OPINION

How do microtubules guide migrating cells?

J. Victor Small, Benjamin Geiger, Irina Kaverina and Alexander Bershadsky

Microtubules have long been implicated in the polarization of migrating cells, but how they carry out this role is unclear. Here, we propose that microtubules determine cell polarity by modulating the pattern of adhesions that a cell develops with the underlying matrix, through focal inhibitions of contractility.

Cell migration is essential to life; it is an integral feature of development, repair and defence processes. To invade new territory, a cell must protrude a front, attach it to its substrate and then retract its rear. As such, it must set up an asymmetric, or polarized, form, in which the protruding and retracting zones are more or less diametrically opposed. The processes of protrusion, adhesion and retraction are driven directly by the actin cytoskeleton. However, polarization usually requires the co-operation of microtubules, as it is lost or impaired when microtubules are disrupted[1]. So, what are the molecular and cellular mechanisms by which microtubules influence cell polarity in migrating cells? The answers to this long-standing question are now emerging from findings that implicate microtubules in the spatial control of the adhesion patterns and tractional forces that cells develop with their substrate.

Establishing asymmetry

Protrusion and retraction. A cell moves by first protruding a thin leaflet of cytoplasm — typically 2–5 μm wide — which is known as a lamellipodium[2]. This often occurs together with the protrusion of finger-like projections called filopodia. Protrusion is based on the polymerization of a dense network of actin filaments, which form the lamellipodium core. Polymerization of actin filaments at the front of the lamellipodium is followed by the depolymerization of a proportion of the filaments that are at its base. Actin filaments that are not disassembled at the lamellipodium base contribute to the network throughout the cell that forms the body of the actin cytoskeleton. Depending on the type of cell, this network can either be relatively dispersed, or be organized into a variable number of compact bundles, which are known as stress fibres (FIG. 1). Retraction of the rear of the cell is mediated by contraction of parts of the actin cytoskeleton network, which is driven by the motor protein myosin II (REFS 3,4).

Adhesion asymmetry and polarization. The formation and reorganization of the actin cytoskeleton is coupled to substrate adhesion. This adhesion is achieved by the linkage of parts of the actin cytoskeleton to transmembrane receptors for the extracellular matrix — which are known as integrins — at specialized focal sites, or adhesion complexes. Adhesion complexes consist of assemblies of structural, adaptor and signalling proteins[5], and they originate in association with the lamellipodia and filopodia as punctate 'focal complexes'. Their formation is induced by the small Rho-family GTPases Rac and Cdc42 (REF. 6). Focal complexes support protrusion — they can either be short-lived or can develop into larger 'focal adhesions' from which actin-filament bundles emanate, and which assemble from the actin-filament network behind the lamellipodium boundary (FIGS 1,2a; Online Video 1). The transition from focal complexes to focal adhesions is stimulated by the activation of Rho[7].

To polarize, a cell persistently extends lamellipodia on one edge and reduces its net protrusive activity elsewhere. In those regions where lamellipodia retract, the cell edge becomes tethered by the focal adhesions

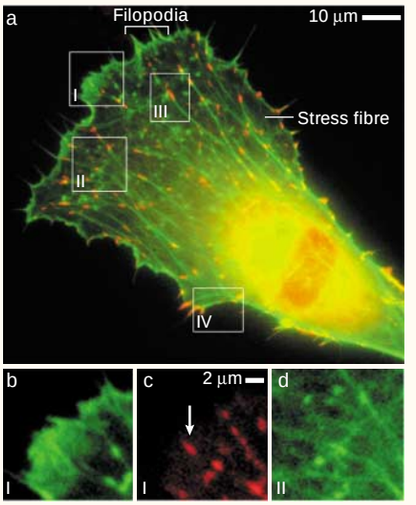

Figure 1 | **The actin cytoskeleton and adhesion sites in a migrating cell. a** | A frame selected from a video sequence (Online Video 1) of a goldfish fibroblast that is expressing green fluorescent protein (GFP)-tagged actin (green) and that was microinjected with rhodamine-tagged vinculin (red). Vinculin localizes to adhesion complexes. **b** | An enlargement of the boxed area I (in **a**), which shows a region of the lamellipodium in the GFP channel (actin). **c** | The boxed area I in the rhodamine channel (vinculin), which shows early adhesion complexes (arrow). **d** | An enlargement of the boxed area II in **a**, which shows a region of the actin network that is behind the lamellipodium. Images courtesy of O. Krylyshkina, Austrian Academy of Sciences, Austria.

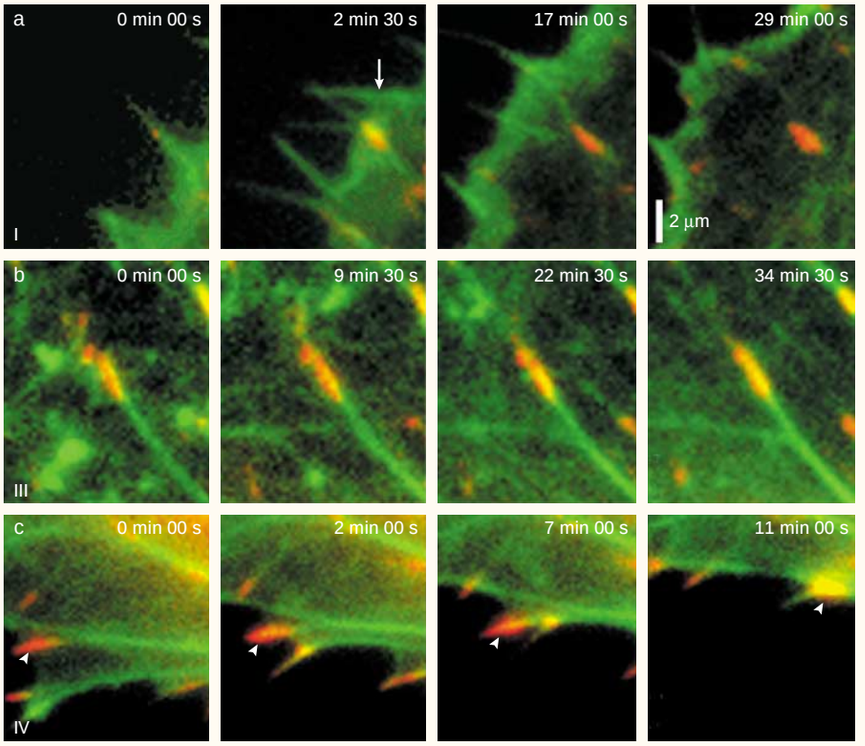

Figure 2 | **Changes in the actin cytoskeleton and adhesion sites in a migrating cell.** The panels in **a**–**c** correspond to the boxed areas I, II and III that are marked in FIG. 1a and show sequential frames from Online Video 1. The times indicated correspond to the individual sequences for each boxed area and they do not have the same starting point. **a** | The creation of a focal complex in association with a filopodium (at 0 min 00 s), and its development into a focal adhesion behind the lamellipodium boundary. The arrow in **a** (at 2 min 30 s) marks the same adhesion site as the arrow in FIG. 1c. **b** | Focal adhesions behind the front of the cell remain stationary relative to the substrate. **c** | Sliding adhesions at a trailing edge of the cell (arrowheads). Images courtesy of O. Krylyshkina, Austrian Academy of Sciences, Austria.

that are formed immediately behind it. In the simplest case, the cell front is marked by lamellipodia and focal complexes (some of which differentiate into focal adhesions), and the cell rear is marked by large focal adhesions (FIG. 1,2). Time-lapse analysis of motile cells shows that the focal adhesions at the front of the cell are mostly stationary relative to the substrate, whereas those at the rear and flanks of the cell can translocate inwards with the retracting edge, before disassembling[8–12] (FIG. 2c; Online Video 1). For cells to move, an asymmetric pattern of substrate adhesions and protrusions must therefore be created and maintained. As we shall see, the microtubule cytoskeleton has a central role in determining this asymmetry.

