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Cofilin-mediated filament softening and crosslinking counterbalance to enhance network flexibility

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Abstract

F-actin crosslinking within the cell cytoskeleton mediates the transmission of mechanical forces, enabling changes in cell shape, as occurs during cell division and cell migration. Crosslinking by actin binding proteins (ABPs) generally increases the connectivity of the F-actin network, but also increases network rigidity. As a result, there is a narrow range in the concentration of crosslinker protein at which F-actin networks are both connected and labile. Another ABP, cofilin, severs F-actin filaments at high pH through increasing their bending flexibility and concentrating mechanical stress, inducing fragmentation. By contrast, at low pH, cofilin increases filament flexibility yet does not sever. Instead, it forms disulfide bonds, which crosslink F-actin into bundles, and bundles into networks. Here, we combine light microscopy and rheology to determine the impact of two potentially opposing effects on the mechanics of F-actin networks— increased flexibility at the filament level, and increased connectivity at the network level. Indeed, by linear rheology, we find that these mechanisms are counterbalanced, such that cofilactin network moduli are only weakly dependent on cofilin concentration over a broad range, in contrast to the dramatic stiffening that occurs with F-actin crosslinking protein. Further, by non-linear rheology, the network stiffens at a higher stress than crosslinking protein, indicative of a broader range in which the material remains flexible. These results may enable F-actin networks to increase connectivity without heavy penalties to rigidity, and thus provide a new route to modulating active polymer mechanics unseen using traditional F-actin accessory proteins.

The actin cytoskeleton mediates the generation and transmission of mechanical stresses, critical for modulating cell shape, as occurs for essential behaviors from cell migration and division[1,2]. The actin cytoskeleton is composed of polymerized actin monomers (G-actin) that form a helical filament (F-actin). F-actin is highly dynamic, constantly undergoing polymerization (addition of actin monomers) and depolymerization (removal of actin monomers). This dynamic ‘turnover’ is regulated by actin-binding proteins (ABPs) like profilin, which promotes polymerization, and ADF/cofilin which promotes depolymerization. This activity is critical for the turnover and recycling of actin filaments in the cell. Further, there are also F-actin crosslinkers, which are proteins that bind to F-actin and link them together into complex, three-dimensional networks. These proteins play critical roles in organizing the actin cytoskeleton. Typically, F-actin crosslinking increases the connectivity of the network, while also increasing its rigidity[3-5]. By contrast, turnover decreases connectivity, thereby decreasing rigidity and increasing fluidity[6]. Thus, to date, network connectivity constrains the deformability of F-actin networks, restricting the range at which active stresses can determine the shape of the cell. Differentially modulating filament mechanical properties and network connectivity separately may circumvent this limitation.

Cofilin initiates depolymerization of F-actin by severing filaments through a multi-step process that involves binding to the filament and altering its structure, leading to filament breakage[7-10]. First, cofilin binding to actin is cooperative - the binding of one cofilin molecule increases the likelihood of additional cofilin molecules binding nearby[9-11]. This leads to localized clusters of cofilin molecules on the actin filament, which creates interfaces between bare actin, and cofilin-coated actin (‘cofilactin’). Upon binding to F-actin, cofilin induces a change in the twist of the actin filament[12]. This conformational change weakens the interactions between actin subunits in the filament, especially at the junctions between cofilin-decorated and undecorated segments. The weakened regions are more susceptible to mechanical forces, leading to filament breakage (severing). Severing creates new ends on the actin filament, and thereby provide additional opportunity to polymerize or depolymerize. As severing reduces F-actin length, it also impacts network connectivity, leading to altered mechanical properties of F-actin networks, as measured *in vitro* [13] and observed *in vivo* [14]. However, severing is known to be strongly pH dependent, occurring for pH >7.3 [6,7,9-11,15-18]. By contrast, at low pH (<7.3), the severing of cofilin is

strongly inhibited, mainly due to an increase in the binding affinity of cofilin to actin, leading to fewer interfaces between the cofilin coated and uncoated region on the actin filaments[19]. In this case, the cofilin coated region is protected against spontaneous depolymerization (Fig. 1a) [19-23]. Further, cofilactin forms bundles through the creation of disulfide bonds between cofilin monomers [24,25]. These bonds are covalent, and thus highly stable. Thus, the conformational changes that lead to weakening of the filament may be combined with F-actin crosslinking, leading to new pH-dependent mechanical properties of F-actin networks unachievable with other F-actin binding proteins.

