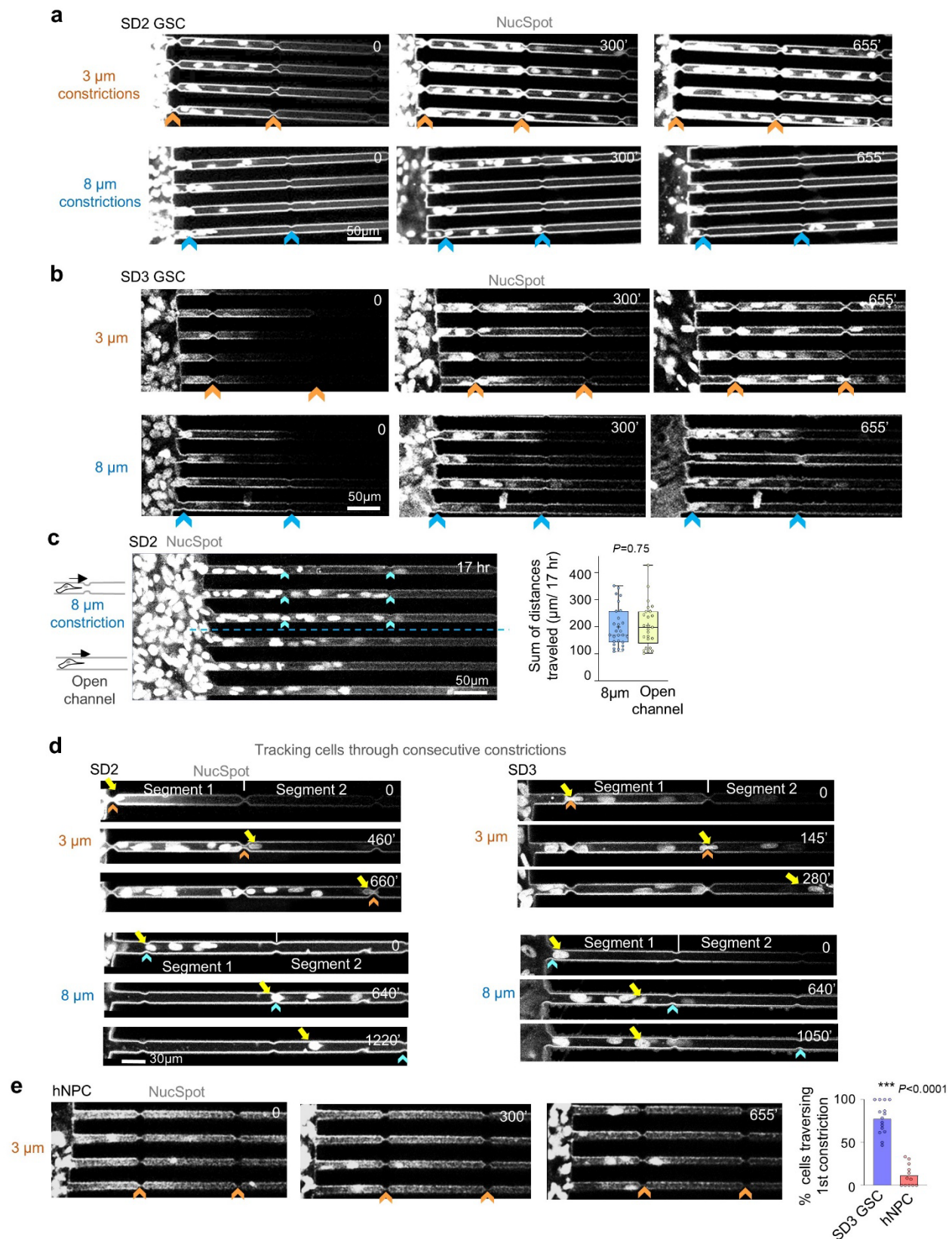




**Figure S1. Patient-derived GSCs are adept at confined migration.**

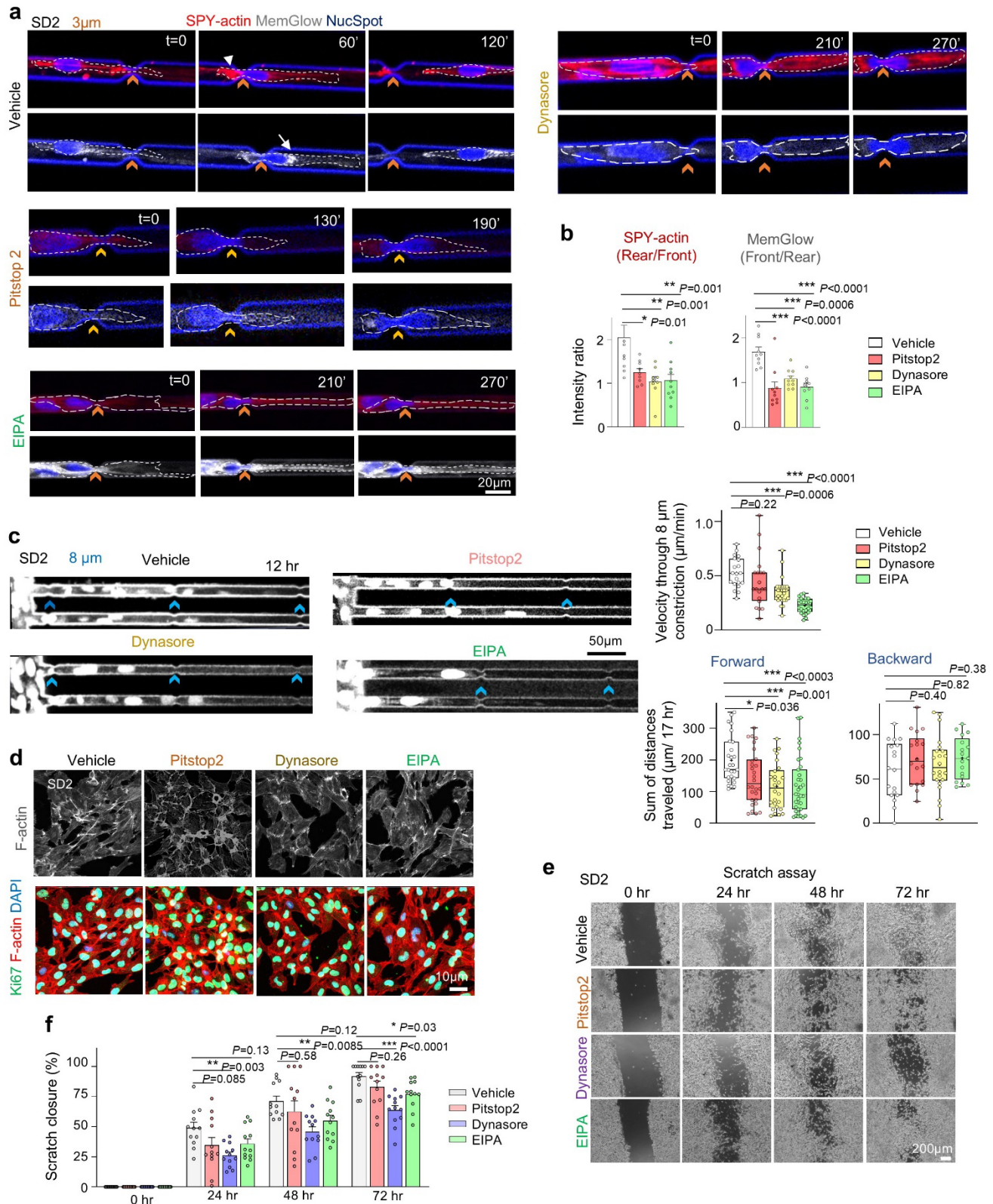
**a, b** Additional still frames from live-cell imaging show that SD2 (**a**) and SD3 (**b**) GSCs labeled with NucSpot passing through microchannels with 3  $\mu\text{m}$  constrictions (orange chevrons) more efficiently than 8  $\mu\text{m}$  constrictions (blue chevrons). Time stamps in minutes in relation to start of imaging were labeled.

**c** Still frame from live-cell imaging of SD2 GSCs migrating through channels with 8  $\mu\text{m}$  constrictions or open channels with no constrictions. Quantifications show comparable sum of distances traveled over 17 hr for GSCs migrating through channels with 8  $\mu\text{m}$  constrictions (n=27 cells) or open channels (n=25 cells). Box plots show 25–75% quartiles, median (line), mean (plus sign). Two-sided unpaired t-test.

**d** Tracking of individual SD2 or SD3 GSC traversing consecutive constrictions (chevrons). Passage through the first 3  $\mu\text{m}$  constriction led to accelerated passage through the second one. Note longer time frames for cells traversing the 8  $\mu\text{m}$  constrictions.

**e** Still frames from live-cell imaging show slower migration of hNPCs in the microchannels with 3  $\mu\text{m}$  constrictions, with few hNPCs reaching the second constriction by 655 minutes (compare to Fig. S1a and b). Bar graphs show the percentage of cells that had successfully traversed the first 3  $\mu\text{m}$  constriction upon entering the first segment of microchannels within the timeframe. Each data point represents cells in one microchannel. For SD3 GSC, n=170 cells from 15 channels; for hNPCs, n=132 cells from 12 channels. Bar graphs show mean  $\pm$  SEM. Two-sided unpaired t-test.

Source data are provided as a Source Data file.



**Figure S2. Endocytosis inhibitors compromise confined migration of GSCs.**

**a** Still frames from live-cell imaging show that migration of SD2 GSCs in microchannels with 3  $\mu$ m constrictions (orange chevrons) was impaired by endocytosis inhibitors, with disruption of accumulation of SPY-actin at rear (arrowhead) and MemGlow at cell front (arrow).

**b** Quantifications of fluorescence intensity ratio of SPY-actin or MemGlow at rear vs. front during confined migration of SD2 treated with vehicle or endocytosis inhibitors.  $n = 9-10$  cells/condition. One-way ANOVA followed

by Dunnett's multiple comparisons test. Data represent mean  $\pm$  SEM.

