Current Biology 19, R781–R789, September 15, 2009 ©2009 Elsevier Ltd All rights reserved DOI 10.1016/j.cub.2009.06.054

Conformational Changes and Signaling in Cell and Matrix Physics

Review

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Physical factors drive evolution and play important roles in motility and attachment as well as in differentiation. As animal cells adhere to survive, they generate force and 'feel' various mechanical features of their surroundings, with mechanosensory mechanisms based in part on force-induced conformational changes. Single-molecule methods for *in vitro* nano-manipulation, together with new *in situ* proteomic approaches that exploit mass spectrometry, are helping to identify and characterize the molecules and mechanics of structural transitions within cells and matrices. Given the diversity of cell and molecular responses, networks of biomolecules with conformations and interactions sculpted by force seem more likely than singular mechanosensors. Elaboration of the proteins that unfold and change structure in the extracellular matrix and in cells is needed — particularly with regard to the force-driven kinetics — in order to understand the systems biology of signaling in development, differentiation, and disease.

Introduction

Evolutionary and developmental pressures, such as outpacing pursuants, pumping essential fluids, or resisting mechanical stress, all physically select for beneficial changes in expressed genes. These examples involve forces of contact that must be sustained within a tissue by an ensemble of molecules that can also transduce — directly or indirectly — signals to the tissue-integrated cells. Since the beginnings of cell biology, various micro-manipulation tools have been developed to push or pull on cells and determine effective responses in terms of elastic, viscous, and yield parameters [1], although often without much molecular insight. Brownian and non-Brownian motions of particles within cells have also been tracked to distinguish passive, thermally-scalable properties of cells from active, energy-driven characteristics [2]. The latter responses often involve signaling and exhibit molecular specificity: when adhesions between a cell and the extracellular matrix (ECM) are stretched by an external force, for example, select proteins are post-translationally modified and actively enriched at the site of applied force [3]. When cells test the mechanics of their microenvironment through adhesive engagement and ATP-driven contraction of actin–myosin 'stress' fibers [4], gene expression can change over hours to days, with recent examples including matrix-elasticity-directed lineage specification of stem cells [5] (Figure 1) and malignant transformations of breast epithelial cells on stiff substrates [6]. Regardless of process, however, the underlying molecular mechanics need to be clarified.

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A number of signaling pathways have been described that transduce mechanical signals into biochemical responses, which could then lead to complex cell behaviors [7,8]. Interactions within or between molecules that are directly modulated by force or by some other mechanical property of the microenvironment are likely to be critical in any putative mechanosensory circuit. Force-induced conformational changes have been thought to be important, but it is only relatively recently that such processes have been observed *in vitro*. Now there is also growing experimental evidence that force-induced conformational changes indeed occur within matrix and cells [9].

Of the various mechanisms that have been proposed for mechanosensation [7,8,10,11], here we focus on tension-induced changes in protein structure (Figure 2A). Forced unfolding can be localized to a part of a domain or loop, or it can involve complete domains; force can also re-orient domains or straighten unstructured regions, such as hinges between domains. We review the experimental methods that have been used to probe transitions and also review how these observations fit into a cellular context. From outside to inside a cell, we first consider the ECM, which defines a mechanical substrate for cells (Figure 2B) and which interacts with cell-surface receptors, such as integrins, to couple the matrix to the cell cytoskeleton (Figure 2C). We then focus on the cytoskeleton itself, including the contractile myosin components with which a cell exerts pulling forces and probes its surroundings (Figure 2D). More physical tools and biochemical insights are certainly needed, but it seems fair to claim that 'molecular mechanobiology' is not just emerging but is perhaps burgeoning.

Proteins Unfold Under Force

Most proteins fold and fluctuate around an average three-dimensional structure that can be influenced by cell signaling processes [12]. Unfolding through changes in temperature or solvent, with denaturants such as urea or guanidine hydrochloride, has taught us much about the determinants of protein structure. However, normal body temperatures tend to remain within tight limits in mammals and birds (especially in terms of the absolute temperature in Kelvin, K), and denaturants are not especially physiological. On the other hand, mechanical stresses are unavoidable in locomotion, flowing biofluids, and even just sitting in the Earth's gravitational field. Forced unfolding of single proteins was first shown about a decade ago [13,14]. A 'grip and break' approach is perhaps the most intuitive way to think about disrupting any structure, but force is a vector (with magnitude and direction) that is difficult to apply to proteins tumbling in solution. Immobilization is required, as occurs with structural proteins within a cell or matrix, and there is also a need for nanoscale techniques to apply a force and monitor the effects of force at the molecular level.

The most commonly used tools today for single-molecule force spectroscopy are atomic force microscopy (AFM; Figure 3A) and optical tweezers (as reviewed elsewhere [15]). Based on forced unfolding measurements of many proteins, forces (f) in the range of 10 to 200 pN will unfold

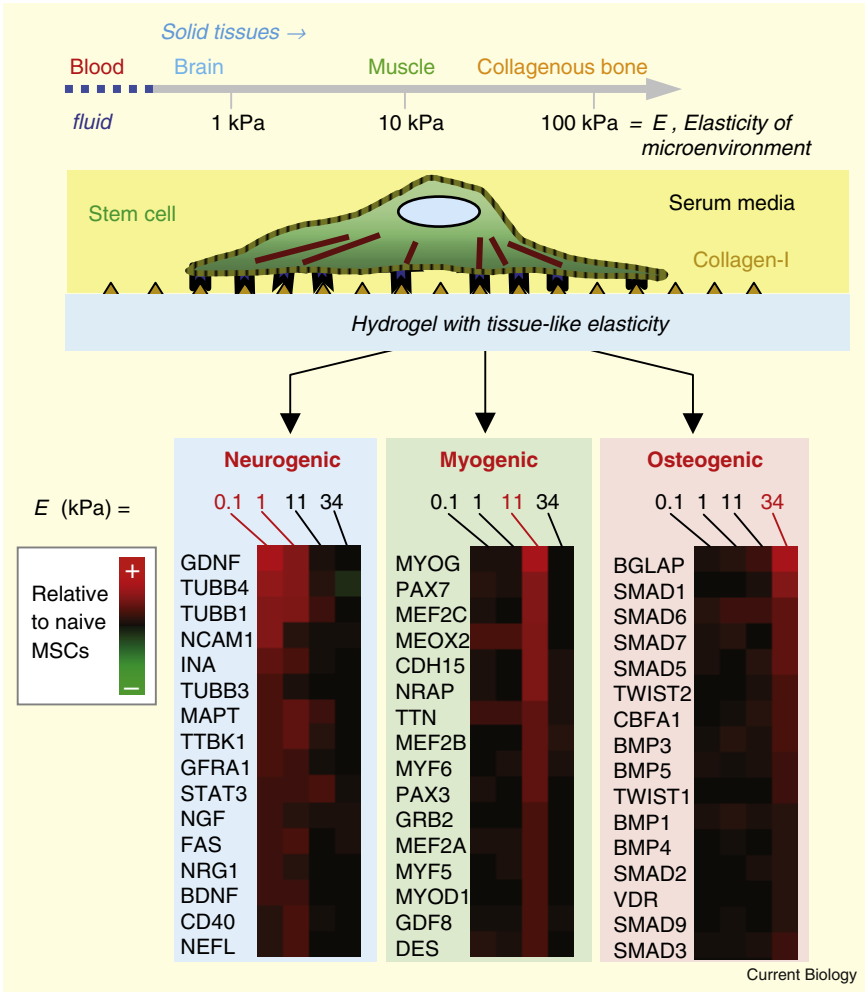

Figure 1. Cells can 'feel' the physical properties of their microenvironment.

