Syndecan-1 controls cell migration by activating Rap1 to regulate focal adhesion disassembly

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SUMMARY
After injury, residual epithelial cells coordinate contextual clues from cell-cell and cell-matrix interactions to polarize and migrate over the wound bed. Protrusion formation, cell body translocation and rear retraction is a repetitive process that allows the cell to move across the substratum. Fundamental to this process is the assembly and disassembly of focal adhesions that facilitate cell adhesion and protrusion formation. Here, we identified syndecan-1 as a regulator of focal adhesion disassembly in migrating lung epithelial cells. Syndecan-1 altered the dynamic exchange of adhesion complex proteins, which in turn regulates migration speed. Moreover, we provide evidence that syndecan-1 controls this entire process through Rap1. Thus, syndecan-1 restrains migration in lung epithelium by activating Rap1 to slow focal adhesion disassembly.
INTRODUCTION

Re-epithelialization is arguably the hallmark event characterizing successful repair following disruptive injury. Pathological conditions (e.g., tumor invasion and metastasis, chronic inflammation, fibrosis, etc.) arise when the epithelium either cannot repair or loses the contextual controls that shut down the migration process (Ridley et al., 2003; Crosby and Waters, 2010; Nathan and Ding, 2010). Cell migration involves multiple signaling and cytoskeletal changes and is modified by various, specific cell-matrix and cell-cell interactions (Montell, 2008). For migration to occur, cells must balance leading edge protrusion and attachment with trailing edge release and retraction (Huttenlocher and Horwitz, 2011). Cell migration would be limited if not for the traction provided by the adhesion to the matrix. Rear retraction is equally important and can limit the migration speed by restraining forward progress.

Once stimulated to migrate, cellular protrusions engage the substratum primarily through integrin receptors, which cluster and recruit adaptor proteins to form nascent adhesions (Kaverina et al., 2002; Wehrle-Haller, 2012). Nascent adhesions contain many components of classical focal adhesions and are generally short-lived and turnover while within the lamellipodia or filopodia (Parsons et al., 2010; Webb et al., 2002). However, some nascent adhesions do not turnover, but instead, mature into focal adhesions (referred to as FA from here on) (Broussard et al., 2008). Whereas nascent adhesions tightly bind the substratum to generate traction forces for forward migration, FAs are positioned at the termini of stress fibers and act as anchors for cell contraction (Beningo et al., 2001; Galbraith and Sheetz, 1997; Galbraith et al., 2002; Broussard et al., 2008).

Disassembly of FAs in the leading edge of migrating cells facilitate formation of new protrusions and adhesions (Webb et al., 2002; Huttenlocher and Horwitz, 2011). Leading edge FAs play an important role in regulating cell migration speed so controlling assembly and disassembly must be tightly regulated (Webb et al., 2004; Gupton and Waterman-Storer, 2006; Millon-Frémillon et al., 2008; Lawson et al., 2012; Webb et al., 2002). Assembly requires actin polymerization and is largely regulated by the Rho family GTPases (Alexandrova et al., 2008; Choi et al., 2008; Vicente-Manzanares et al., 2009; Raftopoulou and Hall, 2004). Disassembly of leading edge FAs is an equally complicated process that mechanistically converges through focal adhesion kinase (FAK) and Src signaling (Huttenlocher and Horwitz, 2011).
We recently identified syndecan-1 as a regulator of lung epithelial migration in vitro and re-epithelialization in vivo (Chen et al., 2009). Syndecan-1, a transmembrane heparan sulfate proteoglycan, mediates many effects on cellular function by coupling with integrins and regulating their allosteric state (Couchman, 2003; Morgan et al., 2007; Chen et al., 2009; Beauvais et al., 2009; Beauvais and Rapraeger, 2003). Given the importance of integrins in regulating FA turnover, we investigated if syndecan-1 controls cell migration through FA maturation and disassembly. We determined that syndecan-1 regulates FA disassembly in lung epithelial cells to control migration speed. Additionally, syndecan-1 mediated its effects via the small GTPase, Rap1, independently of integrin activation.
RESULTS

Syndecan-1 Attenuates FA Disassembly

We reported that cell migration is slower in cells expressing syndecan-1 compared to cells with shRNA-mediated downregulated expression (Chen et al., 2009). Our previous studies only found this effect on collagen matrices so all the following studies are performed on a Type I collagen matrix.

Because FA turnover is a major determinant of cell migration speed (Webb et al., 2002), we compared the presence and pattern of paxillin-positive FAs between migrating lung epithelial cells with syndecan-1 (B2b<sup>shRNA.scr</sup>) and cells lacking syndecan-1 expression (B2b<sup>shRNA.hSdc1</sup>) (Fig. 1). Nascent adhesions lined the cell front in all conditions. Additionally, B2b<sup>shRNA.scr</sup> cells at the wound front had notable leading edge FAs. In contrast, B2b<sup>shRNA.hSdc1</sup> cells had fewer FAs that were much less prominent when compared to B2b<sup>shRNA.scr</sup> cells.

