A Critical Role for Tetraspanin CD151 in $\alpha 3\beta 1$ and $\alpha 6\beta 4$ Integrin–dependent Tumor Cell Functions on Laminin-5^V

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The basement membrane protein laminin-5 supports tumor cell adhesion and motility and is implicated at multiple steps of the metastatic cascade. Tetraspanin CD151 engages in lateral, cell surface complexes with both of the major laminin-5 receptors, integrins $\alpha 3\beta 1$ and $\alpha 6\beta 4$. To determine the role of CD151 in tumor cell responses to laminin-5, we used retroviral RNA interference to efficiently silence CD151 expression in epidermal carcinoma cells. Near total loss of CD151 had no effect on steady state cell surface expression of $\alpha 3\beta 1$, $\alpha 6\beta 4$, or other integrins with which CD151 associates. However, CD151-silenced carcinoma cells displayed markedly impaired motility on laminin-5, accompanied by unusually persistent lateral and trailing edge adhesive contacts. CD151 silencing disrupted $\alpha 3\beta 1$ integrin association with tetraspanin-enriched microdomains, reduced the bulk detergent extractability of $\alpha 3\beta 1$, and impaired $\alpha 3\beta 1$ internalization in cells migrating on laminin-5. Both $\alpha 3\beta 1$ - and $\alpha 6\beta 4$ -dependent cell adhesion to laminin-5 were also impaired in CD151-silenced cells. Reexpressing CD151 in CD151-silenced cells reversed the adhesion and motility defects. Finally, loss of CD151 also impaired migration but not adhesion on substrates other than laminin-5. These data show that CD151 plays a critical role in tumor cell responses to laminin-5 and reveal promotion of integrin recycling as a novel potential mechanism whereby CD151 regulates tumor cell migration.

INTRODUCTION

Laminin-5 is a major basement membrane component that can facilitate either stable epithelial cell attachment or rapid cell motility, depending on its state of proteolytic processing (Hintermann and Quaranta, 2004). Although this functional flexibility is well suited to laminin-5's dual physiological roles of maintaining epithelial integrity and restoring integrity during wound healing (Colognato and Yurchenco, 2000; Nguyen *et al.*, 2000b), metastatic carcinoma cells appear to coopt laminin-5 promigratory functions for invasion, adhesion, and motility. For example, laminin-5 disrupts intercellular adhesion and triggers scattering of some types of squamous carcinoma cells (Miyazaki *et al.*, 1993; Kikkawa *et al.*, 1994; Kawano *et al.*, 2001), and laminin-5 interaction with collagen VII may provide a necessary mechanical link for tumor cell invasion into the interstitial matrix (Ortiz-Urda *et al.*, 2005). At a later stage of metastasis, pre-existing patches of exposed, laminin-5-rich basement membrane in the pulmonary vasculature may provide adhesive sites for circulating tumor cells during extravasation (Wang *et al.*, 2004).

The two major cellular receptors for laminin-5 are integrins $\alpha 6\beta 4$ and $\alpha 3\beta 1$. Integrin $\alpha 6\beta 4$ mediates stable anchorage of epithelial cells to laminin-5 in the basement membrane, whereas $\alpha 3\beta 1$ integrin mediates laminin-5-dependent cell spreading and migration (Nguyen *et al.*, 2000b; Hintermann and Quaranta, 2004). Consistent with its role in motility on laminin-5, several studies have documented $\alpha 3\beta 1$ function in carcinoma (Morini *et al.*, 2000), glioma (Tysnes *et al.*, 1996; Fukushima *et al.*, 1998), rhabdomyosarcoma (Kubota *et al.*, 1997), melanoma (Melchiori *et al.*, 1995), and fibrosarcoma (Okada *et al.*, 1994) cell invasion of basement membrane or endothelial cell layers, and increased $\alpha 3\beta 1$ expression has been linked to tumor progression (Ziobler *et al.*, 1996), increased invasiveness, and propensity to metastasize (Giannelli *et al.*, 2002). However, $\alpha 6\beta 4$ signaling through PI 3-kinase may have a positive (Nguyen *et al.*, 2000a) or negative (Hintermann *et al.*, 2001) impact on $\alpha 3\beta 1$ function, depending on the experimental paradigm, and $\alpha 6\beta 4$ itself may be capable of switching from an intermediate filament-associated state, involved in stable cell adhesion, to an actin filament-associated, promigratory state (Mercurio *et al.*, 2001). Furthermore, despite its predominant role in laminin-5-dependent migration, $\alpha 3\beta 1$ also contributes to basement membrane integrity (DiPersio *et al.*, 1997), and keratinocyte survival (Manohar *et al.*, 2004). Thus, $\alpha 6\beta 4$ and $\alpha 3\beta 1$ may each mediate both migratory and nonmigratory responses to laminin-5, and the interplay between these two receptors may determine the extent to which carcinoma cells can utilize laminin-5 for invasion and dispersal.

One protein with the potential to regulate both $\alpha 6\beta 4$ and $\alpha 3\beta 1$ function in tumor cell motility is tetraspanin CD151. CD151 engages both integrins in lateral cell surface complexes (Yauch *et al.*, 1998; Sincocock *et al.*, 1999; Sterk *et al.*, 2000), and the $\alpha 3\beta 1$ -CD151 interaction in particular is nearly

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Abbreviations used: TEMs, tetraspanin-enriched microdomains.

stoichiometric in many cell types and resistant to harsh detergents (Yauch *et al.*, 1998). Anti-CD151 antibodies (Stipp and Hemler, 2000) or expression of CD151 mutants (Kazarov *et al.*, 2002; Zhang *et al.*, 2002) can selectively inhibit $\alpha 3$ and $\alpha 6$ integrin-dependent cell migration in some experimental settings, whereas overexpression of wild-type CD151 enhances experimental metastasis of colon carcinoma and fibrosarcoma cells (Kohn *et al.*, 2002). CD151 also emerged from a subtractive immunization strategy as the target of an anti-metastatic antibody (Testa *et al.*, 1999), and elevated CD151 expression in lung, colon, and prostate cancers correlates with poor clinical outcome (Tokuhara *et al.*, 2001; Hashida *et al.*, 2003; Ang *et al.*, 2004).

As a member of the tetraspanin family, CD151 possesses four transmembrane domains, cytoplasmic amino and carboxyl termini, and two extracellular loops, the larger of which contains the distinctive pattern of cysteine residues that help to define the family. Tetraspanins may act as adaptors or organizers by assembling multimolecular complexes of cell surface proteins and linking them to specific cytoplasmic signaling enzymes such as classical protein kinase C (PKC) isoforms and PI 4-kinase (Berdichevski, 2001; Boucheix and Rubinstein, 2001; Stipp *et al.*, 2003b; Hemler, 2005). Individual tetraspanins may preferentially associate with specific cell surface partners such as integrins, growth factor receptors, and immunoglobulin superfamily proteins, and link these partners, via tetraspanin-tetraspanin interactions, into extended complexes. The organization of extended tetraspanin complexes has been envisioned as a web (Boucheix and Rubinstein, 2001), and more recently, as tetraspanin-enriched microdomains (TEMs), a novel type of cell surface microdomain that is biochemically distinct from lipid rafts (Claas *et al.*, 2001; Hemler, 2005).

Unlike $\alpha 3$ and $\alpha 6$ integrin-null mice, CD151-null mice are viable and fertile (Wright *et al.*, 2004), indicating that despite its physical and functional association with $\alpha 3$ and $\alpha 6$ integrins, CD151 is not essential for $\alpha 3$ - or $\alpha 6$ -dependent morphogenesis during development. However, CD151-null mice do exhibit defects in platelet aggregation and keratinocyte migration (Wright *et al.*, 2004), as well as wound healing *in vivo* (Cowin *et al.*, 2006), and CD151-deficient human patients develop kidney disease and epidermolysis later in life (Karamatic Crew *et al.*, 2004) that are reminiscent of some of the severe developmental defects observed in mice lacking $\alpha 3$ or $\alpha 6$ integrin (Dowling *et al.*, 1996; Georges-Labouesse *et al.*, 1996; Kreidberg *et al.*, 1996; DiPersio *et al.*, 1997).

Despite the abundance of evidence linking CD151 to $\alpha 3$ and $\alpha 6$ integrin functions in cell motility and metastasis, the CD151 loss-of-function phenotype of transformed cells has not been described. Here we developed an RNA interference (RNAi) strategy to efficiently silence CD151 expression in A431 epidermoid carcinoma cells. We find that near total loss of CD151 has no effect on steady state cell surface expression of $\alpha 3$ or $\alpha 6$ integrin, but that CD151 plays a critical role in $\alpha 3$ integrin-dependent cell motility and $\alpha 6$ integrin-dependent attachment and spreading on laminin-5. Our data definitively identify CD151 as a key regulator of $\alpha 3$ and $\alpha 6$ integrin functions in transformed cells.