In this letter, we explore for the first time, the combined structural and rheological properties of cofilactin networks. For the former, we utilize fluorescent light microscopy to assess the impact of cofilin on the bundling and bending of single filaments. For the latter, we use a cone-and-plate rheometer to measure the mechanical properties on the network scale. Thus, we measure the storage modulus ($G'(\omega)$) and loss modulus ($G''(\omega)$) as a function of **angular** frequency, ω , for different concentrations of cofilin (See Supplemental Material [url], which includes [26,27]). In comparing single filament to network scale measurements, we learn how cofilin binding and crosslinking affects the material properties of the F-actin network. We then contrast these results with those of traditional crosslinking proteins such as α -actinin and fascin to highlight the novel, pH-dependent material properties of cofilactin.

The addition of cofilin decorates and crosslinks the F-actin network over time (Fig. 1b-c, Supplement). From microscopy images, the cofilactin network becomes more bundled during polymerization (Fig. 1c, Supplement). To quantify the degree of bundling, we perform a fluorescence intensity linescan over 20~30 μm on the image across the bundles, and calculate the bundling parameter λ , as:

$$\lambda = \frac{\nu}{\mu} \quad (1)$$

, in which μ is the mean fluorescence intensity along the linescan, and ν the standard deviation (Fig. 1d, Supplement)[28]. For experiments with the same actin concentration (same magnitude of total fluorescence intensity), a less bundled and more uniformly distributed network will result in

lower λ (as the intensity approaches uniformity, $\lambda \rightarrow 0$). By contrary, if the network is extremely bundled, then ν increases while μ remains the same, and $\lambda \rightarrow \infty$. The bundling/crosslinking of F-actin through cofilin-mediated disulfide bonds is reversed by adding 20 mM of dithiothreitol (DTT) (Fig. 1e). DTT is a reducing agent, which breaks disulfide bonds between cysteine residues[29,30]. We find that λ increases with Φ_c , suggesting an increase in bundling of the filaments in the network as more cofilin is introduced (Fig. 1d, f). Accordingly, the network also becomes more porous as Φ_c increases with cofilin concentration (Supplement). To understand physical properties of cofilactin, we explore the mechanical changes on the filament and network scale respectively.

First, we measure mechanical changes to individual F-actin by cofilin. At pH > 7.3, the binding of cofilin changes the twist of F-actin and softens the filaments[8,10,12,31,32]. However, it is unknown if the same impacts on filament mechanics are observed at a lower pH. Therefore, we investigate the mechanical properties of cofilactin networks at pH = 6.5. To do so, we measure the change in persistence length (L_p) of individual actin filaments under varying pH. Thus, we polymerize cofilactin filaments at different Φ_c , and add the filaments at low density on a supported lipid bilayer surface (Supplement). The bilayer surface is fluid, and should not deter the fluctuations of the filaments, yet enable us to visualize the filaments in two dimensions. The thermal fluctuations cause lateral bending of the filaments, and we can calculate the angular correlation function[7]:

$$C_L(s) = \langle \cos[\theta(s) - \theta(0)] \rangle = \exp\left(-\frac{s}{2L_p}\right) \quad (2)$$

, where s is the contour length, $\theta(s)$ is the angle at contour length s , and L_p is the persistence length. Indeed, we observe that as Φ_c increases, L_p decreases from $\sim 12 \mu\text{m}$ to $7 \mu\text{m}$, consistent with results at neutral to basic pH (Fig. 1f, Supplement). In our experiments, we avoid using phalloidin to stabilize F-actin as it binds to actin, increases the stabilized filament length and persistence length[33,34], but also competes with ABPs such as cofilin[12,35].

