Sensing the mechano-chemical properties of the extracellular matrix

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Abstract

The ability of cells to adhere and sense their mechano-chemical environment is key to many developmental, postnatal homeostatic and pathological processes; however, the underlying molecular mechanisms are still poorly understood. Here, we summarize recent progress that indicates how cell adhesion, mechanotransduction and chemical signaling are coordinated in cells, and we discuss how the combination of novel experimental approaches with theoretical studies is currently utilized to unravel the molecular mechanisms governing mechano-chemical coupling during cell adhesion.

Introduction

Many biological processes depend on the ability of cells to sense and respond to the chemical as well as mechanical cues of the extracellular matrix (ECM). The differentiation of stem cells, for instance, is sensitive to matrix composition and rigidity [1,2]. Cell migration, which is important to a wide range of homeostatic processes, is modulated by ECM rigidity gradients in a process termed durotaxis [3]. The proliferation rates of many cell types are strongly influenced by the extent of their spread area which, in turn, depends on the chemical, mechanical and topographical properties of the extracellular environment [4]. The increased mechanical stress in fibrotic tissues is felt by resident cells that transdifferentiate into highly contractile myofibroblasts [5], and the mechanical properties of healing scars, for example in skin or heart muscle, determine tissue function after injury [6,7]. Moreover, increased ECM stiffness drives the progression of certain tumors such as breast cancer [8], and the inability of cells to properly sense their mechanical environment has been associated with a range of pathologies including muscular dystrophies or kidney malfunction [9,10].

The mechanisms by which cells detect and process mechanical information depend on the nature of the mechanical signal and the subcellular structures transmitting it. Mechanosensitive ion-channels, for instance, detect changes in plasma membrane tension [11,12], whereas cadherin-based adherens junctions transduce intercellular stresses [13]. By contrast, the chemical composition and mechanical properties of the ECM are sensed by integrin-associated, multi-molecular complexes called focal adhesions (FAs), and a conceptual understanding of FA function has been developed [14,15]. However, given the complex nature of the ECM [16], it appears increasingly important to consider the molecular complexity of FAs as well [17–22]. To aid this process, we here discuss the inner life of these enigmatic subcellular adhesion structures in more detail.

The architecture of focal adhesions

FAs display a complex, 3-dimensional organization with vertically and horizontally arranged substructures (Fig. 1). The vertical layers consist of an outer FA layer