MATERIALS AND METHODS

Antibodies and Extracellular Matrix Proteins

Anti-integrin monoclonal antibodies (mAbs) used in this study were anti- $\alpha 2$, A2-IIIE10 (Bergelson *et al.*, 1994); anti- $\alpha 3$, A3-X8 and A3-IIIF5 (Weitzman *et al.*, 1993); anti- $\alpha 5$, P1D6 (Covance Research Products, Madison, WI); anti- $\alpha 6$, A6-ELE (Lee *et al.*, 1995) and GoH3 (GeneTex, San Antonio, TX); anti- αv ,

69-6-5 (Bioscience International, Saco, ME), and anti- $\beta 1$, TS2/16 (Hemler *et al.*, 1984). Also used was an anti- $\alpha 3$ integrin polyclonal antibody, D23 (Kazarov *et al.*, 2002). Anti-tetraspanin mAbs used were anti-CD9, ALB6 (Chemicon International, Temecula, CA) and C9-BB (Berdichevski *et al.*, 1996); anti-CD81, M38 (Fukudome *et al.*, 1992); and anti-CD151, 5C11 (Yauch *et al.*, 1998) and 11B1.G4 (Sincok *et al.*, 1997). Anti-EWI-2 mAb was 8A12 (Charrin *et al.*, 2003a). Anti-laminin-5 mAb, 6F12 (also called K140; Marinkovich *et al.*, 1992), was used for laminin-5 affinity purification. Anti-tubulin mAb was DM1B (Calbiochem, La Jolla, CA). Secondary reagents were HRP-conjugated goat-anti-mouse IgG and goat anti-rabbit IgG antibodies (Jackson ImmunoResearch, West Grove, PA), Alexa 680- and Alexa 594-conjugated goat anti-rabbit antibodies (Invitrogen, Carlsbad, CA), and Cy2 goat anti-mouse antibody (Jackson ImmunoResearch).

Rat tail collagen I and human plasma fibronectin were purchased from BD Biosciences (San Diego, CA); human plasma vitronectin was obtained from Takara Bio (Tokyo, Japan.) Human laminin-5 was purified from SCC-25 squamous cell carcinoma-conditioned medium as described (Marinkovich *et al.*, 1992), with some modifications. Freshly confluent SCC-25 cells were rinsed with phosphate-buffered saline (PBS) and then refed with a 1:1 serum-free mixture of DME and F12 medium containing 0.4 μ g/ml hydrocortisone (H0888, Sigma-Aldrich, St. Louis, MO) and 1 μ g/ml each aprotinin and leupeptin (Roche Diagnostics, Indianapolis, IN). After 2 d, conditioned medium was harvested, clarified by centrifugation at 16,000 $\times$ g for 20 min, and supplemented with Tris-HCl, pH 7.4 (50 mM final), Tween-20 (0.1% final; Sigma-Aldrich), and phenylmethylsulfonyl fluoride (PMSF; 2 mM final, Sigma-Aldrich). Conditioned medium was passed over a gelatin-Sepharose column (Sigma-Aldrich) before loading onto an affinity column consisting of the 6F12 anti-laminin-5 antibody coupled to Affigel-10 matrix (Bio-Rad Laboratories, Richmond, CA). After washing with 50 column volumes of 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween-20, laminin-5 was eluted with 1 M acetic acid, 0.1% Tween-20 and neutralized with 1.5 M Tris-HCl, pH 8.8, 0.1% Tween-20. Protein concentration of pooled peak fractions was determined by BCA assay (Pierce Biotechnology, Rockford, IL), and purity was confirmed with silver-stained SDS-polyacrylamide gel electrophoresis (PAGE) gels.

Cell Culture, RNAi, and Retroviral Transduction

A431 epithelial carcinoma cells and retroviral packaging cell lines, GP2-293 and PT67 (BD Biosciences), were cultured in high glucose DME. SCC-25 squamous carcinoma cells were cultured in a 1:1 mixture of DME and F12 media supplemented with 0.4 μ g/ml hydrocortisone. All cultures were supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin (all from Invitrogen). For RNAi, double-stranded oligonucleotides encoding short hairpin RNAs (shRNAs) targeting the human CD151 mRNA were annealed and then cloned into the pSIREN-RetroQ retroviral vector (BD Biosciences). Three shRNAs targeting different sequences were tested: the sh1 targeting sequence is TGCTGGAGAT-CATCGCTGGTA; the sh2 targeting sequence is GCGAGACCATGCTC-CAACAT, and the sh3 targeting sequence is AGTACCTGCTGTTTAC-CTACA. These constructs were cotransfected with the pVSV-G retroviral coat protein expression vector into GP2-293 packaging cells using the calcium phosphate method. At 48 h and again at 96 h after transfection, virus-containing medium was collected, 0.45 μ m filtered, supplemented with 4 μ g/ml polybrene (Sigma-Aldrich), and then used to transduce A431 cells. Stable A431 transductants were selected with 3 μ g/ml puromycin (Sigma-Aldrich) and maintained in 0.1 μ g/ml puromycin.

For CD151 reexpression experiments in A431 sh3 cells, recombinant PCR was used to construct a CD151 cDNA bearing two silent mutations within the sh3 targeting sequence. This CD151 cDNA, which we named CD151 Rx, was cloned into the LXIN retroviral vector and cotransfected with the pVSV-G retroviral coat protein vector into GP2-293 packaging cells, as above. The resulting virus-containing medium was used to transduce PT67 retroviral packaging cells, and stably transduced cells were selected with 0.5 mg/ml G418 (Invitrogen). Virus-containing medium from these stably-transduced PT67 cells was then used to transduce A431 sh3 cells, which were selected with 0.5 mg/ml G418 while maintaining selection with 0.1 μ g/ml puromycin to retain the sh3 shRNA retroviral vector. Restoration of CD151 expression in these cells, which we named A431 sh3 Rx, was confirmed by flow cytometry.

Immunoprecipitation and Immunoblotting

A431 cells were lysed by scraping into 20 mM HEPES, pH 7.5, 150 mM NaCl, 5 mM MgCl₂ (HBSM) supplemented with 1% detergent, and protease inhibitors (2 mM PMSF, 10 μ g/ml aprotinin, 5 μ g/ml leupeptin, and 5 μ g/ml E-64; Roche Diagnostics). Detergents were Brij 96V or Triton X-100 (both from Sigma-Aldrich). In some experiments, cells were biotinylated with 0.1 mg/ml Sulfo-NHS-LC biotin (Pierce Biotechnology) in HBSM for 1 h at room temperature and then rinsed three times with HBSM before lysis. After a 1-h extraction at 4°C with rocking, insoluble material was removed by centrifugation at 16,000 $\times$ g for 15 min, and lysates were precleared for 1 h at 4°C with protein G-Sepharose (Pierce Biotechnology) and then centrifuged as before. Protein concentrations in lysates of different cell types were normalized according to the results of a BCA assay (Pierce Biotechnology), specific antibodies plus protein G-Sepharose were added, and immune complexes were collected overnight at 4°C. After rinsing four times with lysis buffer,

immune complexes were eluted by boiling in sample buffer, resolved by SDS-PAGE, and transferred to nitrocellulose. Blots were blocked with 5% nonfat milk in PBST (PBS with 0.1% Tween-20) and probed with one of the following antibodies, diluted in blocking buffer: 8A12 anti-EWI-2 ascites (1:2000), C9-BB anti-CD9 (10 μ g/ml), biotinylated M38 anti-CD81 (10 μ g/ml), or D23 polyclonal anti- α 3 integrin (5 μ g/ml). Blots were developed with HRP-goat anti-mouse or HRP-goat anti-rabbit secondary antibodies (1:6000) followed by chemiluminescence (Western Lightning reagent, Perkin Elmer-Cetus, Norwalk, CT). Biotinylated proteins were detected with Extravidin-HRP (Sigma-Aldrich), diluted 1:2000 in PBST, followed by chemiluminescence.

Flow Cytometry and Cell Sorting

A431 cells were stained on ice for 1 h with negative control or specific primary antibodies at 5 μ g/ml in blocking buffer (PBS with 10% heat-inactivated goat serum and 0.02% sodium azide). After three rinses in cold PBS, cells were stained on ice for 45 min with a Cy2-conjugated goat anti-mouse secondary antibody (Jackson ImmunoResearch) diluted 1:200 in blocking buffer. After three additional rinses, cells were analyzed on a FACScan flow cytometer (Becton Dickinson, Lincoln Park, NJ). For cell sorting, sodium azide was omitted during staining, and cells were sorted on a fluorescence-activated cell sorting (FACS) DiVa instrument (Becton Dickinson).

Time-Lapse Motility Assays

A431 cells were plated at 5×10^5 cells per 25 cm² flask on day 1 and starved overnight on day 2 in SFM (DME with 5 mg/ml cell culture grade BSA; Sigma-Aldrich) and 25 mM HEPES, pH 7.2). On day 3, cells were harvested by treating with trypsin-EDTA for 3 min at 37°C and collected in SFM supplemented with 0.1 mg/ml soybean trypsin inhibitor and 20 μ g/ml DNase I (Worthington Biochemical, Lakewood, NJ). After centrifugation, cells were resuspended in SFM, and 2.3×10^5 cells were plated to 35-mm dishes that had been coated overnight with 1 μ g/ml laminin-5, 40 μ g/ml collagen I, or 50 μ g/ml fibronectin. In some experiments, function blocking antibodies were added to 10 μ g/ml for 10 min before plating cells in the continued presence of the antibodies. Cultures were maintained on a Leica DMIRE2 inverted microscope (Deerfield, IL) in a stage incubator (20/20 Technology, Wilmington, NC) providing a humidified, 5% CO₂, 37°C atmosphere. OpenLab software (Improvision, Lexington, MA) running on an Apple iMac computer controlled illumination and image acquisition (Cupertino, CA). After allowing 10 min for cells to settle, images were acquired at a rate of 1 frame/min for 3–6 h, using a Hamamatsu ORCA-285 CCD camera (Bridgewater, NJ) and a 20 $\times$ C Plan phase objective. NIH Image 1.63 software was used to record the XY position of cell centroids in frames 10 min apart. Custom Java software (code available upon request) was then used to calculate for each cell 1) the distance traveled in each 10-min interval, 2) cumulative distance traveled versus time, 3) total distance traveled, and 4) mean velocity. In initial quantification of the assays, every cell in each field was followed for as long as it remained in view. All cells that could be tracked for at least 1 h were included in the final calculation of velocity. Subsequently, cells that remained free of cell–cell contact for at least 1 h were analyzed separately during contact-free intervals. In addition, selected videos were analyzed hour-by-hour to determine whether average velocities varied during the 3-h assay. To measure directional persistence, cells were allowed to attach and begin migrating for 1 h. The ratio of net migration distance divided by total migration distance for all the individual cells in the field was then calculated during two subsequent 1-h intervals. This ratio could range from 1.0 (for cells that moved unidirectionally for a whole 1-h interval) to 0.0 (for cells that arrived precisely back at their starting location at the end of a 1-h interval). Only cells that remained in view for an entire 1-h interval were used in the calculations.

