Tetraspanin CD151 regulates RhoA activation and the dynamic stability of carcinoma cell-cell contacts

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Summary

Tetraspanins regulate integrin-dependent tumor cell interactions with the extracellular matrix. Here we show that tetraspanin CD151, which plays critical roles in regulating the adhesion and motility of individual tumor cells, is also an important regulator of collective tumor cell migration. Near total silencing of CD151 destabilizes E-cadherin-dependent carcinoma cell-cell junctions and enhances the collective migration of intact tumor cell sheets. This effect does not depend on reduced E-cadherin cell-surface expression or intrinsic adhesivity, or on obvious disruptions in the E-cadherin regulatory complex. Instead, the loss of CD151 causes excessive adhesivity, or on obvious disruptions in the E-cadherin carcinoma cell-cell junctions and enhances the collective metastasis might be triggered by aberrant reactivation of a developmental switch that includes a downregulation of E-cadherin-dependent cell-cell adhesion, similar to the epithelial-mesenchymal transitions of embryogenesis (Brabletz et al., 2005; Hugo et al., 2007; Thiery and Sleeman, 2006). However, the invasive capacity of certain E-cadherin-positive carcinomas (Cowin et al., 2005; Cowin and Welch, 2007; Jang et al., 2007) indicates that additional regulatory mechanisms, beyond simply extinguishing E-cadherin expression, are likely to come into play in some settings.

Several lines of research suggest that regulation of cell-cell interactions could be an important additional mechanism by which CD151 might influence metastasis. Humans and mice lacking CD151 show loss of epithelial integrity in the skin and kidney (Karamatic Crew et al., 2004; Sachs et al., 2006), and CD151-null mice also show defective wound healing (Cowin et al., 2006). In kidney epithelial cells from mice lacking α3β1 integrin (a major CD151 partner), E-cadherin localization and function appeared perturbed, and association of α3β1 integrin with CD151 might be important for the ability of α3β1 integrin to regulate E-cadherin in this system (Chattopadhyay et al., 2003). Conversely, overexpression of CD151 enhanced carcinoma cell-cell association (Shigeta et al., 2003), whereas an anti-CD151 antibody interfered with E-cadherin localization in HaCat cells and promoted their dispersal (Chometon et al., 2006). Collectively, these data suggest that CD151 might regulate cell-cell interactions between tumor cells; however, the effect of CD151 loss of function on E-cadherin in transformed cells has not been determined.

We recently reported that near-total, RNAi-mediated silencing of CD151 in epidermal carcinoma cells resulted in impaired adhesion and migration mediated by the CD151-associated integrins,
α3β1 and α6β4 (Winterwood et al., 2006). In this previous study, we examined the behavior of individual, dissociated tumor cells. Here, we extended our analysis to conditions where cell-cell contacts were maintained. Our data uncover an important role for CD151 in promoting the stability of carcinoma cell-cell junctions, and reveal complexities that would have to be considered in any potential strategy that targets CD151 to inhibit tumor cell motility. In addition, the data help to illuminate a mechanism whereby the organization of intercellular junctions can be perturbed even though E-cadherin cell surface expression is maintained.

Results
Enhanced migration of CD151-silenced carcinoma cell sheets
To determine how CD151 might regulate tumor cell motility when cell-cell contacts are maintained, we compared parental A431 epidermal carcinoma cells (WT) to CD151-silenced cells (A431 sh3 cells) in gap-filling assays. Freshly confluent monolayers were inscribed with a gap using a micropipet tip, and gap closure was monitored by time-lapse microscopy (Fig. 1A; supplementary material Movie 1). Both cell types closed the gap as continuous sheets. However, in contrast to the reduced single-cell velocity that we previously observed for the CD151-silenced sh3 cells (Winterwood et al., 2006), the sh3 cell sheets closed the gap significantly faster than their wild-type counterparts. Quantification indicated that the gap closure rate for sh3 cells was over twice as fast as for wild-type cells (Fig. 1B). The enhanced closure rate of the sh3 cell sheets was not due to a difference in proliferation, because frame-by-frame analysis of mitoses occurring during assay revealed no differences (Fig. 1C).

We used a spheroid assay to further investigate how CD151 might regulate collective tumor cell migration. Spheroids, created by culturing cells overnight in tubes coated with non-adhesive poly(2-hydroxyethyl methacrylate) (poly-HEMA), were plated on laminin-5, an α3β1 integrin ligand. Both wild-type and sh3 cell spheroids expanded as intact sheets, and once again the sh3 cell sheets migrated significantly farther than wild-type cell sheets (Fig. 1D). Re-expressing CD151 in sh3 cells (creating cells designated A431 sh3 Rx) reversed the enhanced migration of the sh3 cell spheroids (Fig. 1D). Quantification showed that sh3 cell spheroids expanded over twice as far as wild-type or Rx cell spheroids (Fig. 1E). Thus, despite the fact that the motility of individual cells is significantly reduced upon silencing CD151 (Winterwood et al., 2006), the collective migration of intact, CD151-silenced monolayers is significantly enhanced. These results are summarized as linear velocities in Table 1.

