

AD_____

Award Number: W81XWH-06-1-0580

TITLE: Targeting of CD151 in Breast Cancer and in Breast Cancer Stem Cells

PRINCIPAL INVESTIGATOR: Martin E. Hemler, Ph.D.

CONTRACTING ORGANIZATION: Dana-Farber Cancer Institute
Boston, MA 02115

REPORT DATE: September 2007

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				<i>Form Approved</i> OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE (DD-MM-YYYY) 01-04-2007		2. REPORT TYPE Final		3. DATES COVERED (From - To) 1 SEP 2006 - 31 AUG 2007	
4. TITLE AND SUBTITLE Targeting of CD151 in Breast Cancer and in Breast Cancer Stem Cells				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-06-1-0580	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Martin E. Hemler, Ph.D. E-Mail: Jane_MacDonald@dfci.harvard.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Dana-Farber Cancer Institute Boston, MA 02115				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT A mouse model for spontaneous breast cancer has been set up to analyze the role of CD151 during breast cancer progression. Using this model, which involves amplification of the ErbB2 oncogene, preliminary data was then obtained indicating that the absence of CD151 causes a substantial delay in the appearance of mouse mammary tumors. To confirm these preliminary results, we next set up a larger scale experiment to evaluate the role of CD151 during mammary tumor progression. Results from this larger scale experiment are pending further growth of the mice. In another experiment, a subpopulation of human MCF7 cells was isolated that has "stem-cell" like properties. Removal of CD151 from this "stem cell"-like population dramatically diminished cell invasion and responsiveness to EGF. In addition, the CD151 gene was successfully ablated from several different human mammary carcinoma cell lines, by using an siRNA strategy. As a consequence of CD151 removal, human mammary epithelial cells show diminished invasion, migration, signaling, adhesion, and EGFR collaboration. Together these results establish that CD151 plays a major role in the progression of breast cancer.					
15. SUBJECT TERMS CD151 protein, breast cancer progression, ErbB2 amplification, breast carcinoma invasion, laminin-binding integrins					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			USAMRMC
			UU	34	19b. TELEPHONE NUMBER (include area code)

Table of Contents

	<u>Page</u>
Introduction.....	4
Body.....	4-5
Key Research Accomplishments.....	5
Reportable Outcomes.....	5
Conclusion.....	5
References.....	5
Appendices.....	7-34

INTRODUCTION:

a. Subject- Our research addressed the importance of the CD151 protein, which is associated with malignancy in breast cancer and other types of cancer. Others had found that CD151 knockout mice show normal development, but deficiencies in wound healing. This supported our prediction that CD151 targeting will affect pathological, but not normal physiology. Furthermore, tetraspanin-type proteins (e.g. CD151) had never before been targeted in the context of breast cancer. We hypothesized that the CD151 protein plays a critical role during mammary tumor progression, and also carries out vital functions on mammary stem cells. Our proposed experiments were aimed at providing a definitive test of this hypothesis. Also we wanted to determine the feasibility of targeting CD151 to disrupt mammary tumor progression.

b. Purpose- An abundance of suggestive evidence supported the hypothesis that CD151 protein plays a critical role during mammary tumor progression. Furthermore, we strongly suspected that $\alpha 6$ integrins are not just markers on the surface of mammary stem cells, but also carry out vital functions on those cells. Here we proposed to carry out definitive in vivo and in vitro testing of these hypotheses. Also our goal was to determine the feasibility of targeting CD151 to disrupt mammary tumor progression.

c. Scope of the work- Using a mouse model of mammary carcinogenesis, we first aimed to confirm that CD151 is indeed critical for breast tumor progression in vivo. Then our goal was to isolate mammary tumor stem cells and determine the consequences of ablating CD151 on tumor regeneration. Finally, we intended to use in vitro invasion and cell morphology assays to determine whether CD151 antagonists can disrupt mammary carcinoma functions.

BODY:

a. Establishing a mouse model for breast cancer- We attempted to cross our CD151 null mice (backcrossed into FVB strain) with FVB-Tg(MMTV-ErbB2) mice (Jackson Laboratories), a transgenic strain expressing activated an *ErbB2* (*c-neu*) oncogene that induces mammary hyperplasia and tumorigenesis. In initial experiments, we discovered that CD151 knockout mice that had been highly backcrossed into the FVB strain (for 5 or more generations) failed to produce mice that were homozygous null for CD151, when the ErbB2 gene was amplified. We suspect that amplification of the ErbB2 oncogene, together with the absence of CD151, prevents normal development at an early embryonic stage. As an alternative strategy, we generated CD151-null mice expressing MMTV-ErbB2 on a mixed FVB-129Sv genetic background. Under these conditions we were able to produce both CD151-null and CD151-positive mice expressing the amplified ErbB2 gene.

b. Functional consequences of CD151 expression- In a pilot experiment, we monitored tumor progression in 11 MMTV-ErbB2 mice that express CD151. These began to show mammary tumors by 200 days after birth. By contrast, 6 MMTV-ErbB2 mice that lack CD151 did not begin to show tumors until 300 days after birth. Hence, tumor appearance was substantially delayed when CD151 was absent (see Figure 1 in Supporting Data). Due to the small numbers of mice involved, these results are not statistically significant. A larger experiment is underway, which involves 30 mice expressing CD151 and 30 mice not expressing CD151. Results from this experiment should be available in early 2008.