Contractility and traction

Contractility. An important determinant of focal-adhesion development and maintenance is the contractility of the actin cytoskeleton, which is mediated by the interaction of myosin II with actin[13,14]. Myosin-II-induced contractility is stimulated by Rho, and the inhibition of either Rho or myosin II leads to the disassembly of stress fibres and focal adhesions[13,15,16].

Intuitively, a cell needs the contractile forces that are generated between actin and myosin II to break its contacts with the substratum during locomotion[17]. However, the integrin-mediated contacts have a surprising feature: when a force is applied that does not exceed a certain threshold level, the contacts do not disassemble, but, rather, they grow rapidly. An effective illustration of the requirement of mechanical forces for the development of adhesion was provided by experiments in which cells were mechanically manipulated with microneedles[18]. In fibroblasts that have focal complexes and small focal adhesions at their periphery, the application of tension induced the bundling of actin filaments[19] and the conversion of the small peripheral adhesions into elongated focal adhesions[18] (FIG. 3). Interestingly, this effect required neither active myosin II, nor the downstream target of Rho, Rho kinase, which potentiates myosin activity. However, a second Rho effector Dia1 — which, together with Rho, has a role in stress-fibre assembly[20] — was required[18]. We return to this observation below.

Traction. For locomotion to occur, traction is required to transport the cell body forwards. Recently, flexible substrates that incorporate position markers were used to compare the traction forces that are exerted by static and moving fibroblasts[21,22] (for a review, see REF. 23). These experiments showed that stationary cells exert traction forces primarily at focal adhesions, but, in migrating cells not all of the focal adhesions are equal in terms of traction. Specifically, those focal adhesions that are behind the front of the lamellipodium exert more force per unit area than those at the rear of the cell[21] and, in contrast to focal adhesions, focal complexes under lamellipodia seem to exert much less traction. This is shown, for example, by the lack of distortion of flexible substrates by fibroblasts that are treated with a Rho-kinase inhibitor[18] — which inhibits myosin activity, and stimulates lamellipodia and focal-complex formation[7]. The lamellipodia of rapidly migrating epidermal keratocytes, which develop only punctate adhesion complexes[12], likewise exert minimal forces on the substrate[24]. The process of protrusion therefore involves minimal traction, whereas the translocation of the cell body requires large tractional forces, which are exerted between the focal adhesions that have developed at the front, sides and rear. The regional changes in contractility in a cell therefore have a profound influence on the pattern and turnover of adhesion complexes, and therefore on cell shape and traction.

In view of this dependence on traction forces, focal adhesions could be regarded as mechanosensory devices — namely, structures that can respond to mechanical perturbations by triggering signalling processes[14]. This property allows a cell to distinguish between a rigid and a soft substratum. An increase in anchorage leads, in turn, to the retardation of cell migration and, as discussed below, it is this feature that necessitates the involvement of microtubules to promote adhesion-site turnover during directional movement.

Microtubules suppress contractility

In fibroblasts, when microtubules are disrupted the formation of stress fibres and focal adhesions increases within minutes[25–29]

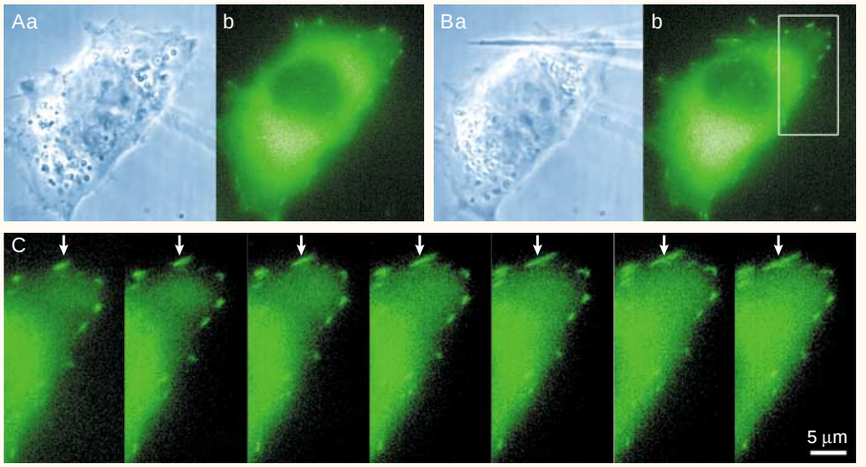

Figure 3 | **Focal adhesion growth in response to the local application of external force.** The panels show a green fluorescent protein (GFP)–vinculin-transfected human SV-80 fibroblast **Aa**, **b** | 3 min before and **Ba**, **b** | immediately after the application of a local centripetal force with a micropipette in the region of the punctate focal complexes at the cell edge (**Aa**, **Ba** phase-contrast microscopy; **Ab**, **Bb** fluorescence microscopy). **c** | The subsequent dynamics of the focal adhesions in the affected cell region shown in the boxed area in (**Bb**) are shown in successive images that were taken at 1-min intervals. A growing focal adhesion is indicated by the arrows. Reproduced with permission from REF. 18 © (2002) The Rockefeller University Press.

(FIG. 4; Online FIG. 1). These changes are paralleled by a rapid increase in cell contractility, as shown by an increase in stress at anchorage sites that is enough to cause the 'wrinkling' of a flexible substrate[30]. The increase in contractility that is seen when microtubules are disrupted is, in fact, a general phenomenon that is observed in various cell types (BOX 1). When microtubules are allowed to repolymerize, the effect on contractility and focal adhesions is reversed[30,31]. Moreover, the inhibition of cell contractility by various treatments abolishes the effect of microtubule disruption on focal-adhesion growth[16,25]. These findings indicate that microtubule-dependent processes act to suppress actomyosin contractility, which in turn influences the pattern of substrate adhesions.

Insights into the spatial influence of microtubules on contractility and adhesion have come from video microscopy of living cells, which has shown that microtubules specifically target adhesion sites[32] (FIG. 5; Online Video 2). Repetitive targeting events were found to correlate with the disassembly or 'sliding' of adhesion sites[31] (Online FIG. 2). The same process of sliding of peripheral adhesion sites has been mimicked by the local, external application of actomyosin inhibitors[11,31], which indicates that microtubules might destabilize individual adhesions by mediating local relaxation at the ends of actin bundles where they enter adhesion foci. If microtubules can exert such relaxing effects in an asymmetric manner in a cell they could, by this route, influence the polarity of the actin cytoskeleton. Consistent with this idea, fibroblasts that are rendered apolar and immotile by microtubule depolymerization can be induced to polarize and move by the asymmetric application of actomyosin inhibitors[11] (FIG. 6).

