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Chapter 4

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redistribution and contractile shape

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Binding of soluble fibronectin to integrin $\alpha 5 \beta 1$ – link to focal adhesion redistribution and contractile shape

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Summary

Focal adhesions are randomly distributed across the ventral surface or along the edge of epithelial cells. In fibroblasts they orient centripetally and concentrate at a few peripheral sites connecting long F-actin stress fibers, causing a typical elongated, contractile morphology. Extensive remodeling of adhesions in fibroblasts also takes part in fibronectin fibrillogenesis, a process that depends on Rho-mediated contractility and results in the formation of a fibronectin matrix. Our current study shows that all these fibroblast characteristics are controlled by the ability of integrin $\alpha 5 \beta 1$ to bind soluble fibronectin molecules in their compact inactive conformation. The hypervariable region of the ligand-binding I-like domain of integrin $\alpha 5 \beta 1$ supports binding of soluble fibronectin. This supports the distribution of centripetally orientated focal adhesions in distinct peripheral sites, Rho activation and fibronectin fibrillogenesis through a mechanism that does not depend on Syndecan-4.

Integrin $\alpha v \beta 3$, even when locked in high affinity conformations for the RGD recognition motif shows no appreciable binding of soluble fibronectin and, consequently, fails to support the typical fibroblast focal adhesion distribution, Rho activity and fibronectin fibrillogenesis in the absence of integrin $\alpha 5 \beta 1$. The ability of $\alpha 5 \beta 1$ integrin to interact with soluble fibronectin may thus drive the cell-matrix adhesion and cytoskeletal organization required for a contractile, fibroblast-like morphology, perhaps explaining why $\alpha 5 \beta 1$ integrin, similarly to fibronectin, is essential for development.

Supplementary material available online at
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Introduction

Integrins consist of non-covalently linked α - and β -subunits. Ligands, including extracellular matrix (ECM) components such as fibronectin, are bound to the integrin extracellular globular head domain whereas a multitude of cytoskeletal adaptor proteins interact via the cytoplasmic tail. Thus, integrins create a link between the ECM and the actin cytoskeleton. Ligand binding can be modulated at the level of integrin clustering (avidity) or by activation of integrins through conformational changes in the extracellular ligand-binding domains (affinity) (Calderwood, 2004; Takagi and Springer, 2002). For instance, binding to soluble ligands through integrin $\alpha 5 \beta 1$, $\alpha v \beta 3$ or $\alpha IIb \beta 3$ can be enhanced more than 20-fold by divalent cations or stimulatory antibodies, leading to firm adhesion of cells that show no appreciable adhesion to immobilized ligands in the absence of such stimuli (Danen et al., 1995; Mould et al., 1995; Smith et al., 1994). In turn, integrin-mediated adhesion can modulate various intracellular signaling cascades (Hynes, 2002). Following cell adhesion to the ECM, integrins and associated proteins assemble in cell-matrix adhesions, which organize the actin cytoskeleton. In epithelial cells, focal adhesions are randomly distributed across the ventral surface or along the cell border. In mesenchymal cells such as fibroblasts, they orient centripetally and concentrate in a few peripheral sites connecting long F-actin stress fibers, causing a typical elongated, contractile morphology.

Fibronectin is essential for embryonic development and is abundantly present in the ECM associated with wound healing and

angiogenesis (Hynes and Zhao, 2000). Mesenchymal cells secrete compact and inactive soluble fibronectin dimers, which are assembled into insoluble fibronectin fibrils following their interaction with cell surface receptors of the integrin and syndecan families (Wierzbicka-Patynowski and Schwarzbauer, 2003). Fibronectin fibrils are assembled into a matrix that is important for anchorage of cells and guides cell migration during embryonic development and wound healing (Hynes and Zhao, 2000). Integrins $\alpha 5 \beta 1$, $\alpha 8 \beta 1$, $\alpha v \beta 1$, $\alpha v \beta 3$, $\alpha IIb \beta 3$, $\alpha v \beta 5$, $\alpha v \beta 6$ and $\alpha v \beta 8$ recognize the common integrin-binding motif Arg-Gly-Asp (RGD) that is found in many ECM components, including fibronectin (Danen and Sonnenberg, 2003). Of these integrins, $\alpha 5 \beta 1$ is particularly efficient in mediating fibronectin matrix assembly, although others can compensate for its absence to some extent (Wennerberg et al., 1996; Wu et al., 1996; Yang and Hynes, 1996). Fibronectin fibrillogenesis requires extensive remodeling of cell-matrix adhesions (Pankov et al., 2000), and contractility of the actin-myosin cytoskeleton, which is stimulated by the small GTPase Rho through activation of myosin-II. Contractility drives fibronectin fibrillogenesis by creating sufficient tension to stretch the compact fibronectin dimers and expose intermolecular fibronectin-binding sites (Zhong et al., 1998).

Integrin-mediated cell adhesion triggers a rapid inactivation of the small GTPase RhoA (Ren et al., 1999), which is important to relieve contractility and allow cell spreading. As cell spreading ends, Rho-mediated cytoskeletal contractility gradually increases, which coincides with maturation of focal adhesions and initiation of

of $\alpha\beta3$ in these processes using mouse embryonic fibroblasts (MEFs) isolated from *Itgb3*-knockout mice. The levels of GTP-RhoA in $\beta3$ -integrin-null MEFs were similar to those in MEFs isolated from wild-type littermates, indicating that RhoA activation does not require $\alpha\beta3$ integrin (Fig. 1B, supplemental material Fig. S1A). Similarly, fibronectin matrix assembly mediated by $\beta3$ -integrin-null MEFs occurred equally efficiently as that mediated by wild-type MEFs (Fig. 1C). Ectopic expression of $\beta3$ integrin did not further stimulate RhoA activity, whereas increased expression of $\beta1$ integrin led to enhanced activation of RhoA (Fig. 1B). Increased expression of $\beta1$ integrins in $\beta3$ -integrin-null MEFs also led to a more elongated, contractile cytoskeletal organization, whereas the presence or absence of $\beta3$ integrin did not affect morphology (Fig. 1D). Moreover, suppression of endogenous $\beta1$ integrins using *Itgb1*-specific siRNAs caused a phenotypic switch in wild-type MEFs characterized by increased cell spreading, more random distribution of focal adhesions and formation of cell-cell adhesions (Fig. 1E,F). We were unable to completely silence $\beta1$ integrin expression in MEFs and the level of suppression reached did not lead to detectable reduction of RhoA-GTP levels (not shown). Nevertheless, the conversion to a more epithelial morphology (strongly resembling that of $\beta1$ -integrin-deficient GE13 cells; Fig. 1A) upon $\beta1$ integrin silencing was in complete agreement with our previous findings using $\beta1$ -integrin-null cells. Silencing expression of $\beta1$ integrins in *Itgb3*-knockout MEFs caused cell rounding but the cells that remained attached resembled GE11 $\beta1$ -integrin-knockout cells, growing in islands with extensive cell-cell contacts (Fig. 1E,F; compare with Fig. 3A, left image).