c. Isolation of mouse mammary tumor stem cells- CD151-null mouse tumors were not initially available, due to breeding problems mentioned above. Hence, to begin the process of studying mammary stem cells, we carried out experiments using human mammary carcinoma cell line MCF7s. These cells were subjected to a Hoechst 33342 dye exclusion experiment, and a small subpopulation of “side population” cells (0.2-0.5%) was isolated. These “side population” cells are

enriched for expression of the EGF receptor. Interestingly, upon deletion of CD151, these cells lose their ability to invade through Matrigel in vitro, and also lose their responsiveness to stimulation by EGF (see Fig. 3D in reference #1).

d. Testing of CD151 antagonists. Experiments proposed in this section, involving antibody and recombinant protein antagonists for CD151, have been delayed. It took longer than we anticipated to obtain mouse tumor cells in the presence and absence of the CD151 gene (see above). However, we have had considerable success in ablating CD151 in human mammary cell lines, by the use of siRNA. In this regard, we have demonstrated a role for CD151 during mammary tumor cell invasion, migration, signaling, adhesion, and EGFR collaboration (see reference #1 in Appendix).

KEY RESEARCH ACCOMPLISHMENTS:

- A mouse model for spontaneous breast cancer has been set up to analyze the role of CD151 during breast cancer progression.
- Preliminary data has been obtained indicating that the absence of CD151 causes a substantial delay in the appearance of mouse mammary tumors.
- To confirm these preliminary results, a larger scale experiment has been set up to evaluate the role of CD151 during mammary tumor progression – results are pending further growth of the mice.
- A subpopulation of human MCF7 cells has been isolated that has “stem-cell” like properties.
- Removal of CD151 from this “stem cell”-like population dramatically diminished cell invasion and responsiveness to EGF.
- CD151 has been successfully ablated from several different human mammary carcinoma cell lines, by using an siRNA strategy.
- As a consequence of CD151 removal, human mammary epithelial cells show diminished invasion, migration, signaling, adhesion, and EGFR collaboration.

REPORTABLE OUTCOMES:

A manuscript has been prepared, entitled “CD151 accelerates breast cancer by regulating $\alpha 6$ integrin functions, signaling, and molecular organization”. This manuscript has been reviewed favorably by the journal *Cancer Research*, and should soon be accepted, pending relatively minor revisions. The full reference is indicated in the “REFERENCES” section, and a copy is included in the APPENDIX.

CONCLUSIONS: We have obtained multiple lines of evidence supporting our main conclusion that CD151 plays a key positive role during breast cancer progression. Not only did absence of CD151 delay tumor progression in a mouse model in vivo, but also the absence of CD151 impaired migration, invasion, adhesion, signaling, and EGFR collaboration in human mammary epithelial cells. Together our results point to CD151 as being a novel target, which may possibly be used for treating breast cancer, with possible advantages over inhibition of laminin-binding integrins.

REFERENCES:

1. Yang XH, Richardson AL, Torres-Arzayus MI, Zhou P, Sharma C, Andzelm MM, Strominger JL, Brown M, Hemler ME. CD151 accelerates breast cancer by regulating $\alpha 6$ integrin functions, signaling, and molecular organization. **Cancer Research**. 2007; (pending minor revisions).

APPENDICES:

A manuscript corresponding to reference #1 is appended.

SUPPORTING DATA:

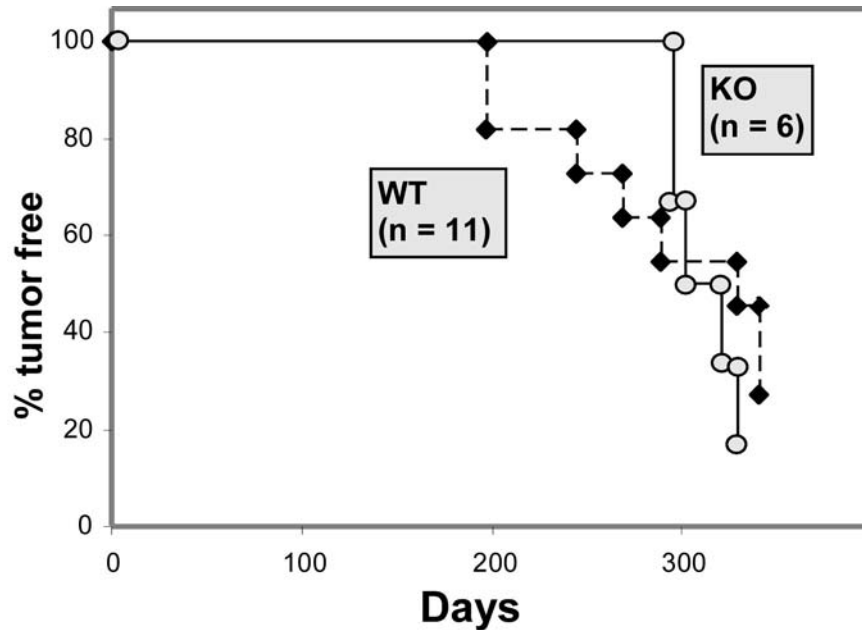


Figure 1. Absence of CD151 delays ErbB2-driven mouse mammary cancer. CD151 null mice were originally prepared in the mouse 129/Sv strain and then were backcrossed into the FVB strain for 3 generations. These mice were then bred with FVB-Tg(MMTV-ErbB2 mice (Jackson Laboratories). This is a transgenic strain expressing activated an *ErbB2* (*c-neu*) oncogene that induces mammary hyperplasia and tumorigenesis. Mice that were CD151-null (6 mice), or CD151-wild type (11 mice), while simultaneously expressing the MMTV-ErbB2 transgene, were then monitored for over 300 days, and tumor appearance was recorded.

