Environmental sensing through focal adhesions

Benjamin Geiger, Joachim P. Spatz‡ and Alexander D. Bershadsky**

Abstract | Recent progress in the design and application of artificial cellular microenvironments and nanoenvironments has revealed the extraordinary ability of cells to adjust their cytoskeletal organization, and hence their shape and motility, to minute changes in their immediate surroundings. Integrin-based adhesion complexes, which are tightly associated with the actin cytoskeleton, comprise the cellular machinery that recognizes not only the biochemical diversity of the extracellular neighbourhood, but also its physical and topographical characteristics, such as pliability, dimensionality and ligand spacing. Here, we discuss the mechanisms of such environmental sensing, based on the finely tuned crosstalk between the assembly of one type of integrin-based adhesion complex, namely focal adhesions, and the forces that are at work in the associated cytoskeletal network owing to actin polymerization and actomyosin contraction.

Extracellular matrix
(ECM). The complex, multimolecular material that surrounds cells. The ECM comprises a scaffold on which tissues are organized, provides cellular microenvironments and regulates multiple cellular functions.

Environmental sensing by living cells displays many features that are usually attributed to 'intelligent systems'. A cell can sense and respond to a wide range of external signals, both chemical and physical, it can integrate and analyse this information and, as a consequence, it can change its morphology, dynamics, behaviour and, eventually, fate. This phenomenon, which involves a rich range of sensory mechanisms, is widespread in almost every cell type, from prokaryotes to multicellular organisms.

Living cells grow and function while being tightly associated with the diverse connective tissue components that form the extracellular matrix (ECM). In recent years, it has become increasingly apparent that the cellular response to environmental signalling goes far beyond the ability of the cell to chemically sense specific ECM ligands, and encompasses a wide range of physical cues that are generated at, or act on, the adhesive interface between cells and the surrounding matrix. Thus, cells can react to internally generated or externally applied forces[1–3] and can sense the topography of the underlying ECM[4–6], its rigidity[7,8] and anisotropy[9,10], among other characteristics. The term sensing is used metaphorically and refers to those environmental features that can exert measurable effects on cell dynamics, function and fate following specific modulation. As shown herein, cells demonstrate an extraordinary capacity to respond to a wide range of physical signals, either locally (thereby affecting adhesion sites directly) or globally (activating signalling pathways that regulate processes such as cell growth, differentiation or programmed cell death). FIG. 1 shows the capacity of cells to respond to variations in multiple surface parameters, including ECM specificity, adhesive ligand density, surface compliance and dimensionality, by altering cell shape and cytoskeletal organization, and by modulating the adhesion sites. Naturally, the cellular sensory machinery is capable of integrating this complex information into a coherent environmental signal.

Transmembrane adhesion receptors of the integrin family have a primary role in such recognition processes. Numerous studies that are summarized in a series of recent reviews[11–15] clearly show that the biochemical characteristics of the substrate, as well as its rigidity and spatial organization, are recognized by cells through differential signalling from integrin-based molecular complexes. Moreover, these complexes are also involved in the sensing and processing of external mechanical stimuli, such as substrate stretching and fluid shear flow.

The mechanisms that underlie adhesion-mediated signalling events raise many intriguing questions. How do adhesion receptors (in particular, integrins) that lack enzymatic activity trigger downstream signalling cascades following interaction with their ECM ligands? What is the molecular sensitivity of the adhesive interactions? At what spatial, temporal and compositional resolutions does adhesion-mediated signalling occur? How are diverse molecular interactions at the adhesion site regulated? How do the physical features of the adhesive surface activate specific signalling pathways?

*Weizmann Institute of Science, Rehovot 76100, Israel.
‡Max Planck Institute for Metals Research, Stuttgart, and the University of Heidelberg, Grabengasse 1, D-69117, Heidelberg, Germany.
Correspondence to B.G.
e-mail: benny.geiger@weizmann.ac.il
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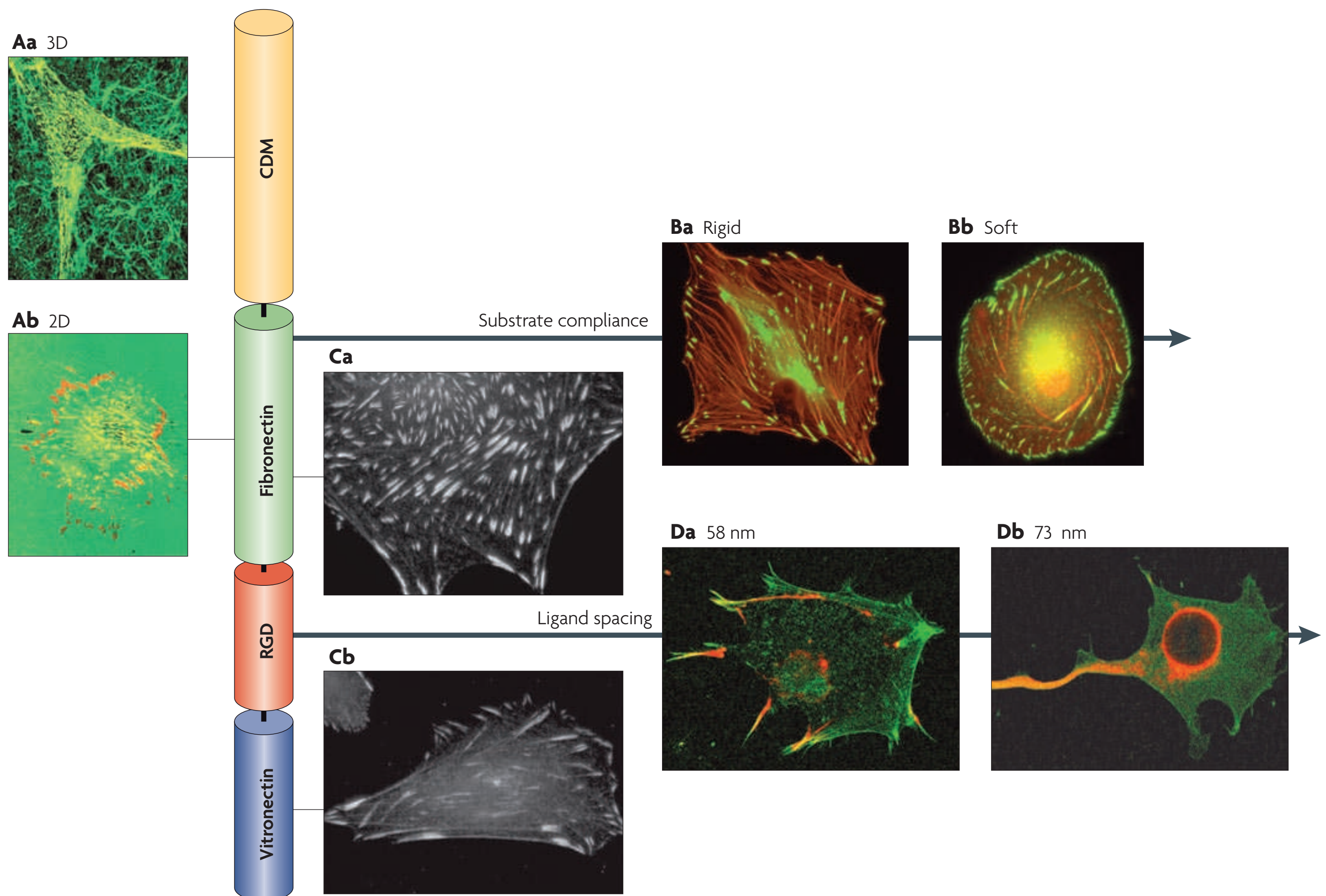