Of the $\beta1$ integrins, $\alpha5\beta1$ integrin most efficiently supports fibronectin matrix assembly but integrins $\alpha8\beta1$, $\alpha4\beta1$ and $\alpha\beta1$ can also mediate cell adhesion to immobilized fibronectin. To determine whether $\alpha5\beta1$ integrin is the $\beta1$ integrin responsible for the typical fibroblast-like characteristics described above, we used differentiated *Itga5*-knockout ES cells (EA5). RhoA-GTP levels were very low in EA5 cells and restoring expression of $\alpha5$ integrin induced efficient RhoA-GTP loading (Fig. 2A, supplementary material Fig. S1B). Expression of an $\alpha5\alpha\gamma$ integrin chimera, consisting of the $\alpha5$ integrin extracellular and transmembrane domains and the $\alpha\gamma$ integrin cytoplasmic domain similarly induced RhoA-GTP loading, indicating that $\alpha5$ -integrin-specific sequences in the cytoplasmic tail are not required for enhancing RhoA activation (Fig. 2A). The very low RhoA-GTP levels in EA5 cells were accompanied by a flat circular cell shape with dispersed focal adhesions. Expression of $\alpha5$ integrin or $\alpha5\alpha\gamma$ integrin induced a reorganization of the cytoskeleton with long F-actin stress fibers connecting peripheral focal adhesions (Fig. 2B). Likewise, fibronectin fibrillogenesis was strongly enhanced in the presence of $\alpha5$ integrin or $\alpha5\alpha\gamma$ integrin in EA5 cells (Fig.

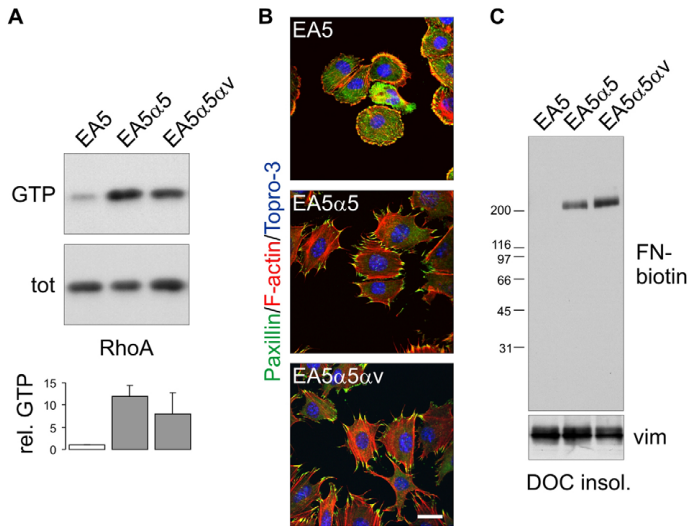


Fig. 2. Integrin $\alpha5$ is required for efficient RhoA activation and fibronectin fibrillogenesis. (A) Western blot analysis of RhoA activity assay on lysates of EA5 cells expressing indicated integrins. Quantification shows relative RhoA activation $\pm$ s.d. compared with EA5 cells from two independent experiments. (B) EA5 cells expressing indicated constructs stained for paxillin (green) and F-actin (red), with the nucleus in blue. Scale bar: 10 μ m. (C) Western blot analysis of assembled fibronectin-biotin and vimentin (loading control) in DOC-insoluble lysates of EA5 cells expressing indicated constructs. Locations of molecular size markers (in kDa) are indicated.

2C), which is consistent with previous findings (Sechler et al., 1997; Wu et al., 1993).

Together, these results demonstrate that the typical fibroblast-like elongated, contractile morphology that is associated with high levels of Rho-GTP and fibronectin matrix assembly do not require $\alpha\beta3$ integrin and are primarily stimulated by $\alpha5\beta1$ integrin.

The extracellular ligand-binding domain of $\alpha5\beta1$ integrin specifies fibroblast morphology, RhoA activation and fibronectin fibrillogenesis

In addition to the obvious essential role of the extracellular domain in ligand binding, the integrin transmembrane and cytoplasmic domains regulate ligand affinity and avidity by controlling integrin conformation and clustering (Calderwood, 2004; Li et al., 2003; Luo et al., 2007). The $\beta1$ integrin cytoplasmic domain is exchangeable with that of $\beta3$ integrin with no effect on Rho activation, fibronectin fibrillogenesis or fibroblast morphology when expressed in $\beta1$ -integrin-deficient GE11 cells (Danen et al., 2002). To test whether specific amino acid residues in the transmembrane domain of $\beta1$ integrin are required for $\alpha5\beta1$ -integrin-mediated support of fibroblast morphology, Rho activity, and fibronectin fibrillogenesis, we generated a chimeric $\beta1^{3^{trt}}$ subunit, consisting of a $\beta1$ integrin extracellular domain and $\beta3$ integrin transmembrane and cytoplasmic domains. When expressed in $\beta1$ -integrin-deficient GE11 cells (supplementary material Fig. S1C), this chimera promoted focal adhesion distribution and enhanced RhoA activity similar to wild-type $\beta1$ (Fig. 3A,B). Moreover, similarly to wild-type $\beta1$ integrin, $\beta1^{3^{trt}}$