The data in Table 1 indicate that all three cell types migrated significantly slower as intact monolayers than as single cells. However, whereas wild-type and Rx monolayer velocities were only ~6% of their respective single-cell velocities, the sh3 monolayer velocity was nearly 50% of the sh3 single-cell velocity. An analysis of wild-type and sh3 collective migration velocity in the gap-filling experiments yielded very similar results (data not shown). These data suggested that: (i) the maintenance of cell-cell contacts during collective migration imposes a restraint on migration velocity, and (ii) this restraint on velocity is manifested much more in wild-type and Rx monolayers than in sh3 monolayers.

Table 1. Single cell vs intact sheet linear velocities

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Single cell velocities (μm/h)</th>
<th>Intact sheet velocities (μm/h)</th>
</tr>
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<tbody>
<tr>
<td>WT</td>
<td>116.7±5.3 (n=5)</td>
<td>7.0±0.9 (n=8)</td>
</tr>
<tr>
<td>sh3</td>
<td>52.8±7.5 (n=3)</td>
<td>24.4±2.8 (n=8)</td>
</tr>
<tr>
<td>Rx</td>
<td>113.0±8.3 (n=3)</td>
<td>7.0±1.7 (n=8)</td>
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Fig. 1. Enhanced migration of CD151-silenced carcinoma cell sheets. (A) Time-lapse microscopy of gap closure by wild-type (WT) and CD151-silenced (sh3) A431 cells. Scale bar: 125 μm. (B) Average gap closure rate in three independent trials ± s.e.m. WT closure rate was significantly lower than in sh3 cells; *P<0.005, unpaired t-test. (C) The cumulative number of mitoses was scored for wild-type and sh3 cells during the assay in 175-μm wide strips on either side of initial gap. (D) Tumor cell spheroids formed from wild-type (WT), CD151-silenced (sh3) or CD151 rescue (Rx) cells were plated in serum-free medium in laminin-5-coated wells. Photographs were taken 1 hour and 18 hours after plating; the extent of spheroid dispersal at 18 hours is indicated with black outlines. (E) Pooled data from eight spheroids per cell type ± s.e.m. sh3 spheroids dispersed over twofold more than either wild-type or Rx cell spheroids. *P<0.001, ANOVA with Bonferroni t-test. (F) Wild-type spheroids (as in D) were cultured on laminin-5 in the presence of 10 μg/ml anti-E-cadherin antibody, SHE78-7. (G) Pooled data from wild-type, sh3 or Rx spheroids (at least nine spheroids per cell type, mean ± s.e.m.) plated on collagen I. *P=0.01, ANOVA with Bonferroni t-test.
a large halo of single cells around the spheroids (Fig. 1F; see also supplementary material Fig. S1 for an expanded view). Although these data were consistent with the view that cell-cell contact can restrain cell motility, they suggested that wholesale disruption of E-cadherin adhesive function was unlikely to explain the phenotype of CD151-silenced monolayers. Instead, the enhanced migration of intact sh3 monolayers suggested that cell-cell contacts were maintained, but might be perturbed, in sh3 cell monolayers.

Lastly, to test whether regulation of collective migration by CD151 depends on the extracellular substrate, we analyzed spheroids on collagen I, a ligand for α2β1 integrin. The loss of CD151 enhanced collective migration on collagen I to a similar extent as on laminin-5 (Fig. 1G). These data indicate that the ability of CD151 to act as a negative regulator of collective migration in this assay might not be strongly dependent on the exogenous extracellular substrate supplied.

**E-cadherin and its partners are mis-localized in CD151-silenced carcinoma cells**

We hypothesized that CD151 might regulate collective migration by regulating the organization of carcinoma cell-cell junctions. Therefore, we next examined the localization of E-cadherin and associated proteins in wild-type, sh3 and Rx cells. Whereas E-cadherin strongly localized to cell-cell contact sites in wild-type cells (Fig. 2A), its junctional localization was substantially perturbed in CD151-silenced cells (Fig. 2B). Re-expression of CD151 in A431 sh3 Rx cells restored normal E-cadherin localization (Fig. 2C). The junctional localization of E-cadherin regulatory proteins β-catenin (Fig. 2D-F), α-catenin (Fig. 2G-I), plakoglobin (Fig. 2J-L) and p120ctn (Fig. 2M-O) were also perturbed in the sh3 cells. CD151 itself localized to cell-cell contact sites (Fig. 2P,R), which was consistent with a role in regulating cell-cell contacts, and was specifically absent in the sh3 cells, as expected (Fig. 2Q). Lastly, the receptor protein tyrosine phosphatase PTPμ, whose expression might be regulated by integrin-tetraspanin complexes in some cells (Chattopadhyay et al., 2003), appeared equally expressed in all three cell types (Fig. 2S-U). These data indicated that E-cadherin-based cell-cell junctions are less well organized in CD151-silenced cells.

