Supplementary information for:

Negative durotaxis: cell movement toward softer environments

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Isomursu et al., Figure S1.

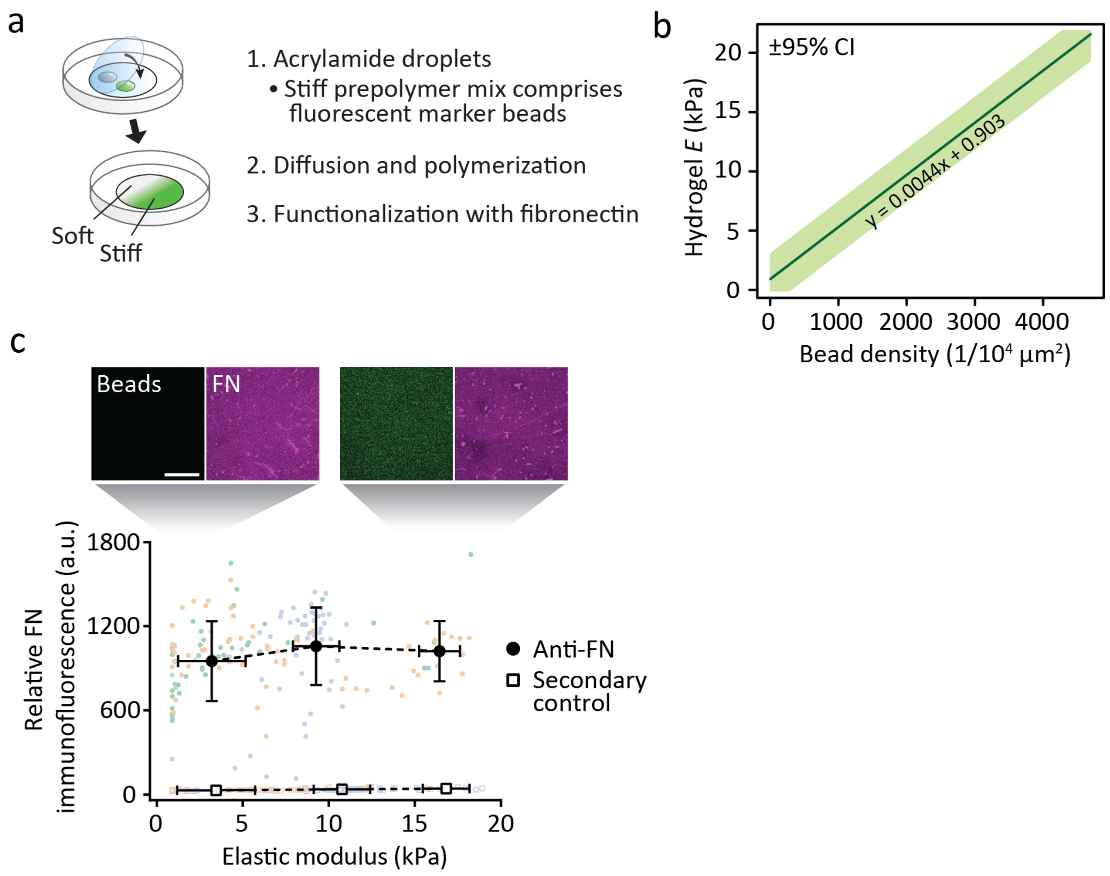

Figure S1. Preparation of diffusion-based stiffness gradient hydrogels. (a) Schematic representation of stiffness gradient preparation. Two acrylamide solutions are polymerized and mixed together on a glass bottom dish to create a continuous gradient in the range of 0.5–22 kPa. The hydrogel is activated and functionalized with fibronectin before use. (b) Calibration curve connecting fluorescent marker bead density, measured using a confocal microscope, to the elastic modulus of the hydrogel. Adapted from Ref.[31] (c) Fibronectin density across the stiffness gradient hydrogels, measured via immunofluorescence. Green, orange and blue colors denote measurements from three individual experiments, overlaid with binned data. Squares depict measurements from secondary controls, stained by omitting the anti-fibronectin antibody. Mean ± SD of n = 19–86 (anti-FN) or 9–42 (control) regions of interest (ROIs).

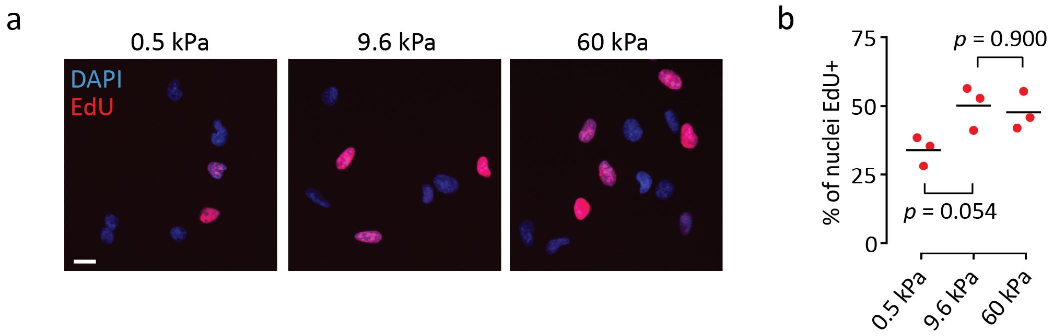

Figure S2. Mechanosensitivity of U-251MG proliferation. (a–b) Fluorescence images (a) and quantification (b) depicting EdU incorporation by U-251MG cells on 0.5–60 kPa substrates. Scale bar, 20 µm. Mean values from three independent experiments. Analyzed by one-way ANOVA and Sidak's *post hoc* test.

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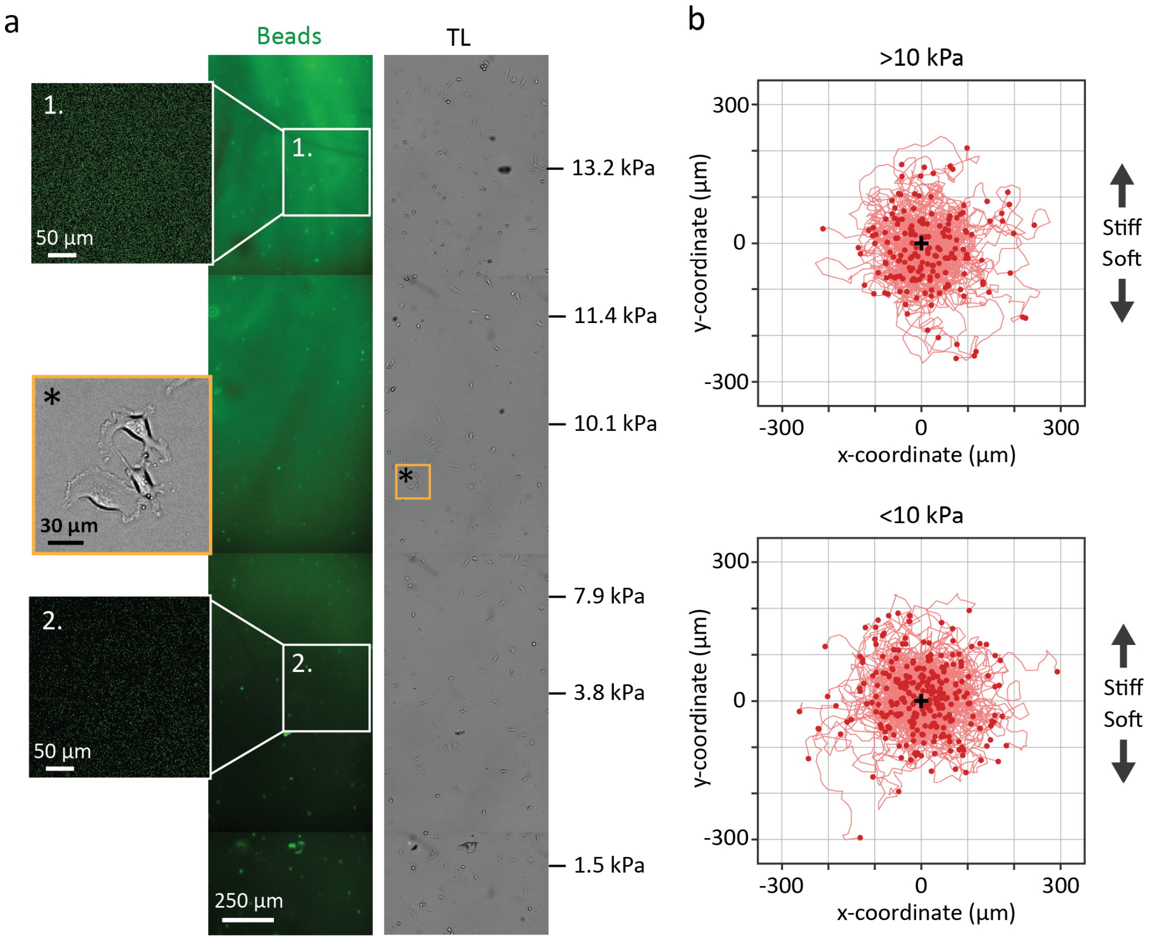

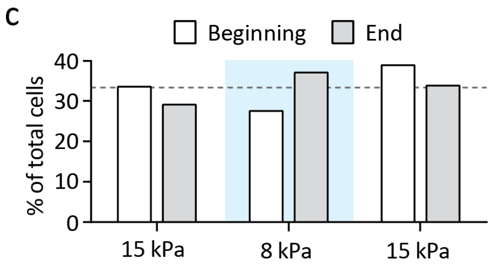

d

Time point	Location (n,%)			*p*-value (Chi-squared)
	Stiff, left	Soft	Stiff, right	
Beginning	319 (33.5)	262 (27.5)	371 (39.0)	
End	921 (29.1)	1176 (37.1)	1070 (33.8)	**< 0.0001**

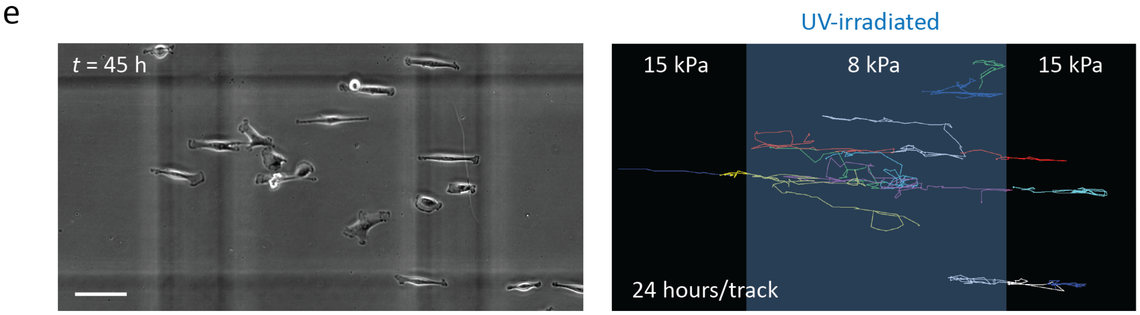

Figure S3. Tracking individual U-251MG cells on stiffness gradients. (a) Representative fluorescence image of a stiffness gradient hydrogel (left) and live U-251MG cells adhering to the substrate (right). (Insets 1, 2) Example confocal images of fluorescent beads, acquired as described in Ref.[31] The images correspond to the indicated gradient regions and were used for calculating the substrate elastic moduli. (*) Close up of the cells. (b) Tracks from individual U-251MG cells migrating on the stiffer (>10 kPa, top) and softer (<10 kPa, bottom) regions of a 0.5–22 kPa stiffness gradient for 10 hours. The tracks correspond to the data in Fig. 1d and the origo (0, 0) is highlighted by a black (+). n = 174–264 cells per condition, from three independent experiments. (c–d) Total number of cells in the different gradient regions in Fig. 1f–g. (c) Bar graph, n = 952–3,167 cells per time point, from two individual experiments. (d) Contingency table summarizing the data, analyzed by chi-squared test. (e) Endpoints (left) and 24-hour tracks (right) depicting the migration of individual cells on the photoresponsive stiffness gradients. Scale bar, 100 µm.