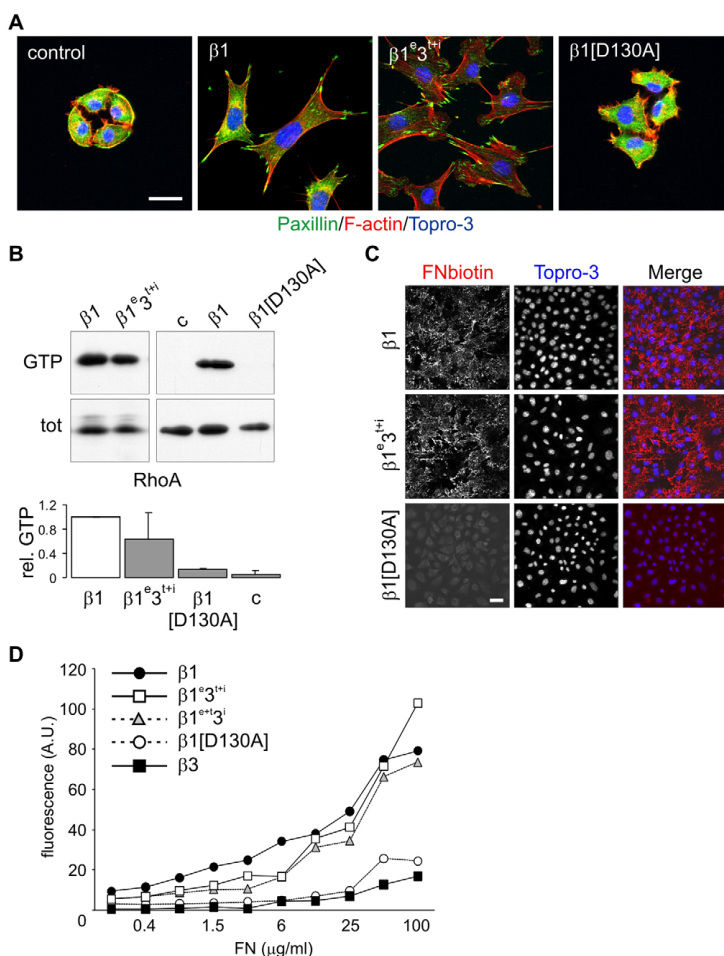


Fig. 3. Ligand binding to the extracellular domain of $\beta 1$ integrin is required for RhoA activation and fibronectin fibrillogenesis. (A) GE11 cells expressing indicated $\beta 1$ integrin subunits stained for paxillin (green), F-actin (red). Nuclei are stained blue with Topro-3. Scale bar: 10 μm . (B) Western blot analysis of RhoA activity assay on lysates of GE11 cells expressing indicated $\beta 1$ integrin subunits. Quantification shows relative RhoA activation $\pm$ s.d. compared with GE11 cells from two experiments. (C) Assembled fibronectin-biotin on GE11 cells expressing indicated $\beta 1$ integrin subunits; fibronectin is red and nuclei are blue. Scale bar: 50 μm . (D) Mean fluorescence analyzed by flow cytometry, demonstrating binding of indicated integrins to different concentrations of soluble FITC-fibronectin.

efficiently supported fibronectin matrix assembly (Fig. 3C), arguing against a specific role of the transmembrane and cytoplasmic domains of $\beta 1$ integrin in these processes. By contrast, expression of a $\beta 1[D130A]$ integrin subunit, containing a mutation in the extracellular I-like domain that abrogates ligand binding (Takada et al., 1992), failed to induce RhoA activation, a mesenchymal morphology, or fibronectin fibrillogenesis (Fig. 3, supplementary material Fig. S1C).

These findings indicate that the support of a fibroblast-like, contractile morphology associated with Rho activity and fibronectin fibrillogenesis by $\alpha 5\beta 1$ integrin is strictly dependent on ligand binding to its extracellular domain. Other regions of this integrin can be exchanged with corresponding regions of $\alpha v\beta 3$ integrin without consequence and surface expression of $\alpha 5\beta 1$ integrin without ligand interaction, which could affect (expression of) other surface receptors, is insufficient to support these processes.

The ability to bind to soluble fibronectin dimers correlates with RhoA activity and fibronectin fibrillogenesis

Many different integrins (including $\alpha 5\beta 1$ integrin and $\alpha v\beta 3$ integrin) can recognize the RGD motif and mediate cell adhesion to immobilized (stretched) fibronectin. However, we observed that all integrin β -subunits that supported a fibroblast-like cytoskeletal organization, Rho activity and fibronectin fibrillogenesis ($\beta 1$, $\beta 1^{e3tr}$ and $\beta 1^{e3}$), efficiently bound to soluble (compact and inactive) fibronectin dimers whereas the other β -subunits tested ($\beta 3$, $\beta 1[D130A]$) did not (Fig. 3D). This suggested that differences in binding to soluble fibronectin between $\alpha 5\beta 1$ integrin and $\alpha v\beta 3$ integrin could explain not only the particular efficiency with which $\alpha 5\beta 1$ integrin mediates fibronectin matrix assembly but also the different abilities of these integrins to support a fibroblast-like, contractile cytoskeletal organization and Rho activation.

We used mutants of $\beta 3$ integrin, which, in the context of $\alpha 1 \beta 3$, are locked in a low- or high-affinity conformation for the RGD sequence in fibrinogen (Luo et al., 2003; Luo et al., 2004), the integrin recognition sequence that is also present in the central cell-binding domain of fibronectin. Expression of a low-affinity $\beta 3$ [T329C; A347C] did not affect the poorly spread GE11 cell morphology (Fig. 4A center image; compare with Fig. 3A left image). Expression of the high-affinity $\beta 3$ [N305T] and $\beta 3$ [V332C; M335C] mutants stimulated cell spreading but distribution of focal adhesions in these cells was similar to those expressing wild-type $\beta 3$ integrin (Fig. 4A; compare with Fig. 1A right image). In line with these morphological similarities, the high-affinity mutants failed to enhance RhoA-GTP levels or fibronectin fibrillogenesis in the absence of $\beta 1$ integrin as also seen with wild-type $\alpha \beta 3$ integrin (Fig. 4B,C). As expected, based on findings in CHO cells (Luo et al., 2003; Luo et al., 2004) the $\beta 3$ [N305T] and $\beta 3$ [V332C; M335C] mutations strongly increased $\alpha \beta 3$ -integrin-mediated binding of GE11 cells to soluble RGD (Fig. 5A). However, even in the presence of the activating divalent cation, manganese, locking $\alpha \beta 3$ integrin in a high affinity state for RGD failed to induce binding to soluble fibronectin (Fig. 5B,C). Again, $\alpha 5 \beta 1$ integrin efficiently bound soluble fibronectin in these experiments which could be competed with unlabeled fibronectin (Fig. 5B,C).