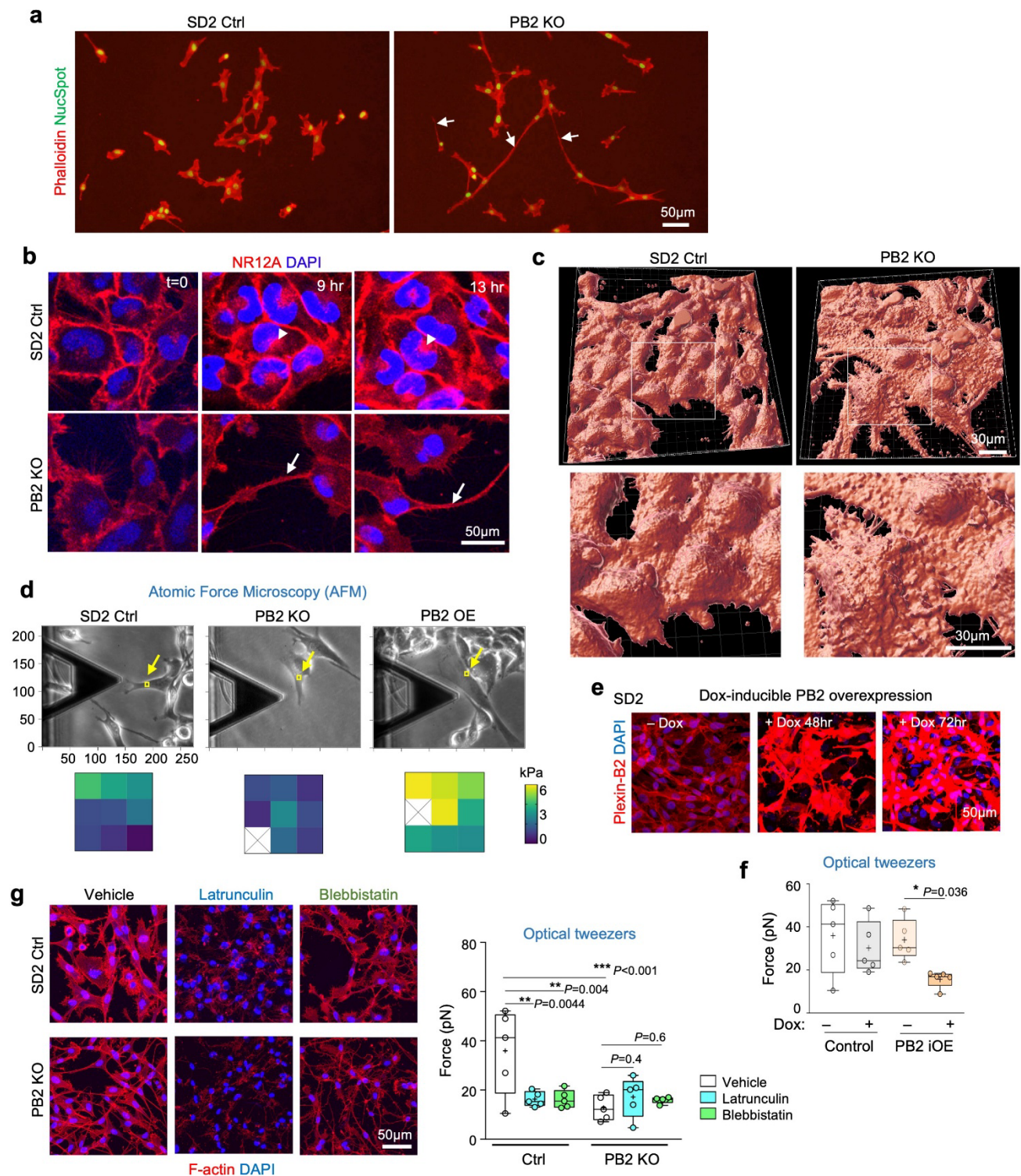
**c** Still frames from live-cell imaging show impaired migration of SD2 treated with endocytosis inhibitor at 8  $\mu$ m constrictions. Box plots of velocity through constrictions and sum of forward and backward movements as affected by endocytosis inhibitors. n=15 to 35 cells/condition. One-way ANOVA followed by Dunnett's multiple comparisons test.

**d** IF images of SD2 in 2D cultures show no major effect on F-actin (phalloidin) or proliferation (Ki67) by endocytosis inhibitors. DAPI for nuclear staining.

**e, f** Images and bar graphs of scratch assay show overall similar wound closure performance of SD2 treated with vehicle or endocytosis inhibitors, except for a modest delay by Dynasore (24-72 hr) or EIPA (only at 72 hr). n=5 fields/group from 3 independent replicates. One-way ANOVA followed by Dunnett's multiple comparisons test. Data represent mean  $\pm$  SEM.

Source data are provided as a Source Data file.





**Figure S3. Plexin-B2 regulates cytoskeletal stiffness and membrane tension in GSCs.**

**a** IF images of SD2 GSCs show long processes from PB2 KO cells (arrows) containing F-actin (Alexa 568 phalloidin), which were not detected in Ctrl cells. NucSpot for nuclear staining.

**b** Still frames from live-cell imaging of SD2 labeled by Nile red (NR12A, a solvatochromic plasma membrane-targeting dye) show reduced NR12<sup>+</sup> endocytic vesicles in PB2 KO compared to Ctrl GSCs (arrowheads). Arrows point to long processes of PB2 KO cells. NucSpot for nuclear staining.

**c** Top panels, 3D surface rendering of GSCs labeled with Nile red (NR12A) dye using IMARIS software (Bitplane). Bottom panels are enlarged images of boxed areas above, showing more pronounced cell membrane ruffling or

wrinkles in PB2 KO relative to Ctrl cells.

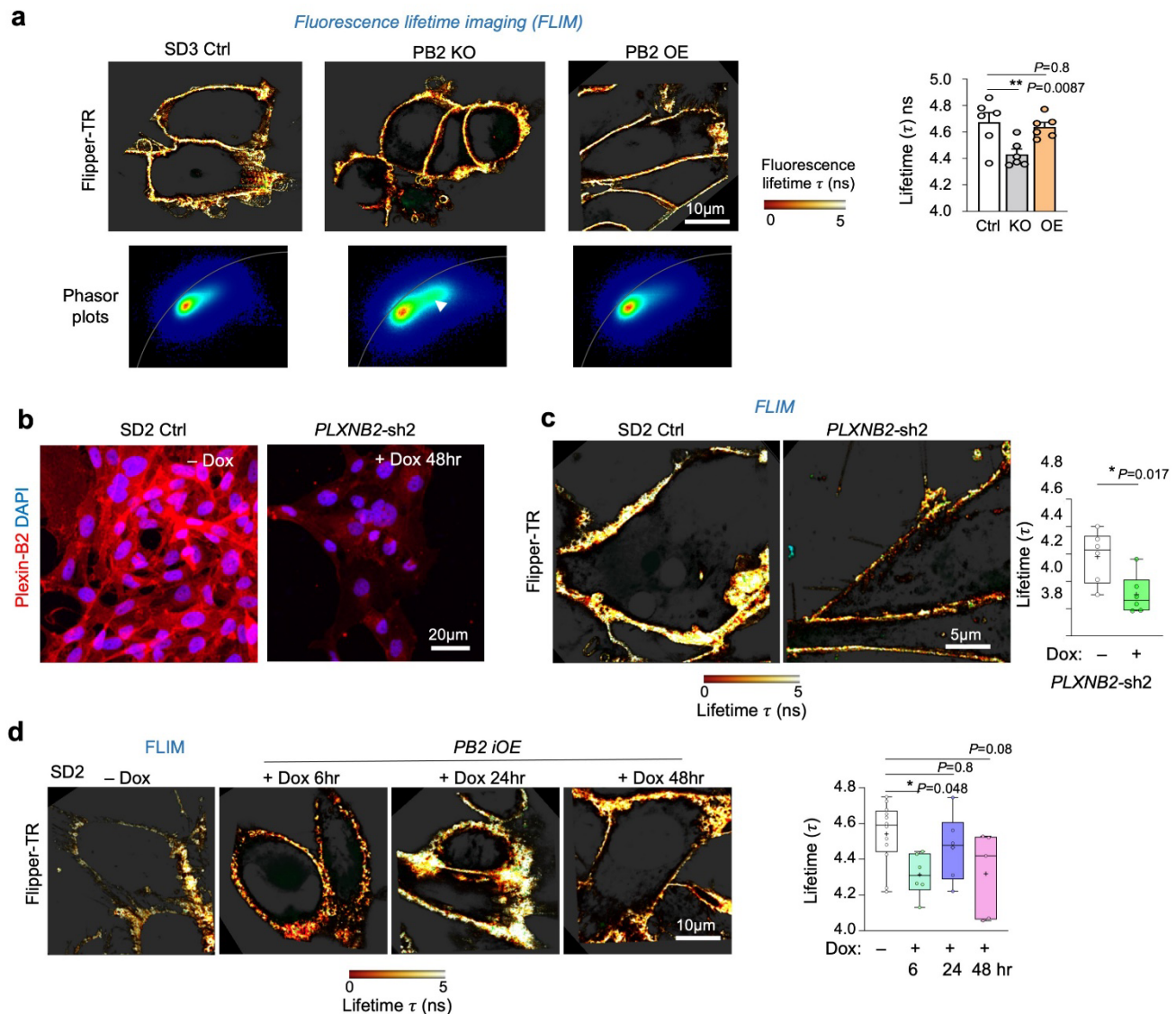
**d** Top panels, phase-contrast images show the area of GSC (yellow box, arrow) measured by atomic force microscopy (AFM) elastography. Bottom panels, corresponding AFM stiffness heatmaps of boxed area above.

**e** IF images show induced overexpression of Plexin-B2 in SD2 GSCs after 48 and 72 hr of Dox induction. DAPI for nuclear staining.

**f** Quantifications of membrane tension measured by optical tweezers of SD2 GSCs with or without Dox induction (72 hr) for control or PB2 iOE. Box plots show 25–75% quartiles, median (line), mean (plus sign). Data collected from 5 cells per group. Kruskal–Wallis test followed by Dunn’s multiple comparisons test.

**g** Left, IF images of Ctrl or PB2 KO SD2 GSCs stained for phalloidin and DAPI after treatment with vehicle or actomyosin inhibitors latrunculin or blebbistatin. Right, box plots of membrane tension measured with optical tweezers, 25–75% quartiles, median (line), mean (plus sign). Data collected from 5 cells per group. One-way ANOVA followed by Dunnett’s multiple comparisons test.

Source data are provided as a Source Data file.



**Figure S4. Plexin-B2 regulates membrane tension in GSCs measured by Flipper-TR dye.**

**a** Top, representative fluorescence lifetime images (FLIM) of SD3 GSCs labeled with the Flipper-TR membrane dye. Heatmap of lifetime values is shown on right. Bottom, phasor plots of lifetime data from FLIM, with arrowhead indicating a shift to shorter lifetime values for PB2 KO cell. Right, bar graphs of average fluorescence lifetime.  $n=6$  images per genotype. One-way ANOVA followed by Dunnett's multiple comparisons test. Data represent mean  $\pm$  SEM.