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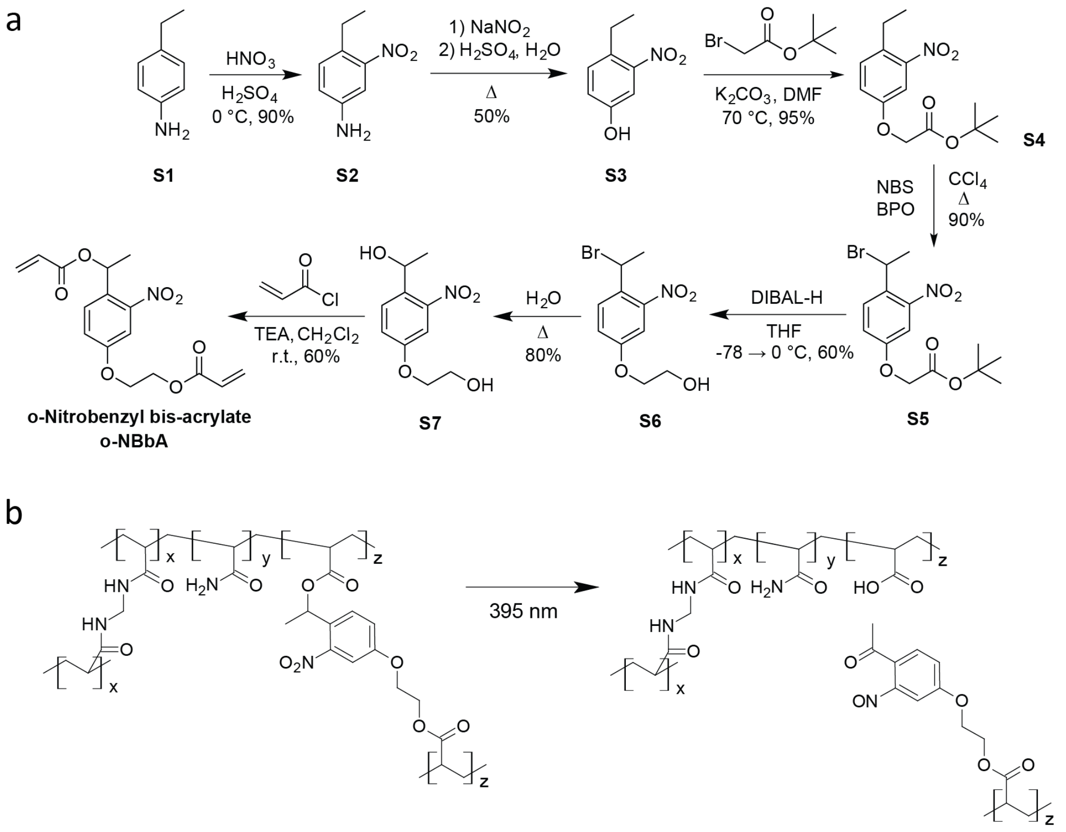

Figure S4. Synthesis and photochemistry of o-nitrobenzyl bis-acrylate. (a) Schematic of the synthesis of o-NBbA. (b) Copolymerization of o-NBbA with acrylamide and bis-acrylamide yields hydrogels composed of strands of polyacrylamide crosslinked by either o-NBbA or bis-acrylamide. UV irradiation cleaves the photolabile o-NBbA, resulting in gels with lower crosslinking density and hence lower stiffness. The process does not release any byproducts to the gel environment.

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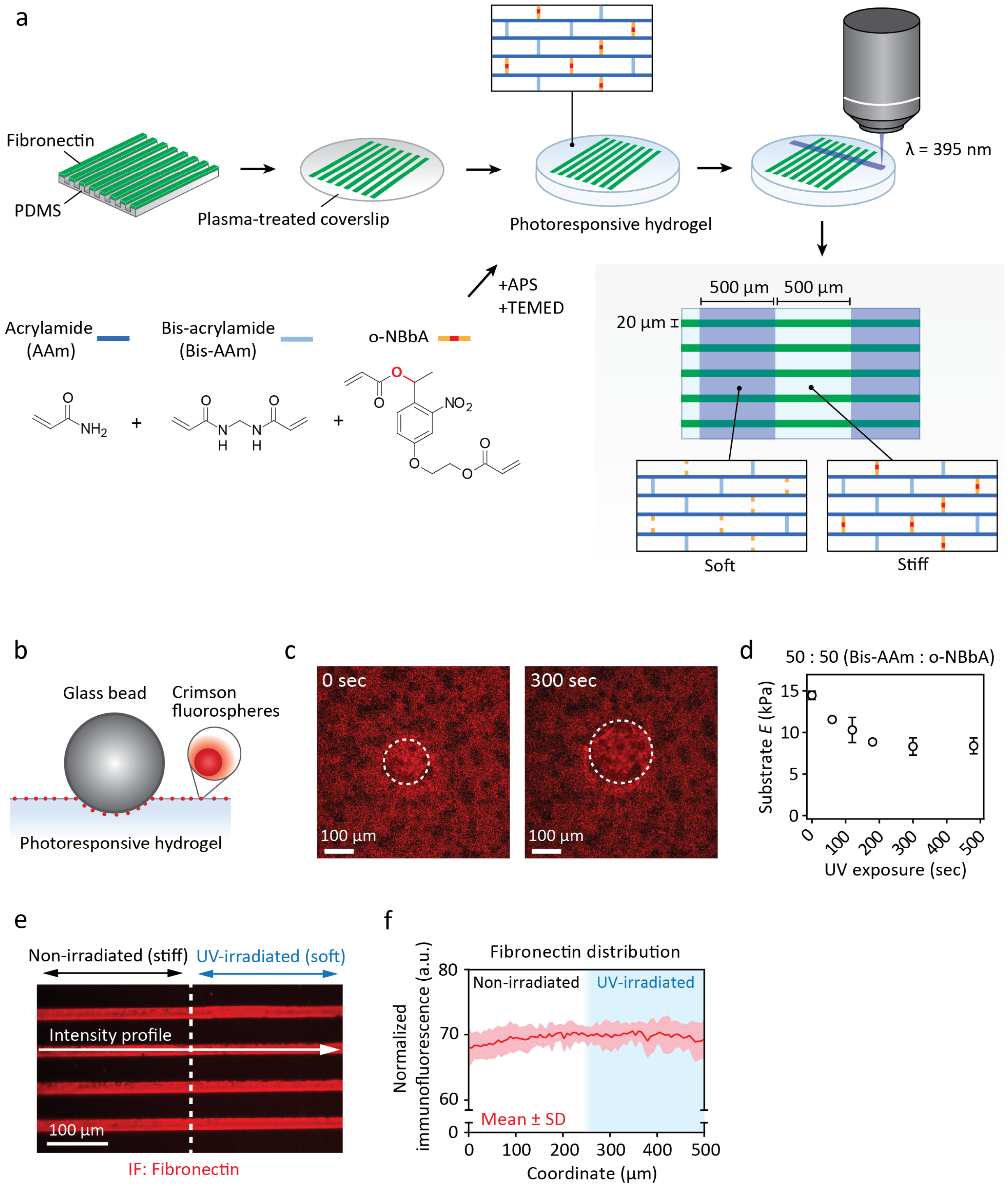

Isomursu et al., Figure S5.

Figure S5. Preparation and characterization of photoresponsive hydrogels. (a) Schematic representation of stiffness gradient preparation for migration experiments. The photocleavable carbon-oxygen bond in o-NBbA is indicated by red color. (**b**–d) Stiffness characterization by bead indentation. A schematic representation of the technique (b), representative fluorescence images (c) and quantified results (d) depicting hydrogel elasticity as a function of UV exposure. Dashed lines highlight indented, out-of-focus areas in the gel. Mean ± SD of n = 3 measurements. (**e**–f) Validation of the microcontact printed fibronectin patterns. Immunofluorescence image (e) and quantification (f) showing fibronectin distribution on non-irradiated and UV-exposed regions of the hydrogel. Mean ± SD of n = 24 intensity profiles.

