The inner lives of focal adhesions

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In focal adhesions of eukaryotic cells, transmembrane receptors of the integrin family and a large set of adaptor proteins form the physical link between the extracellular substrate and the actin cytoskeleton. During cell migration, nascent focal adhesions within filopodia and lamellipodia make the initial exploratory contacts with the cellular environment, whereas maturing focal adhesions pull the cell forward against the resistance of 'sliding' focal adhesions at the cell rear. Experimental approaches are now available for analysing the dynamics and interior structure of these different focal adhesions. Analysing focal-adhesion dynamics using green-fluorescent-protein-linked integrin leads us to propose that the acto-myosin-controlled density and turnover of integrins in focal adhesions is used to sense the elasticity and spacing of extracellular ligands, regulating cell migration by mechanically transduced signaling.

A supplementary movie is available at: http://archive.bmn.com/supp/tcb/imhof.mov

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During recent years our knowledge about focal adhesions and their role in cell spreading, migration and survival has increased vastly. The ever-increasing number of proteins being found to participate in focal adhesions makes them one of the most complex protein aggregates formed in a cell [1]. Focal adhesions fulfil mechanical and sensing functions that involve reversible anchorage of the actin cytoskeleton to the extracellular matrix during migration and monitoring intracellular or extracellular tension. Understanding the molecular mechanisms that account for these distinct functions of focal adhesions is a major challenge.

Eukaryotic cells have differently sized and shaped cell–substrate adhesion sites, which we here generally refer to as 'focal adhesions'. In fibroblasts, these different focal adhesions are commonly referred to as focal complexes, focal contacts and fibrillar adhesions [2]. Many attempts have been made to classify focal adhesions using descriptive features such as shape, size, cellular location, GTPase dependency and protein composition [3–7]. Unfortunately, some of these characteristics vary depending on the environment of the cells [8]. Here, we propose the use of functional criteria to classify focal adhesions according to their physiological role, such as sensing the environment or providing mechanical support, and give new definitions to distinguish focal complexes from focal contacts.

The recent use of chimeras comprising green fluorescent protein (GFP) attached to various focal adhesion proteins has made important contributions to our understanding of focal adhesions. Owing to the stoichiometric fusion of GFP to focal adhesion proteins, such GFP chimeras can be used not only as markers for cellular attachment sites but also to provide dynamic and quantitative information about the composition of focal adhesions [6,9–13]. In parallel, progress has been made in measuring the mechanical traction forces exerted by cells when they interact with elastic surfaces [9,10,14–16]. One of the emerging ideas from these studies is that focal adhesions are mechanical transducing devices with a mechanical sensor function. Hence, they relay changes of intra- and extracellular tension into signaling pathways that, in turn, modify the composition and behaviour of focal adhesion, directly influencing the migratory and contractile state of the cell [17,18].

Although this model provides a major breakthrough and contributes significantly to the understanding of focal adhesion and cellular behaviour, the underlying molecular mechanism and the concept behind mechanically transduced signaling remains obscure. Here, we review the topology of focal adhesions and propose that they are sensors for the elasticity and spacing of extracellular ligands. Our model is based on the quantitative analysis of GFP-tagged focal adhesion proteins associated with the two-dimensional (2D) plane of the plasma membrane that give dynamic insight into the interior structure of focal adhesions.

The second dimension of focal adhesions

In focal adhesions, the actin cytoskeleton is linked through various adaptor proteins to heterodimeric receptors of the integrin family (Fig. 1a) [19]. Integrin receptors bind to extracellular matrix proteins organized in either basement membranes (Fig. 1a) or connective tissues (Fig. 1b). Importantly, whether a focal adhesion is formed on a flat surface (e.g. glass coverslip) or within a network of extracellular-matrix proteins, the integrin receptors are confined to the 2D plane of the plasma membrane, in which they can diffuse laterally [20]. By contrast, the actin cytoskeleton and adaptor proteins are recruited from a cytoplasmic pool and aggregate in complex ways to form ~60-nm-thick focal contacts [21]. Therefore, one can classify focal adhesion components that are confined to or entrapped within the 2D plasma membrane, such as integrins, as 2D focal adhesion markers. By contrast, adaptor proteins that are stacked on top of each other within the actin backbone of focal adhesions, such as vinculin and paxillin, represent markers of the focal adhesion volume.

