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Syndecan-4 independently regulates multiple small GTPases to promote fibroblast migration during wound healing

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Keywords: syndecan-4, Arf6, RhoG, Rac1, RhoA, integrin, wound healing, migration

Abbreviations: 50K, 50 KDa, integrin-binding fragment of fibronectin; Arf, ADP-ribosylation factor; AP2, clathrin adaptor complex 2; CRIB, Cdc42/Rac interactive binding motif; ELISA, enzyme-linked immunosorbant assay; Fn, fibronectin; GAP, GTPase activating protein; GEF, guanine exchange factor; GGA3, Golgi-localized, γ -ear-containing Arf-binding protein 3; GPI, glycosylphosphatidylinositol; GST, glutathione-S transferase; H/0, heparin-binding fragment of fibronectin; HSP70, heat shock protein 70; IFN γ , interferon gamma; MEF, murine embryonic fibroblast; PI3K, phosphoinositide-3-kinase; PKC α , protein kinase C alpha

Upon wounding, syndecan-4 detects the appearance of fibronectin in the wound bed and mediates regulation of the small GTPases, Rac1, RhoA and RhoG. Cohesive regulation of these molecules results in cycles of membrane protrusion and cytoskeletal contraction, and triggers the endocytosis of $\alpha_5\beta_1$ -integrin, which collectively lead to immigration of fibroblasts into the wound bed. In this manuscript we identify the regulation of a fourth GTPase, Arf6 that is responsible for $\alpha_5\beta_1$ -integrin recycling and thereby completes the cycle of syndecan-4-regulated integrin trafficking. We demonstrate that each of the GTPase signals can be regulated by syndecan-4, but that they are independent of one another. By doing so we identify syndecan-4 as the coordinating center of pro-migratory signals.

Introduction

Wound healing consists of several important, overlapping phases—the inflammatory response, wound contraction, closure of the epidermis and finally resolution (reviewed in Shaw et al.¹). Each phase is triggered by appearance of chemical signals and involves immigration of cells into the defect, under the control of small GTPases. For example, migration of keratinocytes that make up the neo-epidermis of wounded skin is impaired by knockout of Rac1 and results in delayed re-epithelialisation.² One of the best-studied processes is the recruitment to the wound of fibroblasts, which then differentiate into myofibroblasts and are responsible for wound contraction.³ Fibroblasts are attracted to fibronectin that leaks from damaged capillaries and is laid down by invading leukocytes, and indeed topical application of fibronectin to a wound

accelerates appearance of fibroblasts and myofibroblasts.⁴

The two key receptors that bind and mediate fibroblast migration toward fibronectin are $\alpha_5\beta_1$ -integrin and syndecan-4. $\alpha_5\beta_1$ -integrin provides the mechanical connection between the fibroblast and fibronectin and is necessary for migration of fibroblasts toward fibronectin-rich extracellular matrix.⁵ However, syndecan-4 is necessary for a complete adhesion-dependent signaling response and acts as the initial fibronectin sensor.⁵⁻⁷ More importantly, syndecan-4 expression is necessary for an efficient healing response, as syndecan-4-knockout mice exhibit a delay in wound closure that is caused by compromised fibroblast migration.⁸ A large number of molecules have now been identified that are regulated by syndecan-4 and $\alpha_5\beta_1$ -integrin, and the key challenge is to understand how these signals are coordinated.

Syndecan-4 engagement triggers the regulation of a number of Rho-family GTPases that, in turn, regulate fibroblast migration. Syndecan-4-engagement activates Rac1,⁶ while simultaneously suppressing RhoA activity,⁹ thus instigating membrane protrusion and nascent focal complex formation. Subsequently, RhoA is activated to drive maturation of focal adhesions and contraction of the cell body.¹⁰ More recently, syndecan-4 has been found to activate RhoG. Engagement of syndecan-4 by either fibroblast growth factor or the heparin-binding fragment of fibronectin causes release of RhoG by the sequestering molecule, RhoGDI.^{11,12} RhoG is reported to drive lamellipodium extension, cell migration and phagocytosis by interaction with the Elmo/Dock180 complex that in turn activates Rac1.¹³ In addition, RhoG is known to be involved in the regulation of receptor internalization by caveolar endocytosis.^{14,15}

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Syndecan-4-dependent activation of RhoG reduced cell avidity for fibronectin by the triggering of dynamin- and caveolin-dependent $\alpha_5\beta_1$ -integrin endocytosis.¹¹ The result was unexpected for two reasons: first, most of the previous data have reported syndecan-4-dependent focal adhesion reinforcement;^{6,7,10} second, all previous models have described syndecan-4 as an integrin co-receptor, rather than a regulator of the integrin itself.