To quantify lateral and trailing edge morphology, movies were scanned manually frame by frame for the appearance and persistence of lateral and trailing edge adhesive contacts, defined operationally as a region of cell–substratum contact that became visually distinct from the remainder of the lateral and trailing edges (see Figure 6A). An event was defined as an adhesive contact that persisted for ≥ 4 min, and the number of events per cell and the mean duration of the events was determined by analyzing the behavior of every cell in the movie field over the 3-h duration of the movie. Four movies per cell type were analyzed.

Detergent Solubility Experiments

Cells, 1.5×10^6 , were seeded into the wells of six-well plates. The next day, cells were biotinylated as above, rinsed with HBSM, and gently overlaid with ice-cold 1% Brij 98 (Sigma-Aldrich) in HBSM with protease inhibitors. Cells were extracted for 20 min on ice, with gentle swirling every 5 min, and then the Brij 98 extracts were removed to separate tubes. Next, Brij 98–insoluble material was collected by scraping the cell layers into 1% Triton X-100, 0.1% SDS in HBSM with protease inhibitors and extracting for 1 h at 4°C. All extracts were then clarified and precleared with protein G-Sepharose as described above, before α 3 β 1 integrin immunoprecipitation. Immunoprecipitates were resolved by SDS-PAGE, blotted with Extravidin-HRP, and visualized by chemiluminescence. For each well, the ratio of Brij 98–soluble to Brij 98–insoluble α 3 β 1 was determined by semiquantitative densitometry of transilluminated films using GeneTools software (Corvallis, OR) on digitized images

captured with a CCD camera in a GeneFlash imaging cabinet (SYNGENE, Frederick, MD).

α 3 β 1 Integrin Internalization Experiments

Internalization and recycling assays were performed as described (Fabbri *et al.*, 1999), with minor modifications. Cells were starved overnight in SFM and harvested as in time-lapse motility assays above. Cells, 5×10^5 , were plated in 35-mm dishes that had been coated with 1 μ g/ml laminin-5 and blocked with 5 mg/ml BSA. Cells were returned to the 37°C 5% CO₂ incubator for 45 min to allow cells to attach and begin migrating. Dishes were then placed on ice, and cells were rinsed twice with ice-cold HBSM before labeling for 45 min on ice with 0.5 mg/ml Sulfo-NHS-SS-biotin (Pierce Biotechnology). After rinsing twice with cold HBSM, individual dishes were returned to the 37°C incubator at staggered time points for total periods ranging from 0 to 30 min. All dishes were then returned to ice and rinsed once with ice-cold HBSM to stop any further trafficking. To strip biotin from labeled proteins remaining on the cell surface, cells were treated twice for 20 min with 42 mM glutathione (reduced form), 75 mM NaCl, 75 mM NaOH, and 1% BSA. One dish for each cell type was left untreated for measuring total labeled α 3 β 1 integrin. Cells were rinsed two more times with cold HBSM and then lysed in 1% Triton X-100 in HBSM with protease inhibitors, as described above. Integrin α 3 was then immunoprecipitated, visualized with Extravidin-HRP and chemiluminescence, and analyzed with semiquantitative densitometry, as above. In a separate trial, samples were prepared, electrophoresed, and transferred to nitrocellulose as described above. Samples were then visualized by blotting with 1) the D23 polyclonal anti- α 3 integrin antibody followed by Alexa 680–conjugated goat anti-rabbit secondary, and 2) IRDye 800–conjugated avidin (Rockland, Gilbertsville, PA). The blot was imaged and quantified using the 700- and 800-nm channels of a LiCor infrared fluorescence gel imaging system (Li-Cor, Lincoln, NE), and the ratio of biotinylated α 3 signal to total α 3 signal was calculated.

Immunostaining

A431 cells were starved overnight in SFM, as for time-lapse assays, and then plated in SFM on acid-washed glass coverslips coated with 1 μ g/ml laminin-5. Cells were allowed 1 h to attach, spread, and begin migrating and then fixed 15 min with 10% Formalin (Sigma-Aldrich) in PBS with 2 mM MgCl₂ and 4% sucrose. Cells were rinsed with Tris-buffered saline, pH 8, and then blocked with 10% heat-inactivated goat serum in PBS. Cells were then stained with 2 μ g/ml primary antibody in blocking buffer, rinsed, and stained with Cy2 goat anti-mouse secondary antibody, as for flow cytometry. Alternatively, for colocalization experiments, cells were permeabilized with 0.1% saponin during the blocking step and then stained with D23 polyclonal anti- α 3 integrin and 5C11 monoclonal anti-CD151 antibodies, followed by Alexa 594 goat anti-rabbit and Cy2 goat anti-mouse secondary antibodies. Coverslips were mounted in FluorSave reagent (Calbiochem) and photographed with the video system described above. Positive and negative controls were imaged with identical parameters and processed identically with Adobe Photoshop 7.0 (San Jose, CA). Image overlays for colocalization were performed with Adobe Photoshop.

Adhesion Assays

Substrates for adhesion assays were 1 μ g/ml laminin-5, 40 or 5 μ g/ml collagen I, 50 or 5 μ g/ml fibronectin, 20 μ g/ml vitronectin, 100 μ g/ml poly-L-lysine (PLL; positive control) or 10 mg/ml heat-inactivated (HI) BSA (negative control). After overnight coating, wells were rinsed and blocked with 10 mg/ml HI BSA. Cells were starved overnight, harvested as for time-lapse motility assays, and resuspended at 3×10^5 cells per ml in serum-free medium, with or without 20 μ g/ml specific function blocking antibodies. After allowing 10 min for antibody binding, 100 μ l of cell suspension was plated to each of four substrate-coated wells per condition in a 96-well plate. After various times at 37°C, 5% CO₂, wells were rinsed three times using a multichannel pipette with warm DME, 40 mM HEPES, pH 7.2. Positive control PLL wells were gently rinsed once. Cells remaining after rinses were fixed for 30 min at room temperature with warm 10% Formalin in PBS with 2 mM MgCl₂ and then stained for 20 min at room temperature with freshly filtered 0.1% crystal violet in ddH₂O. Cells were destained by rinsing three times with tap water, air-dried 10 min, and solubilized overnight with 100 μ l/well of 1% Triton X-100 in ddH₂O. Absorbance at 595 nm was determined with a plate reader. For each cell type, adhesion was expressed as fraction of input cells using absorbances in positive control PLL wells to determine total input. Values for total cells input always varied by <10% for the different cell types.

RESULTS

Efficient Silencing of CD151 Expression by RNAi

To establish a transformed cell line silenced for CD151, we transduced A431 epidermoid carcinoma cells with retroviral vectors encoding shRNAs targeting three different sites on

Figure 1. Efficient silencing of CD151 expression in A431 epidermal carcinoma cells. A431 cells were transduced with retroviral vectors encoding three different CD151 shRNA constructs. Stably transduced cells were selected for 2 wk in puromycin and maintained as uncloned populations. (A) CD151 expression in wild-type parental A431 cells and A431 cells bearing CD151 shRNA constructs was evaluated by flow cytometry. Isotype neg., background staining of wild-type cells with nonimmune mouse IgG1; A431 WT, staining of wild-type cells with 5C11 anti-CD151 antibody; A431 sh1-sh3, staining of cells bearing different CD151 shRNA constructs with the 5C11 antibody. The sh3 construct efficiently silenced CD151 expression in >95% of the cells in the A431 sh3 population. (B) A431 wild-type, sh1, sh2, and sh3 cells were lysed in 1% Triton X-100 and lysate protein concentrations were normalized by BCA assay. CD151 (lanes 1–4) or $\alpha 3$ integrin (lanes 5–8) were immunoprecipitated with the 5C11 antibody or the A3-X8 anti- $\alpha 3$ integrin antibody, followed by blotting with the 11B1.G4 anti-CD151 mAb. (C) The same cell populations as in B were cell surface-labeled with biotin and extracted in 1% Triton X-100 followed by CD151 or $\alpha 3\beta 1$ immunoprecipitation. Biotin-labeled $\alpha 3\beta 1$ integrin was detected by blotting with Extravidin-HRP. Bottom panel in lanes 5–8 shows a shorter exposure time.

the CD151 mRNA. After selecting stably transduced cells, we evaluated CD151 expression in the uncloned cell populations by flow cytometry (Figure 1A and Table 1). Compared with wild-type control cells, cell lines bearing two of the CD151 shRNAs (sh1 and sh2) displayed no decrease in CD151 expression; however, cells bearing the third shRNA (sh3) displayed a near total loss of cell surface CD151.

Conceivably, cell surface expression of CD151 could be more sensitive to suppression by RNAi than total CD151 expression. To test this, we immunoprecipitated total CD151 from Triton X-100 cell lysates and immunoblotted for CD151 (Figure 1B). As with cell surface expression, total levels of CD151 in wild-type, sh1, and sh2 cells appeared comparable (lanes 1–3), whereas CD151 was almost completely extinguished in sh3 cells (lane 4).