**The E-cadherin regulatory complex appears intact in CD151-silenced carcinoma cells**

To begin to explore the basis of the altered cell-cell junctions in CD151-silenced cells, we examined E-cadherin association with components of the cadherin regulatory complex. In E-cadherin immunoprecipitations, the amounts of co-precipitating β-catenin, α-catenin, p120ctn, α-actinin and plakoglobin all appeared unchanged in CD151-silenced cells (Fig. 3A-E). In addition, the amounts of E-cadherin or p120ctn that co-precipitated with β-catenin were unchanged, as was the amount of E-cadherin that co-precipitated with p120ctn (Fig. 3F,G). Because many E-cadherin partners are regulated by phosphorylation, we examined the profile of phosphoproteins co-precipitating with E-cadherin, focusing on a size range that encompasses α-, β- and p120 catenin, as well as plakoglobin and α-actinin. No obvious differences were observed in any of the cell types (Fig. 3H, lanes 2-4). However, treatment of cells with epidermal growth factor (EGF) confirmed that increased phosphorylation of protein bands corresponding to β-catenin and plakoglobin (Hoschuetzky et al., 1994) could be detected by our assay system (Fig. 3I, lane 1). Moreover, repeated experiments that directly assessed β-catenin phosphorylation failed to reveal any differences (data not shown).

We also observed that E-cadherin surface expression was maintained at similar levels in CD151-silenced cells as in wild-type or Rx cells (Fig. 3I), and we observed no changes in the detergent extractability of E-cadherin in CD151-silenced cells (Fig. 3J). Next, we tested for a possible association between E-cadherin and CD151 or other tetraspanins. As shown in Fig. 3K, although E-cadherin was readily detected in a β-catenin immunoprecipitate (lane 2), no E-cadherin could be detected co-precipitating with any of several different tetraspanins (lanes 3-6) or with α2- or α3-integrin subunits (lanes 7 and 8). Conversely, α3 integrin was detected in tetraspanin immunoprecipitates (Fig. 3L, lanes 1-3), but not in a β-catenin immunoprecipitate (lane 4). Lastly, we tested for cell-surface expression of PTPμ and observed no differences in wild-type, sh3 or Rx cells (Fig. 3M), consistent with the staining data in Fig. 2S-U. Collectively, these data indicate that major biochemical disruptions in the E-cadherin regulatory complex or changes in
PTPμ expression are unlikely to explain the disorganized junctions in our CD151-silenced cells. In addition, E-cadherin itself might not directly interact with CD151 or other tetraspanins.

E-cadherin-dependent adherens junctions initially form, but fail to remain organized in CD151-silenced cells

To further define how E-cadherin localization and function might be altered in CD151-silenced cells, we tested wild-type and CD151-silenced sh3 cells in a short-term adhesion assay on purified E-cadherin-Fc fusion protein. As shown in Fig. 4, both cell types adhered equally well to E-cadherin-Fc, and adhesion could be blocked substantially with anti-E-cadherin antibody and completely with the calcium chelator, EGTA. These data suggested that initial E-cadherin-dependent adhesion events might proceed normally in CD151-silenced cells. To further test this, we examined the localization of E-cadherin during a calcium switch assay. To facilitate these experiments, we introduced an E-cadherin-GFP fusion protein (EcadGFP) into A431 wild-type and sh3 cells and sorted GFP-positive cells. In cells cultured overnight, EcadGFP strongly localized to cell-cell contacts in wild-type cells, but much less so in sh3 cells (Fig. 5A,B). After 15 minutes of exposure to low calcium, both cell types rounded up and cell-cell junctions were largely disrupted (Fig. 5C,D). Strikingly, 30 minutes after calcium restoration, EcadGFP was strongly localized to the re-forming cell-cell junctions in sh3 cells, very similar to the situation in wild-type cells (Fig. 5E,F). However, after 3 hours, EcadGFP localization in sh3 cells had begun to deteriorate (Fig. 5G,H). Collectively, these data indicated that E-cadherin-based adherens junctions can form in CD151-silenced cells but they appear unstable and fail to remain organized. The data also suggested that the adhesive function of E-cadherin, per se, might not be impaired in our CD151-silenced cells.

Excessive RhoA activity and formation of stress fibers in CD151-silenced cells

The formation and maintenance of E-cadherin-based adherens junctions is known to depend on the activity levels of Rho family small GTPases, which regulate the organization of the actin cytoskeleton (Arthur et al., 2002; Braga, 2002; Fukata and Kaibuchi, 2001; Yap and Kovacs, 2003). Therefore, we examined F-actin localization in relation to E-cadherin using confocal microscopy. We observed copious actin stress fibers near the basal surface of
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The sh3 cells, whereas F-actin was more concentrated at the cell perimeter in wild-type cells (Fig. 6A,E, and overlaid with actin in Fig. 6A”,E”). At more lateral and apical planes in sh3 cells, some F-actin and E-cadherin was observed at some cell-cell junctions (Fig. 6B,D,B’-D”, overlaid in Fig. 6B”-D”), but overall the junctional staining of both proteins was less prominent and less well-organized than in wild-type cells (Fig. 6F,H,F’-H”, overlaid in Fig. 6F”-H”). Confocal analysis of β-catenin revealed a similar pattern: in wild-type cells, β-catenin was prominently localized at most cell-cell junctions (supplementary material Fig. S2A-C), whereas in sh3 cells, β-catenin localized to some junctions, but in general appeared more diffuse and less well-organized (supplementary material Fig. S2D-F). Re-expression of CD151 in Rx cells restored the junctional localization of β-catenin (supplementary material Fig. S2G-I).