Second, we measure mechanical changes to F-actin networks by cofilin. We polymerize the F-actin network on a stress-controlled rheometer and between a 1° cone-and-plate geometry with $140 \mu\text{m}$ gap spacing (Supplement). As the cofilactin network polymerizes, the network's elastic

and viscous moduli, $G'(\omega)$ and $G''(\omega)$ respectively, increase moderately, and both reach plateau within 2 hours (Supplement). Once the network's polymerization is complete ($G'(\omega)$ and $G''(\omega)$ reach plateau), to explore the viscoelasticity of the network in the linear regime, we perform strain-controlled ($\gamma = 1\%$) frequency sweep. The storage modulus (G') of the network has a moderate increase of roughly an order of magnitude compared to the entangled F-actin network (Fig. 2a-b). Again, crosslinking of cofilin through disulfide bonds is reversed by adding 20 mM of DTT (Fig. 2c). In the linear regime, the elastic modulus G' scales with **angular** frequency as $\sim \omega^\beta$. As the ratio between cofilin and actin (Φ_c) increases, G' of cofilactin network becomes less dependent of ω , and the exponent β decreases from 0.35 to 0.15 (0.16 to 0.09 for α -actinin and 0.2 to 0.085 for fascin), which suggests an increase in crosslinking (Fig. 2d)[36-39]. Actin networks crosslinked by α -actinin or fascin have a crosslinker concentration-dependent plateau modulus (G_0) from intermediate to high crosslinking regime as increase in connectivity almost always leads to a more rigid network[38-42] (Fig. 2e). However, the plateau modulus G_0 of the cofilactin network is weakly dependent of Φ_c at low crosslinking regime ($\Phi_c < 0.1$) with a power law of $G_0 \sim \Phi_c^{0.1}$, and becomes independent of Φ_c at the high regime ($\Phi_c > 0.1$) (Fig. 2e). Thus, there is an increase in connectivity of the network without a significant increase in rigidity. This result is distinct from those of which the network is crosslinked by crosslinkers such as scruin, α -actinin, fascin, filamin, or transient active crosslinkers (TAC), e.g. heavy meromyosin[38,40-49]. We hypothesize that weakly Φ_c -dependent network moduli arise from the counterbalance effect of cofilin crosslinking at the network scale (mesh size ξ) and softening at the filament scale (bending flexibility κ_0 , and inter-crosslinker distance l_c) (See Supplemental Material [url], which includes [50]). As F-actin networks in the cytoskeleton are often under active mechanical stress imparted by myosin motors at ~ 100 pN per thick filament[51,52], large deformations can occur (e.g. astor formation or network rupture) which significantly alter **their** mechanical properties. F-actin crosslinked networks can exhibit highly nonlinear elasticity when driven by nonequilibrium athermal forces during complex biological activities[53,54]. Therefore, the elasticity of the network is highly dependent of the stress that is applied to the network[38-41,53,55,56]. Thus, to understand how cofilactin network's mechanical properties change under large stress to gain further insights on cell mechanics during complex cytoskeletal activities, we explore the cofilactin network in the nonlinear regime.

The complexity of Large Amplitude Oscillatory Shear (LAOS) may not be required for the purpose of this study [57-61] (See Supplemental Material [url], which includes [57,59-61]). Therefore, we utilize the ‘prestress’ method[36,38-43,53].

For the prestress method, we apply a step shear prestress, σ_0 , and superimpose with a small oscillatory stress, $\delta\sigma = |\delta\sigma|e^{i\omega t}$, which is less than 10% of the prestress in the linear regime so that linear response theory still holds (Supplement) [38,41,42] ($|\delta\sigma| \leq \sigma_0/10$). The complex differential modulus K^* is:

$$K^*(\omega, \sigma_0) = \frac{\delta\sigma(\omega)}{\delta\gamma(\omega)}|_{\sigma_0} \quad (3)$$