The range of GTPases regulated by syndecan-4, and possible conflicts in function mean that it is now essential that we understand the temporal relationships between the signals and whether each GTPase is responsible for regulation of the others. In this manuscript we identify a fourth GTPase, Arf6, that is regulated by syndecan-4 and examine the relationship between each of them. We find that although Rac1, RhoA, RhoG and Arf6 are each regulated by the interaction between syndecan-4 and fibronectin, none of them is directly dependent on the others in the fibronectin-signaling pathway.

Our previous work has demonstrated that engagement of syndecan-4 by fibronectin triggers rapid endocytosis of $\alpha_5\beta_1$ -integrin, due to activation of RhoG.¹¹ Atomic force microscopy was used to measure the energy required to detach a fibroblast following short contact (5 sec) with either an integrin-binding fragment of fibronectin (50K) or complete fibronectin (Fn), which acts as a ligand of both $\alpha_5\beta_1$ -integrin and syndecan-4. Despite similar density of $\alpha_5\beta_1$ -integrin ligand, less energy was required to detach the fibroblast from fibronectin, than from integrin ligand alone, and this was found to be due to caveolin-dependent endocytosis of $\alpha_5\beta_1$ -integrin.¹¹ Interestingly, the reduction in unbinding energy was reversible. Using the same fibroblast to make a series of contacts with 50K, then fibronectin, before returning to 50K revealed almost complete recovery of unbinding energy, which is indicative of rapid recycling of the endocytosed integrin (Fig. 1A). Arf6 is one of the key regulators of endosomal membrane trafficking¹⁶ and actin remodelling at the cell surface.¹⁷ It has been suggested that Arf6 regulates recycling of $\alpha_5\beta_1$ -integrin.¹⁸ Depletion of Arf6 expression by RNAi (Fig. 1C) had

no effect on the reduction in unbinding energy when fibroblasts were moved from 50K to fibronectin (Fig. 1B), indicating that Arf6 is not required for the endocytic phase. However, unbinding energy did not recover upon the return to 50K (Fig. 1B), demonstrating that Arf6 mediates recycling of $\alpha_5\beta_1$ -integrin during syndecan-4-regulated integrin trafficking. The next question was whether syndecan-4 actively regulates the recycling process.

Syndecan-4-dependent activation of Arf6 was measured by effector pull-down using GST-GGA3 as the bait protein. Fibroblasts were pre-spread on 50K-coated plastic, stimulated with the syndecan-4-binding fragment of fibronectin (H/0) and lysed for precipitation of active Arf6 over a time course. Arf6 was indeed activated upon syndecan-4 engagement, peaking at 90 min (Fig. 1D). Recovery of unbinding energy, when syndecan-4 engagement is removed during the atomic force experiments, occurs within 10 min so is much faster than induction of Arf6 by syndecan-4. This result suggests that Arf6 plays two crucial roles in syndecan-4-dependent trafficking. First, constitutive, low-level Arf6 activity allows restoration of $\alpha_5\beta_1$ -integrin to the plasma membrane when the endocytic stimulus is removed. Second, there is a later syndecan-4-dependent peak in Arf6 activity. To investigate how the later Arf6 peak relates to integrin trafficking, association of β_1 -integrin with the plasma membrane was measured by membrane fractionation. Fibroblasts spread on 50K and then stimulated with H/0 were fragmented by low-power sonication and separated into large plasma membrane and small vesicle fractions by centrifugation. As reported previously in reference 11, β_1 -integrin was rapidly lost from the plasma membrane fraction (Fig. 1E). However, plasma membrane-associated $\alpha_5\beta_1$ -integrin recovered at 90 min, despite the continued presence of H/0 in the media. Importantly, the time of integrin recovery coincided with the peak in Arf6 activity, indicating that syndecan-4 actively regulates both the endocytic and recycling phases of $\alpha_5\beta_1$ -integrin trafficking. Collectively, these experiments demonstrate that Arf6 is not involved in the syndecan-4-dependent endocytosis of

integrin, but is responsible for recycling the integrin back to the membrane.