Because of the abundant stress fibers in sh3 cells, we next compared sh3 and wild-type cells for the levels of active RhoA, which controls formation of stress fibers. As shown in Fig. 7A, in confluent wild-type cell cultures, the level of active, GTP-loaded RhoA was significantly reduced (lane 3) compared to that in rounded wild-type cells (lane 1), which served as a positive control for RhoA activation. By contrast, the level of active RhoA in confluent sh3 cell cultures (lane 4) was significantly elevated and similar to the rounded cell positive control (lane 2). Restoring CD151 expression in Rx cells reversed the elevation of levels of active RhoA observed in sh3 cells (Fig. 7B). These data suggested that elevated RhoA activity is probably responsible for the elevated formation of stress fibers in CD151-silenced cells.

Monolayers of CD151-silenced cells are more dynamic than wild-type monolayers

Elevated RhoA activity has been associated with a destabilization of adherens junctions (Jou and Nelson, 1998; Zhong et al., 1997) and cell scattering in response to factors such as hepatocyte growth factor (Wells et al., 2005). We therefore examined the behavior of individual cells within intact wild-type and CD151-silenced monolayers by time-lapse microscopy. These experiments revealed that sh3 cell monolayers appeared much more dynamic, with individual cells moving within the monolayer to a much greater extent than in wild-type monolayers.

Fig. 5. Adherens junctions form but fail to remain organized in CD151-silenced cells. Wild-type and CD151-silenced sh3 cells expressing E-cadherin-GFP were compared in a calcium switch assay. (A,B) Cells cultured overnight in normal calcium (con, control). (C,D) Cells placed in low Ca²⁺ (5 μM) for 15 minutes (after overnight culture under normal conditions). (E-H) Cells treated with low Ca²⁺ and then restored to normal conditions for 30 or 180 minutes.

Fig. 6. Elevated stress fiber formation and disorganized junctions in CD151-silenced cells. (A-H”) CD151-silenced cells (sh3) and wild-type cells (WT) were double-labeled for F-actin and E-cadherin and examined by confocal microscopy. For each cell type, optical sections spanning from basal to apical surfaces are displayed from top to bottom.
Elevated RhoA activity in CD151-silenced cells. (A) Confluent wild-type or CD151-silenced sh3 cells were detached and held in suspension for 5 minutes (susp.) or left attached (attach.) prior to lysis. Active RhoA, recovered by pull-down with GST-Rhotekin (upper panel), and total RhoA in the lysates (lower panel) were quantified by immunoblotting. (B) The levels of active RhoA in lysates of confluent A431 wild-type cells, CD151-silenced sh3 cells or CD151-rescued Rx cells were measured as in A. The numbers above each lane indicate the amount of active RhoA corrected for total RhoA in each lysate, with the amount of active RhoA in lane 1 set to 1.0.

Fig. 8. Enhanced dynamics and decreased junctional stability within CD151-silenced monolayers. (A,B) Individual cells within wild-type and sh3 monolayers were tracked by time-lapse microscopy and their trajectories overlaid, setting the initial location of each cell at the center of the graph. Fifteen tracks per cell type are shown for an 8.5-hour experiment. Scale bar: 100 μm. (C) Mean velocities ± s.e.m. for wild-type and sh3 cells moving within confluent monolayers; 35 cells of each type were measured in two separate trials. The sh3 cells migrated three- to fourfold faster than wild-type cells. *P<0.0001, unpaired t-test. (D) A sequence of frames 30 minutes apart depicting two cells within an sh3 cell monolayer that initially shared a junction, but which pulled apart during the observation period. Asterisks mark the two cells, and an arrowhead tracks the junction as it is disrupted. (E,F) The lengths of individual cell-cell contacts within wild-type and sh3 cell monolayers were measured every 5 minutes for 2 hours. Fluctuations in relative cell-cell contact size are plotted, with the original size of each contact set to 1.0. (G) The average rate of change of cell-cell contact length ± s.e.m. The sh3 cell junctions changed size at nearly three times the rate of wild-type junctions. *P<0.0001, unpaired t-test, n=10 junctions/cell type. (H) The fate of 30 wild-type and sh3 cell-cell junctions was followed for 12 hours, and the percentage of the original junctions remaining intact at 15 minutes intervals is plotted. The sh3 cell junctions had a significantly reduced lifetime, with a median survival time of 226 minutes versus >720 minutes for wild-type cells. P=0.0112, log rank test. (I) The mean velocities of individual cells within wild-type and sh3 cell monolayers were measured for 3 hours before and 3 hours after the addition of 2 μg/ml cell-permeable C3 transferease. *P<0.01, **P<0.001, ANOVA with Bonferroni t-test. Values are mean ± s.e.m. of 17 cells per condition.

