Supplementary Materials and Methods

Identification of slow-cycling population using CFSE and flow cytometry. As reported previously (10), carboxyfluorescein diacetate succinimidyl ester (CFSE) can be loaded into brain tumor-initiating cells to identify slow-cycling subpopulations. A Cell Trace CFSE Cell Proliferation Kit (Invitrogen) was used to load the dye into the tumor cells. TICs were trypsinized before fluorescence was measured using an FC-500 flow cytometer (Beckman Coulter), and results were analyzed by using FlowJo. The top 5% of CFSE bright cells were defined as the slow-cycling subpopulation.

Synthesis and functionalization of extracellular matrices. Polyacrylamide 2D matrices were synthesized following our previously described protocol (4) and functionalized with natural mouse laminin (10 mg/ml, Invitrogen). Matrices with elastic moduli of 0.08, 0.80, and 119 kPa contained final acrylamide/bis-acrylamide (A/B) percentages of 3% A/0.05% B, 5% A/0.1% B, and 15% A/1.2% B, respectively, as measured in Urlich et al. (2009). All analyses were conducted on data pooled from at least three technical and three biological (gel) replicates.

Phase and epifluorescence imaging and immunofluorescence staining. All live-cell and fluorescence imaging was performed using either an inverted Nikon TE2000-E2 or a Nikon Ti-E microscope equipped with a motorized, programmable stage (Prior Scientific, Inc. and Applied Scientific Instrumentation), an incubator chamber to maintain constant temperature, humidity, and CO₂ levels (In vivo Scientific), a digital camera (Photometrics Coolsnap HG II, Roper Scientific), and SimplePCI (Hamamatsu Corporation) and NIS Elements (Nikon Instruments, Inc.) software. Cells were fixed and stained for filamentous actin (F-actin), vinculin, and the nucleus as previously described (4). Cell spreading measurements were obtained by quantifying
the area of cells using Image J software (NIH). Epifluorescence images obtained in Figs. 1, 2, 3, 7, and S1 were enhanced by adjustments to brightness and contrast as necessary to reduce background signal. The following primary antibodies were used for immunofluorescence: anti-vinculin (1:500, Sigma-Aldrich), anti-glial fibrillary acidic protein (1:500, Dako), anti-human Nestin (1:500, Millipore), TUJ1 (1:1000, Promega), and anti-human SOX2 (1:500, R&D). F-actin and nuclei were stained with phalloidin (1:200, Invitrogen) and 4',6-diamidino-2-phenylindole (1:200, Invitrogen), respectively.

Measurement and analysis of cell motility. Following a previously established protocol, we measured cell motility using 10X phase contrast timelapse images acquired every 15 minutes over a 12h period (4). At least 10 representative fields of view per substrate and at least three substrates per stiffness condition were used for analysis. ImageJ software (NIH) was used to track the centroid of each cell throughout the time sequence.

Measurement of cell proliferation. Cell proliferation was measured using a BrdU Flow Kit (BD Biosciences) according to the manufacturer's directions and analyzed with an FC-500 flow cytometer and FlowJo software. At least 3 technical and 3 biological replicates were analyzed per stiffness condition for each cell line. In vivo proliferation was measured using Ki67 antibody (1:500, Leica Biosystems) staining of tissue sections.

Constitutively active cell lines. Myc-tagged RhoA Q63L (44), Flag-tagged MLCK ED785-786KK (45), and Myc-tagged ROCK1 D3(46) were subcloned into the lentiviral vector pSLIK containing the TRE tight doxycycline-inducible promoter, the reverse tetracycline transactivator (rtTA), and the YFP variant Venus(47). Viral particles were packaged in 293T cells and used to infect L0 and L2 brain tumor-initiating cells at a multiplicity of infection of 1 IU/cell. Cells expressing the pSLIK vector were sorted on a DAKO-Cytomation MoFlo High Speed Sorter.
based on Venus fluorescence. Control cell lines were created with the same method using empty vectors. Doxycycline was added at a concentration of 100 ng/ml 2-3 days prior to all experiments to activate the constitutively active constructs.