Arf6 predominantly regulates recycling pathways,¹⁶ including recycling of β_1 -integrin,¹⁹ the interleukin-2 receptor and GPI-anchored proteins,¹⁷ but has also been linked to endocytic processes. Arf6 initiates clathrin-dependent endocytosis of transferrin receptor by activating phosphatidylinositol-4-phosphate-5-kinase, which changes the lipid composition of the membrane, and recruiting the clathrin adaptor complex, AP2.^{16,20} Arf6 also associates with caveolin-enriched membrane fractions.²¹ Our data indicate that Arf6 does not mediate syndecan-4-triggered endocytosis of integrin as the fall in unbinding energy is unaffected by reduction of Arf6 expression (Fig. 1B) and syndecan-4-dependent activation of Arf6 occurs after endocytosis (Fig. 1D and E). Combined with our previous observations,¹¹ this suggests that RhoG rather than Arf6 is the relevant switch during syndecan-4-dependent integrin endocytosis. However, syndecan-4 has also been found to trigger clathrin-dependent endocytosis as part of a Wnt signaling pathway,²² and it is possible that syndecan-4-mediated Arf6 regulation plays a role in the clathrin trafficking events. As integrins are themselves subject to clathrin-dependent endocytosis during focal adhesion disassembly,²³ the link between syndecan-4 and Arf6 might be relevant. If syndecan-4 does influence caveolin-dependent and clathrin-dependent endocytosis as well as Arf6-dependent recycling pathways, the composition of protein complexes associated with target receptors will be tremendously important to determining the fate of different matrix receptors.

The relationship between endocytic and recycling signals is logical and we have demonstrated the role of RhoG in the process.¹¹ Due to their role in cytoskeletal regulation, the three prototypic Rho-family members, RhoA, Rac1 and Cdc42 have also been implicated in regulation of membrane trafficking.²⁴ Cdc42 is not regulated by syndecan-4,^{6,25} however several lines of evidence point to a relationship between syndecan-4 and RhoA. Syndecan-4 binds and activates PKC α ,^{26,27} which in turn phosphorylates RhoGDI causing RhoA