This observation is important when the fluorescence intensity of such GFP chimeras is measured by light microscopy, which is unable to resolve the depth of focal adhesions (Fig. 1c) [22]. For example, if one observes an increase in the fluorescence intensity of a given GFP marker within a focal adhesion, the following conclusions can be

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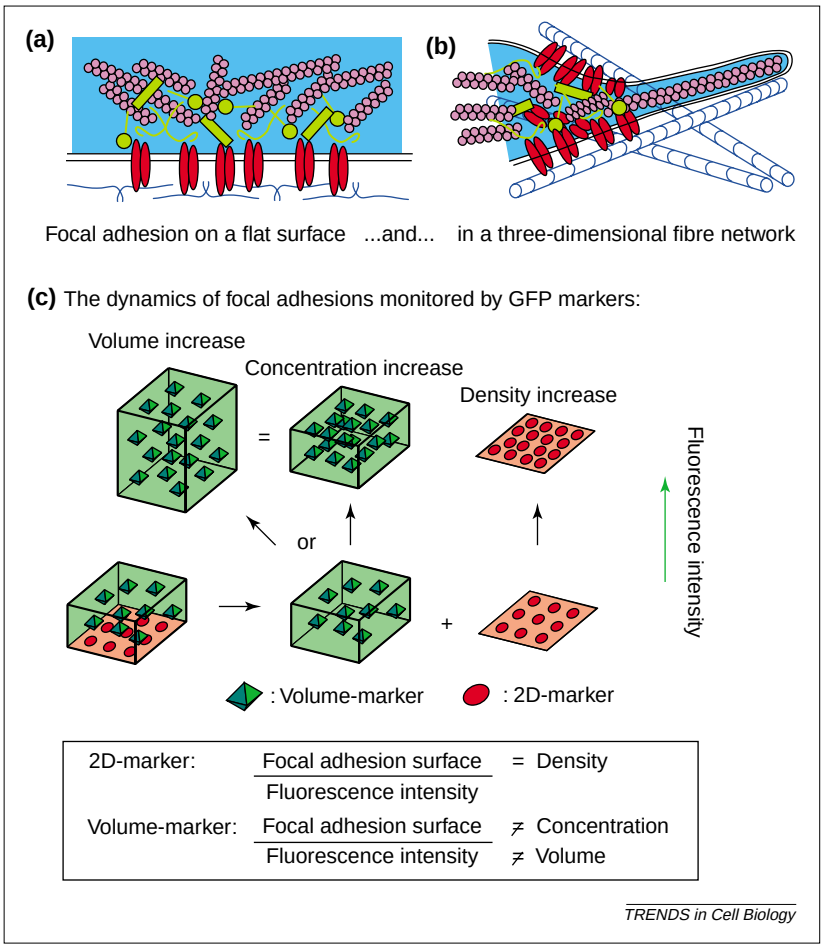

Fig. 1. The topology of focal adhesions and their components on a flat surface such as a fibronectin-coated glass coverslip (a) or formed within a three-dimensional network of collagen fibres (b). The topology of focal adhesion is identical in (a) and (b), linking the extracellular substrate (blue) via integrin heterodimers (red) embedded in the plasma membrane (thin lines) and various adaptor proteins (green) to the actin cytoskeleton (purple). Although focal adhesions are very flat structures, they nevertheless have a certain volume occupied by many adaptor proteins stacked on top of each other. This volume can be considered as a box (c) in which the adaptor proteins appear as volume markers that fill the inside of the focal adhesion. By contrast, transmembrane constituents of focal adhesions such as integrins will behave as two-dimensional (2D) markers labelling just the lower surface of the box. The increase in the fluorescent intensity of the 2D marker indicates a higher packing density within the focal adhesion. By contrast, owing to the missing resolution along the *z* axis, an increase in fluorescence intensity of the volume marker does not allow us to distinguish between an increase in packing density (concentration) or an increase in focal adhesion volume. This makes a 2D marker a better diagnostic instrument to analyse the interior physical structure of focal adhesions.

drawn (Fig. 1c). For a 2D marker, the equation is simple: an increase in fluorescence intensity indicates a higher packing density in the focal adhesion. For a volume marker, the situation is more complex: higher fluorescence intensity indicates more GFP molecules, but this yields no structural information about the organization of the extra molecules recruited to the focal adhesion. The issue is further complicated by the fact that certain focal adhesion adaptor proteins can, owing to their multiple protein–protein interactions [1], behave as 2D or volume markers depending whether they interact with plasma-membrane- or actin-backbone-associated focal adhesion components. In general, however, a 2D marker has a higher diagnostic potential than a volume marker for interpreting changes in fluorescence intensity.

