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ORIGINAL PAPER

Brushes, cables, and anchors: Recent insights into multiscale assembly and mechanics of cellular structural networks

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Abstract The remarkable ability of living cells to mechanical stimuli in their environment depends on the rapid and efficient interconversion of mechanical and chemical energy at specific times and places within the cell. For example, application of force to cells leads to conformational changes in specific mechanosensitive molecules which then trigger cellular signaling cascades that may alter cellular structure, mechanics, and migration and profoundly influence gene expression. Similarly, the sensitivity of cells to mechanical stresses is governed by the composition, architecture, and mechanics of the cellular cytoskeleton and extracellular matrix (ECM), which are in turn driven by molecular-scale forces between the constituent biopolymers. Understanding how these mechanochemical systems coordinate over multiple length and time scales to produce orchestrated cell behaviors represents a fundamental challenge in cell biology. Here, we review recent advances in our understanding of these complex processes in three experimental systems: the assembly of axonal neurofilaments, generation of tensile forces by actomyosin stress fiber bundles, and mechanical control of adhesion assembly.

Keywords Cytoskeleton · Neurofilaments · Phosphorylation · Stress fibers · Actin · Focal adhesions · Zyxin · Fluorescence recovery after photobleaching · Rho GTPase · Laser

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Introduction

An exciting new paradigm has emerged over the past two decades which suggests that mechanical forces in the extracellular environment can inherently influence cell and tissue behavior in profound ways. Signal transduction cascades inside cells are traditionally understood to be triggered by the binding of a soluble ligand to its cognate receptor which in turn induces a conformational change in the receptor. Similarly, mechanical forces can directly alter the conformational and other biophysical properties (e.g., binding kinetics) of specific ''mechanosensitive'' molecules and thereby give rise to biochemical signals. Importantly, these transduction events are not non-specific. Mechanical stresses imparted through the extracellular matrix (ECM) are sensed preferentially through transmembrane integrin receptors [16, 34, 118]. Integrin engagement and clustering promote nucleation of intracellular focal adhesion complexes which facilitate local anchorage of cytoskeletal structures and couple the ECM to the deepest portions of the cell. Moreover, biochemical alterations in cytoskeletal networks may significantly influence the structure and mechanics of these networks and thereby change the shape, stiffness, and mechanosensitivity of the whole cell. Thus, while mechanically-induced biochemical signaling is analogous to receptor–ligand initiated signaling inasmuch as both trigger intracellular signaling pathways, a key and unique challenge in understanding signaling induced by mechanical forces is dissecting the connection between mechanics and biochemistry over multiple time and length scales.

The vast majority of efforts to understand molecular-scale coupling between mechanics and biochemistry has come from studies with purified single molecules. For example, atomic force microscopy (AFM) unfolding

studies have contributed much to our understanding of how mechanical force might unfold individual protein molecules, alter energy landscapes, and break intra- and intermolecular bonds [26, 53, 83]. At the cellular level, much is known about intracellular signaling initiated by well-defined mechanical stresses, including fluid shear [20, 21, 51, 52, 94, 105, 112–115] and substrate stretch [63–65]. However, complete comprehension of the mechanisms underlying mechanical control of cellular physiology will require an understanding of how molecular-level forces that alter protein biochemistry influence whole cell behavior.

In this review, we discuss recent advances in forging these connections in three systems: phosphorylation-dependent control of neurofilament (NF) assembly, stress fiber mechanics and cell shape control, and tension-dependent control of focal adhesion dynamics. These seemingly disparate experimental systems share several mutually defining features. First, and perhaps most superficially, all three systems relate in some way to the maintenance and control of cellular structure, mechanics, and motility. While this may seem obvious, it reflects an emerging recognition of (and increasing interest in) the need for multiscale approaches to begin to understand these fundamental and life-defining processes in molecular biophysical terms [17, 32, 103, 107]. Second, all three systems represent examples of macromolecular networks in which relatively subtle changes in molecular-scale interactions can give rise to microscale or even cell-scale changes in macromolecular organization. For example, NF phosphorylation directly contributes to graded expansion or condensation of the unstructured protein domains in which they are found, which in turn determines NF–NF spacing and therefore network organization throughout the cell. Similarly, local changes in tensile forces on focal adhesions can alter the binding kinetics of zyxin, which in turn bears direct implications for cell-ECM adhesion throughout the cell, and disruption of these same local tensile forces borne by stress fibers can promote wholesale rearrangements in cellular structure and cytoskeletal organization. Third, all three examples involve a clear interplay between mechanics and chemistry- for NF organization, altered phosphorylation translates into changes in NF–NF repulsive forces; for stress fiber mechanics, activation and inhibition of myosin motors along the body of the stress fiber translate into changes in mechanical tension; and for focal adhesion dynamics, changes in mechanical tension on focal adhesions translate into altered binding kinetics of specific proteins. While it is clear that these systems are differ in a number of important details, our goal is to highlight the commonalities in each system as we introduce recent experimental advances.