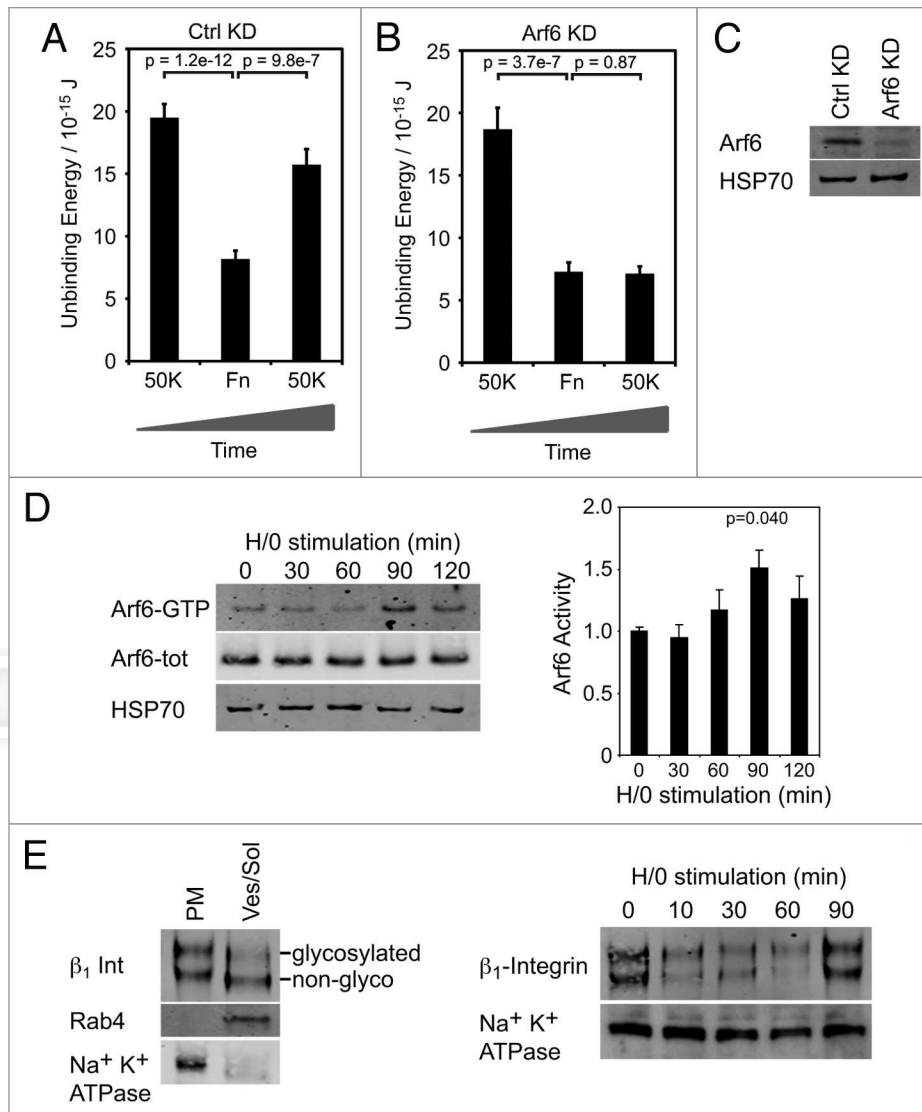


Figure 1. Syndecan-4 regulates both endocytic and recycling phases of integrin trafficking. Contacts were made sequentially with 50K, then Fn, then 50K again to follow $\alpha_5\beta_1$ -integrin endocytosis upon syndecan-4 engagement and recovery upon removal of syndecan-4 ligand. (A) Control fibroblasts exhibit reduction in unbinding energy upon syndecan-4 engagement, and recover high unbinding energy upon return to 50K. (B) Reduction of Arf6 expression by RNAi has no effect on integrin endocytosis, but blocks recovery upon return to 50K. (C) Arf6 expression of fibroblasts transfected with control or Arf6-targeted siRNA. (D) Engagement of syndecan-4 triggers activation of Arf6 in fibroblasts prespread on 50K. (E) Redistribution of β_1 -integrin between plasma membrane and vesicle fractions when fibroblasts, prespread on 50K, were stimulated with H/O over a time course. Segregation of membrane was confirmed by blotting fractions for Na⁺K⁺ATPase and Rab4. Energy values represent at least 80 measurements per condition from four experiments, western blot data represent eight experiments. Error bars represent SEM, significance was tested by ANOVA.

to be released and subject to further regulation by GEFs or GAPs.²⁷ At the same time, engagement of $\alpha_5\beta_1$ -integrin causes tyrosine phosphorylation of p190^{RhoGAP}, while syndecan-4 engagement causes serine/threonine phosphorylation via PKC α .⁹ The two phosphorylation events cause p190^{RhoGAP} to redistribute between membrane-bound and cytosolic fractions, leading to early inhibition of RhoA,⁹ followed by the reactivation of RhoA.¹⁰ The positive and negative regulation of RhoA

allows repeated cycles of protrusion and contraction that are necessary for migration. The chief effector of RhoA is Rho kinase, which phosphorylates myosin light chain phosphatase and LIM kinase, resulting in stabilization and contraction of actomyosin filaments.²⁸ We tested the necessity of Rho kinase activity for integrin trafficking by making atomic force measurements of fibroblast detachment in the presence of the Rho kinase inhibitor, Y27632. Addition of Y27632

to the media reduced the energy required to detach the fibroblast from 50K (Fig. 2A), indicating that cytoskeletal integrity is a factor in matrix engagement. However, comparison between contacts with 50K and fibronectin demonstrated that engagement of syndecan-4 caused a reduction in unbinding energy, regardless of the presence of Y27632. Engagement of syndecan-4 with soluble H/O reduced energy required for detachment from 50K to a level that was comparable with

