

Adhesion signaling – crosstalk between integrins, Src and Rho

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Summary

Interactions between cells and the extracellular matrix coordinate signaling pathways that control various aspects of cellular behavior. Integrins sense the physical properties of the extracellular matrix and organize the cytoskeleton accordingly. In turn, this modulates signaling pathways that are triggered by various other transmembrane receptors and augments the cellular response to growth factors. Over the past years, it has become clear that there is extensive crosstalk between integrins, Src-family kinases and Rho-family GTPases at the heart of such adhesion signaling. In this Commentary, we discuss recent

advances in our understanding of the dynamic regulation of the molecular connections between these three protein families. We also discuss how this signaling network can regulate a range of cellular processes that are important for normal tissue function and disease, including cell adhesion, spreading, migration and mechanotransduction.

Key words: Adhesion, Spreading, Engulfment, Migration, Invasion, Integrins, Rho-GTPases, Src-family kinases, Extracellular matrix, Mechanotransduction

Introduction

Adhesion of cells to the extracellular matrix (ECM) is crucial for development and tissue homeostasis. Cells interact with ECM components through various receptors, including integrins. These heterodimeric transmembrane receptors act as bidirectional signal transducers: interactions with cytoskeletal adaptor proteins that associate with the cytoplasmic tails of integrins control the ligand-binding activity of integrins and, conversely, the extracellular ligand-binding, clustering or pulling on integrins triggers the recruitment of cytoskeletal adaptor proteins (Hynes, 2002). Such signaling is important for the formation and turnover of cell-matrix adhesions, in which integrins and associated proteins are concentrated to mediate local, firm contact to the ECM.

Cell-matrix adhesions function not only as structural anchor points that organize the actin cytoskeleton, but are also coupled to components of the actin-assembly machinery, such as actin-related protein 2/3 (Arp2/3). In addition, integrin engagement regulates the activity of several members of the Rho family of small GTPases, which control the growth or contraction of filamentous actin (F-actin) fibers through proteins such as Arp2/3 and myosin (Demali and Burridge, 2003). Members of the Src family of tyrosine kinases (SFKs) also localize in cell-matrix adhesions. In addition to regulating protein-protein interactions and thereby augmenting cell-matrix adhesion turnover (Webb et al., 2002), downstream of integrins SFKs control guanine-exchange factors (GEFs) and GTPase-activating proteins (GAPs) that act on Rho-GTPases (Fig. 1). In this Commentary, we discuss recent advances in our understanding of the physical and functional connections between integrins, SFKs and Rho-GTPases. We focus on evidence that shows that SFKs are crucial mediators of integrin signaling pathways that regulate the activation of Rho-GTPases, either directly or through the engagement of other classes of receptors. Together, these proteins act in complex networks with extensive crosstalk pathways to orchestrate various aspects of cellular behavior. Here, we

describe the roles of these signaling networks in cell adhesion, membrane protrusion, engulfment, migration, invasion and mechanotransduction.

Crosstalk in cell adhesion and spreading

Integrin-mediated cell adhesion regulates the activation of Rho-GTPases

Initial cell adhesion and spreading occurs in parallel with an inhibition of RhoA-GTP levels and the simultaneous activation of Ras-related C3 botulinum toxin substrate 1 (Rac1) and cell division cycle 42 (Cdc42), which results in suppressed actomyosin contractility and enhanced actin-mediated protrusion (Fig. 2). At later phases, the activities of Rac1 and Cdc42 decrease, whereas the activity of RhoA gradually increases, which drives the formation of stress fibers and the maturation of focal adhesions. Notably, RhoA and Rac1 suppress one another's activity. For example, RhoA antagonizes Rac1 by promoting Rho-associated, coiled-coil-containing protein kinase (ROCK)-mediated phosphorylation of FilGAP, a GAP for Rac1 that localizes to sites of membrane protrusion following the binding of FilGAP to the actin-crosslinking protein filamin A (Ohta et al., 2006). Conversely, Rac1-induced production of reactive oxygen species leads to the inhibition of phosphatases that suppress p190RhoGAP activity thereby antagonizing RhoA (Nimnual et al., 2003). Such crosstalk between RhoA and Rac1 might be important for coordinating F-actin organization during different stages of cell spreading (Fig. 3). In this section, we focus on the role of SFKs in coupling integrins to the actions of Rho-GTPases during cell adhesion and spreading.

The FAK-Src complex in integrin-mediated regulation of Rho-GTPases

Integrin-mediated adhesion induces the autophosphorylation of focal adhesion kinase (FAK) at tyrosine 397, creating a binding site for the Src-homology 2 (SH2) domain of Src, which in turn

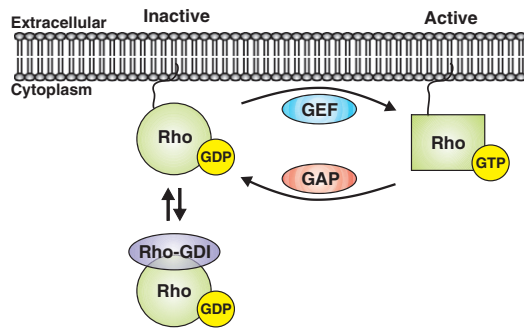


Fig. 1. The Rho-GTPase activation cycle. Model depicting how Rho-GTPases are regulated. Rho-GDP dissociation inhibitors (Rho-GDIs) sequester inactive GDP-bound Rho-GTPases (Rho) in the cytoplasm. When released from Rho-GDIs, Rho-GTPases are targeted to the plasma membrane, where their activation cycle is regulated by GEFs that promote GTP loading and activation of Rho-GTPases. Inactivation of Rho-GTPases is mediated by GAPs that promote GTP hydrolysis to GDP.

phosphorylates other tyrosine residues in FAK and thereby maximizes its kinase activity and creates additional protein-binding sites (Mitra and Schlaepfer, 2006). This active FAK-Src complex (Fig. 3A) stimulates Rac1 activity through the recruitment and phosphorylation of the scaffolding protein p130Cas (also known as Bcr1) (Chodniewicz and Klemke, 2004). Phosphorylated p130Cas recruits Dock180 and engulfment and motility 1 (ELMO1; a *Ced-12* ortholog) through association with the adaptor protein v-crk sarcoma virus CT10 oncogene homolog (Crk). The Dock180-ELMO1 complex functions as an unconventional GEF for Rac1 and promotes formation of membrane protrusions (Brugnera et al., 2002; Kiyokawa et al., 1998). The FAK-Src complex also phosphorylates paxillin, which subsequently recruits the ArfGAP paxillin-kinase linker (PKL, also known as GIT2) and the GEF for Cdc42 and Rac1, Pak-interacting exchange factor-beta (β -PIX, also known as Cool-1 and Gef7). β -PIX recruits and activates Rac1 through a direct interaction within focal adhesions and membrane ruffles (ten Klooster et al., 2006). Interestingly, PKL and β -PIX proteins can be phosphorylated by Src, which might further modulate their activities in response to integrin-mediated adhesion (Brown et al., 2005; Feng et al., 2006). Thus, integrin signaling through SFKs can regulate the localization and activity of GEFs to coordinate membrane protrusion processes.