Phosphorylation-dependent control of neurofilament assembly

The higher-order assembly of neurofilaments (NFs), the most abundant cytoskeletal element of large myelinated neurons, represents one particularly interesting example of multiscale assembly in cytoskeletal systems in which microscale organization is sensitive to molecular-scale forces. In mammals, NFs are obligate heterotrimers composed of a light, medium, and heavy subunit (NF-L, NF-M, and NF-H, respectively). When fully assembled, NFs resemble bottle brushes, with a central backbone that runs parallel to the axis of the axon and hairlike ‘‘sidearms’’ that protrude from the backbone (Fig. 1A). The amino termini of all three subunits contribute to the backbone, whereas the sidearms are formed from the carboxy termini of NF-M and NF-H, both of which contain a multitude of lysine–serine–proline (KSP) repeats (42–51 on human NF-H) which are nearly completely phosphorylated in neurons. NFs in axons are spaced from one another at nonrandom distances, and a wide range of evidence from reconstituted preparations [15, 46, 49, 59] and axons in vivo [54, 116] suggest that the sidearms of adjacent NFs interact with one another to enforce this organization. Several lines of evidence have established that NFs are an important structural and mechanical component of axons, and that the mechanical properties of NFs depends strongly on NF phosphorylation state. For example, NFs reconstituted in vitro form gels consisting of parallel arrays of NFs that structurally resemble axonal NFs, with mechanical compliances that can exceed 100 Pa in the presence of divalent cations [75]. Moreover, NF phosphorylation has been shown to increase both the rate of gel formation and the apparent viscosity of reconstituted NF networks [27, 46]. Unfortunately, experimental evidence linking NF phosphorylation state to the mechanical properties of cultured cells remains limited; however, several studies have demonstrated significant changes in axonal NF network architecture upon crush injury [2, 37, 82], and mice lacking NFs are slow to regenerate axons in response to traumatic nerve compression [128].

Because it bears implications for axonal mechanics, transport, and radial growth, the physical basis of NF–NF interactions has been the subject of great scrutiny—and controversy—for over two decades; for example, mutually exclusive models have been proposed that incorporate direct electrostatic repulsion [116] and noncovalent cross-linking [54] between NFs. This controversy has only been intensified by recent gene replacement studies in mice which argue that the NF-M sidearms are the key contributor to NF–NF spacing, and that the NF-H sidearms play a more modulatory role [93]. Most recently, a new model of NF–NF interactions has emerged based on the idea the

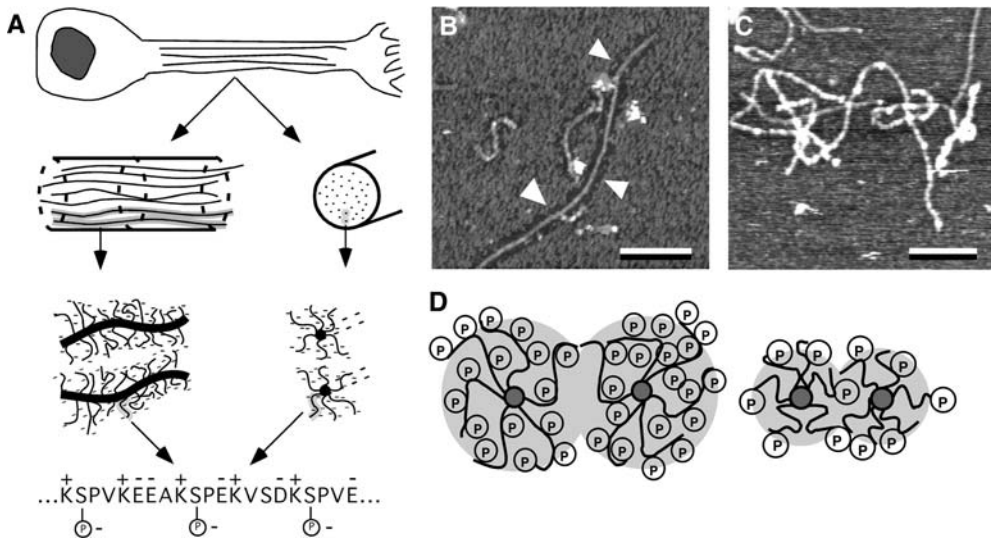

Fig. 1 Phosphorylation-dependent control of neurofilament network assembly. (**A**) Schematic of neurofilament (NF) network assembly. Neurofilaments (NFs) run in parallel along the axon, with regular center-to-center spacings. This organization has been proposed to be due to steric repulsion between the filaments' C-terminal sidearm domains which form a brushlike layer around each NF. Each sidearm is highly negatively charged due to extensive phosphorylation. (**B**) Atomic force microscopy (AFM) imaging of native NFs. The highly phosphorylated sidearms of NFs sweep out a volume which excludes material from the vicinity of the NF, as evidenced by the dark ''exclusion zones'' surrounding the NF backbone (arrows) (Bar = 500 nm). (**C**) AFM imaging of dephosphorylated NFs. When NFs are enzymatically dephosphorylated, the exclusion zones are lost, suggesting condensation and collapse of the sidearms (Bar = 500 nm). (**D**) Model for phosphorylation-dependent control of NF sidearm conformation. Extensive sidearm phosphorylation leads to strong, short-ranged electrostatic repulsion between phosphate groups, swelling the sidearm layer and increasing the effective volume of the NF (left). Dephosphorylation reduces electrostatic repulsion within the sidearm layer, thereby collapsing the sidearm and leading to weaker interfilament repulsion. (**A**) reproduced with permission from [71], Copyright © 2002 Biophysical Society, www.biophysj.org. (**B**) and (**C**) reproduced with permission from [69], Copyright © 2002 Elsevier. (**D**) Reproduced with permission from [72], Copyright © 2002 Wiley-Liss, Inc