Table 1. Flow cytometry of CD151 shRNA cell lines

Cell type	CD151		$\alpha 3$ Integrin		$\alpha 6$ Integrin		$\alpha 2$ Integrin	
	Mfi ^a	% Pos ^b	Mfi	% Pos	Mfi	% Pos	Mfi	% Pos
Wild type	13.8	91	159	100	141	100	139	100
CD151 sh1	12.3	86	143	100	130	100	122	100
CD151 sh2	14.8	92	187	100	140	100	159	100
CD151 sh3	1.2	5	143	100	128	100	142	100

^a Mean fluorescence intensity after subtraction of background fluorescence from a nonimmune negative control antibody.

^b Percentage of cells with fluorescence intensity above background.

Given the unusually stable interaction of CD151 with $\alpha 3\beta 1$ integrin (Yauch *et al.*, 1998), it also seemed possible that RNAi might not deplete the pool of CD151 associated with $\alpha 3\beta 1$ to the same extent as it depleted total CD151. However, immunoblotting CD151 in $\alpha 3\beta 1$ immunoprecipitates revealed that only a trace of CD151 was present in $\alpha 3\beta 1$ immunoprecipitates from sh3 cells (Figure 1B, lane 8). In contrast, the amount of CD151 associated with $\alpha 3\beta 1$ was comparable in wild-type, sh1, and sh2 cells (lanes 5–7). Examining $\alpha 3\beta 1$ coprecipitation with CD151 from Triton X-100 extracts of cell surface-biotinylated cells yielded similar results. Comparable amounts of $\alpha 3\beta 1$ coprecipitated with CD151 in wild-type, sh1, and sh2 cells (Figure 1C, lanes 1–3), whereas very little $\alpha 3\beta 1$ could be detected in a CD151 immunoprecipitate of sh3 cells (lane 4). This last experiment also revealed little if any difference in cell surface $\alpha 3\beta 1$ expression in any of the cell types (lanes 5–8; see bottom panel for a lighter exposure), a result that was confirmed by flow cytometry (Table 1). Likewise, no differences were observed in cell surface expression of $\alpha 6\beta 4$ integrin, another major CD151 partner, or $\alpha 2\beta 1$ integrin, whose association with CD151 is detected in milder detergent conditions (Sincov *et al.*, 1999; Table 1). These data indicated that total, cell surface, and $\alpha 3\beta 1$ -associated pools of CD151 were efficiently silenced in sh3 cells without affecting expression of CD151's weakly or strongly interacting integrin partners.

Loss of CD151 Markedly Impairs $\alpha 3\beta 1$ -dependent Motility on Laminin-5

Because $\alpha 3\beta 1$ and $\alpha 6\beta 4$ both associate with CD151 and both serve as receptors for laminin-5, we first tested the relative contributions of these two integrins to A431 cell motility on laminin-5. In time-lapse videomicroscopy assays, untreated wild-type A431 cells displayed rapid, spontaneous migra-

tion in response to laminin-5 (Figure 2A). An anti- $\alpha 3$ integrin function blocking antibody almost completely abrogated A431 cell motility on laminin-5 (Figure 2B), whereas an anti- $\alpha 6$ function blocking antibody had no obvious effect (Figure 2C), suggesting that $\alpha 3\beta 1$ integrin is primarily responsible for A431 cell motility on laminin-5.

The loss of CD151 in A431 sh3 cells resulted in a striking, $\sim 50\%$ reduction in the velocity of $\alpha 3\beta 1$ -dependent cell motility on laminin-5 (compare Figure 2, A and D; see also Supplementary Video, Figure 2.mov). Because it appeared that A431 sh3 cells may have been slower to adhere fully and spread on laminin-5 (Figure 2.mov), it seemed possible that part of their reduced velocity in our 3-h assay was due to delayed cell spreading at the beginning of the assay. To test this, we compared velocity of wild-type and sh3 cells hour by hour. As shown in Figure 2E, sh3 cells showed the same reduced velocity in hours 2 and 3 as they did in the first hour of the assay. This indicated that delayed attachment and spreading was unlikely to be the major factor in the reduced velocity of A431 sh3 cells. In our initial assays, every cell in each field was measured for as long as it remained in the field, regardless of whether it came into contact with other cells. During the 3-h time course of the assay, cell collisions were almost never observed to result in permanent cell-cell adhesion (i.e., cells collided, but then continued moving without becoming permanently attached to each other). However, to rule out that altered behavior on cell-cell contact was a major contributing factor in the reduced velocity of A431 sh3 cells, we compared the velocities of isolated

wild-type and sh3 cells during intervals that were free of any visible cell-cell contact. As shown in Figure 2F, in the absence of cell-cell contact, we obtained results indistinguishable from results obtained by our initial measurements. These data indicated that the reduced velocity of A431 sh3 cells was due to an altered cellular response to laminin-5, not altered cell-cell interactions.

In several independent trials, wild-type, sh1, and sh2 cells all showed similar, rapid migration on laminin-5, whereas sh3 cells consistently migrated about half as rapidly (Figure 2G). These data provided evidence that the reduced velocity of the sh3 cells was indeed the result of reduced CD151 expression and not the result of nonspecific effects from transduction with retroviral RNAi vectors. To confirm that reduced sh3 cell velocity on laminin-5 reflected altered $\alpha 3\beta 1$ function, we performed several additional assays in the presence of the function-blocking anti- $\alpha 6$ integrin antibody, GoH3. As shown in Figure 2H, these experiments yielded results very similar to the results in Figure 2G, with sh3 cells displaying an $\sim 40\%$ reduction in velocity. Although the velocity of wild-type cells treated with GoH3 appeared modestly reduced ($\sim 15\%$) compared with the velocity of untreated wild-type cells in Figure 2G, this difference did not rise to the level of statistical significance ($p = 0.14$, unpaired t test). Together with the data in Figure 2B, these data indicated that A431 cell motility on laminin-5 is strongly $\alpha 3\beta 1$ -dependent and that reduced sh3 velocity on laminin-5 was likely to be largely due to altered $\alpha 3\beta 1$ integrin function.

Figure 3. CD151-positive A431 sh3 escapers show wild-type motility on laminin-5. (A) A431 sh3 cells were stained with anti-CD151 antibody, 5C11, and subjected to fluorescence-activated cell sorting. The gates used to isolate CD151-silenced cells (NEG SORT) and CD151-positive cells (ESCAPERS) are indicated. (B) Velocities of CD151-silenced A431 sh3 cells and CD151-positive A431 sh3 escapers were measured by time-lapse microscopy, as for Figure 2. Significantly faster than CD151-silenced cells; * $p < 0.0001$, unpaired t test. Results are representative of two separate trials that produced similar results.

The ability of sh1 and sh2 cells to migrate as rapidly as wild-type cells helps to rule out nonspecific effects of our retroviral RNAi vector system as the source of impaired migration in sh3 cells, but as a further control, we used cell sorting to isolate a small population of A431 sh3 cells that retained wild-type CD151 expression levels (Figure 3A). These CD151-positive sh3 “escapers” migrated with wild-type velocity, whereas sh3 cells selected for no CD151 staining above background continued to show the same reduced velocity observed in prior experiments (Figure 3B). These data provided further evidence that the loss of CD151 is specifically responsible for the reduced motility of the A431 sh3 cells.

Loss of CD151 Disrupts $\alpha 3\beta 1$ Association with Tetraspanin-enriched Microdomains

To begin to explore underlying changes in $\alpha 3\beta 1$ biochemistry that might explain altered $\alpha 3\beta 1$ function in CD151-silenced cells, we examined $\alpha 3\beta 1$ association with TEMs in A431 wild-type, sh1, sh2, and sh3 cell populations. A working hypothesis is that, by associating with CD151, $\alpha 3\beta 1$ is localized to TEMs by virtue of CD151’s ability to engage in tetraspanin–tetraspanin interactions. Support for this hypothesis comes from studies of Daudi B lymphoma cells, which naturally lack CD151 (Charrin *et al.*, 2003b), and from cells overexpressing palmitoylation-deficient mutants of CD151 with impaired ability to associate with other tetraspanins (Berdichevski *et al.*, 2002; Yang *et al.*, 2002).

To assess $\alpha 3\beta 1$ association with TEMs, we used coimmunoprecipitation to examine $\alpha 3\beta 1$ interactions with tetraspanins CD9 and CD81 and the IgSF protein EWI-2, a major CD9 and CD81 partner (Clark *et al.*, 2001; Charrin *et al.*, 2003a; Stipp *et al.*, 2003a). These molecules were selected as repre-

sentatives of TEMs because we previously showed that they form functionally relevant complexes with $\alpha 3\beta 1$ integrin in the A431 cell environment (Stipp *et al.*, 2003a). As shown in Figure 4A, $\alpha 3\beta 1$ integrin lost association with EWI-2 (lane 4, middle panel) and CD81 (lane 4, bottom panel) in CD151-silenced sh3 cells, but not in wild-type, sh1, or sh2 control cells (lanes 1–3). In a separate experiment, we also confirmed that $\alpha 3\beta 1$ association with CD9 is disrupted in sh3 cells (Figure 4B, bottom panel, lane 3; note, samples are out of order), but not in wild-type, sh1, or sh2 cells (lanes 1, 2, and 4). Recovery of $\alpha 3$ integrin itself was similar in all cell types (Figures 4, A and B, top panels). These data indicated that $\alpha 3\beta 1$ association with CD9/CD81-containing TEMs is disrupted in the absence of CD151.