This problem with quantifying GFP marker proteins that do not exclusively associate with the plasma membrane of focal adhesions is highlighted in the following two examples. In human foreskin fibroblasts, it has been demonstrated that the amount of tension generated by a focal adhesion correlates directly with focal adhesion size and with the amount of fluorescence of the focal adhesion adaptor protein GFP–vinculin in it [9]. In another study, the fluorescence intensity of GFP–zyxin, another focal adhesion adaptor protein, has been compared with the traction forces exerted by focal adhesions in migrating fish fibroblasts. In contrast to the analysis with vinculin, the fluorescence intensity of GFP–zyxin in focal adhesions demonstrated an inverse correlation with the generated traction stress in the respective focal adhesions [10]. We are led to conclude that the different focal adhesion markers used in these studies have distinct functions and are being recruited by signals that might not be generated by the mechanical forces applied to focal adhesions. Moreover, bearing in mind the complexity and multiple functions of focal adhesions, it is very difficult to assign a specific cause to changes in the fluorescence intensities of any particular GFP marker. Hence, we propose the use of a 2D GFP marker for the quantitative analysis of tension-dependent changes in focal adhesion structure. Preferably, this 2D marker should also serve a mechanical function, for example by being part of the physical link between the extracellular matrix and the actin cytoskeleton.

Using a 2D GFP–β3-integrin marker

The two examples of changing intensities of the focal adhesion markers GFP–vinculin and GFP–zyxin show dramatically that focal adhesions are complex structures that require multiple functional parameters to describe their behaviour, such as fluorescence intensity, traction forces and focal adhesion mobility (also termed 'sliding' [13]). When a 2D GFP–β3-integrin marker is used to study focal adhesions, the respective fluorescence intensity correlates directly with the packing 'density' of this particular integrin in each focal adhesion. The analysis of GFP–β3-integrin in five different focal adhesions in a migrating melanoblast revealed several important features (Fig. 2). First, focal adhesion can be classified into low-density and high-density forms. Second, focal adhesion density can change dramatically with time. Third, high- and low-density contacts are located in different cellular compartments. Fourth, only high-density focal adhesions show mobility ('sliding') [11]. The value of this complex information can be further extrapolated taking into account the fact that low-density focal adhesions form in response to the activity of the GTPases Rac1 and Cdc42, and high-density focal adhesions form in a manner dependent on the GTPase RhoA and acto-myosin contraction [11]. This implies that, at least for

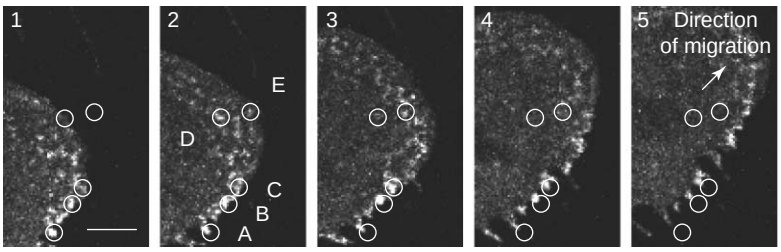

Fig. 2. The two-dimensional (2D) marker green-fluorescent-protein (GFP)–β3-integrin reveals the multidimensionality of focal adhesions. A melanoblast (melb-a [50]) stably transfected with GFP–β3-integrin was stimulated with a growth factor to induce migration. The migration is characterized by the formation of an actin-filament-rich lamellipodium at the cell front and a subsequent coalescence of these actin filaments into actin bundles at the lateral edges of the lamellipodium [41,51]. Focal adhesions A, B and C are located along this lateral edge, whereas focal adhesions D and E are positioned within the advancing lamellipodium. The circles mark the positions of the focal adhesions in the first frame (A, B and C) or where they first appear (D, E) and are kept in a constant position throughout the different frames (1–5). Focal adhesions D and E remain stationary, whereas focal adhesions A, B and C show inward 'sliding'. In addition, notice the differences between the focal adhesion fluorescence intensities of low-density focal adhesions (D, E) within the lamellipodium and high-density focal adhesions (A, B and C) located along the lateral edge of the lamellipodium [11]. Bar, 4.5 μm.

β3-integrins, myosin-dependent actin-cytoskeleton contraction is at the origin of the formation of high-density focal adhesions.

Importantly, the notion that focal adhesions can exhibit different densities would not have been anticipated by the use of GFP markers that are not confined to the 2D plane of the plasma membrane. The observation that the packing densities of β3-integrins can increase two- to threefold [11] has important consequences, bearing in mind that integrins are anchored simultaneously via adaptor proteins to the actin cytoskeleton and their extracellular ligands. Owing to this mechanical link, either density changes in the actin backbone of focal adhesions or changes in the spacing of extracellular ligands (e.g. induced by extracellular tension) will mechanically distort the link between integrins and actin-bound adaptor proteins. Furthermore, the induction of acto-myosin contraction of low-density focal adhesions, as observed in migrating cells (Fig. 2) [11], might have different behavioural consequences depending on whether the bound extracellular ligands form a rigid or elastic surface or whether they exhibit dense or widely spaced integrin binding sites. Some of these theoretical concepts are outlined below with the relevant cellular responses.