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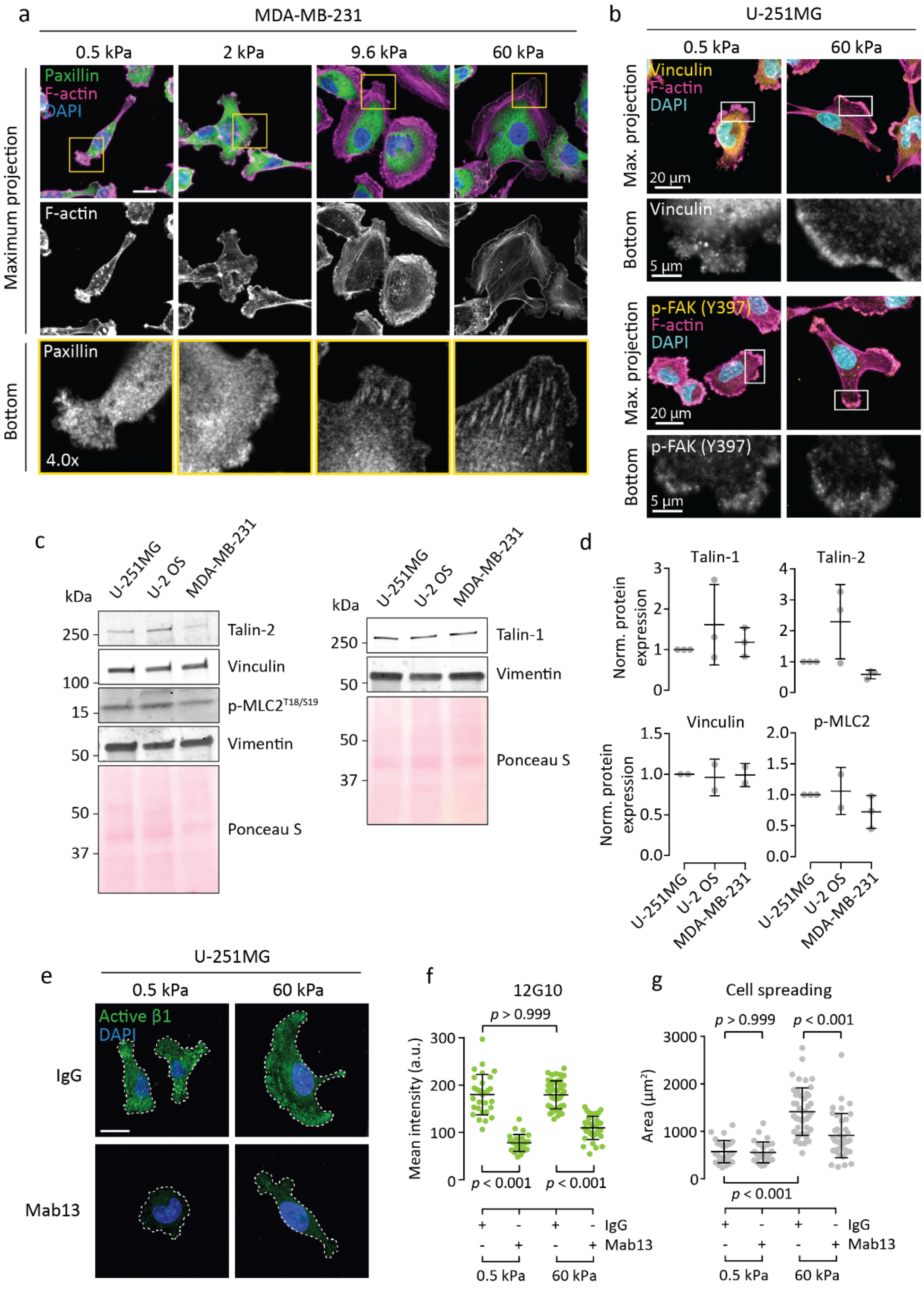

Figure S6. Focal adhesion maturation and adhesion components in U-251MG and other cancer cells. (a) Immunofluorescence images of paxillin and F-actin in MDA-MB-231 cells on 0.5–60 kPa substrates. The bottom panels show individual focal planes from confocal stacks, corresponding to the basal side of each cell. Scale bar, 20 µm. (b) Immunofluorescence images of vinculin (top), p-FAK (bottom) and F-actin in U-251MG cells on 0.5 and 60 kPa substrates. The bottom panels show individual focal planes from confocal stacks, corresponding to the basal side of each cell. (**c**–d) Representative western blots (c) and quantification (d) depicting talin-1/2, vinculin and p-MLC2 levels across three different cell lines. Densitometric measurements were normalized to vimentin, mean ± SD of 2–3 independent experiments. (**e**–f) Immunofluorescence images (e) and quantification (f) showing active β1-integrin (clone 12G10) in U-251MGs on 0.5 and 60 kPa substrates. The cells were treated with a control antibody (normal rat IgG) or β1 function-blocking Mab13 for two hours before fixation. Scale bar, 20 µm. Mean ± SD of n = 27–45 cells, analyzed by Kruskal-Wallis one-way ANOVA and Dunn's *post hoc* test. Representative of two independent experiments. (g) Spreading of U-251MGs on 0.5 and 60 kPa substrates, without or after β1-integrin blocking by Mab13. Mean ± SD of n = 27–45 cells, analyzed by Kruskal-Wallis one-way ANOVA and Dunn's *post hoc* test. Representative of two independent experiments.

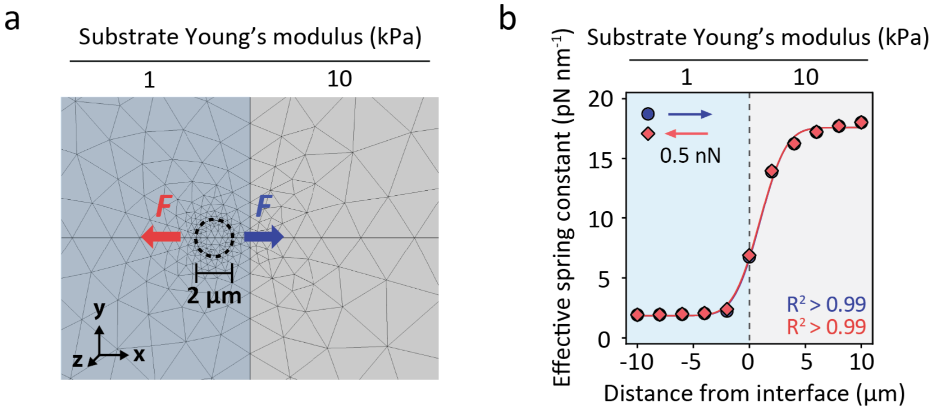

Figure S7. Finite element analysis of polyacrylamide displacement next to a stepwise elastic gradient. (a) COMSOL Multiphysics® model setup (b) The effect of steep elastic gradients on the effective spring constant of polyacrylamide. A lateral 0.5 nN force was exerted on the substrate through a circular adhesion zone (r = 1 µm) as shown in (a). The position of the adhesion zone was adjusted repeatedly at 2 µm steps. The direction of the force was varied by 180° but was always parallel to the gradient. In both cases, normal cumulative distribution function was a good fit to the data.

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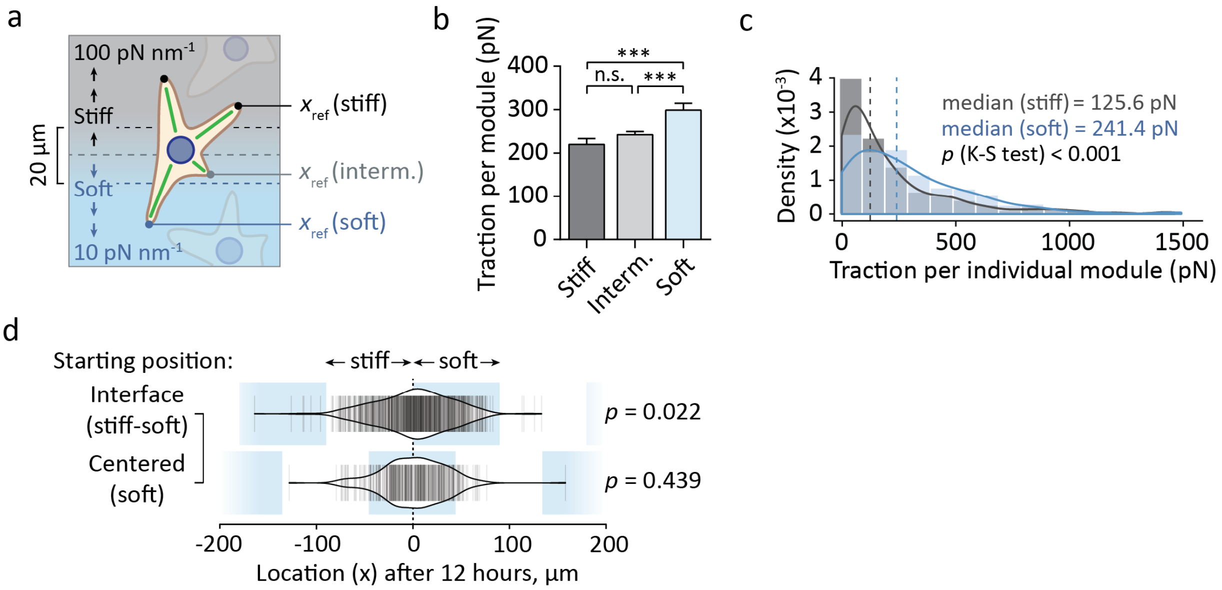

Figure S8. CMS produces asymmetric traction forces in cells that interact with stiffness gradients. (a) When individual cells were on top of a stiffness gradient during the simulations in Fig. 3b–f, their traction forces were recorded. (b–c) Forces exerted by clutch modules on stiff, intermediate and soft substrate, while the cell body is located on a stiffness gradient. (b) Bar graphs depicting mean ± SEM of n = 292–1380 modules. $^{***}p < 0.001$, n.s. = not significant, Kruskal-Wallis one-way ANOVA and Dunn's *post hoc* test. (c) Histograms overlaid with probability density functions, dashed lines indicate medians. n = 292–365 modules, analyzed by Kolmogorov-Smirnov test. (d) Violin plots of accumulated distance migrated by individual cells along the orientation of the gradient and over 12 hours, starting from a gradient (top) or from the middle of a compliant region (bottom). n = 326–759 cells, analyzed by sign test.

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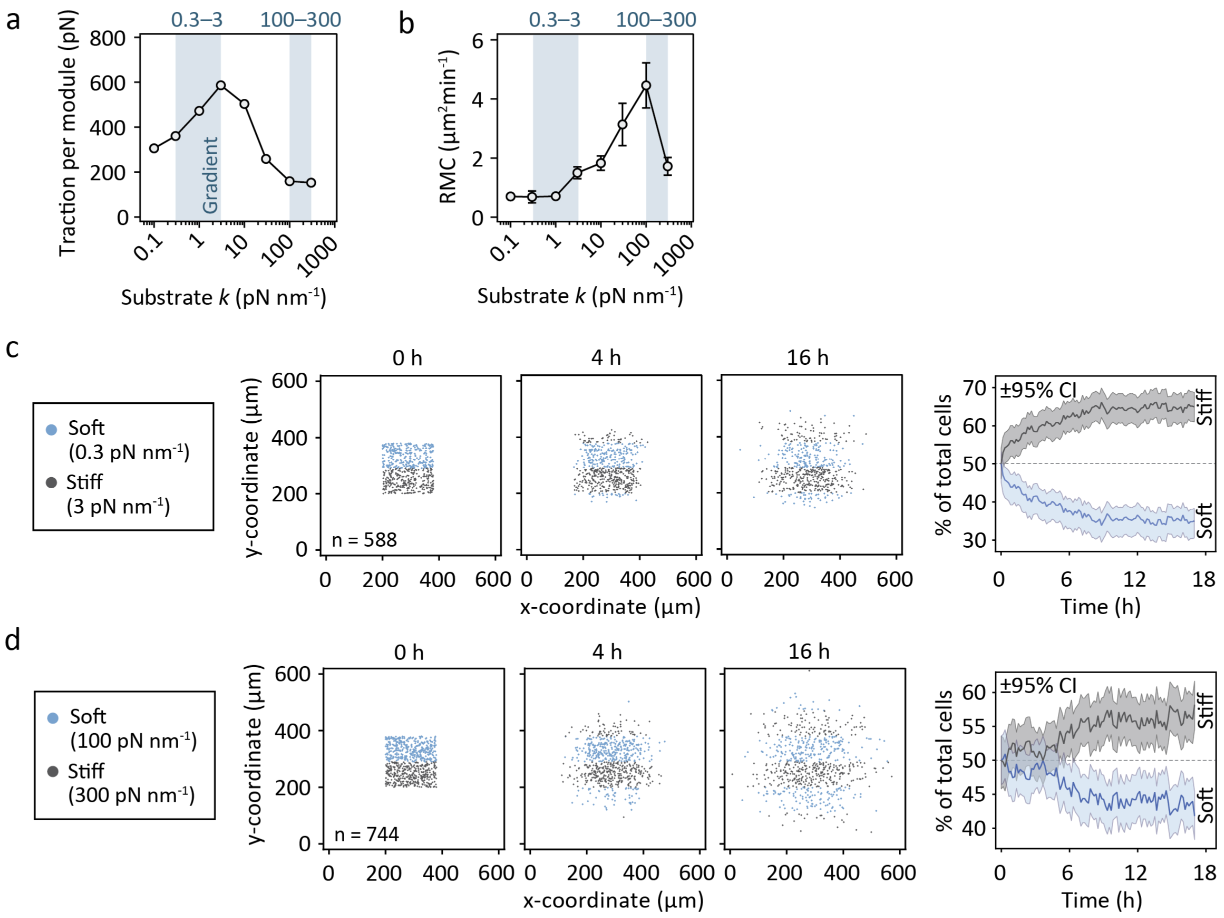