In an earlier study, a CD151 mutant lacking palmitoylation sites simultaneously lost association with other tetraspanins and displayed significantly reduced detergent extractability (Yang *et al.*, 2002), suggesting that proteins that lose association with TEMs may become more difficult to extract with moderately stringent detergents. Therefore, we tested whether $\alpha 3\beta 1$ detergent extractability was diminished in CD151-silenced A431 sh3 cells. We first extracted biotinylated A431 wild-type and sh3 cells for 20 min on ice with 1% Brij 98 detergent and then recovered Brij 98-insoluble material by extracting a second time with 1% Triton X-100, 0.1% SDS. Next, we immunoprecipitated $\alpha 3\beta 1$ from Brij 98 soluble and insoluble fractions. As shown in Figure 4C, significantly less $\alpha 3\beta 1$ could be extracted by Brij 98 from A431 sh3 cells than from wild-type cells. Semiquantitative analysis of three separate determinations revealed a highly reproducible 33% decrease in the ratio of Brij 98-soluble to Brij 98-insoluble $\alpha 3\beta 1$ in sh3 cells (Figure 4D). These data provided additional evidence that $\alpha 3\beta 1$ association with TEMs is disrupted upon loss of CD151.

Impaired Adhesion of CD151-silenced Cells at Early Time Points on Laminin-5

In a recent report, partial siRNA knockdown of CD151 was reported to inhibit cell adhesion on the $\alpha 3$ integrin ligand, laminin-10 (Nishiuchi *et al.*, 2005). Therefore, we tested whether our CD151-silenced cells displayed impaired adhesion on laminin-5. A431 wild-type and sh3 cells were plated on ice in laminin-5-coated wells, allowed to settle, and then placed at 37°C for varying amounts of time. As shown in Figure 5A, after 20 min at 37°, both wild-type and sh3 cells displayed adhesion above background, but adhesion of sh3 cells was modestly but significantly impaired (~30% reduction in specific adhesion above background for sh3 cells). After 30 min, adhesion of both cell types had increased, but sh3 cells continued to display a modest, but significant reduction in adhesion (~18% reduction in specific adhesion above background).

In contrast to the results from short-term assays, the difference between wild-type and sh3 cell adhesion to laminin-5 vanished in extended 2-h adhesion assays (Figure 5B). Interpretation of these extended assays was complicated by the fact that adhesion of both cell types to BSA increased significantly after 2 h (Figure 5B). However, adhesion in BSA control wells after 2 h could be attributed to laminin-5 endogenously produced and deposited by A431 cells, as evidenced by laminin-5 immunostaining (Figure 5C) and by the fact that it could be abolished by a combination of anti- $\alpha 3$ and $\alpha 6$ integrin function-blocking antibodies (Figure 5B). Interestingly, A431 sh3 cell adhesion to this endogenously generated laminin-5 was also diminished compared with wild-type cell adhesion (Figure 5B). Lastly, adhesion

Figure 4. Silencing CD151 disrupts $\alpha 3\beta 1$ integrin association with tetraspanin-enriched microdomains. (A) A431 wild-type, sh1, sh2, or sh3 cells were lysed in Brij 96, and $\alpha 3$ integrin was immunoprecipitated with the A3-X8 antibody. The amount of $\alpha 3$ in each immunoprecipitate was assayed by blotting with the D23 anti- $\alpha 3$ polyclonal antibody, and the amounts of coprecipitating EWI-2 or CD81 were assayed by blotting with 8A12 or biotinylated M38 monoclonal antibodies, respectively. (B) Cells were extracted with Brij 96, and $\alpha 3$ integrin was immunoprecipitated as in A. $\alpha 3$ integrin was blotted with the D23 polyclonal antibody, and coprecipitating CD9 was assayed by blotting the C9BB mAb. (C) Equal numbers of A431 wild-type and sh3 cells were cell surface-labeled with biotin and then extracted on ice with Brij 98 for 20 min. The Brij 98-soluble fraction was gently removed, and Brij 98-insoluble material was recovered by extracting a second time with 1% Triton X-100, 0.1% SDS. $\alpha 3\beta 1$ integrin was immunoprecipitated from each fraction with the A3-X8 mAb, followed by blotting with Extravidin HRP. (D) Results of three separate experiments performed as in C. Data are presented as the mean ratio of Brij 98 soluble to Brij 98 insoluble material $\pm$ SEM. Significantly different from wild type; * $p < 0.0001$, unpaired t test.

after 2 h to wells coated with exogenous laminin-5 was also abolished by anti- $\alpha 3$ and $\alpha 6$ integrin antibodies (Figure 5B), indicating that $\alpha 3/\alpha 6$ integrin-independent ligands had not been deposited in appreciable amounts on the laminin-5 substrate. Thus, sh3 cell adhesion to laminin-5 was initially reduced, but after 2 h it became indistinguishable from wild-type adhesion. In addition, A431 cells produce their own laminin-5, and sh3 cell adhesion on this endogenous laminin-5 is also reduced compared with wild-type cells.

Altered Lateral and Trailing Edge Morphology in A431 sh3 Cells Migrating on Laminin-5

Because A431 sh3 cells adhered as well as wild-type cells to laminin-5 after 2 h, yet continued to display impaired laminin-5-dependent motility at this time point (Figure 2E), we examined motility of sh3 cells on laminin-5 in greater detail. We noticed that sh3 cells migrating on laminin-5 frequently exhibited persistent protrusions at their lateral and trailing edges, in contrast to the typically smooth trailing edges of wild-type A431 cells (Figure 6A, white arrows, see also Supplementary Video, Figure 6.mov). As sh3 cells continued to move, these protrusions appeared to come under significant tension, snapping back into the cell soma upon release, suggesting that they had been maintained by adhesive contact with the substrate. Quantification revealed that the frequency of apparent lateral and trailing edge adhesive events that persisted 4 min or longer was increased nearly twofold in sh3 cells compared with wild-type cells (Figure 6B). Furthermore, the mean lifetime of such events increased by 67%, from 6.5 min in wild-type cells to 10.7 min in sh3 cells (Figure 6C). As seen in Figure 6.mov (and in Figure 2.mov), in conjunction with persistent lateral and trailing edge ad-

hesions, sh3 cells frequently appeared to change their direction of migration. We therefore tested whether directional persistence (defined as net migration distance divided by total migration distance) was reduced for sh3 cells. As shown in Figure 6D, sh3 cells displayed a 25–44% reduction in directional persistence in two independent trials. Collectively, these data suggested that the decreased migration rate observed for sh3 cells might be due in part to altered $\alpha 3\beta 1$ function at the lateral and trailing edges of the cells.

We next compared $\alpha 3\beta 1$ immunolocalization in wild-type and CD151-silenced cells. In unpermeabilized cells, $\alpha 3$ appeared widely distributed over the entire surface of both wild-type and sh3 cells (Figures 7, A and B). CD151 staining was also widely distributed on the surface of wild-type cells (Figure 7C), whereas sh3 cells appeared completely negative (Figure 7D). To study $\alpha 3$ integrin and CD151 distribution simultaneously, we double-labeled permeabilized cells with a CD151 mAb and an anti- $\alpha 3$ cytoplasmic tail polyclonal antibody. In wild-type cells, $\alpha 3$ again appeared widely distributed, with somewhat brighter staining of portions of the trailing edge and leading lamellipodia (Figure 7E). In sh3 cells, the brightest $\alpha 3$ staining was observed in lateral and trailing edge protrusions (Figure 7F). CD151 staining in permeabilized wild-type cells was again widely distributed (Figure 7G). A fraction of CD151 staining codistributed with $\alpha 3$ staining at the trailing edge of wild-type cells (Figure 7I, arrow). In permeabilized sh3 cells, a few cells displayed low levels of residual CD151 expression (Figure 7H), and this codistributed with $\alpha 3$ integrin in lateral/trailing edge protrusions (Figure 7J, arrow). Various cross-reactivity and negative controls revealed a very low level of background staining (Figure 7, K–M). Because both CD151 and $\alpha 3$ integrin are

the 6F12 anti-laminin-5 mAb, followed by Cy2-conjugated goat anti-mouse secondary antibody. Left panels, DIC images; right panels, laminin-5 staining. Negative control staining for this experiment was shared with the experiment in Figure 7 (see Figure 7M).

widely distributed on the cell surface, precise colocalization will require high-resolution confocal analysis; however, these data were consistent with the possibility that CD151 regulates $\alpha 3 \beta 1$ integrin function at the lateral/trailing edge of migrating cells.

Impaired $\alpha 3 \beta 1$ Trafficking in A431 sh3 Cells

The elevated frequency and duration of lateral and trailing edge adhesive contacts in A431 sh3 cells suggested that $\alpha 3 \beta 1$ trafficking might be altered in these cells. Given the previously described localization of CD151 to endocytic compartments (Sincock *et al.*, 1999), and mounting evidence of the importance of integrin recycling for cell motility and invasion (Lawson and Maxfield, 1995; Pierini *et al.*, 2000; Powelka *et al.*, 2004; Strachan and Condic, 2004; Ivaska *et al.*, 2005; Yoon *et al.*, 2005), we hypothesized that $\alpha 3 \beta 1$ internalization might be impaired in cells lacking CD151. To test this, we allowed wild-type and sh3 A431 cells to attach and spread on laminin-5, labeled them on ice with reducible biotin, and then returned them to 37°C to allow migration to resume. At specific time points, individual dishes were treated with cell impermeable alkaline glutathione to strip the biotin from proteins residing on the cell surface, cells were lysed, and internalized $\alpha 3 \beta 1$ integrin retaining the biotin label was recovered by immunoprecipitation. As shown in Figure 8A, $\alpha 3 \beta 1$ accumulated internally significantly faster in wild-type cells than in sh3 cells. Two independent trials yielded very similar results (Figure 8B). Nonlinear regression analysis of the data using pseudo first-order kinetics produced a good fit for both data sets ($R^2 = 0.99$ and 0.98 for wild-type and sh3, respectively). The observed rate constant for $\alpha 3 \beta 1$ in-

ternalization was twofold lower for A431 sh3 cells than for wild-type cells (0.021 min^{-1} vs. 0.041 min^{-1} , respectively).