These results demonstrate that although RGD is a common recognition motif, affinity for RGD does not necessarily indicate binding to all RGD-containing ligands. The central cell-binding domain of compact soluble fibronectin dimers appears to be only available for binding to $\alpha 5 \beta 1$ and not to $\alpha \beta 3$ integrin. Notably, this indicates that efficient binding to soluble fibronectin dimers might in fact underlie the efficiency with which $\alpha 5 \beta 1$ integrin supports a fibroblast-like distribution of focal adhesions, contractile cell shape and Rho-mediated cytoskeletal contractility that drives fibronectin fibrillogenesis.

Specificity in the I-like domain controls binding of soluble fibronectin, focal adhesion distribution, Rho-mediated contractility and fibronectin matrix assembly independently of syndecan-4

Since ligand binding is required for $\alpha 5 \beta 1$ integrin to support a contractile, fibroblast-like morphology and Rho activity ($\beta 1$ [D130A]; Fig. 3) and since its ability to efficiently bind soluble fibronectin dimers appeared to underlie the difference between $\alpha 5 \beta 1$ integrin and $\alpha \beta 3$ integrin in this respect (Fig. 5), we analyzed a more subtle mutation in the I-like domain that participates in ligand binding. Exchanging the CTSEQNC hypervariable sequence in the I-like domain of $\beta 1$ integrin with the corresponding region of $\beta 3$ integrin has been shown to cause a shift in ligand specificity that leads to adhesion to vitronectin, von Willebrand factor and fibrinogen without affecting adhesion to immobilized (stretched) fibronectin (Takagi et al., 1997). We expressed such a $\beta 1$ -3-1 integrin subunit in $\beta 1$ -integrin-deficient GE11 cells (supplementary material Fig. S1D). Although adhesion to immobilized fibronectin was similar, cells expressing this chimera displayed strongly reduced binding of soluble fibronectin compared with cells expressing wild-type $\alpha 5 \beta 1$ integrin (Fig. 6A). Importantly, in addition to causing a marked inhibition of fibronectin matrix

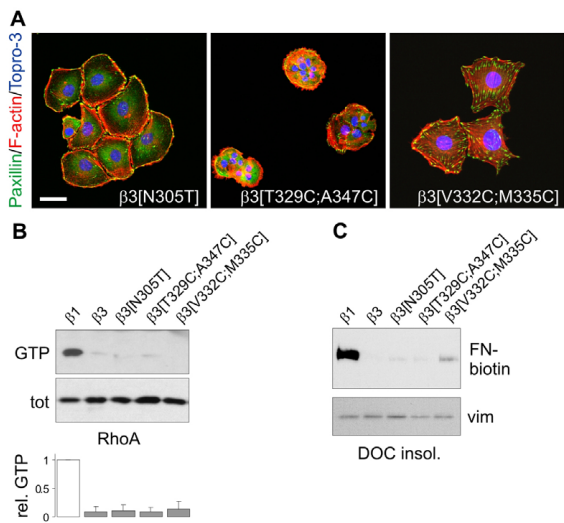


Fig. 4. High-affinity mutants of $\beta 3$ integrin fail to stimulate RhoA activity and fibronectin fibrillogenesis. (A) Images of $\beta 1$ -integrin-deficient GE11 cells expressing indicated $\beta 3$ integrin affinity mutants stained for paxillin (green), F-actin (red) and the nucleus (blue). Scale bar: 10 μ m. (B) Western blot analysis of RhoA activity assay on lysates of GE11 cells expressing indicated constructs. Quantification shows relative RhoA activation $\pm$ s.d. compared with GE11 cells from two experiments. (C) Western blot analysis of assembled fibronectin-biotin and vimentin (loading control) in DOC-insoluble lysates of GE11 cells expressing indicated constructs.

assembly, this was accompanied by a strong reduction in RhoA-GTP levels and by a random (epithelial-like) distribution of focal adhesions (Fig. 6B,C,D).

Rho-mediated contractility is required for fibronectin fibrillogenesis (Zhang et al., 1997; Zhong et al., 1998) and RhoA-GTP levels and fibronectin matrix assembly correlated for all the integrin constructs we tested. High RhoA-GTP levels were also associated with soluble fibronectin binding and fibroblast-like distribution of focal adhesions. We wondered (1) whether the high levels of RhoA-GTP were up- or downstream of soluble fibronectin binding to $\alpha 5 \beta 1$ integrin and the typical fibroblast-like distribution of focal adhesions and (2) whether fibronectin fibrillogenesis couples back to Rho activation in a positive feedback loop. To investigate these possibilities, binding of soluble fibronectin, fibronectin fibrillogenesis and RhoA activity were analyzed in cells expressing $\alpha 5 \beta 1$ under conditions where actomyosin contractility was blocked using ROCK or myosin-II inhibitors. In the presence of these inhibitors, fibronectin fibrillogenesis was strongly suppressed (ROCK) or even completely blocked (myosin-II), but binding of soluble fibronectin and RhoA-GTP levels were not affected (if anything they were slightly enhanced) (Fig. 7). These findings suggest that RhoA activity is downstream of fibronectin binding and they argue against a positive feedback loop from actomyosin contractility or fibronectin fibrillogenesis back to the regulation of Rho activity. Furthermore, the fact that the inhibitors ultimately led to disruption of focal adhesions (not shown) combined with the finding that silencing $\beta 1$ in MEFs leads to a