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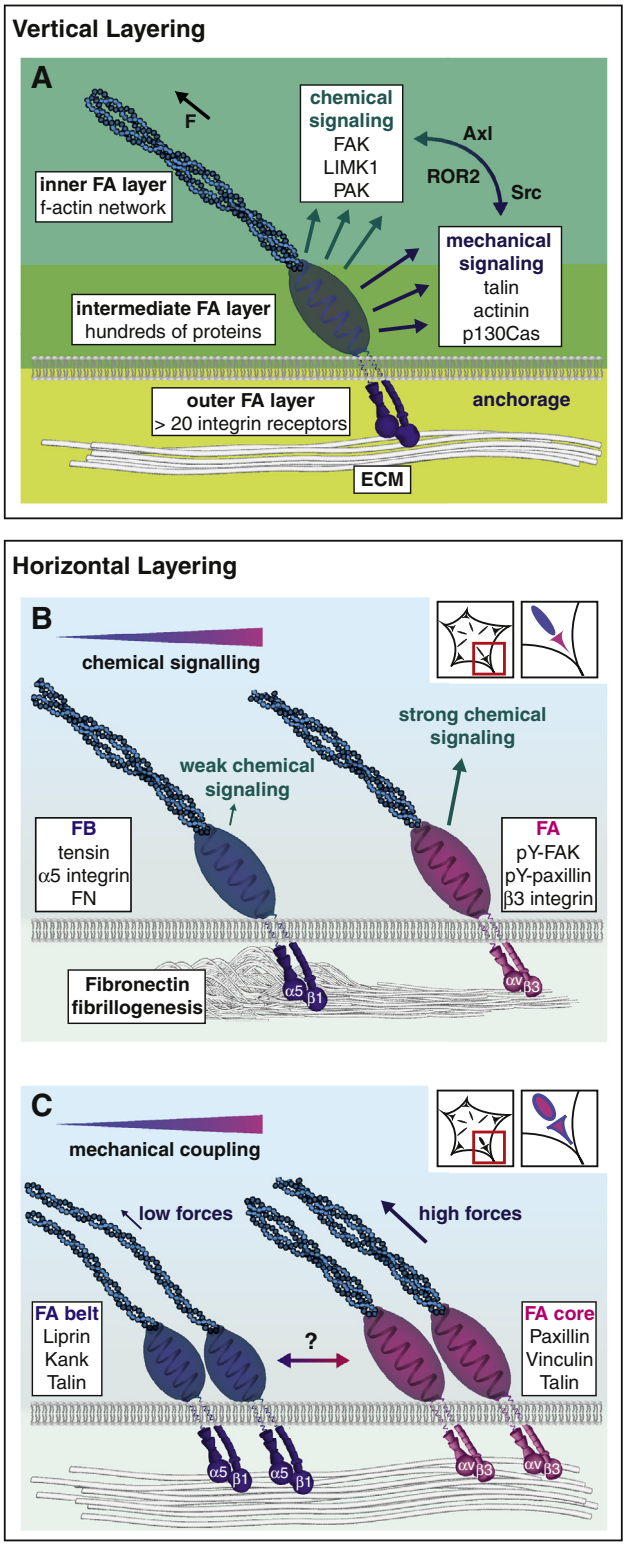
in which integrin receptors associate with the ECM, an intermediate layer where chemical and mechanical signals are processed, and an inner layer that is dominated by the actomyosin cytoskeleton (Fig. 1A). The horizontal layering facilitates the compartmentalization into distinct integrin subtype-dependent regions with different mechano-chemical characteristics (Fig. 1B, C). One fascinating challenge of today's cell adhesion research is to elucidate how the organization and dynamics of these individual FA layers are regulated.



The outer FA layer – ECM-specific anchorage by integrin receptors

The outer FA layer contains the integrin receptors. Integrins are heterodimeric transmembrane proteins that physically connect cells to a wide range of ECM proteins including fibronectin (FN), vitronectin, collagens and laminins but also to other cell surface receptors like VCAM and ICAM [23]. Mammals can express 18 α- and 8 β-integrin subunits to form 24 functionally distinct receptors, and it is clear that many, if not all, integrin subtypes have specific, non-redundant properties [23–25]. As a result, the integrin expression signature determines how FAs assemble, chemical signals are processed and mechanical information is propagated. Cells adhering to collagen type I by integrin α2β1, for instance, induce a very different signaling response as compared to cells binding collagen type IV with

Fig. 1. A vertical (A) and a horizontal (B, C) layering characterize the architecture of FAs. (A) Vertical layering. The outer FA layer, in which integrin receptors bind to extracellular ligands, provides anchorage to the ECM; depending on the expressed integrin receptor subtypes, distinct mechano-chemical signaling networks are activated. The intermediate FA layer comprises hundreds of proteins that respond to mechanical stimuli (e.g. talin, actinin or p130Cas) or mediate chemical signaling (e.g. FAK, LIMK1 or PAK). The crosstalk between FA's mechanical and chemical signaling is, at least to some extent, mediated by tyrosine kinase such as ROR2, Axl and Src. The inner FA layer merges into the actomyosin network, but also acts as a mechanosensitive module during cell adhesion. (B). Horizontal layering – fibrillar adhesions (FBs). The formation of FBs occurs on pliable FN-rich matrices and coincides with FN fibrillogenesis. While αvβ3 integrin receptors and tyrosine-phosphorylated (pY) proteins reside in the FA core, FBs are enriched in α5β1 integrin and tensin. Since FBs display very low levels of tyrosine-phosphorylated proteins as compared to FAs, their chemical signaling is likely to be different. (C). Horizontal layering – the FA belt. This type of horizontal layering is characterized by an accumulation of Kank family of proteins around mature FAs. As Kank proteins blunt integrin force transduction by partially uncoupling talin from f-actin, integrin receptors in the FA belt connect less efficiently to the actomyosin cytoskeleton. In addition, Kank associates with Liprins leading to the formation of cortical microtubule stabilization complexes (not illustrated) that regulate FA turnover. Why Kank proteins are excluded from the FA core remains to be investigated.