Because β -tubulin may not be the best control for $\alpha 3$ integrin immunoprecipitations, we repeated the internalization assay a third time and analyzed the results with a Li-Cor imaging system that allows detection and quantification in two separate fluorescent channels. Total $\alpha 3$ integrin was detected with an anti- $\alpha 3$ polyclonal antibody followed by an Alexa-680-conjugated secondary antibody, and biotinylated $\alpha 3$ was simultaneously detected on the same blot with IRdye 800-conjugated streptavidin (Figure 8C). Quantification revealed that the ratio of biotinylated $\alpha 3$ to total $\alpha 3$ increased more rapidly for wild-type cells than for sh3 cells and that the magnitude of the effect was similar to that which we observed in the first two trials (Figure 8D). These data support the view that the laminin-5 motility defect in CD151-silenced sh3 cells results at least in part from decreased efficiency of $\alpha 3 \beta 1$ internalization during migration.

Reexpression of CD151 in A431 sh3 Cells Reverses Defects in $\alpha 3$ and $\alpha 6$ Integrin Functions

A critical issue in RNAi experiments is the potential for off-target effects. To confirm that the phenotypes of our CD151-silenced cells were indeed due to the specific loss of CD151 expression, we performed reconstitution experiments. We first confirmed that our CD151-silenced cells, which had been sorted to be free of CD151-positive "escapers" (Figure 3), had not reexpressed CD151 over the intervening 23 wk (Figure 9A). We then transduced these cells with a CD151 retroviral expression vector (CD151 Rx), containing two silent mutations within the region targeted by

Figure 6. Lateral and trailing edge defects in CD151-silenced A431 sh3 cells migrating on laminin-5. (A) Individual frames from time-lapse videos comparing morphology of A431 wild-type and sh3 cells migrating on laminin-5. Rapidly migrating wild-type cells typically display smooth trailing edges and a compact but highly active leading lamellipodium. A431 sh3 cells frequently display persistent adhesive contacts that arise at their lateral and trailing edges (white arrows). Elapsed time is indicated to left of each pair of panels. Time 0' in this series corresponds to the 20-min time point in Supplementary Video, Figure 6.mov, which shows a side-by-side comparison of wild-type and sh3 morphology. Lateral/trailing edge adhesive contacts of sh3 cells persist for several minutes and appear to come under tension before snapping back into the cell soma within 1–2 min. (B) The frequency of lateral and trailing edge adhesive events, such as those depicted in A, that lasted 4 min or longer was measured for A431 wild-type and sh3 cells in four separate 3-h videos. Significantly different from wild-type cells; * $p < 0.05$, unpaired t test. (C) The mean duration of lateral and trailing edge adhesive events for A431 wild-type and sh3 cells was measured in four separate 3-h videos. Significantly different from wild-type cells; * $p < 0.01$, unpaired t test. (D) In two independent trials, A431 wild-type and sh3 cells migrating on laminin-5 were tracked for 2 h, and directional persistence for each cell was calculated during each hour. Persistence was defined as the ratio of net distance traveled divided by total distance traveled. The average persistence of sh3 cells was significantly reduced compared with wild-type cells; * $p < 0.03$; ** $p < 0.0001$, unpaired t test.

the CD151 sh3 shRNA construct. These silent mutations defeated RNAi targeting, allowing CD151 to be reexpressed at wild-type levels (Figure 9A). We called these cells A431 sh3 Rx cells. Cell surface labeling experiments confirmed

Figure 7. Localization of $\alpha 3\beta 1$ integrin and CD151. Unpermeabilized A431 wild-type or sh3 cells were stained for $\alpha 3$ integrin with the A3-X8 mAb (A and B) or the 5C11 anti-CD151 mAb (C and D) followed by Cy2 goat anti-mouse secondary antibody. For codistribution studies, cells were permeabilized with 0.1% saponin and stained with the D23 polyclonal anti- $\alpha 3$ integrin antibody, which binds to the $\alpha 3$ cytoplasmic tail, followed by Alexa 594 goat anti-rabbit secondary (E and F) and the 5C11 anti-CD151 mAb followed by Cy2 goat anti-mouse secondary (G and H). Overlays are shown in I and J. Arrows indicate regions of $\alpha 3$ and CD151 codistribution. In negative control experiments, cells were stained with the D23 antibody followed by Cy2 goat anti-mouse (K), 5C11 followed by Alexa 594 goat anti-rabbit (L), or nonimmune mouse IgG followed by Cy2 goat anti-mouse (M).

that the CD151- $\alpha 3\beta 1$ integrin complex, which was virtually absent in A431 sh3 cells, was restored to wild-type levels in the A431 sh3 Rx cells (Figure 9B, lanes 1–3). All three cell types expressed a similar level of $\alpha 3$ integrin on the cell surface (Figure 9B, lanes 4–6).

To test whether reexpression of CD151 in CD151-silenced cells could reverse the effects of CD151 silencing, we first examined cell adhesion on laminin-5. As shown in Figure 9C, first set, adhesion to laminin-5 was moderately but significantly impaired in CD151-silenced cells, as previously observed, but was restored to wild-type levels in A431 sh3 Rx cells. Although our earlier experiments had indicated that motility on laminin-5 was strongly dependent on $\alpha 3$ integrin, $\alpha 6$ integrin is also a major CD151 partner (Kazarov *et al.*, 2002), and thus it seemed likely that CD151 would also contribute to $\alpha 6$ integrin function. Therefore, we tested for $\alpha 6$ integrin contributions to cell adhesion on laminin-5. Although a function-blocking anti- $\alpha 6$ integrin antibody by itself did not measurably reduce adhesion of any of the cell types to laminin-5 (Figure 9C, second set), wild-type parental cells did retain some $\alpha 6$ integrin-dependent adhesion to laminin-5 in the presence of an anti- $\alpha 3$ function blocking antibody (Figure 9C, third set). This $\alpha 6$ -dependent adhesion was completely abolished in A431 sh3 cells but restored in A431 sh3 Rx cells.

The defect in $\alpha 6$ -dependent sh3 cell adhesion was also dramatically illustrated by time-lapse assays of cells plated on laminin-5 in the presence of $\alpha 3$ function-blocking antibody (see Supplementary Video, Figure 9.mov). At the beginning of the assays, which started 15 min after plating, most wild-type cells appeared loosely adherent to the laminin-5 substrate, while many sh3 cells remained nonadherent and were swept along by current flow in the plating medium. At intermediate time points, sh3 cells remained

samples were prepared as in A and B, but biotinylated and total $\alpha 3$ were detected simultaneously on the same blot. Biotinylated $\alpha 3$ was visualized with IRdye 800 avidin and total $\alpha 3$ was visualized with the D23 anti- $\alpha 3$ antibody followed by blotting with Alexa 680 goat anti-rabbit. The blot was analyzed with a Li-Cor infrared gel imager in the 700- and 800-nm fluorescent channels. Lower panel, overlay of two top panels. (D) The blot shown in C was quantified with Li-Cor software, and the ratio of $\alpha 3$ biotin counts to total $\alpha 3$ counts was determined for the 10- and 30-min time points.

loosely adherent and unspread, while many wild-type cells had begun to spread partially, extending short protrusions and/or small lamellipodia. These short protrusions appeared qualitatively different from the persistent lateral/trailing edge protrusions observed in migrating sh3 cells in Figure 6A and Figure 6.mov because 1) they formed circumferentially around the perimeter of the cells, 2) they were not associated with any significant translocation of the cell body, and 3) they were not observed to “snap back” into the soma, as had been observed during sh3 cell migration. Rather, they more closely resembled similar protrusions observed in $\alpha 3$ -null keratinocytes plated on laminin-5 (Choma *et al.*, 2004; see Discussion). Only toward the end of the assay did sh3 cells begin to elaborate protrusions and spread.

Used together, anti- $\alpha 3$ and $\alpha 6$ antibodies blocked adhesion of all cell types on laminin-5 to background levels (Figure 9C, fourth set). Additional time-lapse assays revealed that A431 sh3 Rx cell motility on laminin-5 was restored to a level indistinguishable from that of wild-type cells (Figure 9D). Collectively, these data 1) confirm that adhesion and motility defects in CD151-silenced cells are due specifically to the loss of CD151 and are not the result of off-target effects, 2) reveal CD151 makes a critical contribution to $\alpha 6$ integrin function in cell attachment and spreading on laminin-5, 3) confirm results from Figure 2H that CD151 specifically regulates $\alpha 3$ integrin function when $\alpha 6$ integrin function is blocked, and 4) illustrate how the effect of CD151 silencing is qualitatively different from the loss of $\alpha 3$ function due to antibody blockade (compare wild-type cells in Figure 9.mov to sh3 cells in Figure 2.mov and Figure 6.mov).