Suppression of RhoA activity in CD151-silenced cells restores a more normal E-cadherin localization

We next tested whether the reduced motility within CD151-silenced monolayers upon C3 treatment corresponded to a restored junctional organization. Untreated sh3 cells displayed actin stress fibers and poorly organized junctions at the basal cell surface (Fig. 9A-C). By contrast, C3-treated cells showed fewer stress fibers and a more normal junctional localization of F-actin and E-cadherin (Fig. 9D-F, compared to untreated wild-type cells in Fig. 9G-I). Wild-type cells treated with C3 retained normal F-actin and E-cadherin localization, although F-actin at cell-cell boundaries appeared somewhat depleted, perhaps because RhoA activity in C3-treated wild-type cells was suboptimal for maintenance of F-actin at
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junctions (Fig. 9J-L). At a more lateral plane, some F-actin and E-cadherin did localize to some sh3 cell junctions (Fig. 9A'-C'), but this was more diffuse and less organized than in C3-treated sh3 cells (Fig. 9D'-F') or untreated wild-type cells (Fig. 9G'-I').

Treatment of wild-type cells with C3 again partially depleted F-actin from the junctions, but did not perturb their organization (Fig. 9J'-L'). Collectively, the data in Figs 8 and 9 show that a RhoA inhibitor can suppress the elevated motility within CD151-silenced monolayers and restore a more normal junctional organization.

A CD151 mutant with impaired association of α3β1 integrin fails to restore junctional organization

Given that α3β1 integrin is a prominent CD151 partner (Kazarov et al., 2002; Sterk et al., 2002; Yauch et al., 1998) and might regulate junctional stability in non-transformed cells (Chattopadhyay et al., 2003; Wang et al., 1999), we next began to explore the extent to which α3β1 integrin might be involved in the ability of CD151 to promote junctional organization in carcinoma cells. Using a FLAG-tagged, wild-type CD151 Rx cDNA as a template, we constructed a FLAG-tagged CD151 mutant, CD151VR, in which the ‘variable region’ in the CD151 large extracellular loop (EC2 domain) was replaced with that of tetraspanin TM4SF2 (see the Materials and Methods). In relatively mild Brij detergent lysates (1:1 ratio of Brij 99 to Brij 96), wild-type CD151 readily co-precipitated with α3 integrin (Fig. 10A, lane 1). By contrast, virtually no CD151VR was detected co-precipitating with α3 integrin (Fig. 10A, lane 3).

Conversely, α3 integrin co-precipitated with CD151, but only a trace of α3 integrin co-precipitated with the CD151VR mutant (Fig. 10A, lanes 4 and 5). In CD151-silenced sh3 cell lysates, no specific signal was detected in FLAG blots, and no α3 integrin was retrieved by FLAG immunoprecipitation (Fig. 10A, lanes 2 and 5). The CD151VR mutant migrated as a broad band of ~45 kDa versus the ~32 kDa band observed for wild-type FLAG-tagged CD151, probably reflecting the presence of four glycosylation sites in the TM4SF2 variable region, as compared to the single site in the CD151 variable region.

We next immunoprecipitated FLAG-tagged wild-type CD151 and the CD151VR mutant from extracts of biotin-labeled cells. Simultaneous detection of the biotin label and the FLAG epitope revealed that wild-type CD151 and CD151VR could both be cell-surface-labeled (Fig. 10B). In addition, CD151 and CD151VR both associated with biotin-labeled species with apparent molecular masses (~20-22 kDa) that correspond to tetraspanins CD9 and CD81. Thus, the CD151VR mutant is expressed on the cell surface at a comparable level to wild-type CD151, and appears to retain association with other tetraspanins.

In contrast to wild-type CD151, which was concentrated at cell-cell junctions (Fig. 10C; see also Fig. 2PR), the CD151VR mutant displayed a more dispersed cellular localization (Fig. 10D).

Discussion

CD151 regulates RhoA activation, E-cadherin junctional stability and cell monolayer dynamics

We have shown that CD151-silenced carcinoma cells display enhanced collective migration, despite our earlier observation that the same CD151-silenced cells display reduced single-cell migration velocities (Winterwood et al., 2006). This apparent paradox might be resolved if the factors that limit the much faster single-cell velocity [such as efficient detachment at the lateral or trailing edge (Winterwood et al., 2006)] are not the velocity-limiting factors in slower, collective cell migration. The collective migration velocity of wild-type cells is only ~6% of their single-cell velocity, whereas that of CD151-silenced cells is a full 50% of the single-cell velocity. Thus, for collective cell migration, a major velocity-limiting factor might be the restraint imposed by being part of the collective, a restraint that appears significantly reduced for CD151-silenced cells.
Fig. 10. A CD151 mutant with impaired α3β1 integrin association fails to rescue junctional organization. (A) Brij 99/96 detergent lysates were prepared from CD151-silenced cells rescued with FLAG-tagged wild-type CD151 (Rx) cells, un-rescued cells (sh3) or CD151-silenced cells reconstituted with the CD151VR mutant (VR) (1:1 Brij 99:Brij 96, 1% total detergent by volume). The α3 integrin subunit (lanes 1-3) or FLAG-tagged CD151 constructs (lanes 4-6) were immunoprecipitated, followed by blotting with polyclonal anti-α3 antibody (top panels) or polyclonal anti-FLAG antibody (bottom panels). (B) Rx, sh3, or VR cells were biotinylated and lysed in Brij 99/96, as in A. FLAG-tagged CD151 constructs were immunoprecipitated, and blots were developed simultaneously with IRdye 800-streptavidin and polyclonal anti-FLAG antibody (top panels) or polyclonal anti-FLAG antibody (bottom panels). Top panel shows biotinylated species, middle panel shows with overlay with CD151, CD151VR and CD9/CD81 bands indicated by arrowheads. (C-D) Rx (WT) and VR cells were fixed, permeabilized and stained with anti-FLAG polyclonal antibody followed by Alexa 594 goat anti-rabbit antibody. (E-F) E-cadherin staining of Rx (WT) and VR cells.