FA turnover is a dynamic process that is not well represented by static images. Therefore, we stably expressed paxillin-eGFP in B2b<sup>shRNA.scr</sup> and B2b<sup>shRNA.hSdc1</sup> cells to follow the assembly and disassembly of FAs (Laukaitis et al., 2001). Using time-lapse total internal reflection fluorescent (TIRF) microscopy, FAs assembly and disassembly were observed in migrating cells with a high spatiotemporal resolution (Video 1). B2b<sup>shRNA.hSdc1</sup> cells migrated faster than B2b<sup>shRNA.scr</sup> cells, which is consistent with our previous observations (Chen et al., 2009). Similar to our findings in Fig. 1, both cell lines formed nascent adhesions at the front edge of the cell. However, FAs appeared to be more persistent in B2b<sup>shRNA.scr</sup> compared to B2b<sup>shRNA.hSdc1</sup> cells.

The difference in the leading edge FA lifespan in migrating cells was better illustrated by examining kymographs of individual FAs (Fig. 2A). To quantify the FA dynamics, the intensity of FAs of migrating cells was measured over time to follow adhesion complex assembly and disassembly (Fig. 2B). The FA lifespan was longer in B2b<sup>shRNA.scr</sup> compared to B2b<sup>shRNA.hSdc1</sup> cells (49.5 ± 1.6 vs. 36.3 ± 1.1 min, respectively; p < 0.0001) (Fig. 2C). Additionally, the disassembly rate was slower in migrating B2b<sup>shRNA.scr</sup> cells compared to B2b<sup>shRNA.hSdc1</sup> cells (-3.68 ± 0.23 vs. -5.43 ± 0.30 %max intensity/min, respectively; p < 0.0001) (Fig. 2D). The FA assembly rate and FA size did not differ between cell lines (Fig. S1 and S2). Thus, the shorter FA lifespan was largely driven by a faster disassembly of FAs in conditions lacking syndecan-1.

To further study FA dynamics, we evaluated FA disassembly with a nocodazole washout assay (Millon-Frémillon et al., 2008; Kaverina et al., 1999). Microtubule polymerization after
nocodazole washout induced a rapid disassembly of FAs in lung epithelial cells. Congruous with
the data from migrating cells, syndecan-1 also attenuated FA disassembly after nocodazole
washout (Fig. 2E – H). The FA disassembly rate was more rapid in B2b
\(^{\text{shRNA.hSdc1}}\) cells compared
to B2b
\(^{\text{shRNA.scr}}\) cells (-15.90 ± 1.25 vs. -10.69 ± 1.02 %max intensity/min, respectively; \(p < 0.05\))
(Fig. 2H).

Syndecan-1 facilitates paxillin exchange within the FA

Differences in the exchange of adhesion complex proteins (e.g., paxillin, FAK, zyxin)
between cytoplasmic pools and the stable FA controls cell migration by altering FA stability
(Wichert et al., 2003; Webb et al., 2004). FAs within migrating cells at the wound front were
selectively photobleached, and the recovery of fluorescence was evaluated to quantify this
process (Fig. 3A – B). The amount of the immobilized fraction was similar between the cell lines
(unpublished data). However, the dynamics of paxillin cycling within the FA was different with
B2b
\(^{\text{shRNA.scr}}\) cells having a shorter half-time to recovery of fluorescent signal in comparison to
B2b
\(^{\text{shRNA.hSdc1}}\) cells (19.68 ± 3.03 vs. 29.41 ± 2.63 sec; \(p < 0.05\)) (Fig. 3C). These findings
indicate that syndecan-1 alters the dynamic exchange of adhesion complex proteins in the FA
thus regulating FA disassembly, which ultimately governs cell migration.

Syndecan-1 augments Rap1 activation to restrain cell migration

Small GTPases are vital for cell migration to occur (Raftopoulou and Hall, 2004; Ridley et
al., 2003). Rap1 is a small GTPase that has pleotropic effects on cell migration and has been
specifically implicated in modulating integrin affinity states (Boettner and Van Aelst, 2009).
Because our previous work showed syndecan-1 regulates \(\alpha_2\beta_1\) integrin activation in lung
epithelial cells (Chen et al., 2009), we evaluated if syndecan-1 regulates Rap1 activation.

Rap1 was more activated in cells with syndecan-1 (B2b
\(^{\text{shRNA.scr}}\) cells and wild-type cultures)
compared to cells that lack syndecan-1 (B2b
\(^{\text{shRNA.hSdc1}}\) cells and Sdc1
\(^{-/-}\) cultures) (Fig. 4A and B).
To determine if syndecan-1 augmentation of Rap1 activity affected \(\alpha_2\beta_1\) levels or activity, we
overexpressed a dominant-active Rap1 (Rap1
\(^{G12V}\)) in lung epithelial cells. Surprisingly,
overexpression of Rap1
\(^{G12V}\) had no effect on \(\alpha_2\beta_1\) integrin activation (Fig. S3) indicating that
syndecan-1 does not modulate integrin affinity via Rap1 activation.
Rap1 can affect cell migration independent of effects on the integrin affinity state (Takahashi et al., 2008; Jossin and Cooper, 2011). Therefore, we evaluated if syndecan-1 regulation of Rap1 modulates cell migration. Control conditions (eGFP expression in B2b\textsuperscript{shRNA.scr} and B2b\textsuperscript{shRNA.hSdc1} cells) reproduced the phenotype where B2b\textsuperscript{shRNA.hSdc1} cells migrated faster than B2b\textsuperscript{shRNA.scr} cells (Video 2 and Fig. 4C and E). Co-expression of Rap1\textsuperscript{G12V} and eGFP in B2b\textsuperscript{shRNA.hSdc1} cells slowed the migration of eGFP positive cells only and had no effect on B2b\textsuperscript{shRNA.scr} cells (Video 2 and Fig. 4D and F).