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α1β1 integrin [26,27]. Even similar integrins like the FN-receptors α5β1 and αvβ3 induce adhesion structures with different molecular composition and signaling characteristics [28]. In addition, most cells express various integrin receptor subtypes that may synergize, for example to amplify myosin-dependent signal transduction cascades [28].

Integrin-receptor specificity becomes even more apparent when applying mechanical loads to distinct ECM–receptor linkages. While collagen-bound α2β1 integrins appear to withstand mechanical forces of more than 100 piconewton (pN) [29], FN–α5β1 bonds are likely to break at around 30–50 pN [30], whereas αvβ3 integrins are thought to rupture at even lower forces [31,32]. Interestingly, α5β1 integrin receptors display a so-called 'catch-slip bond' behavior characterized by a transient increase in the FN-bond lifetime between 10–30 pN (Fig. 2C). How many integrin receptors display this rather unusual behavior (i.e. tighter ECM–integrin binding under force) remains to be investigated, but it appears that such catch bond properties are specific to distinct ECM–integrin interactions. Experiments on the fibrinogen–αIIβ3 linkage, for instance, revealed reduced bond lifetimes when pulling forces were increased from 5–50 pN, which is characteristic for a classical slip bond linkage [33].

Together, the available data suggest that the integrin receptor subtypes present in the outer FA layer critically determine how chemical signals and mechanical forces are processed during cell adhesion. Even though morphologically similar, FAs of different cell types may be functionally very dissimilar depending on the set of expressed integrin receptors.

The intermediate FA layer – coupling mechanical and chemical signaling

Integrins, with the exception of the hemidesmosomal β4 integrin [34], are characterized by short