Elasticity and spacing of extracellular-matrix ligands

Anybody who has cultured cells on a plastic dish will have realized that, although cells spread, adhere and divide, they will, in living tissues, encounter non-homogeneous microenvironments consisting of rigid as well as flexible domains. Therefore, experiments in which cells have been cultured on elastic substrates have provided interesting information about the cellular responses within a flexible environment. Fibroblasts plated on a flexible substrate were unable to adhere tightly and migrated much faster than on a rigid substrate [23]. Furthermore, when cells were plated on a gradient of different elasticities, they migrated from the flexible towards the more rigid surface [24]; the term 'durotaxis' is used for this movement towards a more rigid surface. Therefore, cells must be able to measure extracellular resistance by physical contact with their environment.

How do cells measure or sense the physical constraints of their environment? It is plausible that mechanical sensing occurs inside focal adhesions, considering that the sensing organelles of cells – the filopodia and lamellipodia – have low-density focal adhesions (also called focal complexes in fibroblasts) that form in a Rac1- or Cdc42-dependent manner [5,11]. When the cell moves forward (Fig. 2), the low-density adhesions transform into high-density adhesions (also called focal contacts in fibroblasts) in response to RhoA activation and myosin-dependent actin-filament contraction [5,11]. The combination of an increase in focal adhesion density and centripetal acto-myosin contraction is sufficient to probe the resistance of the extracellular environment (Fig. 3). It has been observed that, on elastic substrates, focal adhesions retract, whereas they are reinforced and maintained on a rigid surface, anchoring the cell for forward motion [24].

The mechanism of focal adhesion reinforcement and maintenance on a rigid surface is not well understood but can be observed under different experimental conditions. Fibronectin-coated beads that attach to the dorsal surface of cells show retrograde motion and can be easily removed with optical tweezers. However, holding the bead on the cell surface for only a short period of time is sufficient to reinforce cellular binding to the bead, a process that can be blocked by the tyrosine phosphatase inhibitor phenylarsine oxide (PAO) [25]. Furthermore, it has been demonstrated that focal adhesion loss or reinforcement in response to a local reduction or increase in substrate tension, respectively, requires the presence of focal adhesion kinase (FAK) [26]. In addition, brushing against a moving lamellipodium with a microneedle induces the maturation of lamellipodial focal complexes (low-density focal adhesions) into focal contacts (high-density focal adhesions) [27]. This maturation of focal adhesions in response to extracellularly applied tension depends on RhoA activation and its downstream target Diaphanous (mDia) [18,27,28]. Because mDia acts as an actin polymerization factor, the observed increase in size and density of focal adhesions could be linked to increased amounts of polymerized actin [28,29]. Similarly, in undisturbed cells, the molecular signals involved in transforming low-density into high-density focal adhesions and the synthesis of associated actin stress fibres involves RhoA activation and its downstream targets Rho kinase and mDia [3,5,29,30]. Whereas Rho kinase inhibits the myosin light chain phosphatase, which results in continuous acto-myosin