We quantified the migration speed in all conditions and found in comparison to B2b\textsuperscript{shRNA.scr} and B2b\textsuperscript{shRNA.hSdc1} cells expressing eGFP only, Rap1\textsuperscript{G12V} expression in B2b\textsuperscript{shRNA.hSdc1} cells slowed migration speed (4.45 ± 0.30 vs. 7.26 ± 0.36 vs. 3.80 ± 0.27 μm/hr, respectively; \( p < 0.001 \)) (Fig. 4G). Rap1\textsuperscript{G12V} transduction into B2b\textsuperscript{shRNA.scr} cells did not change migration speed (4.62 ± 0.33 μm/hr) from control B2b\textsuperscript{shRNA.scr} cells (4.45 ± 0.30 μm/hr) suggesting maximal activation of Rap1 in B2b\textsuperscript{shRNA.scr} cells (Fig. 4G). Importantly, expression of Rap1\textsuperscript{G12V} in B2b\textsuperscript{shRNA.scr} cells did not slow migration whereas Rap1\textsuperscript{G12V} expression in B2b\textsuperscript{shRNA.hSdc1} cells did reduce migration speed to the rate of B2b\textsuperscript{shRNA.scr} control conditions, which in essence rescued the migration phenotype and indicated the effect of Rap1 is directly associated with syndecan-1.

**Rap1 slows FA disassembly in syndecan-1 deficient lung epithelial cells**

Our data indicate that syndecan-1 regulates cell migration by slowing FA disassembly (Fig. 2). Using B2b\textsuperscript{shRNA.scr} and B2b\textsuperscript{shRNA.hSdc1} cells that stably express paxillin-eGFP, we transduced Rap1\textsuperscript{G12V} and identified cells by co-expression of mCherry to determine if syndecan-1 regulates FA disassembly via Rap1. FA disassembly occurred faster in control (mCherry expression only) migrating B2b\textsuperscript{shRNA.hSdc1} cells compared to B2b\textsuperscript{shRNA.scr} cells (Fig. 5). However, when B2b\textsuperscript{shRNA.hSdc1} cells expressed Rap1\textsuperscript{G12V}, FA dynamics changed to resemble that of B2b\textsuperscript{shRNA.scr} cells (Fig. 5A – B). Indeed, when compared to B2b\textsuperscript{shRNA.scr} and B2b\textsuperscript{shRNA.hSdc1} cells, FA lifespan was prolonged in B2b\textsuperscript{shRNA.hSdc1} cells expressing Rap1\textsuperscript{G12V} (46.46 ± 2.18 vs. 31.91 ± 2.05 vs. 48.67 ± 5.13 min, respectively; \( p < 0.05 \)) (Fig. 5C), and the FA disassembly rate was slower (-3.90 ± 0.36 vs. -6.40 ± 0.77 vs. -3.71 ± 0.53 %max intensity/min, respectively; \( p < 0.001 \)) (Fig. 5D). Accordingly, B2b\textsuperscript{shRNA.scr} cells and B2b\textsuperscript{shRNA.hSdc1} cells expressing Rap1\textsuperscript{G12V} had FAs identified near the interior of the cell in contrast to B2b\textsuperscript{shRNA.hSdc1} cells, which is consistent with the data presented in Fig. 1 and congruous with the longer FA lifespan.
We also evaluated FA disassembly with the nocodazole washout assay (Fig. 5F – H). After washout, FA disassembly was slower in B2b\textsuperscript{shRNA,scr} cells (-7.67 ± .50 %max intensity/min) compared to B2b\textsuperscript{shRNA,hSdc1} cells (-12.70 ± .75 %max intensity/min; \(p < 0.001\)) (Fig. 5I – J) and was consistent with the data presented in Fig. 2H. In comparison to B2b\textsuperscript{shRNA,hSdc1} cells, Rap1\textsuperscript{G12V} expression in B2b\textsuperscript{shRNA,hSdc1} cells again significantly slowed the FA disassembly rate after nocodazole washout (-8.85 ± .69 %max intensity/min; \(p < 0.001\)).