**Western blot analysis.** Western blots were performed as previously described (32), with minor modifications. Cells were lysed in RIPA buffer with protease inhibitor, phosphatase inhibitor, sodium fluoride, and sodium molybdate. Protein content was measured by BCA assay (Thermo Fisher Scientific) and normalized across samples. Lysates were boiled for 5 minutes, run on a 4-12% Bis-Tris gel, and transferred onto a PVDF membrane (Invitrogen). Primary antibodies used include anti-phospho-myosin light chain 2 (Thr18/Ser19) (1:1,000, Cell Signaling), anti-myosin light chain (1:5,000, Sigma-Aldrich), myosin II heavy chain isoform IIA (1:500,000, Covance), myosin II heavy chain isoform IIB (1:10,000, Covance), myosin II heavy chain isoform IIC (1:10,000, Covance), and anti-glyceraldehyde-3-phosphate dehydrogenase (anti-GAPDH) (1:1,000,000, Sigma Aldrich). Horseradish peroxidase-conjugated secondary antibodies (Invitrogen) and enhanced chemiluminescence reagent (Thermo Fischer Scientific) were used for protein detection on x-ray film or 555 fluorescent secondary antibody (1:1,000, Invitrogen) was used for protein detection using a Typhoon FLA 9500 biomolecular imager (GE Healthcare). Band intensities were quantified using the gel analysis plugin in ImageJ software (NIH) and normalized by GAPDH intensity.

**Atomic force microscopy (AFM) protocol.** AFM indentation measurements were performed as described earlier (32,48) using pyramid-tipped probes (OTR4, Bruker AFM Probes) and fitting force curves with a modified Hertz model. At least 30 cells cultured on glass were measured per condition.
Sphere forming frequency assay and sphere size measurement. Single cells were seeded onto non-adherent 384-well plates (Corning; 10 cells/well; 50µL media/well) using a Multidrop Combi microplate dispenser (Thermo Scientific). After 6-7 days, spheres were stained using Hoescht 33342 (1µg/ml, Sigma Aldrich) and imaged with the ImageXpress Micro cellular imaging system (Molecular Devices) in the CIRM/QB3 Shared Stem Cell Facility. Sphere forming frequency was obtained by dividing the number of observed gliomaspheres in each well by the number of initially plated cells. Sphere sizes were measured using CellProfiler (Broad Institute).

Pharmacologic inhibition of cell motility. Non-muscle myosin-II inhibitor blebbistatin (25µM, Sigma-Aldrich) was added to the cell culture medium after cells were adhered for at least 3 hours.

Live/dead assay. Single cells were incubated with propidium iodide (0.5 mL/sample, BD Biosciences) for 15 min at room temperature as described in the manufacturer instructions. Flow cytometry analysis was conducted to determine percentage of live cells.

Mass spectroscopy quantification of NM II isoform expression. TICs cultured on gels were lysed as described above in Western blot analysis. Lysates with protein concentrations ranging from 0.548-1.306 mg/ml were submitted to the National Heart Lung and Blood Insitute Proteomics Core Facility (Dr. Robert Adelstein, NIH) for analysis by liquid chromatography tandem mass spectroscopy. Peptide numbers for each myosin-II heavy chain isoform were counted (49).

Collagen invasion assay. 1.0 mg/ml collagen I gels (PureCol, Advanced Biomatrix) were assembled in a multiwell plate with 4-5 gliomaspheres implanted per gel. Phase images were taken every 24 hours with an inverted Nikon TE2000-E2 microscope.
Transwell migration assay. Transwell inserts with 3 µm pores (Fluoroblok, Fischer Scientific) were coated with laminin (10 mg/ml, Invitrogen), and a 2x gradient of soluble EGF was used to drive chemotaxis through the pores. Cells were allowed to migrate across the membrane for 24 hours before fixation with 4% paraformaldehyde (Electron Microscopy Sciences). Cell nuclei were fluorescently labeled with propidium iodide and cells adhered to the bottom surface of the membrane were counted using epifluorescence microscopy. At least 3 complete membranes were counted for analysis.

Immunohistochemistry. Mice brains were prepared for sectioning using paraffin, and haematoxylin and eosin staining was used to visualize tissue following standard protocols. Images were taken using an inverted Zeiss Axio microscope with a color camera (AxioCam ERc 5s, Zeiss).

Tumor occupancy measurement. Human GBM TICs were identified using an anti-human Nestin antibody (1:500, Millipore). Immunocomplexes were visualized in 3,3'-diaminobenzidine using the ABC-Elite peroxidase method (Vector Laboratories). Counterstaining of the nuclei was performed using hematoxylin. Tumor occupancy was estimated using the ImageJ (NIH) via calculation of the percent area occupied by the Nestin immunoreactive cells with the implanted brain.
Additional References