Figure S9. Modifying the range of the stiffness gradient can reverse durotaxis *in silico*. (**a**–b) Module traction forces (a) and RMC (b) of the simulated cells as a function of substrate stiffness, as in Fig. 3c–d. Overlays highlight the ranges of the 0.3–3 pN nm^{-1} and 100–300 pN nm^{-1} gradients in (c–d). Mean ± SEM of n = 10 cells. (**c**–d) Evolution of cell density on mechanically heterogeneous substrates over time. (c) Coordinates of individual cells on the 0.3–3 pN nm^{-1} gradient 0, 4 and 16 hours into the simulation (left) and the fraction of cells residing in the stiffer and softer areas over the course of the simulation (right). ±95% CI, n = 588 cells. (d) As above, but for the 100–300 pN nm^{-1} gradient. n = 744 cells.

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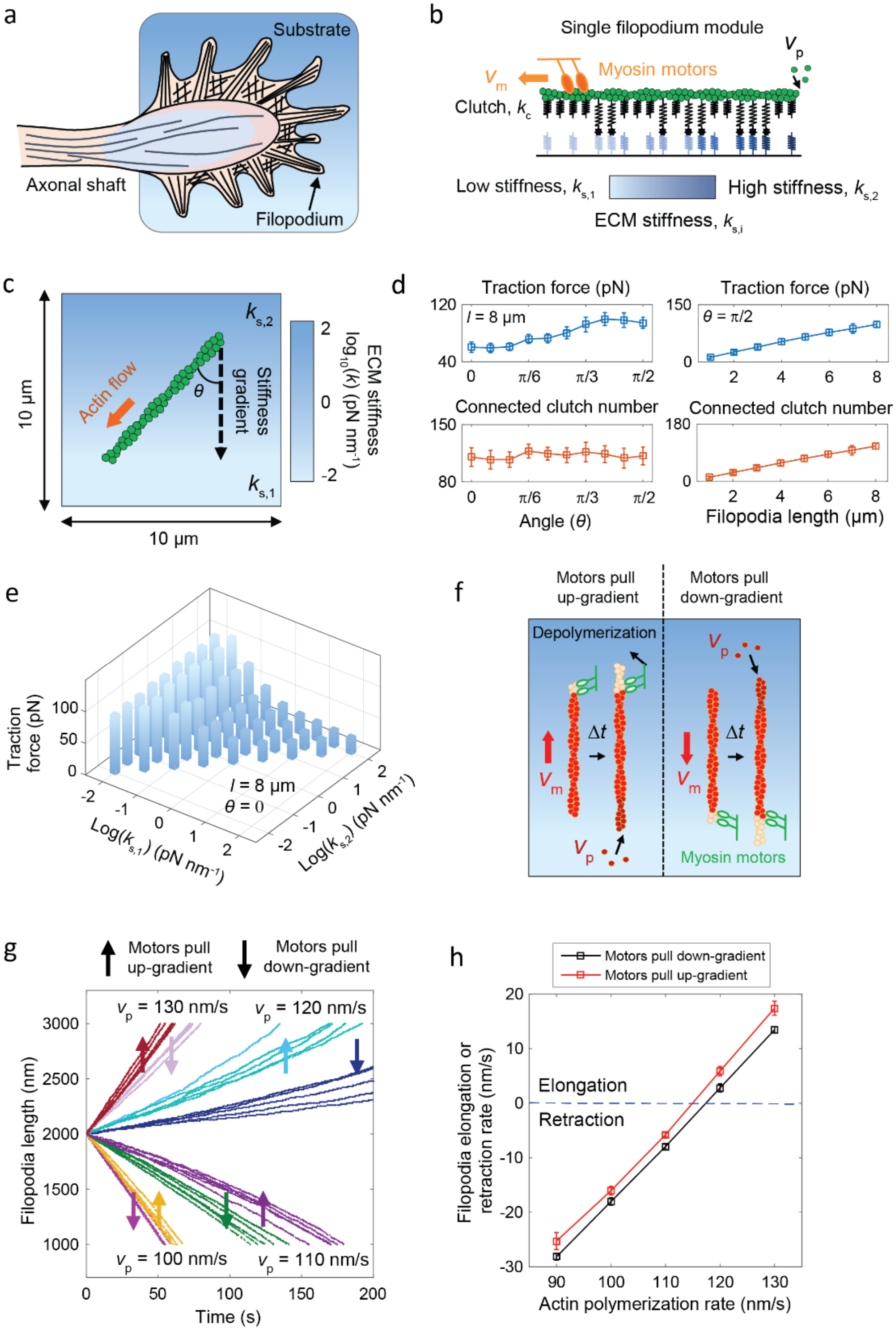

Figure S10. Motor-clutch model of filopodial dynamics. (a) Schematic representation of a neuronal GC. Filopodia, surrounded by a less polarized actin network, reside in the peripheral domain. They are separated from the axon by a thin transitional domain, and a central domain (light blue) that is primarily composed of microtubules. (b) The filopodia in GCs are modeled as individual motor-clutch modules, with adhesion springs (homogeneous stiffness), substrate springs (heterogeneous stiffness) and inward actin flow resulting from active myosin motors. Actin monomers are added into the filaments at a constant rate. (c) Setup used in the single-filopodium simulations. The filopodium interacts with the substrate in a set orientation relative to the linear stiffness gradient. (d) (Left) Traction force exerted by the filopodium increases when the protrusion is pointing down the gradient, toward softer substrate. (Right) Perpendicular to the gradient, traction increases with filopodia length mainly due to more clutches being available to bind with the substrate. Data shown are from *n* = 10 independent simulations. (e) Average traction exerted by a single filopodium on different substrate stiffness gradients. Data represent means of *n* = 10 simulations. (f) Filopodia length is affected by both actin flow, v_m, and the polymerization rate, v_p. Depending on the orientation of the filopodium, the actin may flow toward soft (filopodium pointing up the gradient) or stiff (filopodium pointing down the gradient) substrate. (g) Evolution of filopodia length on stiffness gradients upon different actin polymerization rates. The different combinations of v_p and filopodia orientation are color-coded, while each line represents the temporal variation in the length of a single filopodium. (h) Effect of actin polymerization and orientation relative to a stiffness gradient on the filopodia elongation/retraction rate. Mean ± SEM in (d) and (h).

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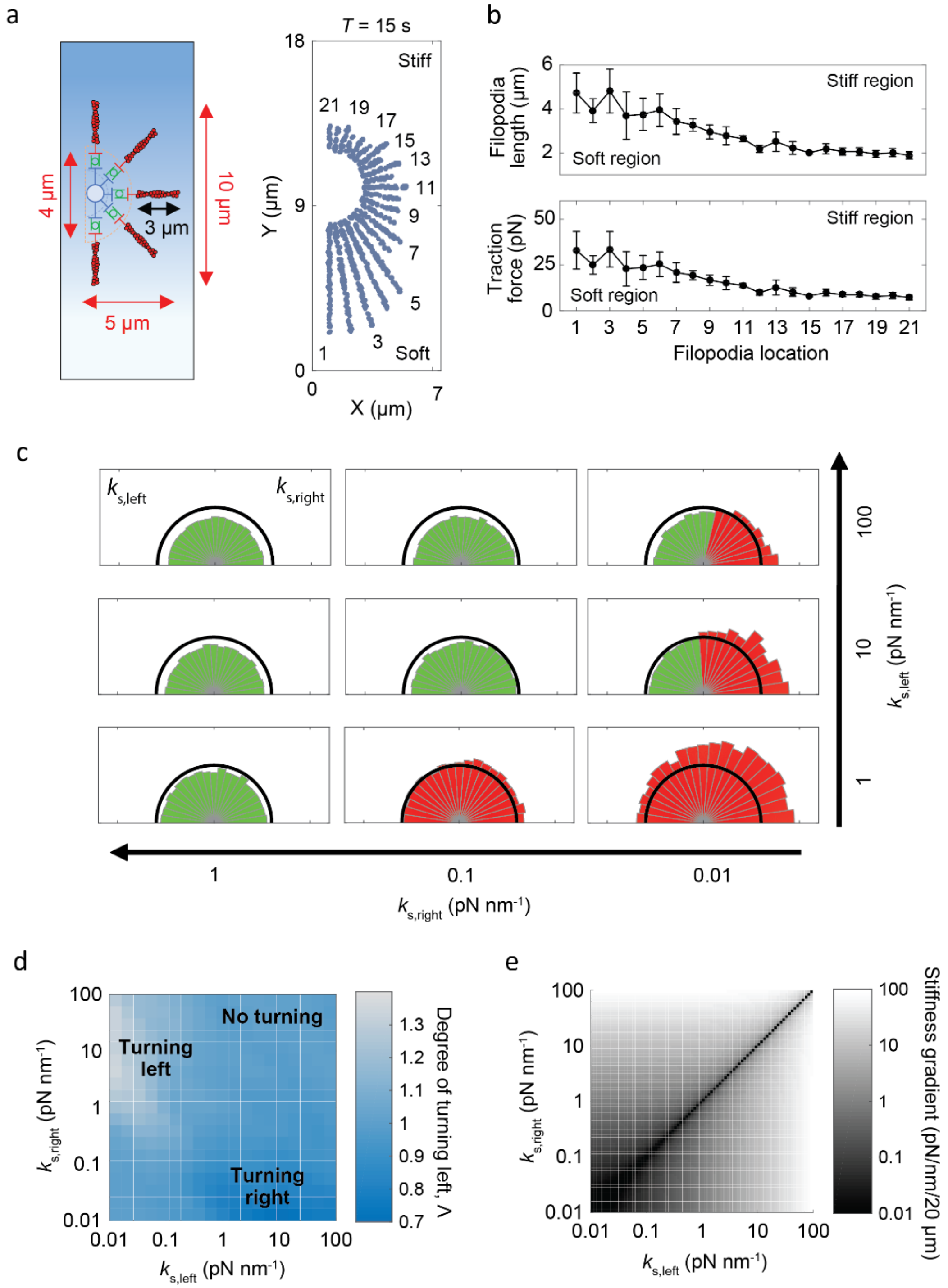