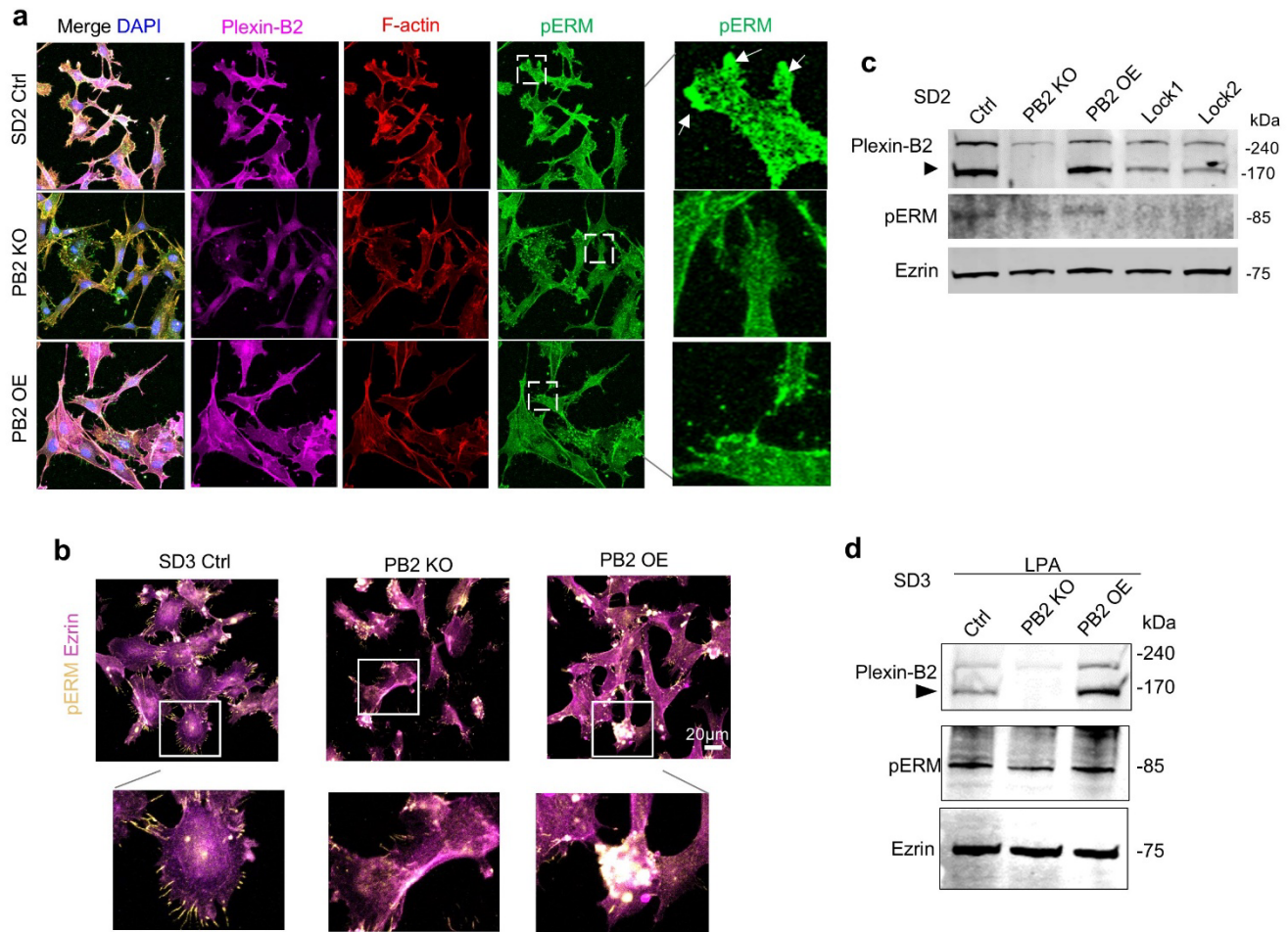
**b** IF images of SD2 GSCs show Dox-induced PB2 knockdown (*PLXNB2*-sh2) after 48 hr. DAPI for nuclear staining.

**c** Left, FLIM of SD2 GSCs labeled by Flipper-TR. Right, box plots show average fluorescence lifetime, with 25–75% quartiles, median (line), mean (plus sign).  $n=6$  cells per group. Two-sided unpaired t-test.

**d** Left, FLIM of SD2 GSCs at different time points with or without Dox-inducible PB2 OE. Right, box plots of average fluorescence lifetime, with 25–75% quartiles, median (line), and mean (plus sign).  $n=12$  cells for -Dox,  $n=5-6$  cells for +Dox groups. One-way ANOVA followed by Dunnett's multiple comparisons test. Data represent mean  $\pm$  SEM.

Source data are provided as a Source Data file.





**Figure S5. Plexin-B2 affects pERM localization in GSCs.**

**a** Confocal IF images of SD2 GSCs co-stained for pERM (phospho-Ezrin/Radixin/Moesin), Plexin-B2, F-actin (Alexa 568 phalloidin), and nuclear staining (DAPI). Enlarged images of boxed areas on the right show high pERM signals in Ctrl cells, particularly at the edge of lamellipodia (arrows) relative to PB2 KO or OE cells.

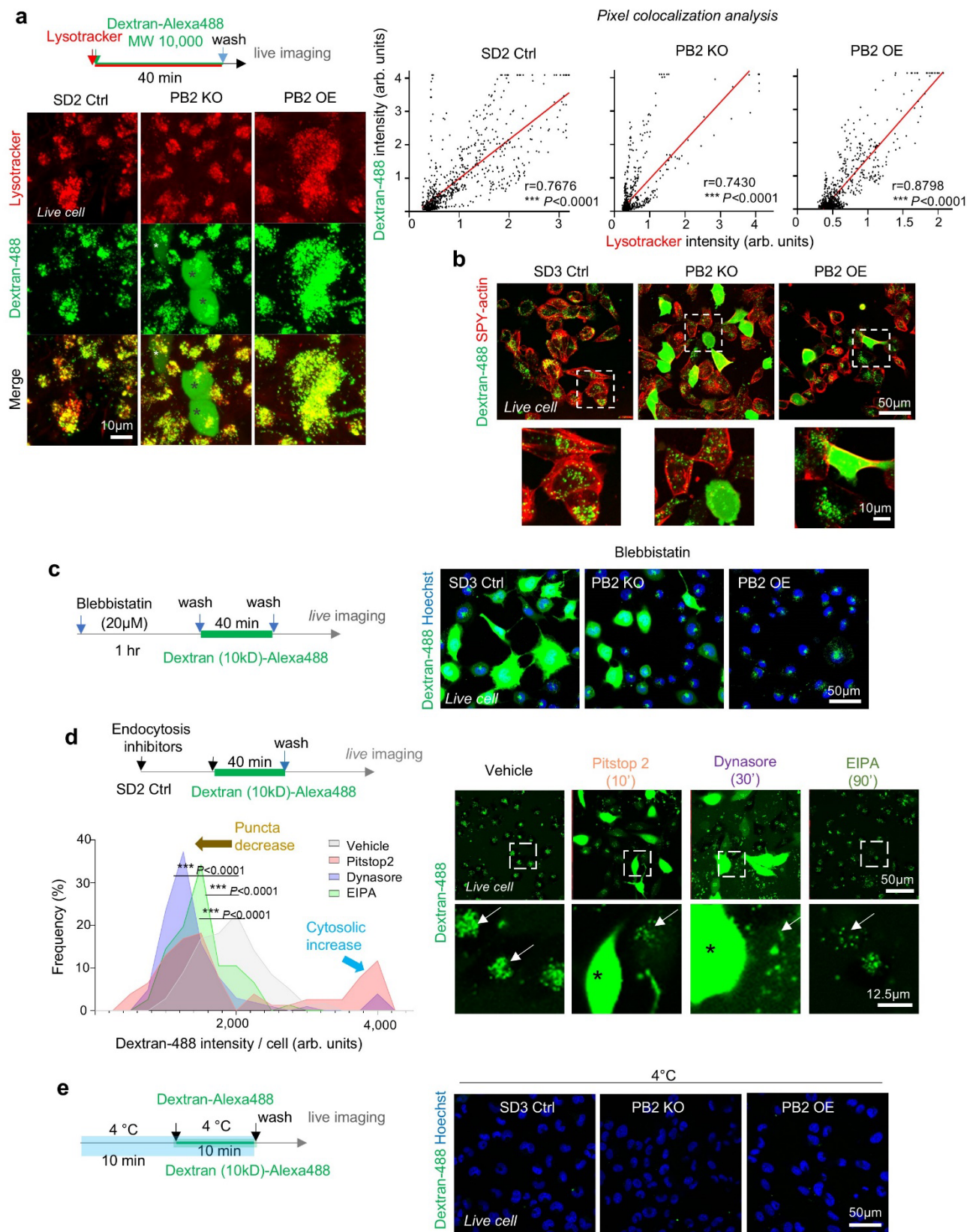
**b** Western blots show expression of Plexin-B2, pERM, and total Ezrin in SD2 GSCs. Note reduced pERM levels in GSCs with PB2 KO or lock mutants compared to Ctrl GSCs. Also note re-expression of PB2 lock1 and lock2 mutants for rescue experiments (see Fig. 7).

**c** Confocal IF images of SD3 GSCs stained for pERM and total Ezrin. Enlarged images of boxed areas on the right show localized pERM signals at tip of cell processes of Ctrl cell, which showed reduction in PB2 KO and abnormal aggregations in PB2 OE GSCs.

**d** Western blots show expression of Plexin-B2, pERM, and total Ezrin in SD3 GSCs treated with oleoyl lysophosphatidic acid (LPA; 5  $\mu$ M) for 5 min to stimulate pERM expression. Note reduced pERM levels in PB2 KO and a slight increase in PB2 OE cells relative to Ctrl GSCs.

Source data are provided as a Source Data file.





**Figure S6. Plexin-B2 affects dextran uptake and membrane internalization in GSCs.**

**a** Top, experimental scheme for dextran uptake assay co-labeled with Lysotracker dye. Bottom, live cell images of GSCs show co-localization of Lysotracker with dextran puncta, but not with the diffuse cytoplasmic dextran (asterisks). Right, scatterplots of pixel co-localization of Lysotracker (red) and Dextran (green) intensities. Three images were measured per group. The linear correlation was determined as Pearson correlation coefficient ( $r$ ).