. In the linear regime, the differential elastic modulus $K'(\omega, \sigma_0)$ and loss modulus $K''(\omega, \sigma_0)$ are equal to $G'(\omega)$ and $G''(\omega)$ respectively[39]. Once σ_0 exceeds critical value, σ_c (onset of the nonlinear regime, determined by 5% deviation from plateau value), K' increases for all cases as shown in Fig. 3a[38-42,53,54,57,62]. This is indicative of strain hardening behavior, typically a result of filaments forming stress-bearing bundles along the axis of shearing[63-65]. For both α -actinin and fascin crosslinked network, the critical stress σ_c decreases as crosslinker density increases, results in rigidifying of the network and less room for filament reconfiguration[44,56,66] (Fig. 3b). By contrast, σ_c for cofilactin increases with Φ_c , potentially from the filament mechanical property change of F-actin due to cofilin binding (Fig. 3b). The yield stress, σ_{max} , the onset at which the network ruptures, indicates the brittleness of the material. As Φ_c increases, σ_{max} shows significant change (Fig. 3c), different from α -actinin or fascin case as reported in other work [45] (Fig. 3c).

The differential elastic modulus, K' , scales as $K' \sim \sigma_0^\xi$, in which the exponent ξ contains information about the biomechanical mechanism behind strain hardening/softening. Extensive *in vitro*, *in vivo*, and *in silico* studies on the power law exponent of the strain hardening of the biopolymer network suggest that the exponent can vary from 1/2 to 3/2 depending on the crosslinker mechanical properties[38,41-45,49,67,68]. As Φ increases, the exponent ξ for fascin crosslinked case decreases (Fig. 3d). This is because of crosslinker unbinding from filaments under tension, which results in the network transitioning from strain hardening to softening[45,49]. ξ for

α -actinin crosslinked network increases with Φ to 3/2 as the crosslinker density increases (Fig. 3d). The crosslinker's unbinding rate decreases exponentially as it experiences force from shearing, and as a result, the strain hardening behavior is predominantly due to filament stretching[41,42,67]. For cofilin crosslinked network, ξ remains <1 from low to high Φ_c , and increases to 3/2 at $\Phi_c = 1$. Therefore, the mechanism behind strain hardening of cofilactin switches from linker unbinding dominant to filament stretching dominant as Φ_c increases. The opposite trends of fascin and α -actinin crosslinked network are reminiscent of 'slip' and 'catch' bond behavior respectively due to their intrinsic crosslinker properties[69-72] (Fig. 3d). Interestingly, cofilin's binding property is agnostic to its concentration over a broad range, suggesting neither 'slip' nor 'catch' bond behavior, until the saturating concentration (Fig. 3d).

Frequency dependence of the viscoelasticity of the network is indicative of crosslinker binding and unbinding rates[46,47,73]. We observe a decrease in K' at low frequency and a clear peak and trough for K'' when performing prestressed frequency sweep (Fig. 4a). K' and K'' scale as $K' \sim \omega^{-2}$, and $K'' \sim \omega^{-1}$, which follows Maxwellian model, indicative of a single relaxation mode within the system[56,73]. This suggests that the network experiences crosslinker binding/unbinding with off-rate k_{off} , which is reflected by the onset of K' decay as well as the peak observed in K'' [46,47]. We use the equations proposed by Lieleg et al.[46,47] on transient linkers to fit experimental data, in which:

$$G'(\omega) = G_0 - a \cdot \frac{N \cdot k_{off}}{\frac{k_{off}^2}{4\pi} + \omega^2} + b \cdot \left(\frac{\omega}{f_0}\right)^{\frac{3}{4}} \quad (4)$$

, and

$$G''(\omega) = c \cdot \frac{N \cdot \omega}{\frac{k_{off}^2}{4\pi} + \omega^2} + d \cdot \left(\frac{\omega}{f_0}\right)^{\frac{3}{4}} \quad (5)$$

. Here G' and G'' can be elastic/differential elastic modulus or viscous/differential viscous modulus respectively. Each parameter is defined in Lieleg et al.[46,47], in which N denotes the number of crosslinks formed, k_{off} denotes the off rate of the crosslinkers, a and c are the coefficients for the

energy dissipation terms, and b and d are the coefficients for single filament fluctuation terms. In the nonlinear regime, cofilin binding affinity on F-actin decreases significantly as stress increases, as reported by other studies[74]. The timescale reflected by the peak of K'' ($1/k_{off} \sim 30s$) is on the same order of magnitude of cofilin unbinding as reported by other studies at high pH[6,75]. Therefore, cofilin unbinds from actin filaments as mechanical stress in the system increases (Fig. 4a).

