Optogenetic control of RhoA reveals zyxin-mediated elasticity of stress fibres

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Cytoskeletal mechanics regulates cell morphodynamics and many physiological processes. While contractility is known to be largely RhoA-dependent, the process by which localized biochemical signals are translated into cell-level responses is poorly understood. Here we combine optogenetic control of RhoA, live-cell imaging and traction force microscopy to investigate the dynamics of actomyosin-based force generation. Local activation of RhoA not only stimulates local recruitment of actin and myosin but also increased traction forces that rapidly propagate across the cell via stress fibres and drive increased actin flow. Surprisingly, this flow reverses direction when local RhoA activation stops. We identify zyxin as a regulator of stress fibre mechanics, as stress fibres are fluid-like without flow reversal in its absence. Using a physical model, we demonstrate that stress fibres behave elastic-like, even at timescales exceeding turnover of constituent proteins. Such molecular control of actin mechanics likely plays critical roles in regulating morphodynamic events.
A diverse array of essential physiological processes, ranging from the subcellular to the multicellular, depend on the spatial and temporal regulation of contractile forces. This regulation drives changes in cell shape and mediates interactions with the extracellular environment. Changes in contractility can furthermore alter gene expression and impact development. The molecular machinery required for generating contractile forces is well conserved and dominated by the actin cytoskeleton, myosin II activity and associated regulatory proteins. Specifically, actin filaments dynamically organize into distinct contractile architectures, including the cortex and stress fibres. Contractile forces are transmitted across the cell by actin arrays and ultimately to the extracellular matrix by focal adhesions.

The regulation of cellular force transmission is controlled by the mechanical properties of actomyosin assemblies. Cellular mechanics has been explored extensively both experimentally and theoretically. At timescales up to those of typical kinetic processes, the actin cytoskeleton behaves like an elastic solid. Such elasticity enables rapid force transmission across the cell and reversible deformations to preserve cytoskeletal architecture. In contrast, at longer timescales, it is thought that dynamic processes make the cytoskeleton behave predominately like a viscous fluid, enabling cytoskeletal flows and remodelling. These dynamic processes, including exchange of proteins from the cytosol, are typically on the order of tens of seconds in structures like the cortex and on the order of a minute in stress fibres. The molecular regulation underlying the competition between elastic and viscous processes in cells is not well understood.

Cellular contractility is largely controlled by the activity of the small GTPase RhoA, which in adherent cells is preferentially active at the cell periphery. RhoA regulates contractility through the promotion of actin polymerization and myosin light chain (MLC) phosphorylation via the downstream effectors Diaphanous-related formins and ROCK, respectively. RhoA activity is required for stress fibres and focal adhesions. Little, however, is known about how small changes in activity can regulate cell contractility, actin architecture and adhesion.

Optogenetics has been used before to control cell migration and cell forces. Here we have used an optogenetic probe to locally activate RhoA in adherent fibroblasts. Plasma membrane recruitment of the RhoA-specific guanine exchange factor (GEF) LARG induces local RhoA activation and recruitment of actin. Local activation of RhoA leads to an increase in actin polymerization and myosin activity in the region of activation, but it does not stimulate local activation of ROCK. Exogenous RhoA activation leads to an immediate increase in both the local and global contractility of the cell, followed by a rapid relaxation after GEF recruitment is stopped. The local increase in stress fibre contractility drives an actomyosin flow towards regions of increased RhoA activity. Surprisingly, these flows reverse direction as soon as GEF recruitment ceases. Using physical modelling, we show this behaviour is consistent with stress fibres behaving as predominately elastic-like over timescales much longer than minutes. We find that actin stress fibres become predominately fluid-like even at second timescales. These results suggest that stress fibre mechanics are sensitive to small changes in composition, which has significant implications for regulation of force transmission and cytoskeletal organization.

Results

Spatiotemporal control of RhoA and its downstream effectors.