Silencing of CD151 Inhibits Migration But Not Adhesion on Non- $\alpha 3\beta 1$ Integrin-dependent Substrates. $\alpha 3\beta 1$ integrin has been reported to participate in motility on a wide variety of

substrates for which it is not the primary receptor and has been proposed to act as a secondary receptor on a number of extracellular matrix proteins (DiPersio *et al.*, 1995). We therefore tested the effects of silencing CD151 expression on adhesion and migration on other extracellular matrix ligands. As shown in Figure 10A, silencing of CD151 had no effect on $\alpha 2\beta 1$ integrin-dependent adhesion to collagen I, αv integrin-dependent adhesion to vitronectin, or $\alpha 5/\alpha v$ integrin-dependent adhesion to fibronectin.

In contrast to the adhesion assays, motility experiments on collagen I and fibronectin did reveal an effect of the loss of CD151 (Figure 10B). Compared with wild-type cells, A431 sh3 cells migrated ~45% slower on collagen I. On fibronectin, the average velocity of A431 sh3 cells was ~38% slower than that of the wild-type cells. Similar results were obtained in two independent trials.

Because we had observed that A431 cells are capable of depositing and adhering to their own laminin-5 (Figure 5), it appeared quite possible that sh3 cell migration defects on collagen I and fibronectin were in fact due to impaired migration on newly deposited laminin-5. However, analysis revealed that sh3 cell velocity on collagen I was equally impaired during the first hour as it was in the 3-h assay as a whole (Figure 10B). This suggested that sh3 cell migration on collagen I was impaired even before significant amounts of laminin-5 might have been deposited. More importantly, similar results on collagen I were obtained in the presence or absence of an anti- $\alpha 3$ integrin function-blocking antibody (Figure 10B), indicating that collagen I migration did not require $\alpha 3$ integrin ligand binding. It is possible that laminin-5 newly deposited by A431 cells is in a form that supports adhesion, but not rapid cell migration, as has been described for laminin-5 secreted by 804G and MCF10A cells (Goldfinger *et al.*, 1998).

Figure 9. Reexpression of CD151 in A431 sh3 cells reverses defects in $\alpha 3$ and $\alpha 6$ integrin functions. A CD151 cDNA (CD151 Rx) containing silent mutations that defeat RNAi was used to restore CD151 expression in A431 sh3 cells (*Materials and Methods* for details). (A) Flow cytometry using the 5C11 anti-CD151 antibody to stain A431 wild-type cells, A431 sh3 cells, or A431 sh3 cells reconstituted with CD151 (Rx). neg. con., wild-type cells stained with an isotype-matched negative control antibody. (B) A431 wild-type, sh3, and sh3 Rx cells were cell surface-labeled with biotin and extracted with 1% Triton X-100. CD151 or $\alpha 3$ integrin were immunoprecipitated with 5C11 or the A3-X8 anti- $\alpha 3$ integrin antibody, respectively, and $\alpha 3\beta 1$ integrin was detected by blotting with Extravidin-HRP. (C) A431 wild-type, sh3, or sh3 Rx cells were plated in laminin-5- or BSA-coated wells, in the presence or absence of 10 $\mu\text{g}/\text{ml}$ function-blocking antibodies. After 25 min, nonadherent cells were removed by rinsing, and adherent cells were stained and quantified as in Figure 5. Function blocking antibodies were anti- $\alpha 6$ integrin, GoH3, and anti- $\alpha 3$ integrin, A3-IIF5. Values represent the means $\pm$ SEM of eight wells per condition from two experiments that yielded very similar results. Values are reported as the fraction of total input cells measured in PLL-coated-wells, as in Figure 5. Significantly different from either wild-type or sh3 Rx cells ($*p < 0.001$) ANOVA with Bonferroni post-tests. The A431 sh3 cell defect in $\alpha 6$ integrin-dependent adhesion shown in the third set of C is also illustrated by time-lapse assay in Figure 9.mov, in which cells treated with A3-IIF5 anti- $\alpha 3$ antibody are plated on laminin-5. (D) A431 wild-type, sh3, and sh3 Rx cells motility on laminin-5 was measured in multiple separate time-lapse assays as in Figure 2 (at least 5 trials per cell type). Significantly different from either wild-type ($*p < 0.001$) or sh3 Rx cells ($*p < 0.05$), ANOVA with Bonferroni post-tests.

Finally, because migration on collagen I and fibronectin was relatively slow, we used comparatively high concentrations of these ligands to stimulate motility. Therefore, it seemed possible that the lack of A431 sh3 cell adhesion defects on these ligands was due to higher concentrations used in adhesion assays. However, even when the plating concentration was reduced 10-fold, sh3 cells still adhered to both substrates just as well as wild-type and Rx cells (Figure 10C). Thus, diminished adhesion is unlikely to contribute to diminished migration velocity of sh3 cells on collagen I or fibronectin. Collectively, these results suggest that CD151 may contribute to motility via both $\alpha 3$ -dependent and $\alpha 3$ -independent mechanisms. Alternatively, they may reflect an $\alpha 3$ contribution to motility that is independent of direct ligand engagement.

Figure 10. CD151 silencing impairs migration but not adhesion on $\alpha 3\beta 1$ integrin-independent substrates. (A) A431 wild-type, sh3, and sh3 Rx cells were plated in wells coated with 40 $\mu\text{g}/\text{ml}$ collagen I (Col I), 20 $\mu\text{g}/\text{ml}$ vitronectin (VN), 50 $\mu\text{g}/\text{ml}$ fibronectin (FN), or BSA, in the presence or absence of various function blocking monoclonal antibodies (10 $\mu\text{g}/\text{ml}$). After 25 min, nonadherent cells were removed by rinsing, and adherent cells were stained and quantified as in Figure 8. Function blocking antibodies were anti- $\alpha 2$ integrin, A2-IIE10, and anti- αv integrin, 69-6-5, and anti- $\alpha 5$ integrin, P1D6. (B) A431 wild-type and sh3 cell motility on collagen I and fibronectin was measured in time-lapse assays as in Figure 2. Velocity on collagen I during the first hour of the assays was also calculated separately (1st h). In additional experiments, velocity on collagen I in the presence of 10 $\mu\text{g}/\text{ml}$ A3-IIF5 anti- $\alpha 3$ integrin function-blocking antibody was measured. Significantly different from wild-type cell motility under the same conditions, $*p < 0.0002$, unpaired t test. Results in B are representative of two independent trials that yielded similar results. (C) A431 wild-type, sh3, and sh3 Rx cells were plated in wells coated with 5 $\mu\text{g}/\text{ml}$ fibronectin or collagen I and adhesion was measured as in A. Results show the mean $\pm$ SEM for 3 independent trials.

DISCUSSION

Silencing of CD151 Markedly Impairs $\alpha 3\beta 1$ Integrin-dependent Carcinoma Cell Motility on Laminin-5

CD151-null keratinocytes display significantly impaired outgrowth from skin explants (Wright *et al.*, 2004), and CD151-null mice display impaired wound healing in vivo (Cowin *et al.*, 2006). However, the CD151 loss-of-function phenotype in transformed cells had not yet been described. Here we show that A431 carcinoma cells in which CD151 has been almost completely silenced display markedly reduced $\alpha 3\beta 1$ inte-

grin-dependent cell motility, consistent with the strong, detergent-resistant CD151- $\alpha 3\beta 1$ interaction (Yauch *et al.*, 1998). CD151 silencing was stable over several months in culture and could be reversed by reexpressing wild-type CD151 from a cDNA containing silent mutations that defeat RNA interference. Thus, this system should be useful for detailed functional analysis of CD151 in transformed cells, upon reconstitution with specific CD151 mutants.

The impact of CD151 silencing on carcinoma cell motility, not only on laminin-5 but also on other substrates, raises the question of why the phenotype of CD151-null mice is not more severe. Potentially, within multivalent tetraspanin-integrin complexes, other tetraspanins may be able to compensate for CD151 during development of CD151-null mice. Another possible explanation is that, although we examined single cell motility on purified extracellular ligands, morphogenesis of multicellular tissues during embryonic development is controlled by multiple, collaborating adhesion systems. Indeed, even $\alpha 3$ and $\alpha 6$ integrin-null mice complete embryonic development, dying as early neonates with defects in various epithelial structures (Hynes, 2002). That CD151-deficient human patients present with lesions in some of these same tissues later in life (Karamatic Crew *et al.*, 2004) may reflect CD151's role as an enhancer of integrin function, whose phenotype is uncovered by multiple rounds of $\alpha 3$ or $\alpha 6$ integrin-dependent tissue repair and maintenance. The relatively overt effects of CD151 overexpression or CD151 antibody blockade on metastatic colonization in vivo (Testa *et al.*, 1999; Kohno *et al.*, 2002) could reflect the importance of single cell motility on basement membrane ligands in in vivo models of tumor cell dissemination.

In a recent study, Garcia-Lopez *et al.* (2005) found that a partial siRNA knockdown of CD151 actually enhanced melanocyte motility toward fibronectin in a transwell assay. However, an inhibitory $\beta 1$ integrin antibody also enhanced motility in their assay. Thus, although the mechanisms controlling melanocyte motility appear different from those controlling carcinoma cell motility in our study, in both systems loss of CD151 produces a result that correlates with impaired $\beta 1$ integrin function.

Silencing of CD151 Disrupts $\alpha 3\beta 1$ Integrin Association with Tetraspanin-enriched Microdomains

We found that $\alpha 3\beta 1$ integrin's ability to associate with TEMs was disrupted in the absence of CD151. These data complement recent observations that 1) CD151 transfection of CD151-negative Daudi B lymphoma cells promotes association of cotransfected $\alpha 3\beta 1$ integrin with other tetraspanins (Charrin *et al.*, 2003b), and 2) palmitoylation-deficient CD151, which has impaired ability to associate with other tetraspanins, has a dominant inhibitory effect on $\alpha 3\beta 1$'s association with TEMs (Berdichevski *et al.*, 2002). Thus, our data contribute to the emerging view that direct *primary* interactions of individual tetraspanins with their major partners allow these major partners to be linked via *secondary* tetraspanin-tetraspanin interactions into extended complexes within TEMs.