In one recent example with mesenchymal stem cells (MSCs), matrix elasticity is seen to direct lineage specification [5]. Elasticity of microenvironments is measured in units of kPa and differs between different solid tissues. Hydrated gels, or hydrogels, coated with collagen-I can be made to mimic the crosslinking that effectively sets tissue elasticity. Culturing stem cells on such gels in the presence of serum alone, without added soluble factors, is found to induce cell morphology and gene expression that typify the various soft tissues. Inhibition of the cell's contractile tension largely blocks this mechanosensation process.

most proteins within a second or less. At lower forces, transitions occur more slowly [16,17], which one can understand from the simplest possible expression, widely attributed to Bell [16], for the rate k of reaction that is accelerated by force above a basal rate k_o:

$$k = k_o exp(fd/k_B T) \quad (1)$$

The distance d is specific to a protein transition, and $k_B T$ (= 4 pN nm) is the standard Boltzmann constant multiplied by absolute temperature (~300 K in many biological systems). The exponential factor (i.e. Boltzmann factor) shifts and sculpts the free energy landscape, which has the form of a folding funnel (Figure 3B). Because cells are filled with myosin motors that each typically generates forces of around 1–10 pN in 1–10 nm steps, unfolding reactions are expected to be accelerated and, therefore, more prominent within active cells.

With full unfolding of a typical repetitive domain within a multidomain structural protein, extensions of 5–10-fold correspond to distensions of 20–40 nm, which is large compared with the dimensions of most other cytosolic proteins. Although extension can serve as a strain-release mechanism that allows some biological scaffolds to distend without dissociating, for a growing list of proteins unfolding seems to have additional functional consequences, such as exposure of previously hidden assembly or binding sites.

Unfolding in the ECM Influences Assembly

Adherent cell types are viable only when they are attached to a solid substrate, which *in vivo* is the ECM, a crosslinked hydrogel-like complex of protein and polysaccharide that is produced by either the cell it surrounds or specialized cells such as fibroblasts. ECM proteins are the most abundant proteins in animals and play critical mechanical roles for tissues by providing them with form and substantial tensile strength and, in the case of bone, through calcification, compressive strength. The role of ECM in regulating cell behavior, and in particular how the ECM can be modulated by force, is emphasized here.

Fibronectin is one well-studied ECM component to which cells adhere via surface receptors called integrins. Fibronectin consists of a series of immunoglobulin (Ig)-like domains that form a chain-like structure, and it further assembles into fibrous bundles and a network (Figure 2B). Assembly depends on tension, whether it is produced by cells [18] or is applied externally [19,20], and this has led to the suggestion that cryptic self-assembly sites in fibronectin are exposed by cell contractility [18]. The hypothesis that this exposure is due to force-induced unfolding was plausible, given that fibronectin unfolding under tension had already been predicted theoretically [21]. This early prediction was shortly followed up by molecular dynamics simulations [22,23] and single-molecule experiments on fibronectin domains from the related ECM protein tenascin [24]. These force spectroscopy experiments also showed that the domains could refold in seconds after the removal of force. Further evidence for some type of conformational change in the matrix under tension was provided by Förster resonance energy transfer (FRET) changes between fluorescent dyes attached to Cys and Lys residues in fibronectin [25,26]. Although such Cys labeling tends to block disulfide formation that is essential for matrix crosslinking within the oxidative extracellular space, the FRET results are quantitative and compelling.

The results for fibronectin suggest that unfolding is activated by cell tension in the ECM, contributing to assembly,

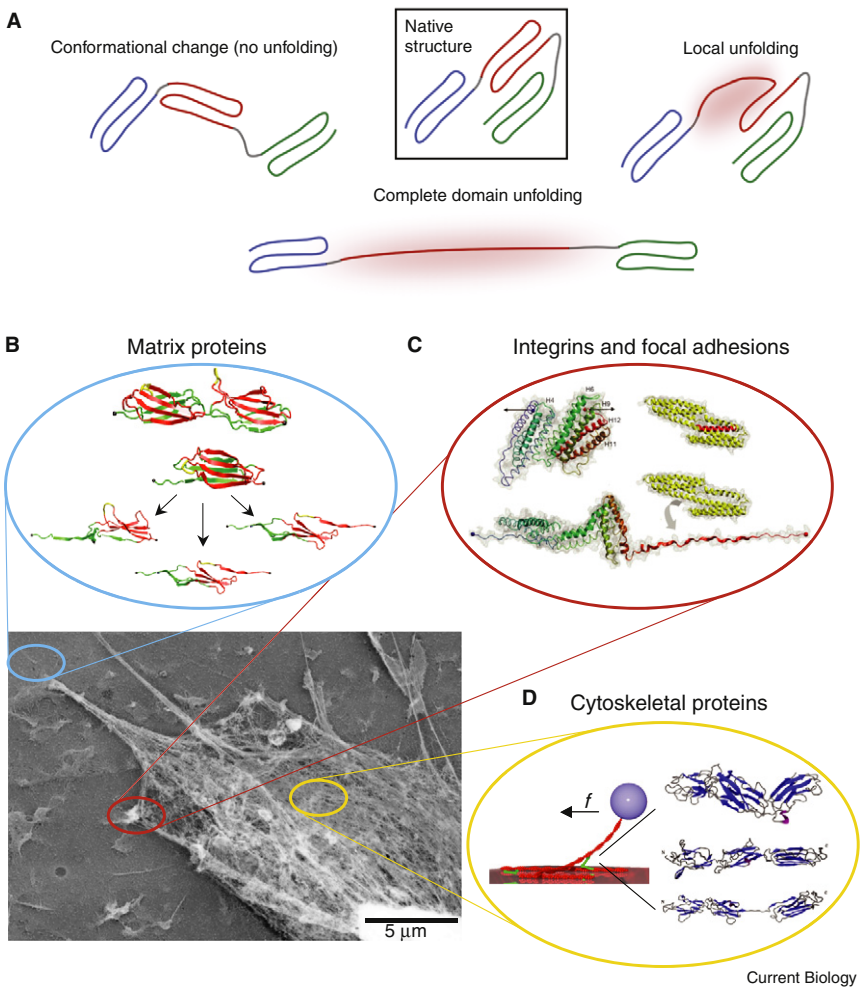

Figure 2. Types of conformational change and cellular contexts.

(A) When force is applied to proteins their native structure can be perturbed. This can involve a conformational change, in which only quaternary structure is perturbed, or unfolding, in which secondary or tertiary structure are disrupted locally or over an entire domain. (B–D) Adherent cells attach to the ECM, spread, and apply contractile forces using actomyosin stress fibers. The scanning electron microscope image in the lower left shows the detergent-extracted cytoskeleton of a mesenchymal stem cell adhering to collagen-coated glass. Adhesion can, in principle, activate several force-sensitive processes that may be mediated by protein unfolding. (B) ECM proteins, such as fibronectin, are extended, exposing cryptic binding sites that promote fiber assembly as well as cell adhesion (inset adapted from [23]). (C) Focal adhesions sense applied force and respond by recruiting additional proteins to shore up the cell–substrate interaction. A possible contributory mechanism is the force-induced exposure of vinculin-binding sites in the talin rod domain, shown here to unfold under force in molecular dynamics simulations (adapted from [55]). (D) The cytoskeleton is actively contractile and these forces impact not only the ECM and focal adhesions, but also the cytoskeleton itself. The inset shows simulations of the forced unfolding of the actin-binding protein filamin (adapted from [76]). The schematic (adapted with permission from the original by Hyungsuk Lee, Jorge M. Ferrer, and Matthew J. Lang) to the left shows how filamin unfolding and actin unbinding can be simultaneously studied in single-molecule experiments [78].

but fibronectin interacts with many other proteins, including the ECM components fibrin and collagen [27], as well as cell-surface integrins [28]. Fibronectin's multi-modular structure might, therefore, have evolved to serve as a multi-purpose mechanosensor in the ECM [29], with different binding events controlled by distinct mechanical stabilities of the different domains: at low forces, sites involved in fibril assembly of fibronectin could be exposed; while later, if the fibrils are put under higher tension, new binding sites could be exposed when the more stable domains are unfolded, as suggested already by single-molecule experiments [30]. A current challenge is to elaborate these mechanically propagating interactions.