CD151 Accelerates Breast Cancer by Regulating $\alpha 6$ Integrin Functions, Signaling, and Molecular Organization

Xiuwei H. Yang^{1,6}, Andrea L. Richardson^{2,6}, Maria I. Torres-Arzayus³, Pengcheng Zhou⁴, Chandan Sharma¹, Milena M Andzelm⁵, Jack L. Strominger⁵, Myles Brown³, Martin E. Hemler¹

¹Department of Cancer Immunology and AIDS, Dana-Farber Cancer Institute and Harvard Medical School;

²Department of Pathology, Brigham and Women's Hospital and Harvard Medical School

³Department of Medical Oncology, Dana-Farber Cancer Institute and Harvard Medical School

⁴Department of Pediatric Oncology, Dana-Farber Cancer Institute and Harvard Medical School

⁵Department of Molecular and Cellular Biology, Harvard University, Cambridge, MA

Contact information: Martin E. Hemler, Dana-Farber Cancer Institute, Rm D1430
44 Binney St., Boston, MA 02115. Phone: 617-632-3410; Fax: 617-632-2662; E-mail:
Martin_Hemler@DFCI.Harvard.EDU

Additional footnotes: ⁴These authors contributed equally to this work.

Running Title: CD151 and $\alpha 6$ integrins in breast cancer

Abstract

CD151, a master regulator of laminin-binding integrins ($\alpha 6\beta 4$, $\alpha 6\beta 1$, and $\alpha 3\beta 1$), assembles these integrins into complexes called tetraspanin enriched microdomains (TEMs). We show elevated CD151 gene expression in ~31% of human breast cancers, especially in high grade and/or basal-like subtypes. CD151 ablation markedly reduced basal-like mammary tumor migration, invasion, signaling (through FAK, Rac1, Ick), adhesion strengthening and/or adhesion on laminin, while disrupting EGFR- $\alpha 6\beta 4$ integrin collaboration. Underlying these defects, CD151 ablation redistributed $\alpha 6\beta 4$ integrins subcellularly, and severed molecular links between integrins and TEMs. In a prototypical basal-like mammary tumor line, CD151 ablation notably delayed tumor progression, in ectopic and orthotopic xenograft models. These results a) establish that CD151- $\alpha 6\beta 4$ complexes play a functional role in basal-like mammary tumor progression, b) emphasize that $\alpha 6$ integrins function, via CD151 linkage, in the context of TEMs, and c) point to potential relevance of CD151 as a high priority therapeutic target, with relative selectivity (compared to laminin-binding integrins) for pathologic rather than normal physiology.

Introduction

CD151 [SFA-1, PETA-3], one of 33 proteins in the mammalian tetraspanin protein family¹, is widely expressed on the surface of many cell types, where it associates strongly with laminin-binding integrins ($\alpha 3\beta 1$, $\alpha 6\beta 1$, $\alpha 6\beta 4$, $\alpha 7\beta 1$) and more weakly with a few additional integrins². Hence, CD151 is well positioned to modulate integrin-dependent cell spreading, migration, signaling, and adhesion strengthening³⁻⁵. CD151 may function by linking laminin-binding integrins to other tetraspanins (e.g. CD9, CD81, CD82, CD63), signaling molecules (PtdIns 4-kinase, PKC), and other proteins, within tetraspanin-enriched microdomains (TEMs)^{1,6}.

CD151-associated integrins ($\alpha 3\beta 1$, $\alpha 6\beta 1$, $\alpha 6\beta 4$) play critical roles in kidney and skin development⁷. CD151 itself may support kidney and skin development, and other functions in humans⁸. By contrast, mice lacking CD151 were viable and fertile, with no obvious developmental defects in kidney or skin⁹. Under pathological conditions, CD151-null mice showed in vivo defects in wound healing¹⁰ and angiogenesis¹¹. Ex vivo analyses of CD151-null cells and tissues revealed selected alterations in cell outgrowth, migration, aggregation, proliferation, morphology, and signaling^{9,11,12}.

Whereas other tetraspanins suppress tumor cell invasion and metastasis¹³, CD151 promotes tumor malignancy¹⁴, and the CD151 gene is upregulated in human keratinocytes during epithelial-mesenchymal transition (EMT)¹⁵. Also, CD151 expression correlated with poor prognosis, enhanced metastasis, or increased motility in several cancer types (e.g.¹⁶). Removal of CD151, either by antisense, siRNA-knockdown or knockout, may affect PI3K, Akt, and Rac1 pathways^{11,17}. Also, CD151 depletion may either increase^{11,18} or decrease^{11,19} cell motility, while effects on cell adhesion vary from minimal to substantial^{11,12,19,20}, perhaps due to effects on integrin activation²⁰ and/or internalization¹⁹. Thus, CD151 has diverse and unpredictable functions in different cellular environments.

At present, little has been done regarding CD151 in breast cancer. The $\alpha 6\beta 4$ integrin (after disconnection from hemidesmosome intermediate filaments) promotes mammary tumor cell motility and invasion by activating the phosphoinositide 3-kinase (PI3K)/AKT pathway or small GTPase Rac1/NF- κ B^{21,22}. Also $\alpha 6\beta 4$ may promote mammary tumorigenesis by amplifying signaling of ErbB family members²³. In human breast cancers, expression of integrin $\alpha 6$ and/or $\beta 4$ is associated with the estrogen receptor-negative basal-like subtype, high tumor grade, and increased mortality²⁴⁻²⁶. Given CD151 association with laminin-binding integrins, we hypothesized that CD151 influences mammary tumor progression. Indeed, we found elevated CD151 in high grade and basal-type human breast cancers. CD151 ablation yielded marked alterations in integrin-mediated cell invasion, migration, and sometimes adhesion in mammary cell lines (MCF-10A, MDA-MB-231) with basal-like gene expression patterns²⁷. Also we gained new insights into CD151 effects on integrin signaling, distribution, and EGFR collaboration. Supporting the relevance of these findings, CD151 ablation delayed human mammary tumor progression in mouse xenograft models.