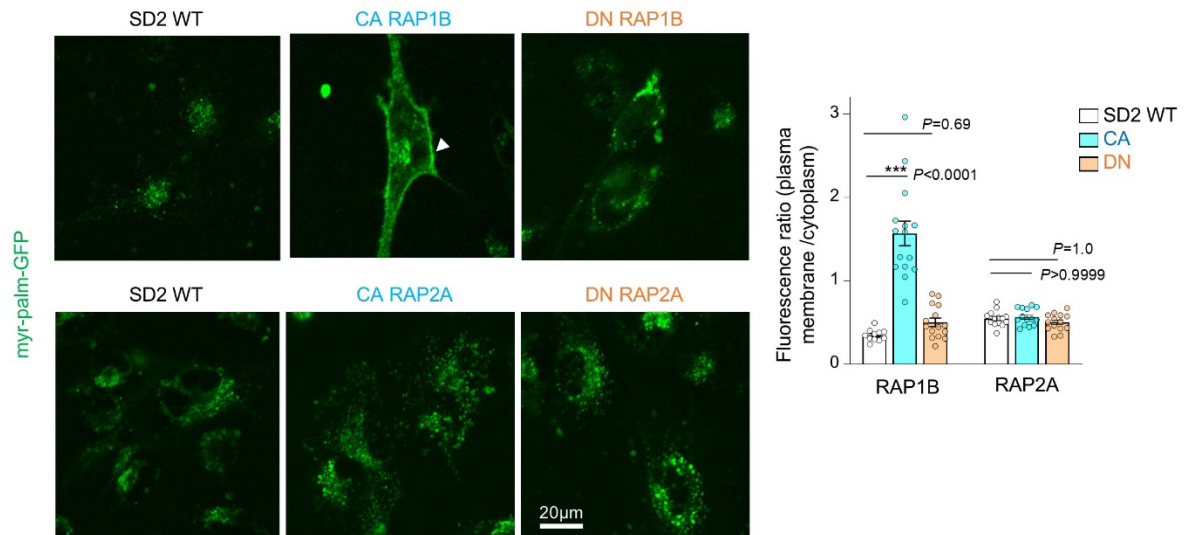
**b** Live-cell imaging of SD3 exposed to dextran-Alexa488 for 40 min and labeled with SPY-actin. Enlarged images of boxed areas below highlight that some of PB2 KO and OE cells harbor diffuse cytoplasmic dextran fluorescence. PB2 KO GSCs also contained less dextran puncta (endosomes) than Ctrl.

**c** Left, dextran uptake assay of SD3 pre-treated with myosin inhibitor blebbistatin (20  $\mu$ M). Right, live cell imaging shows appearance of diffuse cytoplasmic dextran-Alexa488 in Ctrl SD3 treated with blebbistatin, phenocopying PB2 KO, while PB2 OE cells treated with blebbistatin did not harbor cytoplasmic dextran. Hoechst for nuclear visualization.

**d** Top left, experimental scheme of dextran uptake assay. Right, confocal live-cell images show that endocytosis inhibitors reduced dextran puncta but increased diffuse cytoplasmic dextran in Ctrl SD2. Histograms shows reduced dextra puncta (leftward shift, brown arrow), and appearance of bimodal distribution of dextran-Alexa 488 fluorescence (blue arrows) by endocytosis inhibitors. For Ctrl GSCs: vehicle, n=177 cells; Pitstop 2, n= 77; Dynasore, n=102; and EIPA, n=76. One-way ANOVA followed by Dunnett's multiple comparisons test.

**e** Left, experimental scheme for dextran uptake assay of SD3 maintained at 4°C. Right, confocal live images show that 4°C culture blocked the cytoplasmic dextran fluorescence in GSCs of all genotypes.

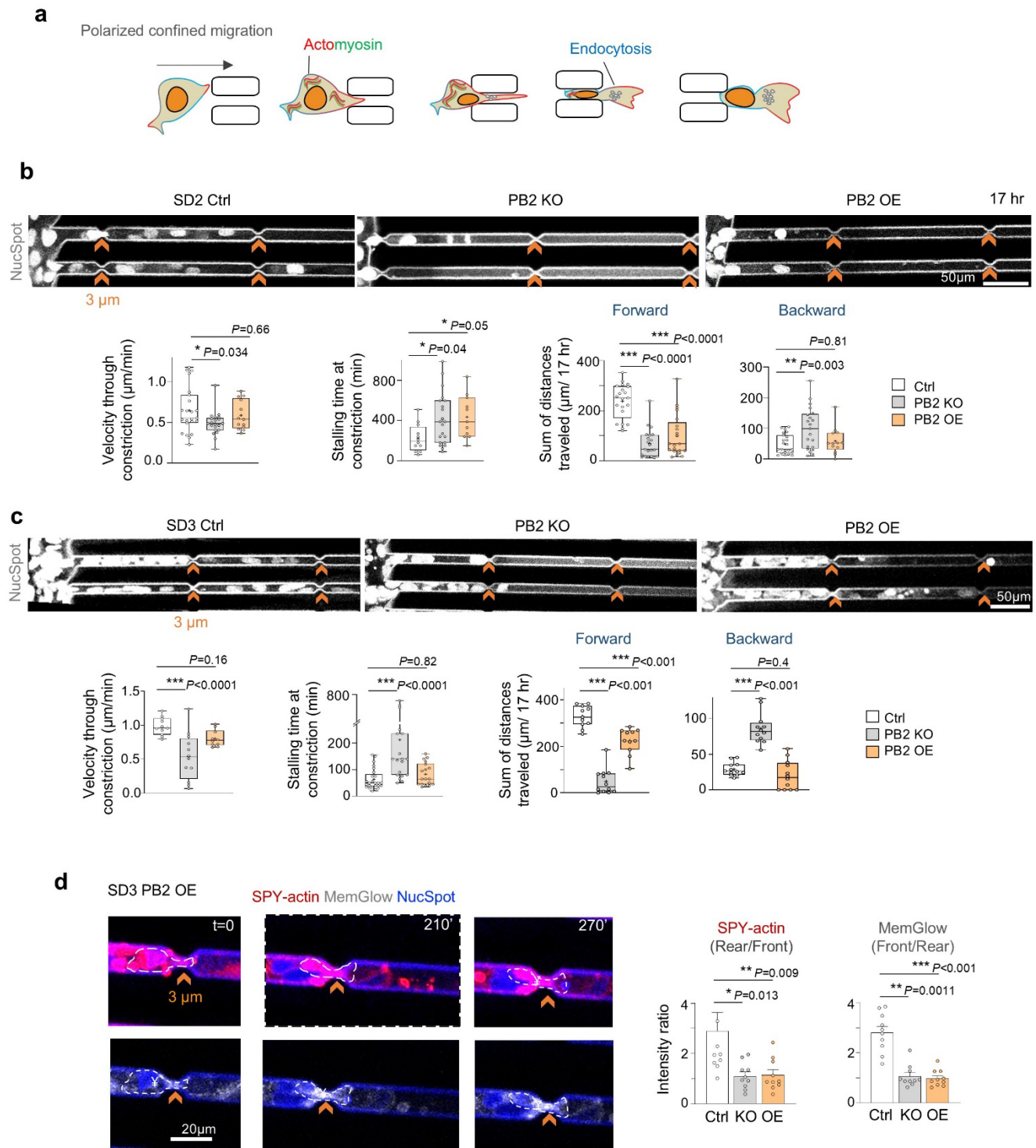
Source data are provided as a Source Data file.



**Figure S7. Rap1 activity regulates membrane internalization in GSCs.**

Live-cell images and quantifications show effects of constitutive active (CA; V12) or dominant-negative (DN; N17) RAP1B or RAP2A on myr-palm-GFP internalization in SD2 GSCs.  $n=11-15$  cells/group. One-way ANOVA followed by Tukey's multiple comparison test. Bar graphs represent mean  $\pm$  SEM.

Source data are provided as a Source Data file.



**Figure S8. Plexin-B2 manipulation compromises confined migration of GSCs.**

**a** Schematic of accumulation of endocytic vesicles at cell front and F-actin at cell rear during confined migration of GSCs.

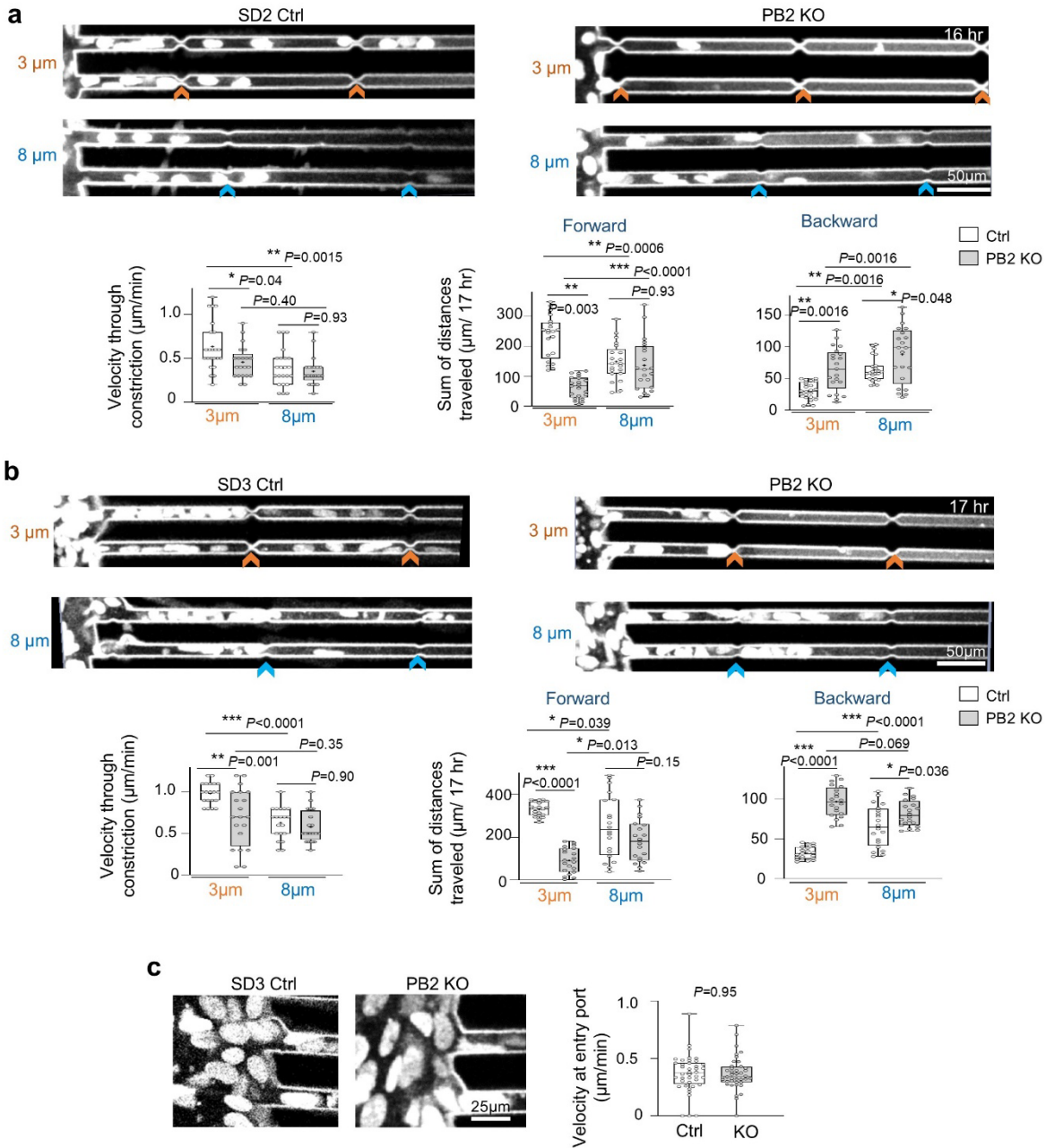
**b** Still frames from live-cell imaging of NucSpot-labeled SD2 GSCs shows reduced migration of PB2 KO or OE cells into the microchannel with 3  $\mu$ m constrictions as compared to Ctrl. Quantifications show velocity through constrictions (n=23 cells for Ctrl, n=21 for KO, n=13 for OE), stalling time at constrictions (n=12 cells for Ctrl, n=21 for KO, n=11 for OE), sums of distances traveled for forward (n=21 each) or backward movement (n=23 cells for Ctrl, n=21 for KO, n=13 for OE) over 17 hr. One-way ANOVA followed by Dunnett's multiple comparisons test. Box plots show 25–75% quantiles, minimal and maximal values (whiskers), median (line), and mean (cross).