We provide evidence that the basis of this effect lies with the excessive activation of the RhoA small GTPase in CD151-silenced cells, leading to elevated actin stress fiber formation, and with a far more dynamic cell monolayer in which cell-cell contacts undergo constant remodeling and have a significantly shorter mean lifespan.

The role of RhoA in the regulation of epithelial morphology is complex. Although Rho activity is required for the formation and stability of E-cadherin-dependent cell-cell junctions (Braga et al., 1997; Takaishi et al., 1997; Yamada and Nelson, 2007), as epithelial cell monolayers reach confluence or re-establish contacts after dissociation, and slowed migration rate in gap-filling experiments (Shigeta et al., 2003). In addition, an anti-CD151 antibody perturbed F-actin and E-cadherin localization. Rac and Cdc42 activities were elevated in CD151-overexpressing cells, but no change in Rho activation was observed. However, Shigeta et al. performed Rac, Cdc42 and Rho pulldown assays in subconfluent cultures, in which the basal Rho activation level was likely to be high (Noren et al., 2001) and might mask any effect of CD151 overexpression on Rho activation. In preliminary experiments, we did not observe any obvious changes in Rac or Cdc42 activation in our CD151-silenced cell cultures (data not shown). Potentially, Rac and Cdc42 regulation by CD151 are more easily uncovered by CD151 overexpression in A431 cells. Overall, our data generally agree with those of Shigeta and colleagues, with the consensus being that overexpression of CD151 enhances junctions and loss of CD151 destabilizes them.

Further support for this view comes from another recent study in which CD151 was silenced in HSSC carcinoma cells, resulting in redistribution of cortical actin and enhanced stress fibers (Hasegawa et al., 2007). Lastly, our results contrast with a recently published study that demonstrated decreased gap filling in vitro by MCF-10A cells treated with a CD151 siRNA (Yang et al., 2008). Multiple factors could account for this difference, including the use of immortalized instead of tumorigenic cell types, the presence of exogenous EGF in the MCF-10A assay, and possible differences in the balance between the ability of CD151 to enhance cell-substrate adhesion strengthening (Lammerding et al., 2003) and its ability to regulate junctional stability in MCF-10A cells versus A431 cells.
Implications for CD151 function in development and disease

Genetic evidence from mice and humans has identified important roles for CD151 in epithelial integrity, wound healing and pathological angiogenesis elicited by tumor formation (Cowan et al., 2006; Karamatic Crew et al., 2004; Sachs et al., 2006; Takeda et al., 2007). How might the Rho-suppressive activity of CD151 be involved in these processes? Elevated Rho activity upon loss of CD151 could diminish the stability of epithelial adherens junctions, contributing to the structural failure in skin and kidney epithelia of human patients and mice lacking CD151 (Karamatic Crew et al., 2004; Sachs et al., 2006). Wound healing could also be impaired by an overly dynamic epithelium in which RhoA was deregulated and adherens junctions were destabilized. This could help to explain the seeming paradox that CD151-silenced cells can fill a gap in vitro more rapidly than wild-type cells (Fig. 1), whereas wound healing in vivo is impaired in CD151-null mice (Cowan et al., 2006). A less stable, CD151-null epithelium on a provisional culture monolayer. Interestingly, genetic deletion of stresses than in the relatively quiescent environment of a tissue context interfering with either protein might be destabilized. This could help to explain the seeming paradox that CD151-silenced cells can fill a gap in vitro more rapidly than wild-type cells (Fig. 1), whereas wound healing in vivo is impaired in CD151-null mice (Cowan et al., 2006). A less stable, CD151-null epithelium on a provisional wound matrix might be more easily disrupted by in vivo mechanical stresses than in the relatively quiescent environment of a tissue culture monolayer. Interestingly, genetic deletion of α3 integrin in keratinocytes led to an enhanced rate of wound healing in vivo and of gap closure in vitro, and this was ascribed to a loss of anti-migratory α3-integrin-dependent adhesion on laminin-5 (Margadant et al., 2009). However, in contrast to α3-integrin-null keratinocytes, which also migrated more rapidly than wild-type keratinocytes in single-cell assays (Margadant et al., 2009), single-cell migration of our CD151-silenced carcinoma cells was slower than that of wild-type cells (Winterwood et al., 2006). Thus, an enhanced intrinsic migration rate of individual cells due to reduced α3-integrin-dependent adhesion would be unlikely to explain the increased rate of collective migration that we observed for the CD151-silenced monolayers.

