Supplemental Materials

*Molecular Biology of the Cell*

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Supplementary Figures

Supplementary Figure 1 | Additional trap calibration examples. a, Frequency response function (FRF) from two representative samples. The best fit to the FRF of the simple viscoelastic model described above is shown in red. b, Power spectrum from the corresponding sample as in a. Power spectrum of the model is shown in red. On the left, direct data acquisition at 20kHz is used, aliasing can be seen at frequencies above ~2500 Hz with the noise floor being reached at frequencies past ~3000 Hz. The part of the power spectrum that is fit is shown in grey and stops before reaching the aforementioned frequencies. On the right, data acquisition at 200 kHz downsampled to 20 kHz through averaging is used. A filtering effect from the averaging can be seen starting at ~2500 kHz with a second apparent corner frequency. This filtering was corrected during the fit by adding a low pass filter with corresponding corner frequency.
Supplementary Figure 2 | Calibrated $k_{\text{trap}}$ and $\beta_{pd}$ for all experiments. Left: $k_{\text{trap}}$ and $\beta_{pd}$ vary between cells and experimental conditions. Error bars indicate SEM. Right: the product of $k_{\text{trap}}$ and $\beta_{pd}$ clusters around 15 pN/V. The mean values are plotted (lines) and indicate that most of the $k_{\text{trap}} \times \beta_{pd}$ fall between 10 and 20 pN/V.
Supplementary Figure 3 | Viscoelastic response of purified actin networks. The elastic (G', black) and viscous (G'', black) moduli obtained from microrheological measurement with the optical trap and in situ calibration in 10 µM (~0.4 mg/mL) actin networks. Below -30 Hz, the elastic modulus weakly scales with frequency (~ f^{0.03}) and above -50 Hz, both G' and G'' strongly scale with frequency (~ f^{0.75}). At frequencies below 1 Hz, G' clearly dominates, indicating a mostly elastic response and no apparent viscous relaxation. These results are in excellent agreement with previous reports on actin networks mechanics at similar actin concentrations (blue^1, purple^2, red^3, connected green dots^4 and connected cyan dots^5, both in terms of frequency scaling and magnitudes.
Supplementary Figure 4 | Micro and bulk rheology of 20% Polyethylene Glycol (PEG).