Figure 1 | **A multidimensional space of environmental parameters.** An 'axis' that consists of coloured cylindrical segments depicts the biochemical diversity of the extracellular matrix (ECM). Fibronectin is shown in green, vitronectin in blue, the Arg-Gly-Glu (RGD) peptide in red and a cell-derived natural composite matrix (CDM) in yellow[25]. Each of these matrices can be arranged into structures that differ in their physical and geometrical properties. For example, the matrices can vary according to rigidity (substrate compliance can be rigid or soft), ligand spacing (58 nm or 73 nm) and dimensionality (two-dimensional (2D) or three-dimensional (3D) network). The axes highlight this diversity by showing the values of corresponding parameters. Several possible cellular responses are shown. **A** | Cells that are attached to 3D matrices assume an elongated morphology (**Aa**) that is similar to the shapes of mesenchymal cells *in vivo*, whereas cells on 2D substrates tend to radially spread onto the substrate (**Ab**). The $\alpha5$ integrin (red) localizes to focal adhesions in cells on 2D substrates that are coated with fibronectin (green), whereas it is organized into thin, elongated adhesions in the 3D matrix. **B** | The response of human fibroblasts to rigid (**Ba**; Young's modulus (E) = 100 kPa) or soft (**Bb**; E = 10 kPa) fibronectin-coated polydimethylsiloxane substrates. The organization of green fluorescent protein (GFP)–paxillin-labelled focal adhesions (green) and phalloidin-labelled filamentous actin (red), as well as overall cell shape, strongly differ in cells that are plated onto the two substrates. **C** | The organization of focal adhesions differs in cells on 2D, rigid matrices of which the biochemical nature varies. Human fibroblast cells were plated on coverslips that are coated with fibronectin (**Ca**) or vitronectin (**Cb**), and the cells were immunostained for paxillin. Note that paxillin in vitronectin-attached cells is organized into elongated peripheral structures, whereas classical focal adhesions are observed in cells that are attached to fibronectin. **D** | B16 melanoma cells attached to nanopatterned surfaces, the adhesive nanodots of which are spaced at varying distances. Confocal micrographs of cells expressing GFP–$\beta3$ integrin (green) and stained for focal adhesion kinase (red) indicate the successful spreading and formation of focal adhesions on the 58 nm surface (**Da**) and the failure to do so on the 73 nm surface (**Db**). Images in part **A** are courtesy of K. Yamada and from REF. 147. Images in part **B** are courtesy of M. Prager-Khoutorsky. Images in part **C** are courtesy of B. Zimerman. Images in part **D** are reproduced, with permission, from REF. 20 © (2004) Wiley-VCH.

Insights into these issues can, in principle, be approached from two distinct angles. One might examine the adhesion machinery of the cell and its capacity to sense an external matrix, to integrate the incoming signals and to respond to them. Alternatively, one might focus on the diverse chemical and physical properties of adhesive surfaces, and study their capacity to trigger specific cellular responses.

Attempts to examine the molecular arsenal of adhesion sites, which are thought to be the main surface-sensing organelles, have thus far focused primarily on the molecular organization of focal adhesions and related structures.

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Such studies have revealed an extraordinary degree of molecular complexity, which is manifested by the many intrinsic components in these adhesions and the rich array of regulatory molecules that are capable of modulating the structure and dynamics of these sites.

An *in silico* survey of the adhesome network has revealed some interesting features and design principles that apparently govern molecular interactions at the adhesion sites[16] (BOX 1). Thus, for example, many of the reported physical interactions between molecules that occur at these sites are switchable, and can be regulated by signalling events, such as Tyr phosphorylation (or dephosphorylation) or the binding of specific lipids[13,17,18]. Moreover, a search of the adhesome network for network motifs has revealed a common scaffolding motif, in which one protein binds to another protein that then binds to a third molecule, the activity of which is modulated by the first. This feature suggests that the adhesome network has a crucial role in the recruitment of signalling enzymes, as well as their substrates, to the same scaffolding molecule, thereby triggering an adhesion-dependent signalling process[16].

The aim of this Review is to discuss how understanding of the interplay between adhesion and the cytoskeleton, together with advanced surface nanoengineering technology, might help us to understand cellular sensing of the microenvironments and nanoenvironments.

Engineering of nanopatterned surfaces

Diversity of substrate features. Studies that combine cutting-edge surface chemistry and molecular cell biology have shown the enormous sensitivity of cells to various features of their environment. These features include the chemical nature of the surface adhesive molecules[19], their precise spatial distribution at the nanometre and micrometre levels[20,21], and the physical properties of the surface, such as its topography[22], stiffness[7] and dimensionality[23–25].

It is known that cells respond differentially to variations in surface chemistry and can specifically distinguish between proteins or even peptides of a few amino acids, which vary by only a single chemical group or by a particular molecular conformation. The cell-specific combination of integrin receptors, for example, might be controlled by the presence of different ECM molecules (for example, fibronectin or vitronectin), by differing structures of the Arg-Gly-Glu (RGD)-based adhesive epitope[19], or by the degree of folding of the particular ECM component[6]. Nevertheless, the complexity of the natural ECM and the uncertainty that surrounds the state of exposure and reactivity of its adhesion-mediating domains render it difficult to define the sensing mechanisms that underlie cellular interactions with such surfaces, and indicate the need to develop synthetic adhesive surfaces with well-defined structures.

Focal adhesion
An integrin-mediated cell–substrate adhesion structure that anchors the ends of actin filaments (stress fibres) and mediates strong attachments to substrates. It also functions as an integrin-signalling platform.

Chemistry, mechanics and geometry. Indeed, a wide range of biomimetic adhesive surfaces have been synthesized in recent years and have been tested for their ability to support multiple cellular functions. A prerequisite for studying molecularly defined cell adhesion is the availability of a non-adhesive, passivated background surface that enables the attribution of specific cellular responses entirely to the interaction of the particular cell-surface receptors with specific adhesion-mediating ligands[26]. Among these, polyethylene glycol (PEG)-based substrates are widely used as biologically inert interfaces. Specific approaches that have been developed thus far for surface passivation include the grafting of high-molecular-weight, linear PEG[27] or star-shaped PEG macromolecules to substrates[28], as well as the use of oligo(ethylene oxide) functionalized, self-assembled monolayers[29].

The average surface concentrations and spatial density of cell-adhesive ligands on such PEG-passivated surfaces might be controlled statistically, by mixing bioactive macromolecular systems with unsubstituted molecules[30]. Chemical grafting of adhesion-associated ligands onto a PEG-based polymer has also been used to create unique surface properties for adhesive cells[31]. These studies indicate that a higher RGD surface density is essential for triggering a pleiotropic cellular response to the adhesion, which is manifested by an increase in cell spreading, the activation of survival signalling pathways and the activation of focal adhesion assembly. These observations made use of PEG molecules in particular conformations, such as star-shaped PEG chains, which enable the control of the number of RGD ligands per macromolecule[28]. One advantage of the direct functionalization (that is, the introduction of chemical functional groups, in this case adhesive RGD groups, to a surface) of the PEG chains is that these large and flexible polymers might account for various cell-binding activities that are probably caused by the local enrichment of ligands at the cell membrane, coupled to anchoring compliance. These studies provide compelling evidence for the importance of ECM flexibility and adaptability in stimulating adhesion-mediated signalling. However, this ligand template is too flexible, and is insufficiently ordered, to determine the precise interligand (and, most likely, interreceptor) spacing that is needed for the induction of specific adhesion signalling.