The turnover of adhesion sites is regulated by the activity of a diverse range of signalling factors, including kinases and phosphatases, that are concentrated in, or recruited by, adhesion assemblies[5]. Through the targeted delivery of additional components, microtubules function to modulate the activity of these 'primary regulators'. The net result of this modulation seems to be the destabilization of adhesion assemblies through highly localized relaxation[31]. In this context, inhibition of the microtubule motor kinesin 1 induced the same increase in the size of adhesion sites, as seen after microtubule depolymerization[28,29], without influencing microtubule targeting interactions. These findings implicate conventional kinesin in the delivery of putative modulator — factors that destabilize adhesion.

Feedback regulation

The crosstalk between microtubules and the actin cytoskeleton also involves a feedback control on microtubule dynamics. The

Box 1 | Microtubules and cell contractility

The occurrence of cell contractility after microtubule disruption is a general phenomenon. Fibroblasts and other cell types that are attached to a flexible substrate or embedded in a collagen gel show an increased ability to deform these substrates on treatment with microtubule-depolymerizing drugs[30,52,53]. The ATP-induced contraction of detergent-extracted 'cell models' is also enhanced by previous microtubule disruption[54]. Likewise, microtubule disassembly causes nerve cells to retract their protrusions[55] and, in *Xenopus laevis* oocytes, rapidly enhances the actomyosin-based cortical flow[56]. After contraction, cells usually do not relax until the microtubule system recovers[57]. In some cases, microtubule disruption induces rhythmic oscillations of the contractile actomyosin system[58,59]. The disruption of microtubules also enhances the contractility of 'professional' contracting cells, such as smooth muscle cells and cardiomyocytes[60–62]. And the excess of microtubules that is observed in some forms of cardiomyopathies leads to contractile dysfunction, which can be cured by microtubule-disrupting drugs[63]. In all of the above-mentioned cases, microtubules seem to control myosin II activity, as shown by the increased phosphorylation of the myosin light chain (MLC)[57,64].

Alternative mechanisms have been proposed to account for the inhibition of contractility by microtubules. First, in the framework of the 'tensegrity' model[64,65] microtubules might function as rigid struts that resist actomyosin contraction. Recent measurements of the mechanical characteristics of microtubules *in vitro*[66,67] show, however, that individual microtubules are far too pliable to assume a role of struts (for discussion, see REF. 68). So, the direct effect of microtubules on tension is applicable only to microtubule arrays with associated motors and other cytoskeletal elements, and not to single microtubules.

Another possible mechanism is that microtubules control cell contractility indirectly, by sequestering signalling molecules in the cytoplasm. It was shown recently that two Rho exchange factors, p190RhoGEF and GEF-H1, are associated with microtubules[69,70]. Moreover, the activity of GEF-H1 is suppressed when it binds to microtubules and is increased when it is released[45]. This could explain why microtubule disruption activates Rho[45,71], and subsequently brings about cell contractility by a Rho kinase-dependent increase of MLC phosphorylation[72]. Another contractility-activating factor is Ca^{2+}, the concentration of which increases on microtubule disruption in some cells[61].

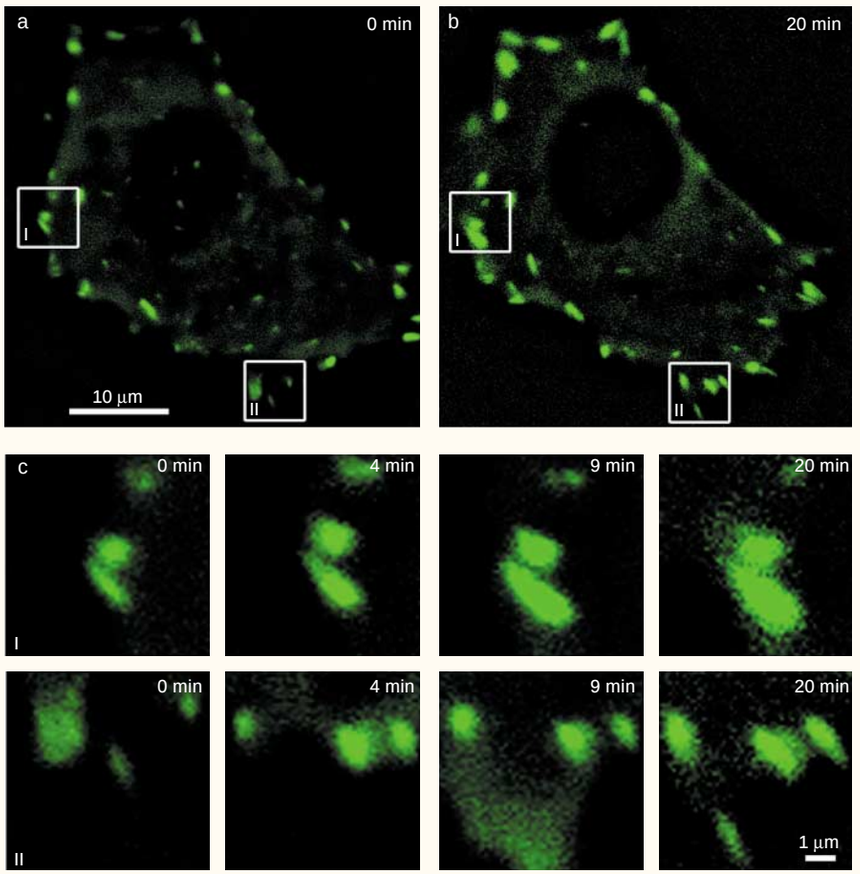

Figure 4 | **Microtubule disruption triggers focal adhesion growth.** A serum-starved SV80 cell that is expressing green fluorescent protein (GFP)–vinculin is shown **a** | immediately before and **b** | 20 min after the addition of the microtubule-disrupting drug nocodazole (10 μM). **c** | Time courses of the focal-adhesion assembly events that correspond to the boxed areas I and II of panels **a** and **b**. Images courtesy of J. Kirchner, Weizmann Institute, Isreal.

main Rho effector, Dia1, which is involved in the formation of focal adhesions and stress fibres, influences the stability of microtubules as well as their effect on the actin cytoskeleton. It induces the alignment of microtubules along stress fibres[33] and increases the number of stable (long-living) microtubules, as seen by the presence of detyrosinated α-tubulin[34]. Recent studies have shown that Dia1 influences microtubule dynamics (C. Ballestrem *et. al.*, unpublished observations). In cells that were transfected with constitutively active Dia1, microtubules grew at half the rate of control cells, their growth was often interrupted and oscillations at the cell periphery lasted longer than in controls. In addition to altering peripheral plus-end dynamics, active Dia1 protected microtubules that were not associated with the centrosome from depolymerization at their minus ends. These modulatory effects of Dia1 could facilitate the targeting of microtubules to peripheral cortical structures including focal adhesions. An effect of Rho on microtubule dynamics is supported by the ability of focal adhesions to capture microtubules and stabilize them transiently against depolymerization by nocodazole and by the observation that microtubules are more resistant to depolymerization in fibroblasts that have numerous focal adhesions, as compared with those that have few[32]. This activity could also constitute an early event in the longer-term stabilization of microtubules that is mediated by Rho.