Figure S11. Motor-clutch model predicts growth cone steering toward soft matrix. (a) Schematic representation of the GC model. (Left) Dimensions of a newly initialized GC. (Right) Each GC consists of multiple filopodia, distributed between $-\pi/2$ and $\pi/2$ relative to the axon. On stiffness gradients ($k_{s,1}$ = 0.01 pN nm^{-1}, $k_{s,2}$ = 100 pN nm^{-1}), filopodia on the more compliant side of the substrate rapidly outgrow the others, leading to effective turning of the GC. (b) Filopodia length (top) and traction (bottom) based on their orientation around the GC central domain. On stiffness gradients, filopodia pointing toward the softer substrate elongate faster and generate more traction. Data shown are from n = 10 independent simulations. (c) Examples of GC behavior on different stiffness gradients. Green denotes filopodia that are retracting during the course of the simulation, red denotes filopodia that are elongating. Depending on the gradient, individual GCs may retract or enlarge isotropically, or steer toward the softer substrate. Displayed are means of n = 10 simulations. (d) Phase diagram of GC turning to left, Λ, on different mechanically graded substrates. (e) Phase diagram depicting the strength of the stiffness gradient for varying $k_{s,1}$ and $k_{s,2}$. Gradient strength alone cannot explain the magnitude of Λ, if the whole substrate is stiffer than the optimal range for individual filopodia (Fig. S7e).

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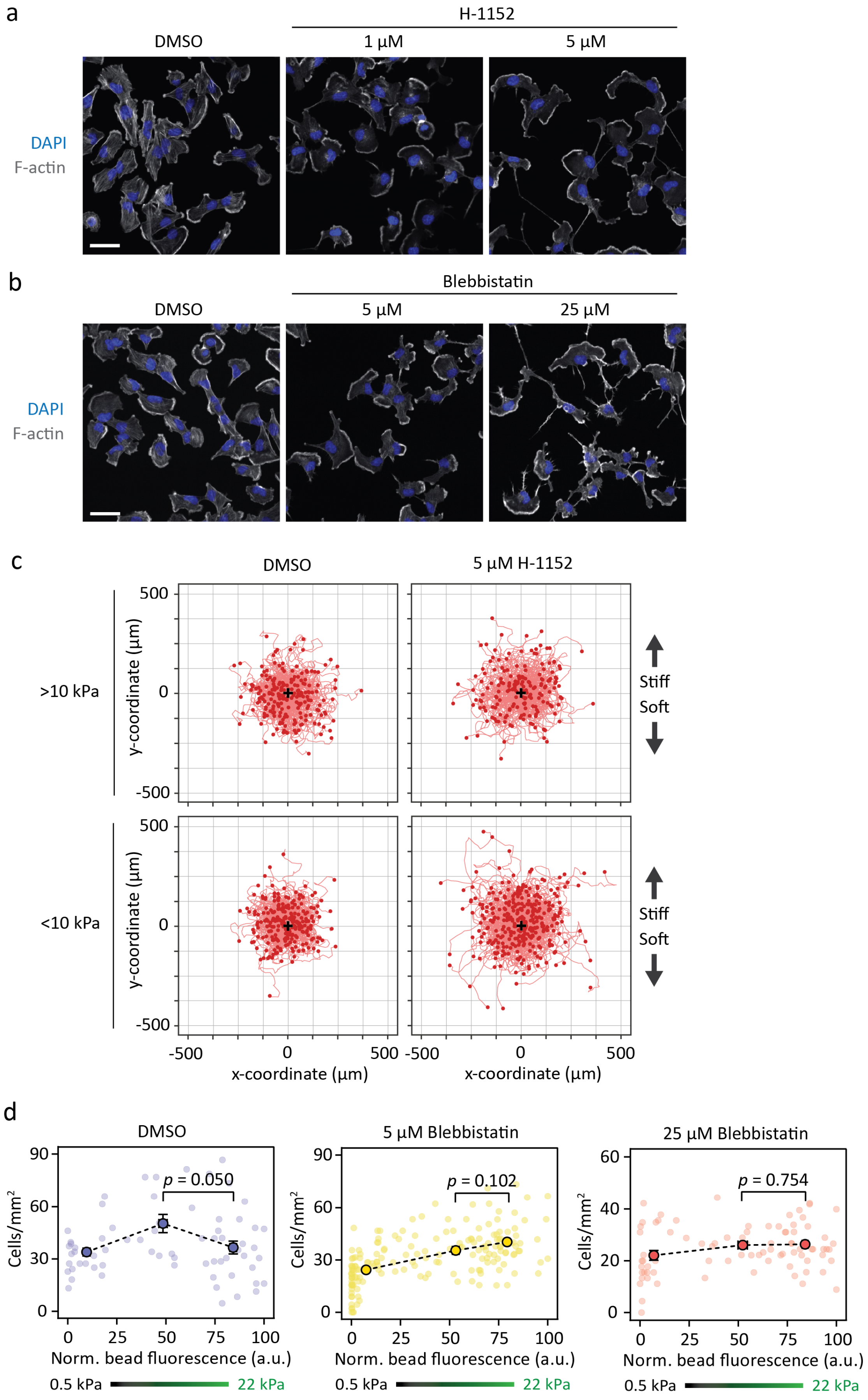

Isomursu et al., Figure S12.

Figure S12. Morphology and migration of U-251MG cells during ROCK and myosin II inhibition. (**a–**b) Validation of different actomyosin-targeting compounds using U-251MG cells. Fluorescence images of the actin cytoskeleton after treatment with intermediate and high doses of ROCK inhibitor H-1152 (a) or myosin II inhibitor blebbistatin (b) for two hours. Scale bar, 50 µm. (c) Tracks from individual U-251MG cells migrating on the stiffer (>10 kPa, top) and softer (<10 kPa, bottom) regions of a 0.5–22 kPa stiffness gradient for 10 hours, treated with 5 µM H-1152 or vehicle (DMSO). The tracks correspond to the data in Fig. 4g and each origo (0, 0) is highlighted by a black (+). n = 177–327 cells per condition, from one (DMSO) to two (H-1152) independent experiments. (d) Cell densities in different parts of 0.5–22 kPa gradients, 48 hours after being seeded and supplemented with varying concentrations of blebbistatin. Mean ± SEM of n = 13–85 ROIs per bin, from two gradient hydrogels per condition, representative of two independent experiments. Analyzed by Mann-Whitney test.

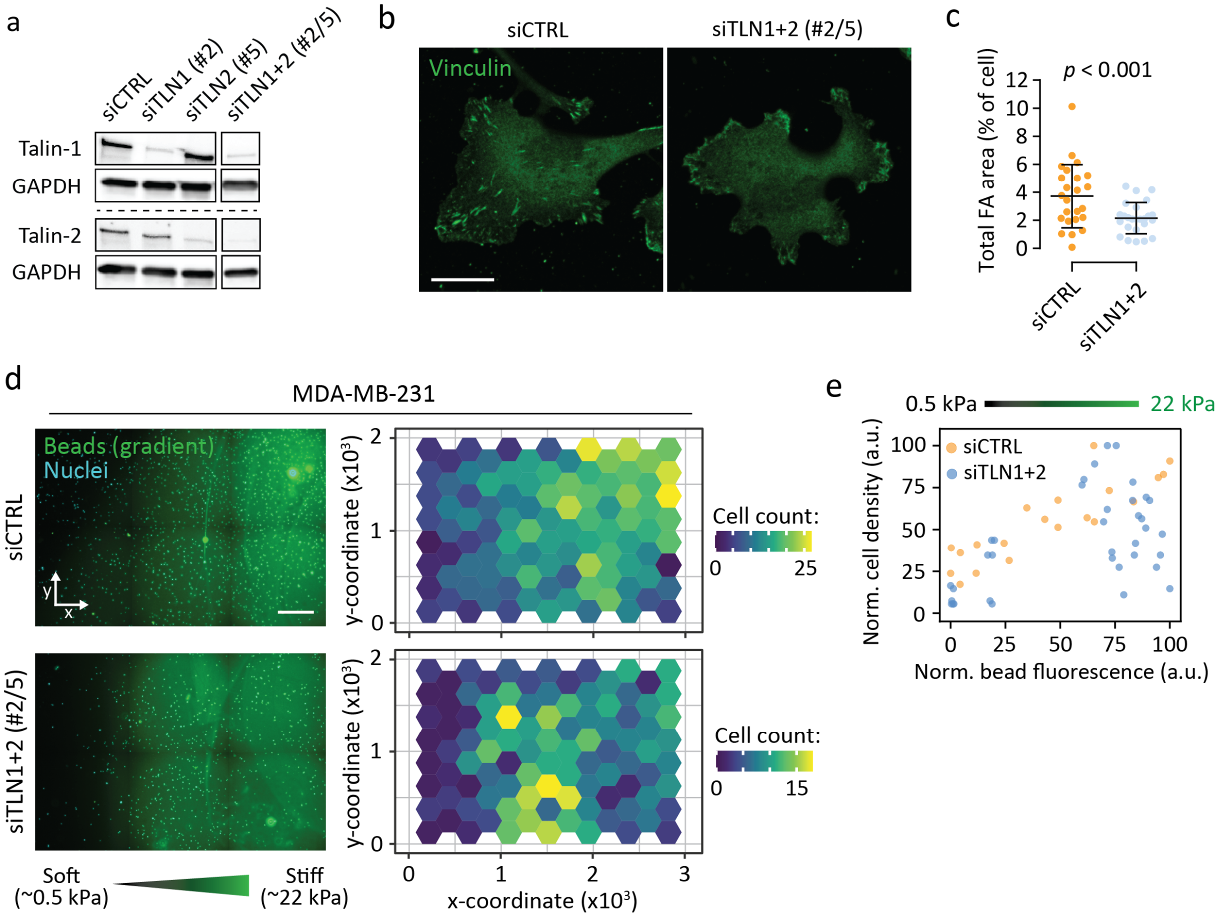

Figure S13. Additional talin-1/2-targeting siRNAs recapitulate the loss of mature adhesions and promote a switch from positive to negative durotaxis in MDA-MB-231 cells. (a) Representative western blot depicting talin-1 and talin-2 knockdown in MDA-MB-231 cells using siRNA oligos that are different from the ones used in Fig. 5. The fourth band on each row, depicting a double knockdown, was cropped from a different site in the same membrane. (**b**–c) Immunofluorescence images (b) and quantification (c) of vinculin-positive focal adhesions in MDA-MB-231s on 60 kPa substrate, without and after talin knockdown. Scale bar, 20 µm. Mean ± SD of n = 24–25 cells, analyzed by Mann-Whitney test. (d) (Left) Representative regions of two 0.5–22 kPa polyacrylamide stiffness gradients, 72 hours after being seeded with MDA-MB-231 cells. Scale bar, 500 µm. (Right) Quantification of cells across the gradients. (e) Relative MDA-MB-231 cell densities in different parts of the stiffness gradients. n = 20–36 ROIs, representative of two independent experiments.