Indeed, a precisely localized, predefined spatial distribution of ligands on an inert background could shed light on the biological read-out of the adhesion signalling machinery. To fabricate such patterning of cell-adhesive surfaces with both adhesive and non-adhesive epitopes, microcontact printing was successfully applied to flat surfaces to geometrically control cell shape and viability. These experiments indicate that the geometry of the adhesive field not only controls integrin distribution and cell shape, but also specific gene expression programmes and, ultimately, cell survival[21].

Ligand nanospacing. Although microcontact printing provides valuable information on the role of matrix geometry in regulating adhesive interactions, more precise control of the spacing between adhesive ligand molecules in a 10–200 nm range is needed to mimic the length scale at which physiological adhesive proteins expose their epitopes at focal adhesions[32–34].

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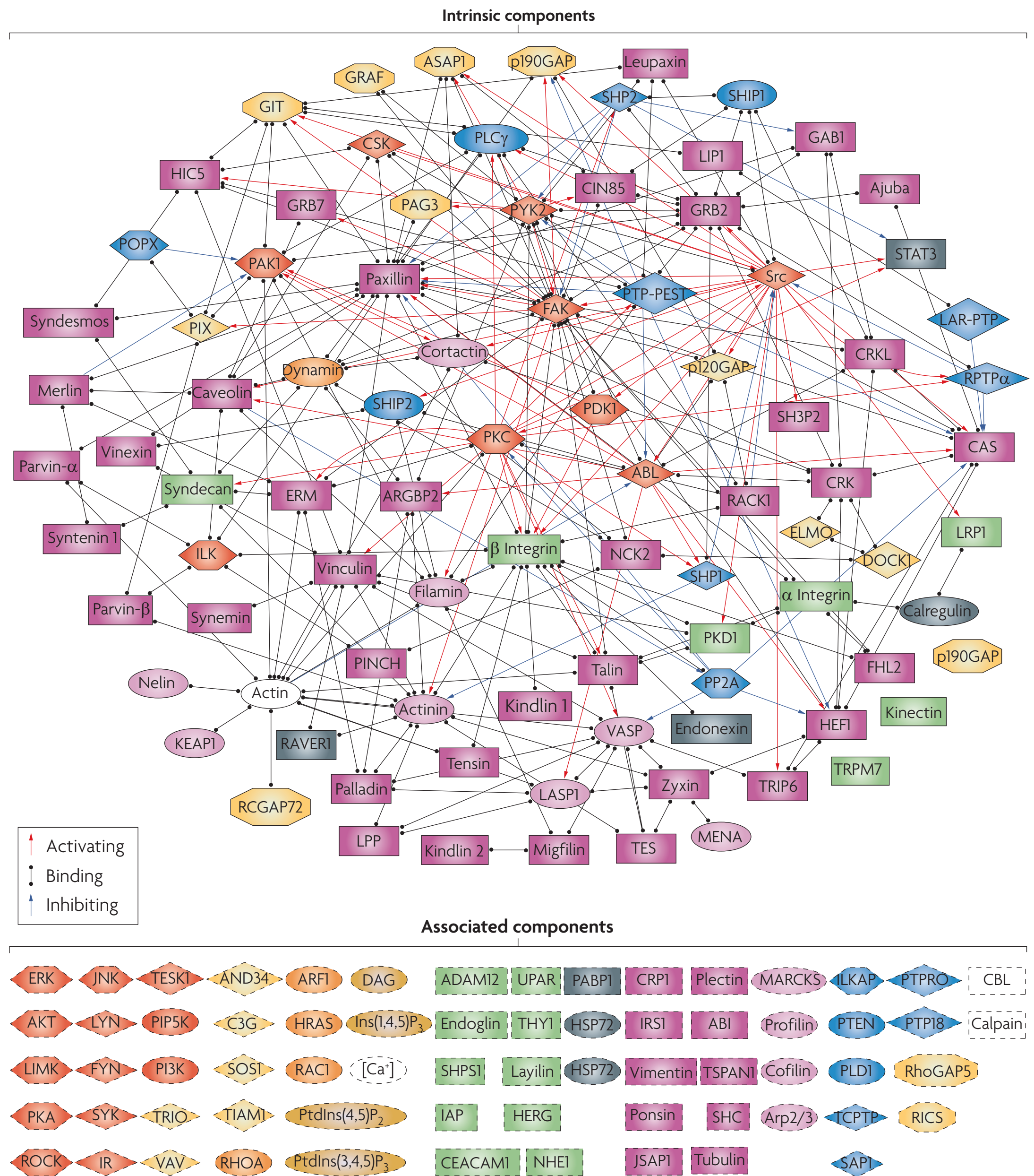
Box 1 | The integrin adhesome network: complexity, robustness and sensitivity

Integrin-mediated adhesions are multiprotein complexes that link the extracellular matrix to the actin cytoskeleton. Molecular analyses of these adhesion sites indicate that the integrin adhesome consists of ~160 distinct components (see the figure). Most of these components are intrinsic constituents of the adhesion sites (boxes surrounded by a black frame), whereas others are transiently associated with the adhesion site and affect its structure or signalling activity (surrounded by a dashed frame). Examination of the molecular interactions that take place between the different constituents of the adhesome points to an extraordinary connectivity. The entire network contains nearly 700 links, most of which (~55%) are binding interactions and the rest are modification interactions, whereby one component affects (for example, activates or inhibits) the activity of another component. The biological activities of the adhesome components are diverse and include several actin regulators that affect the organization of the attached cytoskeleton, many of the adaptor proteins that link actin to integrins either directly or indirectly, and a wide range of signalling molecules, such as kinases, phosphatases and G proteins and their regulators. Examination of the adhesome network topology reveals a prominent, three-node network motif that consists of a signalling scaffold, in which an enzyme and its substrate are recruited to the same molecular complex by a third, binding molecule. It seems likely that the tight association between the structural and signalling elements of the adhesome provides to the adhesion machinery its unique properties as a sensitive environmental sensing system. For further information, see REF. 16 and the Adhesome FA Network web site. Figure is modified, with permission, from *Nature Cell Biology* REF. 16 © (2007) Macmillan Publishers Ltd. All rights reserved.