Of direct relevance to the present discussion is the finding that microtubule-polymerization dynamics are influenced by changes in tension in the actin cytoskeleton. This was first implied by the rapid depolymerization of microtubules away from the edges of fibroblasts that were exposed locally to myosin inhibitors[31] (Online FIG. 3). Conversely, when peripheral stress in the actin cytoskeleton was increased — using the same mechanical manipulation regime that induces the growth of focal adhesions[18] — a stimulation of microtubule polymerization towards the cell periphery and into the enlarged focal adhesions was observed[19]. The targeting of microtubules towards beads that are coated with cell-adhesion molecules and placed at the periphery of growth cones[35] is probably another example of the same phenomenon. These findings have two implications: first, that stress in the actin cytoskeleton stimulates microtubule polymerization and, second, that actin filaments under stress are required for the guidance of microtubules into adhesion sites. Evidence in support of a role of actin filaments in guiding microtubule polymerization was provided recently by the demonstration that microtubules track along the microspike bundles in neuronal growth cones[36] and sometimes move with actin in the cell body[37].

Retraction-potentiated protrusion

Protrusion, the first phase of the motility cycle, occurs independently of microtubules. Nevertheless, several mechanisms for the involvement of microtubules in protrusion formation have been proposed (BOX 2).

Among these, we draw attention to the relationship between retraction and protrusion. It has been shown previously that the retraction of a fibroblast tail leads to accelerated protrusion at the cell front[3,38], and this effect could be mimicked in the forced-polarization experiment that was described above[11] (FIG. 6). This seems to be a general phenomenon, in that the area of a cell that is spread on a substrate is maintained by the opposing actions of retraction and protrusion — retraction activity in one area of a cell precedes protrusion in another[39]. Protrusion is promoted after the disassembly of actin-filament bundles[7], which is presumably facilitated by the replenishment of the cytoplasmic pool of actin and its associated proteins. In keratocytes, in which the processes of retraction and protrusion are tightly linked, the contraction of actin bundles at the cell rear is followed by their disassembly and the recycling of actin into the protruding lamellipodium; in this system, myosin contractility functions to perpetuate the recycling process[40]. So, phases of actin-cytoskeleton contraction that are followed by disassembly and protrusion seem to be a general feature of motile cells. As discussed above, the process of retraction follows the disassembly of trailing adhesions, which is potentiated by microtubules[31,41].

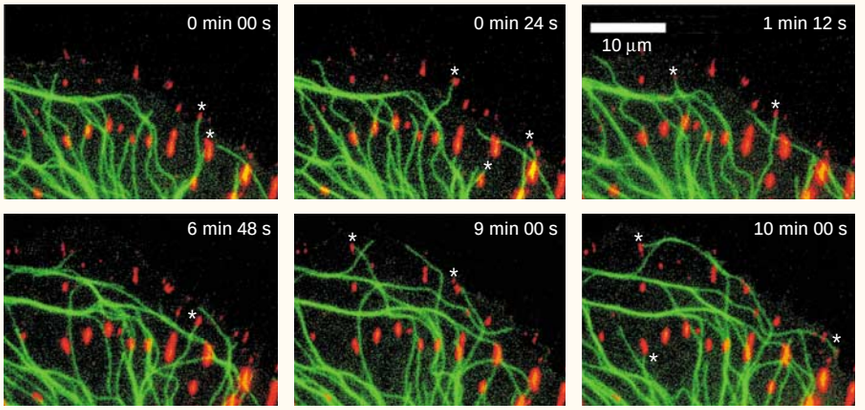

Figure 5 | **Microtubules target substrate adhesion complexes.** The panels show video sequences (Online Video 2) of the periphery of a goldfish fibroblast that was transfected with green fluorescent protein–tubulin and DsRed–zyxin. Each frame is an overlay of images that were taken sequentially, 1 s apart, in different fluorescence channels. Asterisks indicate targeting events.

A parade of dynamic instabilities?

A notable feature of microtubule behaviour is that of 'dynamic instability'[42], in which microtubule ends at the cell periphery undergo successive phases of growth and shrinkage. What consequence might this dynamic instability have on the process of crosstalk between substrate adhesion sites and microtubules? The repetitive targeting of adhesion sites by microtubules could facilitate the pulsed delivery, through kinesin[29], of a modulator that promotes adhesion-site disassembly (for some candidate modulators, see REFS 43,44). At the same time, microtubules might bind factors that promote adhesion-site growth, which could be absorbed locally, inactivated during microtubule polymerization and released during phases of shrinkage. This idea is consistent with the increased contractility and the growth of substrate adhesions that is induced by the global depolymerization of microtubules[25,30], an effect that was recently attributed to the release from microtubules of an exchange factor for Rho[45] (BOX 1).

If these two antagonistic processes occur, the polymerization of a microtubule into an adhesion site would promote local relaxation, whereas depolymerization and withdrawal would be associated with the release of a positive signal that promotes an increase in tension. In this speculative scenario, withdrawal could lead to either retraction and dislocation of the adhesion, which would be determined, for example, by a preceding polymerization phase or, alternatively, to an increase in the size of the adhesion. So, dynamic instability of microtubules could promote the dynamic instability of a population of focal adhesions and potentiate a Darwinian 'survival of the fittest' among them. However, evidence for fluctuations in the size of adhesion sites has yet to be presented.

Maintaining polarization

In a moving cell, adhesion sites are created continually around the periphery by the protrusion of lamellipodia and filopodia. This protrusive activity is far less pronounced in trailing regions — here, protrusion is sporadic and followed rapidly by the retraction and the conversion of focal complexes into focal adhesions (J.V.S. and I.K., unpublished observations). Cell polarization is maintained by microtubule-potentiated sliding and release of these trailing adhesions. But how do we explain the fact that microtubules also target adhesion sites behind the front of a moving cell[31,32]? In this case, the targeting seems to be necessary for promoting the turnover of adhesions to support actin cytoskeleton remodelling for protrusion. Without microtubules, the normal turnover of the anterior focal adhesions of fibroblasts is retarded[11] (FIG. 6). We have already noted that adhesions at the front of these cells are stationary and exert more traction[21], whereas those at the rear can slide, as would be required for the forward translocation of the cell body. This implies that the dosing of putative relaxing signals into adhesion foci is differentially regulated at the front and the rear of the cell. Indeed, the frequency with which focal adhesions in retracting zones are targeted by microtubules has already been shown to be several-fold higher than for the focal adhesions that are behind an advancing front[31], which indicates that the level of the signals might be regulated by targeting frequency. However, other factors, in particular the state of maturation of the adhesions, as well as local differences in the prevailing signalling environment[23,44], must also function in adhesion modulation, rendering, for example, focal adhesions at the rear of the cell more disposed to translocation than those at the front. As already indicated, microtubules polymerize into regions of increased stress and this could be a principal determinant that influences the distribution and action of microtubules. Elsewhere, there has been speculation about the many candidate proteins that could take part in the exchange of signals between microtubules and the actin cytoskeleton[5,43,44].

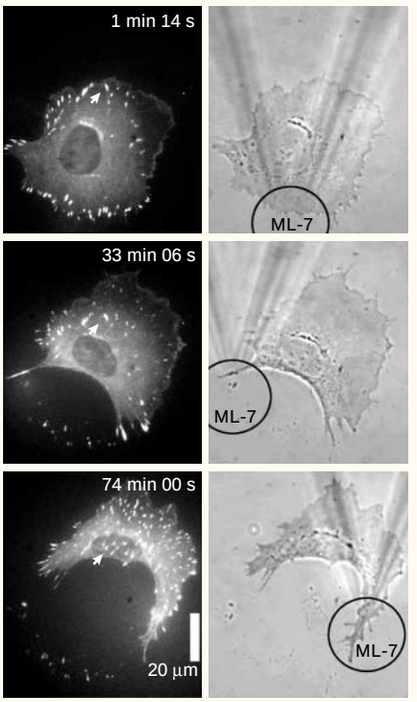

Figure 6 | **Asymmetric relaxation of contractility restores polarization in fibroblasts lacking microtubules.** Apolar fibroblasts that lack microtubules can be induced to polarize and move by the asymmetric application of a relaxant of contractility. Panels show sequences of a video of green fluorescent protein–zyxin expressing goldfish fibroblast that was treated with nocodazole to depolymerize microtubules. At time zero, a myosin relaxant (ML-7) was applied to one side of the cell through a micropipette (circled region). As the cell retracted, the drug was applied to the trailing regions that developed. Although polarized motility was induced in this way, adhesion-site turnover under the body of the cell was impaired (arrows). Left panel, fluorescence microscopy; right panel, phase-contrast microscopy. Modified with permission from REF. 11 © (2002) Elsevier Science.