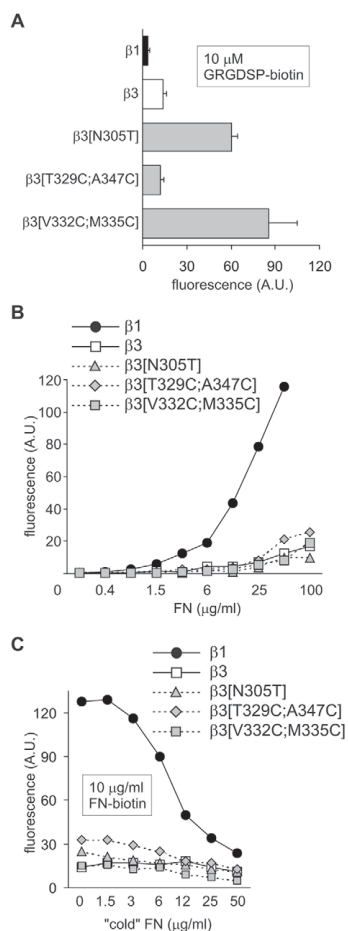


Fig. 5. Integrin binding of soluble GRGDSP and fibronectin. (A) Mean fluorescence $\pm$ s.d. demonstrating GRGDSP-biotin binding (10 μ M) to indicated integrins expressed on GE11 cells analyzed by flow cytometry. (B) Mean fluorescence analyzed by flow cytometry, demonstrating binding of indicated integrins to different concentrations of soluble FITC-fibronectin. Binding to integrin β 3 was shown previously in Fig. 3D. (C) Mean fluorescence demonstrating binding of soluble fibronectin-biotin (10 μ g/ml) to indicated integrins upon competition with increasing concentrations of unlabeled fibronectin analyzed by flow cytometry.

more epithelial distribution of focal adhesions (Fig. 1F) while in both cases RhoA levels remain high, argues against the possibility that the fibroblast-like cytoskeletal organization acts upstream of RhoA.

It has been reported that the transmembrane proteoglycan syndecan-4 acts in concert with integrins to mediate fibronectin matrix assembly (Chung and Erickson, 1997) and can modulate the formation of cell-matrix adhesions and the activities of Rho-

GTPases, including Rac1 (Bass et al., 2007) and RhoA (Dovas et al., 2006; Saoncella et al., 1999). We investigated whether syndecan-4 played a role in the support of fibroblast-like cytoskeletal organization, Rho activity or fibronectin fibrillogenesis by α 5 β 1 integrin. For this purpose, we silenced syndecan-4 expression in GE β 1 cells using syndecan-4 specific siRNAs. A complete knockdown of surface expression of syndecan-4 was achieved within 48 hours, whereas syndecan-4 expression was unaffected in control siRNA transfected GE β 1 cells (Fig. 8A). The formation and distribution of cell-matrix adhesions and organization of the actin cytoskeleton remained unaltered in cells without syndecan-4 (Fig. 8B). Similarly, fibronectin matrix assembly and binding of soluble fibronectin by α 5 β 1 integrin was still intact in GE β 1 cells lacking syndecan-4 (Fig. 8C,D). Finally, α 5 β 1-integrin-supported RhoA activation was also unaffected by syndecan-4 silencing (Fig. 8E) although for this assay, cells had to be expanded to obtain sufficient lysate resulting in incomplete syndecan-4 downregulation (not shown). Notably, a knockdown of syndecan-4 expression levels in GE β 3 cells induced a dramatic morphological change, and GE β 3 cells lacking syndecan-4 were unable to form their typical flattened circular shape but instead formed an irregular cell border containing several cytoskeletal extensions (Fig. 8F,G). These experiments indicate that syndecan-4 can act in concert with α v β 3 integrin to regulate cell-matrix adhesion distribution when α 5 β 1 integrin is absent but syndecan-4 is not required for cytoskeletal organization, focal adhesion formation/distribution, Rho activity or fibronectin matrix assembly in the presence of α 5 β 1 integrin.

Taken together, our data demonstrate that following the initial drop in RhoA activity during cell adhesion, the ability of α 5 β 1 integrin to bind compact soluble fibronectin dimers drives the typical fibroblast-like distribution of focal adhesions and the accumulation of Rho activity, which, in turn, stimulates fibronectin fibrillogenesis (Fig. 9). Interactions of fibronectin with α v β 3 integrin or syndecan-4 are dispensable for all these processes.

Discussion

Fibronectin fibrillogenesis requires integrin-mediated binding of soluble fibronectin dimers and depends on Rho-mediated cytoskeletal contractility to stretch integrin-bound fibronectin molecules exposing cryptic fibronectin-binding sites (Geiger et al., 2001; Mao and Schwarzbauer, 2005; Zhong et al., 1998). The α 5 β 1 integrin mediates fibronectin matrix assembly with particular efficiency although other integrins can substitute for α 5 β 1 integrin to some extent (Wennerberg et al., 1996; Wu et al., 1996; Yang and Hynes, 1996). Our current study indicates that α 5 β 1 integrin not only promotes fibronectin matrix assembly, but also stimulates the contractile, fibroblast-like morphology. The ability of α 5 β 1 integrin to efficiently bind compact soluble fibronectin dimers appears to drive the appropriate redistribution of focal adhesions to support this cell shape that is associated with increased RhoA activity, which, in turn, stimulates fibronectin fibrillogenesis. Such dynamic behavior of α 5 β 1 integrin was previously implicated directly in fibronectin fibrillogenesis (Pankov et al., 2000). Our findings indicate that an analogous process underlies the distribution of focal adhesions leading to the typical contractile cell shape observed in fibroblasts. Integrins that cannot bind soluble fibronectin stimulate cell spreading and focal contact formation but these adhesions distribute randomly and are connected by short F-actin fibers, leading to an overall non-contractile, flat, epithelial-like cell shape. These findings point to