Fibrin, fibronectin, and collagens (types 1 and 3) are linked physically through binding — perhaps with tension dependent mechanisms — and they are linked functionally during wound healing. An initial fibrin clot is replaced by ECM components that include fibronectin and then more permanent (and stiffer [31]) crosslinked collagen [32]. Fibrinogen binds several proteins in addition to fibronectin, and some of these interactions might also be regulated by force. The coiled-coil domains of fibrinogen have already been shown to unfold under force [33,34]. More recent studies of fibrin gel stretching further indicate that, at small strains (<50%), a volumetric phase transition occurs with a large negative compressibility that represents loss of water and association of unfolded protein, and then a reversible order–disorder transition occurs as seen in small angle X-ray scattering [35]. Despite such macroscopic evidence of coiled-coil unfolding, it is not yet clear that binding sites found in the globular end domains of fibrinogen undergo changes in conformational exposure under force. Mechanically labile coiled coils might protect the globular domains from unfolding and thus play the opposite role of unfolding in fibronectin. Another possibility is that force regulates the conformation of the carboxy-terminal end of the γ-chain in fibrinogen, a natively unstructured region that contains an AGDV amino-acid sequence involved in platelet adhesion [36,37].

Collagen, like fibronectin, has a cryptic cell-binding site that can be exposed by enzymatic degradation. Exposure of this site has been shown to influence angiogenesis [38] and to play a role during enzyme-mediated matrix degradation, but it is not known whether this site can also be exposed by force. Although the tightly aligned nature of the collagen triple helix makes unfolding seem less likely, simulations have shown a variety of failure modes at high forces [39]. This could be relevant after traumatic injury, even if not under normal circumstances from cell-generated tension, but an active role of cells in any aspect of ECM remodeling should not be underestimated. Growth factors — so essential in development and differentiation — provide a valuable lesson in that many have long been known to bind to the ECM, but more recent experiments have now shown also that release of at least transforming growth factor-β (TGF-β) is directly

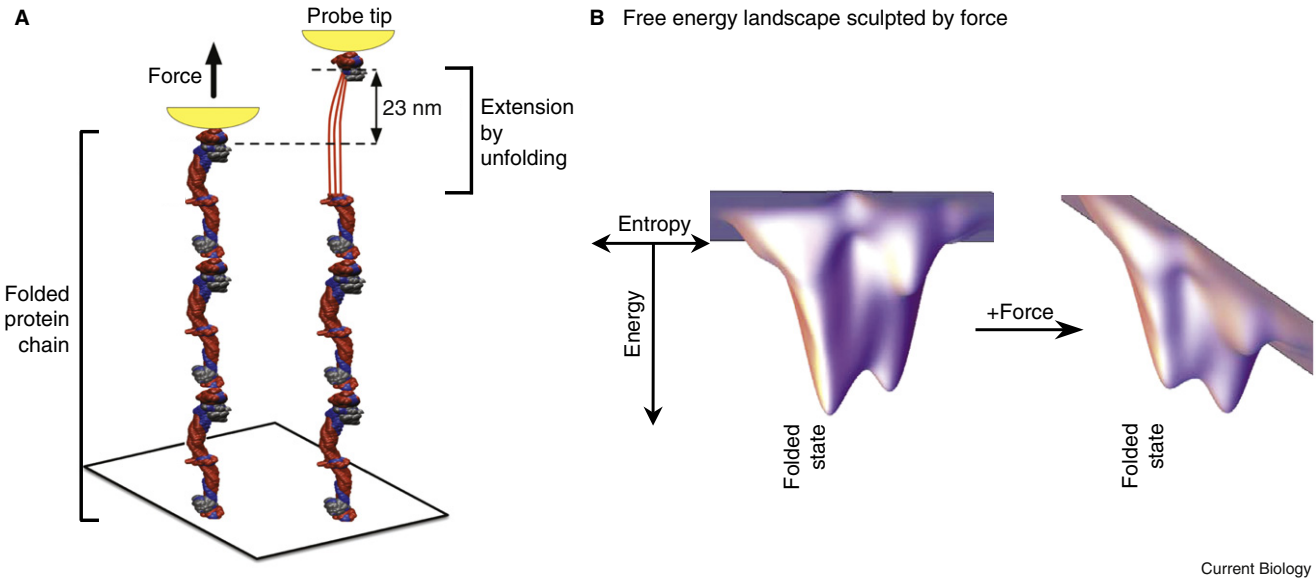

Figure 3. Nano-manipulation tools for protein folding and funnels.

(A) In studies using AFM or optical tweezers, proteins can be extended and unfolded by force using a probe (shown in yellow; adapted from [33]). (B) The energy of an extended protein conformation is typically higher than the folded native state and this is often conceptualized as an energy landscape in the form of a folding funnel where chain entropy dominates, with the many unfolded states and energetic interactions pulling the protein into one or a few well-defined structures. With force, the energy of more extended conformations is decreased, thereby accelerating unfolding.

modulated by the elasticity of the matrix and by tension generated by cells [40].

Force Modulates Cell–Matrix Interactions

Cells have evolved many ways of modulating their physical interaction with each other and with the surrounding ECM. Most cells possess integrin receptors that bind to specific ECM peptides and mediate stable adhesion [28]. Forced unfolding with exposure (or protection) of these sequences is not the only way for mechanics to influence cell behavior; integrins and perhaps other focal adhesion proteins are also sensitive to force through changes in protein structure (Figure 2C). Methods for interrogating forces from single adhesions on living cells [41] continue to be developed [42] and should provide more insight into the mechanics of adhesion that accompany these conformational changes [43].

With integrins, a conformational change from a more compact to a more extended conformation is thought to strengthen binding to ECM [44,45]. Force sensitivity is suggested by recent work with fluid shear forces imposed on adherent cells [46]. With increasing stress, the average bond force per integrin increases, but this increase depended on exposure of a synergy site in fibronectin. This scenario is consistent with the formation of 'catch-bonds', which exhibit an increased binding strength when put under mechanical load. This behavior has been directly observed at the single-molecule level in other proteins [47–49], and similarly well-controlled single-molecule experiments with integrins are anticipated.

In addition to regulating adhesion strength, integrin clustering acts as a signal involved in the formation of focal adhesions, which are currently estimated to contain around 160 component proteins [50]. When cells are stretched with a glass microneedle, they respond by locally recruiting focal adhesion proteins to shore up their attachment to substrate [3]. In this sense, focal adhesions are mechanosensitive complexes; however, changes in focal adhesion size and even the recruitment of specific proteins or the activation of particular kinases do not by themselves explain how mechanical cues are sensed, as it is not yet clear how these processes could be modulated by force. Protein unfolding is one possibility.

Vinculin is one of the proteins recruited to focal adhesions when cells are stretched [51,52] and talin, another focal adhesion component, is known to have a cryptic vinculin-binding site [53]. Thus, a possible mechanism for the force-dependent recruitment of vinculin to focal adhesions is the exposure of this site under force. Molecular dynamics simulations explored how this exposure could occur [54] and the hypothesis has recently gained experimental support with the finding that the rod domain of talin can be unfolded under moderate forces of around 10–100 pN in single-molecule experiments [55]. Importantly, single-molecule fluorescence analyses during magnetic trapping and extension of proteins reveals that vinculin is more likely to bind the talin rod domain when the rod domain is under tension. The number of binding events increased in constructs with tandem talin rod domains, while the low number of binding events to α-actinin was independent of the force, helping to rule out some possible experimental artifacts. These results are suggestive, but there are still several critical questions that remain unanswered. For example, what is the nature of the talin–vinculin binding? Simulations have provided insight [56], but experiments are lacking; rod domains with mutations in the binding sequence could help address whether the force-activated binding is similar to that observed in a talin construct that has been mutated to adopt the active conformation [57]. More importantly, a central open question is whether this mechanism is in fact operative in focal adhesions in cells. This question is now becoming addressable

with new methods for the *in situ* labeling of exposed protein sites [9], as discussed in more detail below.