**Rap1 accelerates the exchange of adhesion complex proteins in syndecan-1 deficient lung epithelial cells**

Migrating B2b\textsuperscript{shRNA,scr} and B2b\textsuperscript{shRNA,hSdc1} cells stably expressing paxillin-eGFP had FAs photobleached to determine if the dynamic cycling of adhesion complex proteins is regulated by Rap1 activation. B2b\textsuperscript{shRNA,hSdc1} cells expressing Rap1\textsuperscript{G12V} changed the dynamic exchange of paxillin within the stable FA to behave similarly to B2b\textsuperscript{shRNA,scr} cells (Fig. 6A – B). Compared to the half-time of B2b\textsuperscript{shRNA,hSdc1} cells (35.40 ± 1.45 sec), Rap1\textsuperscript{G12V} expressed in B2b\textsuperscript{shRNA,hSdc1} cells significantly shortened the half-time of fluorescent recovery (26.77 ± 2.37 sec; \(p < 0.01\)) (Fig 6C).
DISCUSSION

Syndecan-1 modulates re-epithelialization of the wounded lung epithelium (Chen et al., 2009). Here, we show that syndecan-1 controls the turnover of the leading edge FAs in lung epithelial cells to regulate cell migration. Our data demonstrate that syndecan-1 governs FA dynamics by regulating the exchange of adhesion complex proteins, which in turn slows FA disassembly and restrains migration. Moreover, we show that syndecan-1 controls this process by activating Rap1.

FA turnover in migrating cells is integral for generating traction forces as well as maintaining cell-matrix contacts (Broussard et al., 2008). Our data reveal that the syndecan-1 prolongs the leading edge FA lifespan and is consistent with other reports that demonstrate decreased FA turnover slows cell migration (Webb et al., 2004; Gupton and Waterman-Storer, 2006; Millon-Frémillon et al., 2008; Lawson et al., 2012). Maximal cell migration involves a complicated interplay between the cell and extracellular matrix. Indeed, intermediate levels of cell adhesion are ideal for maximal migration velocity (Palecek et al., 1997; DiMilla et al., 1993; Gupton and Waterman-Storer, 2006). However, the optimal substrate concentration can be shifted by altering the FA turnover dynamics (Gupton and Waterman-Storer, 2006; Millon-Frémillon et al., 2008).

Syndecan-1 had no effect on the assembly rate and primarily regulates the turnover of leading edge FAs in migrating cells by controlling FA disassembly. Furthermore, our data indicate that syndecan-1 restricts FA disassembly by increasing the trafficking of adhesion complex protein within the stable FA. This finding is consistent with others that have showed faster exchange of FA proteins stabilizes the FA and slows cell migration (Webb et al., 2004; Wichert et al., 2003; Hamadi et al., 2005; Deramaudt et al., 2011). Phosphorylation of adapter proteins such as paxillin and FAK alter the kinetics of cycling and work in concert to stabilize the FA (Webb et al., 2004; Zaidel-Bar et al., 2007). FAs can also be destabilized by external factors such as targeting by microtubules to induce disassembly (Kaverina et al., 1998; Kaverina et al., 1999; Ezratty et al., 2005; Bhatt et al., 2002). Syndecan-1 could be modulating one or more of these factors in stabilizing the FA in migrating cells.

Our findings indicate that syndecan-1 mediates its effects on FA disassembly through Rap1. Rap1 is a Ras-family GTPase that associates with various effectors to change the way the cell interacts with the environment (Boettner and Van Aelst, 2009; Kooistra et al., 2007). By associating with either RapL or RIAM, Rap1 switches integrins to a high affinity state to govern
cell adhesion (Han et al., 2006; Carmona et al., 2009; Katagiri et al., 2003). Rap1 did not affect α2β1 integrin activation in our studies. However, syndecan-1 can also regulate the allosteric state of the αvβ3 and αvβ5 integrins (Beauvais et al., 2009; Beauvais et al., 2004). Our previous work demonstrated that syndecan-1 primarily regulates cell migration via effects on the collagen binding integrin, α2β1, and was not dependent on the αvβ3 and αvβ5 integrins (Chen et al., 2009). Plus, our experiments were all conducted on collagen matrices corroborating the idea that Rap1 is facilitating FA disassembly independent of direct effects on integrin activation. However, we cannot rule out Rap1 effects on other integrins in the lung epithelium independent of syndecan-1. Re-organization of adherens junctions, which can be mediated by Rap1, is a large determinant of collective cell migration (Montell, 2008; Knox and Brown, 2002; Price et al., 2004; Hogan et al., 2004). In fact, cadherin and integrin mediated signals are highly interconnected so changes to the cell-cell junctions indirectly alters cell-matrix dynamics (Weber et al., 2011). Syndecan-1 has been functionally linked to E-cadherin so it is plausible that syndecan-1 modifies cell-cell contacts through Rap1 to circuitously control FA turnover (Leppä et al., 1996; Kato et al., 1995).

Syndecan-1 forms multimeric complexes to transduce intracellular signaling and alter cellular function (Beauvais and Rapraeger, 2010; Beauvais and Rapraeger, 2003; Beauvais et al., 2009; McQuade et al., 2006; Hayashida et al., 2008). Therefore, syndecan-1 could be directly interacting with Rap1 to facilitate its activation. Indeed, Rab5, a small GTPases, has been shown to associated with syndecan-1 through the syndecan-1 cytoplasmic domain to control syndecan-1 shedding (Hayashida et al., 2008). Alternatively, syndecan-1 could be associating with guanine nucleotide exchange factors (GEFs) and/or GTPase-activating proteins (GAPs). Rap1 is controlled by cycling between a GTP-bound (on) state and a GDP-bound (off) state, which is governed by GEFs and GAPs (Gloerich and Bos, 2011). Several Rap-1 GEFs and GAPs have PDZ domains that could associate with the PDZ binding domain on syndecan-1 to spatiotemporally control Rap1 (Nourry et al., 2003; García-Mata and Burridge, 2007).