**c** Still frames from live-cell imaging of NucSpot-labeled GSCs show reduced migration of PB2 KO or OE SD3 GSCs in microchannels with 3  $\mu\text{m}$  constriction (orange chevrons) as compared to Ctrl. Quantifications of velocity through constrictions, stalling time at constrictions, sums of distances traveled forward or backward over 17 hr are shown. For stalling time at constrictions: n=25 cells for Ctrl, n=19 for KO, n=18 for OE. For the rest, n=10-12 cells. One-way ANOVA followed by Dunnett's multiple comparisons test.

**d** Left, still frames from live-cell imaging of PB2 OE SD3 stalled at 3  $\mu\text{m}$  constriction. Dashed lines delineate cell boundary. Note disorganized SPY-actin and MemGlow<sup>+</sup> endosomes. Right, bar graphs show the ratio of fluorescence intensity for SPY-actin or MemGlow at rear vs. front of cells during confined migration. Data represent mean  $\pm$  SEM. Of note, Ctrl and PB2 KO quantifications are the same as in Fig. 4B. n=10 cells per group. Kruskal–Wallis test followed by Dunn's multiple comparisons test.

Source data are provided as a Source Data file.

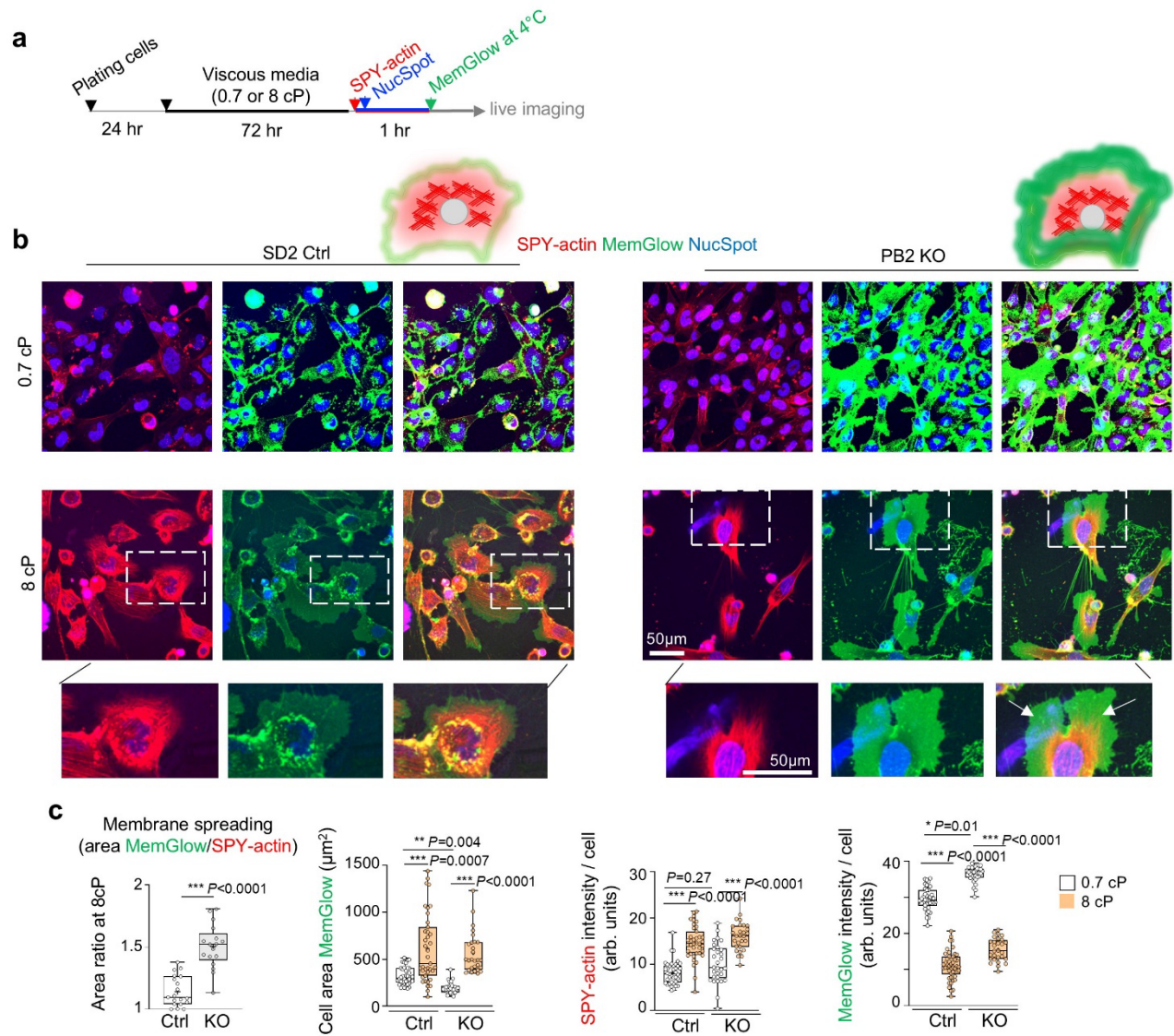


**Figure S9. Plexin-B2 is mainly required for confined migration.**

**a, b** Still frames from live-cell imaging of NucSpot-labeled SD2 (a) or SD3 GSCs (b) show that PB2 KO reduced velocity and forward migration only in channels with 3  $\mu\text{m}$ , but not with 8  $\mu\text{m}$  constrictions. However, directional persistence was affected for both. Box plots show velocity through constrictions and sums of distances traveled for forward ( $n=21$  each) or backward movement over 17 hr ( $n=20-23$  each). One-way ANOVA followed by Tukey's multiple comparison test. 25–75% quantiles, minimal and maximal values (whiskers), median (line), and mean (cross).

**c** Still frames from live-cell imaging of NucSpot-labeled SD3 GSCs seeded into entry port show that PB2 KO did not affect cell movement at non-constricted areas. Box plot show 25–75% quartiles, median (line), mean (plus sign).  $n=25-27$  cells each. Two-sided unpaired t-test.

Source data are provided as a Source Data file.



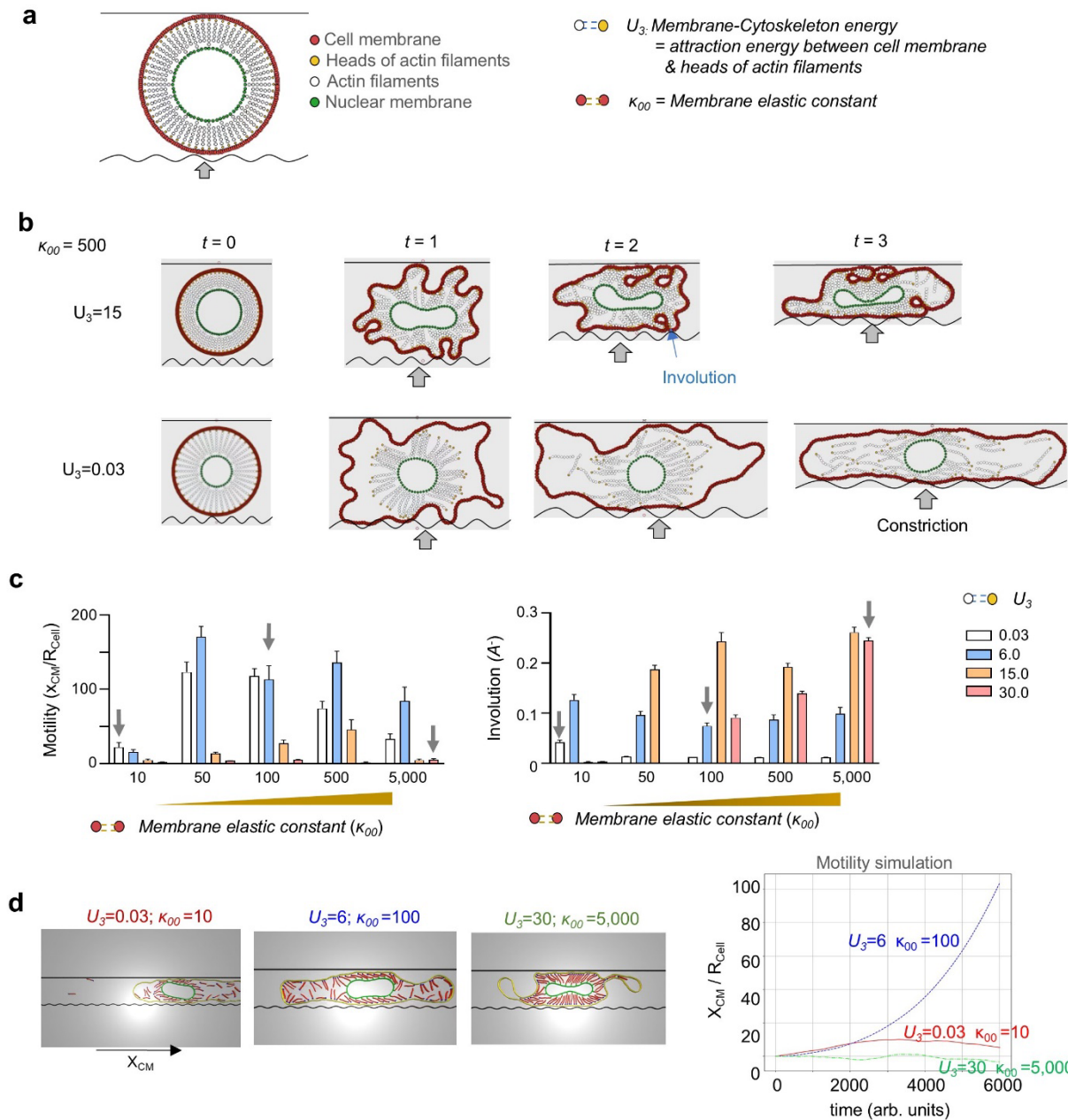
**Figure S10. Viscosity induced actin contraction is independent of Plexin-B2.**

**a** Experimental scheme of increasing viscosity in culture media, followed by live cell imaging.

**b** Confocal live cell images show increased cortical actin (SPY555-actin) in response to higher viscosity in both Ctrl and PB2 KO cells, but the membrane spreading (MemGlow) was more pronounced in KO than Ctrl cells. Cartoon illustrations on top show typical observations.