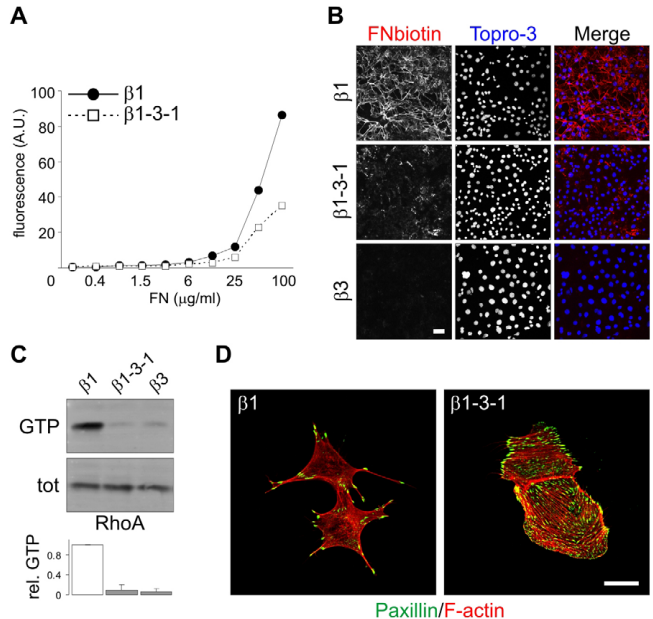


Fig. 6. High-affinity binding to the hypervariable region of the $\beta 1$ I-like domain controls signaling to fibronectin fibrillogenesis. (A) Mean fluorescence analyzed by flow cytometry, demonstrating binding of integrin $\beta 1$ and $\beta 1$ -3-1 to different concentrations of soluble FITC-fibronectin. (B) Images of assembled fibronectin-biotin on GE11 cells expressing $\beta 1$ or $\beta 1$ -3-1; fibronectin is red and nucleus blue. Scale bar: 50 μ m. (C) Western blot analysis of RhoA activity assay on lysates of GE11 cells expressing indicated integrins. Quantification shows relative RhoA activation $\pm$ s.d. compared with GE11 cells from two independent experiments. (D) Images of GE11 cells expressing $\beta 1$ integrin or $\beta 1$ -3-1 stained for paxillin (green) and F-actin (red). Scale bar: 10 μ m.

a critical role for $\alpha 5\beta 1$ integrin as an efficient mechano-transducer, which couples binding of soluble fibronectin dimers to a cytoskeletal organization that supports the assembly of a fibronectin matrix (Fig. 9).

In agreement with our findings, $\alpha 5\beta 1$ integrin has recently been demonstrated to support ROCK-mediated contractility in fibroblasts (Gaggioli et al., 2007; White et al., 2007). However, others have shown that overexpression of $\alpha v\beta 3$ integrin can stimulate RhoA activity in leukocytes (Butler et al., 2003) and CHO cells (Miao et al., 2002). Notably, these cells express endogenous $\beta 1$ integrins making it difficult to compare these studies with our own. In addition, multiple different cell surface receptors can regulate the activity of Rho-GTPases and may contribute to the difference between these studies. Nevertheless, in our previous studies we were unable to observe any stimulation of RhoA activity by $\alpha v\beta 3$ integrin in the absence of $\beta 1$ integrins (Danen et al., 2002; Danen et al., 2005). Here we use *Itgb3*-knockout MEFs to demonstrate directly that $\alpha v\beta 3$ integrin is dispensable for RhoA activation. Wild-type and $\beta 3$ -integrin-null MEFs also display the same fibroblast-like cytoskeletal organization that is very similar to that in $\beta 1$ -deficient cells expressing high levels of $\alpha v\beta 3$ integrin (GE $\beta 3$). Thus, although expression of $\beta 1$ integrin induces a morphological switch that resembles an 'epithelial-to-mesenchymal transition' in $\beta 1$ -integrin-null cells (Danen, 2002), silencing $\beta 1$ integrin in MEFs induces what appears like a 'mesenchymal-to-epithelial transition'.

We observe a tight correlation between high RhoA-GTP levels and focal adhesion dynamics, contractile morphology, and fibronectin matrix assembly (Danen 2002; Danen, 2005) (this study). Experiments using inhibitors argue against a positive feedback loop

from cytoskeletal organization to RhoA GTP loading but we do not know at present how the ability of $\alpha 5\beta 1$ integrin to bind soluble fibronectin supports the activity of RhoA. Recently, the guanine nucleotide exchange factors (GEFs) Lsc and LARG were shown to link cell adhesion on fibronectin to RhoA GTP loading and focal adhesion and stress fiber formation (Dubash et al., 2007). However, the integrins tested in our study that do not bind soluble fibronectin and do not stimulate RhoA activity do in fact efficiently support the formation of focal adhesions and short stress fibers. RhoA-mediated contractility appears to be more important for the distribution of focal adhesions and orientation of stress fibers. If regulation of GEFs and GAPs (GTPase-activating proteins) is involved, our experiments using chimeric integrins indicate that obligate interactions of such proteins or their regulators with $\alpha 5\beta 1$ -integrin-specific residues in the cytoplasmic domains do not underlie $\alpha 5\beta 1$ -integrin-stimulated RhoA activation. However, it is possible that binding of soluble fibronectin to $\alpha 5\beta 1$ integrin induces conformational alterations and/or clustering of the integrin that affects the localization and/or activity of such regulatory proteins.

Syndecan-4 can act in concert with integrins to regulate cytoskeletal organization during cell adhesion to fibronectin through interactions at the HepII domain in fibronectin. Using an RNAi approach we rule out the idea that crosstalk with syndecan-4 is involved in the fibroblast-like cytoskeletal organization, Rho activity and fibronectin matrix assembly that is supported by $\alpha 5\beta 1$ integrin. Our findings do not seem to support the previously reported role of syndecan-4 in stimulation of RhoA-mediated processes (Saoncella et al., 1999). However, others have recently shown that during adhesion to fibronectin, syndecan-4 is required for regulation of Rac1, but not RhoA (Bass et al., 2007). This might also explain why syndecan-4 knockdown strongly affects cytoskeletal

