A. Additional methods

Protein preparation

Actin was purified from rabbit skeletal muscle acetone powder (Pelfreez, Inc.) [s1], labeled on random lysines with NHS-rhodamine (Pierce Biotechnology, Inc.), and cycled repeatedly to ensure polymerization competency. *Xenopus laevis* extract was prepared as previously described [s1]. Following isolation, crude extract was aliquoted and stored at -80°C. Extract mix was prepared by diluting crude extract to 50% with *Xenopus* buffer (XB) (100 mM KCl, 0.1 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM EGTA, 10 mM HEPES pH 7.7), spining through Durapore® 100 nm centrifugation filters (Millipore) to remove large particulates, and adding 37.5 µl of 50% extract to 1.7 µl of energy mix (150 mM creating phosphate, 2mM EGTA, 20 mM $MgCl_2$, 20 mM ATP pH 7.4) and 1.7 µl of rhodamine-actin (1.7 mg/ml).

Dual-cantilever AFM

A dual-cantilever AFM has been developed to provide increased long-term stability for actin network measurements [s2]. Briefly, two silicon nitride cantilevers, supported by the same rigid support (Veeco Metrology, Inc.), are monitored with separate optical levers and position sensitive detectors (PSD, Pacific Silicon Sensor, Inc.) to allow accurate long timescale measurements of actin network growth. The PSD signals were calibrated before each experiment by incrementally driving the glass (bottom) surface into the cantilevers with an extremely accurate (resolution better than 0.05 nm) piezoelectric positioner (Physik Instrumente, GmBH) and subsequent fitting of the resulting PSD signal-position curve. Calibration data were used for real-time software feedback while experimental data signals from each PSD were anti-alias filtered with an 8-pole Butterworth filter (Krohn-hite, Inc.), recorded at 100 Hz with a PCI-6053E data acquisition board (National Instruments, Inc.), and saved for offline processing. Custom software written in LabView 7 (National Instruments, Inc.) was used to control data acquisition, calibration, cantilever feedback, and piezoelectric stage position for the instrument. Epi-fluorescence imaging was accomplished using a 32X 0.4NA objective (Carl Zeiss, Corp.) and a low-light cooled charge coupled device (CCD) (QImaging, Corp.).

During each experiment, the instrument was operated as a force-clamp and microrheometer. In a force-clamp, one cantilever was used as a reference to the bottom surface while the other cantilever was functionalized to nucleate actin network growth. Software feedback was used to hold the actin-associated cantilever's deflection, and therefore force, constant while the signal from the other cantilever reflects drift-corrected changes in network length under the specified load. As a microrheometer, the force-clamp was disengaged, and the bottom surface was sinusoidally driven at specified frequencies while both cantilever positions were recorded at 1 kHz and saved for offline processing.

Experimental setup

Mechanical property experiments were performed with a cantilever having a mean stiffness of 100 pN/nm. This cantilever was dipped into a solution of 0.4 mg/ml ActA as described previously [s2] followed by immersion in 10 mg/ml bovine serum albumin (BSA) to prevent nonspecific adsorption of proteins from the extract mix during the experiments. No other cantilever was dipped in ActA. The cantilever was then loaded into the fluid cell, placed in the AFM, positioned near a piezoelectric-driven glass surface and immersed in extract mix. The surface was then finely adjusted, the PSD signals were calibrated, and a force-clamp was engaged to hold the network under a constant stress (~ 6 Pa) at the outset of each experiment.

Cantilever preparation

Uncoated silicon nitride cantilevers (Veeco Metrology, Inc.) were cleaned of organic contaminants using Piranha (3:1 sulphuric acid: hydrogen peroxide), rinsed in deionized water, and dried. Drying was followed by chemical vapor deposition (CVD) of amino-propyl-trimethoxy-silane (APTMS, Sigma Aldrich, Inc.) onto the cantilevers, after which cantilevers were stored under vacuum and used in experiments within 3 days.

Data Analysis for Fig. 3B

For the averaged normalized nonlinear elasticity trace shown in Fig. 3B of the text, the normalization parameters E_{min}, E_{max}, and σ_c for each individual set of measurements (corresponding to one nonlinear elasticity trace) was first determined from a sliding 5-point boxcar window for increasing stress. Then the traces (both for increasing and decreasing stresses) were normalized by these parameters. Each set of measurements was binned into 40 log-spaced bins between $0.005\,\sigma_c$ and $3\,\sigma_c$. All individual data sets for increasing stresses were then averaged together to produce the final averaged normalized (black) trace shown in Fig. 3B of the manuscript, as were all data sets for decreasing stresses (Fig. 3B, red trace). Each point represents data from at least 4 measurements though most represent data from more than 10 measurements.