**c** Quantifications of membrane/cortical actin membrane spreading ratio, cell area, and SPY-actin and MemGlow fluorescence intensities in SD2 GSCs in response to high vs. low viscosity media (8 vs. 0.7 cP) for 72 hr. Box plots show 25–75% quantiles, minimal and maximal values (whiskers), median (line), and mean (cross). For membrane/actin spread ratio:  $n=17-19$  cells/genotype, two-sided unpaired t-test. For the rest,  $n=28-39$  cells per condition, Kruskal–Wallis test followed by Dunn's multiple comparisons test.

Source data are provided as a Source Data file.



**Figure S11. Molecular dynamics simulations illustrate the requirement of orchestrated cytoskeleton contraction and membrane tension for optimal confined migration.**

**a** Cell components for molecular dynamics simulation, based on a coarse-grained bead-spring model. Two virtual walls are on opposing side of the cell, with the top wall fixed and frictionless, and the bottom wall movable with a friction factor to simulate external resistance and cell confinement.

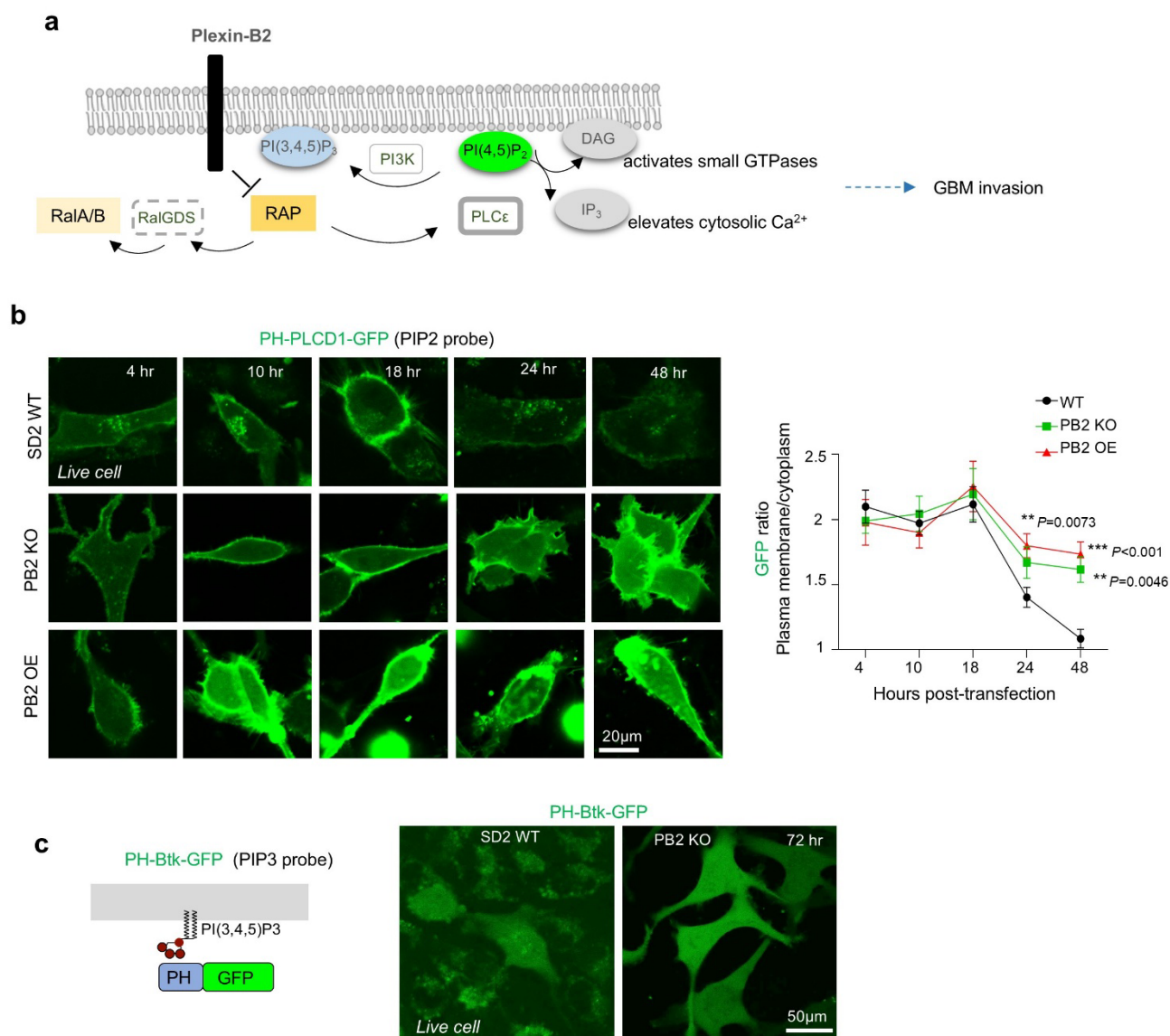
**b** Still images from simulation show cells being constricted between the virtual walls, with membrane tension set at  $\kappa_{00} = 500$ . At  $t=0$ , the distance between the walls is 1.2x cell diameter. At  $t=1$  to 3, the cells are increasingly compressed. Note involution of plasma membrane (arrow) during constriction only in cells with high ( $U_3=15$ ) and not low ( $U_3=0.03$ ) membrane-cytoskeletal energy.

**c** Left graphs show travelled distance by simulated cells (motility), measured as center of mass (CM) in the x direction ( $x_{CM}$ ) as a function of varying membrane tension  $\kappa_{00} = 500$  and membrane-cytoskeleton energy  $U_3$ . Right graphs show cell negative areas (involutions;  $A^-$ ) as a function of varying  $\kappa_{00} = 500$  and  $U_3$  conditions.  $n=10$  iterations per group. Data represent mean  $\pm$  SEM. Arrows point to specific  $\kappa_{00}$  and  $U_3$  conditions used for motility simulation shown in (d).



**d** Left panels, still images of simulated cells migrating under constriction for three different  $\kappa_{00}$  and  $U_3$  conditions corresponding to arrows in (c). Right panels, graphs of travelled distance by cell (motility) measured as center of mass (CM) in x direction (xCM) for three different  $\kappa_{00}$  and  $U_3$  conditions. Note that cell motion is predicted to be minimal and lack directionality for high or low values ( $U_3=30$ ,  $\kappa_{00}=5,000$ ;  $U_3=0.03$ ,  $\kappa_{00}=10$ ), but for intermediate values ( $U_3=6$ ,  $\kappa_{00}=100$ ), the cell moves continuously under constriction.

Source data are provided as a Source Data file.



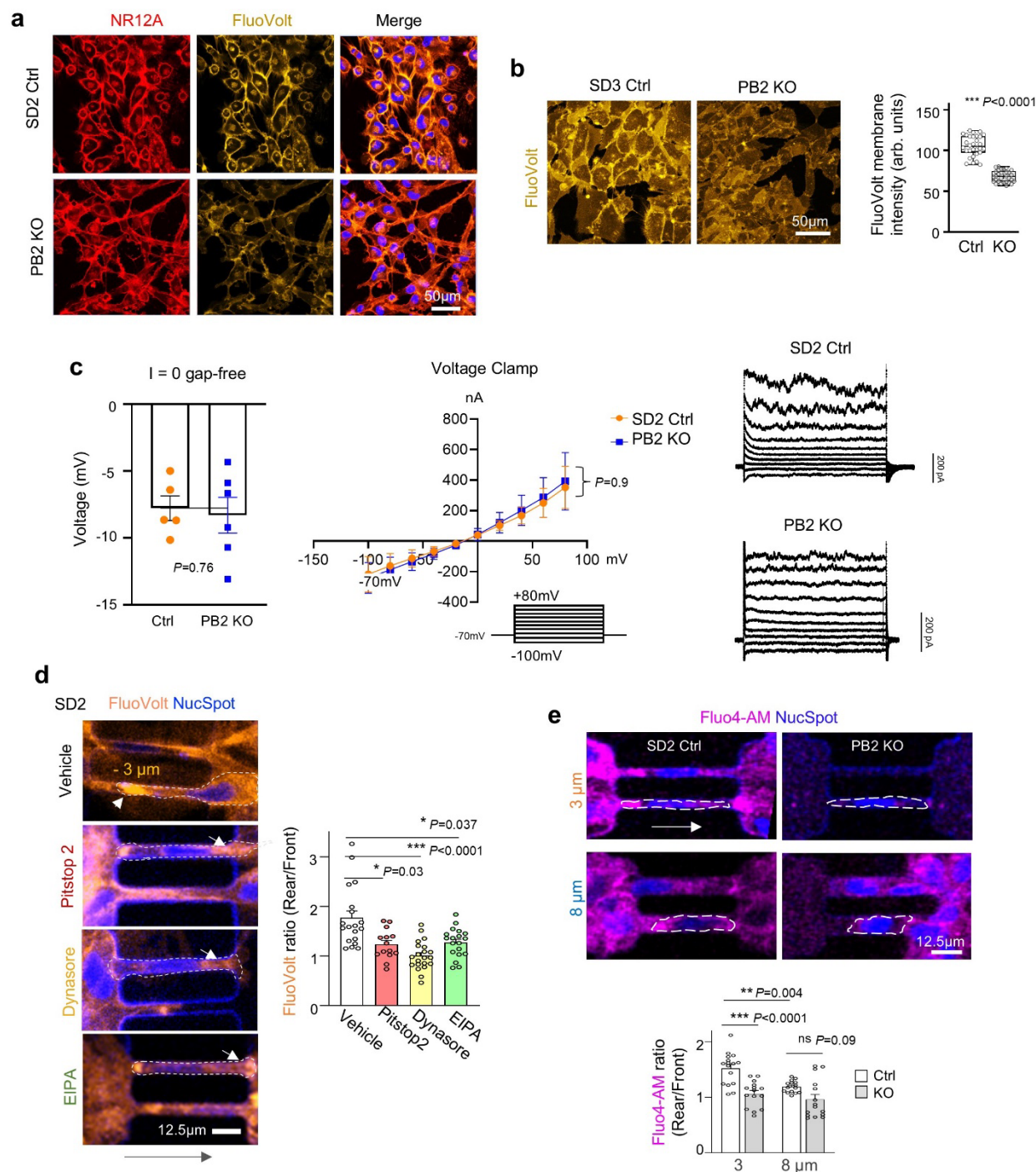
**Figure S12. Plexin-B2 affects anionic membrane lipid composition.**

**a** Diagram of regulation of PI(4,5)P<sub>2</sub> by PLC epsilon under the control of Plexin-B2/Rap1 signaling. Activation of Ral GTPases via Rap1/RalGDS as a potential alternative pathway (Ral GTPases are associated with control of exocytosis).