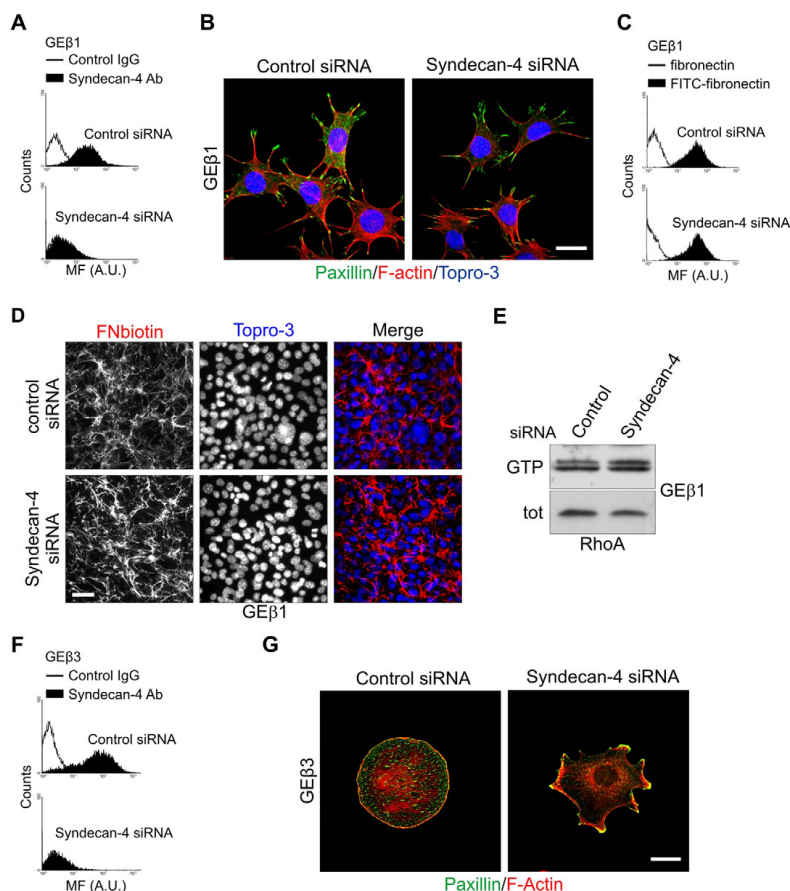


Fig. 8. Syndecan-4 is not required for $\alpha 5\beta 1$ -integrin-supported RhoA signaling. (A) Flow cytometry analysis of endogenous syndecan-4 surface expression on GE $\beta 1$ cells transfected with syndecan-4 or control siRNA. (B) Images of GE $\beta 1$ cells transfected with syndecan-4 or control siRNA stained for paxillin (green), F-actin (red) and the nucleus (blue). (C) Flow cytometry analysis of soluble FITC-fibronectin (10 $\mu\text{g}/\text{ml}$) binding to GE $\beta 1$ cells with or without syndecan-4 knockdown. (D) Images of assembled fibronectin-biotin on GE $\beta 1$ transfected with syndecan-4 or control siRNA; fibronectin is red and nucleus is blue. Scale bar: 50 μm . (E) Western blot analysis of RhoA activity assay on lysates of syndecan-4 knockdown and control GE $\beta 1$ cells. (F) Flow cytometry analysis of endogenous syndecan-4 surface expression on GE $\beta 3$ cells transfected with syndecan-4 or control siRNA. (G) Images of GE $\beta 3$ cells transfected with syndecan-4 or control siRNA stained for paxillin (green) and F-actin (red).

et al., 2002). cDNA encoding human $\alpha 5$ (provided by Erkki Ruoslahti, Cancer Research Center, The Burnham Institute, La Jolla, CA) was cloned into LZRS-neo. To generate a cDNA encoding a chimeric human $\alpha 5\alpha v$ integrin subunit, a fragment containing the extracellular and transmembrane domain of $\alpha 5$ integrin was digested from LZRS- $\alpha 5$ as a *SwaI/HindIII* fragment using the internal *HindIII* site immediately downstream of the transmembrane region. This was ligated to a *HindIII/SnaBI* cDNA fragment derived from LZRS- αv by PCR amplification of the αv integrin cytoplasmic domain in which the *aggatg* sequence immediately upstream of the cytoplasmic tail of αv integrin was changed to *aagctt*, creating a *HindIII* site. To generate a cDNA encoding a chimeric human $\beta 1^{c3^{+1}}$ subunit, the *Igfb1* sequence downstream of the internal *SnaBI* site (70 nucleotides upstream of the transmembrane region) was replaced with the corresponding sequence derived from LZRS- αv by PCR amplification in which the *ccagta* sequence was changed to *tacgta*, creating a *SnaBI* site. These cDNAs, as well as cDNAs encoding $\beta 3$ [N305T], $\beta 3$ [T329C;A347C] and $\beta 3$ [V332C;M335C] (provided by Bing-Hao

Luo and Timothy A. Springer, CBR Institute for Biomedical Research, Boston, MA) and cDNA encoding $\beta 1$ [D130A] (provided by Yoshikazu Takada, University of California Davis Medical Center, Sacramento, CA) were re-cloned into LZRS-neo. The $\beta 1$ -3-1 expression plasmid was provided by Yoshikazu Takada. Retroviral constructs were transfected into ecotrophic packaging cells to generate virus-containing culture supernatants. $\beta 3$ knockout MEFs, EA5 and GE11 cells were transfected with cDNA using effectene (Qiagen) or transduction with retroviral supernatants and positive cells were bulk sorted at least twice by FACS for the human integrin expressed.

Antibodies and other materials

Monoclonal antibodies used were anti-human $\alpha 5$ NK1-Sam1 (provided by Carl Figdor, Nijmegen Centre for Molecular Life Sciences, Nijmegen, The Netherlands), anti-human $\beta 1$ TS/2/16, clone 18 (Transduction Laboratories), anti-human $\beta 3$ C17 (provided by Ellen van der Schoot, Sanquin, Amsterdam, The Netherlands), anti-