Disruptions in Rho family GTPase signaling could also interfere with pathological angiogenesis. For example, excessive RhoA signaling, such as might occur upon loss of CD151, has been linked to loss of bipolarity, detachment, cell death and sprout retraction in an organotypic model of angiogenesis (Mavria et al., 2006). Such a mechanism could help to explain the reduced pathological angiogenesis observed in CD151-null mice (Takeda et al., 2007).

The above considerations indicate that the role of CD151 in metastasis is probably complex. Several studies have identified CD151 as a potential promoter of metastasis (Ang et al., 2004; Hashida et al., 2003; Kohno et al., 2002; Testa et al., 1999; Tokuhara et al., 2001; Yang et al., 2008). However, if downregulation of CD151 activates RhoA, destabilizes adherens junctions and enhances collective migration, then the potential exists for CD151 to be a suppressor of invasion in some settings. Recently, an anti-metastatic CD151 antibody was shown to prevent tumor-cell detachment from the primary tumor site (Zijlstra et al., 2008). Activation of the ability of CD151 to stabilize cell-cell contacts might represent another mode of action by this anti-metastatic antibody, in addition to its ability to upregulate integrin-dependent adhesion at the trailing edge (Zijlstra et al., 2008).

The potentially opposing roles for CD151 in metastasis could help to explain why its major partner, α3β1 integrin, might act as either a promoter or a suppressor of metastasis, depending on the context (Giannelli et al., 2002). Thus, future studies in which the roles of α3β1 integrin and CD151 are carefully examined in specific stages and types of malignancy will be important to determine in which contexts interfering with either protein might be advantageous. It will also be important to determine whether the ability of CD151 to promote integrin-dependent cell motility and its ability to promote stable cell-cell junctions are separable functions. If so, factors regulating the balance between these opposing functions might determine whether CD151-integrin complexes act as promoters or suppressors of metastasis in specific settings.

Materials and Methods

Antibodies and laminin-5

Monoclonal antibodies used were anti-E-cadherin (BD Biosciences #610182), SHE78-7 (Zymed Laboratories, Invitrogen), DECMA-1 (Sigma), αβ-catenin (BD Biosciences #601514), αβ-catenin (BD Biosciences #610194), anti-α-catenin (BD Biosciences #610134), αβ-actin (BD Biosciences #612577), α-plakoglobin (BD Biosciences #610253), anti-phosphotyrosine PY20 (BD Biosciences #610000), anti-CDF, ALB6 (Chemicon International), anti-PTPb BK2 (Santa Cruz Biotechnology #sc-33651), anti-FLAG epitope tag M2 agaarse (Sigma) and anti-RhoA (Cytoskeleton #ARHO1). Monoclonal antibodies specific for CD81 (M38), CD151 (SC11 and 1B1.G4), α2 integrin (A2-HE10) and α3 integrin (A3-X8 and A3-HE5) were previously referenced (Winterwood et al., 2006). Also used were rabbit polyclonal antibodies recognizing E-cadherin (gift of Jack Lilien and Jamie Balsamo, University of Iowa Department of Biology, Iowa City, IA), α3 integrin (Kazarov et al., 2002) and FLAG epitope tag (Sigma). Secondary reagents were Alexa 680-goat anti-mouse and IRDye 800-streptavidin (Rockland), and CY2-goat anti-mouse and FITC-goat anti-rabbit antibodies (Jackson ImmunoResearch). Human laminin-5 was purified from SCC-25 cell-conditioned medium as described (Winterwood et al., 2006).

RNA interference and rescue with wild-type and mutant CD151

The production of fluorescence activated cell sorting (FACS)-sorted A431 epithelial carcinoma cells, with near-total silencing of CD151 (A431 sh3 cells), and the A431 sh3 Rx rescue cells, in which wild-type CD151 is re-expressed, was described previously (Winterwood et al., 2006). The wild-type CD151 Rx cDNA contains two silent mutations near the center of the site targeted by the sh3 CD151 shRNA. Using the CD151 Rx cDNA as a template, the CD151VR mutant was created with two rounds of recombinant PCR to (i) add a FLAG tag at the amino terminus, (ii) convert the endogenous imitator mitogene to leucine, (iii) retain the silent Rx mutations that allow re-expression in CD151-silenced cells and (iv) swap the region of the CD151 EC2 domain from seme 158 to glycine 207 with the corresponding region from human tetraspanin TM4SF2/A15. This region corresponds to the ‘variable region’ of CD151 and contains sequences critical for CD151 interaction with α3β1 integrin (Shipp et al., 2003). The CD151VR mutant is not bound by anti-CD151 antibodies 5C11 or 1B1.G4, and was recognized weakly or not at all by available anti-TM4SF2 antibodies. CD151VR is readily detected by anti-FLAG antibody. We also created a FLAG-tagged version of wild-type CD151 for use in comparison with the CD151VR mutant.