**b** Live imaging of SD GSCs expressing PIP2 probe (PH(PLCD1)-GFP) at 4 to 48 hr after transfection. Note the appearance of membrane GFP signals at 18 hr in control GSCs followed by internalization, but sustained membrane GFP signals in PB2 KO and OE cells. Quantifications show the ratio of membrane vs. internalized GFP signals. For 4 and 10 hr: n=15 cells/condition. For 18 hr: n=27 for control, n=15 for PB2 KO or OE. For 24 hr: n=30 for control or OE, n=15 for KO. For 48 hr: n=24 for control, n=15 for PB2 KO or OE. Two-way ANOVA followed by Dunnett's post hoc test.

**c** Left, diagram of PIP3 probe (PH(Btk)-GFP). Right, live cell imaging shows low GFP levels in both WT and PB2 KO GSCs.

Source data are provided as a Source Data file.



**Figure S13. Plexin-B2 affects the membrane electric field, but not transmembrane potential.**

**a** Live-cell images show comparable Nile red (NR12A) fluorescence intensity (solvatochromic plasma membrane-targeting dye), but reduced FluoVolt fluorescence intensity in PB2 KO compared to Ctrl GSCs.

**b** Live-cell images show reduced FluoVolt fluorescence in plasma membrane of PB2 KO SD3 (i.e., higher negative charge of inner membrane). Box plots show 25–75% quartiles, median (line), mean (plus sign).  $n=30$  cells per group. Two-sided unpaired t-test. Data represent mean  $\pm$  SEM.

**c** Whole-cell patch-clamp recordings of SD2 GSCs. Left, currents were clamped at 0 A and bar graphs show comparable transmembrane potentials of Ctrl ( $n=5$ ) and PB2 KO ( $n=6$ ) cells. Two-sided unpaired t-test. Middle, I/V curves reveal similar membrane conductance of Ctrl ( $n=4$ ) and PB2 KO ( $n=5$ ) cells. The voltage step is shown below. Right, representative whole cell current traces from Ctrl and PB2 KO cells. Cells were clamped in 20 mV steps relative to holding potential ( $-70$  mV), ranging from  $-100$  to  $+80$  mV. Two-way ANOVA followed by

Bonferroni's post hoc test.

**d** Still images from live-cell imaging show higher FluoVolt signals at the rear zone (arrowhead) of vehicle-treated SD2 traversing micro-tunnels, a pattern disrupted by endocytosis inhibitors, leading to FluoVolt fluorescence signals also appearing at cell front (arrows). Dashed lines delineate cell boundary. Long arrows denote migration direction. Bar graphs show the ratio of FluoVolt intensity at rear vs. front during confined migration. n=14-21 cells/condition. Kruskal–Wallis test followed by Dunn's multiple comparisons test. Data represent mean  $\pm$  SEM.

**e** Still images capture calcium localization (Fluo4-AM fluorescence) at rear zone of Ctrl GSCs when traversing tunnels, more so in 3 than 8  $\mu$ m tunnel, but not in PB2 KO cells. Migration direction is denoted by long arrow. Bar graphs showing Fluo4-AM intensity ratio at rear vs. front in GSC during passage through tunnels. n=15-16 cells. One-way ANOVA followed by Tukey's multiple comparison test. Data represent mean  $\pm$  SEM.

Source data are provided as a Source Data file.



**a**

LV-sgSEMA4 BCG-Cas9-Hygro (vector 1)



LV-sgSEMA4 ADF-Cas9-Neo (vector 2)

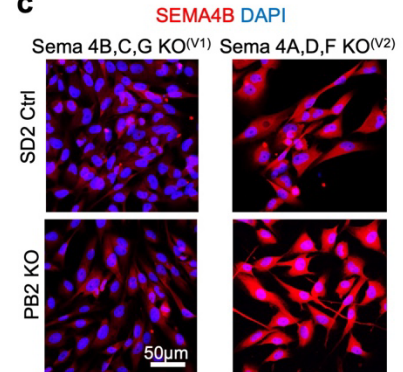


| Target gene | gRNA sequence           | Peptide in target | Position | Domain |
|-------------|-------------------------|-------------------|----------|--------|
| SEMA4B      | ggacttgaaatcgggtcga     | FDPNFKS           | ~190 aa  | Sema   |
| SEMA4C      | cttcacgggggacgacgaca(-) | FTGDDD            | ~230 aa  | Sema   |
| SEMA4G      | tggcggctccggcgatgtc     | DIRRSRH           | ~200 aa  | Sema   |
| SEMA4A      | cttcaacgtcatccgccacg    | FNVIH             | ~300 aa  | Sema   |
| SEMA4D      | gaccctgtcatcctcgccgt(-) | DGEDDRV           | ~230 aa  | Sema   |
| SEMA4F      | atgaaggtgagtagcggtc     | DRVLTFI           | ~400 aa  | Sema   |

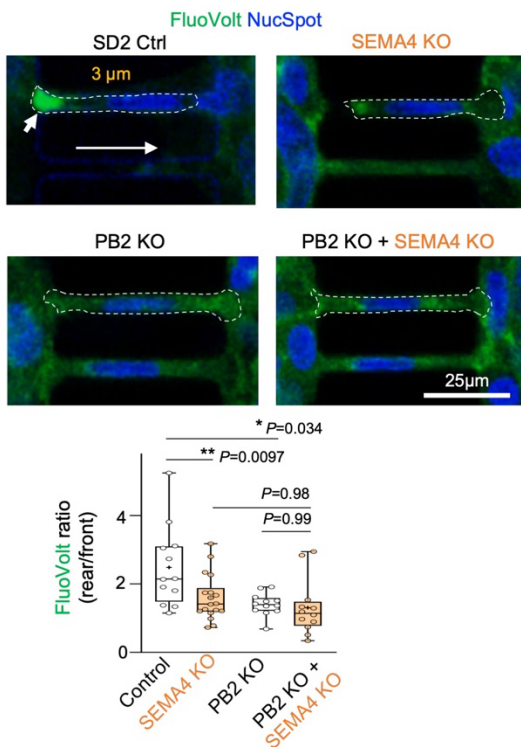
**b**



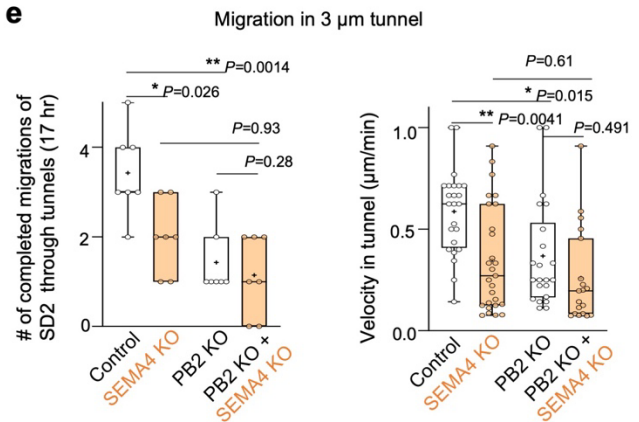
**c**



**d**



**e**



**Figure S14. Pan-SEMA4 KO impairs confined migration of SD2 GSCs.**

**a** Diagram of two lentiviral vectors for CRISPR/Cas9-mediated *SEMA4* knockout (KO). Vector 1 has three small guide (sg) RNAs targeting *SEMA4B*, *C*, and *G*. Vector 2 has three sgRNA targeting *SEMA4A*, *D*, and *F*. Right, table containing gRNA sequences, and the respective peptide sequence and domain positions.

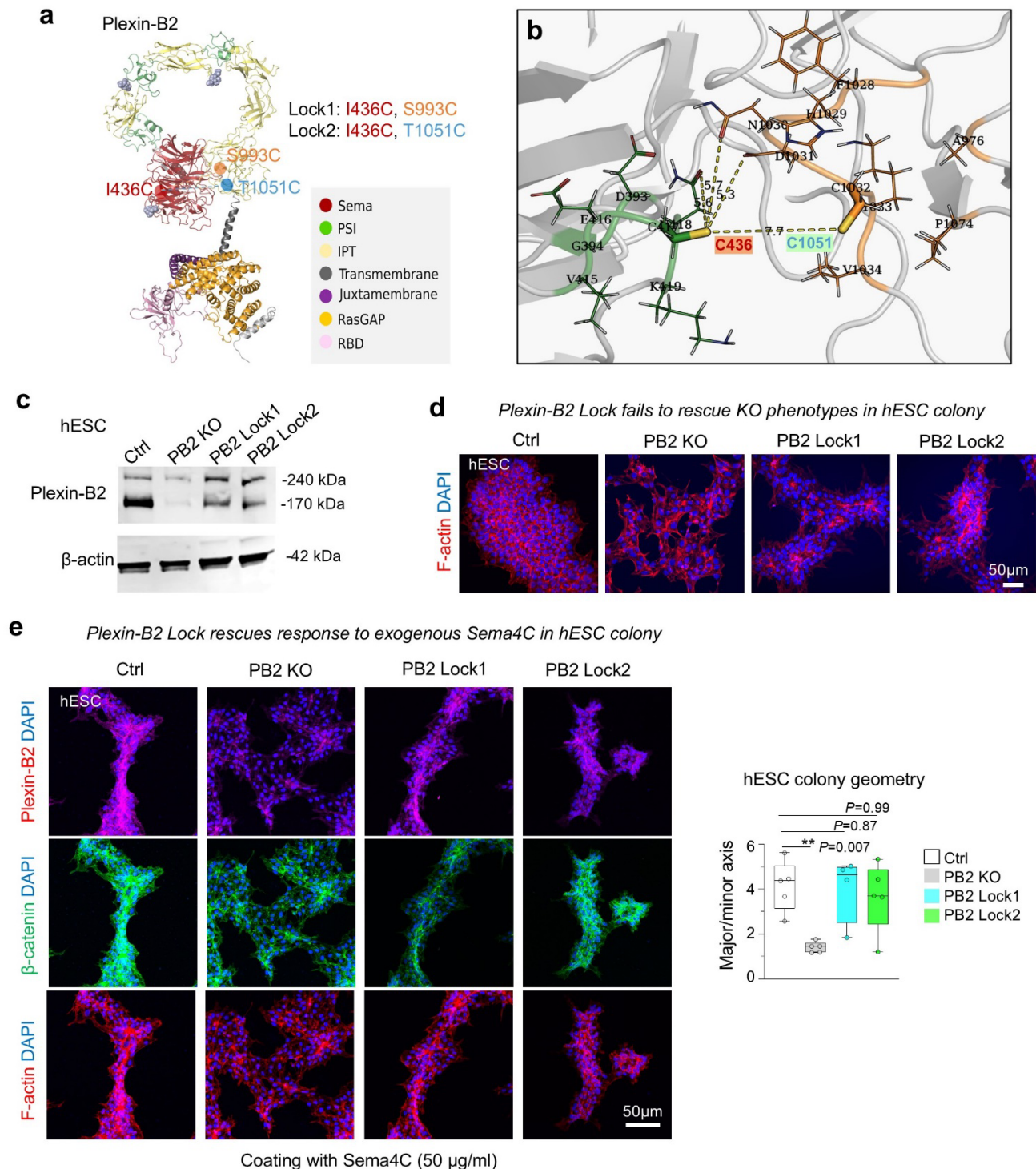
**b** Western blots show absence of Sema4B and Sema4C protein in pan-*SEMA4* KO SD2 Ctrl and PB2 KO GSCs. Note the absence of mature Plexin-B2 (170 kDa) in PB2 KO GSCs.  $\beta$ -actin serves as a loading control.