The elastic ($G'$, solid circles) and viscous ($G''$, open squares) moduli from a rheometer (magenta, MCR 302, Anton Paar) are compared to moduli obtained from the optical trap (black). The values obtained from bulk rheology show $G'' \sim f^{1.098}$ (magenta line, magenta number) at frequencies below ~7 Hz, in agreement with the $G''$ scaling measured from microrheology, with $G'' \sim f^{0.946}$ (black line, black number). At frequencies past ~7 Hz, a stronger $G''$ scaling is observed for the bulk data, with $G'' \sim f^{2.810}$ (blue line, blue number). This is likely due to inertial effects coming from the instrument and the sample itself that can show an apparent increased frequency dependence at higher frequencies\(^6\). For optical trapping measurements at the microscale, inertial forces remain negligible at these timescales. In terms of magnitude, the data from the rheometer show significantly greater viscous and elastic moduli across the whole frequency spectrum. This is likely due to differences between the length scales investigated in bulk and microrheology that result in a different mechanical response to deformation\(^7,8\). From the loss tangent ($G''/G'$, inset), before 1 Hz, the rheometer is unable to probe the elastic modulus reliably, resulting in very low elastic moduli (out of the plotted region in the main plot) and jumps in the loss tangent. Between 1 Hz and 10\(^3\) Hz, the rheometer data show lower loss tangent, indicating that there is a greater elastic contribution, likely from surface tension\(^6\). Above 10 Hz the loss tangent increases due to inertial effects as discussed above.
Supplementary Figure 5 | Difference in Bayesian Information Criteria (BIC) values. (A) The distribution of the difference in BIC (∆BIC) values from the loss tangent (G'/G'') fit between the dynamic crosslinking model and power law model (see Fig. 3b) is obtained at each bootstrap sample for all four conditions and plotted vertically (y-axis and x-axis are both arbitrary units). The number next to the distribution indicates the mean ∆BIC values. A downward shift from zero (dashed red line) indicates a smaller BIC value for the dynamic crosslinking model, i.e. a better fit using that model. Wild type (WT), Blebbistatin(BB)-treated WT (WT BB) and mutant (K255E+/-) cells show a downward shift suggesting that the dynamic crosslinking model fits better (blue). Surprisingly, K255E+/- BB show an upward shift, suggesting that the power law model captures the loss tangent better (purple), possibility due to the inability of the dynamic crosslinking model to capture the slowest and fastest frequencies. (B) The distribution of ∆BIC values obtained from the fit to G' and G'' show an upward shift for all conditions, suggesting that the power law model captures G' and G'' better (purple). This may be due to single frequency point magnitude shifts that contributes to increased error terms. The loss tangent is less sensitive to these sources of error and is likely better suited for BIC values comparison. Overall, while the ∆BIC values distributions comparing the power law and dynamic crosslinking model are not statistically significant, the power law model is unable to capture the relaxation dynamics at low frequencies as seen in the loss tangent plots (see Figs. 3a and 4a in main text and Supplementary Figure 6) where a distinct V-shape is observed.
Supplementary Figure 6 | Relaxation dynamics after actin filament disruption. The elastic (G', solid circles) and viscous (G'', open squares) moduli from untreated WT (gray) and cells treated with 100nM Latrunculin A (Lat A, red). The best fit to the Blebbistatin-treated WT cells is shown in black for comparison. The Lat A-treated cells show faster relaxation (k_{off} = 0.80 Hz) compared to untreated WT (k_{off} = 0.24 Hz), as indicated by the earlier drop in G' and earlier rise in G'' at ~0.4 Hz. This is also visible in the loss tangent (inset), with a minimum observed at ~1 Hz, and an earlier rise in loss tangent with decreasing frequency, overall indicating an earlier transition to the fluid-like regime. Lat A-treated cells also show very similar relaxation compared to the Blebbistatin-treated WT cells (k_{off} = 0.89 Hz), as indicated by the near overlap between the red and black curves. Lat A concentrations greater than 100nM lead to complete cell death (data not show) while at 100 nM, only a small fraction of the cells showed severe signs of actin depolymerization and were avoided for mechanical measurements.
Supplementary Figure 7 | Immunofluorescent images of the WT and K255E+/- cells in various conditions.