Materials and Methods

Cell culture and reagents. Human basal-like mammary epithelial cell lines (MCF-10A, MDA-MB-231, BT549, Hst578²⁷) and J110 (ER-positive metastatic mouse mammary line²⁸) were cultured in DMEM or RPMI 1640 with 10% fetal calf serum (GIBCO BRL, Rockville, MD), 10 mM HEPES and antibiotics (penicillin, streptomycin). A Hoechst dye effluxing mammary cell subline (sMCF-7) was selected for high sensitivity to EGF). In vivo passaged

pMDA-MB-231 cells were from nude mouse tumors, treated with control siRNA (clones C1, C2) or CD151 siRNA (clones K1, K2, K3).

Anti-CD151 monoclonal antibodies include 5C11, 1A5¹⁴; and FITC-conjugated IIG5a (GeneTex Inc., San Antonio, CA). Anti-CD9 mAb MM2/57 (unconjugated and FITC-conjugated) was from Biosource, Camarillo, CA). mAbs to tetraspanins CD81 (M38) and CD82 (M104), to integrins $\alpha 2$ (A2-IIE10), $\alpha 3$ (A3-X8), $\alpha 6$ (GöH3), $\beta 1$ (TS2/16) and $\beta 4$ (3E1, ASC-8); and rabbit polyclonal antibodies to the integrin $\alpha 3A$ and $\alpha 6A$ cytoplasmic domains, were referenced elsewhere^{5,11}. Anti- $\beta 1$ mAb 9EG7 was from Pharmingen (San Diego, CA). Antibodies to FAK, Y397-phosphorylated FAK, fyn, src and p130Cas were from Santa Cruz Biotechnology, Inc. Antibodies to phosphorylated Src, Lck and FAK (Y925) were from Cell Signaling Technology (Beverly, MA). PI 3-kinase inhibitor (Ly294002) was from Calbiochem (San Diego, CA) and Mitomycin C was from Sigma.

Human tissue array analyses. Formalin-fixed paraffin-embedded tumor samples annotated with pathological and pre-diagnosis clinical data were obtained under an IRB approved protocol (Partners IRB #2000-P-001448) from Brigham and Women's Hospital. Immunohistochemistry was performed on 4 paraffin tissue microarrays (TMAs) of 124 primary human breast tumors containing two representative 0.6 mm cores of each tumor and representative cores of normal breast tissues. For CD151 immunohistochemistry, primary antibody (Clone RLM30, Novacostra) was used at 1:50 dilution, and detected using DAKO EnVision™+ System (Dako, Carpinteria). Immunoreactivity was scored semiquantitatively by a breast pathologist (A.R.) using a scale (0 to 3+), where 1+ staining approximates that in normal breast myoepithelial cells. Each single intensity score is based on two tissue cores, with 0-1+ = low and 2-3+ = high/over-expressed.

siRNA and shRNA targeting. siRNA's were purchased from Dharmacon (Lafayette, CO), and used to target human CD151 (GCAGGUCUUUGGCAUGA, #4; CCUCAAGAGUGACUACAUCUU, #2), $\alpha 6$ integrin (CAAGACAGCUCAUAUUGAUUU), $\alpha 3$ integrin (UUACAGAGACUUUGACCGAUU), CD9 (CCAAGAAGGACGUACUCGAUU), CD81 (CCACCAACCUCUGUAUCUUU) and CD82 (a pool of four siRNAs). Cells were seeded (1.0×10^5 /ml, 12-20 h) prior to siRNA transfection, using lipofectamine 2000. To enhance knockdown, cells were typically transfected again, 2 days later.

For stable knockdown of human CD151, oligo AGTACCTGCTGTTTACCTACA was cloned into lentivirus expression vector plenti-U6BX (CelloGenetics, Inc) and verified by DNA sequencing. Viral titers were determined by HEK 293T cell infection. Infected cells were sorted by flow cytometry (using mAb 5C11) for CD151 absence.

Matrigel Invasion, Migration and Cable Formation Assays. To assess invasion, cells were detached using non-enzymatic EDTA-containing dissociation buffer (Gibco). Then, cells ($3\text{--}5.0 \times 10^4$) in serum-free DMEM with 0.02% BSA were added to transwell chambers, containing 8 μM membranes pre-coated with Matrigel (BD Biocoat, Bedford, MA). Chamber bottoms contained serum-free medium $\pm$ 10 ng/ml EGF. After invasion through Matrigel (12-18 h, 37°C), membranes were washed, dried, fixed, stained (Giemsa, Sigma), and cells counted.

For monolayer scratch assays, 20-30% confluent cells, in 24 well plates, were transfected with siRNA for five days. Confluent cells were starved for ~12 hr, and gaps were scratched by pipette tip. After removing loose cells, DMEM/F12 medium was added, containing MCF-10A-specific supplement at 1% dilution, $\pm$ 10 ng/ml EGF. Cell images were acquired by monochrome CCD camera (RT SPOT, Diagnostic Instruments, Sterling Heights, MI), on an Axiovert 135 inverted microscope (Zeiss Co., Thornwood, NY), and were controlled by IP Lab software (Scanalytics) running on a G4 Macintosh computer. Cell gaps were quantitated using Scion Image vs.62 (Scion Corp.).