sidearms are natively disordered (unstructured) protein domains that interact through steric repulsive forces [12, 69, 71, 72, 86]. This model is inspired by synthetic ''polymer brushes,'' which are layers of polymers grafted or adsorbed onto planar surfaces and colloidal particles to create an excluded volume that prevents fouling and aggregation [11]; in these systems, when two brush-coated surfaces are brought into close proximity, they repel one another as the chains encounter and exclude one another. In the steric repulsion model of NF organization, NFs are conceptualized as cylindrical polymer brushes whose lateral spacing is maintained by repulsive interactions between the unstructured sidearms. This model is supported by circular dichroism measurements and secondary structure predictions of isolated sidearm domains, both of which suggest that the sequence forms largely random-coil structures in solution [1, 46], and AFM force measurements of assembled sidearms which detect a repulsive force ~50 nm from the NF core, consistent with an extended conformation for the sidearms [12] (Fig 1B). Quantitative statistical analysis and computer simulations of NF spatial distributions in mice reveal that this organization is consistent with an entropic repulsion model, but not a model based solely on NF–NF crosslinking [71]. Moreover, Monte Carlo simulation [71] and AFM and gel-volume measurements of variably-phosphorylated NFs [69] reveal that the degree of sidearm phosphorylation controls the range and strength of the repulsive interactions, consistent with a model in which phosphate groups within each sidearm electrostatically repel one another, thereby swelling the sidearms and expanding the effective excluded volume. This model is also consistent with observed NF organization in axons, in that NF phosphorylation correlates with NF–NF spacing in a wide variety of axonal systems [56, 116]. In other words, graded changes in sidearm biochemistry (in this case, sidearm phosphorylation) produce nanoscale conformational changes (swelling and collapse of the sidearms) which in turn profoundly impact the microstructural organization of the NF cytoskeleton and the structure, mechanics, and physiologic properties of the whole axon (Fig. 1C).

There is mounting evidence that many other biomacromolecular systems incorporate unstructured protein domains that function by providing steric barriers. Microtubule-associated proteins (MAPs) decorate cytosolic microtubules with hairlike projections that influence rates of microtubule polymerization and depolymerization [80]; in microtubule bundles, the length of the amino acid

sequence that creates these projections directly controls the spacing between the microtubules. Indeed, when MAPs are assembled onto a planar surface and compressed by an AFM probe, they produce a long-range repulsive force like NFs [85]. Many MAPs are also heavily phosphorylated, suggesting the presence of conformational control mechanisms similar to those found in NFs. These systems are not limited to the cytoskeleton. β- and κ-casein are amphipathic proteins which protrude from the surface of fat droplets in milk thereby preventing droplet aggregation and phase separation. Light scattering of these proteins suggest that they are also unstructured and that their biophysical properties are strongly influenced by chain phosphorylation [23, 68]. More recently, unstructured protein domains have been proposed to play a central role in gating nuclear pore complexes (NPCs), the structures which permit exchange of materials between the nucleus and cytoplasm. Here, a class of proteins known as nucleoporins has been postulated to protrude from nuclear pores into the cytoplasm, where they occupy an excluded volume that prevents cargo from entering the nucleus in an unregulated fashion. This model is supported by immunoelectron microscopy and cryoelectron tomography of NPCs in cells [7, 99] and AFM force spectroscopy of purified nucleoporins which demonstrate a long-range repulsive force similar to that observed with NFs and MAPs [76].

NF network assembly represents a multiscale structural system within the cell in which changes in subunit biochemistry—in this case, phosphorylation—alter molecular-scale mechanical interactions between NFs, which would be expected to impact NF organization throughout the whole cell and, presumably, cellular mechanics. An important challenge in this area will be to define to what extent these molecular-scale changes actually govern cellular mechanics and motility, and to develop a quantitative framework that relates the two. We now examine the mechanics of actomyosin stress fiber bundles, hierarchically-assembled cytoskeletal structures in which the biochemical state of the components (e.g., myosin motors) governs the mechanical properties of the microscale fiber, which in turn contributes to whole-cell structure and mechanics.

Stress fiber mechanics and cell shape control

A central principle that underlies cellular structure and mechanics is the ability of a cell to generate contractile forces on its extracellular matrix (ECM), which is in turn balanced by the ECM's material resistance to deformation (i.e., its stiffness). This balance between cellular tension and ECM compression supports the ability of cells to adhere and spread on substrates, extend processes, and locomote in a directed fashion. The cell-ECM force balance may be controlled by either manipulating the cellular contractile machinery, the biophysical properties of the ECM, or both. Indeed, it is becoming clear that this force balance can serve as an intrinsic signal that regulates cellular physiology in the most fundamental ways. For example, the differentiation trajectory of mesenchymal stem cells (MSCs) may be controlled by allowing cells to spread to large areas [81] or by culturing them on progressively stiffer ECMs [25], thereby bolstering the tractional forces that the cell can exert on the ECM [109]. In some cases, these ECM-derived cues may be reversed through reciprocal changes in cellular tension; e.g., through the use of drugs or genetic constructs which intensify or abrogate cellular tension. Moreover, cellular locomotion is highly sensitive to ECM elasticity in that there exists an optimal ECM stiffness at which cell speed is maximized [92], and the turnover of cell-ECM adhesion complexes accelerates as the ECM is made more compliant [90].