To spatially and temporally control contractility in adherent cells, we adapted a previously established optogenetic probe to act on the RhoA signalling pathway. During stimulation by blue light, a cytosolic fusion protein, photo-recruitable GEF (prGEF), consisting of tandem PDZ domains fused to the DH domain of the RhoA-specific GEF LARG, is recruited to the plasma membrane where it activates RhoA. To illustrate the local recruitment of prGEF, we tagged it with the fluorophore mCherry and imaged an NIH 3T3 fibroblast expressing the constructs on a glass coverslip. A digital micromirror device was used to spatially control the illumination of the blue-activating light (orange box) and was pulsed before each image acquisition during the recruitment period. Recruitment of the prGEF to the activation region was rapid and reversible upon extinguishing the stimulating blue light.

To investigate whether recruitment of the prGEF resulted in activation of proteins downstream of RhoA, we tracked the dynamics of actin and MLC during recruitment. Both actin and myosin accumulated in the activation regions, resulting in an increase in fluorescence intensity during the 15 min activation period. At the end of the activation period, fluorescence intensities of both actin and myosin returned to baseline levels. These results indicate that exogenous RhoA activation via LARG recruitment is not sufficient to maintain elevated RhoA activity and the concomitant increases in local actin and myosin concentrations. To confirm that local activation of RhoA was acting on actin and myosin through its downstream effectors, formin and ROCK, the experiments were repeated in the presence of either SMIFH2, a pan formin inhibitor, or Y-27632, a ROCK inhibitor. Local recruitment of actin and MLC were significantly inhibited by the presence of SMIFH2 and Y-27632, respectively. These results illustrate that RhoA activity and recruitment of its downstream effectors can be spatially and temporally controlled via light.

Adhesion morphology is unperturbed by local RhoA activity.

Previous work has suggested that focal adhesion formation and maturation are tension-dependent processes driven by increased RhoA activity at adhesion sites. To test these hypotheses, we examined how local RhoA activation affected traction forces and focal adhesions. Cells expressing mCherry-vinculin, a marker of focal adhesions, were plated on polyacrylamide gels coated with fibronectin and traction stresses were measured via traction force microscopy. During local activation of RhoA, traction stresses increased at focal adhesion sites, on a similar timescale to that of myosin localization. Despite the increased force, the total number of adhesions remained essentially constant. Individual adhesion morphology and vinculin intensity were also unaffected, despite large increases in stress at a majority of previously established adhesion sites during local RhoA activation. The lack of change in adhesion morphology is consistent with previous results showing that adhesion size and tension are only correlated during the initial growth phase of the adhesion and that adhesion maturation is driven by proximal actin stress fibre assembly.

To determine the effect of local RhoA activation on the overall contractility of the cell, we used traction force microscopy to measure the total strain energy, which includes both near and far field deformations and reflects the total amount of mechanical
work done by the cell on its environment. Activation induced a rapid increase in both traction stresses and strain energy. At the end of the activation period, the strain energy decreased to its original baseline value. Interestingly, traction stresses were mostly seen to increase at the cell periphery, where traction stresses were already established, and in areas immediately adjacent to the activation region. No change was seen in the activation area itself. This suggests that locally generated forces balance within the activation region and only unbalanced forces at the edge of this region are turned into productive traction forces. Thus a local increase of tension leads to globally distributed traction forces at pre-existing focal adhesions.

Cells maintain a contractile set point. That cells return to a similar baseline contractility following a period of exogenous RhoA activation is consistent with previously established ideas of tensional homeostasis. To explicitly probe this behaviour, we performed a series of local activations of different sizes on a single cell (Fig. 2e and Supplementary Movie 5). After measuring the strain energy at an initial steady state, a cell was exposed to different concentrations of SMIFH2 (pan formin inhibitor; n = 7) or 1 μM Y-27632 (ROCK inhibitor; n = 6). Inhibition of either Dia or ROCK results in reduced average increases in local intensity during RhoA activation. Error bars represent s.d. **P < 0.01, two-sample Student’s t-test. Time is min:s. Scale bars are 15 μm.
three 15 min periods of local RhoA activation of increasing size with relaxation periods between each activation (Fig. 2e,f). The strain energy increased concomitant with the size of the activation region. During activation, both local stresses immediately surrounding the activation region, and long-range stresses at the cell periphery could be seen to increase (Fig. 2e). Following each activation, the strain energy returned to the initial baseline level (Fig. 2f).