Syndecan-1 shedding from the cell surface is an important process that regulates multiple cellular functions including stimulating cell migration (Bass et al., 2009; Endo et al., 2003; Chen et al., 2009). Therefore, we propose that syndecan-1 shedding from injured lung epithelium induces a migratory phenotype that is mediated by effects on Rap1. Our data indicate that the loss of syndecan-1 on lung epithelial cells causes Rap1 to assume an inactive state. In turn, the
loss of Rap1 activity slows adhesion complex proteins exchange, which accelerates FA disassembly causing faster cell migration and facilitating wound closure.
MATERIAL AND METHODS

Cloning

AAV vectors were used for transient transduction of exogenous genes in cultured epithelial cells. The AAV expression vector (AAV-DJ Helper Free Expression System; Cell Biolabs; San Diego, CA) was modified to include an internal ribosomal entry site (IRES)-eGFP immediately 3’ to the multiple cloning site by subcloning from the pBM-IRES-eGFP plasmid (Addgene; Cambridge, MA; deposited by Garry Nolan) with BamHI (5’) and SalI (3’) to create an AAV-IRES-eGFP vector. To create an AAV-IRES-mCherry vector, we subcloned into the AAV-IRES-eGFP vector used a KpnI restriction site that is located within the IRES and the SalI site 3’ to eGFP. The cDNA sequence for a 5’ KpnI restriction site, intervening IRES sequence, mCherry cDNA (GenBank: AB512478.1), and a 3’ SalI restriction site was synthesized (Genscript; Piscataway, NJ) and subcloned into the AAV-IRES-eGFP.

The mRNA sequence for human Rap1 (NM_001010935) was modified with a single point mutation (G → T) at position 307 resulting in a glycine (G) to valine (V) mutation of the 12th amino acid to create a constitutively active Rap1 (Rap1G12V) (Han et al., 2006). The cDNA sequence for Rap1G12V flanked by BamHI (5’) and XhoI (3’) restriction sites was commercially synthesized (Genscript) and subcloned into the AAV-IRES-eGFP and AAV-IRES-mCherry plasmids. The plasmid sequence of the final expression vectors were confirmed by DNA sequencing.

AAV vectors were created by transfecting HEK cells with calcium phosphate precipitation using 10 μg each of the expression, capsule and helper plasmids. HEK cells were collected 2 days after transfection, and AAV vectors were released by three freeze-thaw cycles. The viral supernate was added to cells for 24 hours two days prior to the desired experiment.

Cell culture

Subclones of BEAS-2b cells, a non-malignant immortalized bronchial epithelial cell line, were created by stably expressing shRNA toward syndecan-1 (B2bshRNA.hSdc1) or a scramble control (B2bshRNA.scr) as previously described (Chen et al., 2009). Cultures were maintained in bronchial epithelial growth medium (BEGM) supplemented with growth factors and retinoic acid (Lonza; Walkersville, MD). Selection of B2bshRNA.scr and B2bshRNA.hSdc1 cells were ensured by supplementing the culture medium with puromycin (5 μg/ml; Thermo Fisher Scientific;
Waltham, MA) and periodic characterization of cell surface human syndecan-1 levels (clone BA38-RPE; Serotec; Raleigh, NC) with a Guava bench top flow cytometer (Millipore; Billerica, MA).

Paxillin tagged with a c-terminal eGFP in the pEGFP-N3 vector was purchased from Addgene (deposited by Rick Horwitz). B2b\textsuperscript{shRNA.scr} and B2b\textsuperscript{shRNA.hSdc1} cells were transfected with the paxillin-eGFP plasmid with Lipofectamine 2000 (Invitrogen; Carlsbad, CA), and stable clones were sorted by FACS for GFP positive cells (Aria II; BD Biosciences; San Jose, CA). Additionally, B2b\textsuperscript{shRNA.scr} and B2b\textsuperscript{shRNA.hSdc1} cells that stably express paxillin-eGFP were confirmed to maintain the respective high and low expression of human syndecan-1 by flow cytometry (clone BA38-Alexa 647; Serotec) (Fig. S4).

Primary cultures of airway epithelial cells grown at an air-liquid interface (ALI) were created from wild-type and Sdc1-null (Sdc1\textsuperscript{-/-}) C57BL/6 mice as previously described (Chen et al., 2009). Cultures were maintained in mouse tracheal epithelial cell (MTEC) growth medium supplemented with 2% Nuserum (BD Biosciences) (You et al., 2002).

Immunofluorescence

For immunofluorescence and live-cell assays, B2b\textsuperscript{shRNA.scr} and B2b\textsuperscript{shRNA.hSdc1} cells were plated into chambered #1.5 glass coverglass (Nunc International; Rochester, NY) coated with rat tail Type I collagen (BD Biosciences) at 2 \(\mu\text{g/cm}^2\).