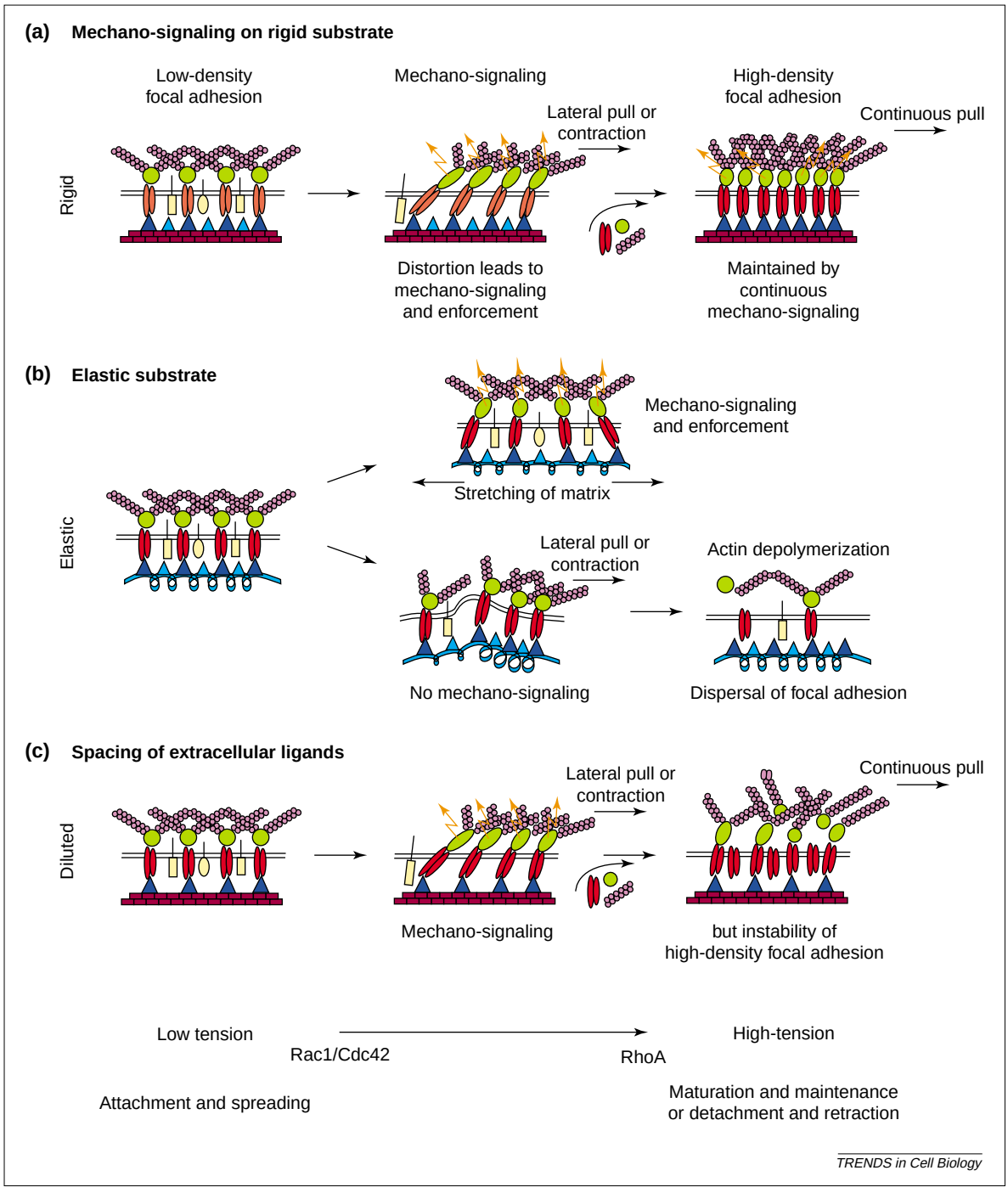

Fig. 3. The influence of substrate elasticity and spacing on focal adhesions formed on rigid [brick wall; (a, c)] and elastic [blue ribbon (b)] substrates. The integrin-binding sites are represented by blue triangles in a dense (a, b) or wider-spaced (c) configuration. When focal adhesions are formed within filopodia or lamellipodia, they assume a low-density configuration that does not necessarily occupy all available extracellular-matrix-binding sites (left-hand side). In addition, the loose integrin spacing might leave space for additional non-integrin transmembrane receptors within the adhesion site (yellow). Owing to the absence of acto-myosin contraction, adaptor proteins are in a relaxed configuration (green circles). In response to RhoA activity and increased tension, the actin cytoskeleton will contract, generating a lateral pull. We propose that the distortions created in integrins (red) and adaptor proteins (green ovals) between the rigid extracellular binding sites and the contracting actin cytoskeleton induces mechanical signaling ('zigzag' orange arrows) by exposing so-far-unidentified stress-sensitive binding sites for adaptor or signaling proteins [31]. The subsequent recruitment of new integrins and adaptor proteins together with actin polymerization will lead to high-density focal adhesions that, however, need to be continuously pulled to maintain mechanical signaling. When the cell establishes a low-density focal adhesion on an elastic substrate, the subsequent acto-myosin-dependent contraction will not be able to create a distortion within the condensing focal adhesion because of the lack of resistance of the substrate (b). In the absence of a mechanical distortion, no mechanical signaling will occur. The lack of focal adhesion reinforcement will result in the dispersal of the focal adhesion site. By contrast, when an elastic substrate is locally pulled or stretched, mechanical signaling is initiated by the distortion of the focal adhesion site, resulting in a high-density adhesion (b). When low-density focal adhesions on widely spaced extracellular-matrix ligands are contracted by RhoA-induced acto-myosin activity, mechanical signaling will be initiated, leading to the formation of high-density adhesions (c). However, when the spacing of extracellular ligands is too wide, only a suboptimal amount of integrins will be engaged in substrate adhesion, insufficient for the formation of stress fibres and resistance to a further increase in tension (c).

contraction, mDia acts as an actin polymerization factor within focal adhesion sites.

Most importantly, however, it has recently been demonstrated that the mechanically stretched, Triton-resistant cytoskeleton of fibroblasts recruits signaling molecules such as paxillin and FAK to stretched focal adhesions [31]. These signaling molecules are recruited to the focal adhesions and not to the actin cytoskeleton extended between them. This is of particular importance because it suggests that the mechanical distortion of focal adhesions itself is at the origin of mechanical signaling. However, it has to be demonstrated whether the mechanical distortion of integrin receptors or the specific adaptor proteins such as FAK [26] or paxillin, which extend between integrins and the actin cytoskeleton, is involved in mechanical sensing. Interestingly, when the extracellular tension is reduced, focal adhesion sites loose the ability to recruit paxillin [31] and will detach from the relaxed substrate [26], suggesting that continuous generation of intracellular tension (and hence high-density focal adhesions) is required to maintain mechanical signaling. Figure 3b demonstrates the different fates of low- and high-density focal adhesions with respect to the elasticity of the substrates. The absence of mechanical signaling on an elastic substrate is proposed to be because of the lack of physical distortion during the contraction of focal adhesions.