Altered $\alpha 3\beta 1$ Integrin Trafficking in CD151-silenced Cells. Several tetraspanins have been implicated in the trafficking of their cell surface partners (Odintsova *et al.*, 2000; Duffield *et al.*, 2003; Stipp *et al.*, 2003a; Hu *et al.*, 2005), but direct evidence linking CD151 to integrin trafficking had not been described. We found that, during cell migration on laminin-5, the $\alpha 3\beta 1$ integrin internalization rate was significantly reduced in CD151-silenced cells. Concurrently, these cells displayed persistent adhesive contacts at their lateral and trailing edges, suggesting that impaired $\alpha 3\beta 1$ trafficking

resulted in reduced efficiency of de-adhesion at the trailing edge of migrating cells. These results are reminiscent of data showing that interfering with $\alpha 5$ and αv integrin trafficking impairs neutrophil migration on fibronectin and vitronectin (Lawson and Maxfield, 1995; Pierini *et al.*, 2000), and that recycling of $\alpha 6$ integrin is important for rapid motility of cranial neural crest cells on high concentrations of laminin-1 (Strachan and Condic, 2004). Previously, $\alpha 3\beta 1$ integrin had been suggested to participate little if at all in recycling (Bretscher, 1992), but this study used cells in suspension in the absence of laminin-5, which may explain why $\alpha 3\beta 1$ recycling was not observed. Indeed, recycling of $\alpha 6$ integrin and the L1 cell adhesion molecule are both strongly influenced by ligand engagement (Kamiguchi and Lemmon, 2000; Strachan and Condic, 2004).

Interestingly, although CD151-silenced A431 cells appeared completely negative for cell surface CD151 (after CD151-positive escapers had been removed by cell sorting), at least some of these cells expressed low levels of residual CD151 detectable upon cell permeabilization. This small, residual pool of CD151 strongly codistributed with $\alpha 3\beta 1$ integrin at lateral/trailing edge adhesive contacts. We suggest that, when CD151 is severely limited, residual CD151 is preferentially allocated to sites of $\alpha 3\beta 1$ accumulation, but remains insufficient to fulfill its role in $\alpha 3\beta 1$ trafficking.

CD151 contains in its C-terminal cytoplasmic tail a YXX Φ motif, which has the potential to interact with AP adaptor complexes that mediate cargo selection during clathrin-dependent endocytosis (Robinson, 2004). The function of the CD151 YXX Φ motif is unknown, but up to 66% of the total cellular CD151 may be localized to intracellular compartments corresponding to endosomes and/or lysosomes (Sincok *et al.*, 1999). This observation led to the proposal that CD151 might regulate the trafficking of its integrin partners (Sincok *et al.*, 1999), an idea for which our new data provide substantial support. CD151's ability to mediate $\alpha 3\beta 1$ association with TEMs may be important for regulating $\alpha 3\beta 1$ trafficking. Within TEMs, $\alpha 3\beta 1$ is linked to both classical PKC isoforms (Zhang *et al.*, 2001a), which may regulate integrin endocytosis and integrin-dependent cell motility (Ng *et al.*, 1999; Zhang *et al.*, 2001b), and type II PI 4-kinases (Yauch *et al.*, 1998; Yauch and Hemler, 2000), which have the potential to regulate a variety of vesicular traffic (Guo *et al.*, 2003; Wang *et al.*, 2003; Salazar *et al.*, 2005).

Relationship between Altered Adhesion, Altered $\alpha 3\beta 1$ Integrin Trafficking, and Altered Migration in CD151-silenced Cells. Our finding that $\alpha 3\beta 1$ integrin-dependent cell adhesion was decreased in CD151-silenced cells might seem to be contradictory to the increased lateral and trailing edge adhesive contacts of these cells during migration on laminin-5. However, initial attachment and spreading on laminin-5 may also depend on proper $\alpha 3\beta 1$ trafficking. Indeed, interfering with the recycling of $\alpha 5\beta 1$ or $\alpha v\beta 3$ integrin (by expressing dominant negative rab4 or constitutively active glycogen synthase kinase-3) inhibits cell spreading and adhesion on their respective ligands, fibronectin and vitronectin (Roberts *et al.*, 2001, 2004).

Nishiuchi *et al.* (2005) recently proposed that CD151 may act by stabilizing the activated conformation of $\alpha 3\beta 1$. Although our results are not necessarily incompatible with this kind of function for CD151, we have observed in preliminary experiments that the stimulatory anti- $\beta 1$ integrin antibody, TS2/16 (Chan and Hemler, 1993), actually inhibits the migration of wild-type A431 cells on laminin-5 and fails to rescue the migration defect of CD151-silenced cells (A. Varzavand and C. Stipp, unpublished data). Perhaps more

importantly, we found that A431 sh3 cells continued to display impaired motility even 2 h after plating, a time point at which their adhesion to laminin-5 was indistinguishable from that of wild-type cells. Thus, promoting $\alpha 3 \beta 1$ ligand binding appears unlikely to be a complete explanation of CD151 function in cell migration. In this regard, the phenotype of CD151-silenced cells appeared quite distinct from that of cells in which $\alpha 3$ function had been blocked with an inhibitory antibody. The short, circumferential protrusions that formed upon antibody blockade of $\alpha 3$ in wild-type A431 cells resembled those previously described for nonmigratory, $\alpha 3$ -null keratinocytes plated on laminin-5 (Choma *et al.*, 2004). In contrast, the lateral/trailing edge protrusions we observed in CD151-silenced cells occurred in cells that were actively migrating. Thus, we propose that CD151 plays an important role in regulating $\alpha 3 \beta 1$ internalization during cell migration in addition to any function it may have in regulating $\alpha 3 \beta 1$ ligand binding. An additional, nonmutually exclusive possibility is that CD151 somehow contributes to the ability of $\alpha 3 \beta 1$ to trigger persistent polarization of migrating cells via activation of the small GTPase, Rac1 (Choma *et al.*, 2004).

Effects of CD151 Silencing on Integrins Other than $\alpha 3 \beta 1$.

Although $\alpha 6 \beta 4$ integrin did not appear to make a strong contribution to A431 cell motility on laminin-5, an $\alpha 6$ contribution to cell adhesion and spreading was uncovered in the presence of an inhibitory anti- $\alpha 3$ integrin antibody, and CD151 made a critical contribution to these $\alpha 6$ functions. The inability of an $\alpha 6$ function-blocking antibody by itself to interfere with adhesion, even though $\alpha 6$ can clearly contribute to adhesion, may seem contradictory. However, similar results have been described for αv integrin, in a study that showed an anti- αv integrin antibody only inhibited adhesion to fibronectin in cells lacking $\alpha 5$ integrin (Yang and Hynes, 1996). Indeed, we obtained a similar result for αv and $\alpha 5$ integrins in Figure 10A, in which inhibiting either integrin alone had little or no effect on adhesion to fibronectin. Clearly, the ability to detect the contribution of individual integrins to cell adhesion can depend on the activity of other integrins with similar ligand binding specificities. In that regard, our data predict that in cell types such as MDA-MB-231 breast carcinoma cells, where $\alpha 6$ integrin contributions to motility are more apparent (Yoon *et al.*, 2005), the loss of CD151 would have profound effects on $\alpha 6$ -dependent migration.

Our data also suggest that, in short-term adhesion assays, there is a correlation between the strength of CD151 interaction with its integrin partners and its ability to influence their function. Thus, CD151-silenced cells displayed defects in $\alpha 3$ and $\alpha 6$ integrin-dependent adhesion, but not in adhesion dependent on $\alpha 2$, $\alpha 5$, or αv integrins, whose interactions with CD151 are only observed in milder detergent conditions. However, we found that loss of CD151 *does* affect cell motility on the $\alpha 2$ integrin ligand, collagen I, and the $\alpha 5 / \alpha v$ integrin ligand, fibronectin. These data contribute to the growing appreciation that tetraspanin-integrin interactions observed in milder detergents can be just as functionally relevant as more detergent-resistant interactions. Although the spectrum of integrins associating with CD151 in milder detergents is broader, it is nonetheless specific because other abundant cell surface proteins, such as PECAM-1, CD71, CD34, CD44, and VE-cadherin, fail to associate with CD151 under the same conditions (Sincock *et al.*, 1999).

Implications for CD151 Function in Metastasis. Four years after its initial discovery as a platelet antigen (Fitter *et al.*,

1995), CD151 was “rediscovered” as the target of an anti-metastatic mAb (Testa *et al.*, 1999). Anti-CD151 antibodies inhibited metastatic colonization *in vivo* in this study, whereas forced expression of CD151 enhanced chemotactic cell migration. A role for CD151 in human cancer is supported by recent clinical studies, in which increased CD151 expression correlated with poor prognosis in non-small cell lung cancer (Tokuhara *et al.*, 2001), colon carcinoma (Hashida *et al.*, 2003), and prostate cancer (Ang *et al.*, 2004), with the CD151 expression level actually superior to traditional histologic grading in predicting patient outcomes in some cases (Ang *et al.*, 2004). Our data suggest that CD151 could function at multiple steps in the metastatic cascade, contributing to motility during early invasive steps, to adhesion of hematogenously circulating tumor cells, and again to motility during extravasation. Comparing the *in vivo* metastatic colonization potential of wild-type and CD151-silenced tumor cells is an important next step.

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