To elucidate the underlying mechanical principles, we built a physical model that would capture this physical response (see Supplementary Note for details). We constructed a model of the cell as a continuum of contractile elements in series, each in parallel with one elastic and one viscous element in series (Fig. 2g). Such a model is known as an ‘active Maxwell fluid’ and is used here because we are interested in long timescales, when the system is expected to flow21,25,48. Contractility was assumed...
to increase with an exponentially plateauing ramp in the activated region, consistent with the observed accumulation profiles for actin and myosin (Fig. 1g), and the substrate was represented as an elastic spring coupled to the cell by a friction element. The model parameters for the elastic modulus, viscosity, friction and contractility were found by fitting the model to the strain energy data, while the value of the substrate stiffness was fixed. This procedure resulted in a curve in good agreement with the experimental data (Fig. 2f).

We find that both the viscous and elastic elements are necessary to accurately capture the behaviour of the system over the whole time course of ramping, plateau and relaxation. The ratio of viscosity to elasticity defines a viscoelastic relaxation time of approximately 60 min; this timescale determines the transition from when the cytoskeleton behaves predominantly elastic-like (<60 min) to predominately fluid-like (>60 min). Our results thus indicate that stress fibres are predominantly elastic on the scale of tens of minutes, despite proteins within the stress fibre turning over on timescales of tens of seconds. This strong elastic behaviour is also consistent with the immediate increase in cell traction stresses at the cell periphery (Fig. 2b,e) upon local activation of RhoA in the centre of the cell.

Stress fibres contract independent of the background network. To investigate the cytoskeletal architectures that give rise to this strong contractility, we tracked myosin dynamics during local RhoA activation. In the steady state, as new actomyosin is polymerized and incorporated into stress fibres, there is a retrograde flow of actomyosin from the periphery towards the cell centre. Using particle image velocimetry, we measured both the local direction and magnitude of myosin flow. We found that myosin flow rates along the stress fibre increased as myosin accumulated in the activation region creating a local contraction and that this flow was directed along the orientation of the stress fibres spanning the activation region (Fig. 3a,b). The flow direction was independent of the activation region geometry, with the direction always being determined by the stress fibre orientation (Supplementary Movie 7).

The cytoskeleton of a strongly adherent cell is typically thought to be a two-dimensional (2D) material comprised of stress fibres embedded in an isotropic actin network. Since flows induced by local RhoA activation appear to track the orientation of the stress fibres, there is a one-dimensional (1D) continuum model described above (Fig. 3c; Supplementary Note). The model consists of a triangular mesh with the contractile elements again connected in parallel to the viscous and elastic elements, with lines of increased contractility representing the stress fibres. Using a simple rectangular cell, we first verified that, without stress fibres, this model recapitulates the results from the 1D continuum model (Fig. 3d). Similar to the 1D model above, the contractile components in a region in the centre of the cell were slowly increased with an exponentially plateauing ramp. The parameters were then adjusted so that the model both qualitatively and quantitatively recapitulated the expected flow patterns of the 1D continuum model (Fig. 3d).

To explore the relative contributions of the background mesh and the stress fibres, we considered two test cases: (1) If both the mesh and the stress fibres contained contractile elements, the stress fibres pinched inward transverse to their orientation during local activation (Fig. 3e); and (2) when contractile elements were only included in the stress fibres (as depicted in Fig. 3c), the cytoskeletal flow was restricted to directions along the stress fibre (Fig. 3f), consistent with our experimental results (Fig. 3a,b, Supplementary Fig. 3 and Supplementary Movies 6 and 7). Since transverse deformations were never seen in experiments, it is clear that the stress fibres must be the predominant contractile elements observable at this resolution that respond to local RhoA-induced contractions. Furthermore, this result illustrates that it is appropriate to think of a stress fibre as a 1D contractile element with viscous and elastic components embedded in a passive viscoelastic network, which is consistent with recently published image-based observations.