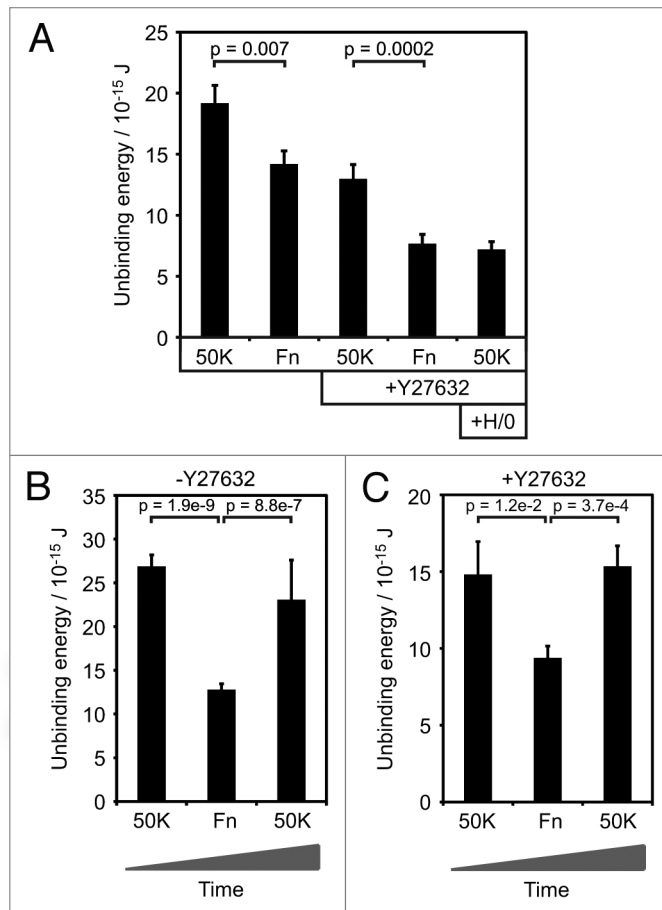


Figure 2. Syndecan-4-regulated integrin trafficking is independent of Rho kinase activity. Energy required to detach fibroblasts from 50K or Fn was measured by atomic force microscopy. (A) Energy measurements of untreated and Y27632-inhibited fibroblasts reveal that Rho kinase activity affects cell avidity itself, but is not required for syndecan-4-induced integrin endocytosis. (B and C) Contacts were made sequentially with 50K, then Fn, then 50K again to follow $\alpha_5\beta_1$ -integrin endocytosis upon syndecan-4-engagement and recovery upon removal of syndecan-4 ligand. (B) Untreated fibroblasts exhibit reduction in unbinding energy upon syndecan-4 engagement, and recover high unbinding energy upon return to 50K. (C) Treatment with Y27632 blocks neither the endocytic nor recovery phases caused by contact with fibronectin. Values represent at least 80 measurements per condition from 4 experiments. Error bars represent SEM, significance was tested by ANOVA.

detachment from fibronectin, confirming that energy differences were dependent on syndecan-4 ligand rather than density of integrin ligand. To distinguish effects on endocytosis and recycling we analyzed sequential contacts. With each cell we made a series of contacts with 50K, then fibronectin, then 50K again, both before and after addition of Y27632. As seen in **Figure 1**, untreated cells recovered high unbinding energy when detached from 50K after contact with fibronectin (**Fig. 2B**). Importantly, cells also recovered from contact with fibronectin in the presence of Y27632, demonstrating that neither syndecan-4-dependent endocytosis

or recycling of $\alpha_5\beta_1$ -integrin rely on Rho kinase activity (**Fig. 2C**).

The next step was to examine the potential involvement of Rac1. Our previous work has demonstrated that syndecan-4 is essential for the activation and spatial localization of Rac1 to the leading edge.⁶ Syndecan-4-null fibroblasts migrate randomly due to high, delocalized Rac1 activity, a behavior also observed *in vivo*. Depletion of syndecan-4-expression by morpholinos compromises the directional movement of migrating neural crest cells during embryonic development of zebrafish,²⁹ while fibroblast-specific deletion of the Rac1 gene in mice reduces fibroblast

migration, myofibroblast formation and therefore causes a wound closure delay.³⁰ Curiously, fibroblasts deficient in the endocytic mediators, RhoG and caveolin exhibit random migration that resembles the effect of syndecan-4 deletion, and RhoG- and caveolin-deficient mice suffer healing defects that closely resemble those observed in syndecan-4-deficient mice or the mice with a fibroblast-specific Rac1 deletion.^{8,11,31} The similarities in phenotype might suggest that syndecan-4, Rac1, RhoG and caveolin-dependent endocytosis lie on a single pathway. Rac1 activation and trafficking pathways were distinguished by stimulating fibroblasts with the different fragments of fibronectin. Engagement of syndecan-4 with H/0 triggered Rac1 activation in cells already spread on 50K (**Fig. 3A and C**). However, H/0-stimulation of fibroblasts in suspension was not sufficient for Rac1 activation (**Fig. 3B and C**), indicating that engagement of both syndecan-4 and $\alpha_5\beta_1$ -integrin is necessary for Rac1 regulation. By contrast, H/0-stimulation of fibroblasts in suspension did cause endocytosis and subsequent recycling of β_1 -integrin (**Fig. 3D**), demonstrating that the syndecan-4 trigger is sufficient. The redistribution of integrin under conditions that are insufficient for Rac1 regulation demonstrates that Rac1 does not mediate the syndecan-4 trafficking signal. It also demonstrates that integrin trafficking alone is not the sole cause of Rac1 activation, although we cannot rule out the possibility that it is a contributory factor. Certainly, integrin complexes play a role in localizing active Rac1 to the membrane,³² but whether activation or localization is the first event, and whether integrin trafficking is linked to either remains to be resolved.