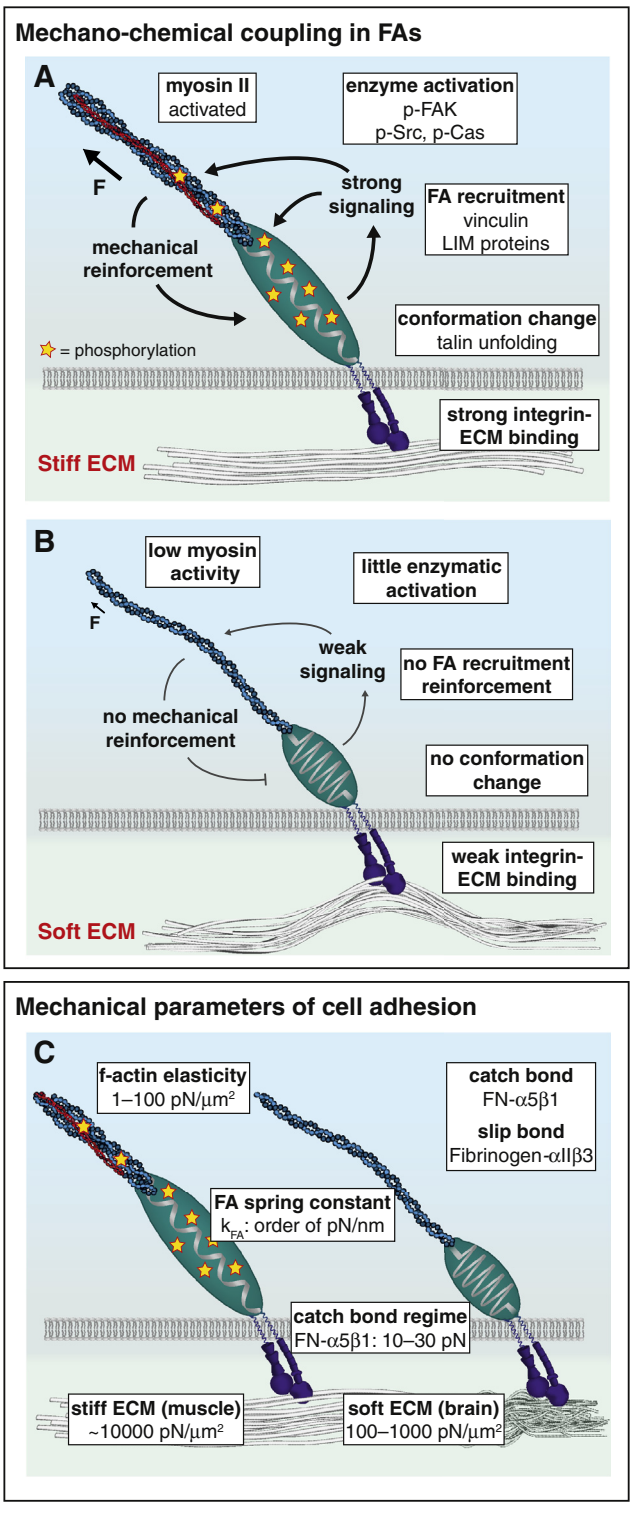

Fig. 2. FAs couple chemical and mechanical signaling. (A) Stiff ECM. Cells adhering to rigid matrices enforce their FAs under load through mechano-chemical feedback mechanisms. For instance, high mechanical tension across integrin receptors leads to partial talin unfolding and subsequent vinculin recruitment; also LIM domain proteins are recruited to FAs in response to mechanical tension but the underlying mechanisms are still unclear [17]. Mechanical force may also activate FA-resident kinases that amplify chemical signaling in FAs through protein phosphorylation [49]. Modulation of RhoGTPase signaling pathways leads to increased actomyosin contractility and stress fiber formation. (B) FAs in cells on soft matrices may not experience high enough stresses to activate force-sensitive proteins that, as a result, remain insensitive to chemical signals. (C). Typical mechanical parameters of cell-matrix adhesions. The elastic modules of the extracellular matrix typically ranges from hundreds–thousands of pN/μm^2; the actomyosin network is comparably soft [120]. Typically, FA spring constants are modelled in the order of tens of pN/nm [121]. Force of 10–30 pN increase the lifetime of the α5β1–FN linkage [30], whereas the fibrinogen–αvβ3 bond displays slip bond characteristics [33].

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cytoplasmic tails of 13–70 amino acids that have neither enzymatic activity nor binding sites to directly associate with the actomyosin cytoskeleton [35]. Instead, intracellular proteins assemble at the cytoplasmic tails leading to integrin clustering and the formation of the second FA layer, where most of the chemical and mechanical signals are integrated.

Crucial for the formation of the intermediate FA layer are the integrin regulators talin and kindlin both of which are required for inducing and then maintaining the active, ligand-binding competent state of integrins [36]. Talin comprises three f-actin and eleven vinculin-binding sites and mediates one of the first mechanical connections to the actomyosin network [37]. Interestingly, a similar f-actin binding function of kindlin-2 has been recently proposed [38], but it remains to be tested whether kindlin can bear mechanical forces during cell adhesion. Other important integrin interactors are the f-actin binding proteins filamin, α-actinin and tensin, the adaptor protein paxillin and focal adhesion kinase (FAK) [39], but also integrin inactivators like ICAP1 [40] or the recently described pan-integrin inactivator SHARPIN [24,41]. These integrin-binding proteins engage further cytoplasmic molecules such as the ternary ILK-PINCH-Parvin (IPP) complex, actin-binding proteins like vinculin [42], as well as protein kinases, phosphatases, and other protein modifying enzymes to form the adhesome comprising hundreds of distinct proteins [17,18]. How these individual FA components associate in space and time to transduce mechanical and chemical information in the dynamic context of a living cell has remained poorly understood.

Yet, it is believed that chemical and mechanical signals synergize to enforce distinct signal transduction pathways (Fig. 2). Tension across the integrin activator talin, for example, leads to the exposure of cryptic vinculin binding sites and enhanced recruitment of vinculin [43,44] that then becomes phosphorylated [45,46]. Likewise, mechanical tension across the adaptor protein p130Cas promotes phosphorylation of specific tyrosine residues by Src family kinases [47]. Since many proteins are recruited to FAs in a force-dependent manner – numerous LIM-domain containing proteins localize to FAs only when cell adhesion forces are high [17,18,48] – it is likely that other proteins are regulated in a similar fashion. Furthermore, the catalytic activities of certain FA components are thought to be modulated by a mechanical allosterism; the enzymatic activity of Src, for instance, rapidly increases upon mechanical stimulation [49]. As other tyrosine kinases like Axl and ROR2 are critical for FA-mediated ECM rigidity sensing [50,51], it is clear that this class of enzymes plays a key role in FAs' mechano-chemical regulation. In addition, many growth factors induce pathways that modulate FA-resident tyrosine kinases such as FAK [52]; therefore, these enzymes are also key to the integration of FA- and growth factor-dependent signaling cascades during cell adhesion.