Stress fibres flow due to local RhoA induced strain. Having identified the stress fibre as the main contractile unit responding to exogenous RhoA activation, we next sought to address whether stress fibres undergo deformation during contraction. Since stress fibres can be considered as 1D structures, we analysed myosin flow along the fibre using kymographs. A kymograph drawn along a single stress fibre illustrates that myosin puncta flowed from both ends towards the activation regions when RhoA was activated locally (Fig. 4a,b). Similarly, a kymograph drawn by projecting the flow speed along the stress fibre from the velocity field created by our particle image velocimetry analysis illustrates even more clearly how cytoskeletal flow was perturbed by local RhoA activation. Flow of myosin from both ends of the stress fibre reoriented towards the recruitment regions and increased from approximately 0.1 nm s\(^{-1}\) on average to >3 nm s\(^{-2}\) during activation (Fig. 4c and Supplementary Fig. 5). Strikingly, the flow was also seen to reverse direction, flowing away from the recruitment region and towards the cell periphery, during the relaxation period following the local activation (Fig. 4b,c). This flow reversal is reminiscent of the restoring force in elastic objects that restores its original shape after removal of external force (for example, recoil of an elastic band after stretch). In active systems, flow reversal could also arise from spatial variations in tension. Specifically, this would either require the myosin stress within the activation region to fall below its preactivation level or increased myosin activity distal to the activation site. However, we do not observe such changes in actomyosin density, indicating that a passive elastic-like element may be sufficient in describing this recoil.

We next developed a protocol to measure the magnitude of the stress fibre displacement during these periods of contraction and relaxation (Fig. 4d). The displacement in a given fibre was determined by measuring the relative position of puncta along the fibre following 15 min of local RhoA activation and 15 min after it ceased. During contraction, puncta on either side of the activation region contracted on average ~3 μm from their original position before relaxing back to ~1 μm from their original position (Fig. 4d). The relaxation response across many stress fibres from multiple cells could be further clustered into two groups, one which exhibited strong reversal (~80% of the original position) and one which exhibited little to no reversal (~25% of the original position) (Fig. 4d).

To determine whether stress fibres were stretching due to the local contraction, we used cells expressing mApple–α-actinin, an actin crosslinker that localizes to well-defined puncta on stress fibres (Fig. 4e and Supplementary Movie 8). We created kymographs of α-actinin flow during local activation of RhoA and tracked paths of individual puncta (Fig. 4f). The velocity of individual puncta was determined from the slope of the tracks in the kymograph and plotted as a function of distance from the activation zone (Fig. 4g). Puncta along the stress fibre moved at similar speeds, indicating that, in general, the stress fibre was translating as a rigid rod during the local contraction (Fig. 4g).
contraction pinches together the stress fibres. If only the stress fibres contract, the flow profile is restricted to the orientation of the fibres, mimicking the
above. (Fig. 5b). Surprisingly, we found that zyxin also accumulated along stress fibres in the region of local activation (Fig. 5c,d). Given the myosin accumulation and direction of flow, this suggests that zyxin might be recruited to both sites of compression and extension. Paxillin, another mechanosensitive LIM domain protein that responds to stress56,57, behaved similarly to zyxin (Supplementary Fig. 4).