During the strain hardening process, studies show that force bearing chains that scale across the entire network can form[65,68]. As these force chains bear the majority of the stress, cofilin's unbinding probability greatly increases. As a result, cofilin unbinds from the filaments that bear more stress, and may be redistributed to regions that bear lower stress (Fig. 4b). When $\Phi_c = 1$, cofilin is uniformly distributed all over the network (Supplement). As the network undergoes large stress, the distribution of stress is more uniform, thus the scaling reflects uniform filament stretching.

In summary, we find that: 1. For $pH < 7.3$, the moduli of the cofilin crosslinked network are weakly dependent of Φ_c , potentially due to the counterbalanced effect between the increase in network connectivity and filament flexibility; 2. Cofilin extends the linear mechanical regime (**stress or strain** range) of the network, strain hardens **with** Φ_c -dependent **mechanism**; 3. Cofilin shows transient linker properties when the network is under stress. The *in vitro* system presented here provides insights on how cofilin, a traditional actin severing protein, can act as a crosslinker at low pH. Thus, cofilin proves to be a unique F-actin crosslinker that can increase the connectivity of the network without compensating with significant stiffness increase. Our recent work suggests that a planar cofilactin network crosslinked by α -actinin can lead to out-of-plane deformation under active stress imparted by myosin thick filaments[28]. This may illustrate a mechanism by which cells can modulate their mechanical properties in ways separate from those driven by F-actin crosslinking protein.

Furthermore, the existence of cofilactin in the cell is highly relevant to physiological and neurodegenerative diseases such as Alzheimer's and Parkinson's [14,76-79]. Recent studies suggest that the dynamics of forming filopodial structures [80] can be a result of the interplay

between cofilin and other crosslinkers such as fascin or α -actinin as cofilin is pH sensitive in intracellular environment[23,24,81,82]. These mechanical properties of cofilactin may serve as important factors in understanding cofilactin rods biologically and provide values from the physics perspective in understanding the cause of neurodegenerative diseases[14,76-79]. The findings in this work show to date novel, pH-dependent mechanical properties of an active biopolymer network. Further, we present a novel mechanism, that a single protein differentially impacts connectivity and flexibility simultaneously. The results from this work shed light on the path of discovery and synthesis of biomaterials and bio-inspired materials respectively that exhibit similar counterbalancing mechanical properties, and further nourish the field of research in neurodegenerative diseases from a unique biophysical perspective.