Cultures were fixed with 4% paraformaldehyde for 15 minutes at 37\(^{\circ}\text{C}\) before being processed for immunofluorescence. Focal adhesions were identified by immunostaining for paxillin (clone 5H11; Millipore) followed by an anti-mouse Alexa-488 secondary antibody (Invitrogen). F-actin was labeled with phalloidin-Alexa 568 (Invitrogen). Immunofluorescent pictures were obtained with a DeltaVision Olympus IX71 inverted microscope using a 1.35 40x/1.35 NA U Plan Apo oil immersion objective.

Focal Adhesion Turnover Assays

Live cell time-lapse microscopy was performed using a Nikon TiE inverted widefield fluorescence microscope that has a chamber to maintain cells at 37\(^{\circ}\text{C}\) and 5% CO2. Images were obtained by total internal reflection fluorescent (TIRF) microscopy using a CFI 60x/1.49 NA Apo TIRF oil immersion objective.
Monolayers of B2b\textsuperscript{shRNA.scr} and B2b\textsuperscript{shRNA.hSdc1} cells that stably express paxillin-eGFP were scratched with a p-100 pipet tip to simulate a wound. Cells were allowed to initiate migration for at least 2 hours before starting time-lapse TIRF microscopy. Images were captured every 2 minutes for up to 6 hours. Each experiment was repeated at least 3 times. For each independent experiment, FAs at the leading edge of migrating cells were evaluated in at least 10 different randomly chosen cells. Within each cell, we analyzed between 1 – 3 FAs over time. Therefore, we evaluated approximately 100 focal adhesions per condition.

FA disassembly was induced with a nocodazole washout assay (Millon-Frémillon et al., 2008; Kaverina et al., 1999). Sub-confluent B2b\textsuperscript{shRNA.scr} and B2b\textsuperscript{shRNA.hSdc1} cells that stably express paxillin-eGFP were incubated with 10 μM of nocodazole in BEGM for 4 hours. Cells were then thoroughly washed with warm BEGM immediately before the start of time-lapse TIRF microscopy. Images were captured every 45 seconds for 30 minutes. Each experiment was repeated a minimum of 3 times. In each independent experiment, at least 5 different cells and 2 FAs per cell was evaluated. In all, we evaluated at least 30 FAs per condition.

FA assembly and disassembly rates were determined as previously described (Webb et al., 2004; Millon-Frémillon et al., 2008). Fluorescent intensity of FAs in the cells was measured in raw images with ImageJ (NIH). The background intensity was subtracted from the intensity values and was then normalized so that all values are a percent of the maximum intensity of the FA. The rate of the FA disassembly was determined by linear regression analysis of the linear portion of the descending fluorescent intensity. The slope of the declining FA fluorescent intensity represents the rate of FA disassembly with a more negative slope equating to faster disassembly.

**Fluorescent Recovery after Photobleaching**

Fluorescence recovery after photobleaching (FRAP) experiments were performed on a Nikon A1R scanning laser confocal microscope using a CFI 60x/1.40 NA Plan Apo VC oil immersion objective. A Tokai Hit stage-top incubator was installed to maintain cells in a humidified chamber at 37°C and 5% CO2.

Monolayers of B2b\textsuperscript{shRNA.scr} and B2b\textsuperscript{shRNA.hSdc1} cells that stably express paxillin-eGFP were scratched with a p-100 pipet tip to simulate a wound. Cells were allowed to initiate migration for at least 2 hours before starting FRAP experiments. Leading edge FAs in cells at the wound front
were photobleached with both the 405 and 488 laser at 50% intensity for 1 second, which consistently achieved approximately 80% photobleaching. After photobleaching, sequential images were obtained with a low laser intensity (i.e., <3%) to minimize any further photobleaching. The frequency of image acquisition was variable to optimize the resolution during the recovery period. Images were acquired every 0.5 s for 10 s, 1.0 s for 20 s, 5 s for 1 min, and 10 s for 1 min. Raw images were evaluated with the Nikon Elements AR software. The intensity of the FA was normalized so that all values are a percent of the initial intensity of the FA immediately prior to photobleaching.

The recovery half-time ($T_{1/2}$) of the FRAP intensity curve was determined for each experiment. The fluorescent recovery curve is best described by the equation: $I(t) = I_E(1-e^{-\tau t})$, where $I_E$ is the maximum fluorescent intensity recovered after photobleaching, $t$ is time, and $\tau$ is a constant that is determined after fitting the fluorescent intensity recovery curve to the exponential equation (Prism; Graphpad Software; La Jolla, CA). The half-time is calculated by the following equation: $T_{1/2} = \ln0.5/-\tau$. Experiments were repeated a minimum of 3 times with at least 5 FAs evaluated per experiment.

**Rap1 Assays**

The activated and total Rap1 levels in cell lysates were determined by western blot using the Rap1 Activation Assay Kit (Millipore). In time-lapse microscopy experiments, control or Rap1$^{G12V}$ transduced cells were identified by either eGFP or mCherry expression.