WT (top three rows) and K255E+/- mutant (bottom three rows) immunofluorescent images under normal conditions, after 50µM Blebbistatin (BB) treatment and after 100nM Latrunculin A (Lat A) treatment. ACTN4 was stained with 1:350 primary anti-ACTN4 (ab59468, abcam), incubated overnight at 4°C, followed by 1:350 secondary anti-rabbit (Anti-Rabbit Atto 488, Sigma-Aldrich) incubated for 30 mins along with 1:100 phalloidin (Alexa Fluor™ 647 Phalloidin, Thermo Fisher Scientific). Images show the maximum intensity projection of 3 Z stacks 200 µm apart acquired using near-Total Internal Reflection Fluorescence (near-TIRF) illumination. In normal conditions, K255E+/- cells are slightly larger and show more developed actin stress fibers compared to WT cells (untreated, actin column) in agreement with observations on homozygous K255E+/- mutant cells. K255E+/- cells show less specific ACTN4 localization to both actin stress fibers and regions of increased actin enrichment (untreated, ACTN4 and merged column), suggesting that K255E ACTN4 does not preferentially bind to actin filaments under tension or active reorganization, in agreement with its binding kinetics. K255E+/- cells also show little preferential actin enrichment at the cell leading edge suggesting an impaired cell motility machinery, consistent with the mutant’s reduced motility reported previously. For both WT and K255E+/- cells, Blebbistatin treatment reduces the number of visible stress fibers and causes highly irregular cell membrane shape at the cell periphery (Blebbistatin treatment, actin column), indicating a disruption in the actomyosin machinery. Under Lat A treatment, actin is most visible at the cell periphery (Lat A treatment, actin column), indicating that thick bundles and dense actin networks at the cell periphery are able to resist Lat A better compared to single or weakly bundled actin filaments in the cytosol, consistent with Lat A’s expected effect. Overall, we observe only subtle differences between the WT and K255E+/- mutant cells, which may also explain the subtle differences we measure in cytoplasmic viscoelasticity. Careful quantification of these actin and ACTN4 localization differences in the future would help in further interpreting the mechanical measurements. Finally, it is important to note that cells under both Blebbistatin and Lat A treatment only show mild signs of actin cytoskeleton disruption. Indeed, the concentrations of Blebbistatin and Lat A were chosen to cause a mild disruption of the actin cytoskeleton to ensure cells were viable for mechanical measurements and no large-scale morphological changes were observed.
Supplementary Figure 8 | Loss tangent change over time of different cells after the one-hour recovery from hypotonic shock. Each point represents the change in loss tangent after an additional 25-45 mins (red, $t = 25$-45 min) or 45 mins (blue, $t = >45$ min) has elapsed since the completion of the one-hour recovery (not shown for clarity, $t = 0$ min). Each point is the ratio of the loss tangent over its corresponding initial value as indicated at the top of the plot. A set of experiment with WT cells (circles) shows the change in loss tangent of two measurements performed after 25-45 mins (red circles) and one measurement performed after over 45 mins (blue circle). Within this set of experiments, the red and blue circles do not show any consistent patterns with the change in time. Similarly, within a set of experiment with K255E cells (diamonds) the red and blue diamonds also do not show any consistent patterns. Moreover, the changes in loss tangent of both experimental sets taken together (all red and all blue symbols) do not show any observable patterns and they cluster at ~1, indicating little changes in mechanics over time. Both sets of experiments were performed under control conditions (no drug treatments).
Supplementary Figure 9 | Raw frequency response measurements. The frequency response measurements (mean +/- SEM) recorded for each condition (WT: gray, K255E +/-: light blue, WT blebbistatin: black, K255E +/- blebbistatin: dark blue). The moduli (Fig. 3,4) were calculated using these frequency responses (see Methods). The mechanical responses are sensitive to perturbations to crosslinking of the actin network, particularly at long timescales.
Supplementary Information

Data handling. To collect data over several periods of excitation, a single measurement typically lasts 220 seconds or longer and active oscillations across all ~17 frequencies are continuously being applied. The frequency response function (FRF), i.e. the transfer function $H(\omega)$, is obtained using Welch’s method, implemented in MATLAB with the “tfestimate” function. The Hamming window function is used and gives a slightly better response in terms of magnitude and coherence compared to the Hann window. The transfer function estimate is obtained at each input frequency separately with separate window lengths (NFFT). The window length is chosen to be an integer multiple of the input frequency to minimize signal leakage into adjacent frequency bins. The per cent overlap between windows is based on empirical observations to yield the maximum magnitude and maximum coherence. At the lowest frequencies, less periods of excitation are contained in the measurement total time and the per cent overlap is thus chosen to be larger to allow more averaging over the ~4 windows. At higher frequencies, the overlap is chosen to be 50% over the 6-8 windows. All windows and overlap amounts are chosen such that at most, ~15 % of the data is truncated at the end. Indeed, as we have a finite sample length, truncation of the data occurs when the windows don’t exactly add up to the total length. Overall, the aforementioned window lengths and overlaps yield the best balance between frequency resolution and averaging. However, a more detailed and quantitative characterization may help for future application of this method. The magnitude and phase obtained are fit to the active part of the viscoelastic model discussed further below.