In addition to promoting the activation of Rac1 and Cdc42 to stimulate membrane protrusions, the FAK-Src complex mediates the transient suppression of RhoA-GTP levels following integrin ligation (Fig. 3A). This role of FAK-Src is important for transiently relieving cytoskeletal tension during cell spreading and is mediated through regulation of p190RhoGAP (also known as Grf1) (Arthur et al., 2000; Ren et al., 2000). In cells that are adhering to fibronectin, p190RhoGAP regulation can involve extensive cooperation between different classes of receptors: engagement of integrin α 5 β 1 stimulates Src-mediated p190RhoGAP tyrosine phosphorylation, whereas binding of the proteoglycan receptor syndecan-4 is required in some cell types to stimulate protein kinase C α (PKC α)-dependent translocation of p190RhoGAP to the cell membrane (Bass et al., 2008). As a consequence, both types of receptors coordinate the coupling of p190RhoGAP activity to the suppression of RhoA activity. Thus, in addition to

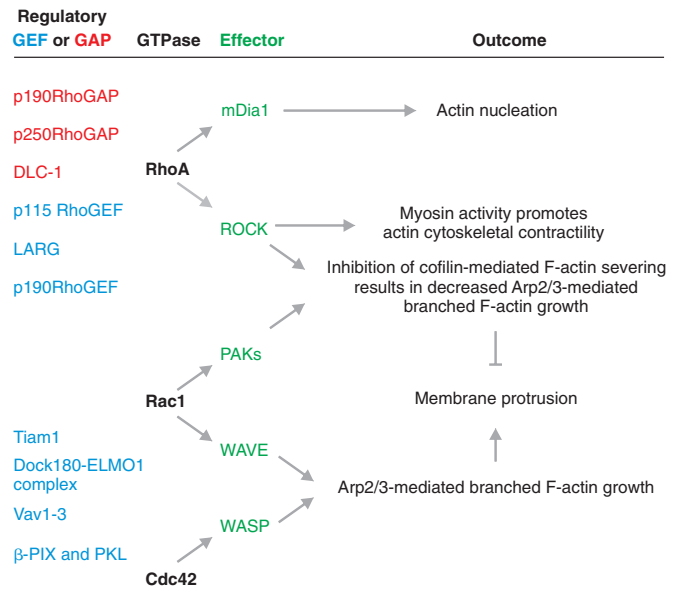
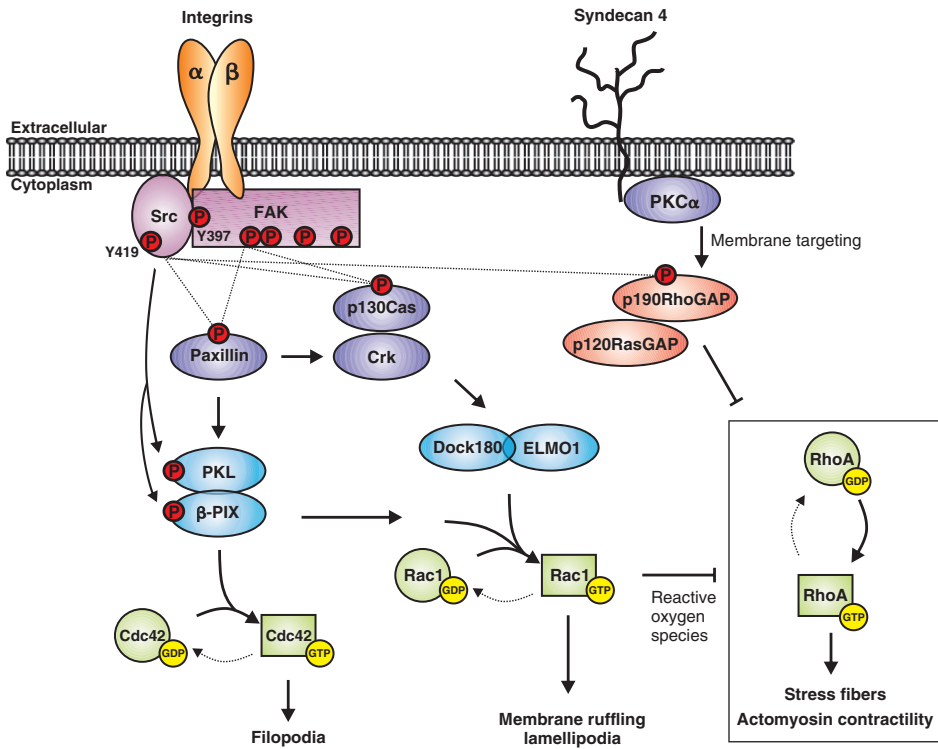


Fig. 2. Overview of Rho-GTPases, the GAPs and GEFs that regulate them and the effector pathways that act downstream of integrins. An overview of the currently known GEFs (indicated in blue) and GAPs (indicated in red) that control the activation of RhoA, Rac1 and Cdc42 downstream of integrins is shown. Through the recruitment and activation of different effector proteins (indicated in green) these Rho-GTPases regulate the actin cytoskeletal dynamics that are required for membrane protrusions and/or cytoskeletal contractility. See main text for additional details.

stimulating cell-matrix adhesion turnover through initiating local tyrosine phosphorylation events, integrin-mediated activation of the FAK-Src complex also stimulates membrane protrusion and inhibits cytoskeletal contractility, thereby facilitating cell spreading.

As spreading ends, RhoA activity gradually increases, concomitant with the formation of stress fibers and the maturation of focal adhesions (Fig. 3B). The GEFs p115RhoGEF (also known as Lsc and Gef1), LARG (also known as Gef12), and p190RhoGEF have been implicated in these processes, but it is unclear what triggers their activity (Dubash et al., 2007; Lim et al., 2008). One possibility is that physical limitations on spreading create tension that initiates RhoA activation, perhaps through local mechanical signals that trigger Src-mediated activation of these GEFs in cell-matrix adhesions. Integrin α 5 β 1 is particularly effective at promoting this second phase during spreading on fibronectin (Danen et al., 2002). Although overexpression of integrin α v β 3 can stimulate RhoA activity in some cell types (Miao et al., 2002), integrin- α v β 3-deficient fibroblasts display normal RhoA signaling. Moreover, when integrin α 5 β 1 is deleted, the overexpression of other fibronectin-binding integrins, including α v β 3, fails to compensate, and RhoA activity remains low (Huvneers et al., 2008b). This lack of redundancy seems not to involve specific interactions necessary at the α - or β -cytoplasmic tails of α 5 β 1 integrin. Instead, the particularly complex receptor-ligand interactions that are required for mechanocoupling through integrin α 5 β 1 (Garcia et al., 2002), or the ability of integrin α 5 β 1 to bind folded, 'inactive' fibronectin molecules promotes the turnover – and perhaps affects the clustering – of cell-matrix adhesion components (Danen et al., 2002; Huvneers et al., 2008b). It is possible that this leads to different conformations in mechanoresponsive cell-matrix