The inner FA layer – force generation and mechanosensing

The actomyosin network dominates the inner FA layer that does not only generate mechanical forces but also senses mechanical and chemical stimuli. For instance, the stability of the actin–myosin bond is sensitive to applied mechanical loads [53,54], and the probability of f-actin to be severed by cofilin decreases when the filaments experiences mechanical tension [55]. In addition, growth factor receptor pathways modulate the actin cytoskeleton through controlling Rho-like GTPases [56] as well as non-muscle myosin II activity [57], and certain GTPase-activating proteins seem to bind f-actin directly [58]. Thus, the actomyosin network acts as a mechanosensitive force generator in FAs [59].

The function of this innermost FA layer is dominated by the inherently dynamic nature of the actomyosin network, which is regulated in a spatially and temporally highly sensitive fashion [60]. While FAs in the lamellipodium of cells are exposed to a fast Arp2/3-dependent retrograde flow of actin filaments, more centrally located adhesions connect to stress fibers that can be classified, according to their morphology and myosin content, into distinct elements such as dorsal and ventral stress fibers, transverse arcs and the perinuclear cap [61]. Moreover, cells can express different actin- and myosin-isoforms as well as a myriad of f-actin associated proteins, all of which affect actomyosin assembly and activity to very different extents [61–63]. Therefore, the organization and functional role of the inner FA layer is cell-type dependent and sensitive to the FA's subcellular location.

The horizontal layering of FAs

In addition to the vertical layering, FAs frequently undergo a horizontal segmentation (Fig. 1B). The FN-binding integrin receptors α5β1 and αvβ3, for instance, segregate into distinct FA-substructures when adhering to pliable FN-rich surfaces. Under these conditions, α5β1 integrins translocate from FAs into elongated fibrillar adhesions (FBs), while αvβ3 integrin remains associated with the FA core [64,65]. In addition to the assembly of FN and the associated α5β1 integrin receptor, FBs accumulate the actin-crosslinking protein tensin, which binds to the same NPxY motif in β-integrins like talin. Since NPxY phosphorylation impairs talin binding but does not affect the tensin–integrin interaction, this modification may act as a molecular switch that regulates the horizontal compartmentalization of αvβ3–talin

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and α5β1–tensin enriched adhesion domains [66]. Both compartments are likely to have very different biochemical properties, as FBs are devoid of tyrosine-phosphorylated proteins such as pY397-FAK indicating rather weak or at least very different signaling activity [50]. In addition, FAs and FBs have different mechanical characteristics. Matured FBs are rather insensitive to myosin inhibition, whereas FA maintenance as well as FB formation strictly depend on actomyosin contractility [65]. Interestingly, the composition of FBs depends on whether cells are cultured in 2D or 3D environments [67], and it has been recognized that distinct cell types express different tensin isoforms (tensin-1, -2 and -3) displaying various degrees of FB enrichment [68]. Thus, FB formation and the physiological role of the FB–type of horizontal layering remains somewhat enigmatic and needs further investigation.

Another form of horizontal layering has been identified in a morphologically distinct structure, called the FA belt (Fig. 1C), which is characterized by the evolutionary conserved Kank proteins. Kank assembles in the immediate vicinity of FAs through talin-binding thereby promoting the active conformation of talin and thus integrin activation [69,70]. In parallel, Kank impairs talin's ability to associate with f-actin and to transduce mechanical forces. As a result, adhesions surrounded by a FA-belt display reduced force transmission leading to FA sliding and slower cell migration [69]. Intriguingly, Kank proteins also associate with cortical microtubule stabilization complexes (CMSC) through liprins that recruit microtubule plus-end tracking proteins like CLASPs [71]. Since microtubule targeting coincides with FAs turnover [72,73], this Kank-dependent type of horizontal layering is likely to play an important role in regulating FA stability through microtubule plus-end capture. Why Kank proteins bind to talin exclusively at the β1-rich FA border and not in the FA core is unclear.