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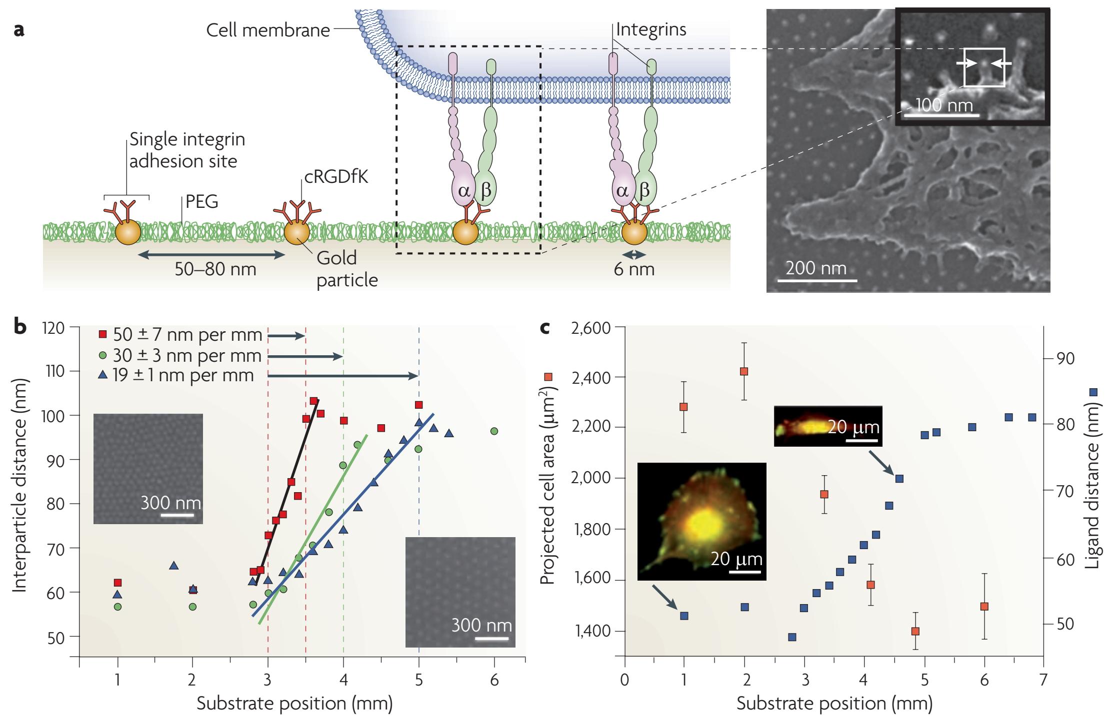

Figure 2 | **Signalling by nanopatterned substrates. a** | A schematic of a biofunctionalized gold particle substrate in contact with a cell membrane (left panel) and a scanning electron micrograph of a cell that is adhering to a gold particle (right panel). To enable the specific interaction of gold nanoparticles with integrins, the nanoparticles were functionalized with a cyclic adhesive peptide (c(RGDfK)-thiol). A functionalized gold particle with a diameter of ~6 nm on a polyethylene glycol-passivated background is small enough to allow the binding of only a single integrin protein. **b** | Particle spacing gradients with varying gradient strengths (19 ± 1, 30 ± 3 and 50 ± 7 nm per mm). The inset panels are electron micrographs that show the gold particles at different spacings. **c** | Projected cell area along a 2-mm long cRGDfK patch spacing gradient on a sample covering spacings from 50 to 80 nm after 23 h in culture. Inset panels show immunofluorescence optical microscopy images of Mc3T3 osteoblasts 23 h after plating on a homogeneous nanopatterned surface with 50 nm cRGDfK patch spacing and along the spacing gradient (large image). The small image shows a cell at a section of this spacing gradient with a ~70 nm cRGDfK patch spacing. A smaller patch spacing appears towards the left side of the image. Cells are immunostained for vinculin (green) and actin (red).

To achieve such resolution, a technology that enables the nanoscale positioning of ECM ligand molecules was developed using block copolymer micelle nanolithography. This technique involves the positioning of 1–15 nm-sized metal particles (usually gold) in a quasi-hexagonal pattern, with a tunable interparticle spacing of 10–200 nm. Besides its unique ability to precisely position single molecules at this length scale, it enables the fabrication of large surfaces that are suitable for the analysis of large numbers of cells[35–38]. A functionalized gold particle with a diameter of ~6 nm on a PEG-passivated background, for example, is small enough to allow the binding of only a single receptor protein (for example, integrin)[20,26] (FIG. 2a).

To enable the specific interaction of gold nanoparticles with integrins, the nanoparticles were functionalized with a cyclic adhesive peptide (for example, c(RGDfK)-thiol; see FIG. 2)[19,20]. Plating of cultured fibroblasts on these surfaces indicated that the cells were sensitive to variations in the spacing of the functionalized nanoparticles. Although the cells spread, multiplied and displayed restrained migratory behaviour on surfaces that have nanogold spacings of <58 nm, they spread poorly, migrated rapidly and erratically, and eventually underwent apoptosis on surfaces with interparticle spacings of >73 nm[20,39]. Apparently, there is a maximal distance (in a range of 50–70 nm) between binding sites of individual integrin molecules, above which normal integrin signalling and adhesion cannot take place. This suggests that integrin nanoclustering is essential for effective integrin-mediated signalling.

The exquisite sensitivity of cells to nanoscale variations in adhesive patch spacing might be further appreciated by offering cells nanoparticle spacing gradients along the substrate[40] (FIG. 2b). By varying the fabrication parameters, the strength of the gradient could be controlled over a rather broad range. Examination of cell behaviour on such surfaces indicated that the weakest gradient to which cells responded had a strength of ~15 nm per mm, provided that the gradient included interparticle spacings of 58–73 nm. The response to this gradient was manifested by cell elongation in the direction of the gradient and a strong tendency to migrate in this direction (FIG. 2c). Given a typical cell length of ~60 µm, this finding implies that cells can respond to a difference of ~1 nm in average ligand patch spacing between the front and rear of the cell. This sensitivity to such small variations in interparticle spacing is remarkable, and is

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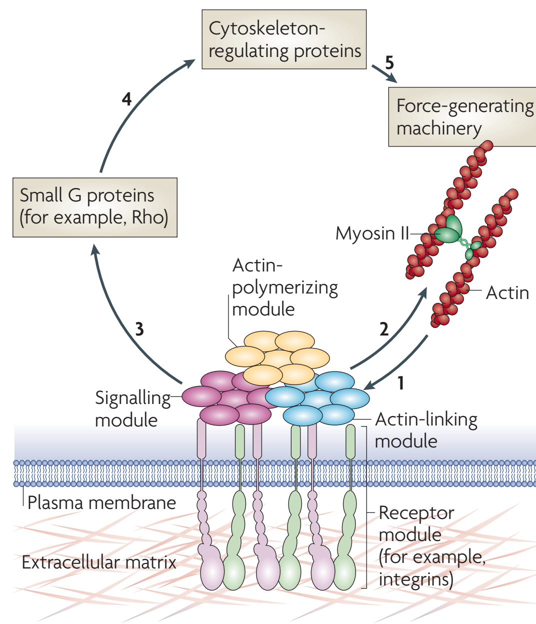

Figure 3 | **Actin cytoskeleton–focal adhesion interplay.** A schematic depicting the feedback loops that interconnect the actin machinery and integrin-mediated adhesions. Forces that are generated by actin polymerization and myosin II-dependent contractility (step 1) affect specific mechanosensitive proteins in the actin-linking module (perhaps talin and vinculin), the receptor module (represented by integrins, such as $\alpha5\beta1$ integrin and $\alpha v\beta3$ integrin) and co-receptors (such as syndecan 4 (REF. 148)), the associated actin-polymerizing module (for example, zyxin and formins) and the signalling module (represented by, for example, focal adhesion kinase and p130CAS). Acting in concert, these interacting modules, with their particular mechanosensitive components, form a mechanoresponsive network. The effect on the actin cytoskeleton (step 2) depends on the integrated response of the entire system to interactions with the matrix (FIG. 1) and to applied mechanical forces. Stimulation of the signalling module eventually leads to the activation of guanine nucleotide-exchange factors and GTPase-activating proteins, leading to activation or inactivation of small G proteins, such as Rho and Rac (step 3). These G proteins affect actin polymerization and actomyosin contractility through cytoskeleton-regulating proteins (step 4), thus modulating the force-generating machinery (step 5).

probably achieved in a time-integrative manner. These variations are far smaller than the typical variations in interligand spacing that are found on nanopatterned surfaces with uniform ligand spacing. The physiological significance of this spacing sensing and the mechanisms whereby the cells measure the particular interligand distance remain unclear, but this exquisite cellular sensitivity might arise from conditions that prevail *in vivo*, such as the 67 nm banding periodicity that is observed in collagen fibres[33] and a nanoscale order of epitope presentation found on fibronectin fibres[41,42]. Therefore, these findings might also bear relevance to the precise conditions under which natural cellular environments are constructed, in order to sustain the structure and function of living tissues.