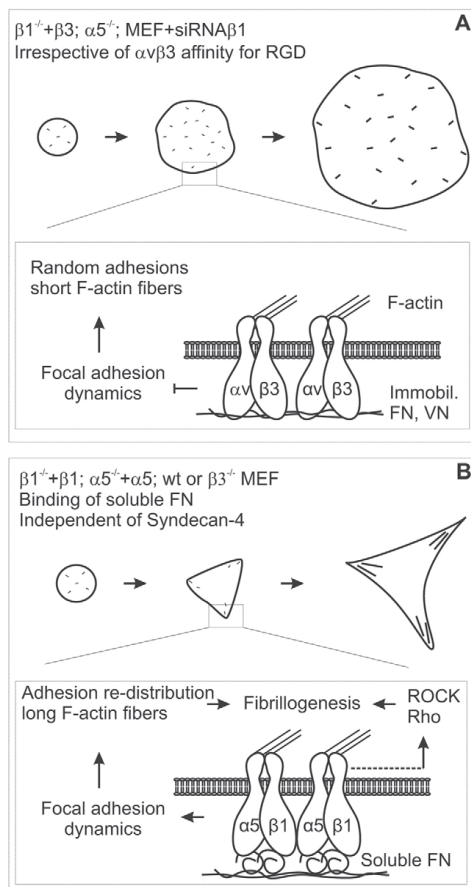


Fig. 9. A model for the role of $\alpha 5\beta 1$ integrin in reorganization of focal adhesions, contractility, cell morphology and fibronectin-matrix assembly. (A) Adhesion to immobilized (stretched) RGD-containing ECM components such as vitronectin (VN) or fibronectin (FN) through $\alpha v\beta 3$ integrin in the absence of $\alpha 5\beta 1$ integrin promotes the formation of randomly distributed cell-matrix adhesions leading to an epithelial-like, flat, circular morphology. (B) In the presence of $\alpha 5\beta 1$ integrin, binding of compact soluble fibronectin dimers to this integrin promotes the dynamic centripetal redistribution of focal adhesions. Together with the high activity of RhoA that is supported in the presence of $\alpha 5\beta 1$ integrin, this allows the cytoskeletal organization that drives fibronectin fibrillogenesis.

mouse $\beta 3$ (clone 2C9.G2, Pharmingen), anti-paxillin (clone 349, BD Transduction Laboratories), anti-RhoA (clone 26C4, Santa Cruz), anti-Syndecan-4 (clone KY/8.2, Pharmingen), anti-vimentin (clone K36, provided by F. Ramaekers, University of Maastricht, Maastricht, The Netherlands). Texas-Red-conjugated phalloidin and Topro-3 were purchased from Molecular Probes. Texas-Red-conjugated streptavidin was purchased from Pierce Chemical Co. Human plasma fibronectin and biotinylated-fibronectin were prepared as described previously (Danen et al., 2002). GRGDSP peptide was generated at the Netherlands Cancer Institute and biotinylated using EZ-link Sulfo-NHS-Biotin (Pierce Chemical Co.) according to the manufacturer's protocol. Y27632 and blebbistatin were obtained from Calbiochem.

RhoA activity assays

Cells were plated overnight to subconfluency before lysis in Nonidet P-40 lysis buffer [0.5% Nonidet P-40, 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM $MgCl_2$, 10% glycerol, supplemented with a protease inhibitor mix (Sigma-Aldrich)], and lysates were clarified by centrifugation at 20,000 g for 20 minutes at 4°C. A 1% aliquot was removed for determination of total quantities of RhoA. Clarified lysates were then incubated for 45 minutes at 4°C with a GST fusion protein of the Rho-binding domain of the Rho effector protein RhoGAP (Ren et al., 1999). Complexes were bound to glutathione-conjugated beads, and washed three times in Nonidet P-40 lysis buffer. The samples were analyzed by SDS-PAGE and western blotting.

Immunofluorescence and flow cytometry

For immunofluorescence, cells were fixed in 2% paraformaldehyde, permeabilized in 0.4% Triton X-100, blocked with 2% BSA, and incubated with anti-paxillin antibody, followed by FITC-labeled secondary antibody, phalloidin-Texas-Red and Topro-3 staining. To visualize fibronectin fibrillogenesis, cells were plated on fibronectin-coated coverslips for 4 hours and subsequently incubated for an additional 20 hours in medium containing 10% fibronectin-depleted serum supplemented with 10 $\mu g/ml$ biotinylated fibronectin. Cells were fixed in 2% paraformaldehyde, blocked with 2% BSA and stained with streptavidin-Texas-Red. Subsequently, coverslips were permeabilized in 0.4% Triton X-100 and stained with Topro-3. Preparations were mounted in Mowiol 4-88 solution supplemented with DABCO (Calbiochem) and analyzed using a confocal Leica TCS-NT microscope. Images were obtained using a 40 $\times$ or 63 $\times$ oil objective and imported into Adobe Photoshop.

For flow cytometry of integrin expression and cell sorting, cells were trypsinized, collected in culture medium, washed with PBS, and incubated with primary antibodies in PBS containing 2% serum for 1 hour at 4°C. For flow cytometry of syndecan-4 expression, cells were harvested using enzyme-free Dissociation buffer (Gibco), washed with 0.1% BSA/0.1% sodium azide followed by primary antibody incubation. Cells were then washed in PBS, incubated with FITC-, PE- or APC-conjugated secondary antibodies for 1 hour at 4°C, washed in PBS, and analyzed on a FACSCalibur or sorted on a FACSstar plus[®] (Becton Dickinson).

DOC insolubility assays

Cells were labeled with biotinylated fibronectin as described above and lysed in DOC buffer [1% sodium deoxycholate (DOC), 20 mM Tris-HCl pH 8.5, 2 mM N -ethylmaleimide, 2 mM iodoacetic acid, 2 mM EDTA and 2 mM PMSF]. Lysates were passed through a 23 GA needle and DOC-insoluble material was collected by centrifugation at 20,000 g for 20 minutes at 4°C. The pellet was washed once with DOC buffer, resolved in reduced sample buffer and analyzed by SDS-PAGE and western blotting.

RGD and fibronectin binding assays

Cells were harvested, resuspended in DMEM supplemented with 0.5% BSA and 2 mM $MnCl_2$, and incubated with 10 μM biotinylated GRGDSP-peptide or 10 $\mu g/ml$ biotinylated human plasma fibronectin for 1 hour at 4°C in the absence or presence of increasing concentrations of unlabeled fibronectin. The cell pellet was washed in 0.9% NaCl and 2 mM $MnCl_2$ and subsequently labeled with PE-conjugated streptavidin for 30 minutes at 4°C. To monitor dose-responsive fibronectin binding, cells were incubated with different concentrations of FITC-conjugated human plasma fibronectin for 1 hour at 4°C. Binding of the RGD-peptide or fibronectin was determined by flow cytometry.

siRNA transfection

Cells were plated at 40% confluency and were transfected the following day using DharmaFECT 1 reagent and a final concentration of 100 nM of integrin $\beta 1$ SMARTpool siRNA (M-040783-00), Syndecan-4 ON-TARGET plus SMARTpool siRNA (L-044221-00), or siCONTROL non-targeting siRNA#2 (D-001210-02) purchased from Dharmacon. After replating, the cells were analyzed 48 hours post-transfection for integrin $\beta 1$ or syndecan-4 surface expression, and used for immunofluorescence, RhoA activity assays and fibronectin binding assays.

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