Box 2 | Microtubules and protrusion formation

The basic machinery that is responsible for the protrusion of the lamellipodium does not depend on microtubules. Nevertheless, in many cell types, the formation and dynamics of lamellipodia are apparently modulated by microtubules. Microtubule disruption not only leads to the random distribution of lamellipodia over the cell perimeter[1], but also apparently decreases the average amplitude of lamellipodial activity, which reduces the areas of both the protruded and retracted lamellipodia per unit of time[73–75].

Several hypotheses aim to explain these microtubule-dependent effects. First, formation of lamellipodia depends on, in addition to actin polymerization, the delivery and insertion of the new membrane into the lamellipodium tip[76]. A proposed function of microtubules is the delivery of new membrane material from the *trans*-Golgi network to the plasma membrane[77]. So, microtubules could facilitate lamellipodia formation by enhancing the supply of the new membrane material. This explanation is consistent with the observation that disruption of the Golgi complex — by either brefeldin A[78] or blocking kinesin motor activity[79] — mimics the effects of microtubule disruption on the lamellipodial activity, although these treatments do not affect the integrity of the microtubule network. The problem with these models is that microtubules lag far behind rapidly protruding cell fronts and, indeed, their complete loss in such situations does not impede protrusion[41,46].

Another explanation is that microtubules control the process of actin assembly in lamellipodia through the delivery, or local activation, of some regulatory components. In particular, microtubule re-polymerization was shown to induce the activation of Rac1 (REF. 75), an important molecular switch that triggers lamellipodia formation. However, the delivery of activators along microtubules into lamellipodia seems unlikely for the same reasons as above, and Rac activation during microtubule re-polymerization is more likely explained as an antagonistic response to the parallel downregulation of Rho[7,32].

Finally, a global balance exists in the cell between the processes of protrusion and retraction. So, as discussed in the text, microtubules might affect protrusion formation indirectly, through their effect on cell contractility and retraction.

Wittmann and Waterman-Storer[44] have also discussed alternative ideas about how microtubules might regulate protrusion (BOX 2). They propose that microtubules exert a global control of Rho-protein activity, which renders Rac more active at the front of the cell and Rho more active at the rear. One theme is that Rac is activated at the front of the cell by the penetration of microtubules into advancing regions. However, one caveat that is associated with this idea is that microtubules generally lag behind protruding regions, and in rapidly migrating cells they might not enter them at all[41,46]. Nevertheless, there is a general consensus that microtubules impinge on the Rho pathways; our view is that this occurs in a highly site-specific manner at focal adhesion sites.

Who needs microtubules?

It is evident that most cells are microtubule dependent, but some are more dependent than others. At one extreme, the polarity of rapidly migrating keratocytes is unaffected by microtubule disassembly[46]. These cells move one cell-length in less than 2 min and do not develop typical focal adhesions: instead, focal complexes that are formed in the sides of the lamellipodium collect into sliding adhesions that are lateral to the cell body[12,24]. The polarization of keratocytes can be explained by a feedback mechanism that maintains the asymmetry of the contractile machinery, which is induced initially by a mechanical stimulus[40]. The case of the keratocyte indicates to us that microtubules might be required to assist in adhesion release only when the stress at the adhesion sites exceeds a certain threshold, and that this threshold is not reached in gliding keratocytes. Microtubules do in fact enter the lateral adhesions of keratocytes and also regions in lamellipodia when stress is increased by mechanical manipulation[19], which indicates that tension-linked microtubule responses could assist in establishing the initial polarity of the cell.

According to this theme, the extent of the engagement of microtubules seems to depend on the adhesion parameters of a particular cell — namely on the extent of focal-adhesion formation. Fibroblasts are strongly adherent and they depend crucially on microtubules for polarization and locomotion[1]. Conversely, neutrophil leukocytes are less adherent and can move even faster without microtubules[47], whereas more adherent macrophages can still move, but with variable persistence[48], which indicates the requirement for microtubules to tune adhesion release, mainly in trailing regions. In accordance with this idea, strongly and weakly adherent sublines of the Walker carcinosarcoma cells respond to microtubule disruption in a way that is similar to fibroblasts and leukocytes, respectively[49]. And although melanoma cells can migrate without microtubules, tail retraction is then incomplete[41]. In rapidly migrating melanoma cells, few microtubules penetrate to the front of the cell[41] but many invade the rear. This correlates with the formation of trailing focal adhesions at the rear and predominantly focal complexes at the front (K. Rottner, J. V. S. and I. K., unpublished observations).

Which direction now?

We have highlighted, here, one example of the crosstalk between microtubules and actin in cell morphogenesis[50]. In migrating cells, microtubules sense points of traction between the actin cytoskeleton and the substrate, and deliver signals to antagonize adhesion at these sites. Through the spatially selective targeting of adhesion sites, microtubules can potentiate retraction in one region of a cell and influence protrusion in another, to induce and maintain polarization (FIG. 7; Online Video 3).

The challenge ahead is to establish the nature of the crosstalk between microtubules and actin. The complexes of proteins at the tips of microtubules[51] are clearly suspects in this collusion, but the facts are still outstanding. Likewise, the functional states of different types of substrate adhesion — in terms of their pre-disposal to anchorage, sliding and release — are poorly understood[14,23]. And the nature of the signal

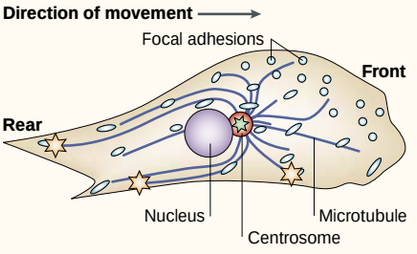

Figure 7 | **Microtubule–adhesion-site crosstalk in a migrating cell.** New adhesion sites form in regions of protrusion, independently of microtubules. Targeting of focal adhesions behind the cell front by microtubules promotes their turnover to support further protrusion. At the rear of the cell, adhesion-site sliding is driven by stress-fibre contraction: microtubules potentiate this process and mediate the release of the cell from the substrate through several targeting events (as depicted by the orange stars). Courtesy of G. Resch, Austrian Academy of Sciences, Austria (for more information, see Online Video 3).

from adhesions that stabilizes microtubules is just as elusive. We also have to tackle the problem of how membranes are recycled in a moving cell (BOX 2). Nevertheless, important, general principles about cell guidance have been unveiled and they provide the guidelines for where to look next.

J. Victor Small and Irina Kaverina are at the Institute of Molecular Biology, Austrian Academy of Sciences, Billrothstrasse 11, Salzburg 5020, Austria.

Benjamin Geiger and Alexander Bershadsky are at the Department of Molecular Cell Biology, The Weizmann Institute of Science, Rehovot 76100, Israel.