In summary, we have demonstrated that the small GTPase signaling events, particularly the activation of Rac1, RhoA and RhoG leading to integrin endocytosis are independent of one-another. Furthermore we have identified a fourth component, Arf6, that is regulated by syndecan-4 and closes the loop in syndecan-4-dependent integrin trafficking. Finally, to better understand how the different signals are coordinated, we conducted time-course analysis of activation of the

different components in the same cell type, in response to H/O. Signals could be divided broadly into early or late signals (Fig. 3E). The early signals included activation of RhoG, which leads to endocytosis of $\alpha_5\beta_1$ -integrin, activation of Rac1, which promotes membrane protrusion, and suppression of the RhoA contractile signal (Fig. 4). All of these signals would promote the initiation of cell migration as syndecan-4 senses fibronectin that appears in the wound bed. The late signals included activation of Arf6, which returns $\alpha_5\beta_1$ -integrin to the membrane, reactivation of RhoA to promote cytoskeletal contraction and the maturation of adhesions, and inactivation of the protrusive signals. Therefore, although the different GTPases can be viewed as independent of one-another, they pull together in a cohesive fashion to promote cell migration.

Methods and Materials

Antibodies and ECM proteins. Mouse monoclonal antibodies raised against Arf6 (A5230) and tubulin (T6199) (Sigma), Rac1 (610651) and Rab4 (610889) (Transduction Labs), RhoG (Santa Cruz, sc-80015), HSP70 (Affinity BioReagents, MA3-028), α_1 Na⁺ K⁺ ATPase transporter (Millipore, 05-369) and polyclonal antibodies against α_5 integrin (Santa Cruz, sc-10729). Monoclonal antibody for blotting β_1 integrin (JB1A) was a gift from J.A. Wilkins (University of Manitoba, Canada). Alexa Fluor680-conjugated IgGs were from Molecular Probes (Invitrogen), IR800-conjugated IgGs from Rockland. Recombinant fibronectin polypeptides encompassing type III repeats 6–10 (50K) and 12–15 (H/O) were expressed as recombinant polypeptides as described previously in reference 6. Bovine plasma fibronectin (F1141) was purchased from Sigma.

Cell culture. Mouse embryonic fibroblasts were cultured at 33°C in DME, 10% fetal bovine serum, 4.5 g/l glucose, 2 mM L-glutamine, 20 U/ml IFN γ (Sigma, I4777). For all experiments, to prevent de novo synthesis of fibronectin and other syndecan-4 ligands, cells were treated with 25 μ g/ml cycloheximide (Sigma, C7698) for 2 h prior to detachment and throughout the experiment.

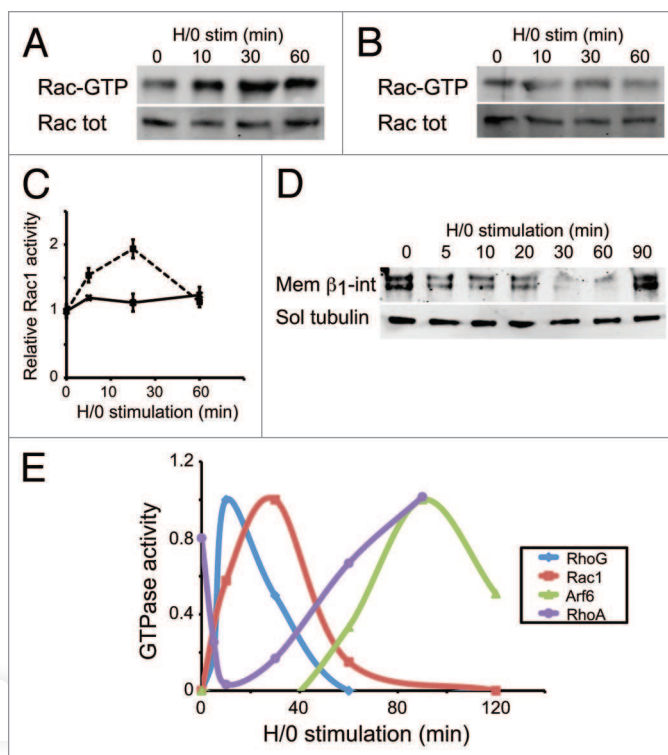


Figure 3. Relationships between integrin and GTPase signaling. (A–C) Engagement of syndecan-4 triggers Rac1 activation in cells spread on 50K (A and C, dotted line) but not cells in suspension (B and C, solid line), demonstrating that Rac1 activation is integrin dependent. (D) Engagement of syndecan-4 still triggers loss of $\alpha_5\beta_1$ -integrin from the plasma membrane fraction when fibroblasts are in suspension. (E) Kinetics of regulation of the multiple GTPases regulated by syndecan-4. Data represent eight experiments. Error bars represent SEM.