A Early spreading



B Late spreading

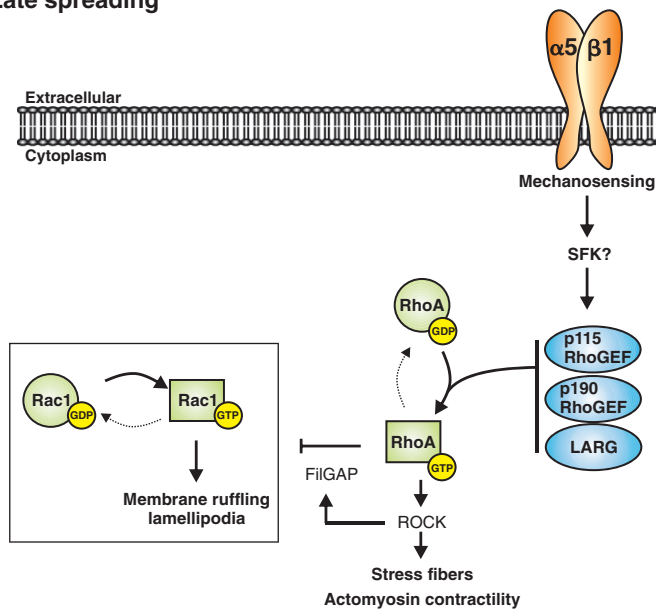


Fig. 3. Integrin regulation of Rho-GTPases during early and late stages of cell spreading. (A) During early stages of cell spreading, the FAK-Src complex activates several pathways that lead to protrusive activity via Rac and Cdc42 GTPases at sites of integrin ligation. At the same time, this complex, together with syndecans, mediates suppression of actomyosin contractility by keeping the activity of RhoA low. Broken lines indicate protein-protein interactions. (B) At later stages of cell spreading, integrins stimulate the activity of several GEFs, which leads to a shift in the balance between RhoA and Rac1 activity in favor of RhoA, thereby enhancing RhoA-mediated actomyosin contractility. Integrin $\alpha 5 \beta 1$ is particularly efficient at promoting this second phase of cell spreading, which might involve force-induced activation of SFKs. See main text for additional details. Broken arrows indicate transition not supported by presented pathway or not supporting presented output.

adhesion proteins, such as vinculin or p130Cas (Puklin-Faucher and Sheetz, 2009), when adhesions are composed of different integrins. Through the exposure or hiding of binding sites for signaling molecules, such as Src or GEFs and GAPs for Rho-GTPases, conformational changes would have effects upstream of RhoA activation. Indeed, RhoA controls actin cytoskeletal remodeling but, in turn, p190RhoGAP-mediated suppression of RhoA activity is itself regulated by the cytoarchitecture (Mammoto et al., 2007).

Integrin-SFK signals regulate Rho-GTPases

A selective direct interaction between the SH3 domain of Src and the integrin- $\beta 3$ cytoplasmic domain can regulate cell spreading (Arias-Salgado et al., 2005). Engagement of integrin $\alpha 1 \beta 3$ or integrin $\alpha v \beta 3$ promotes the release of Src tyrosine kinase (Csk) from the inhibitory tyrosine 530 residue of Src, which might be triggered by the recruitment of the protein tyrosine phosphatase-1B (PTP-1B). This is followed by phosphorylation of the tyrosine 419

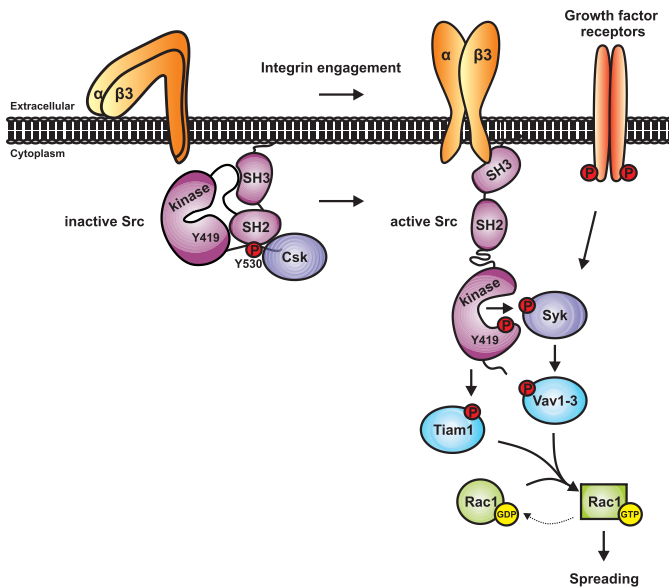


Fig. 4. Src binding to $\beta 3$ integrins influences cell spreading via regulation of Rac1 GTP loading. Following ligand binding, α Ib $\beta 3$ and α v $\beta 3$ integrins can directly interact with the SH3 domain of Src via the cytoplasmic domain of integrin $\beta 3$. These interactions result in a release of Csk from tyrosine 530 and induce the activation of Src. Clustering of $\beta 3$ integrins promotes transphosphorylation in the kinase domain of Src. This is followed by the recruitment and phosphorylation of Syk, which can occur in concert with signaling downstream from growth-factor receptors. Activated Syk phosphorylates Vav proteins, which act as GEFs for Rac1. Alternatively, activated Src can phosphorylate and regulate the activation of the RacGEF Tiam1. In turn, activated Rac1 induces membrane protrusions and spreading. Broken arrows indicate transition not supported by presented pathway or not supporting presented output. See main text for additional details.

residue in the activation loop of the Src kinase domain, probably resulting from enhanced cross-phosphorylation that is triggered by integrin clustering (Arias-Salgado et al., 2003; Huveneers et al., 2007). In hematopoietic cells, this integrin- $\beta 3$ -Src complex recruits and phosphorylates spleen tyrosine kinase (Syk) (Woodside et al., 2001), which in turn phosphorylates the RacGEF Vav1, and stimulates Rac1 activation, lamellipodia formation and cell spreading (Fig. 4) (Oberfell et al., 2002). Integrins can promote Rac1 activation and cell spreading in non-hematopoietic cells by stimulating Src-dependent phosphorylation of the related RacGEF Vav2 (Marignani and Carpenter, 2001). Activated Src can also phosphorylate T-cell lymphoma invasion and metastasis 1 (Tiam1) (Servitja et al., 2003), a RacGEF that promotes integrin- $\alpha 3\beta 1$ -mediated cell spreading on laminin (Hamelers et al., 2005).

Integrins can be coupled to the SFK family member Fyn through association with the membrane adaptor protein caveolin-1 (Wary et al., 1998). During oligodendrocyte differentiation, ligation of integrins activates Fyn, which phosphorylates p190RhoGAP, leading to decreased RhoA-GTP levels (Wolf et al., 2001); this decrease in RhoA activity might indirectly support GTP loading on Rac1 and Cdc42 (Liang et al., 2004). Furthermore, phosphorylation of p250RhoGAP by Fyn can lead to further downregulation of RhoA activity (Taniguchi et al., 2003).

Thus, in addition to inducing an active FAK-Src complex that regulates GEFs and GAPs for Rho-GTPases, there are several alternative routes linking integrin ligation with SFK activity that have been implicated in regulating Rho-GTPases during cell spreading.