Given the complexity of the network of interactions in focal adhesions, it would be surprising if this were the only process regulated directly by force. The hypothesis that there are other critical binding interactions that are directly regulated by force, possibly through unfolding to expose cryptic sites, is made more attractive by studying the type of network motifs that are found in the 'adhesome' — the set of proteins comprising the focal adhesion complex. One common network motif that appears 181 times by itself, and is also frequently embedded in larger motifs, consists of two enzymes that bind to the same scaffold protein [50]. Any time such a scaffold protein is incorporated structurally into a focal adhesion where it experiences an applied force, it could play a mechanosensitive role in which the affinity of one or both of its binding partners is altered when it completely or partially unfolds. Recent evidence suggests that this situation occurs for the mechanical activation of p130Cas [58]. An important future task, perhaps best suited to higher throughput efforts, will be to determine which of the many candidate interactions in the adhesome are indeed directly modified by force. Interaction forces or 'strengths' will not provide immediate answers, however, because entropy dictates that more frequent structures or pathways will tend to be affected more often than the unitary Boltzmann factor of equation 1 would predict. In other words, a more appropriate weight or probability P in comparing processes α and β that respectively exist N_α or N_β times is:

$$P_{\alpha/\beta} = [N_\alpha k_{o\alpha} exp(f_\alpha d_\alpha / k_B T)] / [N_\beta k_{o\beta} exp(f_\beta d_\beta / k_B T)] \quad (2)$$

where f is the force applied to the protein a distance d along the reaction coordinate. Analogies to the width of a protein folding funnel (Figure 3B), which represents the number of chain conformations, could perhaps be usefully formalized to multi-domain proteins in multi-protein network modules.

The Cytoskeleton Responds to and Actively Applies Force

'Integrin' can be considered to be a linguistic contraction for tissue 'integration' in that these membrane proteins in focal adhesions provide a continuous, physical (if kinetic) and conformationally dynamic link between the external ECM and the internal actin cytoskeleton. The latter not only supports and stabilizes the cell and helps determine its shape, but also contains at least a few proteins that clearly respond to force and are likely to be involved in mechanosensation (Figure 2D). The cytoskeleton also possesses a unique feature not present in the ECM or most focal adhesion components: it contains active assemblies that consume energy and are therefore capable of doing mechanical work [59]. This means that the cytoskeleton not only responds to force but also applies force to its surroundings [60]. This closes the mechanical loop described above and allows a cell to alter its surroundings by the forced remodeling of proteins like fibronectin and also to probe the mechanics of its substrate [4].

One of the central components of the cytoskeleton is actin. Monomeric actin polymerizes to form filaments that are an important structural component of cells. For example, gels made from purified filamentous actin and actin crosslinkers recapitulate aspects of cellular mechanics [61]. Actin polymerization consumes ATP and, because it is directional, it can apply forces to the cell periphery that drive cell motility [62]. In order to control cell morphology and to help regulate these complex actin dynamics, a host of actin-binding proteins regulate its assembly and interact with other pathways [63]. An interesting example that has received significant attention is filamin.

Filamin consists of a tandem array of Ig-like domains with an actin-binding domain on one end and a homodimerization domain on the other. When mixed with filamentous actin, filamin forms cross-links between filaments and readily promotes gelation [64]. Actin–filamin gels are more resilient and stiffer than actin gels alone or gels formed with other actin-binding proteins [65,66], suggesting that one important role for filamin is the regulation of the mechanical properties of the actin cytoskeleton. Indeed, several studies have found that filamin impacts cell mechanics [67–69] and filamin A expression has been shown to be required for active cell stiffening in response to substrate mechanics, but not for passive stiffening in response to an external force [70]. For *Dictyostelium discoideum* filamin (ddFLN), regulation of actin-cytoskeletal mechanics seems to be its main function, as it is only known to bind to one other protein in addition to actin [71]. In contrast to ddFLN, human filamin has a more elaborate structure, including hinge regions with important mechanical consequences and 24 instead of 4 Ig-like repeats. More than 20 binding partners have been identified for human filamin [72,73], including proteins with a role in signaling. Particularly relevant here, filamin has also been shown to compete with talin in binding to the cytoplasmic domain of integrin [74]. Thus, there is a direct link between filamin and proteins known to be critical for mechanosensation. Given this connection with focal adhesions and the cytoskeleton as well as filamin's structural similarity via its Ig domains to proteins like fibronectin, it is natural to ask how filamin behaves under force and whether this behavior has functional consequences.

The mechanics of filamin have been measured at the single-molecule level using AFM, and the Ig domains were found to unfold in an abrupt all-or-nothing manner at forces of around 100 pN [75], consistent with molecular dynamics simulations [76]. An important finding from work on ddFLN is that the force required to break the dimerization interaction is larger than the force required for unfolding [77], suggesting that filamin could unfold *in vivo*. However, these earlier AFM experiments could not address the strength of the filamin–actin interaction under load and, therefore, could not rule out the possibility that this interaction is the weakest link.

More recently, the mechanics of filamin have been measured in an elegant native-like system in which single filamin molecules are connected at one end to a fixed actin filament and at the other to a filament that is connected to a bead [78]. When the bead is trapped using optical tweezers and the sample stage is moved, the force required either to break the filamin–actin bond or to unfold one or more of the domains of filamin can be measured. One reason that this experiment is significant is that it gets directly at the issue of competition between alternative stress-release mechanisms. In this geometry, at the rates studied, filamin was found to unbind most of the time, but unfolding was also observed. The similarity in the force required for unfolding and unbinding could have functional consequences in regulating cytoskeletal remodeling under force and highlights the importance of thinking about timing, rates, and kinetics. Furthermore, it is not known whether any of the proteins that bind the filamin repeats affect the stability of

these regions or, conversely, whether applied stress can affect the binding affinity of the filamin-binding proteins. This question becomes especially interesting in light of recent work that suggests that filamin can serve to trap a transcription factor (i.e. PEBP2/CBF) in the cytoplasm [79], thereby regulating its action in the nucleus. If these results are confirmed, force-induced shifts in transcription factor binding could provide an elegant mechanism for mechanics to alter gene expression, which is known to occur in (filamin-expressing) mesenchymal stem cells in response to substrate stiffness [5].

Mechanical regulation of a transcription factor has already been observed in a cardiomyocyte [80], which is one type of mesenchymal cell. A prototypical kinase domain in the sarcomeric protein titin can be activated by cell contractility to bind a signaling complex which ultimately regulates a transcription factor that is well known to be regulated by the cytoskeleton — namely, serum response factor (SRF). Molecular dynamics calculations [81] and single-molecule AFM provide insight into a possible mechanism, suggesting that the kinase domain unfolds at lower force than individual Ig domains of titin [82], although equation 2 would suggest some of titin's approximately 200 Ig domains are likely to unfold if the kinase domain unfolds. Mutations in the titin kinase domain interfere with the signaling pathway and lead to a hereditary muscle disease in humans [80], which implicates force-controlled unfolding of this domain and perhaps also of some of titin's Ig domains in signaling and disease.

Another class of actin-binding proteins that has been relatively well studied also contains an actin-binding domain followed by a series of compact repeats, but, instead of the β-sheet Ig-like domains, these repeats consist predominantly of α-helical bundles. The spectrin superfamily of proteins with their triple-helical bundle domains is prototypical: α-actinins are the shortest isoforms and are found ubiquitously, while spectrins — originally isolated from red blood cell membranes — are generally found at all cell membranes and are well known to be essential for membrane stability. Additional members of the family include dystrophin, which links the contractile apparatus of striated muscle to ECM, and nesprins, which link the nuclear envelope to the actin cytoskeleton. Dystrophin deficiency is the major cause of muscular dystrophy, with symptoms emerging at a few years of age when mechanical stresses of growth become critical. Many proteins play mechanical roles *in vivo*, and a series of single-molecule experiments has been directed at understanding the molecular bases of these functions [83–87]. All spectrin family proteins tested thus far have been found to be remarkably labile, unfolding at forces of around 15–30 pN, even at the high loading rates typical in AFM experiments. Compared with β-sheet Ig-like domains, the force required to separate the helices of the spectrin bundles is relatively small and the helices themselves unfold easily because the hydrogen bonds holding them together are arranged in series in the direction of the applied force. In contrast, the bonds holding β-strands together are arranged in parallel and must be simultaneously sheared apart when the domains are pulled at their amino and carboxyl termini [88]. Given that proteins soften at higher temperatures [89,90], spectrin domains will unfold at even lower forces *in vivo*. In fact, an exhaustive study of the thermal stability of the domains of spectrin in humans found that several domains have melting temperatures of $\leq 37\,^{\circ}C$ [91]; most single-molecule experiments have been carried out at room temperature and will miss such effects. Strong effects of temperature (beyond k_BT) and the stability of multiple serial versus parallel bonds are important to consider in future experiments with single molecules as well as in extrapolations to, or the direct study of, cellular structures.