Correspondence to J.V.S.
e-mail: jvsmall@imb.oeaw.ac.at

doi:10.1038/nrm971

1. Vasiliev, J. M. *et al.* Effect of colcemid on the locomotory behaviour of fibroblasts. *J. Embryol. Exp. Morphol.* **24**, 625–640 (1970).
2. Small, J. V., Stradal, T., Vignal, E. & Rottner, K. The lamellipodium: where motility begins. *Trends Cell Biol.* **12**, 112–120 (2002).
3. Chen, W.-T. Mechanism of retraction of the trailing edge during fibroblast movement. *J. Cell Biol.* **90**, 187–200 (1981).
4. Jay, P. Y., Pham, P. A., Wong, S. A. & Elson, E. L. A mechanical function of myosin II in cell motility. *J. Cell Sci.* **108**, 387–393 (1995).
5. Geiger, B., Bershadsky, A., Pankov, R. & Yamada, K. M. Transmembrane extracellular matrix–cytoskeleton crosstalk. *Nature Rev. Mol. Cell Biol.* **2**, 793–805 (2001).
6. Nobes, C. D. & Hall, A. Rho, Rac, and Cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell* **81**, 53–62 (1995).
7. Rottner, K., Hall, A. & Small, J. V. Interplay between Rac and Rho in the control of substrate contact dynamics. *Curr. Biol.* **9**, 640–648 (1999).
8. Smilenov, L. B., Mikhailov, A., Pelham, R. J., Marcantonio, E. E. & Gundersen, G. G. Focal adhesion motility revealed in stationary fibroblasts. *Science* **286**, 1172–1174 (1999).
9. Zamir, E. *et al.* Dynamics and segregation of cell–matrix adhesions in cultured fibroblasts. *Nature Cell Biol.* **2**, 191–196 (2000).
10. Ballestrem, C., Hinz, B., Imhof, B. A. & Wehrle-Haller, B. Marching at the front and dragging behind: differential αVβ3-integrin turnover regulates focal adhesion behavior. *J. Cell Biol.* **155**, 1319–1332 (2001).
11. Kaverina, I. *et al.* Enforced polarisation and locomotion of fibroblasts lacking microtubules. *Curr. Biol.* **10**, 739–742 (2000).
12. Anderson, K. I. & Cross, R. Contact dynamics during keratocyte motility. *Curr. Biol.* **10**, 253–260 (2000).
13. Chrzanowska-Wodnicka, M. & Burridge, K. Rho-stimulated contractility drives the formation of stress fibers and focal adhesions. *J. Cell Biol.* **133**, 1403–1415 (1996).
14. Geiger, B. & Bershadsky, A. Assembly and mechanosensory function of focal contacts. *Curr. Opin. Cell Biol.* **13**, 584–592 (2001).
15. Ridley, A. J. & Hall, A. The small GTP-binding protein Rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell* **70**, 389–399 (1992).
16. Helfman, D. M. *et al.* Caldesmon inhibits nonmuscle cell contractility and interferes with the formation of focal adhesions. *Mol. Biol. Cell* **10**, 3097–3112 (1999).
17. Webb, D. J., Parsons, J. T. & Horwitz, A. F. Adhesion assembly, disassembly and turnover in migrating cells — over and over and over again. *Nature Cell Biol.* **4**, E97–E100 (2002).
18. Riveline, D. *et al.* Focal contacts as mechanosensors: externally applied local mechanical force induces growth of focal contacts by an mDia1-dependent and ROCK-independent mechanism. *J. Cell Biol.* **153**, 1175–1186 (2001).
19. Kaverina, I. *et. al.* Tensile stress stimulates microtubule outgrowth in living cells. *J. Cell Sci.* **115**, 2283–2291 (2002).
20. Watanabe, N., Kato, T., Fujita, A., Ishizaki, T. & Narumiya, S. Cooperation between mDia1 and ROCK in Rho-induced actin reorganization. *Nature Cell Biol.* **1**, 136–143 (1999).
21. Beningo, K. A., Dembo, M., Kaverina, I., Small, J. V. & Wang, Y. L. Nascent focal adhesions are responsible for the generation of strong propulsive forces in migrating fibroblasts. *J. Cell Biol.* **153**, 881–888 (2001).
22. Balaban, N. Q. *et al.* Force and focal adhesion assembly: a close relationship studied using elastic micropatterned substrates. *Nature Cell Biol.* **3**, 466–472 (2001).
23. Beningo, K. A. & Wang, Y. L. Flexible substrata for the detection of cellular traction forces. *Trends Cell Biol.* **12**, 79–84 (2002).
24. Oliver, T., Dembo, M. & Jacobson, K. Separation of propulsive and adhesive traction stresses in locomoting keratocytes. *J. Cell Biol.* **145**, 589–604 (1999).
25. Bershadsky, A., Chausovsky, A., Becker, E., Lyubimova, A. & Geiger, B. Involvement of microtubules in the control of adhesion-dependent signal transduction. *Curr. Biol.* **6**, 1279–1289 (1996).
26. Enomoto, T. Microtubule disruption induces the formation of actin stress fibers and focal adhesions in cultured cells: possible involvement of the rho signal cascade. *Cell Struct. Funct.* **21**, 317–326 (1996).
27. Liu, B. P., Chrzanowska-Wodnicka, M. & Burridge, K. Microtubule depolymerization induces stress fibers, focal adhesions, and DNA synthesis via the GTP-binding protein Rho. *Cell Adhes. Commun.* **5**, 249–255 (1998).
28. Pletjushkina, O. J. *et al.* Maturation of cell–substratum focal adhesions induced by depolymerization of microtubules is mediated by increased cortical tension. *Cell Adhes. Commun.* **5**, 121–135 (1998).
29. Krylyshkina, O. *et al.* Modulation of substrate adhesion dynamics via microtubule targeting requires kinesin-1. *J. Cell Biol.* **156**, 349–360 (2002).
30. Danowski, B. A. Fibroblast contractility and actin organization are stimulated by microtubule inhibitors. *J. Cell Sci.* **93**, 255–266 (1989).
31. Kaverina, I., Krylyshkina, O. & Small, J. V. Microtubule targeting of substrate contacts promotes their relaxation and dissociation. *J. Cell Biol.* **146**, 1033–1044 (1999).
32. Kaverina, I., Rottner, K. & Small, J. V. Targeting, capture, and stabilization of microtubules at early focal adhesions. *J. Cell Biol.* **142**, 181–190 (1998).
33. Ishizaki, T. *et al.* Coordination of microtubules and the actin cytoskeleton by the Rho effector mDia1. *Nature Cell Biol.* **3**, 8–14 (2001).
34. Palazzo, A. F., Cook, T. A., Alberts, A. S. & Gundersen, G. G. mDia mediates Rho-regulated formation and orientation of stable microtubules. *Nature Cell Biol.* **3**, 723–729 (2001).
35. Suter, D. M., Errante, L. D., Belotserkovsky, V. & Forscher, P. The Ig superfamily cell adhesion molecule, apCAM, mediates growth cone steering by substrate–cytoskeletal coupling. *J. Cell Biol.* **141**, 227–240 (1998).
36. Schaefer, A. W., Kabir, N. & Forscher, P. Filopodia and actin arcs guide the assembly and transport of two populations of microtubules with unique dynamic parameters in neuronal growth cones. *J. Cell Biol.* **158**, 139–152 (2002).