Adhesion, Spreading and Detachment Assays. To measure static adhesion, 96 well plates were coated (5-20 $\mu\text{g/ml}$, 12 hours) with laminin-1, Matrigel or fibronectin; cells were added, and attached cells were quantitated using a Cytofluor 2300 system (Millipore). For spreading assays, cells (suspended at 37°C, 45 min) were plated onto 48 well plates precoated with ECM substrates, and photographed after 45 minutes, as indicated above. Cells defined as spread showed flattened morphology that was not phase bright by light microscopy. To assess detachment, MCF-10A cells were pretreated with siRNAs (5 days) with replating (days 4-5) to accumulate endogenously produced substrates (mostly laminin-5). Cells were then incubated with varying trypsin-EDTA (37°C) with gentle shaking. Upon release of >80% of plated cells, detachment time was recorded.

Flow cytometry and immunofluorescence microscopy. For flow cytometry, suspended cells were incubated with primary antibodies (10-20 $\mu\text{g/ml}$) on ice (30-60 min), stained with FITC- or PE-conjugated secondary antibodies, followed by FITC-conjugated secondary antibody

(Biosource, Camarillo, CA), then analyzed using FACSCalibur (Becton Dickinson, Bedford MA). For confocal analyses, cells cultured on coverslips were treated with siRNAs (5-6 days), stained with various primary antibodies, then incubated with secondary antibody (FITC- or Alexa-594-conjugated goat anti-mouse or anti-rat) alone or combined (Molecular probes, Portland, OR). Cells were visualized using a Zeiss LSM 510 laser-scanning confocal microscope. Using LSM510 Meta software, z axis images were acquired at 0.5 to 1 μ M increments.

Immunoprecipitation, [3H]-palmitate labeling, and signaling assays. For metabolic labeling, siRNA-treated cells (80-90% confluent) were washed in PBS, serum-starved (3-4 h), pulsed for 1-2 h in medium containing 0.2-0.3 mCi/ml [3 H]-palmitic acid plus 5% dialyzed fetal bovine serum, then lysed in 1% Brij-96 for 5 h at 4°C. Immunoprecipitation and detection of [3 H]-palmitate-labeled proteins was as described¹¹. To assess protein phosphorylation, cells were lysed in RIPA (1% Triton X-100, 1% deoxycholate, 0.1% SDS), and then phosphorylated proteins were either immunoprecipitated and blotted with anti-phosphotyrosine antibody (4G10, UBI) or directly blotted in cell lysates, using phospho-specific antibodies. Rac1 activation was assessed using a GST-PBD pull-down assay kit (UBI).

Nude mouse xenograft assays. For ectopic analysis, five mice were each injected subcutaneously at two sites with MDA-MB-231 cells (1×10^6 cells/site) and then tumor sizes were measured using calipers, and volumes were calculated. For orthotopic analysis, MDA-MB-231 cells were injected into mammary fat pads of 10 mice (2 sites each, 7.5×10^5 cells/site) and animals were maintained in a “non-terminated” state until tumors reached a width of 2 cm, or mice became moribund.

Results

CD151 on normal and malignant human mammary epithelial cells. Samples of normal and malignant human breast tissues were analyzed for CD151 protein expression (Fig. 1A). In normal breast tissue, CD151 was in the basal-myoepithelial cell layer surrounding both ducts and lobular alveolae (1A.a and 1A.b). Staining was predominantly cytoplasmic with regions of basal or basolateral membrane accentuation. By comparison, a range of CD151 patterns was seen in human breast tumor tissue microarray samples. Some tumors showed absent (score 0, Fig. 1A.c)

or only modest CD151 protein (score 1+, Fig. 1A.d). Others showed moderate to high CD151 (score 2+, Fig. 1A.e, f; score 3+, Fig. 1A.g, h). CD151 localization varied from predominantly membranous (1A.e, g), to mostly cytoplasmic (1A.f, h). CD151 was overexpressed in 31% of invasive breast tumors, and significantly correlated with high tumor grade, and estrogen receptor negativity (Fig. 1B and Supplemental Table 1). ER-negative/HER2-negative tumors (i.e. basal-like subtype) showed the highest proportion of CD151 overexpression, whereas luminal tumors (ER-positive and HER2-negative) had the lowest proportion (46% vs. 17% respectively, $p < 0.003$). CD151 expression was not associated with patient age, tumor size, ductal or lobular histology, lymph node metastasis, the presence of peritumoral lymphovascular invasion (LVI), or with HER2 overexpression in this cohort of patients. Long-term outcome and distant metastatic recurrence data is not yet available.

CD151 effects on mammary cell invasion and motility. We next examined the role of CD151 in myoepithelial-basal derived mammary cell lines. Treatment with siRNA reduced CD151 protein levels by >90% in MCF-10A cells, as seen by blotting (Supplemental Fig. 1A), metabolic labeling (see Fig. 5B), or flow cytometry (not shown). Mobilization of MCF-10A cells into a gap (scratched into a confluent cell monolayer) was reduced by nearly 50% for CD151-siRNA-treated cells, compared to control cells (Fig 2A). Similar results were obtained when CD151 was stably silenced (>95%) by stable shRNA expression in MCF-10A cells (not shown). Knockdown of integrin $\alpha 6$ (but not $\alpha 3$) essentially eliminated motility (not shown). Because $\alpha 6$ mostly associates with $\beta 4$ in MCF-10A cells (Supplemental Fig. 2, lane 3), motility in Fig. 2A must depend on integrin $\alpha 6\beta 4$. Neither proliferation nor survival of MCF-10A cells was affected by CD151 silencing (not shown).