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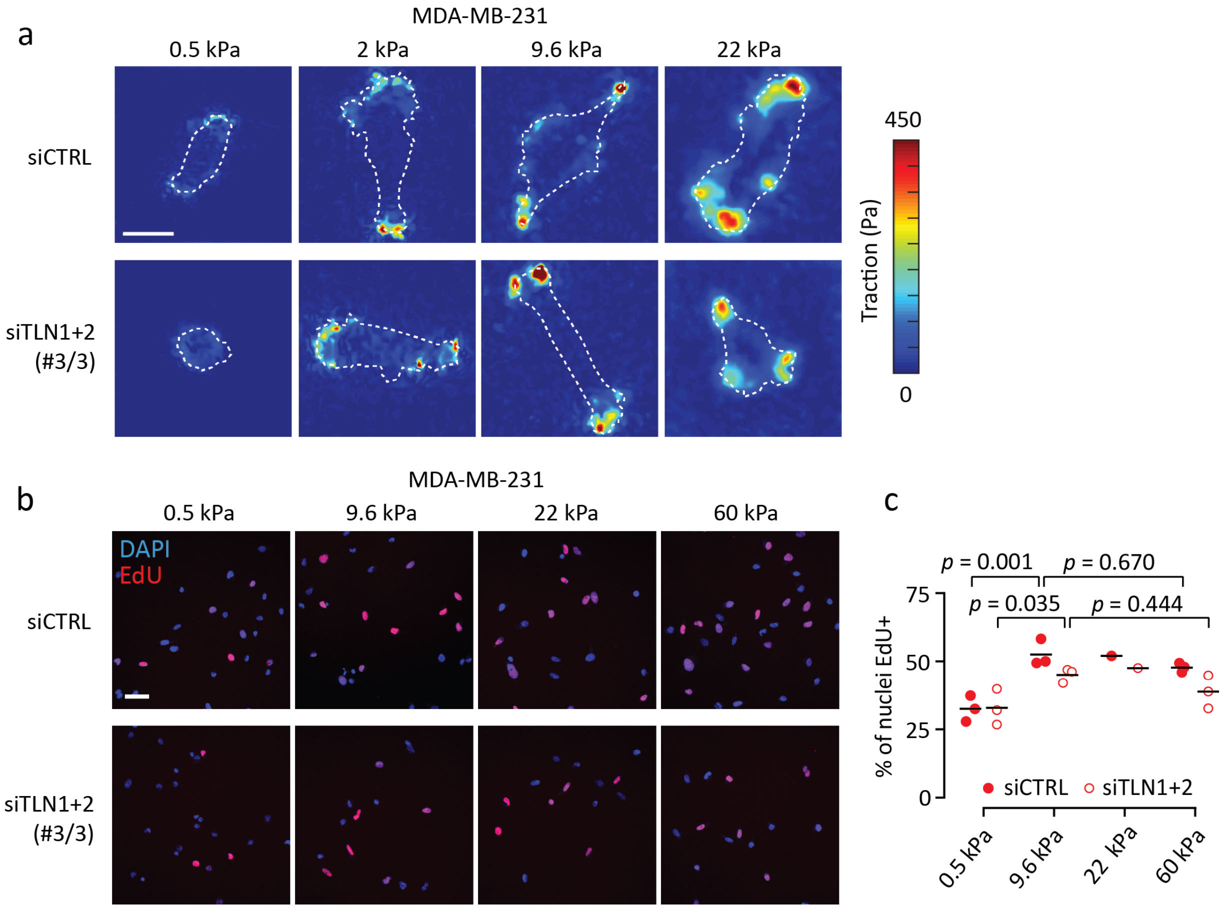

Figure S14. Mechanosensitive traction and proliferation of MDA-MB-231 cells. (a) Representative traction maps from MDA-MB-231 cells on 0.5–22 kPa substrates, corresponding to the data in Fig. 5f. Cell outlines are indicated by white dashed lines. Scale bar, 20 µm. (b–c) Fluorescence images (a) and quantification (b) depicting EdU incorporation by control and talin-low MDA-MB-231 cells on 0.5–60 kPa substrates. Scale bar, 50 µm. Mean values from one to three independent experiments. Analyzed by one-way ANOVA and Sidak's *post hoc* test.

Isomursu et al., Figure S15.

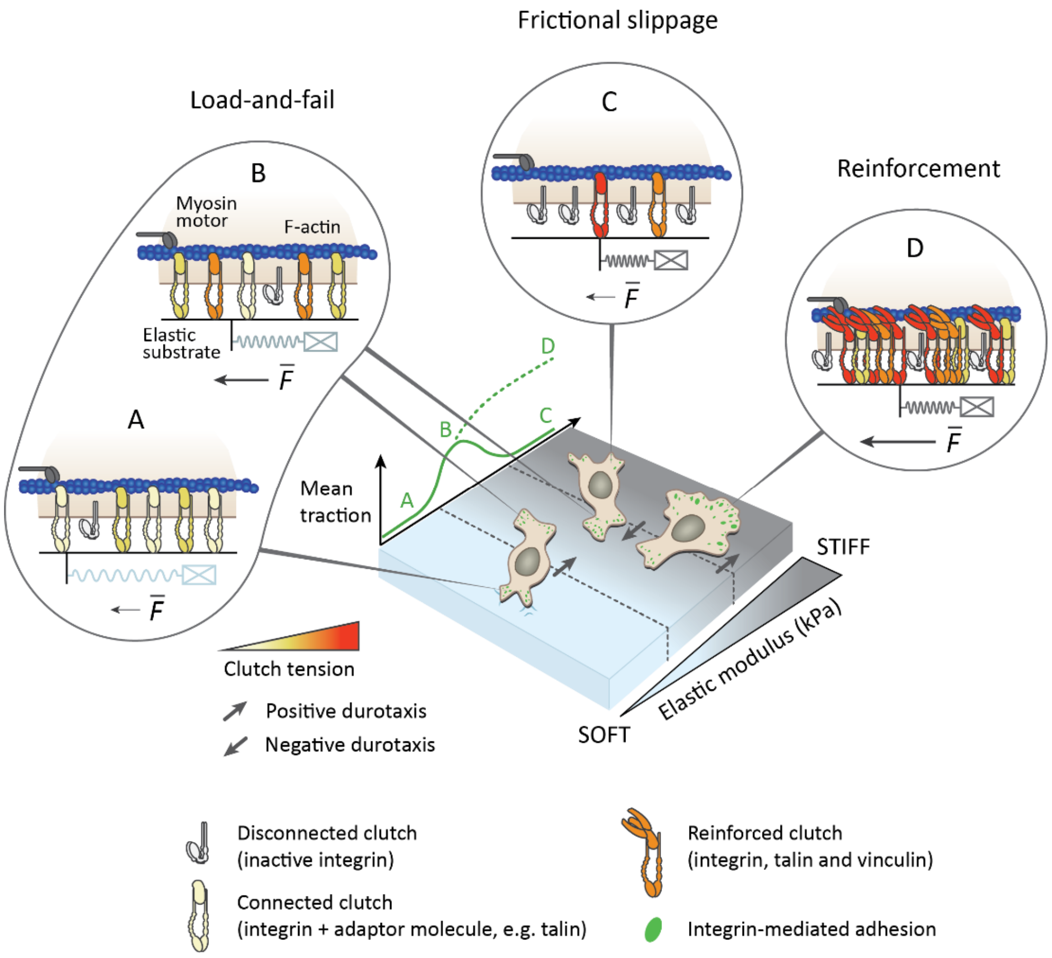

Figure S15. Schematic representation of the regulation of positive and negative durotaxis by motor-clutch dynamics. Cell-intrinsic molecular machinery dictates the cell's capacity to exert force on mechanically heterogeneous substrates, driving positive or negative durotaxis. Without clutch reinforcement (mechanosensitive FA formation, D), the motor-clutch model predicts a biphasic relationship between traction force and substrate stiffness (A–C)[18,19,26–30]. This fundamental relationship, and the physical reinforcement of cell-matrix adhesion by FAs, are likely to be further influenced by biochemical signaling pathways and feedback loops that modulate the expression, activity and localization of individual cytoskeletal and clutch components, in a cell type-dependent manner.

Supplementary Text 1: Chemistry of o-NBbA and photoresponsive polyacrylamide hydrogels

Polyacrylamide was selected as the base material for the stiffness gradients used in this study, as it is the most widely employed model system for investigating the role of substrate stiffness in directing cell behavior. This is partly due to the ease of obtaining elastic moduli in a wide, physiologically relevant range[51,52]. While other types of gels (e.g. collagen or hyaluronic acid) are known to interact directly with cell surface receptors, including integrins, polyacrylamide gels are inert to such interactions. This allows more control over the types and densities of ligands that will be presented to the cells, making the material ideal for mechanobiological studies. Various methods have been developed to fabricate stiffness gradients in gels to study the durotactic behavior of cells. Some examples exploit the diffusion of two prepolymer solutions[13,31], tilted-superposition of two hydrogels[53], freeze-thaw-induced crosslinking of polyvinyl alcohol[54], or toehold-mediated strand displacement of DNA[55]. Aiming for high-resolution spatiotemporal control over the mechanical properties of the gel, we chose light as the external stimulus[56–60]. Therefore, we aimed to design and synthesize a new, minimalistic and photocleavable crosslinker that contains acrylate moieties.

Among various photolabile functionalities that are available, o-nitrobenzyl (o-NB) was chosen due to its high one-photon photolysis efficiency and high deprotection yields[61,62]. o-NB based compounds have been used widely in hydrogel-based studies to achieve controlled release or immobilization of payloads[63–67], photodegradation of gels[68–70], or modulation of gel stiffness[58,71]. However, many of these studies have focused on polyethylene glycol-based gels rather than polyacrylamide, or complete degradation of the gel rather than controlling the Young's modulus. To our knowledge, there has been only one report to date where o-NB-based crosslinkers have been used to fabricate photoresponsive polyacrylamide hydrogels[72]. While the study demonstrates the feasibility of o-NB based crosslinking, the method itself requires multiple steps to crosslink chains of polyacrylamide through the o-NB moiety, which made its application here unwieldy.

In this study, a simple one-step synthesis of photoresponsive polyacrylamide gels was enabled by the functionalization of an o-NB group with two acrylate moieties to yield a crosslinker that would cleave upon photolysis. The photocleavable crosslinker, o-nitrobenzyl bis-acrylate (o-NBbA), was synthesized in seven steps from p-ethyl aniline (Fig. S4a) and designed so that its cleavage would not release any byproducts in the medium (Fig. S4b). Based on a previously reported polyacrylamide recipe[46], a photoresponsive gel with an initial stiffness of 20 kPa that can be reduced down to 10 kPa was designed by replacing 50 mol % of bis-acrylamide with o-NBbA. The resulting gel exhibited a Young's modulus of ~15 kPa that was reduced down to ~8 kPa after complete cleavage of the o-NBbA crosslinker by exposure to 395 nm light for 5 min (Fig.