All of these findings represent examples of a growing recognition that cells and the ECM represent a mechanobiological continuum in which cytoskeletal mechanics adjust in a homeostatic fashion to match ECM mechanics, and in which the cytoskeleton and ECM modulate one another in a reciprocal and dynamic fashion. The MSC studies described above reveal that stem cells confronted with an ECM of a defined compliance tend to differentiate into a cell type whose tissue microenvironment best matches that compliance; extremely compliant substrates promote neurogenesis, whereas extremely stiff substrates promote osteogenesis [25]. Similarly, proliferating myoblasts differentiate most readily on ECMs whose compliance best matches that of skeletal muscle tissue [24]. Finally, ECM stiffness may be used to select for specific cell types in mixed cortical culture, with neurons preferring soft substrates and glial cells preferring rigid substrates [35]. Intriguingly, more recent studies have suggested that neurons are in fact slightly stiffer than glial cells, revealing additional complexities in the mechanical interplay between cytoskeletal and ECM mechanics [78].

Forces are transmitted between cells and the ECM at nascent adhesions called focal complexes as well as more mature cell-ECM adhesions called focal adhesions [3, 8, 33]. In stationary cells, actomyosin stress fiber bundles are anchored into focal adhesions, which transmit stress-fiber generated contractile forces onto the ECM [36]. Stress fibers are rich in actin, a variety of actin-bundling and crosslinking proteins, and myosin, whose motor activity contributes to the contractile nature of these structures. The question of how these nanoscale motor elements collectively contribute to cell-ECM traction forces remains an important and unsolved problem. Recent studies have sought to address this question by quantifying the mechanical properties of stress fibers [22]. Data verifying

the contractile nature of stress fibers come primarily from observations of fluorescently-labeled stress fibers in whole cells. For example, immunofluorescence imaging of cultured cells demonstrates that a variety of actin-binding proteins including α-actinin [91, 100] and myosin [77], distribute themselves along stress fibers in a periodic fashion, reminiscent of sarcomeres in skeletal muscle. Indeed, when stress fibers are isolated from cells and treated with agonists that stimulate the contraction of muscle (e.g., magnesium and ATP), these periods decrease, reflecting a concerted contraction of the entire stress fiber [62]. The situation appears to be more complex in living cells; recent observations of the distribution of green fluorescent protein-tagged actin-binding proteins in living cells during contraction shows that in some stress fibers, the contractile activity is localized to peripheral portions of the structure, leading to an apparent contraction at the ends of the stress fiber and passive thinning (extension) at the center [91].

In addition to these cellular studies, stress fibers have been isolated from living cells with the goals of both rigorously determining tensile properties in general and dissecting differences in contractile activity in different portions of the cell. For example, Katoh and co-workers developed a method to isolate stress fibers from fibroblasts by chemically extracting the rest of the cell, leaving the stress fibers attached to the culture substrate [62]. Immunofluorescence and immunoblotting of the isolated stress fibers revealed retention of several key stress fiber and focal adhesion proteins including vinculin, α-actinin, and myosin light chain kinase (MLCK). Treatment of these isolated fibers with magnesium and ATP contracted the fibers by 80% over the course of 5 min, demonstrating the presence of functionally intact motor units. A subsequent study leveraged this method to study intracellular regional variations of stress fiber sensitivity to drugs which inhibit various elements of the Rho GTPase signaling pathway. This work revealed the presence of two populations of stress fibers in the cell: central stress fibers sensitive to the activity of rho-associated kinase (ROCK) and peripheral stress fibers sensitive to MLCK [61], a finding consistent with earlier observations in living cells [111]. More recently, stress fibers isolated in this way have been manipulated with microcantilever systems to obtain quantitative tensile properties, revealing mean Young's moduli of approximately 1.45 MPa [22]. Interestingly, this value is much higher than typical cellular elasticity values on the order of 1–1000 Pa measured by atomic force microscopy [78, 97], magnetic bead microrheometry [6, 119], micropipette aspiration [55, 110], and particle-tracking microrheometry [87, 122]. This suggests that while stress fibers are important contributors to the ability of living cells to stabilize their shape and generate contractile forces, they do not dominate the measured elasticity of the whole cell.