CD151 was also silenced in MDA-MB-231 cells (>80-90%, Supplemental Fig. 1B), resulting in 78% reduction of invasion through Matrigel-coated transwell chambers (Fig. 2B). Stable silencing of MDA-MB-231 CD151 (by shRNA) yielded similar results (not shown). By contrast, silencing of tetraspanins CD82 and CD9 minimally reduced invasion (Fig. 2B). Although CD9 silencing slightly elevated MDA-MD-231 invasion (Figs. 2B, 2C), silencing of CD9 and CD151 together mimicked the CD151 effect (Fig. 2C), suggesting that CD151 silencing is dominant. We also silenced CD151 (by ~90%) in malignant mouse breast cancer J110 cells. Again invasion through Matrigel was significantly reduced (Fig. 2D). Knockdown of $\alpha 6$ integrin protein (by 80-90%, not shown) also caused a >50% reduction in invasion by J110

cells (Fig. 2D), and $\alpha 6$ integrin silencing caused >47% decrease in invasion by MDA-MB-231 cells (not shown). Hence, CD151 contributes considerably to $\alpha 6$ integrin-dependent motility and invasion in multiple mammary cell lines.

CD151 effects on integrin adhesion, adhesion strengthening, and EGF stimulation

To understand CD151 effects on cell motility, we analyzed MCF-10A cell static adhesion. Adhesion to neither laminin-5 (mediated by $\alpha 3\beta 1$ and $\alpha 6\beta 4$) nor fibronectin (mediated by $\alpha 5\beta 1$) was appreciably altered (Supplemental Fig. 2A) by CD151 knockdown. Nonetheless, CD151-depletion did cause MCF-10A cells to lift more quickly from tissue culture dishes, upon exposure to varying trypsin-EDTA solutions (Supplemental Fig. 2B). During the 48 hours of cell culture prior to trypsinization, CD151 may contribute to laminin matrix production and adhesion strengthening, thus enhancing trypsin resistance. In parallel with diminished adhesion, MCF-10A cells also showed diminished spreading on laminin-1 (Fig. 3A,B). Silencing of CD151 in other malignant basal-like mammary cell lines (BT549 and HS578T, by >90%) again diminished spreading on laminin-1, by >70% (not shown). Effects of CD151 silencing on static cell adhesion to laminin-1 were more obvious for MDA-MB-231 cells (Supplemental Fig. 2C). CD151-depleted MDA-MB-231 cells were also tested in a 3D Matrigel assay, which correlates with adhesion strengthening⁵. In that assay, knockdown of CD151 caused markedly diminished alignment of cells into a branching network (Supplemental Fig. 2D).

We considered that EGF, an activator of integrin functions, might rescue defects in integrin-dependent cell spreading and invasion. Indeed, spreading on laminin 1 increased (~31% to 63%) upon EGF stimulation of control MDA-MB-231 cells (Fig. 3A-B). By contrast, cells lacking CD151 showed lower initial spreading (~8%) that was not stimulated by EGF (Fig. 3A-B). CD151 ablation did not affect cell spreading on fibronectin (Fig. 3A-B), and cells did not spread on BSA-coated surfaces (not shown). EGF stimulation did not rescue defective Matrigel invasion in multiple mammary cell lines, including MDA-MB-231 cells (Fig. 3C), an MCF7 subline (Fig. 3D), and BT549 cells (not shown). However, EGF did stimulate cells that had been treated with control siRNAs (Figs. 3C, 3D). Two additional stimulators, PMA and IGF-1 (which also activate integrins via inside-out signaling) showed similar inability to overcome CD151 depletion effects on MDA-MB-231 cell invasion and spreading (not shown).

CD151 affects cell signaling. Treatment of MDA-MB-231 cells with PP2, a specific inhibitor of src family kinases (SFKs), completely abolished cell spreading on laminin-1

substrate (not shown), suggesting a role for SFK-mediated tyrosine phosphorylation. Within 30-60 minutes after plating on laminin-1, CD151-silenced MDA-MB-231 cells showed reduced tyrosine phosphorylation of proteins of 60-120 kDa (Fig. 4A). One of these proteins is FAK (Fig. 4B), a kinase crucial for tumor invasion²⁹. Surprisingly, CD151 ablation did not affect activation of *src* (which typically modulates FAK), as assessed by blotting with anti-pY416 (present in *src*, *fyn*, and *yes* kinases), after cell plating on laminin-1 or fibronectin (Fig 4A, lower panel). Phosphorylation of FAK-Y925, which is mediated by *src*³⁰, was also unaffected (not shown), even though overall FAK tyrosine phosphorylation was diminished (Fig. 4B). However CD151 ablation did diminish activation (at Y505) of *lck*, an SFK member implicated in mammary tumor progression³¹ (Fig. 4B, lower panels).

Rac and Akt signaling pathways exert major influence on cell morphology, motility, and migration^{32,33}. Consistent with this, CD151 ablation markedly reduced integrin-dependent Rac1 activation in MDA-MB-231 cells plated on Matrigel for 30 min (Fig. 4C). However, Akt activation was not notably altered in CD151-silenced cells on Matrigel (Fig. 4C), even though CD151 and associated integrins modulate Akt activation in other cell types (see discussion), and the PI3-kinase/Akt pathway is critical for MDA-MB-231 cell invasion³⁴.