S5b–d). The slight discrepancy between the expected and measured substrate stiffness could be due to the relatively low water solubility and partial phase separation of o-NBbA in the prepolymer solution, which would result in softening of the hydrogel post-polymerization[73].

The light source used for the photocleavage was an LED from a SpectraX light engine instrument installed in a Nikon TiE microscope, originally intended for epifluorescence imaging. This method had several advantages: spatial control can be achieved easily, as the location of the substrate can be precisely chosen via phase-contrast imaging and the area of irradiation can be controlled with the field diaphragm and objectives. For instance, the diameter of the LED-irradiated area could be adjusted to as low as 59 µm using a 40x objective with a nearly closed field diaphragm, or as high as 978 µm under a 10x objective with a fully opened field diaphragm. Stiffness patterns could also be created using the ‘time lapse movie’ function of the NIS-Elements software (Nikon). Here, alternating stiffness gradients were created by initiating a time-lapse movie between two regions of the gel, a method that could be modified to yield more complex 1D patterns or even 2D shapes. Although not explored here, temporal control would be equally possible: for example, stiffness gradients could be introduced in gels at various time points during live cell culture, while simultaneously observing cellular behavior and responses.

To conjugate fibronectin to the surface of the gel via covalent interaction, acrylic acid N-hydroxysuccinimide (NHS) ester was used as the tethering agent. While two methods, addition of acrylic acid NHS ester in the pregel solution followed by stamping of fibronectin, or stamping of the pregel solution with fibronectin preincubated with acrylic acid NHS ester, both produced fibronectin-patterned hydrogels, the former was chosen since it yielded more consistent results. Once the gel had been fabricated, any remaining NHS ester moieties in the gel were passivated with bovine serum albumin (BSA) in PBS to prevent any non-specific interactions between the gel and cells.

Supplementary Text 2: Implementation of the cell migration simulator using mechanically heterogeneous substrates

To establish whether our observations of negative (and positive) durotaxis could be explained through a single set of principles, namely the motor-clutch dynamics of cell-matrix adhesions, we developed a modified version of the cell migration simulator (CMS) that can be used for modeling cell migration on mechanically heterogeneous substrates. The detailed governing equations and algorithms of the original CMS were described previously[29]. Briefly, the CMS comprises multiple motor-clutch models (i.e. modules) that mimic cellular protrusions found in U-251MG glioblastoma cells over the stiffness range used in the study. These include multiple lamellipodial protrusions distributed at the leading edge and around the perimeter of the migrating cell (Fig. 2c, Ref.[29]). Cell motion is determined by a force balance between the modules and a central cell body (Fig. 3a). In the CMS, new modules are nucleated stochastically, module length increases over time via actin polymerization that is simultaneously counteracted by myosin-induced retraction of actin fibers, and modules are removed when they become too short. In addition, total actin and numbers of clutches and motors are kept constant in accordance with the conservation of mass.

In each motor-clutch system, adhesion clutches bind to elastic substrate springs with a constant rate of k_{on}. Connected clutches form a direct mechanical link from the intracellular cytoskeleton to the extracellular substrate – forces are borne from active myosin motors and transmitted by the resulting inward actin flow. The unbinding rate of a connected clutch i, $k_{off,i}$, varies with force F_i according to the Bell model[74]:

$$k_{off,i} = k_{off}^{*}\, exp(F_i / F_b) \qquad \text{(S1)}$$

$$F_i = k_c x_i$$

where k_{off}^{*} is the clutch unbinding rate in the absence of loading, F_b is the characteristic clutch rupture force, and x_i is the elongation of the spring representing the i^{th} connected clutch with a spring constant k_c. The actin filaments are pulled by n_m myosin motors, each capable of exerting a force F_m, and balanced by the traction force F_s, resulting in inward actin flow with the effective actin flow rate (v_m) based on

$$v_m = v_m^{*}\left(1 - \frac{F_s}{F_{stall}}\right) \qquad \text{(S2)}$$

where v_m^{*} is the unloaded rate, $F_{stall} = n_m F_m$ is the stall force of the ensemble of myosin motors, and the traction force F_s transmitted by all the connected clutches is given by:

$$F_s = \sum_{i=1}^{n_{c,on}} F_i \tag{S3}$$

in which $n_{c,on}$ is the number of connected clutch bonds. Actin monomers are added to the barbed ends of actin filaments in the cellular protrusions (modules) at a polymerization rate v_p, constrained by the total actin length A_{tot} in the cell according to the relation:

$$v_p = v_p^*\left(A_{free}/A_{tot}\right) \tag{S4}$$

where A_{free} is the amount of available G-actin and v_p^* is the maximum polymerization rate. Module elongation and retraction both result from this actin polymerization and the actin flow rate (v_m). New modules are nucleated at a nucleation rate k_{mod}, also constrained by actin availability:

$$k_{mod} = k_{mod}^*\left(A_{free}/A_{tot}\right) \tag{S5}$$

where k_{mod}^* is the maximum module nucleation rate. Actin filaments are depolymerized into actin monomers when they pass through the position of the myosin motors. Filaments can also be capped and polymerization arrested by actin capping proteins at a capping rate k_{cap}. Actin filaments, and the corresponding modules, are removed from the simulation when their length falls below l_{min}.

Monte Carlo simulations were conducted using a direct Gillespie Stochastic Simulation Algorithm[50], with each time step determined based on total event rates, including k_{on}, $k_{off,i}$, k_{mod}, and k_{cap}, and the event execution determined based on accumulated event rates. The CMS C++ version, described in[38], was modified to account for variations in substrate stiffness (described below), and simulations were conducted in Mesabi computer cluster at the Minnesota Supercomputing Institute (MSI).

After the simulated cells had reached a dynamic steady state (60 min), they were displaced randomly to a 180 µm x 180 µm region (Fig. 3b), and the substrate stiffnesses (k_s) experienced by the cell body and each protrusion were determined based on their respective y-coordinates (y). The substrate could be either soft (k_{soft}), stiff (k_{stiff}), or between the two extremes [gradients following a normal cumulative distribution function, described by the following error functions (erf)]:

$$k_{\text{s}} = k_{\text{soft}} \qquad -\frac{1}{2}\Delta y_{\text{gradient}} - \Delta y_{\text{plateau}} < y \le -\frac{1}{2}\Delta y_{\text{gradient}} \tag{S6}$$

$$k_{\text{s}} = k_{\text{soft}} + \frac{1}{2}\left(1 + \mathrm{erf}\left(4y/\Delta y_{\text{gradient}}\right)\right)\left(k_{\text{stiff}} - k_{\text{soft}}\right) \qquad -\frac{1}{2}\Delta y_{\text{gradient}} < y \le \frac{1}{2}\Delta y_{\text{gradient}} \tag{S7}$$

$$k_{\text{s}} = k_{\text{stiff}} \qquad \frac{1}{2}\Delta y_{\text{gradient}} < y \le \frac{1}{2}\Delta y_{\text{gradient}} + \Delta y_{\text{plateau}} \tag{S8}$$

$$k_\mathrm{s} = k_\mathrm{stiff} + \frac{1}{2}\left(1 + \mathrm{erf}\left(4\left(y - \Delta y_\mathrm{gradient} - \Delta y_\mathrm{plateau}\right)/\Delta y_\mathrm{gradient}\right)\right)(k_\mathrm{soft} - k_\mathrm{stiff}) \qquad \frac{1}{2}\Delta y_\mathrm{gradient} + \Delta y_\mathrm{plateau} < y \leq \frac{3}{2}\Delta y_\mathrm{gradient} + \Delta y_\mathrm{plateau} \quad \text{(S9)}$$

where $\Delta y_\mathrm{gradient}$ is the width of a region with stiffness gradient (30 µm) and $\Delta y_\mathrm{plateau}$ is the width of a region with constant stiffness (60 µm). This way, the number of cells in both soft and stiff regions was initially the same. In addition, by repeating the same stiffness pattern *ad infinitum*, the finite amount of cells placed in the finite rectangular region was representative of infinite cells placed on an infinite substrate with the same initial distribution of cells between soft and stiff areas. A normal cumulative distribution function was selected due to a finite element model of polyacrylamide, which demonstrated that the effective spring constant around a true stepwise gradient of elastic modulus follows a similar distribution (Fig. S7). This was valid regardless of the orientation of the applied traction (soft-to-stiff vs. stiff-to-soft).

Here, we adopted the high-motor-clutch parameter values used previously[29] to describe U-251MG migration on mechanically distinct but isotropic substrates (Table S2). Clutch stiffness was further adjusted to 8 pN nm^{-1} to better recapitulate the stiffness-dependence of U-251MG speed *in vitro*[29]. Moreover, the total number of available molecular motors (N_m) was adjusted between 4,000 and 10,000 to evaluate the impact of actomyosin inhibition on the U-251MG stiffness optimum (Fig. 4a–b). During the CMS simulations, cell positions and traction forces were recorded every second. The data collected during the first 60 min were analyzed to ensure that the simulated cells had indeed reached a dynamic steady state. Random motility coefficients (RMC) were calculated as described previously[29]. Briefly, the mean squared displacement, $\langle r^2 \rangle$, was calculated with overlapping time periods Δt = 10 min, 20 min, ..., and plotted as a function of Δt. The first half of the plotted curve was fitted with a straight line (slope = $\langle r^2 \rangle/\Delta t$), and RMC was given by RMC = $\langle r^2 \rangle/4\Delta t$. Module forces were recorded every 10 min and averaged throughout the simulation to yield the average traction force per module. Custom MATLAB scripts were employed to analyze the change in cell numbers in soft and stiff regions over time, to compare module forces in the soft and stiff parts of the gradients, and to track individual cells over time based on their initial location in soft or graded substrate regions.

On 10–100 pN nm^{-1} gradients, we found that the majority of cells translocated away from stiffer regions and toward soft areas (Fig. 3e–f), which were associated with higher traction forces per module and lower overall migration speed, RMC (Fig. 3c–d). We also tested whether altering the range of the gradient would affect the durotaxis. On 0.3–3 pN nm^{-1} gradients, the stiffer side was associated with higher traction forces and higher RMC (Fig. S9a–b). On these substrates, simulated cells displayed rapid accumulation in the stiffer regions (Fig. S9c). Finally, when the gradient was chosen such that there would be no appreciable difference in mean traction forces (100–300 pN nm^{-1}), cells clustered primarily in stiffer regions with lower RMC (Fig. S9a–b,d).