Zyxin is required for stress fibres to behave elastically. To further explore the role of zyxin in stress fibre mechanical behaviour, we used mouse embryonic fibroblast cells derived from zyxin\(^{-/-}\) mice58. Despite the loss of zyxin, these cells form actin stress fibres and focal adhesions and are highly contractile59. When we locally activated RhoA in the zyxin\(^{-/-}\) cells, myosin accumulated in the activation region (Fig. 5e and Supplementary Movie 10). This accumulation drove a contractile flow into the local activation area that was indistinguishable from wild-type cells, indicating that loss of zyxin did not impede myosin activity. Upon stopping the GEF recruitment in zyxin\(^{-/-}\) cells, cytoskeletal flow returned to preactivation rates, consistent with the reduced local contraction, but did not reverse direction (Fig. 5e,i, Supplementary Fig. 5, and Supplementary Movie 10). Expression of enhanced green fluorescent protein (EGFP)-zyxin in this cell line restored the flow reversal (Fig. 5g–i, Supplementary Fig. 5, and Supplementary Movie 11). Together these results indicate that zyxin is required for the flow reversal occurring after local RhoA activation ends.
Using the kymographs produced in the zyin (\(-\)/-) and zyin (\(-/-\)) + EGFP-zyxin cells, we again fit the data to our mechanical model (Fig. 5j-1, Supplementary Fig. 6). For the zyin (\(-/-\)) cells, we found the viscoelastic relaxation time reduced to \(1\ h\), indicating that the stress fibres are predominantly fluid-like at all physiological timescales. Rescue of the zyin (\(-/-\)) cells with EGFP-zyxin resulted in parameter fits that were consistent with the NIH 3T3 fibroblast data. Zyxin is thus important for maintaining the qualitative mechanical response of stress fibres, ensuring that they are predominately elastic at \(1\ h\) timescales.

**Discussion**

This study demonstrates that the mechanical behaviour of adherent cells is strongly shaped by stress fibres and their ability for rapid force transmission even in the face of molecular turnover and flow. Using an optogenetic probe to locally activate RhoA via recruitment of the DH domain of LARG, a RhoA-specific GEF, we find that we can stimulate a local contraction in stress fibres due to an increased accumulation of actin and myosin in the activation area (Fig. 6, 1). This local contraction causes a tension gradient at the boundaries of the activation region and a flow towards it (Fig. 6, 2). The flow of myosin and \(\alpha\)-actinin increases the strain both on the interface coupling the stress fibre to the adhesion and in the activation region, leading to recruitment of the mechanosensitive protein zyxin (Fig. 6, 3). When local activation of RhoA is stopped, the system relaxes to the preactivation state, mainly driven by elastic energy accumulated in the strained regions, and results in a transient cytoskeletal flow of material away from the local activation region (Fig. 6, 4).

This elastic behaviour is dependent on zyxin. Previous reports have shown that zyxin localizes along the stress fibre at the interface of the adhesion \(60,61\). This positioning suggests that previously reported zyxin-mediated stress fibre repair mechanisms \(56,59\) are also occurring at the adhesion interface as actin is assembled and is incorporated into the stress fibre while under tension. The localization of zyxin to potential sites of compression, however, is novel. While it is known that the LIM
domain of zyxin is sufficient for localization\(^6\), the exact mechanism through which zyxin recognizes sites of strain remains unknown. These data further illustrate that RhoA activity and its downstream effectors are tightly regulated by the cell. We see no evidence that RhoA activation alone leads to de novo stress fibre formation or adhesion maturation. Instead these processes likely result from concurrent changes in cytoskeletal architecture\(^4\)\(^3\),\(^6\). More interestingly, the data suggest cells actively regulate total RhoA activity to maintain a constant