As the glycocalxy of cells facilitates the lateral organization of integrin receptors during cell adhesion [74,75], it will be interesting to test whether the different types of horizontal layering within FAs are also glycocalyx-dependent.

Towards a conceptual understanding of FA function

Our current understanding of FA function is strongly influenced by the observation that cell adhesions can strengthen under mechanical loads [76,77]. Indeed, many cell types form large FAs on rigid substrates when cellular traction forces are high, while they display small FAs on soft matrices [77,78]. Similarly, inhibition of myosin activity usually reduces FA size, whereas the induction of intracellular contractility (e.g. by activating RhoA) stimulates FA growth. Consistent with these experiments, certain integrin receptors enforce their binding to extracellular ligands in response to mechanical tension [30,79], and force-induced unfolding of molecules such as talin can increase the recruitment of other FA proteins like vinculin [43]. Together, these experiments have led to the concept that FAs act very much like elastic springs that enlarge and become enforced when mechanical tension increases, but remain small and short-lived when forces are low. In this model, the spring constant assigned to a FA determines the adhesion growth rate under force, which occurs only when the ECM is sufficiently rigid (Fig. 2); on substrates that are significantly softer than the FA spring, adhesions do not enlarge because the ECM deforms and the spring does not stretch [80,81].

While this model correctly describes a large body of experimentation and provides an excellent framework for further theoretical approaches [81–83], some data indicate that the molecular regulation of FAs is far more complex. First and most importantly, FAs do not always grow under force. In fact, the opposite – FA disassembly under mechanical load – frequently occurs during cell migration and is necessary for cells to move forward [84]. Second, small and short-lived FAs termed 'nascent adhesions' (NAs) at the lamellipodium of cells generate comparably high traction forces [85], while large, centrally-located FAs often exert only low stresses [69,86]. Third, the cellular response to distinct matrix rigidities is integrin receptor subtype-dependent [28] and can be extracellularly (e.g. by addition of hyaluronic acid) [87] or intracellularly (by affecting protein tyrosine kinase signaling) modulated [50]. Finally, FAs display a heterogeneous substructure [88,89] and traction forces are often unevenly distributed below individual adhesion sites [21,86]. Thus, further experimentation and theoretical modelling is needed to comprehend FA function in cells.

Experimental approaches to investigate FA function

A number of recently developed technologies including mass-spectrometry methods and high-resolution microscopy techniques have revolutionized cell adhesion research. FAs' molecular composition is analyzed with a depth and the individual components become visualized with a resolution that is mind-blowing and was considered unrealistic just a few years ago. In addition, a set of genetically modified cell lines to investigate molecular mechanisms has become available and Crispr-Cas9 approaches will likely complement this toolbox. In the following, we will discuss how some of

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these techniques may be utilized to evaluate the composition and function of the distinct FA layers.

Probing the outer FA layer – how do distinct integrins propagate mechanical forces?

One of the open questions regarding the outer FA layer is how different integrin receptor subtypes govern FA function. The establishment of integrin-deficient cell lines, in which distinct receptor subtypes can be re-expressed and individually investigated, have been useful to study different FN-binding integrin receptors such as α5β1 and αvβ3 [28]. An exciting alternative is the use of engineered surfaces to which only distinct FN-binding integrin receptor subtypes can bind [90,91]; yet it seems important to extend these systems so that also other integrin receptors subtypes can be studied [92].

Similarly, it will be important to elucidate how distinct ECM–integrin interactions respond to applied mechanical forces, for example by atomic force microscopy (AFM) methods that revealed catch-bond behavior in FN–$\alpha_5\beta_1$ integrin linkages [30,79]. Unfortunately, a comparison with other integrin receptor types has been difficult because different experimental setups or loading rates – the speed at which mechanical forces are applied – were used [30,31,93]. It should be also noted that ECM–integrin linkages might be sensitive to the history of force application such as the FN–α5β1 bond, which is strengthened by repeated applications of mechanical forces [79]. Whether such a force-strengthening mechanism is a general feature of ECM–integrin linkages or specific to the FN–α5β1 integrin bond will be important to determine. Direct evidence that the observed mechanical responses occur in the crowded environment of a living cell may be obtained by methods allowing force measurements across individual integrin-ligand bonds during cell adhesion [94–96].