CD151 affects integrin subcellular distribution, but not expression levels. We assumed that CD151 effects seen in Figs. 2-4 arise due to CD151 effects on laminin-binding integrins. In this regard, immunoprecipitation of $\alpha 3$ and $\alpha 6$ (but not $\alpha 2$) integrins yielded CD151, and immunoprecipitation of CD151 yielded $\alpha 3$ and $\alpha 6$ integrins (Supplemental Fig. 3). Besides integrin subunits ($\alpha 3$, $\alpha 6$, $\beta 4$) and CD151, other known (e.g. CD9) and unknown proteins also appeared, as addressed further in Fig. 5B.

Next we considered that cell surface expression of laminin-binding integrins might be altered upon removal of CD151. However, despite nearly complete removal of CD151 from the surface of MDA-MB-231 cells, we did not see a decrease in cell surface CD82, or integrin $\alpha 6$, $\alpha 3$, and $\beta 1$ subunits (Supplemental Fig. 4). Conversely, tetraspanin CD82 depletion did not affect surface expression of CD151. Furthermore, the percentage of $\beta 1$ expressing the 9EG7 neoepitope (sometimes associated with integrin activation³⁵) was also not decreased (9.9% increased to 11.5%) upon knockdown of CD151. Silencing of CD151 in MCF-10A cells had a similar lack of effect on surface expression of integrins and other tetraspanins (not shown). Hence, although CD151 can associate closely with laminin-binding integrins such as $\alpha 3\beta 1$ and

$\alpha 6\beta 4$, it is not required for their cell surface expression, and appears not to alter integrin activation.

Also we analyzed CD151 ablation effects on integrin distribution in MCF-10A cells. As seen in ventral sections, integrin $\alpha 6$ and CD151 are present in broad patches (Fig 5A, a-c), resembling hemidesmosome-like staining typical for MCF-10A cells³⁶. However CD151 depletion markedly diminished hemidesmosome-like staining, as bands of $\alpha 6$ became thinner and more proximal to cell-cell boundaries, while CD151 staining was greatly diminished (Fig. 5A, d-f). By contrast, CD151 depletion minimally affected integrin $\alpha 3$ staining (Fig. 5A, compare g-i with j-l), and did not affect integrin $\alpha 2$ staining (Fig. 5A; m,n). In a related experiment, CD151-depletion markedly diminished susceptibility of cell surface $\beta 4$ integrin to antibody capping (Supplemental Fig. 5). Hence, CD151 markedly affects the subcellular distribution of $\alpha 6$ integrins (which in this case is mostly $\alpha 6\beta 4$).

CD151 affects integrin associations with other proteins. CD151 may link laminin-binding integrins to other proteins within the plasma membrane^{37,38}. Hence, we tested whether CD151 depletion would disconnect $\alpha 3$ and $\alpha 6$ integrins from cell surface partners. From metabolically labeled MCF-10A lysate, recovery of $\alpha 6\beta 4$ integrin was not diminished upon ablation of CD151 (Fig. 5B, lanes 5-7; see $\beta 4$ in the top panel, and $\alpha 6$ immunoblot in 3rd panel). However, recovery was diminished for CD151 itself, tetraspanin CD9, and at least five other proteins (white arrowheads, lane 6). Similarly, immunoprecipitation of $\alpha 3$ integrin was not diminished (Fig. 5B, 2nd panel, lanes 2-4), but levels of CD151 and nearly all other associated proteins were decreased (Fig. 5B, top panel, lane 3). Diminished recovery of CD9 as an integrin partner, due to CD151 ablation, was confirmed by CD9 immunoblotting (see bottom panels, Figs. 5B, lanes 2,3; 5,6). Also, immunoprecipitation of $\alpha 6$ integrin yielded a small amount of $\alpha 3$ (Fig. 5B, 2nd panel, lanes 5, 7) that was lost when CD151 was ablated (lane 6), while $\alpha 2$ integrin yielded no prominent proteins, consistent with $\alpha 2$ not associating with tetraspanins (Fig. 5B, lane 1. CD151 ablation from MDA-MB-231 cells yielded similar results (Supplemental Fig. 6). These results strongly support a critical role for CD151 in linking $\alpha 3$ and $\alpha 6$ integrins to multiple components within tetraspanin-enriched microdomains.

CD151 accelerates MDA-MB-231 tumor progression in vivo. To test whether CD151 affects tumor progression in vivo, MDA-MB-231 nude mouse xenograft models were used. In a

preliminary ectopic (s.c.) injection experiment, nude mice were injected with MDA-MB-231 cells expressing either control shRNA, or CD151 shRNA. Tumors arising from control MDA-MB-231 cells appeared by 8-9 weeks, whereas CD151-ablated cells did not yield detectable tumors until 11-12 weeks (Fig. 6A). MDA-MB-231 cells were also injected into mammary fat pads, and primary tumor growth was analyzed (Fig. 6B). As indicated, none of the 10 mice injected with CD151-ablated cells reached a terminal state within 12 weeks. By contrast, several mice injected with control cells either developed tumors of 2 cm, or reached a moribund state. Cells were recovered from independent MDA-MB-231 tumors from both control mice (C1, C2), and CD151-knockdown mice (K1, K2, K3), and then cultured in vitro. Blotting of CD151 confirms that control MDA-MB-231 cells indeed contain abundant CD151, whereas CD151-ablated cells express little or no CD151 (Fig. 6C).