**c** IF images of SD2 GSCs with *Sema4B,C,G* KO (vector 1, V1) and *Sema4A,D,F* KO (vector 2, V2) stained for SEMA4B and DAPI.

**d** Still images from live-cell videography show higher FluoVolt fluorescence signals at the rear zone (arrow) of Ctrl SD2 traversing 3  $\mu$ m tunnel, a pattern disrupted by pan-*SEMA4* KO and PB2 KO, or both. Dashed lines outline cell boundary. Long arrows denote migration direction. Box plots show 25–75% quartiles, minimal and maximal values (whiskers), median (line), and mean (cross).  $n=12-23$  cells quantified per group. One-way ANOVA followed by Tukey's multiple comparison test.

**e** Box plots show the number of successful migration events per cell through 3  $\mu\text{m}$  tunnels and velocity in tunnels. Box plots show 25–75% quantiles, minimal and maximal values (whiskers), median (line), and mean (cross). n=19–27 cells/group. One-way ANOVA followed by Tukey’s multiple comparison test.

Source data are provided as a Source Data file.



**Figure S15. Locked ring mutations of Plexin-B2 do not rescue cytoskeletal defects, but restore responsiveness to exogenous semaphorin in PB2 KO human ES cells.**

**a** 3D structural modeling of human Plexin B2, with domains coded by colors. Predicted glycosylations of residues N108, N509 and N714 are depicted as light blue spheres. Image visualization made with Pymol (version 2.3). The locations of two pairs of mutations: Lock1 (I436C and S993C) and Lock2 (I436C and T1051C) are predicted to form intramolecular disulfide bridges that lock the ring structure.

**b** Predicted intramolecular disulfide bond interaction of human Plexin B2 with mutations I436C and T1051C (Lock2). The predicted intramolecular disulfide bridges between C436 with C1051 locks the ring structure (note that the amino acid positions in the figure is shifted, as the signal peptide is excluded). Dashed yellow lines represent the distance between atoms, given in angstroms. Image visualization made with Pymol (version 2.3).

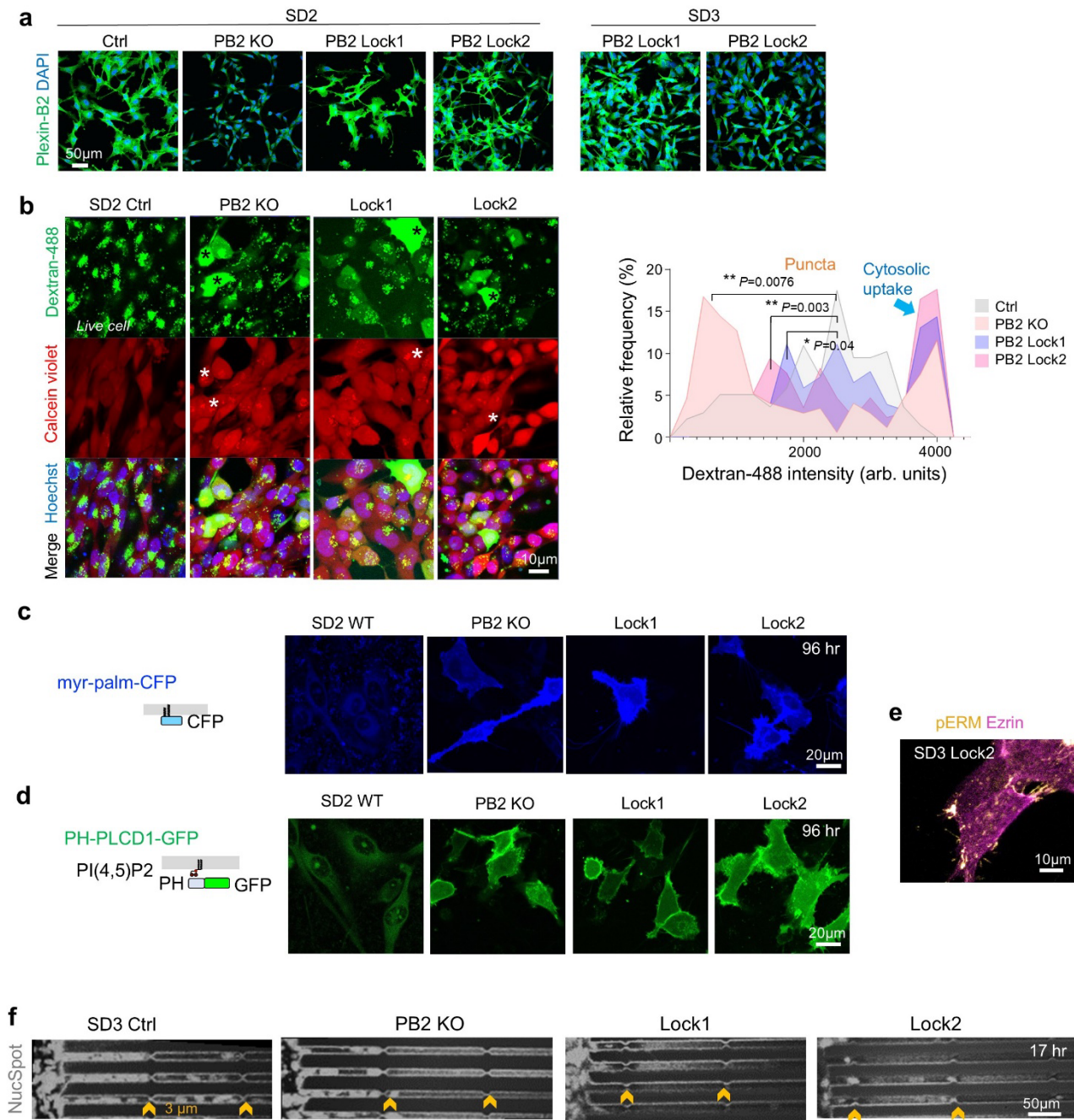
**c** Western blot shows absence of mature Plexin-B2 (170 kDa) in PB2 KO hESCs (H9 cell line) compared to wild-type cells, and re-expression of PB2 lock mutants.  $\beta$ -actin serves as a loading control.

**d** IF images of hESC colonies stained for F-actin (Alexa 568 phalloidin) and DAPI show differences in cell organization, colony geometry, and actin network between Ctrl and PB2 KO, which were not rescued by PB2 Lock1 and Lock2 mutants.

**e** IF images of hESC colonies in culture dishes coated with Sema4C-Fc (50  $\mu$ g/ml) mixed with Matrigel and stained for Plexin-B2,  $\beta$ -catenin, and F-actin. DAPI for nuclear staining. Sema4C affected the colony geometry of Ctrl but not PB2 KO cells, which was rescued by PB2 Lock1 and Lock2. n=5 colonies per group. One-way ANOVA followed by Dunnett's multiple comparisons to compare treatments within the groups.

Source data are provided as a Source Data file.





**Figure S16. Plexin-B2 lock mutants affected membrane internalization and confined migration.**

**a** IF images show absence of Plexin-B2 in PB2 KO GSCs compared to Ctrl SD2 and re-expression of PB2 Lock1 and Lock2 mutants in both SD2 and SD3. DAPI for nuclear staining. Also see WB shown in Fig. S5B.

**b** Live-cell confocal fluorescence images show diffuse cytoplasmic dextran-Alexa488 (asterisks) in PB2 KO cells and both lock mutants. Calcein violet-AM dye was used to indicate live cells, Hoechst for cell nucleus. Histograms show bimodal distribution of dextran-Alexa 488 fluorescent intensities for PB2 KO and both lock mutants (blue arrow), reflective of reduced endocytosis and higher cytoplasmic dextran uptake.  $n=137$  for Ctrl cells,  $n=173$  for PB2 KO,  $n=170$  for Lock1, and  $n=153$  for Lock2. Kruskal–Wallis test followed by Dunn’s multiple comparisons test.

**c** Left, schematic of myr-palm-CFP attached to inner membrane leaflet. Right, live cell imaging at 96 hr after transfection shows membrane retention of the probe in PB2 KO and of both lock mutants, but not control GSCs.

**d** Left, diagram of the PIP2 probe PH(PLCD1)-GFP. Right, live-cell imaging at 96 hr post transfection shows internalization of the PIP2 probe in WT GSCs in contrast to the membrane retention in PB2 KO and Lock mutants.

**e** Confocal IF images of SD3 Lock2 GSCs stained for pERM (phospho-Ezrin/Radixin/Moesin) and total Ezrin. Note failure of rescue by PB2 Lock2 mutant form (see Fig. S5b for comparison).

**f** Still images at the end of 17 hr videography show compromised confined migration of PB2 KO and both lock mutants through microchannels with 3  $\mu$ m constrictions as compared to Ctrl. NucSpot for live cell nuclei staining.

Source data are provided as a Source Data file.