B. Cantilever-surface interaction

To account for hydrodynamic influences between the oscillating surface and cantilever, we performed control measurements in which the surface was held at specific distances from the cantilever and oscillated at all frequencies. Our controls show that the interaction due to hydrodynamic effects is at most a 2 nm oscillation of the cantilever when the standoff distance is 0.5 µm, which is a spacing that was rarely achieved in any microrheology/nonlinear elasticity experiments (Fig. S1). The typical minimum spacing between the cantilever and surface in our experiments is ~ 1.5 µm, a spacing at which the cantilever amplitude of oscillation is less than 1 nm and is approaching the inherent Brownian fluctuations of the cantilever itself. Furthermore, this interaction shows no clear frequency dependence at any standoff distance measured, which would be expected for a hydrodynamic coupling between the surface and cantilever [s3] .

Another possible source of error in our measurements is time-dependent changes in the extract mix. It has been shown that cytoskeletal proteins in *Xenopus* extract will polymerize over time, which may change the mechanical properties [s4]. Control measurements where the cantilever was held 3 µm away from the surface showed little to no change in coupling after 100 minutes of immersion (Fig. S2.).

Figure S1

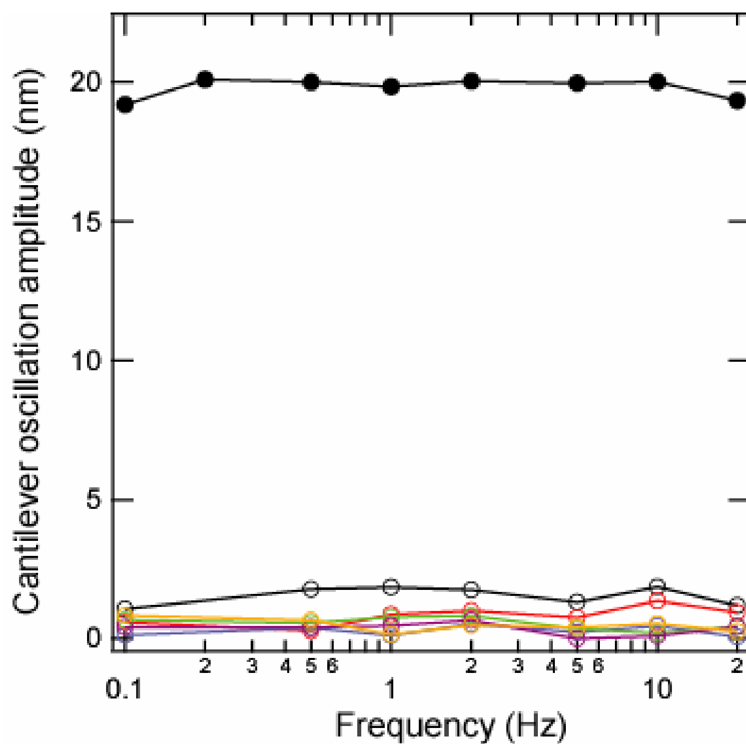

Fig. S1. Control experiment in extract mix showing cantilever-surface coupling as a function of standoff distance. Open circles correspond to standoff distance of 0.500 µm (black), 1.5 µm (red), 2.5 µm (blue), 3.5 µm (green), 4.5 µm (magenta), and 5.5 µm (orange). Filled circles show cantilever deflection when in contact with the bottom surface, which matches the drive amplitude of 20 nm within experimental error. Cantilever oscillation is always less than 1 nm over relevant distances in our experiments when there is no actin network (1.5 µm and greater).