Crosstalk between integrins, SFKs and growth-factor receptors regulates Rho-GTPases

There are multiple mechanisms by which adhesion and growth-factor receptors cooperate to control cell behavior. Growth-factor receptors and downstream signaling intermediates are recruited to sites of integrin ligation (Yamada and Even-Ram, 2002). This might lead to enhanced clustering and autophosphorylation of growth-factor receptors, providing binding sites for SFKs (Bromann et al., 2004). Phosphorylated growth-factor receptors control Rho-GTPases through direct interactions with GEFs or GAPs, or through indirect mechanisms such as activation of phosphatidylinositol 3-kinase (PI3K) (Burrige and Wennerberg, 2004). Notably, an analogous process that involves a different class of adhesion receptors takes place at cell-cell junctions: in this case, cadherins act together with growth-factor receptors to control the stability of cell-cell adhesions by regulating the activity of Rho-GTPases. Here, the cadherin-associated Src substrate catenin (also known as p120-catenin) mediates local suppression of RhoA activity through a transient interaction with p190RhoGAP, following growth-factor-induced Rac1 activation, to regulate cell-cell adhesion (Wildenberg et al., 2006).

Integrin-mediated adhesion can promote the activation of growth-factor receptors independently of growth-factor binding. For example, integrin-mediated activation of Src leads to a phosphorylation pattern in the kinase domain of epidermal growth-factor receptor (EGFR) that is different from that induced by the binding of EGF to EGFR (Moro et al., 2002). Such cross-activation of EGFR following integrin ligation is involved in the induction of Rac1 activity during cell adhesion through PI3K and Vav2 and promotes lamellipodia formation and cell spreading (Fig. 5) (Marcoux and Vuori, 2003). Following EGF stimulation, a protein complex that contains Src, the F-actin and microtubule-organizing formin diaphanous 1 (mDial1) and Dia-interacting protein with SH3 domain (*NCKIPSD*) stimulates Rac1 activity through Vav2 and suppresses RhoA activity through p190RhoGAP (Meng et al., 2004). Because mDial1 is itself a RhoA effector, this represents a potential negative feedback loop downstream of RhoA. This feedback loop requires Src activity and is probably modulated by the integrin-Src-EGFR crosstalk described above, although this has not been shown. EGF also induces tyrosine phosphorylation of integrin $\alpha 6\beta 4$ through an association of EGFR, integrin $\alpha 6\beta 4$ and Fyn (Mariotti et al., 2001). Phosphorylation of integrin $\beta 4$ by Fyn, together with additional serine phosphorylation, disrupts hemidesmosomal adhesions (Mariotti et al., 2001; Wilhelmsen et al., 2007), and the ligation of integrin $\alpha 6\beta 4$ augments EGF-induced activation of Rac1 (Russell et al., 2003); however, the molecular basis for this crosstalk has not been clarified.

Crosstalk in cell adhesion and spreading – implications for physiological processes

Hemostasis

Activated platelets bind to soluble fibrinogen through α Ib $\beta 3$ integrins, and this initiates platelet aggregation and thrombus formation. Src is constitutively associated with α Ib $\beta 3$ integrins and becomes rapidly activated following fibrinogen binding (Shattil, 2005). Activation of Src induces Syk-mediated activation of Vav1 followed by GTP loading on Cdc42 and Rac1, which drives lamellipodia formation and platelet spreading (Vidal et al., 2002). This process seems to be crucial for hemostasis: a $\beta 3$ -integrin mutant that can bind to its ligand but that is impaired in outside-

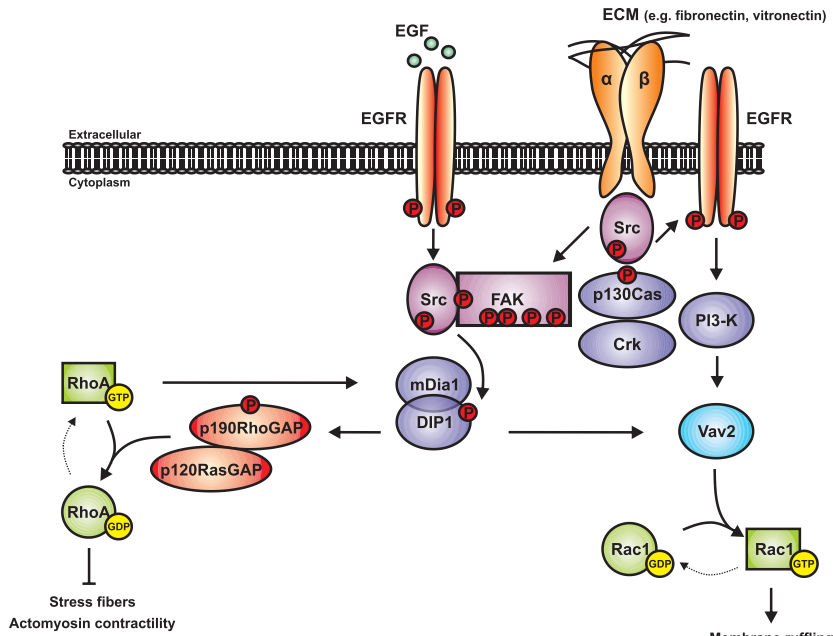


Fig. 5. Crosstalk between integrins and EGFR regulates Src activation to control Rho-GTPases. Integrins can promote Src activation to control Rho-GTPases. Integrins can promote Src-mediated phosphorylation of EGFR in the absence of growth factors, which occurs through a signaling complex that involves Src, p130Cas and Crk. Unengaged but activated EGFR signals through PI3K and Vav2 to promote Rac1 GTP loading. The binding of EGF to EGFR results in the direct activation of Src, which might be enhanced in the presence of integrin-FAK-Src complexes. Src activation then leads to the activation of DIP1, which in turn interacts with p190RhoGAP (leading to the inactivation of RhoA) and with Vav2 (leading to the activation of Rac1). See main text for additional details. Broken arrows indicate transition not supported by presented pathway or not supporting presented output.

in signaling fails to rescue the $\beta 3$ -integrin-knockout phenotype in mice, which is characterized by severe bleeding, similar to the effect of *ITGB3* mutations in humans that cause Glanzmann's thrombasthenia (Law et al., 1999). Little is known about the role of the integrin- α IIb β 3-Src complex in regulating RhoA activity, although there is evidence that RhoA activity is required to maintain stable interactions of integrin α IIb β 3 with the ECM under conditions of shear stress (Schoenwaelder et al., 2002) (Fig. 6A).

Bone remodeling

In a manner analogous to the cooperation between integrin α IIb β 3 and Src in platelets, integrin- α v β 3-mediated Src activation regulates the function of osteoclasts during bone resorption. $\beta 3$ -integrin-deficient or Src-deficient mice display partially overlapping abnormalities that are caused by defective bone remodeling (McHugh et al., 2000; Soriano et al., 1991). Osteoclasts that lack integrin α v β 3 fail to activate Src and display reduced cell spreading and membrane ruffling, which collectively leads to a dysfunctional sealing zone (Feng et al., 2001). Osteoclasts from Src-deficient mice have similar deficiencies and, in addition, contain mislocalized integrin- α v β 3 clusters (Lakkakorpi et al., 2001). The interaction between integrin α v β 3 and Src is required for the activation of Syk. Together, integrin α v β 3 and Fms (the receptor for macrophage-colony-stimulating factor, M-CSF) stimulate Syk-mediated GTP loading on Rac1 via the RacGEF Vav3 in osteoclasts, thereby driving cytoskeletal remodeling, which is required for effective bone resorption (Faccio et al., 2005). Integrin α v β 3 also controls RhoA-mediated regulation of the cytoskeletal remodeling proteins Wiskott-Aldrich syndrome protein (WASP) and gelsolin; this process is crucial for the organization and formation of dynamic and proteolytically active adhesions known as podosomes, which are important for osteoclast function (Chellaiiah, 2006). Although not established, it is probable that this involves Src-mediated regulation of the GEFs and GAPs that regulate Rho-GTPases downstream of integrin α v β 3 (Fig. 6B).