Adhesion-dependent sensory mechanisms

The organization of the cellular machinery that is responsible for exploring microscale to nanoscale environments seems to involve feedback networks of varying complexity that connect the sensory (input) and operational (output) modules. Integrin receptors are unique in that they form an integral part of both the input and output modules. As part of the operational system that is driven by the cytoskeletal machinery, integrins that are associated with the peripheral domains of the cytoskeleton and a range of accessory signalling molecules form multiprotein adhesion complexes that both participate in and regulate multiple cellular features, such as cell anchoring, locomotion, substrate deformation and matrix remodelling.

As part of a sensory system, these integrin receptors, together with a multitude of associated proteins, including bona fide signalling elements (for example, kinases, phosphatases and adaptor proteins), respond to particular biochemical and physical characteristics of the microenvironment by initiating a cascade of events. Such cascades include the activation of phosphorylation- and G-protein-mediated pathways, which result in local alterations in cytoskeletal dynamics and the generation of mechanical force. These, in turn, lead to global changes in cell shape and motility and, ultimately, to long-term changes in transcriptional regulation, cell proliferation, differentiation and survival. These dual functions of integrins are often referred to as inside-out and outside-in signalling activities.

The molecular machinery that responds to the complex chemosensitive and mechanosensitive environmental cues — that is, signals that are generated by the molecular composition of the ECM and its mechanical properties — can be schematically viewed as a network of tightly interconnected modules (FIG. 3). Crosstalk between the actin cytoskeleton and the mechanoresponsive matrix-sensing machinery clearly has a crucial role in all types of integrin-mediated adhesions. However, existing experimental data on mechanosensing in focal adhesions are considerably more detailed than those on the sensory function of any other type of adhesion. It is worth noting that other types of integrin adhesions, namely podosomes and invadopodia, are also mechanosensitive[43,44].

The crucial scaffolding interactions that are responsible for linking the ECM to the actin cytoskeleton include actin-polymerizing and actin-linking modules, and the associated ECM-binding (that is, integrin receptor) module. These interactions regulate, and are regulated by, their associated adhesion signalling molecules (the signalling module). The system as a whole is mechanoresponsive, but probably does not contain a single, structurally distinct, mechanosensitive module. The mechanosensitive elements, namely focal adhesion-associated molecules — the structure or activity of which are modulated by mechanical force — seem to be spread over the entire focal adhesion, so that each of the aforementioned functional–structural modules contains such elements. As described below, all of these elements are integrated into the adhesome network[16].

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Box 2 | **Actin–integrin linkage and integrin activation: roles of talin and kindlin**

Integrin activation, which involves a conformational reorganization of the α integrin–β integrin dimer such that its affinity to the matrix ligand is radically increased, is essential for the initiation of focal adhesions. Two groups of proteins, the talins (talin 1 and 2) and the kindlins (kindlin 2 (also known as FERMT2 and MIG2) and kindlin 3 (also known as FERMT3)), both of which bind to cytoplasmic domains of β integrins and connect them with the actin cytoskeleton, are crucial for integrin activation.

Talins bind actin through an I/LWEQ motif at their carboxy-terminal tail domain[56–58,137]. At the same time, the FERM (four point one, ezrin, radixin and moesin) domain at the amino terminus of talin, which operates as a variant of the classic phosphotyrosine binding (PTB) domain, interacts with an NPXY motif in the conserved cytoplasmic tail of the β integrin subunit[138,139]. Compared with other proteins that link integrins to actin, talin has a special role as it binds to the cytoplasmic domain of the β integrin subunit, thereby triggering the transition of the entire α integrin–β integrin dimer from an inactive to an active conformation that is capable of high-affinity interactions with ECM ligands[140–144].

The binding of talin alone, however, seems to be insufficient for complete integrin activation. It was recently shown that other FERM- or PTB-domain proteins, kindlin 2 and kindlin 3 (which is expressed in platelets and other haematopoetic cells), are required for maximal integrin activation[53–55]. Kindlin 2 and 3 can directly bind, through their FERM or PTB domains, to β integrin tail NPXY motifs that are distinct from those used by talin. Then, in cooperation with talin, kindlins trigger integrin activation. Kindlin 2 was also shown to bind integrin-linked kinase (ILK) and migfilin (also known as FBLIM1), which links kindlin 2 to the actin cytoskeleton[145,146].

Feedback networks in the adhesome that interconnect integrin and actin filaments are absolutely essential to both the sensory and operational functions of focal adhesions. Numerous experiments clearly show that the pattern of cell–matrix adhesion strictly determines the organization of the actin cytoskeleton, whereas disruption or modification of the actin cytoskeleton leads to dramatic changes in the adhesion pattern. In particular, focal adhesions are highly sensitive, not only to inhibitors of actin polymerization but also to inhibitors of myosin II-driven contractility. It seems that focal adhesions can form and grow only if they experience pulling forces through their actin connections. At the whole-cell level, this provides a plausible mechanism for distinguishing between soft and rigid substrates, as well as between mechanically stable and unstable adhesions. Thus, mechanical cross-talk between integrins and the actin cytoskeleton is a key feature of environmental sensing. The major features of the actin–integrin feedback network, as it is presently understood, are described below.

Focal adhesions as actin–integrin links

Actin–integrin-linking proteins. Focal adhesions are dynamic actin–integrin links, the formation and maturation of which are driven by feedback from spatial and temporal interactions between the actin cytoskeleton, and integrin-based molecular constellations of increasing complexity (BOX 1). Actin filaments can be linked to the cytoplasmic domains of β integrin subunits through numerous anchoring proteins[16,45]. Whereas some of these links are redundant, others have proven to be essential to focal adhesion formation. In *Drosophila melanogaster*, for example, Talin (also known as Rhea)[46], integrin-linked kinase (ILK)[47], PINCH (also known as STCK)[48], Tensin[49] and Wech[50] are required for actin–integrin linkage. The function of talin as an anchoring protein was also shown in mammalian cells[18], where it exists in two redundant isoforms, talin 1 and talin 2 (REF. 51). A recent study[52] clearly showed that cells that lack both talin 1 and talin 2 cannot form focal adhesions, and their spreading on the substrate is unstable. Talins have a unique role in the formation and maintenance of focal adhesions, as they not only link integrin to actin filaments but, together with the essential integrin-binding proteins kindlin 2 (also known as FERMT2 and MIG2) and kindlin 3 (also known as FERMT3)[53–55], they are required for integrin activation (BOX 2). The synergistic effect of talin and kindlin on both integrin activation and on the subsequent assembly of adhesion structures is further amplified by mechanical forces that are generated by the associated polymerizing actin or by actomyosin-driven contractility. The signalling pathways in which cytoskeleton-driven forces affect the initiation, assembly and maturation of focal adhesions are outlined below.