A second model based on the different densities of focal adhesions can be extrapolated from the spacing of extracellular ligands. RGD peptides represent the integrin recognition sequence of the major cell binding site of fibronectin [2]. Differently spaced RGD peptides have been used to study the minimal RGD densities required for cell spreading and adhesion [32]. Whereas cells readily spread and attached to an RGD density of 1 fmol cm^{-2}, cells were unable to form focal contacts (high-density focal adhesions) and stress fibres on this substrate. Only at RGD densities of 10 fmol cm^{-2} or above were high-density adhesions and stress fibres formed. This suggests that, beyond a crucial density of integrin ligands (e.g. RGD), cells can only use low-density focal adhesions for attachment, which form in spreading lamellipodia and filopodia, exhibiting wider integrin spacing to establish a stable contact to the substrate.

These data have recently been complemented by an elegant study analysing cell adhesion in a centrifugal force field on a substrate coated either with small clusters of RGD peptides or with monomeric RGD peptides [33]. Interestingly, when pulled by a centrifugal force field, cells plated on the clustered RGD substrate showed reinforcement of cell adhesion, suggesting the formation of high-density focal adhesions. Cells plated on an equimolar amount of monomeric RGD peptides, however, could not resist the centrifugal force and detached. These data suggest that, on the evenly spaced, monomeric RGD substrate, the high-density focal adhesions (which formed in response to mechanical forces) could not be stabilized because of the absence of the appropriate high-density spacing of RGD ligands found only in the clusters.

Based on these data, it is therefore possible (Fig. 3c) that low-density focal adhesions within filopodia or lamellipodia can bind to surfaces with widely spaced extracellular matrix ligands. As soon as a cell generates RhoA-induced acto-myosin contraction of focal adhesion, these sites will only resist if the spacing of the extracellular-matrix ligands matches that of the packing density of the integrins within the high-density focal adhesion. An important prediction of this model is that Rac1- or Cdc42-induced cellular processes such as neuronal growth cones, which move forward by the force generated by actin polymerization in lamellipodia and filopodia, can adhere to and explore substrates that have only widely spaced extracellular ligands. These processes, however, will retract on these substrates when RhoA activity leads to acto-myosin contraction [34,35].

FRAP analysis reveals the dynamic interior of focal adhesions

The model of focal adhesion behaviour and mechanical signaling presented here is based on the notion that the initial stress-induced physical distortion of focal adhesions causes changes in their densities and subsequent recruitment of signaling and structural focal adhesion proteins. Although this model explains many of the experimental findings, it does not explain the observed mobility ('sliding') of focal adhesions [11,13]. One of the best techniques for measuring the internal dynamics of complex structures is fluorescence recovery after photobleaching (FRAP) [36]. This technique can be used to analyse many cellular structures and is particularly suited for use with GFP chimeras. During FRAP, the chromophore in the GFP protein is irreversibly inactivated and the generated radicals are efficiently scavenged by the GFP protein cage, reducing the phototoxicity of the laser pulse. The time and degree of fluorescence recovery within the bleached spot give information about the reorganization and renewal of the analysed structure. When this technique is applied to focal adhesions, each focal adhesion marker will have its own characteristic FRAP 'value' that depends on its intracellular concentration (availability) as well as the number and strength of binding sites to other focal adhesion components. Because integrins are a structural component of the link between the cytoskeleton and the extracellular matrix, FRAP can give information about the dynamic state of the focal-adhesion–substrate interface.

Analysing GFP–β3-integrins by FRAP revealed that high-density focal adhesions undergo renewal that results in the complete exchange of integrins within 5–10 min. Interestingly, low-density focal adhesions that do not exhibit acto-myosin