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Figures

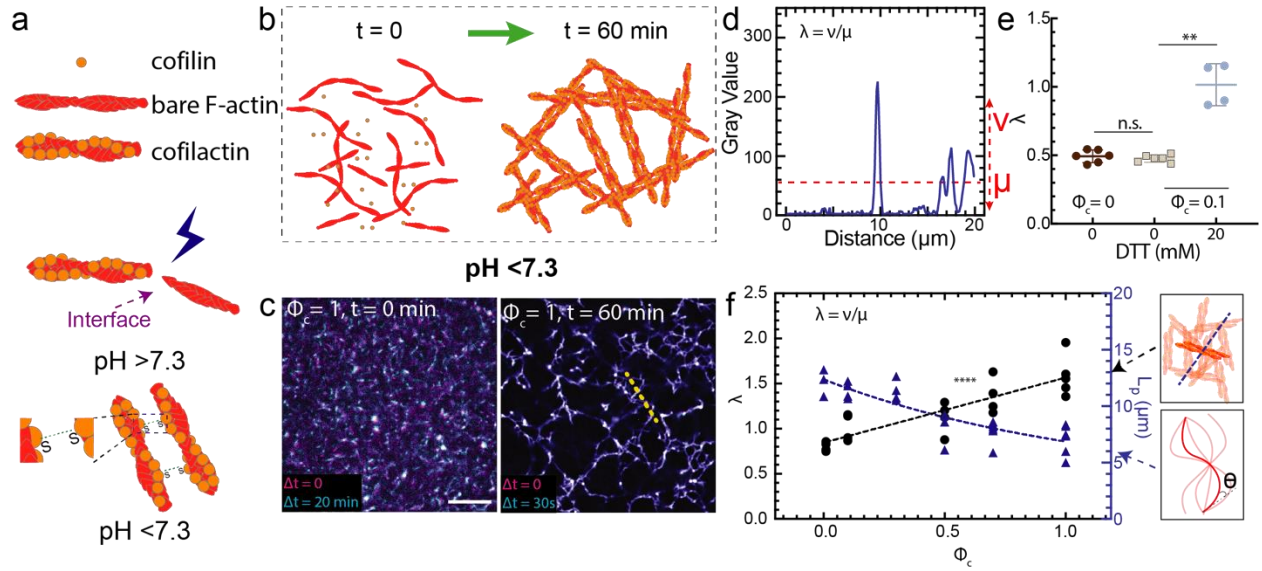


Figure 1. Cofilin bundles and reduces F-actin bending at acidic pH. (a) Schematics of bare F-actin and cofilactin (Top). Cofilin severing F-actin (middle) at pH > 7.3, and cofilactin bundling event through disulfide bond formation (bottom, with zoomed in image of disulfide binding). (b) Schematics cofilactin gelation and bundling overtime. (c) Microscopy images of cofilactin network for $\Phi_c = 1$ at initial stage during polymerization (left), and completion (right). Yellow dashed line shows an exemplary linescan analysis in (d-f). (d) Intensity linescan of the microscopy image in (c). μ is the mean, and v is the s.t.d. (e) λ at $\Phi_c = 0.1 \pm$ DTT, and bare actin case. Two-tailed *t*-test with Welch's correction is performed, $p < 0.01$. (f) λ and L_p as a function of Φ_c . Dashed black line: $\lambda = 0.6529 * \Phi_c + 0.86$. Blue dashed line: exponential fit to L_p . *F*-test is performed, $p < 0.0001$. (top right) Schematic of linescan on F-actin bundles, and (bottom right) schematic of single F-actin filament fluctuations to calculate L_p .