Towards a Characterization of the Cellular 'Unfoldome'

Despite the many insights obtained to date *in vitro*, the difficulty of determining whether any domains are indeed wholly or partly unfolded in either cells or ECM highlights a crucial deficit in our knowledge. New methods are therefore being developed to assess the conformational state of proteins in cells and thus extend the knowledge gained *in vitro* closer to the relevant physiological environment. However, even with the relatively small number of cases that have been examined in some detail so far, it is clear that there is no singular protein transition or master mechanical switch at the heart of cellular mechanical responses. Instead, we are probably at the early stages of discovering what will prove to be a wide variety of force-sensitive proteins and reactions. In terms of signaling networks, we have just begun to identify the mechanosensitive nodes embedded in the cell's broader system of signaling pathways. A more complete characterization of the proteins that are unfolded either by external forces applied to cells or by the cell's own myosin-driven contractility and motility (e.g. [92]) will help to guide efforts to understand the molecular basis of mechanotransduction. Just as the study of focal adhesions will progress by understanding the interactions in the adhesome, the study of mechanotransduction more generally will benefit from a more complete characterization of the cellular 'unfoldome' — the set of proteins that can be unfolded as part of their physiological function. Although the focus of this review is on cell mechanics and transduction mechanisms, the unfoldome concept can be extended more broadly to other processes that induce changes in protein conformation or quaternary structure, including heat shock and other pleiotropic perturbations.

It is possible in principle to characterize the unfoldome using experiments designed to monitor the conformation of a single protein of interest, such as using antibodies against cryptic sites or using FRET, when the requisite double-labeling is possible. For example, an antibody that recognized an extended conformation of p130Cas *in vitro* also bound preferentially to the cell periphery where forces are expected to be highest in spreading cells [58]. However, the time and effort required to develop an assay for every protein to be investigated seems unlikely to provide significant coverage of the unfoldome in the near future. Methods are therefore required to search for a wider variety of proteins whose conformations are sensitive to force.

Advances in mass-spectrometry-based proteomics have set the stage to develop a method to screen the conformational state of a large number of proteins in living cells [9]. The experimental scheme uses cysteine accessibility as a probe (Figure 4A): as a moderately hydrophobic amino acid, cysteines are often buried or partially buried within protein tertiary and/or quaternary structure and are thus not accessible to small molecules in the solvent. If a cysteine-reactive fluorescent dye is introduced into cells, only those cysteines that are exposed at the protein surface will be labeled. If changes in conditions alter the conformational state of a protein with a buried cysteine, a difference in dye labeling will be detected. Thus, the spatial distribution of

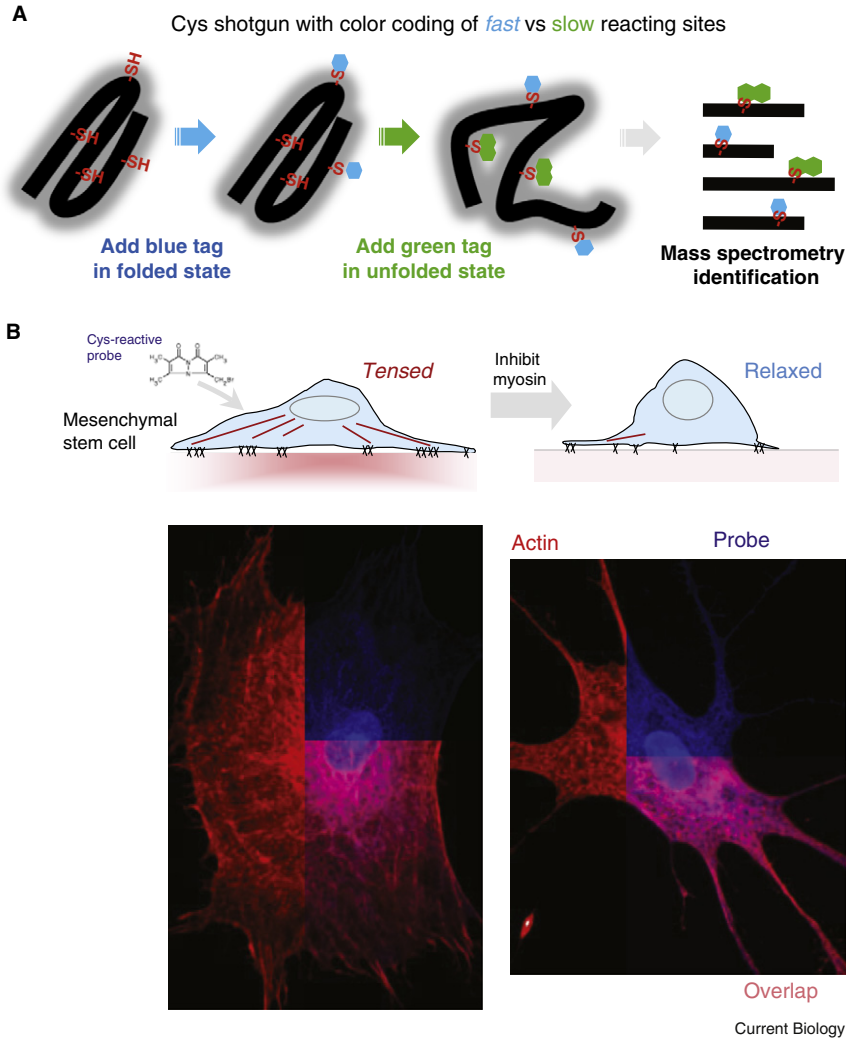

Figure 4. Cysteine shotgun labeling in cells.
(A) Cysteine residues are either buried within a protein fold or interaction site or exposed on the protein surface. The surface sites are rapidly labeled with one color before perturbation, and the buried sites are then exposed and colored differently. This provides a ratiometric signal of unfolding and improves signal-to-noise ratio for exposed sites. (B) Addition of a membrane-permeable dye to adherent cells (in this case, mesenchymal stem cells) labels cysteine residues depending on cell state, and inhibition of myosin (here with blebbistatin) leads to significant differences in protein labeling. Proteins that remain folded or assembled upon relaxation show a site-specific decrease in cysteine accessibility that is pinpointed using mass spectrometry (adapted from [9]).

unfolding can be monitored to some extent in cells using standard fluorescence microscopy. More importantly, mass spectrometry of tryptic fragments from cell lysates can identify not only the proteins that show a change in conformation, but even the precise cysteines that are exposed, so that force-sensitive domains can be mapped within proteins.

When applied to the simplest possible cell, a red blood cell, this 'Cys shotgun' approach identified six spectrin repeats that unfolded in response to physiological shear stress. Many sites in spectrin showed no change, and other membrane skeleton proteins, such as actin and ankyrin, also showed no detectable change. The Cys shotgun technique can also be applied to adherent cells. Some proteins that unfold in response to cell-generated tension are expected to refold when that tension is released (Figure 4B), for example following treatment with blebbistatin, a non-muscle myosin II inhibitor that had been shown not only to suppress cell tension but also to inhibit stem-cell differentiation (Figure 1). Cys shotgun labeling of mesenchymal stem cells attached to collagen-coated substrates in the presence or absence of blebbistatin gave a number of differential 'hits' that included non-muscle myosin IIa (consistent with drug treatment preventing a conformational change in force generation), filamin, and the intermediate filament protein vimentin. Similar mass spectrometry results were obtained with embryonic cardiomyocytes that were labeled while beating spontaneously on a soft, heart-like matrix or while static on a non-physiologically rigid matrix [93].