Phagocytosis and engulfment

Cell spreading on a rigid surface is a membrane protrusion process that can be considered analogous to (and perhaps a 'frustrated attempt at') the engulfment of other cells or particles. Integrins play a pivotal role in phagocytosis and in the movement of leukocytes through individual endothelial cells. Enteropathogenic bacteria such as *Yersinia pseudotuberculosis* express invasin, an outer membrane protein that enables bacterial uptake by non-phagocytotic intestinal cells (Wong and Isberg, 2005). Invasin tightly binds to, and causes clustering of, $\beta 1$ integrins on the cell surface, thereby leading to rapid activation of Rac1 and the induction of membrane protrusions in host cells. Rac1 activity is required for membrane ruffling initiated by integrin-invasin interactions and for uptake of *Yersinia* bacteria by host cells (Alrutz et al., 2001). The FAK-Src complex might transmit signaling from $\beta 1$ integrins to Rac1 GTP loading; this is supported by the findings that invasin-mediated bacterial uptake is impaired in *FAK*^{-/-} cells, and that the expression of a *FAK*^{Y397F} mutant that cannot bind to SFKs does not restore this defect (Alrutz and Isberg, 1998; Bruce-Staskal et al., 2002). Prior to uptake, *Yersinia* inject virulent *Yersinia* outer proteins (Yops) into the host cell. In contrast to bacterial uptake, which depends on Rac1 activity, the delivery of so-called effector Yops depends on the activity of RhoA, RhoB or RhoC (Mejia et al., 2008). Certain Yops act in concert with $\beta 1$ integrins at the plasma membrane to stimulate Rho-GTPase activation in a Src-dependent manner. In a feedback loop, increased Rho-GTPase activity stimulates pore formation, which allows entry of effector Yops; some of these effector Yops interact with and inactivate Rho-GTPases to terminate the delivery of Yops into the host cell (Fig. 6C).

A comparable membrane protrusive process occurs during the transendothelial migration of leukocytes, when leukocyte adhesion to the endothelium induces endothelial membrane ruffles, which surround the leukocytes prior to diapedesis (Carman et al., 2003). Endothelial engulfment is initiated when leukocyte integrins bind to endothelial intercellular adhesion molecule 1 (ICAM-1), which locally activates RhoG in the endothelial cells through Src homology

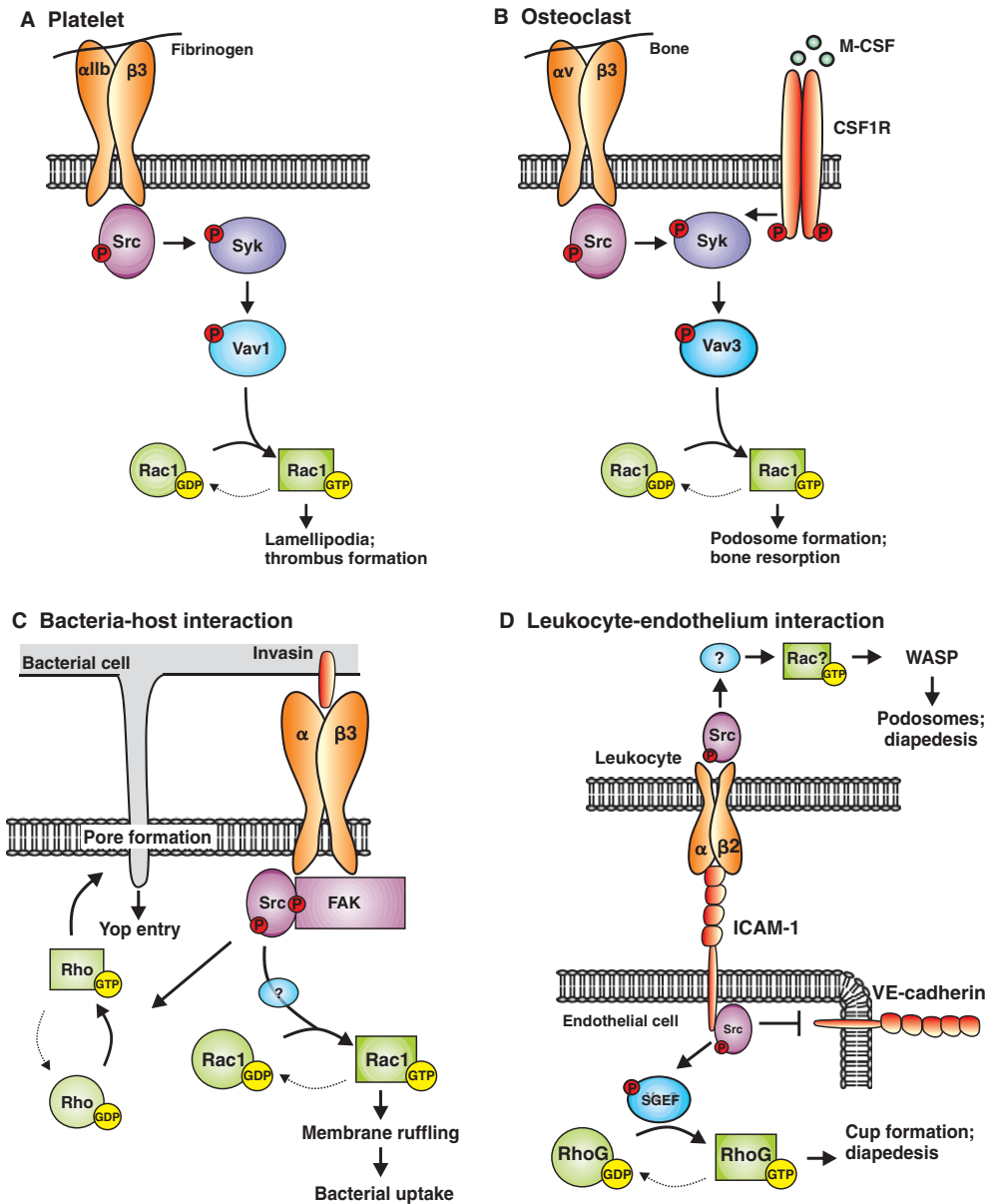


Fig. 6. The involvement of integrin-SFK crosstalk in physiological processes. The ability of integrins to regulate Rac and Rho GTPases through SFKs controls intracellular processes involved in hemostasis (A), bone resorption (B), bacterial uptake by non-phagocytic cells (C) and leukocyte extravasation (D). A schematic overview of the molecular pathways that are known to occur downstream of integrin engagement in each of these processes is shown. M-CSF, macrophage colony-stimulating factor; CSF1R, colony-stimulating-factor 1 receptor. See main text for additional details. Broken arrows indicate transition not supported by presented pathway or not supporting presented output.