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**Figure 5** Zyxin accumulates at sites of strain on stress fibres during local RhoA activation. (a) A NIH 3T3 expressing mCherry-zyxin. The activation region is indicated by the orange box. (b) A kymograph of a representative adhesion marked by the white box in panel a. (c) The average intensity of zyxin in the activation region. (d) A kymograph illustrating the local zyxin accumulation during activation along the green line shown in panel a. (e) A zyxin\((/-)\) MEF expressing mApple-MLC before activation, at peak activation and following relaxation. Myosin accumulates as in 3T3s. (f) A kymograph of myosin intensity and flow speed drawn along the green line indicated in panel e. Zyxin\((/-)\) MEFs exhibit little to no elastic flow reversal. (g) A zyxin\((/-)\) MEF rescued with EGFP-zyxin and expressing mApple-MLC, during an activation sequence. (h) A kymograph of myosin and flow illustrating a strong elastic flow reversal along the line drawn in panel g. (i) Displacement analysis of zyxin\((/-)\) \((n = 40\) from 3 cells) and zyxin\((/-)\) + EGFP-zyxin MEFs \((n = 18\) from 3 cells). Without zyxin, cells do not exhibit an elastic response. (j) A kymograph representing the average fit of the continuum model to the zyxin\((/-)\) data. (k) A kymograph representing the average fit of the continuum model to the zyxin\((/-)\) + EGFP-zyxin data. (l) The elastic \((E)\) and viscous \((Z)\) parameters found from fitting the experimental kymographs to the continuum model \((n = 5\) from 3 cells for condition). Without zyxin, the elasticity increases and the viscosity decreases. Scale bars are 10\(\mu m\) in panels a,e,g. Horizontal scale bars are 10 min in panels b,d,f,h,k. Vertical scale bars are 2\(\mu m\) in panel b and 5\(\mu m\) in panels d,f,h,k. *\(P<0.05\); **\(P<0.01\), two-sample Student’s \(t\)-test.
homeostasis. Specifically, the relaxation kinetics of the downstream effectors match the kinetics of the optogenetic probe, thus indicating that there is no positive feedback loop whereby production of RhoA-GTP alone is sufficient to promote further activation of RhoA. To sustain a given contractile state, therefore, the cell must actively regulate and maintain a specific RhoA-GTP concentration.

By using an optogenetic approach to perturb the local mechanical balance within the cell, we were able to probe the material properties of the cytoskeleton in ways previously inaccessible. It is instructive to consider our results within the context of other approaches that probe stress fibre mechanics. One such method is the cyclic stretch of an elastic substrate, in which Rho activation is involved in reorienting the cell and its stress fibres away from the direction of stretch. It has been argued that the reorientation response might result from a homeostatic set point, that is either passive or active in nature. Our optogenetic experiments also reveal a homeostatic mechanism, which depends on the repair protein zyxin. This suggests that the homeostasis we describe has evolved to be actively maintained and is not simply an elastic response of a passive material. Another interesting comparison can be made with severing of stress fibres via laser ablation. In severing experiments, the mechanical stability of the fibre is compromised while myosin activity on the remaining portion remains unchanged, thus driving a large contraction over seconds. By contrast, we probe the response to small perturbations in tension along an intact stress fibre over tens of minutes. As a result of the differences between the two setups, these experiments are probing different types of stress fibre mechanical response. In contrast to severing experiments, where sarcomeres have been reported to collapse to a minimal length following a reduction in tension, we do not see changes in the spacing of the sarcomeric structure of crosslinkers in the fibre during the increase in tension driven by local RhoA activation. Our experiments thus probe the homeostasis of the system, while severing probes the limits of stability. Interestingly, it has been reported previously that sarcomere size in stress fibres fluctuates on a 20 min timescale, which might be related to the 50 min viscoelastic timescale revealed by our model results. Future insights could be derived from fluctuation analysis during optogenetic activation.

Contraction is driven by the local accumulation of myosin in the region of activation, but the origin of the elastic-like recovery is less clear. We see no evidence of additional myosin accumulation in distal portions of the stress fibre during the relaxation, suggesting that the recoil arises from zyxin-mediated repair mechanisms within stress fibres. This is further supported by the fact that the zyxin(-/-) cells exhibit identical contractile behaviour, but none of the elastic recoil. Zyxin is known to recruit actin polymerization factors such as VASP and actin crosslinkers such as alpha-actinin, and these interactions might be essential to ensure mechanical integrity of stress fibres. The molecular detail of these protein interactions under applied load remains an open question.