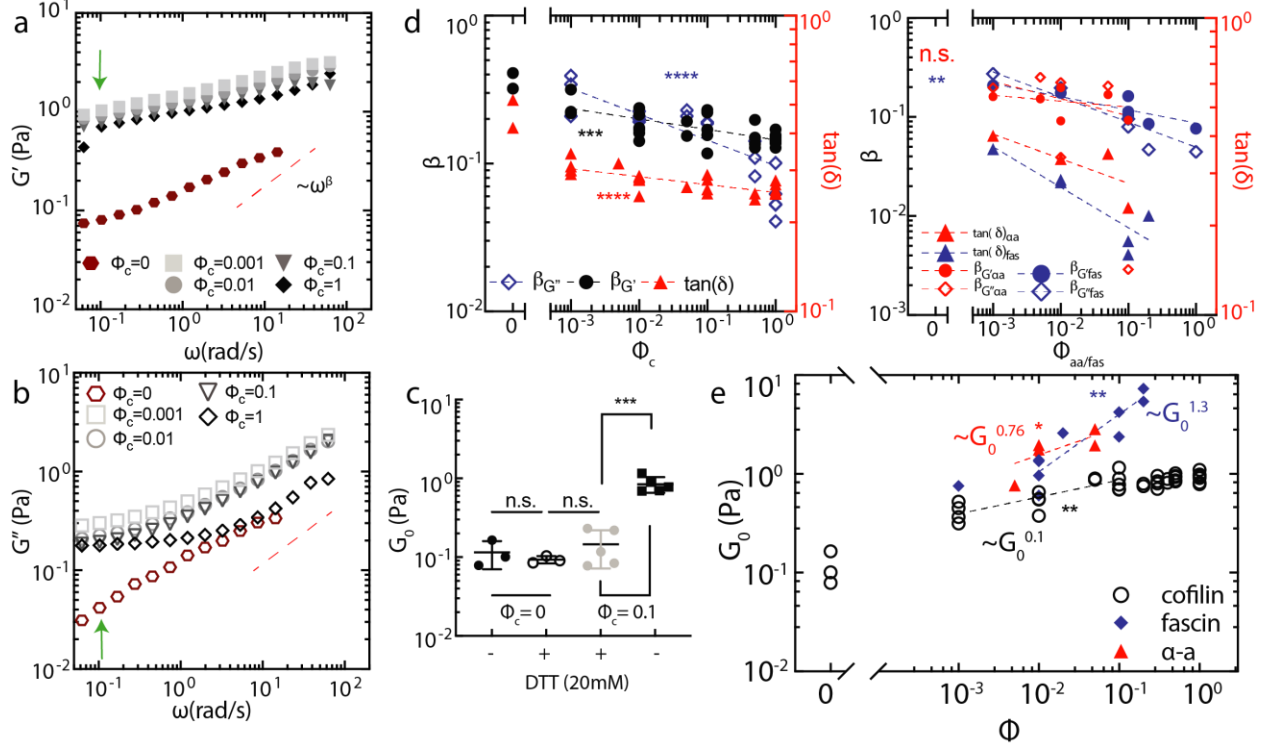


Figure 2. Cofilactin network elasticity depends weakly on cofilin mediated disulfide bonds. (a) Storage modulus (G') and (b) loss modulus (G'') as a function of **angular** frequency ω . Red dashed line shows $\omega^{1/2}$. Green arrows show the **angular** frequency ($\omega = 0.103 \text{ rad/s}$) for calculating plateau modulus $G_0(G_{\omega=0.103 \text{ rad/s}})$. (c) Plateau modulus G_0 of $\Phi_c = 0.1$ cofilactin network with (+DTT, gray circles), without (black squares) 20mM DTT addition, and F-actin network (+/-DTT, open/solid black circles). $p = 0.0005$. Two-tailed t test with Welch's correction is performed. (d) β (left y axis) and $\tan(\delta)$ (right y axis) as a function of Φ_c (left) and $\Phi_{aa/fas}$ (right). $\beta_{G'} \sim \Phi_c^{-0.07}$ and $\beta_{G''} \sim \Phi_c^{-0.175}$. F -test is performed. $p < 0.001$ for $\beta_{G'}$, and $p < 0.0001$ for $\beta_{G''}$ and $\tan(\delta)$ respectively. (Right) $p < 0.01$ for fascin and n.s. for α -actinin. (e) Plateau modulus G_0 of cofilactin network as a function of Φ_c and $\Phi_{aa/fas}$.