**Statistics**

The Student’s T-test was used to determine statistical significance between 2 conditions. The One-Way ANOVA with post-test Bonferroni analysis was used for multiple conditions. Statistical significance was considered when $p < 0.05$. All data bars are mean ± standard error of the mean unless otherwise stated.
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REFERENCES


FIGURE LEGEND

Figure 1. Syndecan-1 increases FAs. B2b^{shRNA.scr} and B2b^{shRNA.hSdc1} cells were allowed to migrate for 2 hours and immunostained for paxillin (green) and dapi (blue). Nascent adhesions were seen at the cell front (arrows). FAs (arrowheads) are seen throughout migrating B2b^{shRNA.scr} cells. In contrast, B2b^{shRNA.hSdc1} cells had fewer and less prominent FAs in migrating cells. These are representative images from reproducible experiments. Scale bar = 20 μm.

Figure 2. Syndecan-1 slows FA disassembly in lung epithelial cells. A – D) Migrating B2b^{shRNA.scr} and B2b^{shRNA.hSdc1} cells stably expressing paxillin-eGFP were observed by time-lapse TIRF microscopy (Video 1). A) Kymographs revealed a longer FA lifespan in B2b^{shRNA.scr} compared to B2b^{shRNA.hSdc1} cells. Horizontal line = 10 min; Vertical line = 5 μm. B) Normalized fluorescent intensity (% of max intensity) of the FAs in panel A over time. C) The total FA lifespan and D) the rate of the FA disassembly were determined for migrating lung epithelial cells. *p < 0.0001 by Student’s T-Test. n = 4 independent experiments. E – H) B2b^{shRNA.scr} and B2b^{shRNA.hSdc1} stably expressing paxillin-eGFP were treated with nocodazole (10 μM; 4 hrs). After washing the cells with fresh medium, FA disassembly was observed with time-lapse TIRF microscopy in E) B2b^{shRNA.scr} and F) B2b^{shRNA.hSdc1} cells. G) The intensity curves of the FAs in panels E and F. H) The FA disassembly rate was determined in B2b^{shRNA.scr} and B2b^{shRNA.hSdc1} cells. *p < 0.05 by Student’s T-Test. n = 3 independent experiments.

Figure 3. Syndecan-1 facilitates the recovery of fluorescence after photobleaching in migrating lung epithelial cells. A scratch wound was created on monolayers of B2b^{shRNA.scr} and B2b^{shRNA.hSdc1} cells stably expressing paxillin-eGFP, and FAs at the wound front were identified for photobleaching. A) Representative FA intensities prior to (-1 second) and after photobleaching. B) The normalized intensity of the representative FA in panel A was plotted over time. C) The FRAP recovery half-time was determined for B2b^{shRNA.scr} and B2b^{shRNA.hSdc1} cells. More than 15 FAs were analyzed across 3 independent experiments. *p < 0.05 by Student’s T-Test.

Figure 4. Syndecan-1 restrains lung epithelial cell migration by activating Rap1. Western blot for active and total Rap1 in A) B2b^{shRNA.scr} and B2b^{shRNA.hSdc1} cells and B) wild-type and
Sdc1−/− primary ALI cultures. Migration of C) B2b shRNA.scr transduced with eGFP, D) B2b shRNA.scr co-transduced with eGFP and Rap1G12V, E) B2b shRNA.hSdc1 cells transduced with eGFP, or F) B2b shRNA.hSdc1 cells co-transduced with eGFP and Rap1G12V (Video 2). The white asterisk identifies the same cell at 0h and 6h after migration. G) Cell migration speed was measured for all conditions. *p < 0.001 by One-Way ANOVA and post-test Bonferroni analysis. n = 3 independent experiments.

Figure 5. Rap1 slows FA disassembly in lung epithelial cells lacking syndecan-1 expression. A – E) A scratch wound was created on monolayers of B2b shRNA.scr and B2b shRNA.hSdc1 cells stably expressing paxillin-eGFP, and FA assembly and disassembly was observed by time-lapse TIRF microscopy. All cells were identified by expression of mCherry. In control conditions (B2b shRNA.scr and B2b shRNA.hSdc1), cells were transduced with mCherry only whereas B2b shRNA.hSdc1 + Rap1G12V cells were transduced with both mCherry and Rap1G12V. A) Kymographs (Horizontal line = 10 min; Vertical line = 5 μm) and B) intensity curves of FAs of the labeled conditions. C) The total FA lifespan and D) the rate of the FA disassembly were determined. *p < 0.05 by One-Way ANOVA and post-test Bonferroni analysis. n = 3 independent experiments. E) FAs (arrowheads) are present in the cell center of B2b shRNA.scr cells and B2b shRNA.hSdc1 cells + Rap1G12V and lacking in B2b shRNA.hSdc1 cells. Green = paxillin; Red = mCherry; Scale bar = 20 μm. F – J) Cells stably expressing paxillin-eGFP were treated with nocodazole (10 μM; 4 hrs). In control conditions (B2b shRNA.scr and B2b shRNA.hSdc1), cells were transduced with mCherry only whereas B2b shRNA.hSdc1 + Rap1G12V cells were transduced with both mCherry and Rap1G12V. After washing with fresh medium, FA disassembly was observed by time-lapse TIRF microscopy in F) B2b shRNA.scr, G) B2b shRNA.hSdc1 and H) B2b shRNA.hSdc1 + Rap1G12V cells. I) The intensity curves of the FAs in panels F – H. J) The FA disassembly rate was determined in all conditions. *p < 0.001 by One-Way ANOVA and post-test Bonferroni analysis. n = 3 independent experiments.