3 domain-containing guanine nucleotide exchange factor (SGEF), which might involve SFK-mediated phosphorylation (van Buul et al., 2007). The ligation of ICAM-1 also leads to the phosphorylation of vascular endothelial (VE) cadherins through the activation of Src, thereby destabilizing cell-cell adhesions and promoting transendothelial migration (Allingham et al., 2007). At the same time, in leukocytes, the ligation of $\beta 2$ integrins induces the formation of Src- and WASP-dependent podosome-like adhesions, which seem to palpate the endothelial membranes in search of a weak spot for diapedesis (Carman et al., 2007). In this case, ligation of the leukocyte integrins might trigger SFK-mediated signaling to Rho-GTPases, similar to the signaling pathway downstream of integrin $\alpha \nu \beta 3$ that supports podosome formation in osteoclasts, as described above (Fig. 6D).

The influence of crosstalk pathways on cell migration

The actin-driven processes involved in cell adhesion and spreading described thus far play an important role in cell migration. To allow

directionality along gradients of soluble factors or ECM components, spatiotemporal coordination of protrusion, contraction, assembly and disassembly of cell-matrix adhesions is required in a moving cell. Because Rho-GTPases control each of these processes, the activity of Rho-GTPases and/or of their downstream effectors must be tightly regulated in space and time for efficient cell migration to occur. The coupling of SFK activity to the regulation of Rho-GTPases at sites of integrin engagement could aid such spatiotemporal coordination.

Integrin-specific regulation of Rho-GTPases controls the directionality of cell migration

The balance between Rho A and Rac1 activities, as discussed above in the context of cell spreading (Fig. 3), is crucial for spatiotemporal coordination of cytoskeletal dynamics in moving cells. The balance between Rac1-mediated membrane protrusion and RhoA-mediated contractility is also modulated by the expression of different integrins (Fig. 7). As a consequence, alterations in the profile of

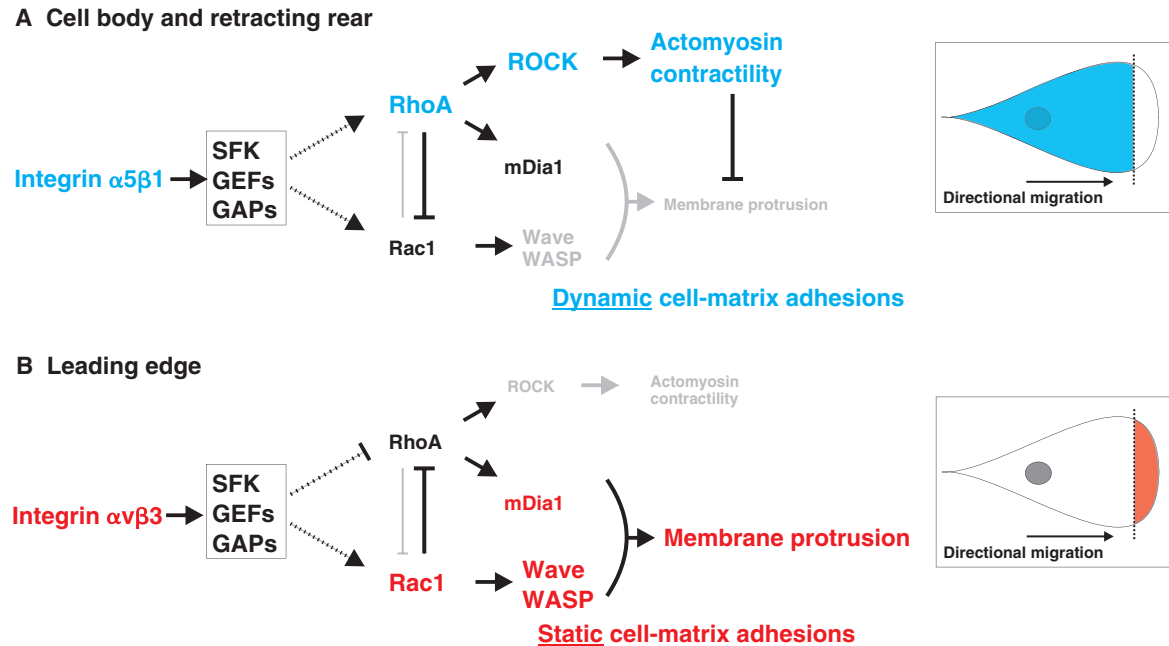


Fig. 7. Integrin–SFK–Rho-GTPase crosstalk in directional migration. The balance between the activity of RhoA and Rac1 is regulated differently in the retracting rear (A) and the protrusive front (B) of the cell body. In A, RhoA activity is required to prevent Rac1-stimulated formation of multiple lamellipodia that would interfere with directionality, and supports the high turnover of cell-matrix adhesions observed in the retracting rear of the cell. In B, RhoA-induced contractility is suppressed while Rac1 activity drives the formation of the lamellipodium at the leading edge. Integrins can regulate this spatial segregation of Rho-GTPase activities through the pathways that involve SFKs, GEFs and GAPs as described in the previous figures. See main text for additional details.

integrin expression can cause dramatic shifts in modes of cell migration. The expression of integrin $\alpha 5 \beta 1$, but not of others, such as integrin $\alpha \nu \beta 3$, is associated with strong RhoA-mediated contractility and random cell migration (Danen et al., 2005; White et al., 2007). In the absence of integrin $\alpha 5 \beta 1$, integrin $\alpha \nu \beta 3$ promotes persistent cell migration that is characterized by a single large lamellipod, relatively static adhesions and low RhoA activity (Danen et al., 2005). In agreement with these observations, activated integrin $\alpha \nu \beta 3$ is preferentially recruited in a Rac1-dependent manner to the leading edge of migrating cells, where cell-matrix adhesions are static compared with those found at the retracting rear (Ballestrem et al., 2001; Kiosses et al., 2001). Moreover, integrin $\alpha 5 \beta 1$ and integrin $\alpha \nu \beta 3$ use distinct endosomal recycling routes, and Rab4-mediated recycling of integrin $\alpha \nu \beta 3$ supports persistent migration by antagonizing the Rab11-mediated recycling of integrin $\alpha 5 \beta 1$ that is coupled to the activity of ROCK, an effector molecule downstream of RhoA (White et al., 2007). Interestingly, Rho-GTPases control the targeting of SFKs to the plasma membrane by regulating their endosomal recycling rate; this seems to occur via the same endosomal routes as those that control integrin recycling, which suggests that endosomes are ‘hot spots’ for crosstalk between integrins, SFKs and Rho-GTPases (Sandilands and Frame, 2008). Directional migration of keratinocytes has been found to require integrin- $\alpha 3 \beta 1$ -induced activation of a FAK-Src-Rac1 signaling axis that is important for cell polarity (Choma et al., 2007). However, the role of epidermal integrin $\alpha 3 \beta 1$ in wound healing in vivo was challenged recently, and might rely on the directional migration of keratinocytes that is mediated through other integrins (Margadant et al., 2009).

Although the above studies indicate that integrins can support persistent (directional) cell migration by promoting Rac1 rather than RhoA activity, too much Rac1 activity can also interfere with

persistent migration (Pankov et al., 2005) or prevent migration altogether (Danen et al., 2005). Moreover, whereas strong RhoA-mediated contractility counteracts persistent migration (Danen et al., 2005; White et al., 2007), too little RhoA activity can apparently have the same result: caveolin-1 knockout fibroblasts fail to migrate in a directional manner owing to an inability to suppress Src-mediated p190RhoGAP activation, which prevents normal RhoA GTP loading (Grande-Garcia et al., 2007). It is also possible that impaired caveolar transport in these cells disturbs cell migration by affecting the endosomal recycling of integrins and/or SFKs, in which caveolin-1 has a sorting function (Pelkmans et al., 2004).