37. Salmon, W. C., Adams, M. C. & Waterman-Storer, C. M. Dual-wavelength fluorescent speckle microscopy reveals coupling of microtubule and actin movements in migrating cells. *J. Cell Biol.* **158**, 31–37 (2002).
38. Dunn, G. A. in *Cell Adhesion and Motility* (eds Curtis, A. S. G. & Pitts, J. D.) 409–423 (Cambridge Univ. Press, Cambridge, UK, 1980).
39. Dunn, G. A. & Zicha, D. Dynamics of fibroblast spreading. *J. Cell Sci.* **108**, 1239–1249 (1995).
40. Verkhovsky, A. B., Svitkina, T. M. & Borisy, G. G. Self-polarization and directional motility of cytoplasm. *Curr. Biol.* **9**, 11–20 (1999).
41. Ballestrem, C., Wehrle-Haller, B., Hinz, B. & Imhof, B. A. Actin-dependent lamellipodia formation and microtubule-dependent tail retraction control-directed cell migration. *Mol. Biol. Cell* **11**, 2999–3012 (2000).
42. Kirschner, M. & Mitchison, T. Beyond self-assembly: from microtubules to morphogenesis. *Cell* **45**, 329–342 (1986).
43. Kaverina, I., Krylyshkina, O. & Small, J. V. Regulation of substrate adhesion dynamics during cell motility. *Int. J. Biochem. Cell Biol.* **34**, 746–761 (2002).
44. Wittmann, T. & Waterman-Storer, C. M. Cell motility: can Rho GTPases and microtubules point the way? *J. Cell Sci.* **114**, 3795–3803 (2001).
45. Krendel, M., Zenke, F. T. & Bokoch, G. M. Nucleotide exchange factor GEF-H1 mediates cross-talk between microtubules and the actin cytoskeleton. *Nature Cell Biol.* **4**, 294–301 (2002).
46. Euteneuer, U. & Schliwa, M. Persistent, directional motility of cells and cytoplasmic fragments in the absence of microtubules. *Nature* **310**, 58–61 (1984).
47. Keller, H. U., Naef, A. & Zimmermann, A. Effects of colchicine, vinblastine and nocodazole on polarity, motility, chemotaxis and cAMP levels of human polymorphonuclear leukocytes. *Exp. Cell Res.* **153**, 173–185 (1984).
48. Glasgow, J. E. & Daniele, R. P. Role of microtubules in random cell migration: stabilization of cell polarity. *Cell Motil. Cytoskeleton* **27**, 88–96 (1994).
49. Sroka, J., von Gunten, M., Dunn, G. A. & Keller, H. U. Phenotype modulation in non-adherent and adherent sublines of Walker carcinosarcoma cells: the role of cell–substratum contacts and microtubules in controlling cell shape, locomotion and cytoskeletal structure. *Int. J. Biochem. Cell Biol.* **34**, 882–899 (2002).
50. Goode, B. L., Drubin, D. G. & Barnes, G. Functional cooperation between the microtubule and actin cytoskeletons. *Curr. Opin. Cell Biol.* **12**, 63–71 (2000).
51. Schroer, T. A. Microtubules don and doff their caps: dynamic attachments at plus and minus ends. *Curr. Opin. Cell Biol.* **13**, 92–96 (2001).
52. Kolodney, M. S. & Wysolmerski, R. B. Isometric contraction by fibroblasts and endothelial cells in tissue culture: a quantitative study. *J. Cell Biol.* **117**, 73–82 (1992).
53. Brown, R. A., Talas, G., Porter, R. A., McGrouther, D. A. & Eastwood, M. Balanced mechanical forces and microtubule contribution to fibroblast contraction. *J. Cell Physiol.* **169**, 439–447 (1996).
54. Lyass, L. A., Bershadsky, A. D., Vasiliev, J. M. & Gelfand, I. M. Microtubule-dependent effect of phorbol ester on the contractility of cytoskeleton of cultured fibroblasts. *Proc. Natl Acad. Sci. USA* **85**, 9538–9541 (1988).
55. Solomon, F. & Magendantz, M. Cytochalasin separates microtubule disassembly from loss of asymmetric morphology. *J. Cell Biol.* **89**, 157–161 (1981).
56. Canman, J. C. & Bement, W. M. Microtubules suppress actomyosin-based cortical flow in *Xenopus* oocytes. *J. Cell Sci.* **110**, 1907–1917 (1997).
57. Kolodney, M. S. & Elson, E. L. Contraction due to microtubule disruption is associated with increased phosphorylation of myosin regulatory light chain. *Proc. Natl Acad. Sci. USA* **92**, 10252–10256 (1995).
58. Bornens, M., Paintrand, M. & Celati, C. The cortical microfilament system of lymphoblasts displays a periodic oscillatory activity in the absence of microtubules: implications for cell polarity. *J. Cell Biol.* **109**, 1071–1083 (1989).
59. Pletjushkina, O. J. *et al.* Induction of cortical oscillations in spreading cells by depolymerization of microtubules. *Cell Motil. Cytoskeleton* **48**, 235–244 (2001).
60. Lampidis, T. J., Kolonias, D., Savaraj, N. & Rubin, R. W. Cardiostimulatory and antiarrhythmic activity of tubulin-binding agents. *Proc. Natl Acad. Sci. USA* **89**, 1256–1260 (1992).
61. Paul, R. J., Bowman, P. S. & Kolodney, M. S. Effects of microtubule disruption on force, velocity, stiffness and $[Ca^{2+}](i)$ in porcine coronary arteries. *Am. J. Physiol. Heart Circ. Physiol.* **279**, H2493–H2501 (2000).
62. Zhang, D., Jin, N., Rhoades, R. A., Yancey, K. W. & Swartz, D. R. Influence of microtubules on vascular smooth muscle contraction. *J. Muscle Res. Cell Motil.* **21**, 293–300 (2000).
63. Koide, M. *et al.* Microtubule depolymerization normalizes *in vivo* myocardial contractile function in dogs with pressure-overload left ventricular hypertrophy. *Circulation* **102**, 1045–1052 (2000).
64. Wang, N. *et al.* Mechanical behavior in living cells consistent with the tensegrity model. *Proc. Natl Acad. Sci. USA* **98**, 7765–7770 (2001).
65. Ingber, D. E. Cellular tensegrity: defining new rules of biological design that govern the cytoskeleton. *J. Cell Sci.* **104**, 613–627 (1993).
66. Elbaum, M., Kuchnir Fygenson, D. & Libchaber, A. Buckling microtubules in vesicles. *Phys. Rev. Lett.* **76**, 4078–4081 (1996).
67. Felgner, H., Frank, R. & Schliwa, M. Flexural rigidity of microtubules measured with the use of optical tweezers. *J. Cell Sci.* **109**, 509–516 (1996).
68. Elbaum, M., Chausovsky, A., Levy, E. T., Shtutman, M. & Bershadsky, A. D. Microtubule involvement in regulating cell contractility and adhesion-dependent signalling: a possible mechanism for polarization of cell motility. *Biochem. Soc. Symp.* **65**, 147–172 (1999).
69. Ren, Y., Li, R., Zheng, Y. & Busch, H. Cloning and characterization of GEF-H1, a microtubule-associated guanine nucleotide exchange factor for Rac and Rho GTPases. *J. Biol. Chem.* **273**, 34954–34960 (1998).