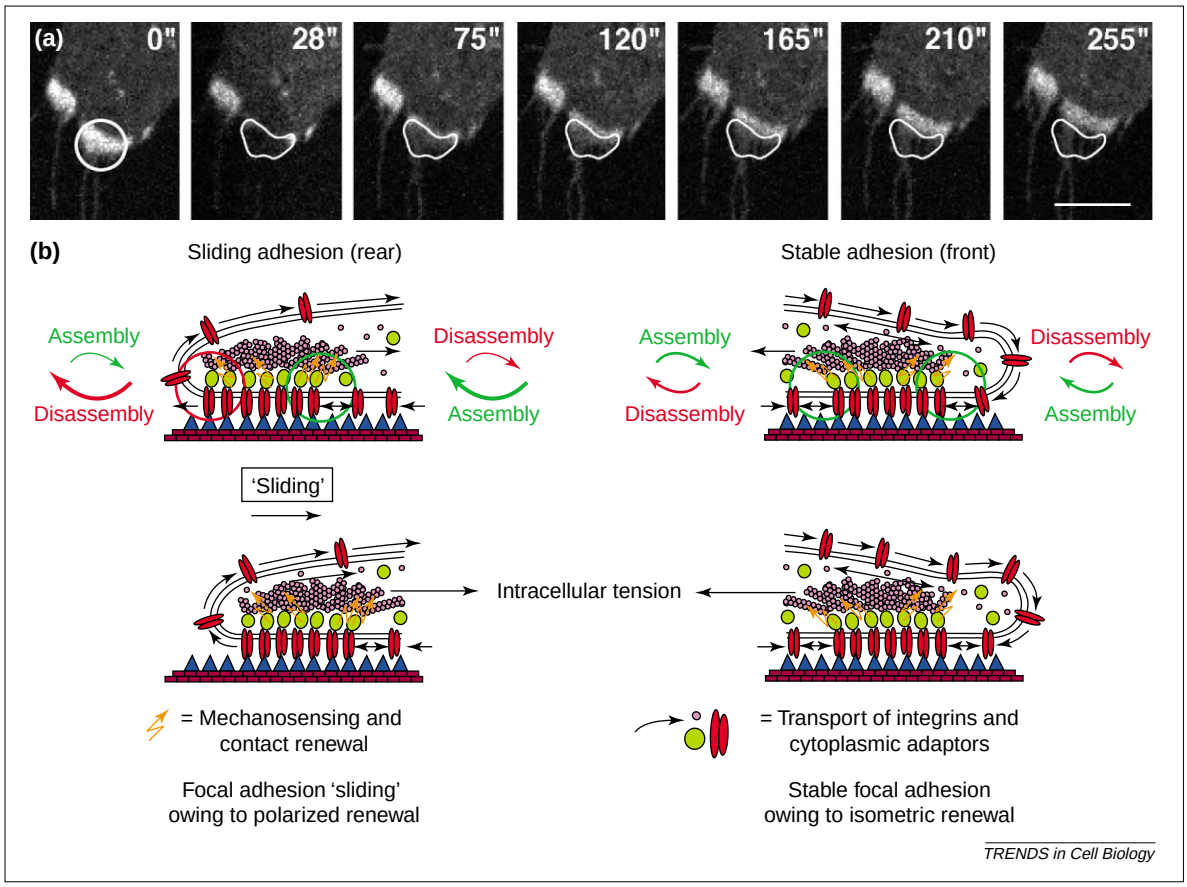

Fig. 4. Fluorescence recovery after photobleaching (FRAP) reveals the dynamic remodelling of high-density focal adhesions. An inwardly 'sliding' high-density focal adhesion of a green-fluorescent-protein (GFP)–β3-integrin-transfected melanoblast (melb-a) was analysed by FRAP [11] (a). Between 0 and 25 sec, the fluorescence has been inactivated within the circled region. In all subsequent frames, the initial outline (at 0′′) of the focal adhesion has been traced to show the relative locations of the reappearance of the GFP fluorescence. Some fluorescence recovers within the former location of the focal adhesion, but most GFP–β3-integrin accumulates at the edge of the bleached focal adhesion facing the interior of the cell. (See also the accompanying supplementary video at: http://archive.bmn.com/supp/tcb/imhof.mov. In the video, the bleached region is indicated by a circle in frame 1. After the fluorescence inactivation (0′), each subsequent frame is labeled with the respective time after bleach. The width of field is 27 μm.) A schematic view of the proposed mechanism of sliding caused by a polarized renewal of high-density focal adhesion is shown (b). High-density focal contacts demonstrate continuous dispersal of old (Disassembly, red) and polymerization and aggregation of new (Assembly, green) focal adhesion components. Local differences in the cellular traffic of these building blocks along the polarized microtubule network creates an imbalance between disassembly and assembly in focal adhesion localized to the retracting rear of migrating cells. This imbalance results in a polarized renewal of focal adhesions, giving the impression of 'sliding' (left-hand side). At the cell front, however, defined by the presence of an advancing lamellipodium, integrins and adaptor proteins ready to become incorporated into the renewing focal adhesion have similar access to all sides to induce a nonpolarized renewal that does not result in sliding (right-hand side). Bar, 5.5 μm.

contraction show a reduced turnover [11]. Focal complexes formed at the cell front do not 'slide' or demonstrate integrin turnover; by contrast, focal contacts at the cell rear slide and renew their integrins at a high rate [11,13]. This suggests that sliding of focal contacts might be a consequence of integrin renewal.