The FAK-Src complex regulates adhesion turnover and membrane protrusion during cell migration

As explained above, the FAK-Src complex acts as an important suppressor of RhoA activity downstream of integrin ligation. Not surprisingly, both *Src*^{-/-} and *FAK*^{-/-} fibroblasts display impaired migration (Ilic et al., 1995; Klinghoffer et al., 1999; Ren et al., 2000). These cells have an increase in the number of peripheral focal adhesions owing to a decrease in focal adhesion disassembly. Tight regulation of p190RhoGAP phosphorylation by the FAK-Src complex maintains RhoA activity at levels that favor cell migration (Arthur et al., 2000; Schober et al., 2007). There is evidence that the signaling pathway that involves RhoA and mDia1 mobilizes active Src to focal adhesions leading to enhanced adhesion disassembly in migrating cells (Yamana et al., 2006). This suggests that there is a negative feedback loop whereby integrin-mediated activation of Src leads to p190RhoGAP-mediated inhibition of Rho-GTP levels, which in turn lowers the amount of active Src in integrin-containing adhesions and thereby modulates focal adhesion turnover during cell migration. The proteolytic cleavage of talin by calpain is important for cell-matrix adhesion turnover (Franco et al., 2004). In addition to RhoA-

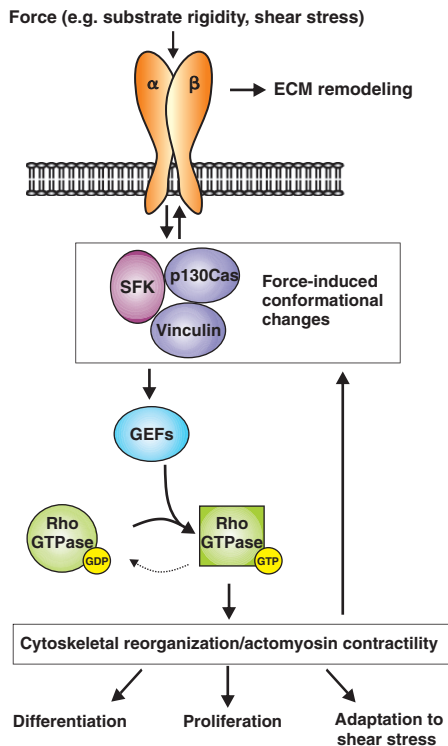


Fig. 8. Crosstalk in mechanotransduction. Integrins are necessary for translating the mechanical properties of the microenvironment (such as substrate rigidity) into intracellular signaling pathways. This is thought to involve tension-dependent conformational alterations in integrin-associated proteins, such as Src, p130Cas and vinculin, and might involve signaling through Rho-GTPases to remodel the actin cytoskeleton. Conversely, cytoskeletal tension might act on this same protein complex to regulate dynamic integrin-ligand interactions and ECM remodeling. Such mechanotransduction pathways regulate various cellular processes, including differentiation and proliferation. See main text for additional details.

mediated effects downstream of FAK and Src, regulation of calpain activity is yet another pathway through which the FAK-Src complex can enhance focal adhesion disassembly in support of cell migration (Frame et al., 2002). Finally, the recruitment of paxillin to the FAK-Src complex can further modulate cell motility; this occurs through the effects of paxillin on signaling pathways that lead to an increase in Rac1-GTP and Cdc42-GTP levels, or through the activation of a signaling pathway [involving paxillin, extracellular-regulated MAP kinase (ERK) and myosin light chain kinase (MLCK)] that regulates adhesion turnover at the front of migrating cells (Klemke et al., 1997; Webb et al., 2004).

As described, the FAK-Src complex also interacts with the adaptor protein p130Cas. Similarly to cells that lack FAK or Src, *Cas*^{-/-} cells display a defect in spreading and migration (Honda et al., 1999), probably in this case owing to impaired Rac1-mediated membrane protrusion. In the absence of p130Cas, integrins might fail to stimulate Rac1-induced lamellipodia formation through the Crk-Dock180-ELMO1 complex or paxillin-PKL-β-PIX signaling (see above).

Crosstalk pathways in tumor-cell invasion

The invasion of tumor cells can be mesenchymal (involving integrin and protease-dependent ECM remodeling) or, alternatively, tumor cells can move in an amoeboid manner and squeeze through the limited spaces between the ECM fibers (Wolf et al., 2003). Src

kinase activity is required for mesenchymal invasion as it controls the turnover of integrin-based adhesions, whereas amoeboid invasion is much less dependent on Src (Carragher et al., 2006). The ability of integrin $\alpha 5 \beta 1$ to bind soluble fibronectin, to promote cell-matrix adhesion turnover and to support RhoA-mediated contractility as discussed above (Huvneers et al., 2008b), might strongly affect invasion. First, inactivation of $\beta 1$ integrins in tumor cells causes suppression of RhoA and enhanced mesenchymal (Rac1-associated) invasion (Sahai and Marshall, 2003; Vial et al., 2003). Second, integrin $\alpha 5 \beta 1$ seems to be essential for the contractility mediated by RhoA and ROCK, which is required for ECM remodeling in invading cancer-associated fibroblasts in order to create invasive tracks that guide the carcinoma cells that follow behind (Gaggioli et al., 2007).

Proteolytic degradation of ECM components during tumor invasion can take place in podosomes or invadopodia (Yamaguchi et al., 2006). Signaling pathways triggered by integrins that regulate the activity of Src and Rho-GTPases are crucial for the formation of these highly dynamic actin-rich adhesions, in which the three types of proteins (integrins, SFKs and Rho-GTPases) are present around the F-actin core. Src-mediated suppression of RhoA following integrin engagement might be important for relaxation of the contractile actin cytoskeleton, which is essential for podosome or invadopodia formation (Clark et al., 2007). Conversely, the ability of activated Src mutants to induce podosomes depends on Src-induced phosphorylation and/or inactivation of $\beta 1$ integrins, which is important for the suppression of RhoA-mediated actomyosin contractility that is stimulated by integrin $\alpha 5 \beta 1$ (Huvneers et al., 2008a). In addition, the constitutive activation of ERK5 induced by activated Src leads to increased expression of the RhoGAP DLC-1, which further facilitates the inactivation of RhoA and supports invasive podosome formation (Schrapf et al., 2008).

Crosstalk in mechanotransduction

Cells are subject to mechanical stresses that are either external (from the ECM) or internal (from cytoskeletal contractility). As receptors that bridge the ECM and the actin cytoskeleton, integrins mediate coupling between the external and internal forces (Fig. 8). This allows cells to structurally modify the ECM through cytoskeletal forces that pull on integrins and to respond to external forces by remodeling of the cytoskeleton. Moreover, a growing body of evidence supports the idea that there is a crucial role for the mechanical aspects of integrin signaling in cellular differentiation and malignant growth. There is evidence indicating that several of the pathways described above have crucial roles in mechanosensing and mechanotransduction. For example, integrin $\alpha \nu \beta 3$ might activate Src in response to increased fibronectin rigidity during tumorigenesis (Huvneers et al., 2007; Jiang et al., 2006), and it has been reported that cells respond to mechanical tension through the activation of integrins, Rho-GTPases and SFKs (Matthews et al., 2006).