Time-lapse video microscopy

A431 cells were cultured in standard medium (DMEM with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin). Gaps were incubated in freshly confluent monolayers using a yellow micropipet tip, and the cultures were refed with standard growth medium. The lifespan of individual cell-cell contacts was followed every 5 minutes for 2 hours, to measure mitoses during the assays, 175-μm wide strips on either side of the initial gap were analyzed frame-by-frame. Mitotic events were clearly visualized as cells rounded up, divided and spread back into the monolayer. Single-cell velocities on laminin-5 were measured for wild-type, CD151-silenced sh3 cells and Rx rescue cells in 3-hour videos, as previously described (Winterwood et al., 2006). In another series of experiments, unwounded monolayers in standard growth medium were imaged as described above, and NIH ImageJ 1.63 software was used to calculate the gap closure rate in μm/h. To measure mitoses during the assays, 175-μm wide strips on either side of the initial gap were analyzed frame-by-frame. Mitotic events were clearly visualized as cells rounded up, divided and spread back into the monolayer. Single-cell velocities on laminin-5 were measured for wild-type, CD151-silenced sh3 cells and Rx rescue cells in 3-hour videos, as previously described (Winterwood et al., 2006).
after plating, and their areas were measured using NIH Image 1.63. The following equation was used to estimate linear velocity (in μm/h) of cell sheet migration in spheroid dispersal assays:

$$\text{Linear velocity} = \sqrt{\frac{Area_2 - Area_1}{17}}$$  

(1)

where Area_2 and Area_1 are the areas (in μm²) of the spheroids measured at 18 hours and 1 hour, respectively.

**Immunostaining**

Cells cultured on coverslips were fixed in 10% formalin in HEPES-buffered saline (HBS) with 4% sucrose and 1 mM CaCl₂, rinsed twice with TBS, and blocked with 10% goat serum in PBS. For intracellular epitopes, cells were permeabilized with 0.1% triton X-100 in PBS for 20 minutes at RT. Cells were then washed three times with HBS prior to lysis. Between 2 and 1:1 mixture of Brij 96V and Brij 99 (all from Sigma-Aldrich). In some experiments, phosphatase inhibitors (Pierce Biotechnology). Detergents were NP-40, Brij 96V or sodium deoxycholate (SDC). In some experiments, cells were biotinylated with 0.1 mg/ml Sulfo-NHS-LC biotin (Pierce Biotechnology) in HBS (20 mM HEPES pH 7.2, 150 mM NaCl) for 1 hour at room temperature and then rinsed three times with HBS prior to lysis. Between 2×10⁵ and 6×10⁵ cells were used for each experiment. Lysates were clarified, separated by SDS-PAGE, and blotted to nitrocellulose as previously described (Winterwood et al., 2006), or by laser scanning confocal microscopy on a Leica SP2 AOBS inverted microscope.

**Immunoprecipitation and immunoblotting**

Cells were lysed in PBS with 1% detergent, protease inhibitors (2 mM PMSF, 10 μg/ml leupeptin and 5 μg/ml E-64) (Roche Diagnostics), and HALT phosphatase inhibitors (Pierce Biotechnology). Detergents were NP-40, Brij 96V or a 1:1 mixture of Brij 96V and Brij 99 (all from Sigma-Aldrich) and protease inhibitors, followed by centrifugation at 16,000×g for 4 minutes at 4°C. Whole cell lysates were then treated with 5 mM EGTA or with anti-E-cadherin function-blocking antibodies for 15 minutes. Then, calcium was restored to 1.8 mM for 30 minutes, 1 hour or 3 hours, respectively. E-cadherin-GFP-expressing cells were selected with G418, and E-cadGFP-expressing cells were sorted by FACS. For analysis of the role of RhoA activation and the cytoskeleton, cells growing on coverslips were placed in low-calcium medium (CaCl₂-free) for 3 days to achieve complete confluence. Cells were then lysed with 500 μg/ml leupeptin and 10 μg/ml EDTA, and the cells were held in suspension for 5 minutes prior to lysis and RhoA pulldown.

**RhoA activity assays**

To assess the role of RhoA activity on junctional stability in CD151-silenced sh3 cells, cells plated on coverslips were treated for 1 hour with 2 μg/ml cell-permeable C3 transferase (Cytoskeleton, Denver, CO). Treated and untreated cells were then fixed and stained for E-cadherin and F-actin, as above. In parallel experiments, freshly confluent monolayers were analyzed by time-lapse microscopy, as described above, for 3 hours before and 3 hours after the addition of 2 μg/ml cell-permeable C3 transferase.

**References**


**Analysis of the role of RhoA activation**

For RhoA activity assays, 5×10⁵ cells were plated in 100 mm dishes and cultured for 3 days to achieve complete confluence. Cells were then lysed with 600 μl of 25 mM HEPES, 0.5% NP-40, 10 mM MgCl₂, 1% glycerol and protease inhibitors, followed by centrifugation at 16,000 × g for 4 minutes at 4°C. Active RhoA was recovered for 30 minutes at 4°C with 30 μg of GST-Rhotekin bound to glutathione Sepharose and analyzed by SDS-PAGE followed by blotting for RhoA. Blots were quantified using a LiCor infrared-fluorescence blot imager. As a positive control for RhoA activation, some dishes were harvested with trypsin plus EDTA, and the cells were held in suspension for 5 minutes prior to lysis and RhoA pulldown.

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