Finally, super-resolution experiments have become indispensable to study the nanostructure of FAs (Fig. 3) and the molecular dynamics of distinct integrin receptors in living cells. Single-protein tracking photo-activated localization microscopy was used to investigate the movement of individual integrin receptors molecules within and outside of FAs [97]. These experiments revealed a rather static behavior for β3 integrin receptors, while β1 integrins appeared more mobile during cell adhesion. Extending such experiments to other integrin subunits should provide valuable insights into the integrin-subtype specific properties of FAs.

Investigating the intermediate FA layer – how are mechano-chemical signals transduced?

The development of efficient mass spectrometry protocols has dramatically improved our understanding of the intermediate FA layer. Hundreds of proteins, many of which with yet unknown function, have been identified to associate with FAs [17–19], and the development of high-throughput proteomics platforms should further facilitate testing how chemical signals are transduced within FAs in a force-dependent fashion [98]. An exciting perspective is that the integration of various such data sets should provide important indications on the relevance and patho-physiological function of newly identified FA-associated molecules [99,100]. However, establishing consistent protocols that permit meta-analyses will be key to those bioinformatics approaches.

In parallel, various microscopy methods are currently being utilized to study FA structure and 3D organization with nanoscale resolution [20,22,101]. Molecular mechanics can be analyzed by FRET-based tension sensors [44,102–104] that

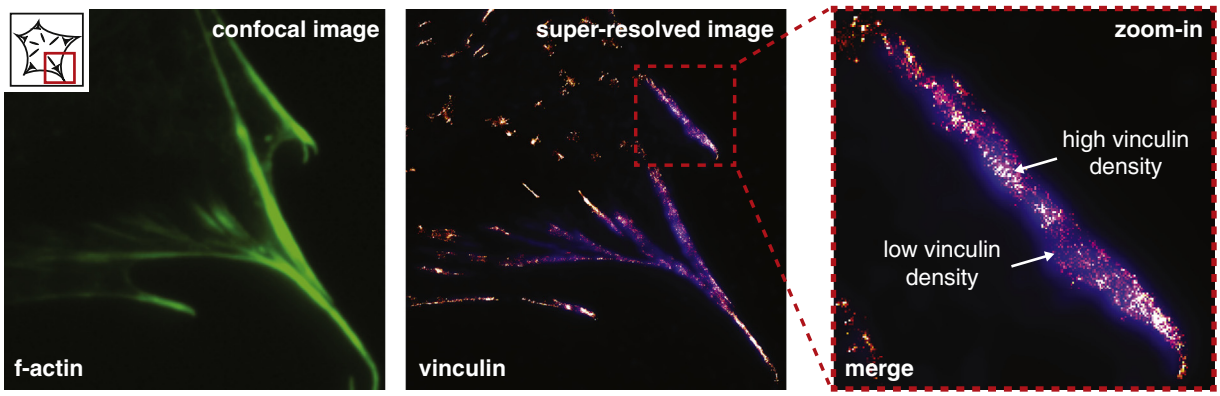

Fig. 3. FA nano-scale organization. Super-resolution microscopy reveals that proteins display an uneven distribution in FAs. The mechanisms governing this complex spatio-temporal organization are still unclear. Here, we show a fibroblast adhering to a FN-coated glass coverslip stained for f-actin; visualization of an expressed vinculin construct by stochastic optical reconstruction microscopy reveals areas of high and low protein density.

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provided first insights into vinculin- and talin-dependent processes of FA force transduction [44,105,106]. Together, these microscopy approaches revealed that FAs assemble in a much more dynamic and heterogeneous fashion than previously appreciated, and understanding how the observed FA substructures are spatio-temporally organized will be crucial. This is likely to require the further development of existing imaging techniques, for example to resolve intracellular force transduction processes with single-molecule resolution in cells.