Figure S2

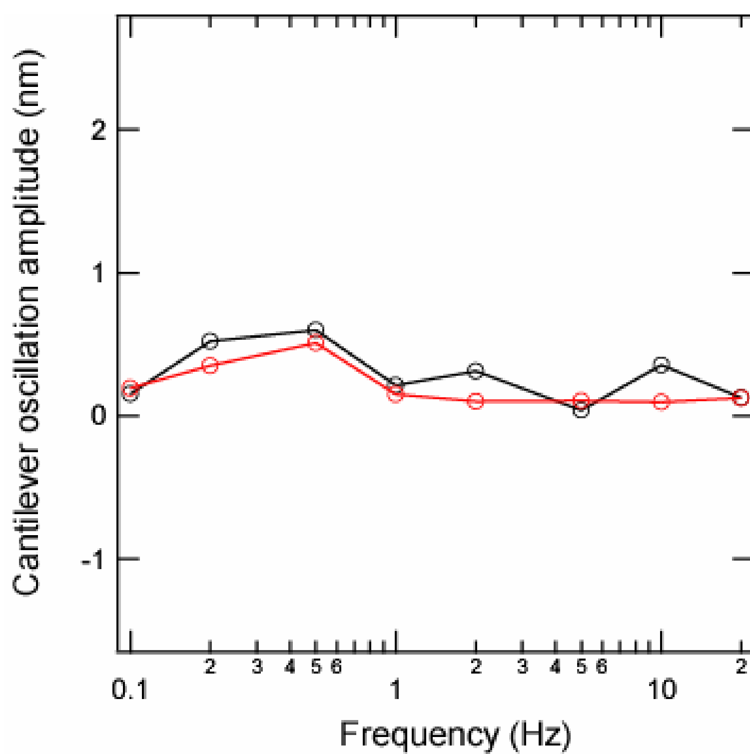

Fig. S2. Cantilever-surface coupling in extract mix does not change with time. Black circles show coupling at t = 0 minutes after extract immersion and red circles show coupling at t = 100 minutes. The standoff distance used in this experiment was 3 µm.

C. Normalization method to determine power law

One particular measurement challenge had to be overcome before mechanical property data could be interpreted. Actin networks exhibit nonlinear elasticity, in which the elastic modulus increases with applied stresses beyond a critical value. We found that network growth during the rheology measurements slowly deflected the cantilever during the frequency sweep measurement thus affecting elasticity measurements due to the nonlinear elasticity of the network. To account for this, we performed the following normalization procedure: each measurement at a particular frequency was preceded and followed by a measurement at 5 Hz. The viscous and elastic moduli obtained for that measurement were then normalized by the average elastic modulus from the two 5 Hz measurements. This method yields relative viscous and elastic moduli that are always compared to the 5 Hz elasticity, and is valid provided that the power law does not change significantly over the range of stresses traversed during one frequency sweep. We found this to be true (Fig. S3). In each frequency sweep, measurements were first done from 20 Hz to .1 Hz and then repeated from 1 Hz to 20 Hz. The power law obtained from the first segment of the sweep (20 Hz – 1 Hz) with an average stress of 11 Pa was found to be $x = 0.108$ (Fig S3, red dotted line), while the power law over the second segment of the sweep (1 Hz – 20 Hz) was found to be $x = 0.104$ (Fig S3, black dotted line) over an average stress of 24 Pa. This validates the application of this normalization procedure in determining the overall power law behavior.

Figure S3

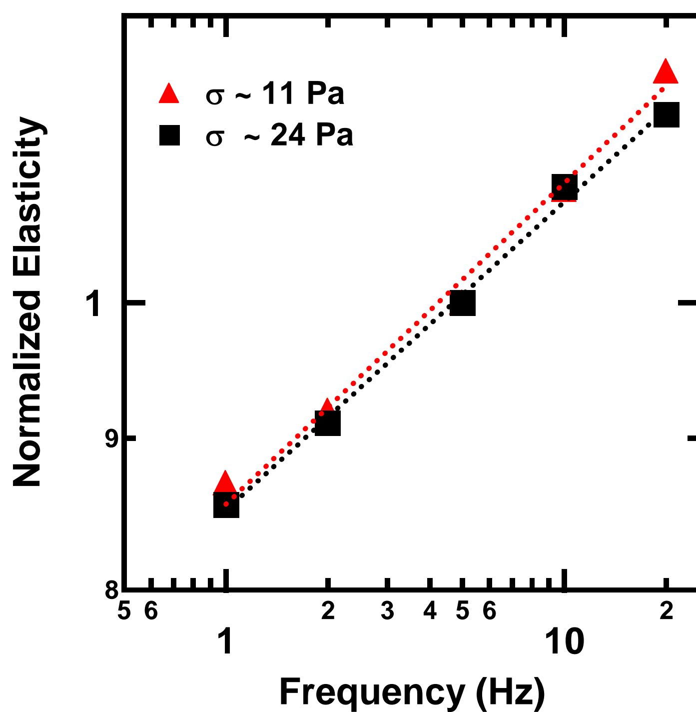

Fig. S3. Power law does not change significantly during a frequency sweep due to growth of the network. Power law for measurements taken over an average stress of 11 Pa (n = 22, red triangles) is $x = 0.108$ (red dotted line) while the power law for measurements taken over an average stress of 24 Pa (n = 22, black squares) is $x = 0.104$ (black dotted line).

D. Myosin inhibition experiments

To determine whether there was any myosin dependent prestressing of the networks in these experiments, we performed frequency dependent microrheology experiments in the presence of 50 μM of blebbistatin (Sigma Aldrich, Inc.). Blebbistatin is a known myosin II inhibitor[s5]. The average elasticity at 5 Hz of these networks was 1.6 kPa (n = 2 trials, 20 measurements), among the higher range of elasticities seen without blebbistatin, demonstrating that myosin dependent prestress does not play a role in the elasticity of the dendritic actin networks studied here.

References

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