A key limitation of the isolated stress-fiber experiments is that they remove these structures from their cellular context, where they are both tightly associated with focal adhesion complexes and other cytoskeletal systems and mechanically coupled to the ECM. Conversely, the whole-cell experiments retain this context but enable neither quantitative measurements of stress fiber mechanics nor selective interrogation of individual stress fibers. To develop a more complete multiscale framework that relates the mechanics of these nanoscale structures to the structure, mechanics, and contractility of the whole cell, methods are needed which permit disruption of single stress fibers in living cells. To this end, a recent study sought to obtain mechanical information about individual stress fibers in their intracellular context by severing single stress fibers with femtosecond laser ablation and then quantifying their recoil dynamics [70]. This study built on earlier work in which femtosecond laser ablation was established as a method capable of disrupting intracellular structures with submicron precision and without compromising the plasma membrane [50, 104]. Here, stress fibers in living endothelial cells were visualized through the expression of yellow fluorescent protein-tagged actin. Laser irradiation of stress fibers led to complete scission and retraction of the severed ends over the course of 15–30 s (Fig. 2A); in some cases, laser irradiation punctured a hole in stress fibers, leading to progressive distension of the hole into an ellipsoid shape as the stress fiber relaxed to accommodate the injury (Fig. 2B). The retraction dynamics of severed fibers followed that of a viscoelastic cable (Fig. 2C) and were highly sensitive to pharmacological inhibition of actomyosin contractility, with partial slowing of retraction in the presence of ROCK inhibitors and complete loss of retraction in the presence of MLCK inhibitors. Intriguingly, the sensitivity of cell shape to stress fiber incision depended strongly on the rigidity of the ECM; incision of one or even multiple parallel stress fibers in cells cultured on rigid glass ECMs did not appreciably change the footprint of the cell, whereas incision of even one stress fiber in cells cultured on compliant (~4 kPa) ECMs led to dramatic cellular elongation, cytoskeletal rearrangements many microns from the site of incision, and dissipation of tractional forces into the ECM as measured by traction force microscopy. Companion studies (discussed in more detail below) demonstrated that dissipation of stress fiber tension by laser ablation accelerated the turnover of specific proteins in associated focal adhesions, suggesting that these proteins are intrinsically mechanosensitive [74].

Importantly, the strengths and limitations of these studies are reciprocal to the mechanical studies of isolated stress fibers described earlier. Whereas the earlier studies proved capable of producing highly quantitative mechanical measurements, they did so at the expense of

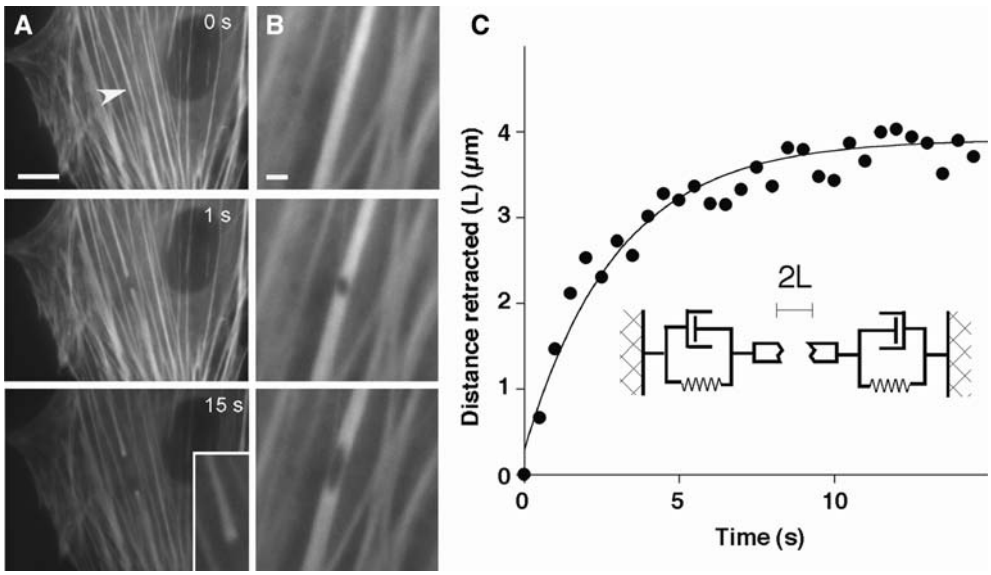

Fig. 2 Tensile properties of single stress fibers in living cells. (**A**) Subcellular laser incision of a single stress fiber in a living cell. This endothelial cell was transfected with YFP-actin in order to visualize the actomyosin stress fiber network. Femtosecond laser irradiation of a selected stress fiber (arrow) leads to immediate retraction of the stress fiber (Bar = 10 μm). (**B**) Puncturing of a single stress fiber. Laser irradiation of a large stress fiber results in puncturing of the fiber, with subsequent distension of the wound into an ellipsoid shape (Bar = 2 μm). (**C**) Viscoelastic retraction of a stress fiber. The retraction dynamics of a stress fiber may be fit with a simple mechanical model that features a viscous and elastic element in parallel and is described by a single relaxation time constant. Reproduced with permission from [70], Copyright © 2006 Biophysical Society, www.biophysj.org

removing the structures from their cellular context. Conversely, the live-cell studies preserved the cellular context, but because of the overwhelming physical complexity of the system had to sacrifice some ability to make quantitative statements about stress fiber mechanics. For example, the former measurements were able to yield a value for the Young's modulus for stress fibers whereas the latter were not. A key challenge will be to develop technologies that combine the strengths of both approaches and in particular enable direct comparison of stress fiber mechanics and whole cell mechanics in the same experimental construct.