These initial studies serve as a proof-of-principle for Cys shotgun labeling of the unfoldome in cells and demonstrate the possibility of taking a proteomic-based approach for the identification of protein conformational changes associated with a wide range of microenvironments or drug treatments. It will be important to explore mechanically perturbed proteins in other adherent cell types and to examine cell responses to changes in applied strain. Cys shotgun investigations will also suggest promising targets and domains for more detailed study by other approaches. In combination with phosphorylation networks, which are now widely studied by mass spectrometry, the approach is poised to clarify the breadth of interplay between molecular mechanics and signaling networks.

Conclusions

A wealth of information has been gained from *in vitro* studies of the behavior of proteins and other macromolecules subjected to force. Some of the distended conformations already seem to have functional consequences, ranging from the regulation of matrix assembly and cell adhesion to cytoskeleton dynamics. Proximal effects of mechanics also have ramifications for more complex cell processes that require changes in gene expression, because these changes in protein conformation couple into other signaling pathways that can have a broad range of downstream effects. Despite some successes in identifying proteins involved in mechanosensation and obtaining an increased understanding of their mechanism of action, it seems likely that there are many more such proteins to be discovered. This search will probably be aided by methods that can broadly pinpoint relevant conformational changes amidst the complex background of mechanically silent cytosolic and cytoskeletal proteins — recognizing that there is no single cellular mechanosensor.

Ultimately, a diverse network of proteins will likely respond in specific ways to different mechanical cues, such as matrix stiffness, applied force, and fluid flow. As hinted at by the species differences in filamin, a variety of sensing mechanisms might reflect the importance of mechanical homeostasis in the evolution of larger multicellular organisms. New perspectives on human diseases — beyond heart disease [94], muscular dystrophy, and cancer [6] — are readily anticipated.

Acknowledgments

Support from the NIH, NSF, and the NSF-NSEC and MRSEC is very gratefully acknowledged. A.L. Zajac and T.M. Svitkina (University of Pennsylvania) are gratefully acknowledged for the cytoskeleton preparation imaged in Figure 2.

References

1. Pelling, A.E., and Horton, M.A. (2008). An historical perspective on cell mechanics. Pflügers Arch. *456*, 3–12.
2. Wirtz, D. (2009). Particle tracking microrheology of living cells: principles and applications. Annu. Rev. Biophys. *38*.
3. Riveline, D., Zamir, E., Balaban, N.Q., Schwarz, U.S., Ishizaki, T., Narumiya, S., Kam, Z., Geiger, B., and Bershadsky, A.D. (2001). Focal contacts as mechanosensors: externally applied local mechanical force induces growth of focal contacts by an mDia1-dependent and ROCK-independent mechanism. J. Cell Biol. *153*, 1175–1186.
4. Discher, D., Janmey, P., and Wang, Y. (2005). Tissue cells feel and respond to the stiffness of their substrate. Science *310*, 1139–1143.
5. Engler, A.J., Sen, S., Sweeney, H.L., and Discher, D. (2006). Matrix elasticity directs stem cell lineage specification. Cell *126*, 677–689.
6. Paszek, M.J., Zahir, N., Johnson, K.R., Lakins, J.N., Rozenberg, G.I., Gefen, A., Reinhart-King, C.A., Margulies, S.S., Dembo, M., Boettiger, D., *et al*. (2005). Tensional homeostasis and the malignant phenotype. Cancer Cell *8*, 241–254.
7. Orr, A.W., Helmke, B.P., Blackman, B.R., and Schwartz, M.A. (2006). Mechanisms of mechanotransduction. Dev. Cell *10*, 11–20.
8. Chen, C.S. (2008). Mechanotransduction - a field pulling together? J. Cell Sci. *121*, 3285–3292.
9. Johnson, C.P., Tang, H.Y., Carag, C., Speicher, D.W., and Discher, D. (2007). Forced unfolding of proteins within cells. Science *317*, 663–666.
10. Ingber, D.E. (2006). Cellular mechanotransduction: putting all the pieces together again. FASEB J. *20*, 811.
11. Bershadsky, A., Kozlov, M., and Geiger, B. (2006). Adhesion-mediated mechanosensitivity: a time to experiment, and a time to theorize. Curr. Opin. Cell Biol. *18*, 472–481.
12. Smock, R.G., and Gierasch, L.M. (2009). Sending signals dynamically. Science *324*, 198–203.
13. Rief, M., Gautel, M., Oesterhelt, F., Fernandez, J.M., and Gaub, H.E. (1997). Reversible unfolding of individual titin immunoglobulin domains by AFM. Science *276*, 1109–1112.
14. Kellermayer, M.S.Z., Smith, S.B., Granzier, H.L., and Bustamante, C. (1997). Folding-unfolding transitions in single titin molecules characterized with laser tweezers. Science *276*, 1112–1116.
15. Neuman, K.C., and Nagy, A. (2008). Single-molecule force spectroscopy: optical tweezers, magnetic tweezers and atomic force microscopy. Nat. Methods *5*, 491–505.
16. Bell, G.I. (1978). Models for specific adhesion of cells to cells. Science *200*, 618–627.
17. Evans, E.A., and Calderwood, D.A. (2007). Forces and bond dynamics in cell adhesion. Science *316*, 1148–1153.
18. Zhong, C., Chrzanowska-Wodnicka, M., Brown, J., Shaub, A., Belkin, A.M., and Burridge, K. (1998). Rho-mediated contractility exposes a cryptic site in fibronectin and induces fibronectin matrix assembly. J. Cell Biol. *141*, 539–551.
19. Ejim, O.S., Blunn, G.W., and Brown, R.A. (1993). Production of artificial-oriented mats and strands from plasma fibronectin - a morphological study. Biomaterials *14*, 743–748.
20. Baneyx, G., and Vogel, V. (1999). Self-assembly of fibronectin into fibrillar networks underneath dipalmitoyl phosphatidylcholine monolayers: Role of lipid matrix and tensile forces. Proc. Natl. Acad. Sci. USA *96*, 12518–12523.
21. Erickson, H.P. (1994). Reversible unfolding of fibronectin type III and immunoglobulin domains provides the structural basis for stretch and elasticity of titin and fibronectin. Proc. Natl. Acad. Sci. USA *91*, 10114–10118.
22. Krammer, A., Lu, H., Isralewitz, B., Schulten, K., and Vogel, V. (1999). Forced unfolding of the fibronectin type III module reveals a tensile molecular recognition switch. Proc. Nat. Acad. Sci. USA *96*, 1351–1356.
23. Gao, M., Craig, D., Vogel, V., and Schulten, K. (2002). Identifying unfolding intermediates of FN-III10 by steered molecular Dynamics. J. Mol. Biol. *323*, 939–950.
24. Oberhauser, A.F., Marszalek, P.E., Erickson, H.P., and Fernandez, J.M. (1998). The molecular elasticity of the extracellular matrix protein tenascin. Nature *393*, 181–185.
25. Baneyx, G., Baugh, L., and Vogel, V. (2002). Fibronectin extension and unfolding within cell matrix fibrils controlled by cytoskeletal tension. Proc. Natl. Acad. Sci. USA *99*, 5139–5143.
26. Smith, M., Gourdon, D., Little, W., Kubow, K., Eguiluz, R.A., Luna-Morris, S., and Vogel, V. (2007). Force-induced unfolding of fibronectin in the extracellular matrix of living cells. PLoS Biol. *5*, e268.
27. Hynes, R.O., and Yamada, K.M. (1982). Fibronectins: multifunctional modular glycoproteins. J. Cell Biol. *95*, 369–377.
28. Hynes, R.O. (2002). Integrins: bidirectional, allosteric signaling machines. Cell *110*, 673–687.
29. Vogel, V. (2006). Mechanotransduction involving multimodular proteins: converting force into biochemical signals. Annu. Rev. Biophys. Biomol. Struct. *35*, 459–488.
30. Oberhauser, A.F., Badilla-Fernandez, C., Carrion-Vazquez, M., and Fernandez, J.M. (2002). The mechanical hierarchies of fibronectin observed with single-molecule AFM. J. Mol. Biol. *319*, 433–447.
31. Berry, M.F., Engler, A.J., Woo, Y.J., Pirolli, T.J., Bish, L.T., Jayasankar, V., Morine, K.J., Gardner, T.J., Discher, D., and Sweeney, H.L. (2006). Mesenchymal stem cell injection after myocardial infarction improves myocardial compliance. Am. J. Phys. Heart Circ. Phys. *290*, H2196–H2203.
32. Singer, A.J., and Clark, R.A. (1999). Cutaneous wound healing. N. Engl. J. Med. *341*, 738–746.
33. Brown, A.E.X., Litvinov, R.I., Discher, D.E., and Weisel, J.W. (2007). Forced unfolding of coiled-coils in fibrinogen by single-molecule AFM. Biophys. J. *92*, L39–L41.
34. Lim, B.B.C., Lee, E.H., Sotomayor, M., and Schulten, K. (2008). Molecular basis of fibrin clot elasticity. Structure *16*, 449–459.
35. Brown, A.E.X., Litvinov, R.I., Discher, D.E., Purohit, P., and Weisel, J.W. (2009). Multiscale mechanics of fibrin polymer: Gel stretching with protein unfolding and loss of water. Science *325*, 741–744.
36. Joel, S.B. (2001). Platelet-fibrinogen interactions. Ann. NY Acad. Sci. *936*, 340–354.
37. Springer, T.A., Zhu, J., and Xiao, T. (2008). Structural basis for distinctive recognition of fibrinogen gammaC peptide by the platelet integrin alphaIIb-beta3. J. Cell Biol. *182*, 791–800.
38. Xu, J., Rodriguez, D., Petitclerc, E., Kim, J.J., Hangai, M., Yuen, S.M., Davis, G.E., and Brooks, P.C. (2001). Proteolytic exposure of a cryptic site within collagen type IV is required for angiogenesis and tumor growth in vivo. J. Cell Biol. *154*, 1069.
39. Buehler, M.J. (2006). Nature designs tough collagen: explaining the nanostructure of collagen fibrils. Proc. Nat. Acad. Sci. USA *103*, 12285–12290.
40. Wipff, P.-J., Rifkin, D.B., Meister, J.-J., and Hinz, B. (2007). Myofibroblast contraction activates latent TGF-β1 from the extracellular matrix. J. Cell Biol. *179*, 1311–1323.
41. Benoit, M., Gabriel, D., Gerisch, G., and Gaub, H.E. (2000). Discrete interactions in cell adhesion measured by single-molecule force spectroscopy. Nat. Cell. Biol. *2*, 313–317.
42. Helenius, J., Heisenberg, C.-P., Gaub, H.E., and Muller, D.J. (2008). Single-cell force spectroscopy. J. Cell Sci. *121*, 1785–1791.
43. Schmitz, J., and Gottschalk, K.E. (2008). Mechanical regulation of cell adhesion. Soft Matter *4*, 1373–1387.
44. Sims, P.J., Ginsberg, M.H., Plow, E.F., and Shattil, S.J. (1991). Effect of platelet activation on the conformation of the plasma membrane glycoprotein IIb-IIIa complex. J. Biol. Chem. *266*, 7345–7352.
45. Hato, T., Pampori, N., and Shattil, S.J. (1998). Complementary roles for receptor clustering and conformational change in the adhesive and signaling functions of Integrin alpha IIbbeta 3. J. Cell Biol. *141*, 1685–1695.
46. Friedland, J.C., Lee, M.H., and Boettiger, D. (2009). Mechanically activated integrin switch controls alpha5beta1 function. Science *323*, 642–644.
47. Marshall, B.T., Long, M., Piper, J.W., Yago, T., McEver, R.P., and Zhu, C. (2003). Direct observation of catch bonds involving cell-adhesion molecules. Nature *423*, 190–193.
48. Yakovenko, O., Sharma, S., Forero, M., Tchesnokova, V., Aprikian, P., Kidd, B., Mach, A., Vogel, V., Sokurenko, E., and Thomas, W.E. (2008). FimH forms catch bonds that are enhanced by mechanical force due to allosteric regulation. J. Biol. Chem. *283*, 11596–11605.
49. Evans, E., Leung, A., Heinrich, V., and Zhu, C. (2004). Mechanical switching and coupling between two dissociation pathways in a P-selectin adhesion bond. Proc. Nat. Acad. Sci. USA *101*, 11281–11286.
50. Zaidel-Bar, R., Itzkovitz, S., Ma'ayan, A., Iyengar, R., and Geiger, B. (2007). Functional atlas of the integrin adhesome. Nat. Cell Bio. *9*, 858–867.
51. Sawada, Y., and Sheetz, M.P. (2002). Force transduction by Triton cytoskeletons. J. Cell Bio. *156*, 609–615.
52. Galbraith, C.G., Yamada, K.M., and Sheetz, M.P. (2002). The relationship between force and focal complex development. J. Cell Bio. *159*, 695–705.