The power spectrum used for the thermal response is obtained using the same data. The peaks that correspond to the active oscillations are cropped out based on the known excitation frequencies. The remaining power spectrum represents the thermal response. For data that is acquired at 200kHz downsampled to 20kHz using a Field Programmable Gate Array, a pole is added to the viscoelastic model (corner frequency ~ 3000 Hz) to account for this low pass filter coming from the downsampling by averaging. Sampling at 200kHz downsampled to 40kHz shifts this filtering to higher frequencies, confirming that the averaging is acting like a low pass filter (data not shown). Data acquired directly at 20kHz show a reduced signal to noise ratio compared to oversampled and averaged data, where the noise floor is visible on the power spectrum at high frequencies (Supplementary Fig. 1b). In these cases, the power spectrum is fit only up to the point where this upwards shift is observed.

In situ calibration. From the mechanical circuit (Fig. 2b), for the case where the stage is stationary and by making the following substitution: $\Delta x(t) = x_{\text{bead}}(t) - x_{\text{trap}}(t)$ and $k_{\text{cyt}}(t) = k_{\text{cyt,0}} + k_{\text{cyt,1}}(t^{(\alpha-1)})$, the following equation of motion can be obtained

$$m(\ddot{x}_{\text{trap}}) + \gamma_{\text{cyt}}(\dot{x}_{\text{trap}}) + (k_{\text{cyt,0}} + k_{\text{cyt,1}}t^{(\alpha-1)})(x_{\text{trap}}) = -m(\ddot{x}) - \gamma_{\text{cyt}}(\dot{x}) - k_{\text{trap}}(\dot{x}) - (k_{\text{cyt,0}} + k_{\text{cyt,1}}t^{-(\alpha-1)})(\Delta x) + F_{\text{thermal}}$$

Substituting the cytoplasmic stiffness ($k_{\text{cyt}}$) by the sum of the constant stiffness ($k_{\text{cyt,0}}$) and the frequency dependent stiffness ($k_{\text{cyt,1}}(t^{(\alpha-1)})$) observed in semiflexible polymer network allows the model to account for some of the viscoelastic properties of the cytoplasm. Looking at the active part only (i.e. $F_{\text{thermal}} = 0$), neglecting inertial forces ($\ddot{x}_{\text{trap}} = 0$) and taking the Laplace transform at steady state ($s = j\omega$), the following transfer function $H(\omega) = \Delta X(\omega) / X_{\text{trap}}(\omega)$ is obtained:

$$H(\omega) = \frac{V_{pd}(\omega)}{X_{\text{trap}}(\omega)} = \frac{1}{\beta_{pd}} \times \frac{\gamma_{\text{cyt}}j\omega + k_{\text{cyt,0}} + \frac{k_{\text{cyt,1}}(j\omega)^{\alpha}}{\Gamma(\alpha)}}{\gamma_{\text{cyt}}j\omega + k_{\text{trap}} + k_{\text{cyt,0}} + \frac{k_{\text{cyt,1}}(j\omega)^{\alpha}}{\Gamma(\alpha)}}$$

Where $V_{pd}(\omega)\beta_{pd} = \Delta X(\omega)$ is used and represents the voltage from the photodiode and the photodiode calibration constant respectively. For visualization, if one ignores the complex cytoplasmic stiffness ($k_{\text{cyt,1}} = 0$), the numerator is a single zero proportional to $1/k_{\text{cyt,0}}$ and the denominator is a single pole proportional to the inverse of the total stiffness, $1/(k_{\text{trap}} + k_{\text{cyt,0}})$. The zero and pole are responsible for the two inflection points in the magnitude response, leading to the sigmoidal curve observed in the magnitude response obtained experimentally (Fig. 2b and Supplementary Fig. 1a). The addition of the frequency dependent stiffness shifts the zero and pole positions and changes the sharpness of the transition between the two. The magnitude and phase of EQ (1) is what we fit to our experimental $H(\omega)$ obtained through tfestimate as outlined above.