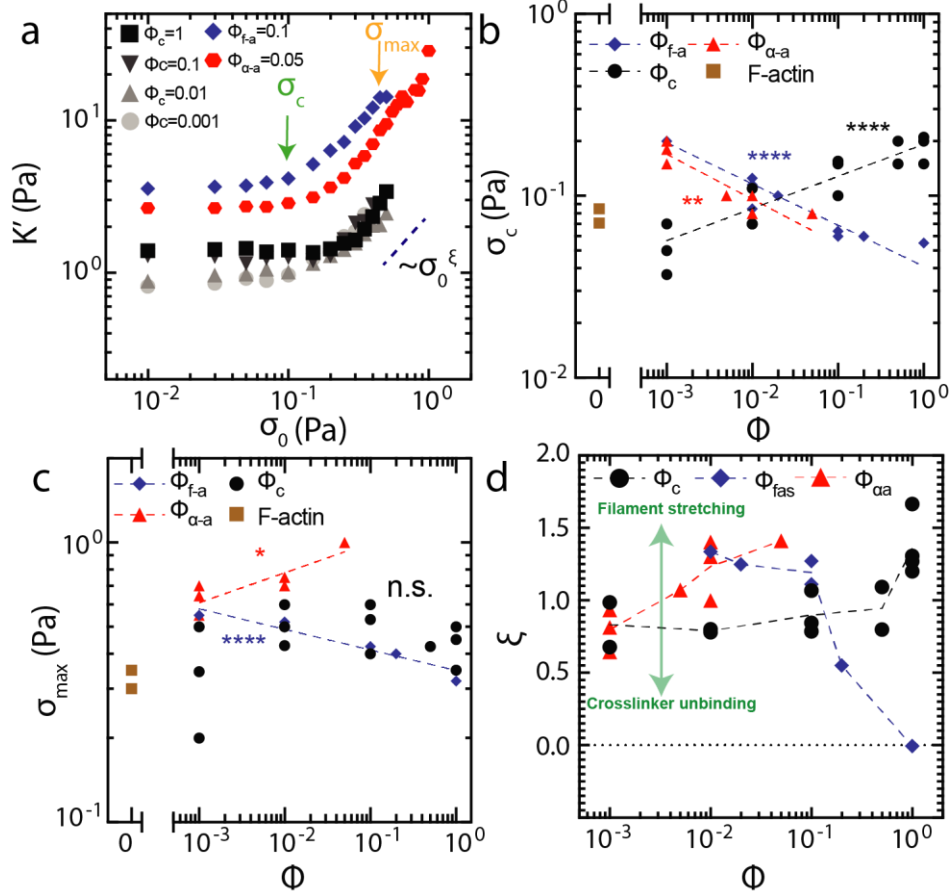


Figure 3. Cofilin crosslinking broadens linear mechanical response. (a) K' as a function of prestress σ_0 at $\omega = 3.14 \text{ rad/s}$ for $\delta\sigma$. Dashed blue line: $K' \sim \sigma_0^\xi$. The green arrow shows the critical stress σ_c , and orange arrow shows the yield stress $\sigma_{\max}$. (b) σ_c as a function of Φ . Black dashed line: $\sigma_c \sim \Phi^{0.185 \pm 0.07}$. (c) $\sigma_{\max}$ as a function of Φ . (d) ξ as a function of Φ . F -test is performed for all cases. * = $p < 0.05$, ** = $p < 0.01$, **** = $p < 0.0001$.

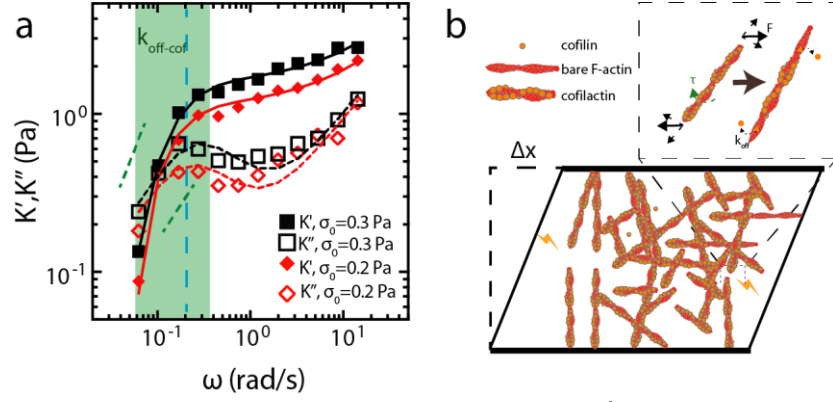


Figure 4. Cofilin as a crosslinker is mechanosensitive. (a) K' and K'' at $\Phi_c = 1$ as a function of **angular** frequency ω at two different prestress values σ_0 . Solid and dashed lines are fits to the Lieleg model. Left of vertical blue dashed line indicates the transient regime. Green dashed lines: slope of -2 (top), and -1 (bottom). Green shaded region shows value range of k_{off} for cofilin. (b) Schematics of possible mechanism of transient behavior during prestress. Red filaments are F-actin, orange circles are cofilin. Enlarged schematics show cofilin unbinding from F-actin.

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