70. van Horck, F. P., Ahmadian, M. R., Haeusler, L. C., Moolenaar, W. H. & Kranenburg, O. Characterization of p190RhoGEF, a RhoA-specific guanine nucleotide exchange factor that interacts with microtubules. *J. Biol. Chem.* **276**, 4948–4956 (2001).
71. Ren, X. D., Kiosses, W. B. & Schwartz, M. A. Regulation of the small GTP-binding protein Rho by cell adhesion and the cytoskeleton. *EMBO J.* **18**, 578–585 (1999).
72. Fukata, Y., Amano, M. & Kaibuchi, K. Rho–Rho-kinase pathway in smooth muscle contraction and cytoskeletal reorganization of non-muscle cells. *Trends Pharmacol. Sci.* **22**, 32–39 (2001).
73. Bershadsky, A. D., Vaisberg, E. A. & Vasiliev, J. M. Pseudopodial activity at the active edge of migrating fibroblast is decreased after drug-induced microtubule depolymerization. *Cell Motil. Cytoskeleton* **19**, 152–158 (1991).
74. Dunn, G. A., Zicha, D. & Fraylich, P. E. Rapid, microtubule-dependent fluctuations of the cell margin. *J. Cell Sci.* **110**, 3091–3098 (1997).
75. Waterman-Storer, C. M., Worthylake, R. A., Liu, B. P., Burridge, K. & Salmon, E. D. Microtubule growth activates Rac1 to promote lamellipodial protrusion in fibroblasts. *Nature Cell Biol.* **1**, 45–50 (1999).
76. Bretscher, M. S. & Aguado-Velasco, C. Membrane traffic during cell locomotion. *Curr. Opin. Cell Biol.* **10**, 537–541 (1998).
77. Toomre, D., Keller, P., White, J., Olivo, J. C. & Simons, K. Dual-color visualization of trans-Golgi network to plasma membrane traffic along microtubules in living cells. *J. Cell Sci.* **112**, 21–33 (1999).
78. Bershadsky, A. D. & Futerman, A. H. Disruption of the Golgi apparatus by brefeldin A blocks cell polarization and inhibits directed cell migration. *Proc. Natl Acad. Sci. USA* **91**, 5686–5689 (1994).
79. Rodionov, V. I. *et al.* Microtubule-dependent control of cell shape and pseudopodial activity is inhibited by the antibody to kinesin motor domain. *J. Cell Biol.* **123**, 1811–1820 (1993).

Acknowledgements
We thank O. Krylyskina and G. Resch for their invaluable help with the videos and animation. A.B. and B.G. are grateful to D. Riveline and J. Kirchner for providing the experimental data for figures 2 and 3. A. Huttenlocher is acknowledged for providing the DsRed zyxin construct that was used in figure 5. This work was supported in part by a grant from the Austrian Science Research Council to J.V.S. and I.K. B.G. is the incumbent of the E. Neter Chair in Cell and Tumor Biology; A.B. holds the J. Moss Chair of Biomedical Research.

Online links

DATABASES
The following terms in this article are linked online to:
Swiss-Prot: http://www.expasy.ch/
Cdc42 | Dia1 | Rho kinase
Access to this interactive links box is free online.

TIMELINE

Protein engineering 20 years on

James A. Brannigan and Anthony J. Wilkinson

It is 20 years since site-directed mutagenesis was first used to modify the active site of an enzyme of known structure and mechanism. Since then, this method has contributed far-reaching insights into catalysis, specificity, stability and folding of proteins. Engineered proteins are now being used in industry and for the improved treatment of human disease.

At the beginning of the 1980s, a major stumbling block to progress in biochemistry was our inability to direct chemistry specifically at macromolecular surfaces in a way that allowed the relationship between structure and activity to be examined in detail. Nowhere was this limitation more acutely felt than in the field of enzymology. The principles that govern enzyme catalysis were understood[1,2] and the number of enzyme structures solved by X-ray crystallography was beginning to grow, albeit slowly[3]. These structures, with their stereochemical clarity, provided a framework for formulating mechanisms of action in which precise roles were attributed to functional groups that were pinpointed in the active sites. The natural way to test emerging hypotheses was to modify these functional groups specifically and to explore the effects on activity. This could only be achieved by painstaking chemical modification with its attendant problems of limited range and poor specificity. In this climate, the arrival of site-directed mutagenesis, a technique that allowed amino-acid sequences in proteins to be altered at will, was the answer to an enzymologist's prayer. The first uses of this technique, to mutate genes that encode enzymes of known mechanism and produce proteins with defined amino-acid residue substitutions, were reported for tyrosyl–transfer RNA synthetase (TyrRS) and β-lactamase at the end of 1982 (REFS 4–6).

These precise changes of only one or two amino-acid residues were later followed by changes of entire loops[7] and even domains[8]. This construction of modified proteins and the analysis of their properties coalesced to form a new field — that of protein engineering. In this article, we have traced the field of protein engineering over the past 20 years, principally following the thread of enzyme engineering that was pioneered in those early papers (TIMELINE). It is not possible in an article of this length to provide comprehensive coverage of this subject. The examples chosen therefore reflect our own experiences and perspectives, and many significant topics and achievements have necessarily been omitted or abbreviated.

Kicking off with TyrRS

TyrRS proved to be a fruitful system for the dissection of enzyme catalysis by site-directed mutagenesis. It is a central enzyme in molecular biology and is responsible for ligating the amino-acid tyrosine (Tyr) to its cognate $tRNA^{Tyr}$ in an ATP-dependent reaction that produces tyrosyl–$tRNA^{Tyr}$. As with all aminoacyl–tRNA synthetases, the accurate selection of the cognate amino acid is important for the faithful translation of the genetic code. In particular, in the living cell, TyrRS discriminates against the most closely related amino acid to tyrosine — phenylalanine (Phe) — which lacks only the phenolic hydroxyl group of tyrosine.

The *Bacillus stearothermophilus* TyrRS was ripe for engineering. Greg Winter's laboratory had determined the sequence of the enzyme by using a combination of classical protein sequencing and DNA sequencing of the cloned gene, and this gene had also been expressed in *Escherichia coli*. David Blow's laboratory had crystallized the enzyme and was in the final stages of solving its structure. Mechanistic studies of the enzyme in Alan Fersht's laboratory had shown the existence of a remarkably stable aminoacyl-adenylate intermediate. This meant that the enzyme

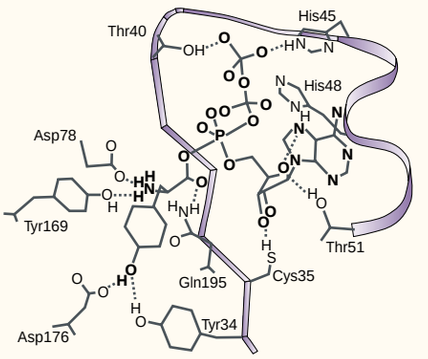

Figure 1 | **The active site of tyrosyl–transfer RNA synthetase.** The modelled structure of the transition state in tyrosyl-adenylate formation is shown. The transition state was extrapolated from the known structure of the enzyme-bound tyrosyl-adenylate. Hydrogen-bonding interactions between the enzyme and transition state species are shown as dashed lines. The roles of threonine (Thr) 40 and histidine (His) 45 are discussed in the main text. Asp, aspartic acid; Cys, cysteine; Gln, glutamine; Tyr, tyrosine. Modified with permission from REF. 10 © (2002) National Academy of Sciences, USA.