The dependence of stress fiber retraction kinetics on the activity of contractile motors illustrates the connection between biochemistry and mechanics in this system, in the sense that activation or inhibition of specific mechanochemical motors alters the mechanics of microscale stress fibers, which in turn governs coupling between the whole cell and the ECM. In the final portion of this review, we describe a case in which mechanical force directly influences biochemistry, specifically tension-dependent regulation of turnover specific molecules within focal adhesion complexes at the cell-ECM interface.

Tension-dependent control of focal adhesion dynamics

Focal adhesions are formed when transmembrane integrin receptors ligate to extracellular matrix (ECM) molecules such as fibronectin. Ligation and clustering of integrins result in a cascade of events that includes the recruitment of a large number of proteins including molecules that couple integrins to the cytoskeleton (e.g., α-actinin, talin), kinases and phosphatases that act on substrates in the adhesion complex (e.g., focal adhesion kinase), and adaptor molecules that allows multiple substrates to ''dock'' in adhesions through binding interactions (e.g., paxillin) [9]. The focal adhesion provides a path for the transmission of cytoskeleton-generated forces via integrins onto the underlying substrate. Indeed focal adhesion assembly is closely regulated by tensile forces exerted by stress fibers that anchor into adhesions (Fig. 3A), which may be related to the role of cell-generated traction forces in cell motility [9]. Focal adhesions are also signaling complexes; for example, focal adhesion assembly is tightly linked to the MAP kinase signaling cascade, which promotes cell growth [14, 96]. Since adhesion assembly is controlled by mechanical forces that are generated through the cellular cytoskeleton, adhesions are a central link that allows cytoskeletal-mediated mechanical control of cell behavior.

Cell-substrate adhesion size correlates directly with the level of traction force that is exerted on the substrate at these sites in stationary cells [3]. In migrating cells, nascent adhesions at leading lamellipodia called focal complexes can also exert significant propulsive forces [8, 33]. When cytoskeletal prestress is dissipated by inhibiting actomyosin contractility, adhesions disassemble [9]. In addition,

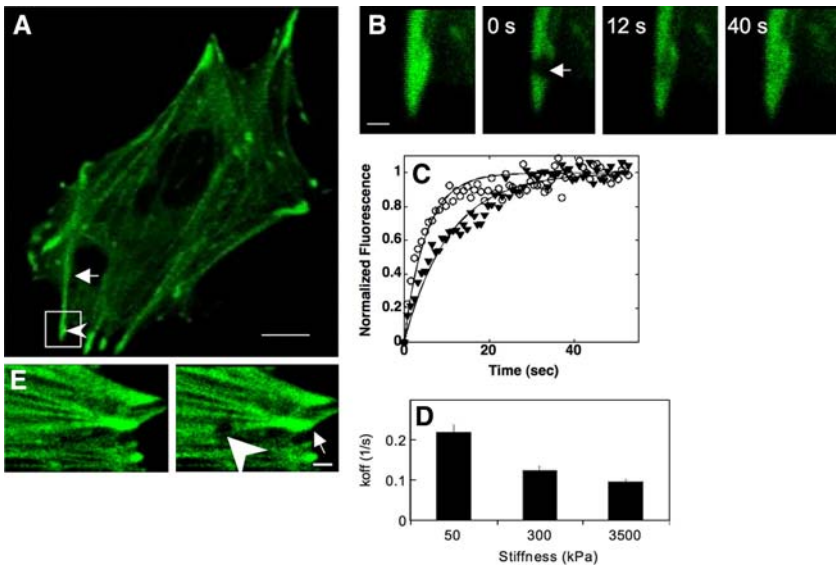

Fig. 3 Tension increases the dissociation rate constant of zyxin. (**A**) Confocal fluorescence micrograph of a single capillary endothelial cell expressing zyxin. Zyxin decorates stress fibers (arrow) which terminate into adhesions (arrowhead). Scale bar is 10 microns. (**B**) A representative FRAP experiment inside a single focal adhesion (inset), in which a ~0.5 square micron area was bleached (white arrow) and subsequent time-dependent fluorescence recovery recorded by capturing confocal fluorescence images (Bar = 1 μm). (**C**) Dependence of zyxin exchange dynamics on intracellular tension. The curves show time-dependent recovery of fluorescence intensity for GFP-zyxin in control cells (open circles) versus cells in which tension was dissipated by treating cells with Y27632 (closed triangles); solid lines are curve-fits to $1 - e^{-k_{OFF}t}$ using the method of least squares to estimate k_{OFF}. (**D**) Zyxin k_{OFF} increases as matrix stiffness is decreased. FRAP measurements were carried out in cells cultured on fibronectin-coated polyacrylamide gel substrates with different Young's moduli. The dissociation rate constants are significantly different from each other ($P < 0.01$). (**E**) Representative experiment indicating femtosecond laser ablation of a zyxin-decorated stress fiber (arrowhead) that is anchored into a zyxin-labeled adhesion site (arrow). This method was used to dissipate tension in the stress fiber, followed by subsequent FRAP analysis of zyxin in the tension-dissipated adhesion site. Reproduced with permission from fs1], Copyright (c) 2006 Wiley-Liss, Inc)

focal adhesion disassembly at the cell base can be overcome by applying external mechanical loads to the apical surface of the cell [95, 115]. This dependence of focal adhesion size, protein localization and signaling on internal cytoskeletal prestress or external mechanical stress has led to the hypothesis that integrins act as mechanoreceptors [16, 118], and that certain focal adhesion proteins function as mechanosensors by altering their conformation and/or binding kinetics in response to stress [9, 57, 58, 66].