Matrix-coated surfaces. For atomic force measurement, poly-d-lysine-coated fluorodishes (World Precision Instruments, FD35PDL-100) were coated with patches of 10 μ g/ml fibronectin (Sigma) in Dulbecco's PBS containing calcium and magnesium (Sigma, D8662) for 1 h at room temperature, and then patches of 100 μ g/ml 50K at 4°C overnight. Equivalent coating of fibronectin and 50K onto fluorodishes was tested by ELISA using the anti-fibronectin mAb 333.¹¹ For biochemical assays, 10-cm tissue culture-treated plastic dishes (Corning, 430167) were coated with 10 μ g/ml 50K at 4°C overnight.

RNAi knockdown. siRNA duplex targeted against Arf6 with ON TARGET™ modification (CUG ACA UUU GAC ACG AAU A, J-004008-05-0050) for enhanced specificity, and an siGLO®, non-targeting control duplex (D-001600-01-05) were purchased from Dharmacon. For knockdown, 160 pmol of oligo was transfected into a 90% confluent

25-cm² flask using Dharmafect2 reagent (Dharmacon, T-2002-01). After 18 h, the cells were passaged and cultured for 2 d, before transfecting again to ensure substantial knockdown. Cells were passaged 18 h after the second round of transfection, and used within 2–3 d. Expression of target proteins, in comparison with mock-transfected cells was tested by western blotting.

Atomic force microscopy. Force measurements were made using a CellHesion200 atomic force head (JPK Instruments) mounted on an Olympus chassis. Cells were mounted on tipless silicon SPM-sensor cantilevers (Nanoworld, ARROW-TL1-50) coated with 50K. The spring constant for each cantilever was calculated in situ by thermal fluctuation analysis. Cells suspended in HAM's F-12 nutrient media, 25 mM HEPES (Sigma, 56659C), 25 μ g/ml cycloheximide were captured before they could adhere firmly to the dish. Cantilever-mounted cells were contacted repeatedly with the