Looking at the passive part only ($X_{\text{trap}} = 0$) and taking the Laplace transform at steady state as before, the following expression is obtained:
This complex response function \( \chi(\omega) \) can be related to the complex shear modulus through Stokes relation:

\[
G_{\text{th}}(\omega) = \frac{1}{6\pi R} \chi(\omega)
\]

Here, \( G_{\text{th}}(\omega) \) represents the theoretical complex shear modulus based on the simple viscoelastic model, and \( R \) is the radius of the spherical probe. From the fluctuation-dissipation theorem, the power spectral density \( \langle x_{\text{pd}}^2 \rangle \) of the bead can be related to this response function \( \chi(\omega) \) or equivalently, to \( G_{\text{th}}(\omega) \) as follows:

\[
\langle V_{\text{pd}}(\omega)^2 \rangle = \frac{1}{\beta^2} \frac{2k_B T}{6\pi R \omega} \frac{G_{\text{th}}''(\omega)}{|G_{\text{th}}(\omega)|^2}
\]

Where the following substitution has been done \( \langle x_{\text{pd}}^2 \rangle = \beta_{\text{pd}}^2 \langle V_{\text{pd}}(\omega)^2 \rangle \), \( V_{\text{pd}} \) is the voltage measured by the photodiode, \( k_B \) the Boltzmann constant and \( T \) the temperature. EQ (4) is the equation fit to the power spectrum, with \( G_{\text{th}}(\omega) \) defined by EQ (2) through EQ (3). The corner frequency in the power spectrum is the result from the sum of the total stiffness of the system and corresponds to the pole in the magnitude response described above. \( \alpha \) dictates the slope after the corner frequency, with \( \alpha=1 \) corresponding to purely diffusive behavior and a slope of 2 in the power spectrum, and \( \alpha<1 \) corresponding to constrained diffusion with a slope < 2 in the power spectrum. For more details on the different parameters of the model, see reference 13.

Once the calibration is done, the complex shear modulus \( G(\omega) \) of the real cytoplasm can be directly obtained using the experimental transfer function \( H(\omega) \) of the measurement following reference:

\[
G(\omega) = \frac{1}{6\pi R} \frac{1 - k_{\text{trap}} A(\omega)}{A(\omega)}
\]

Where \( A(\omega) \) is the apparent complex response function (See equations (4) and (5) in reference 5) which can be directly related to the experimental transfer function \( H(\omega) \).

With the previously calibrated \( k_{\text{trap}} \) and \( \beta_{\text{pd}} \) calibrated from EQ (1) and EQ (4) and the experimental \( H(\omega) \), \( A(\omega) \) can be solved using EQ (6) and the complex shear modulus \( G(\omega) \) of the actual cytoplasm can be obtained using EQ (5).

Simple viscoelastic model fitting. A global fitting algorithm is used to fit simultaneously EQ (1) and EQ (4) to the FRF and power spectrum of the data respectively. As mentioned previously, only frequencies above \( \sim 1 \) Hz in the FRF and frequencies above \( \sim 200 \) Hz in the power spectrum are included in the fit (Supplementary Figure 1). Occasionally, despite the addition of an extra pole compensating for the filtering effect observed when data acquisition was done with downsampling (Supplementary Figure 1b, right), or direct sampling where filtering effect was not observed (Supplementary Figure 1b, left), the FRF at higher frequencies showed the presence of an extra pole, causing a shift in magnitude and phase. As the simple viscoelastic model did not include a term to correct for this shift, fitting of the FRF was stopped before reaching these frequencies. The last point in the magnitude plot in Supplementary Figure 1a left shows such a dip in magnitude, indicative of filtering at higher frequencies. Further experiments and quantification of this phenomenon may help to isolate the source and correct the data above \( \sim 500 \) Hz allowing the higher frequencies to be reliably obtained (frequencies past \( \sim 500 \) Hz were not included in this manuscript). A weight function was applied to the FRF and power spectrum to account for the large difference in number of points (\( \sim 11 \) for the FRF, and thousands for the power spectrum). A second weight function was applied to the FRF to weight the magnitude response more than the phase as the latter appeared to be more sensitive to sources of error. Each fit begins with an initial guess that is manually found to be close to the solution and with tighter bounds to restrict the algorithm from finding a local minimum in a completely separated region in the parameter space. A second fit is done based on the output of the first fit with looser bounds.