Assembly of focal complexes or nascent adhesions. The molecular nature of the earliest integrin adhesion complexes is not clear, but it is plausible that they comprise at least two molecules of talin (which interact through the carboxy-terminal dimerization motif) that connect two α integrin–β integrin dimers with actin filaments[56–58]. Such hypothetical adhesion nanocomplexes resemble the talin-dependent, 2 pN 'slip bonds' that are formed between fibronectin and the cytoskeleton, as detected using laser tweezers[59]. Subsequent steps in focal adhesion assembly include the recruitment of additional components that promote the clustering of elementary nanocomplexes and reinforcement of the integrin–cytoskeleton bonds. In particular, the binding of vinculin to talin triggers the clustering of activated integrins[60] and, through the vinculin tail, their association with actin, thereby strengthening the actin–integrin link[61].

The earliest microscopically visible integrin-containing structures, the so-called focal complexes or nascent adhesions[45,62–64], appear as spots of ~100 nm in diameter that are composed of several hundred protein molecules. Even smaller (30–40 nm) structures that contain integrin and some associated adhesion plaque proteins have recently been detected using photoactivated light microscopy[65,66]. As a rule, the formation of the focal complexes occurs underneath the lamellipodia[62,63,67–69] — thin, flat, cellular extensions that are generated by actin-related protein 2/3 (Arp2/3) complex-mediated actin polymerization[70] and filled with a dynamic branching actin network.

Focal complex
A small (1 μm diameter), dot-like adhesion structure that is formed underneath the lamellipodium.

Lamellipodium
A ribbon-like, flat protrusion at the periphery of a moving or spreading cell that is enriched with a branched network of actin filaments.

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Actin assembly at the submembrane area, near the lamellipodial tip, generates mechanical forces that push the membrane forward[71]. At the same time, the entire actin network in the lamellipodium moves backwards relative to the lamellipodial tip, thus generating a retrograde actin flow[72]. Usually, the velocity of lamellipodial extension is slower than that of actin network assembly (even in rapidly moving cells such as keratocytes), so that the actin network in the lamellipodium moves backwards relative to the substratum[73,74]. The velocity of such movement, driven by actin polymerization in the lamellipodia of fibroblasts or epithelial cells, is several micrometres per minute[62,68,74].

At the boundary between the lamellipodium and the lamella proper (~2–4 μm from the lamellipodial tip), the density of the actin network reduces by approximately tenfold, and its architecture and protein composition change substantially. In particular, Arp2/3 complexes disappear, whereas tropomyosin and myosin II become evident[68,74]. The actin network in the lamella continues to move centripetally, although at a velocity that is at least twofold slower than that in the lamellipodium. This movement depends on myosin II activity[68], particularly on the myosin IIA isoform[75]. Retrograde actin flow in lamellipodia and in lamella apparently brushes against the immobile adhesion complexes, 'massaging' them and thereby transmitting force to them through some of their components[76,77].

Thus, even nascent focal complexes seem to experience mechanical forces that are generated by the centripetal motion of the lamellipodial actin network. Moreover, these forces seem to be required for the formation of focal complexes, as the brief treatment of cells with low doses of cytochalasin D, which does not affect the overall integrity of the actin cytoskeleton but halts the centripetal flow, leads to the complete dissolution of nascent adhesions, as visualized by the disappearance of spots containing the focal adhesion protein paxillin in the lamellipodia[62,63]. These results concur with findings that demonstrate a remarkable correlation between the uncapped barbed ends at actin polymerization sites, and localization of the conformationally active form of the β1 integrin subunits in lamellipodia and filopodia[78].

Attempts to correlate lamellipodial dynamics with nascent adhesion formation revealed that the initiation of new adhesion sites coincides with the periodic uplifting of the lamellipodium and myosin II-driven edge retraction, which suggests that myosin II-dependent contractility, as well as actin assembly, might contribute to the formation of focal complexes[67]. Inhibition of myosin II activity by various means does not, however, prevent the formation of nascent focal complexes[62,63,79–81], which suggests that either low levels of myosin II activity are sufficient for the initiation of focal adhesion or that myosin II is dispensable at this stage.

The transition to focal adhesions. Focal complexes, or nascent matrix adhesions, are transient structures that either disappear or develop into fully grown, mature focal adhesions. The molecular nature of this transition is still enigmatic, even though differences in protein composition, phosphorylation and dynamics were detected in several studies[82–86]. The LIM-domain protein zyxin, for example, constitutes a distinctive protein marker that localizes to focal adhesions but not to the nascent focal complexes[69]. However, it seems that focal adhesions that come from micrometre-sized focal complexes usually undergo maturation at the boundary between the lamellipodium and the lamella[62,63,68]. In motile or spreading cells, the cell edge with the lamellipodium continues to move forward, whereas focal adhesions remain immobile under the lamella but increase in length and thickness by incorporating new integrin molecules and cytoplasmic plaque components.

Myosin-driven contractility in adhesion maturation. Structurally, mature focal adhesions are elongated and localized at the termini of stress fibres. Stress fibres consist of actin filament bundles that contain a multitude of accessory proteins, including actin filament crosslinkers (such as α-actinin and filamin) and myosin II[87]. The presence of myosin II is responsible for the contractile nature of the stress fibres[88–90] such that focal adhesions experience continuous pulling forces, which they then transmit, through the associated integrins, to the ECM[2,91].

The formation and further growth of focal adhesions depend on myosin II and, particularly, on myosin IIA. This is the case in cells that are growing on flat, rigid substrates[81,92]. Notably, the transition from nascent contacts to elongated focal adhesions could be partially rescued in myosin IIA-knockout cells by a myosin IIA mutant that has deficient motor activity, or even by the overexpression of α-actinin[63]. Most likely, focal adhesions at the early stages of maturation still experience the centripetal forces that are generated by actin polymerization in lamellipodium, which can compensate for the lack of myosin IIA-driven contractility. The formation of fully developed, mature focal adhesions, however, requires myosin IIA motor activity[63]. Myosin IIB, which is not essential for the formation of the bulk of focal adhesions, seems to be required for the formation of stable actin filament bundles and adhesions at the rear of the cell[93], as well as for the integrin-dependent translocation of collagen fibres over the upper cell surface[94]. It seems that the difference in function between myosin IIA and IIB, and their cellular distribution, is determined by a small region at the C terminus of the molecule[93,95].

Mechanosensitivity of focal adhesions

It is becoming increasingly clear that each key step in the assembly of focal adhesions depends on, or can be strongly promoted by, the application of mechanical force by the actin system. This principle is shown by the putative force-mediated activation of vinculin binding to talin at the early stages of focal complex formation. The vinculin-binding site is buried in the talin rod, so that substantial talin unfolding is required to facilitate this interaction[96,97]. Recent simulations of the molecular dynamics of focal adhesions suggest that vinculin recruitment might be enhanced by locally applied tensile forces[98,99]. Thus, the application of mechanical force that is generated by the actin system seems to be a prerequisite for the earliest stages of focal adhesion assembly.