53. Gingras, A.R., Ziegler, W.H., Frank, R., Roberts, G.C.K., Critchley, D.R., and Emsley, J. (2005). Mapping and consensus sequence identification for multiple vinculin binding sites within the talin rod. J. Biol. Chem. *280*, 37217–37224.

54. Lee, S.E., Kamm, R.D., and Mofrad, M.R. (2007). Force-induced activation of talin and its possible role in focal adhesion mechanotransduction. J. Biomech. *40*, 2096–2106.

55. del Rio, A., Perez-Jimenez, R., Liu, R., Roca-Cusachs, P., Fernandez, J.M., and Sheetz, M.P. (2009). Stretching single talin rod molecules activates vinculin binding. Science *323*, 638–641.

56. Lee, S.E., Chunsrivirot, S., Kamm, R.D., and Mofrad, M.R. (2008). Molecular dynamics study of talin-vinculin binding. Biophys. J. *95*, 2027–2036.

57. Papagrigoriou, E., Gingras, A.R., Barsukov, I.L., Bate, N., Fillingham, I.J., Patel, B., Frank, R., Ziegler, W.H., Roberts, G.C., Critchley, D.R., *et al*. (2004). Activation of a vinculin-binding site in the talin rod involves rearrangement of a five-helix bundle. EMBO J. *23*, 2942–2951.

58. Sawada, Y., Tamada, M., Dubin-Thaler, B.J., Cherniavskaya, O., Sakai, R., Tanaka, S., and Sheetz, M.P. (2006). Force sensing by mechanical extension of the Src family kinase substrate p130Cas. Cell *127*, 1015–1026.

59. Pellegrin, S., and Mellor, H. (2007). Actin stress fibres. J. Cell Sci. *120*, 3491–3499.

60. Wang, J.H., and Lin, J.S. (2007). Cell traction force and measurement methods. Biomech. Model Mechanobiol. *6*, 361–371.

61. Gardel, M.L., Nakamura, F., Hartwig, J.H., Crocker, J.C., Stossel, T.P., and Weitz, D.A. (2006). Prestressed F-actin networks cross-linked by hinged filamins replicate mechanical properties of cells. Proc. Natl. Acad. Sci. USA *103*, 1762–1767.

62. Rafelski, S.M., and Theriot, J.A. (2004). Crawling toward a unified model of cell mobility: spatial and temporal regulation of actin dynamics. Annu. Rev. Biochem. *73*, 209–239.

63. Revenu, C., Athman, R., Robine, S., and Louvard, D. (2004). The co-workers of actin filaments: from cell structures to signals. Nat. Rev. Mol. Cell Biol. *5*, 635–646.

64. Shizuta, Y., Shizuta, H., Gallo, M., Davies, P., and Pastan, I. (1976). Purification and properties of filamin, and actin binding protein from chicken gizzard. J. Biol. Chem. *251*, 6562–6567.