Bootstrapping and cell mechanics model fitting. Bootstrapping with replacement is done by generating \( k \) numbers randomly drawn from 1 to \( N \). \( N \) is the total number of data sets per condition, and \( k \) is the minimum amount of data available at any given frequency. For example, in the case of untreated WT, the number of data points available per frequency varied from 14 to 17, due to slightly different excitation inputs. In that case, \( k = 14 \), and \( N = 17 \). This
bootstrapping is repeated 1000 times to obtain 1000 bootstrap samples. The mean of these samples is first manually fit to either the power law or dynamic crosslinking model to obtain an initial guess of the parameters. Then, each bootstrap sample is automatically fit to either model using the corresponding initial guess. Each bootstrap sample is fit 3x, starting at the initial guess with stricter bounds, and passing the best parameters found for the next 2 subsequent fits with increasingly looser bounds.

References

1. Atakhorrami, M et al. Scale-dependent nonaffine elasticity of semiflexible polymer networks. Phys. Rev. Lett. 112, 088101, doi:10.1103/PhysRevLett.112.088101 (2014).
2. Gurmessa, B. et al. Counterion crossbridges enable robust multiscale elasticity in actin networks. Phys. Rev. Res. 1, 013016, doi:10.1103/PhysRevResearch.1.013016 (2019).
3. Gittes, F., Schnurr, B., Olmsted, P. D., C., M. F. & F., S. C. Microscopic viscoelasticity: shear moduli of soft materials determined from thermal fluctuations. Phys. Rev. Lett. 79, doi:10.1103/PhysRevLett.79.3286 (1997).
4. Broedersz, C. P. et al. Measurement of nonlinear rheology of cross-linked biopolymer gels. Soft Matter 6, 4120–4127, doi:10.1039/c0sm00285b (2010).
5. Mizuno, D., Head, D. A., MacKintosh, F. C. & Schmidt, C. F. Active and passive microrheology in equilibrium and nonequilibrium systems. Macromolecules 41, 7194-7202, doi:10.1021/ma801218z (2008).
6. Ewoldt, R. H., Johnston, M. T. & Caretta, L. M. in Complex Fluids in Biological Systems Ch. 6, 207-244 (Springer, 2015).
7. Staunton, J. R., Blehm, B., Devine, A. & Tanner, K. In situ calibration of position detection in an optical trap for active microrheology in viscous materials. Opt Express 25, 1746-1761, doi:10.1364/OE.25.001746 (2017).
8. Blehm, B. H., Devine, A., Staunton, J. R. & Tanner, K. In vivo tissue has non-linear rheological behavior distinct from 3D biomimetic hydrogels, as determined by AMOTIV microscopy. Biomaterials 83, 66-78, doi:10.1016/j.biomaterials.2015.12.019 (2016).
9. Feng, D. et al. Disease-causing mutation in alpha-actinin-4 promotes podocyte detachment through maladaptation to periodic stretch. Proc Natl Acad Sci U S A 115, 1517-1522, doi:10.1073/pnas.1717870115 (2018).
10. Ehrlicher, A. J. et al. Alpha-actinin binding kinetics modulate cellular dynamics and force generation. Proc Natl Acad Sci U S A 112, 6619-6624, doi:10.1073/pnas.1505652112 (2015).
11. Hendricks, A. G., Holzbaur, E. L. & Goldman, Y. E. Force measurements on cargoes in living cells reveal collective dynamics of microtubule motors. Proc Natl Acad Sci U S A 109, 18447-18452, doi:10.1073/pnas.1215462109 (2012).
12. Lau, A. W., Hoffman, B. D., Davies, A., Crocker, J. C. & Lubensky, T. C. Microrheology, stress fluctuations, and active behavior of living cells. Phys Rev Lett 91, 198101, doi:10.1103/PhysRevLett.91.198101 (2003).
13. Hendricks, A. G. & Goldman, Y. E. Measuring Molecular Forces Using Calibrated Optical Tweezers in Living Cells. Methods Mol Biol 1486, 537-552, doi:10.1007/978-1-4939-6421-5_21 (2017).