Analyzing FAs' inner layer – quantifying acto-myosin forces in cells

The use of optical or magnetic tweezer techniques has been especially useful to study the mechanics of individual actin fibers and the actin-myosin linkage in vitro [53–55]. As distinct actin modulators and myosin isoforms regulate actomyosin function locally in cells [63,107], it will become increasingly important to complement these studies with live cell experiments. Potentially useful methods include correlational fluorescence speckle microscopy to determine acto-myosin kinetics in cells [108,109] and stochastic optical reconstruction microscopy as well as cryo-electron tomography to visualize and study distinct actin networks in situ [110,111]. Visualizing the mechanics of actomyosin networks in living cells, however, has remained challenging. A FRET-based actin tension sensor was described [112], but its rather diffuse intracellular localization may complicate the extraction of mechanical parameters with sufficient spatio-temporal resolution.

Theoretical approaches to study FA function

Many attempts to describe FA behavior theoretically are essentially captured by the original idea of Mitchison and Kirschner [113], which describe FAs as mechanical clutches that engage with the actin retrograde flow in an ECM stiffness-dependent fashion.

A stochastic simulation that considered ECM stiffness, load-and-fail-behavior of FA clutches and a force-velocity relationship of the actomyosin cytoskeleton predicted that cells tune their sensitivity towards distinct ECM rigidities by two distinguishable mechanisms. For stiff substrates, the model predicted a 'frictional slippage' state in which individual FA springs quickly increase tension and then break before other clutches can be mechanically engaged leading to high retrograde flow rates with low traction forces. On soft substrates, the model assumed a 'load-and-fail' regime where FA springs disengage less frequently resulting in slower retrograde flow velocity, maximal traction forces but frequent failure of all clutches leading to traction force fluctuations [83]. Interestingly, these predictions are consistent with the behavior of neural growth cones, and force fluctuations have been observed in migrating fibroblasts [114]. However, many cell types are known to react differently to distinct ECM rigidities. Neurons, for instance, are easily cultured on soft substrates whereas astrocytes do not spread on compliant surfaces and likely exert very little traction forces under these conditions [115]. Furthermore, distinct cell lines display maximal migration speeds on substrates with different rigidities [116]. Thus, an updated version of the stochastic simulation suggested a second frictional slippage regime on soft substrates where FA clutches failed spontaneously before mechanical loads could build up [116]. This model made the intuitive prediction that cells adjust to different extracellular compliances by modulating the 'clutch' (outer and intermediate FA layer) and 'motor' parameters (inner FA layer).

An independent study predicted and experimentally observed myoepithelial cells adapting to distinct matrix rigidities by regulating their integrin expression profile and thus modulating integrin-ECM binding/unbinding rates [117]. Another model included tension-dependent reinforcement of the FA clutch (through vinculin recruitment to partially unfolded talin molecules) and successfully predicted the frequently observed behavior of cells to increase traction forces monotonically when ECM stiffness is elevated [118]. In the future, it will be fascinating to test how well these models predict the behavior of other FA subtypes. The recent identification of the Kank family of proteins, which bind and activate talin but reduce force transduction in centrally located FAs, suggests are more complex scenario in mature adhesion structures [69]. Then again, the description of NAs, in which talin binds RIAM rather than vinculin [119], may require a different set of parameters.

Outlook

Given the highly complex nature of cell-ECM adhesions that are dependent on the mechano-chemical nature of the extracellular substrate, the integrin expression profile and the composition of the intracellular FA layers, it seems unlikely that, for the time being, one unifying theoretical model will describe the versatile behavior of FAs. Instead, the fascinatingly complex biology of FAs will likely require a set of theoretical descriptions, which consider the molecular complexity of the horizontal and vertical FA layers in more detail. The development of new technologies and their application to physiologically relevant studies should allow the identification of those parameters worth implementing.

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AFM, atomic force microscopy; CMSC, cortical microtubule stabilization complex; ECM, extracellular matrix; FA, focal adhesion; FB, fibrillar adhesion; FN, fibronectin; FRET, Förster resonance energy transfer; NA, nascent adhesions.

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