The mechanisms underlying force-dependent control of adhesion assembly are relatively poorly understood, although several models have been proposed. For example, force-dependent unfolding of proteins may expose cryptic binding sites and promote binding of molecules; e.g., mechanical unfolding of fibronectin may give it the ability to bind to specific partners on the cell surface and give rise to adhesion assembly. In support of this hypothesis, Zhong et al. [127] and Baneyx et al. [4] have convincingly demonstrated that cellular traction may cause local unfolding of fibronectin leading to self-association and fibril assembly. Mechanical forces have also been proposed to induce conformational changes in specific focal adhesion proteins. Molecular simulations suggest that the focal adhesion targeting domain of focal adhesion kinase (FAK) may change conformation in response to mechanical force, and result in an increase in its binding affinity for paxillin [60]. Moreover, stretching detergent-insoluble cytoskeletons causes alterations in the cytoskeletal binding affinities of several cytoplasmic proteins, including the focal adhesion molecules paxillin and FAK [101].

Another potential mechanism underlying adhesion assembly is a change in the binding kinetics of individual molecules that assemble into focal adhesions. For example, talin engages multiple binding partners in the adhesion including integrins, actin and focal adhesion kinase. If the conformation of any of these proteins is altered by mechanical force, then the binding and unbinding rate constants that describe talin binding to these individual molecules may change. A change in rate constants would alter the balance between binding and unbinding rates, giving rise to net assembly or disassembly of specific molecules from adhesions.

There is ample evidence from in vitro experiments to suggest that protein–protein binding energy landscapes can be substantially altered by mechanical force [5, 79, 120, 129, 130]. A recent study tested the hypothesis that bond-dissociation rate constants of adhesion proteins inside living cells depend on force [74]. To do this, the authors

used Fluorescence Recovery after Photobleaching (FRAP) (Fig. 3B) to measure the effective unbinding rate constant k_{OFF} of two adhesion proteins, zyxin and vinculin, and measured their dependence on intracellular traction force incident on the adhesion sites. If adhesions can be selectively bleached (i.e., diffusing cytoplasmic protein is not bleached), then the FRAP measurement can be used to measure the time scale of exchange τ that describes how long it takes for adhesion-bound molecules to exchange with the cytoplasm. The effective unbinding rate constant k_{OFF} is then given by $k_{OFF} = \frac{1}{\tau}$.

By dissipating contractile forces exerted by the actin cytoskeleton using ROCK inhibitors, it was found that zyxin exchange between the bound adhesion and the cytoplasm was accelerated with decreasing tension (Fig. 3C). Similar effects were captured when cells were cultured on substrates varying in their stiffness. Zyxin in adhesions formed on flexible substrates had higher k_{OFF} values than those on stiff substrates (Fig. 3D), implying that reducing global intracellular contractility by decreasing ECM stiffness caused increased rates of exchange between adhesions and the cytoplasm. When traction force at adhesions was physically disrupted by severing a single stress fiber anchored into the adhesion (Fig. 3E, F), a similar effect was observed for the effective unbinding rate constant of zyxin [74]. While these studies relied on tension inhibition, a quantitative correlation between adhesion size, morphology and intracellular location and dissociation kinetics is lacking. Given that adhesions are heterogeneous in size, and focal complexes and focal adhesions differ not only in the magnitude of traction force [3, 9, 95, 102], but also in chemical heterogeneity [124, 125], a quantitative correlation between kinetic parameters for different molecules in adhesions with force, morphology and composition may be extremely useful.

Interestingly, the dissociation rate constant of vinculin remained unchanged when tension was dissipated with Y27632, suggesting that vinculin binding may not be sensitive to force. This finding is supported by at least one other study [101] in which bound vinculin concentrations in triton-extracted cytoskeletons were not found to be sensitive to stretching forces. Our findings suggest that vinculin binding kinetics may not be directly altered by mechanical forces; however, other mechanisms such as the autoinhibitory interaction between the head and tail of vinculin are probably key to its regulatory role in adhesion assembly [18, 19]. This autoinhibitory interaction acts as a barrier to talin–vinculin complex formation [19], thereby regulating turnover dynamics of talin and vinculin. Thus, the role played by vinculin in regulating cell mechanics, adhesion assembly and migration [28, 38–45] may fundamentally be due to the exposure of cryptic binding sites that promote binding interactions with other molecules like talin, thereby regulating the efficiency of mechanical coupling between integrins and the cytoskeleton [28].