Lamella
A flat, sheet-like extension that is found at the cell periphery but is more internal than lamellipodia. A fan-shaped lamella is a prominent feature that characterizes the leading edge of a cell that is undergoing locomotion on a flat surface. Actin networks, also containing myosin IIA, are the principal structures in lamellae.

Filopodium
A thin, transient actin protrusion that extends out from the cell surface and is formed by the elongation of bundled actin filaments in its core.

LIM domain
A repeat of ~60 amino acids that contains Cys and His residues. The LIM domain is thought to be involved in protein–protein interactions.

Stress fibres
Also termed actin-microfilament bundles, these are arrays of parallel filaments that contain filamentous actin and myosin II, and often stretch between cell attachments as if under stress.

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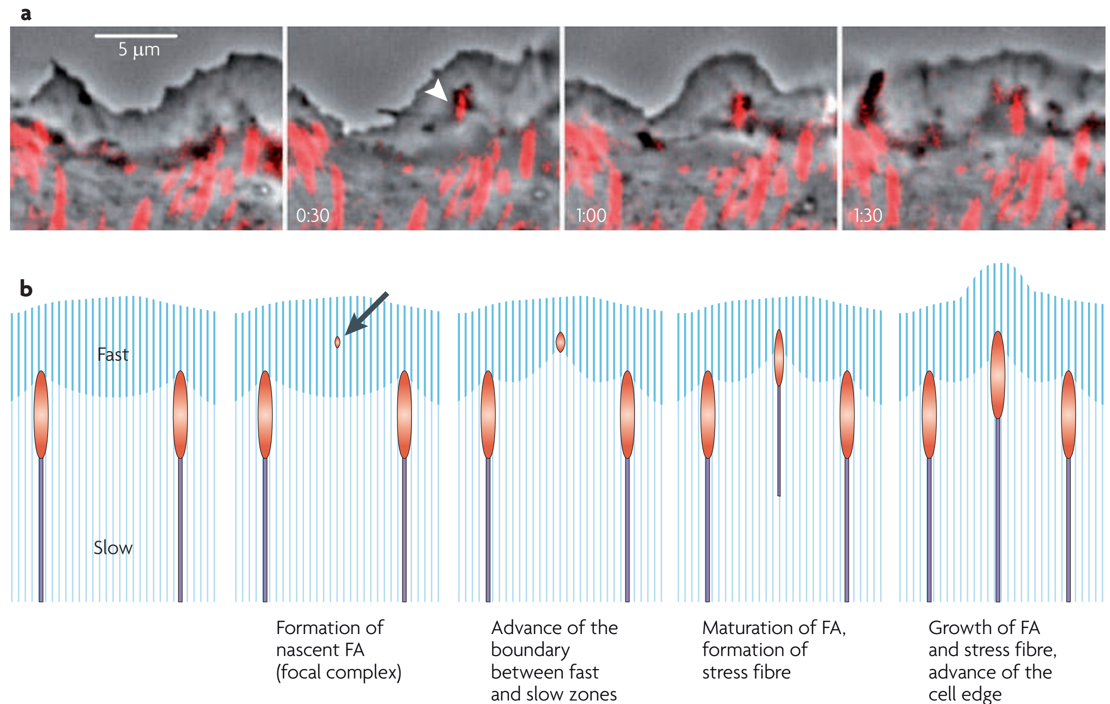

Figure 4 | **Focal adhesion formation and the lamellipodium–lamella boundary. a** | Selected frames from a time-lapse sequence that show the formation of new focal adhesions (FAs), and the associated dynamics of the boundary between the lamellipodium and the lamella. Time is indicated in minutes. Nascent FAs (a paxillin-positive spot is indicated by an arrowhead) form inside the lamellipodia. Disturbance of the flow is seen in the phase-contrast image as a dark zone in front of the adhesion site. Formation of FAs is followed by the advance of the lamellipodium–lamella boundary. The newly formed contact undergoes maturation and elongates in the direction of flow. **b** | A diagram that summarizes the stages of FA formation and maturation, and the simultaneous advancement of the boundary between fast (lamellipodium) and slow (lamella) actin flow zones. Nascent and mature FAs are shown as red ellipses of different sizes; and stress fibres are shown as purple lines of different thicknesses. Note that the process by which the boundary advances between the flows in lamellipodium (fast) and lamella (slow), and that of FA maturation, are presented in different panels, for clarity. In fact, these two processes proceed simultaneously. Parts **a** and **b** are modified from REF. 62.

A growing body of evidence indicates that mechanical perturbation, either external (for example, shear stress or matrix stretching) or internal (for example, driven by actin polymerization or by actomyosin contractility), can affect numerous proteins in the cell[100], thereby triggering a cascade of large-scale protein unfolding events[101]. Such conformational transformations could affect the exposure of binding sites, consequently modulating the recruitment of additional components to the adhesion site.

A particularly interesting component of focal adhesions is p130CAS (also known as BCAR1). The conformation of this molecule can be modified by its mechanical stretching in such a way that potential Tyr phosphorylation sites become exposed[102]. In a similar manner, the ECM protein fibronectin, a prominent integrin ligand, undergoes cell-mediated, force-driven unfolding[41]. Moreover, simulation of the molecular dynamics suggests that the transition of the β integrin subunit from an inactive to an active conformation could be produced by mechanical force[103]. The list of potential molecular mechanosensors that are associated with focal adhesions also includes mechanosensitive Ca^{2+} channels, such that the force developed by contractile stress fibres can induce a local Ca^{2+} influx near focal adhesions[104].

How these diverse molecular mechanosensing devices are indeed integrated into a single mechanosensing module remains a major challenge. Thermodynamic principles suggest that the application of stretching force to an aggregate of protein subunits should promote the growth of the aggregate in the direction of force[105], irrespective of any conformational changes in the subunits. Thus, the focal adhesion mechanosensor might be regarded as a network of tightly interconnected molecular mechanosensing units that operate in a coordinated fashion in response to mechanical forces[15,106,107]. Although these forces might be applied externally, they are usually generated by the actin cytoskeleton, thereby rendering the formation and maturation of focal adhesions actin-dependent.

Focal adhesions regulate actin assembly

The interactions between integrin-mediated adhesions and the actin cytoskeleton are bidirectional: cytoskeletal forces regulate the assembly and maturation of adhesions (see above), and at the same time, the growing adhesions can regulate the assembly of the actin system. This notion is elegantly shown by plating cells onto micropatterned surfaces, which spatially restrict the localization of adhesions[9,10,108–110]. Specifically, cells

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that are plated on flat, triangular adhesive islands form focal adhesions and stress fibres along the edges of the triangles in a reproducible manner[108]. Moreover, plating cells on islands that consist of straight and semicircular (∈-shaped) strips induce the development of a fan-like morphology in the actin cytoskeleton, with an actin-rich lamellipodium (which contains specific, actin-binding marker proteins) that is associated with the curved strip, and a 'tail' located at the end of the straight strip[9]. These simple experiments show that integrin adhesions control the formation of the actin cytoskeleton to a far greater extent than was previously appreciated.