Extracellular-matrix assembly

Fibronectin is essential for embryonic development and is abundant in the ECM that is associated with dynamic cellular processes such as wound healing and angiogenesis (Hynes and Zhao, 2000). Fibronectin is synthesized as a soluble dimer that can be assembled into an insoluble fibrillar matrix on binding to cell-surface receptors of the integrin and syndecan families (Mao and Schwarzbauer, 2005). Integrin $\alpha 5 \beta 1$ binds soluble fibronectin particularly efficiently and promotes fibronectin fibrillogenesis, as well as RhoA activity and the dynamics of cell-matrix adhesions (Huvneers et al.,

2008b). RhoA-mediated actomyosin contractility (or cytoskeletal tension) and the translocation of ligated $\alpha 5\beta 1$ integrins from focal adhesions to fibrillar adhesions promote matrix assembly through force-mediated stretching of soluble fibronectin dimers (Pankov et al., 2000; Zhong et al., 1998). It is not clear whether the particular efficiency with which integrin $\alpha 5\beta 1$ stimulates these processes is related to regulation of FAK-SFK signaling, although the finding that neither *FAK*^{-/-} cells, nor cells deficient for the Src-family kinases Src, Yes and Fyn, can efficiently form fibronectin fibrils suggests that this might be the case (Ilic et al., 2004; Wierzbicka-Patynowski and Schwarzbauer, 2002). Conversely, too much Src activity causes transformation of cell-matrix adhesions into highly dynamic podosomes, in part through the suppression of integrin function and of RhoA-mediated contractility, and leads to a loss of fibronectin-matrix assembly (Huvencers et al., 2008a).

Collagen and fibronectin molecules are direct binding partners, and their fibrils often colocalize in the ECM. In contrast to fibronectin fibrillogenesis, soluble collagen can self-assemble into insoluble fibrils *in vitro* in the absence of a cellular involvement. However, the organization and formation of collagen fibrils *in vivo* strictly depends on interactions with other ECM components and with integrins (Larsen et al., 2006). Collagen-binding integrins promote the organization of collagen matrix, although the initiation of collagen fibrillogenesis is preceded by fibronectin-matrix assembly (Velling et al., 2002). This suggests that collagen-matrix assembly is controlled by the signals from integrins and SFKs that stimulate RhoA-mediated contractility and drive fibronectin fibrillogenesis. Similarly, fibrillin-microfibril assembly is preceded by fibronectin fibrillogenesis and strongly depends on the binding of integrin $\alpha 5\beta 1$ to fibronectin and subsequent RhoA-mediated cytoskeletal contractility (Kinsey et al., 2008).

Angiogenesis

The adhesion-regulated activity of SFKs and Rho-GTPases (either through integrins or cadherins) is crucial for angiogenesis (Mammoto et al., 2008). The activation of Src and RhoA through collagen-binding $\beta 1$ integrins induces the formation of capillary cords and supports vascular sprouting (Liu and Senger, 2004). At later stages of angiogenesis, the stability of endothelial vessels is maintained by SFKs that suppress RhoA-ROCK signaling (Im and Kazlauskas, 2007). Differences in mechanical force that result from shear stress in blood vessels lead to integrin activation, which occurs through a mechanosensing complex of endothelial-cell-specific adhesion receptors and the vascular endothelial growth-factor receptor-2 (VEGFR-2; also known as Flk-1), which results in an accumulation of SFK activity; this, in turn, controls integrin affinity (Tzima et al., 2005). Together, these signaling events regulate the activities of Rho-GTPases and align the actin cytoskeleton of endothelial cells to adapt to differences in shear stress.

ECM rigidity in the control of cell migration, differentiation and tumor growth

The stiffness of the stroma differs between various tissues. It is now clear that such differences can have profound effects on cell-fate decisions: mesenchymal stem cells can be forced to differentiate into different anchorage-dependent cell lineages by changing the stiffness of the matrix in which they are embedded (Engler et al., 2006). Matrix stiffness is increased in tumors and has been shown to control tumor growth (Paszek et al., 2005). Such differences in ECM rigidity lead to changes in the expression, conformation and clustering of integrins, as well as in integrin-

mediated signaling pathways (Larsen et al., 2006). Increased ECM rigidity stimulates RhoA GTP loading and cytoskeletal contractility, which promotes the differentiation of breast epithelial cells (Wozniak et al., 2003). Moreover, a positive feedback loop from RhoA-mediated contractility towards ECM remodeling further contributes to the sustained proliferation of tumor cells in rigid tumor stroma (Paszek et al., 2005). Integrin signaling that leads to the activation of SFKs might be crucial for sensing matrix rigidity. In fibroblasts, receptor-like protein tyrosine phosphatase- α (RPTP α) cooperates with integrin $\alpha \nu \beta 3$ at the leading edge to recruit and activate Fyn in response to increased matrix rigidity (Kostic and Sheetz, 2006). This effect is specific for Fyn owing to its specific palmitoylation sequence and it triggers the recruitment and phosphorylation of p130Cas. As described above, such local activation of p130Cas can be a driving force in cell spreading and migration through the activation of proteins that regulate Rho-GTPases. A similar matrix rigidity response through SFKs has been observed in mammary epithelial cells that are in contact with the basement membrane. However, in this case, the activities of Src, Lyn and Lck are all significantly reduced in response to increased matrix stiffness, whereas RhoA-ROCK-mediated contractility is enhanced under the same conditions (Paszek et al., 2005). Thus, in response to changes in extracellular tension, integrins can selectively regulate the activity of SFKs, which might allow them to modify the activity of Rho-GTPases and cytoskeletal contractility through the pathways described above. Given the established role for RhoA-ROCK-mediated cytoskeletal tension not only in cell migration but also in cell proliferation (Danen and Yamada, 2001), these pathways might crucially regulate both processes during tissue homeostasis and malignant transformation.

Concluding remarks

Signaling pathways downstream of adhesion receptors are highly complex. The regulation of cytoskeletal shape and dynamics through the activation of SFKs and Rho-GTPases has a central role in the cellular responses that are induced by integrin engagement. Important themes in integrin-mediated regulation of Rho-GTPases are the formation of an active FAK-Src complex and the (direct) recruitment of Src to the $\beta 3$ -integrin cytoplasmic tail. In addition, it is now clear that SFKs mediate extensive crosstalk between integrins and other receptors, such as receptor tyrosine kinases, and that this crosstalk is an important aspect of integrin signaling in the regulation of Rho-GTPases. An important outstanding issue is how mechanical forces that are sensed within cell-matrix adhesions are translated into biochemical changes, such as the activation of SFKs. Conformational changes in mechanosensitive cell-matrix adhesion components, such as vinculin and p130Cas, might expose binding sites for SFKs and Rho-GEFs or Rho-GAPs to trigger actin cytoskeletal remodeling through the pathways discussed here. Future advances in high-resolution microscopy might further clarify how such crosstalk pathways are spatiotemporally regulated in living cells when they are challenged with different extracellular cues *in vitro* or *in vivo*. Finally, it is probable that additional GEFs and GAPs will be found that are regulated by integrins, and it might well be that SFKs link cell adhesion to such regulators of Rho-GTPases as well.

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