Figure 6. Rap1 accelerates the recovery of fluorescent after photobleaching in migrating lung epithelial cells lacking syndecan-1 expression. A scratch wound was created on monolayers of B2b shRNA.scr and B2b shRNA.hSdc1 cells stably expressing paxillin-eGFP, and FAs at the wound front were identified for photobleaching. In control conditions (B2b shRNA.scr and B2b shRNA.hSdc1), cells were transduced with mCherry only whereas B2b shRNA.hSdc1 + Rap1G12V cells
were transduced with both mCherry and Rap1\textsuperscript{G12V}. \textbf{A)} Representative FA intensities prior to (-1 second) and after photobleaching. \textbf{B)} The normalized intensity of the representative FA in panel A was plotted over time. \textbf{C)} The FRAP recovery half-time was determined for B2b\textsuperscript{shRNAscr} and B2b\textsuperscript{shRNAhsdc1} cells. More than 15 FAs were analyzed across 3 independent experiments. *\(p < 0.01\) by One-Way ANOVA and post-test Bonferroni analysis.
Supplemental Online Material

Video Legend

**Video 1. Syndecan-1 restrains migration speed and FA turnover.** Migrating B2b<sup>shRNA.scr</sup> and B2b<sup>shRNA.hSdc1</sup> cells stably expressing paxillin-eGFP were observed by time-lapse TIRF microscopy using a Nikon TiE inverted widefield fluorescence microscope. Each time point was acquired every 2 minutes for 3 hours. Scale bar = 20 μm. Time = hr:min.

**Video 2. Rap1 slows migration of lung epithelial cells deficient in syndecan-1 expression.** Migration of B2b<sup>shRNA.scr</sup> cells and B2b<sup>shRNA.hSdc1</sup> cells transduced with eGFP or co-transduced with eGFP and Rap1<sup>G12V</sup> was observed using a Nikon TiE inverted widefield fluorescence microscope. Each time point was acquired every 10 minutes for 14 hours. Scale bar = 20 μm. Time = hr:min.

Supplementary Figures

**Figure S1. Syndecan-1 does not affect FA assembly in migrating lung epithelial cells.** A migrating B2b<sup>shRNA.scr</sup> and B2b<sup>shRNA.hSdc1</sup> cells stably expressing paxillin-eGFP were observed by time-lapse TIRF microscopy, and the rate of the FA assembly was determined. The FA assembly rate was similar between B2b<sup>shRNA.scr</sup> cells (9.04 ± .45 %max intensity/min) and B2b<sup>shRNA.hSdc1</sup> cells (9.55 ± .39 %max intensity/min).

**Figure S2. Syndecan-1 does not affect FA size in migrating lung epithelial cells.** Migrating B2b<sup>shRNA.scr</sup> and B2b<sup>shRNA.hSdc1</sup> cells stably expressing paxillin-eGFP were observed by time-lapse TIRF microscopy. Leading edge FAs were identified and the size measured at the peak FA intensity (and size). The FA size was similar between B2b<sup>shRNA.scr</sup> cells (1.61 ± .06 μm<sup>2</sup>) and B2b<sup>shRNA.hSdc1</sup> cells (1.64 ± .07 μm<sup>2</sup>).

**Figure S3. Rap1 expression does not augment activation of the β<sub>1</sub> integrin subunit in B2b<sup>shRNA.hSdc1</sup> cells.** B2b<sup>shRNA.scr</sup> and B2b<sup>shRNA.hSdc1</sup> cells were infected with AAV-eGFP (control) or AAV-Rap1<sup>G12V</sup>-IRES-eGFP to transduce activated Rap1. Cells were immunostained for the high affinity β<sub>1</sub> integrin subunit (clone 12G10) and all β<sub>1</sub> integrin subunit (clone P5D2). The β<sub>1</sub> subunit antibodies (clones 12G10 and P5D2) were conjugated with Pacific Blue and Alexa-647,
respectively (Zenon labeling kit; Invitrogen) prior to immunostaining cells. Flow cytometry was performed on a BD FACS Canto II. After gating for eGFP positive cells, the geometric mean fluorescent intensity (MFI) was determined for the high affinity and total $\beta_1$ integrin subunit (FlowJo), and the ratio of high affinity (12G10) to total (P5D2) $\beta_1$ integrin subunit is presented in the bar graph. *$p < 0.0005$ by One-Way ANOVA. n = 3.

Figure S4. Syndecan-1 expression levels on control (B2b$^{\text{shRNA.scr}}$) and knockdown (B2b$^{\text{shRNA.hSdc1}}$) cells. Syndecan-1 levels were quantified on B2b$^{\text{shRNA.scr}}$ and B2b$^{\text{shRNA.hSdc1}}$ cells by flow cytometry using anti-syndecan-1 antibody (clone BA38). The geometric mean fluorescent intensity (MFI) was determined for B2b$^{\text{shRNA.scr}}$ cells (16,310 ± 637 MFI) and B2b$^{\text{shRNA.hSdc1}}$ cells (3407 ± 61 MFI).