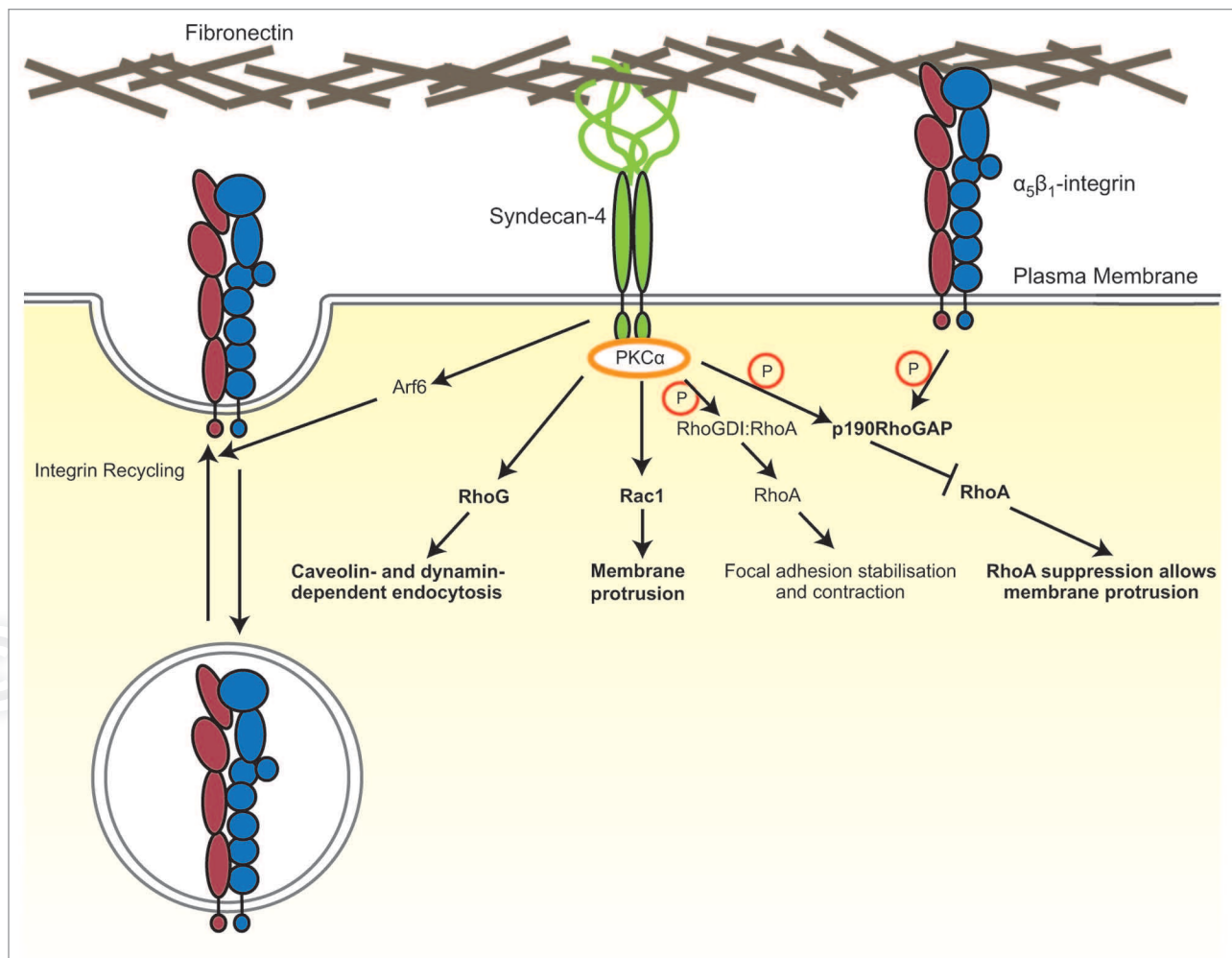


Figure 4. Parallel regulation of multiple GTPases by syndecan-4. Engagement of syndecan-4 triggers early signals (bold): RhoG, Rac1 and RhoA inhibition, followed by late signals: Arf6 and RhoA activation.

ligand-coated fluorodish with an applied force of 1 nN and a 5 sec contact time. Retraction measurements were made over a 90- μ m pulling distance with an extension/retraction speed of 5 μ m/s. 45 sec pauses between measurements allowed the cell to recover. For stimulation experiments, baseline measurements of unbinding from 50K and fibronectin were made before injecting 10 μ g/ml H/0 or 10 μ M Y27632 (Calbiochem, 688000) into the dish. Force curves were analyzed using JPK data analysis software with baseline and tilt correction.

Membrane fractionation. For each time point, 10⁶ fibroblasts were spread on 50K-coated dishes in DME, 25 mM HEPES, 25 μ g/ml cycloheximide at 37°C for 2 h, and then stimulated with 10 μ g/ml H/0 for 0–90 min. Cells were harvested at 4°C by scraping in Dulbecco's

PBS containing calcium and magnesium, cOmplete protease inhibitor (Roche), 5 mM Na₂VO₄, 10 mM NaF. Membranes were fragmented by three 4-Joule pulses using a Vibra-Cell sonicator (Sonics), before removing nuclear debris with a 10 min, 1,000x g centrifugation step. Membranes were separated into plasma membrane pellet and vesicle/soluble supernatant by 10 min centrifugation at 10,000 x g. Proteins were resolved by SDS-PAGE and analyzed using the Odyssey western blotting fluorescent detection system (LI-COR). This involved the use of fluorophore-conjugated secondary antibodies that were detected using an infrared imaging system that allowed both an image of the membrane and an accurate count of bound protein to be recorded.

GTPase activation assays. Active GTPases were affinity precipitated using

the following bait constructs: Arf6: GST-GGA3 (VHS-GAT domain), Rac1: GST-PAK (CRIB domain), RhoG: GST-ELMO2 (amino acids 1–362). For each time point, fibroblasts were spread and stimulated with H/0 as described above before lysis in the appropriate buffer: Arf6: 50 mM Tris (pH 7.5), 10% (v/v) glycerol, 150 mM NaCl, 10 mM MgCl₂, 1% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate, 0.1% sodium dodecyl sulfate, cOmplete protease inhibitor (Roche, 1-697-498). Rac1: 20 mM HEPES (pH 7.4), 10% (v/v) glycerol, 140 mM NaCl, 1% (v/v) NP40, 0.5% (w/v) sodium deoxycholate, 4 mM EGTA, 4 mM EDTA, cOmplete protease inhibitor. RhoG: 50 mM Tris (pH 7.5), 10% (v/v) glycerol, 100 mM NaCl, 2 mM MgCl₂, 1% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate, 0.1% sodium dodecyl sulfate, complete

protease inhibitor. GST-protein-loaded beads were incubated with the lysates for 1 h and then washed 3 times with lysis buffer. Active, precipitated GTPase was analyzed by fluorescent western blotting. Rho ELISA (Tebu-Bio, BK124) was used according to manufacturer's instructions.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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