Our experiments highlight the importance of defining the relevant timescales and perturbations when describing a material as elastic or viscous. Given that typical turnover rates for proteins in the cytoskeleton are on the order of tens of seconds, it is surprising that our model results suggest that stress fibres have elastic-like properties on timescales of ~1 h. The viscous behaviour of cells is typically associated with irreversible changes brought on through remodelling and dynamic activity of proteins (for example, cytoskeletal remodelling during migration). This has informed much of the active fluid theoretical frameworks that have been developed. Conversely, elasticity has typically been used to describe cellular material properties without consideration of the dynamic activity of the components, and has been used in understanding force generation and mechanosensing. Our results suggest that active remodelling within the cytoskeleton can result in elastic-like properties of timescales exceeding those of dynamics of internal components. Moreover, our results indicate that very different viscoelastic timescales might co-exist in the same cell and even in the same cytoskeletal structures. Finally, our results implicate an important role for zyxin in regulating transitions between fluid-like and elastic-like behaviours. This has exciting implications for interpreting the underlying physics of active cytoskeletal materials. The fact that this behaviour can be controlled by the activity of a single protein suggests that there are a number of intriguing potential mechanisms cells can use to regulate their mechanical properties during morphodynamics and development.
Methods

Drug treatments. Cells were treated with either the 10µM SMIFH2, a pan-formin inhibitor66, or 1 µM of Y-27632, which inhibits ROCK (ThermoFisher Scientific), for at least 30 min before imaging.

Plasmids. The optogenetic membrane tether consisting of Stargazin-GFP-LOVpep and prGEF constructs used are previously described6. prGEF-YFP was constructed in an identical manner to prGEF with YFP replacing mCherry. This construct was used in experiments where the effects on various downstream markers were compared groups of data. Statistical significance is indicated by asterisks:

- * represents a P value < 0.05;
- ** represents a P value < 0.01.

Code availability. MATLAB analysis routines can be made available from the corresponding authors upon request.

Data availability. The data that support the findings of this study are available from the corresponding authors upon reasonable request. Stargazin-GFP-LOVpep and prGEF plasmids are available from Addgene.

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Traction force microscopy. Traction force microscopy was performed as described previously40,41. Briefly, polyacrylamide gels embedded with 40-nm fluorescent microspheres (Invitrogen) were polymerized on activated glass coverslips. The shear modulus of the gels used in these experiments was 8.6 kPa. Following polymerization, gels were washed with PBS and crosslinked with the extracellular matrix protein fibronectin (Millipore, Billerica, MA) using the photoactivatable crosslinker sulfo-sapphire (Thermo Fisher Scientific). Cells were plated and allowed to spread for at least 4 h before imaging as described above.

Following imaging, cells were removed from the substrate using 0.5% SDS and a reference image of the fluorescent beads in the unstrained substrate was taken. The image stack was then aligned to correct for drift and compared to the reference image using particle imaging velocimetry to create a displacement field with a grid spacing of 0.86 µm. Displacement vectors were filtered and interpolated using the Kriging interpolation method. Traction stresses were reconstructed via Fourier Transform Traction Cytometry46, with a regularization parameter chosen by minimizing the L2 curve41. The strain energy was calculated as one half the integral of the traction stress field dotted into the displacement field41.

Statistical analysis. All experiments were repeated a minimum of three times. Cells presented in figures are representative samples of the population behaviour. Box plots represent the 25th, 50th and 75th percentiles of the data. Whiskers on the boxplot extend to the most extreme data points not considered outliers. Error bars represent the s.d., except where noted otherwise. Statistical significance was determined using independent two-sample Student’s t-tests of the mean to compare groups of data. Statistical significance is indicated by asterisks: (*) represents a P value < 0.05; (**) represents a P value < 0.01.
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**Author contributions**

P.W.O, E.K.W, M.G. and M.L.G conceived the study and designed the experiments. P.W.O and E.K.W performed experiments. P.W.O, D.P. and M.L. performed data analysis. E.K.W. designed and cloned the molecular constructs. C.A.B., D.P., M.L. and U.S.S. conceived and designed the theoretical model. C.A.B. and D.P. performed simulations. P.W.O., M.G., U.S.S. and M.L.G. wrote the manuscript with feedback from all authors.

**Additional information**

**Supplementary Information** accompanies this paper at [http://www.nature.com/naturecommunications](http://www.nature.com/naturecommunications)

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