65. Tseng, Y., An, K.M., Esue, O., and Wirtz, D. (2004). The bimodal role of filamin in controlling the architecture and mechanics of F-actin networks. J. Biol. Chem. *279*, 1819–1826.

66. Janmey, P.A., Hvidt, S., Lamb, J., and Stossel, T.P. (1990). Resemblance of actin-binding protein/actin gels to covalently crosslinked networks. Nature *345*, 89–92.

67. Cunningham, C.C., Gorlin, J.B., Kwiatkowski, D.J., Hartwig, J.H., Janmey, P.A., Byers, H.R., and Stossel, T.P. (1992). Actin-binding protein requirement for cortical stability and efficient locomotion. Science *255*, 325–327.

68. Tandon, R., Levental, I., Huang, C., Byfield, F.J., Ziembicki, J., Schelling, J.R., Bruggeman, L.A., Sedor, J.R., Janmey, P.A., and Miller, R.T. (2007). HIV infection changes glomerular podocyte cytoskeletal composition and results in distinct cellular mechanical properties. Am. J. Physiol. Renal Physiol. *292*, F701–F710.

69. Coughlin, M.F., Puig-de-Morales, M., Bursac, P., Mellema, M., Millet, E., and Fredberg, J.J. (2006). Filamin-A and rheological properties of cultured melanoma cells. Biophys. J. *90*, 2199–2205.

70. Kasza, K.E., Nakamura, F., Hu, S., Kollmannsberger, P., Bonakdar, N., Fabry, B., Stossel, T.P., Wang, N., and Weitz, D.A. (2009). Filamin A is essential for active cell stiffening but not passive stiffening under external force. Biophys. J. *96*, 4326–4335.

71. Knuth, M., Khaire, N., Kuspa, A., Lu, S.J., Schleicher, M., and Noegel, A.A. (2004). A novel partner for Dictyostelium filamin is an alpha-helical developmentally regulated protein. J. Cell Sci. *117*, 5013–5022.

72. Popowicz, G.M., Schleicher, M., Noegel, A., and Holak, T.A. (2006). Filamins: promiscuous organizers of the cytoskeleton. Trends Biochem. Sci. *31*, 411–419.

73. Stossel, T.P., Condeelis, J., Cooley, L., Hartwig, J.H., Noegel, A., Schleicher, M., and Shapiro, S.S. (2001). Filamins as integrators of cell mechanics and signalling. Nat. Rev. Mol. Cell Biol. *2*, 138–145.

74. Kiema, T., Lad, Y., Jiang, P., Oxley, C.L., Baldassarre, M., Wegener, K.L., Campbell, I.D., Ylänne, J., and Calderwood, D.A. (2006). The molecular basis of filamin binding to integrins and competition with talin. Mol. Cell *21*, 337–347.

75. Furuike, S., Ito, T., and Yamazaki, M. (2001). Mechanical unfolding of single filamin A (ABP-280) molecules detected by atomic force microscopy. FEBS Lett. *498*, 72–75.

76. Kolahi, K.S., and Mofrad, M.R. (2008). Molecular mechanics of filamin's rod domain. Biophys. J. *94*, 1075–1083.

77. Schwaiger, I., Kardinal, A., Schleicher, M., Noegel, A.A., and Rief, M. (2004). A mechanical unfolding intermediate in an actin-crosslinking protein. Nat. Struct. Mol. Biol. *11*, 81–85.

78. Ferrer, J.M., Lee, H., Chen, J., Pelz, B., Nakamura, F., Kamm, R.D., and Lang, M.J. (2008). Measuring molecular rupture forces between single actin filaments and actin-binding proteins. Proc. Natl. Acad. Sci. USA *105*, 9221–9226.

79. Yoshida, N., Ogata, T., Tanabe, K., Li, S., Nakazato, M., Kohu, K., Takafuta, T., Shapiro, S., Ohta, Y., Satake, M., *et al*. (2005). Filamin A-bound PEBP2 beta/CBF beta is retained in the cytoplasm and prevented from functioning as a partner of the Runx1 transcription factor. Mol. Cell. Biol. *25*, 1003–1012.

80. Lange, S., Xiang, F., Yakovenko, A., Vihola, A., Hackman, P., Rostkova, E., Kristensen, J., Brandmeier, B., Franzen, G., Hedberg, B., *et al*. (2005). The kinase domain of Titin controls muscle gene expression and protein turnover. Science *308*, 1599–1603.

81. Gräter, F., Shen, J., Jiang, H., Gautel, M., and Grubmüller, H. (2005). Mechanically induced titin kinase activation studied by force-probe molecular dynamics simulations. *88*, 790–804.

82. Puchner, E.M., Alexandrovich, A., Kho, A.L., Hensen, U., Schäfer, L.V., Brandmeier, B., Gräter, F., Grubmüller, H., Gaub, H.E., and Gautel, M. (2008). Mechanoenzymatics of titin kinase. Proc. Natl. Acad. Sci. USA *105*, 13385–13390.

83. Rief, M., Pascual, J., Saraste, M., and Gaub, H.E. (1999). Single molecule force spectroscopy of spectrin repeats: Low unfolding forces in helix bundles. J. Mol. Biol. *286*, 553–561.

84. Law, R., Carl, P., Harper, S., Dalhaimer, P., Speicher, D.W., and Discher, D.E. (2003). Cooperativity in forced unfolding of tandem spectrin repeats. Biophys. J. *84*, 533–544.

85. Randles, L.G., Rounsevell, R.W.S., and Clarke, J. (2007). Spectrin domains lose cooperativity in forced unfolding. Biophys. J. *92*, 571–577.

86. Law, R., Harper, S., Speicher, D.W., and Discher, D.E. (2004). Influence of lateral association on forced unfolding of antiparallel spectrin heterodimers. J. Biol. Chem. *279*, 16410–16416.

87. Bhasin, N., Law, R., Liao, G., Safer, D., Ellmer, J., Discher, B.M., Sweeney, H.L., and Discher, D.E. (2005). Molecular extensibility of mini-dystrophins and a dystrophin rod construct. J. Mol. Biol. *352*, 795–806.

88. Lu, H., Isralewitz, B., Krammer, A., Vogel, V., and Schulten, K. (1998). Unfolding of Titin immunoglobulin domains by steered molecular dynamics simulation. Biophys. J. *75*, 662–671.

89. Law, R., Liao, G., Harper, S., Yang, G., Speicher, D.W., and Discher, D.E. (2003). Pathway shifts and thermal softening in temperature-coupled forced unfolding of spectrin domains. Biophys. J. *85*, 3286–3293.

90. Schlierf, M., and Rief, M. (2005). Temperature softening of a protein in single-molecule experiments. J. Mol. Biol. *354*, 497–503.

91. An, X., Guo, X., Zhang, X., Baines, A.J., Debnath, G., Moyo, M., Salomao, M., Bhasin, N., Johnson, C., Discher, D., *et al*. (2006). Conformational stabilities of the structural repeats of erythroid spectrin and their functional implications. J. Biol. Chem. *281*, 10527–10532.

92. Brown, A.E.X., Hategan, A., Safer, D., Goldman, Y., and Discher, D.E. (2009). Cross-correlated TIRF/AFM reveals asymmetric distributions of force generating heads along self-assembled, 'synthetic' myosin filaments. Biophys. J. *96*, 1952–1960.

93. Engler, A.J., Carag-Krieger, C., Johnson, C.P., Raab, M., Tang, H.-Y., Speicher, D.W., Sanger, J.W., Sanger, J.M., and Discher, D.E. (2008). Embryonic cardiomyocytes beat best on a matrix with heart-like elasticity: scar-like rigidity inhibits beating. J. Cell Sci. *121*, 3794–3802.

94. Colet, J.P., Allali, Y., Lesty, C., Tanguy, M.L., Silvain, J., Ankri, A., Blanchet, B., Dumaine, R., Gianetti, J., Payot, L., *et al*. (2006). Altered fibrin architecture is associated with hypofibrinolysis and premature coronary atherothrombosis. Arterioscler. Thromb. Vasc. Biol. *26*, 2567–2573.