In order to track individual cells on stiffness gradients, and to calculate the cells’ angular displacements and forward migration indices (FMI), 350 cells were simulated on a continuous 200 µm gradient ranging from 10 to 30 pN nm^{-1}. Cells in a dynamic steady state were positioned randomly within an approximately linear 50 µm region in the middle of the gradient (Fig. 3g) and followed for an additional 14 hours of simulation time. FMIs, defined as Δy divided by the total track length (accumulated distance), and where positive values denote migration toward increasing stiffness, indicated that the cells moved preferentially toward the softer side of the stiffness gradient – in accordance with their predicted stiffness optimum (Fig. 3h–i).

Supplementary Text 3: Modeling axonal pathfinding and mechanosensitive steering of growth cones

Axonal growth cones (GCs) (Fig. S10a) can turn or contract in response to substrate stiffness gradients[24,75] by controlling the dynamics of adhesions, filopodial remodeling, and active contraction[39]. To establish whether motor-clutch dynamics could explain the mechanosensitive turning of neuronal GCs[24], akin to the negative durotaxis exhibited by the U-251MG glioblastoma cells, we modified the CMS to model an individual GC on a functionally graded substrate. A group of i filopodia, each modeled as a single molecular clutch module (Fig. S10b), were attached to a GC central domain. Each module was allocated n_i molecular clutches (linear springs of stiffness k_c) and n_i corresponding substrate clutches (linear springs of stiffness $k_{s,i}$). Substrate clutches were distributed randomly, and had values $k_{s,1} \leq k_{s,i} \leq k_{s,2}$ that varied linearly with position along the gradient.

Monte Carlo simulations were conducted to evaluate filopodial and GC dynamics over time. We modeled a GC as having 21 potential growth sites for filopodia, chosen from a uniform orientation distribution between –π/2 and π/2, relative to the direction of the 'axon'. New protrusions with an initial length l_{in} and width l_{wid}, dictating the effective clutch-ligand binding area, were added into the simulation at a rate k_{mod} and assigned n_m myosin motors; note that we used an actin filament in the schematic diagram (Fig. S11a) to represent the filament bundle in the filopodium. The adhesion and substrate clutches under each filament then evolved according to the clutch binding and unbinding dynamics described above. Unlike the cellular level CMS, our modified model assumes a relatively stable pool of actin monomer in the GC. Thus, the actin polymerization rate v_p remained constant during each simulation. See Table S3 for parameter details.

First, we investigated whether the dozens of filopodia within a GC might enable mechanically directed growth by evaluating the response of an individual 8 μm filopodium to a linear stiffness gradient of 0.01 to 100 pN nm^{-1} (Fig. S10b). The filopodium was placed on a 10 μm x 10 μm square substrate and oriented at an angle $0 \leq \theta \leq \pi/2$ relative to the gradient (Fig. S10c). When the filopodium length was fixed, simply increasing the orientation between the filopodium and the gradient was sufficient to significantly increase traction force generation (Fig. S10d). Conversely, when the orientation was fixed at π/2, i.e. perpendicular to the gradient, we found that both traction force and the number of engaged clutches increased linearly with filopodium length (Fig. S10d).

Next, we investigated the impact of different stiffness gradients for traction force generation using a fixed filopodium length (8 μm) and orientation (0). Maximal traction forces resulted from the filopodium sensing a soft region, in the range of 0.01 to 0.1 pN nm^{-1}. The higher end of the stiffness gradient proved significantly less important for the overall traction (Fig. S10e). This result demonstrates that a filopodium can generate comparatively high forces even if only a part of it is located on softer substrate. Thus, high traction force generation by individual filopodia is favored at a low optimal stiffness and forces drastically drop on stiffer matrices.

Higher traction forces are often accompanied by a decrease in actin retrograde flow, as myosin-borne forces are transmitted to the ECM instead of freely displacing actin. Regardless of filopodia orientation, actin in GCs flows toward the structure's center, and much like traction forces, actin flow rates can also differ for different types of neurons[40]. We therefore investigated how both the speed and direction of actin flow relative to the stiffness gradient affect the dynamics of single filopodia. By studying filopodia oriented at their growing end with either the stiffer or more compliant end of a stiffness gradient (Fig. S10f), we found that orientation toward the compliant end of the substrate (and hence actin retrograde flow toward the stiff end of the substrate) led to increased extension rates and decreased retraction rates (Fig. S10g). In all cases, the overall growth rate of filopodia was a trade-off between growth at the constant actin polymerization rate v_p, and shortening at the actin flow rate v_m, which varied almost linearly with substrate stiffness (Fig. S10h). For an intermediate polymerization rate of v_p = 120 nm s^{-1}, orientation affected filopodia growth rate by a factor of two (Fig. S10h). Together, these results provide a mechanism by which individual filopodia can exert more traction and elongate faster on softer substrates.

We then investigated whether these changes in filopodial dynamics could contribute to GC steering on stiffness gradients. First, we evaluated the degree of GC turning on one type of stiffness gradient ($k_{s,1}$ = 0.01 pN nm^{-1} and $k_{s,2}$ = 100 pN nm^{-1}) by studying an initially semicircular GC with 21 uniformly distributed filopodia (Fig. S11a). Within 15 seconds of simulation, the filopodia pointing toward the compliant end of the substrate outgrew the rest, resulting in an effective turning of the GC (Fig. S11a). As expected from the previous results, filopodia in the softer regions of the gradient were longer and generated higher traction forces (Fig. S11b).

To investigate the effect of different stiffness gradients on GC turning in detail, we repeated our simulations over a broad range of possible substrate stiffnesses, with $k_{s,left}$ and $k_{s,right}$ varying from 0.01 to 100 pN nm^{-1}. To quantify the degree of turning, we defined a parameter Λ = $\bar{l}_{left}/\bar{l}_{right}$, which represents the degree to which the GC has turned left. Here, $\bar{l}_{left}$ and $\bar{l}_{right}$ are the average lengths of filopodia in the left-hand and right-hand sides of the GC after 100 seconds of simulation, respectively. In addition to developing polarity through turning, the GC could enlarge, with all filopodia elongating as compared to their initial length, or retract (Fig. S11c). Enlarged GCs appeared on very compliant substrates (red section, $k_{s,left}$ and $k_{s,right}$ on the order of 0.01 to 0.1 pN nm^{-1}) with a negligible stiffness gradient, and retractile GCs appeared on higher stiffnesses, independent of the actual strength of the gradient (green section, $k_{s,left}$

and $k_{s,right}$ on the order of 1 to 100 pN nm^{-1}). Finally, polarized GCs appeared on compliant substrates with a moderate or high stiffness gradient ($k_{s,left}$ on the order of 0.01 to 0.1 pN nm^{-1}, >1 pN/nm/20 μm). A phase diagram for GC turning illustrates how the structure can either remain straight or turn to the more compliant side (Fig. S11d), and reveals that a stronger gradient may also promote GC turning, unless the range of the gradient as a whole is significantly stiffer than the optimal stiffness range for individual filopodia (Fig. S11e). Thus, the motor-clutch model can recapitulate mechanosensitive GC steering toward softer matrix *in silico*.

Table S1. Relative acrylamide and bis-acrylamide concentrations and corresponding Young's moduli for homogeneous (constant modulus) hydrogels

Final acrylamide %	Final bis-acrylamide %	Volume of (40%) AA stock, µl	Volume of (2%) bis-AA stock, µl	PBS, µl	~Young's modulus, kPa*
5.4	0.04	63	10	397	0.5
5.7	0.08	63	17.5	365	2
7.5	0.2	94	50	356	9.6
12	0.2	150	50	300	22
18	0.4	225	100	175	60

*Values obtained using atomic force microscopy, see Ref.[31]

Table S2. Parameters for the cellular level CMS

Parameter	Symbol	Value	Ref.
Total number of myosin motors	N_m	(4,000–)10,000	[29], adjusted
Total number of clutches	N_c	7,500	[29]
Maximum total actin length	A_{tot}	100 µm	[29]
Maximum actin polymerization rate	v_p^*	200 nm/s	[29]
Maximum module nucleation rate	k_{mod}^*	1 s^{-1}	[29]
Module capping rate	k_{cap}	0.001 s^{-1}	[29]
Initial module length	l_{in}	5 µm	[29]
Minimum module length	l_{min}	0.1 µm	[29]
Cell spring constant	k_{cell}	10,000 pN/nm	[29]
Number of cell body clutches	$n_{c,cell}$	10	[29]
Substrate spring constant	k_s	0.3–300 pN/nm	Adjusted
Maximum number of module motors	n_m^*	1,000	[29]
Myosin motor stall force	F_m	2 pN	[29]
Unloaded actin flow rate	v_m^*	120 nm/s	[29]
Maximum number of module clutches	n_c^*	750	[29]
Clutch on-rate	k_{on}	1 s^{-1}	[29]
Unloaded clutch off-rate	k_{off}^*	0.1 s^{-1}	[29]
Clutch spring constant	k_c	8 pN/nm	Adjusted
Characteristic clutch rupture force	F_b	2 pN	[29]

Table S3. Parameters for the filopodia/GC model

Parameter	Symbol	Value	Ref.
Actin polymerization rate	v_p	90–130 nm/s	[76], adjusted
Module nucleation rate	k_{mod}	1 s^{-1}	[29]
Initial filopodium length	l_{in}	3 µm	Adapted from [29]
Minimum filopodium length	l_{min}	0.1 µm	Adapted from [29]
Filopodium width for ligand binding	l_{wid}	0.2 µm	[77]
Substrate spring constant (soft region)	$k_{s,1}$	10^{-2}–10^{2} pN/nm	Adjusted
Substrate spring constant (stiff region)	$k_{s,2}$	10^{-2}–10^{2} pN/nm	Adjusted
Initial number of module motors	n_m	50	[27]
Myosin motor stall force	F_m	2 pN	[27]
Unloaded actin flow rate	v_m^*	120 nm/s	[27]
Initial number of module clutches	n_c	50	[27]
Clutch on-rate	k_{on}	0.3 s^{-1}	[27]
Unloaded clutch off-rate	k_{off}^*	0.1 s^{-1}	[27]
Clutch spring constant	k_c	1 pN/nm	[27]
Characteristic clutch rupture force	F_b	2 pN	[27]

Captions for Movies S1–S3

Movie S1. Evolution of U-251MG glioblastoma cell distribution on photoresponsive stiffness gradient hydrogels over time. Blue overlay in the middle denotes a softer, UV-irradiated region. Vertical and horizontal lines are out-of-focus markings in the underlying glass. Scale bar, 200 µm.

Movie S2. Migration of individual U-251MG cells on photoresponsive stiffness gradient hydrogels. (Top) Phase-contrast data showing migrating cells over the span of 24 hours. Scale bar, 100 µm. (Bottom) Tracks corresponding to the cells in the top panel. Softer, UV-irradiated hydrogel is marked with gray color.

Movie S3. DMSO- and H-1152-treated U-251MG cells migrating on stiffness gradients. Phase-contrast movies of migrating glioma cells treated with 0.1% DMSO (left) or 5 µM H-1152 (right) for 10 hours. The cells are on continuous 0.5-22 kPa stiffness gradients, in the >10 kPa region (with substrate stiffness increasing toward the top). Scale bar, 50 µm.