The effect of integrin adhesions on actin organization can also be shown by the formation of the lamellipodium–lamella boundary (FIG. 4). As mentioned above, nascent focal complexes form underneath the lamellipodium, whereas maturing focal adhesions are usually found at the boundary between the lamellipodium and the lamella. Simultaneous examination of focal adhesion formation and the dynamics of the lamellipodium–lamella interface clearly show that the appearance of nascent adhesions in the lamellipodium leads to the rapid formation of a new lamellipodium–lamella border that encompasses these newly formed adhesions and moves in the direction of cell migration or spreading[62].

Actin nucleation by focal adhesions. The putative actin-nucleating function of focal adhesions serves as a prime example of adhesion-dependent regulation of the actin cytoskeleton, as shown by pioneering experiments in which the dynamics of fluorescently labelled actin, which had been microinjected into cells, were directly measured[111]. These experiments demonstrated that the actin subunits were predominantly incorporated at the membrane-associated end of the actin filaments[111]. Later studies confirmed that the stress fibres associated with focal adhesions grow and incorporate new components, mainly at the focal adhesion–stress fibre interface[112,113]. Focal adhesions were shown to be enriched with uncapped, actin-barbed ends, which is an indication of their ability to nucleate actin filament growth[114]. Although the underlying molecular mechanism is not entirely clear, the most likely nucleating factors seem to be formins.

This notion is supported by elegant biochemical experiments that show that crude, isolated integrin-based adhesion complexes can nucleate Arp2/3-independent actin polymerization *in vitro*, a process that was found to be sensitive to sequestration of the diaphanous (DIA) family of formins[115]. In line with these results, knockdown or antibody-mediated sequestration of Dia1 (also known as DIAPH1) or Dia2 (also known as DIAPH3) formins led to partial suppression of the actin-nucleating function of focal adhesions[114] and stress fibre formation[113]. As redundancy might exist among the various formins, others besides Dia1 and Dia2 could also be involved[116]. Theoretical considerations imply that formin-mediated actin polymerization could be facilitated by means of a moderate pulling force[117], which suggests that formins could be among those components that confer mechanosensitivity on focal adhesions. However, direct evidence for the association of specific formins with focal adhesions is still lacking.

Zyxin, which is a hallmark of mature focal adhesions[69], was recently shown to be required for force-dependent actin polymerization[118]. Zyxin seems to be a genuine mechanosensory component, whose association with both focal adhesions and stress fibres depends on the application of mechanical force to these structures[69,119,120]. Zyxin functions in the regulation of actin polymerization, and stress fibre remodelling might involve its cooperation with ENA/VASP (enabled/vasodilator-stimulated phosphoprotein) proteins and caldesmon[121], but the mechanisms of these processes remain to be studied. How zyxin and formins function in force-dependent actin polymerization at focal adhesions remains unclear.

Signalling from focal adhesions to the cytoskeleton. Integrin-based molecular complexes contain many bona fide signalling proteins[16], which led to the commonly held notion that they function as signal-transduction organelles. Cell motility, as well as other aspects of integrin-mediated signalling, was recently discussed in considerable detail in several excellent reviews[12–14,122–124]. Herein, we will only briefly touch on the mechanisms involved in environmental sensing that are triggered by focal adhesion-mediated signals.

The master regulators of essentially every aspect of actin cytoskeleton function are the small Rho family GTPases, principally Rho and Rac[125]. The activation of Rho GTPases is mediated by guanine nucleotide-exchange factors (GEFs), which catalyse the exchange of GDP for GTP. The activation of Rac by matrix adhesion occurs through a GEF known as the DOCK180–ELMO complex[126]. This complex is activated by a pathway that involves the focal adhesion proteins paxillin and p130CAS, both of which respond to mechanical stimulation[127]. Several GEFs for RhoA, including p115 RhoGEF (also known as ARHGEF1; LSC in mice), LARG (also known as ARHGEF12)[128] and p190RhoGEF (also known as RGNEF)[129], were recently shown to associate with focal adhesions, and become activated following cellular interaction with the ECM. Indeed, following plating of cells on fibronectin, knockdown of these factors decreases RhoA activation and, consequently, stress fibre formation also decreases. In addition to GEFs, integrin adhesions also negatively regulate RhoA activity through GTPase-activating proteins (GAPs), such as p190RhoGAP[130,131] and GRAF[132].

However, despite the intensive efforts that have been invested in characterizing the differential activity of these factors, their precise specificities and modes of activation are not known. Consequently, several issues remain unresolved. For example, do activated GEFs diffuse from focal adhesions to approach their targets, or do they activate small G proteins locally? What is the size of the region in which the activating or inhibiting effects of the focal adhesion are operative? Is it a small 'cloud' of components that surrounds an individual focal adhesion, or does it encompass a large area that is spread over the entire cytoplasm?

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Factors that transduce integrin signals to GEFs and GAPs and regulate their activity clearly have a crucial role in these processes. The best-studied example is focal adhesion kinase (FAK)[13]. This non-receptor Tyr kinase, which is localized to focal adhesions, is a key intermediary in numerous integrin-originated signalling pathways. In particular, FAK can bind, phosphorylate and activate both GEFs, such as p190RhoGEF[129], and GAPs, such as p190RhoGAP, which FAK controls in cooperation with Src[13,133]. Notably, FAK is one of the regulatory elements that is required for the mechanosensory activity of focal adhesions[13]; several other Tyr kinases and phosphatases that are localized to focal adhesions (for example, FYN, receptor-type Tyr-protein phosphatase-α (RPTPα) and SH2-domain-containing protein Tyr phosphatase 2 (SHP2)) also participate in this regulation[17]. Another important protein that is involved in the interaction of focal adhesions with the actin cytoskeleton is ILK, which, together with the proteins PINCH and parvin, form a ternary complex that has an indispensable role in both the linking of integrins to the actin cytoskeleton and in the regulation of actin dynamics[122].

Conclusions

In this article, we have addressed the issue of environmental sensing by cells from two opposite, yet highly complementary, angles. We propose that a comprehensive understanding of adhesion-mediated signalling requires the precise characterization of both the sensed surface and the sensory machinery of the cell. In recent years, remarkable progress has been made in both areas: surface nanoengineering has opened up new possibilities for the systematic modulation of individual surface features, such as surface chemistry, ligand spacing, geometry and surface rigidity. In parallel, novel techniques of gene modulation enable the selective removal, overexpression and mutation of individual genes. The effects of such perturbations on the cellular response of the sensory machinery can then be assessed.

Although our current understanding of adhesion-mediated environmental sensing is still incomplete, several design principles have emerged from the experiments outlined above. It seems, for example, that surface chemistry (that is, the presence of diverse matrix proteins) has a strong effect on the selection of specific integrin receptors and, consequently, on the initial assembly of the integrin nanocomplexes. Indeed, differential activation of integrins (for example, α5β1 integrin compared with αvβ3 integrin) can result in major differences in both the initiation and the progression of the adhesion process[134–136].

Furthermore, a growing body of evidence implicates mechanical force as central to the regulation of nearly every stage of focal adhesion assembly, from the actin polymerization-dependent assembly of the first visible, nascent adhesions, to the myosin II-dependent growth and maturation of focal adhesions. A deeper understanding of the ongoing interplay between molecular surface design and genetic modulation of the adhesion machinery is likely to reveal the nature of the mechanisms that underlie the exquisite sensitivity of living cells to both the chemical and physical characteristics of the surfaces to which they adhere.

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DATABASES
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