Conclusions and future prospects

Living cells have the remarkable ability to transduce external mechanical inputs into biochemical signals. In response to mechanical forces, cells actively remodel their morphology by tuning molecular-scale interactions within intracellular structural networks. Understanding the details of these mechanochemical conversions, and how they are regulated at specific places and times within the cell, presents significant challenges for cell biologists and bioengineers. We have reviewed recent progress in three such systems: phosphorylation-dependent control of NF network organization, control of cellular structure by actomyosin stress fiber bundles, and tension-dependent control of focal adhesion assembly. All three systems share key common features: direct relevance to cellular structure, mechanics, and motility; connections between molecular-scale and cell-scale organization; and interplay between biochemistry and mechanics. While substantial progress along these lines has been made in the three multiscale structural systems reviewed here, challenges remain which are likely to serve as fertile ground for future investigation.

One critical challenge is the development of computational frameworks capable of integrating experimental findings across multiple length scales that range from portions of molecules to whole cells. For example, local force balances at adhesion sites which are nano-structures (vertical length of ~50–60 nm [31], and an area of a few square microns) regulate the self-assembly of a large variety of proteins of varying sizes and binding affinities. Stress fibers that promote adhesion assembly result from incorporation of individual micro-filaments through the concerted action of crosslinking and bundling proteins to create an ordered, tensile fiber that extends over many microns along the length of the cell. Stress fiber remodeling and reorientation, coupled with adhesion assembly and disassembly, enable cells to migrate hundreds of microns over substrates. These processes are themselves regulated by the Rho family of GTPases [13], which have varying concentrations and activities in different portions of the cell that in turn direct localized stress fiber and adhesion remodeling. Connecting events across all of these length scales may require integration of molecular dynamics simulations that study how proteins may change conformation in response to mechanical forces [60], Monte Carlo simulations that describe mesoscale interactions between cytoskeletal filaments, and constitutive relationships that describe cytoskeletal rheology [29, 67, 106]. Accurate, quantitative measurements of the biophysical properties of

cellular structural elements, such as those reviewed here, should be of value to these approaches.

Second, our ability to experimentally probe multiscale structural systems in the cell is severely limited by a lack of in vitro experimental platforms which realistically approximate key biophysical aspects of the cellular environment in vivo. Specifically, the vast majority of measurements of cellular structural and mechanics are obtained using spatially isolated cells cultured on rigid two-dimensional ECM substrates. However, in their true physiological context, cells exist in complex microenvironments which are often three-dimensional, include ECMs whose topographic, biochemical, and mechanical properties vary dramatically on the microscale, and involve multiple homo- and heterotypic interactions with other cells. Direct comparison of cells cultured in two-dimensional and three-dimensional ECMs illustrates this point well; fibroblasts form very robust stress fibers and focal adhesion complexes when cultured on collagen-coated glass but frequently fail to do so when cultured in three-dimensional collagen matrices [89]. Furthermore, the dependence of cell migration speed on ECM protein density and compliance differs substantially between two- and three-dimensional matrices, and three-dimensional migration requires cell-mediated proteolytic degradation in ways that two-dimensional migration does not [126]. Similar differences in cell structure and mechanics have been found for cells cultured on rigid versus compliant ECMs [70, 92] and isolated cells versus cells in either monolayers [98] or co-cultures [25]. For all of these reasons, there is a need to develop more sophisticated and physiologically relevant cell culture platforms that enable quantitative, high-resolution measurements of the multiscale networks that underlie cell structure and mechanics [47, 123]. Complementary efforts are in progress to develop advanced imaging modalities that are capable of precisely tracking structural changes in three-dimensional tissues, including multiphoton fluorescence imaging [48], higher-harmonic generation imaging [108], and use of molecular constructs which enable measurement of signaling and conformational dynamics in living cells [117].

A third challenge centers around developing a deeper appreciation of the importance of these multiscale structural systems for cell, tissue, and organ physiology and pathology. For example, adhesion assembly at the nanoscale feeds back to regulate global cell behavior through the activation of MAP kinase pathways which regulates cell growth. Similarly, controlling ECM stiffness can direct stem cell differentiation [25], promote myotube assembly [24] and optimally promote neuronal branching [30]. Systematic changes in the mechanical properties of cells have recently been explored as diagnostic tools in cancer [47]. Intriguingly, tumors are frequently stiffer to palpation than normal tissue, and recent work suggests that this may arise in part from elevated levels of Rho-associated signaling in tumor cells which leads to robust assembly of stress fibers and strong cell-ECM adhesions, which in turn enhances growth and interferes with normal tissue assembly [88]. Similarly, actomyosin contractility has been shown to regulate lung morphogenesis in whole lung mouse models [84]. These studies clearly underscore the potential payoff of efforts to ''close the loop'' by relating fundamental biophysical studies and understanding to the control of cell and tissue function.

A defining feature of a living cell is its ability to process and integrate multiple biochemical and physical cues over a wide range of length and time scales to produce a coordinated response. The role of structural networks in establishing and modulating cellular structure embody this principle perfectly. While we have focused on only three of these systems, they are by no means the only examples. Equally interesting studies are underway for microtubule assembly and mechanics [10], nuclear lamin assembly [73], and assembly of cell–cell juntional contacts [121], to name just a few. While our understanding of mechanochemical coupling from the molecular to cellular length scale remains in its infancy, progress is rapid and will continue to accelerate. In time, thanks to these and related efforts, the seemingly impassable gap between molecular-scale forces and cell- and tissue-level behavior should slowly but surely begin to narrow.

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