In Figure 4a, a rapidly sliding focal adhesion has been bleached by a laser pulse (see also the supplementary video at: http://archive.bmn.com/supp/tcb/imhof.mov). Although there is some renewal of the focal adhesion within the bleached region, a much faster *de novo* recruitment of integrins can be observed at the side of the focal adhesion facing the cell centre. This suggests that sliding focal adhesions demonstrate a polarized renewal leading to their displacement (Fig. 4b). The renewal of focal adhesions requires the delivery of new building blocks. The disassembly, at the side of the focal adhesion facing the cell periphery, could therefore be caused by the absence of delivery of new focal adhesion components. In this respect, it is interesting that integrins, like many other membrane components, are transported away from the cell rear towards the front by vesicular transport [12,37–39]. By contrast, when the building blocks required for focal adhesion renewal are transported towards pre-existing focal adhesion sites, for example to form a local lamellipodium (Fig. 4b), focal adhesions can renew at their distal as well as their proximal end. This isometric renewal could explain why retracting focal adhesions temporarily halt their inward sliding at sites of local lamellipodium formation [11] and why maturing focal adhesions that are formed behind the cell front in migrating fibroblasts do not demonstrate sliding [13]. This model is also in agreement with the observation that high-affinity αvβ3 integrins are generated by Rac1 activity, associated with the formation of lamellipodia [40]. To conclude, the

continual renewal of high-density focal adhesions gives the cell the necessary plasticity to continuously adapt pre-existing focal adhesions to modify cellular shape and function.

Concluding remarks

We have proposed here that new models to explain focal adhesion structure, function and regulation require the analysis of functional criteria such as focal adhesion density, renewal and the amount of traction force. Nevertheless, much work remains to be done to identify the regulatory pathways involved in focal adhesion dynamics and mechanical signaling. Although it is plausible that the physical distortion of focal adhesions is at the root of mechanical signaling, it is not known which protein domains can fold in a tension-dependent manner to expose binding sites for signaling proteins.

A second crucial task involves the elucidation of the signals that lead to and control focal adhesion turnover. This is especially important for migrating cells, because the efficient release of focal adhesions at the cell rear allows fast migration [41,42]. It has been demonstrated that stretch-activated calcium channels play an important role in rear detachment [43]. Calcium influx might stimulate RhoA and subsequent Rho-kinase activation, which is required for rear detachment of cells of haematopoietic origin [44,45]. This suggests, however, that acto-myosin contraction within focal adhesions is part of the signaling pathway that leads to focal adhesion disassembly. It remains a paradox that the same signaling pathway is also involved in focal adhesion enforcement. Finding the molecular switch remains a crucial task.

Although the model presented here is mainly based on data generated with the αvβ3 integrin, which is involved in cell migration, the integrin family comprises more than 20 different members with potentially different functions [19]. One remarkable example is the α5β1 integrin, which initially localizes with αvβ3 in focal contacts of fibroblasts but subsequently segregates into fibrillar adhesions involved in fibronectin matrix assembly [6,46]. It has been suggested that this difference is due to the constitutive high-affinity state of α5β1 integrins required to bind to fibronectin, whereas the high-affinity (or ligand-bound) state of αvβ3 integrins is less stable [47]. Alternatively, it has been demonstrated that the non-receptor tyrosine kinase Src selectively suppresses the reinforcement of substrate binding (the high-affinity state) of αvβ3 integrins but not of α5β1 integrins [48]. Whether the high- to low-affinity switch of αvβ3 or other integrins is correlated with focal adhesion turnover and cell migration remains to be shown [49].

What is most pertinent, however, is that volume and 2D focal adhesion markers have to be analysed in parallel, to calibrate focal adhesion density changes with the recruitment or loss of focal adhesion components. For example, RhoA-induced high-density GFP–β3-integrin contacts have much more vinculin (as determined by conventional immunofluorescence) than control focal adhesions (B. Wehrle-Haller, unpublished). This suggests that vinculin is recruited to high-density focal adhesions in response to RhoA signaling and/or tension [9]. However, vinculin is not recruited to stretched and Triton-extracted focal adhesions [31], suggesting that it plays a structural role by supporting the increased tension within focal adhesion but is not directly involved in mechanical sensing.

A careful analysis of the functional role and the topology of several GFP markers is required in order to correctly interpret qualitative changes in fluorescence intensities and FRAP behaviour. This will allow the construction of a structural, dynamic model of focal adhesion. Revealing the regulatory circuits involved in focal adhesion dynamics should facilitate the discovery of new therapeutic approaches for wound healing or to prevent metastasis.

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