Discussion

Functional roles for CD151 in breast cancer had not previously been demonstrated. Mammary tumor initiation and progression are regulated by laminin-binding integrins ($\alpha 3\beta 1$, $\alpha 6\beta 1$, $\alpha 6\beta 4$)^{23,39}. Since CD151 associates closely with laminin-binding integrins, and affects their functions, we surmised that CD151 might also play a role in mammary tumor progression. In support of this, CD151 was overexpressed in all subtypes of breast cancer (ER+, HER2+, and Basal-like) but most frequently in ER-negative and basal-like tumors. Laminin-binding integrins are also elevated in human breast cancer²⁵, especially in ER-negative basal-like breast cancers²⁴, consistent with CD151-integrin complexes playing an important functional role in those tumors. ER-negative tumors frequently overexpress members of the ErbB family tyrosine kinase receptors, HER2/neu (in the HER2+ subtype) and EGFR (in the basal-like subtype)⁴⁰. In this regard, CD151 may play a critical role during integrin-EGFR cross-talk (discussed below).

Not only is CD151 significantly upregulated in basal-like human breast cancer, but also it plays an active role in cell physiology, as seen in studies of human basal-like mammary cell lines (MCF-10A, MDA-MB-231 and others). Upon removal of CD151, but not other tetraspanins, MDA-MB-231 cell invasion through Matrigel was decreased by over 80%. Support of invasion by CD151 is consistent with results seen in other tumor cell types^{16,41}, and with CD151 joining with laminin-binding integrins in contributing to tumor cell invasion. In MCF-10A cells, removal of CD151 markedly impaired cell migration, consistent with a pro-migratory role for

CD151 on epidermal carcinoma cells¹⁹, but contrasting with anti-migratory roles for CD151 on other cells^{11,18}. Because cell migration is biphasic with respect to adhesion strength, removal of CD151 may either impair or enhance cell migration depending on whether initial adhesion strength conditions are optimal or excessive.

Cell migration and invasion involves cycles of cell adhesion, adhesion strengthening, and deadhesion. Here we obtained mixed results with respect to cell adhesion. Effects of CD151 removal on static cell adhesion were more obvious in MDA-MB-231 cells, and less obvious for MCF-10A cells. As seen elsewhere, CD151 effects on adhesion can be substantial¹⁹, marginal²⁰, or minimal^{11,12}. We suspect that CD151 effects may be more or less obvious depending on whether laminin-binding integrin density is low (as in MDA-MB-231) or high (as in MCF-10A). Depletion of CD151 also affected cell spreading (MDA-MB-231 cells), the alignment of cellular “cables” on Matrigel (MDA-MB-231) and cell-substrate detachment (MCF-10A cells). These assays all involve adhesion strengthening, which is known to be regulated by CD151⁵. The contributions of CD151 to cell adhesion, deadhesion and adhesion strengthening all help to explain its effects on migration and invasion in mammary cell lines.

Rac1 and FAK typically play critical roles during invasion and migration. Upon CD151 silencing, we observed diminished signaling through Rac1 and FAK in MDA-MB-231 cells plated on laminin-1, thus helping to explain CD151 silencing effects on mammary cell invasion and migration. Although CD151 can promote FAK signaling, as shown here and elsewhere, it did not affect FAK signaling in endothelial cells¹¹. We suspect that FAK regulation by CD151 may be more obvious when cell adhesion is more dependent on CD151 (e.g. as seen in MDA-MB-231 cells, but not in endothelial cells).

Laminin-binding integrins and CD151 itself¹¹ can markedly affect signaling through a PI3K-Akt pathway. Indeed, treatment of MDA-MB-231 cells with PI3K inhibitor Ly294002 almost completely abolished spreading and migration on laminin-1 substrate (not shown). Hence it was surprising that CD151 depletion did not decrease Akt signaling in MDA-MB-231 cells. Future analysis of specific Akt isoforms may help to explain why CD151 removal diminishes Akt signaling in endothelial cells¹¹, but not in MDA-MB-231 cells.

In another unexpected finding, CD151 ablation led to decreased activation of Lck, but not Src (or Fyn or Yes). Lck was recently implicated as playing a role during mammary tumor progression³¹. Thus, decreased signaling through Lck (as well as FAK and Rac) could help to

explain functional effects of CD151 depletion on MDA-MB-231 cells. Although Src typically contributes to FAK signaling, Lck can also contribute⁴². Indeed, our results suggest that CD151 depletion in MDA-MB-231 cells impairs Lck-FAK rather than Src-FAK signaling.

CD151 associates with, and modulates the functions of laminin-binding integrins³⁻⁵. Indeed, we found CD151 association with $\alpha 3\beta 1$, $\alpha 6\beta 1$ and $\alpha 6\beta 4$ integrins on mammary cell lines, and silencing of CD151 affected cell signaling, spreading, and adhesion on laminin, but not fibronectin. So far, little mechanistic insight has emerged regarding the mechanisms by which CD151 affects integrins. Here we show that CD151 affects the distribution, clustering, and biochemical organization of $\alpha 6$ integrins on mammary cell lines. On MCF-10A cells, removal of CD151 disrupted $\alpha 6$ integrin present in hemidesmosome-like structures. Hence, CD151 is not only present in hemidesmosome-like structures⁴³, but also may play an active role in their maintenance. On MDA-MB-231 cells, removal of CD151 caused diminished antibody-dependent clustering of $\alpha 6$ integrins. If less available for clustering, these integrins are also likely to be less available to mediate cell adhesion, spreading, and migration. Although CD151 expression might affect integrin turnover¹⁹, neither we nor others^{11,19,20} observed any effect on integrin expression levels. Results elsewhere have suggested that CD151 effects on $\alpha 3\beta 1$ integrin activation might underlie its effects on cell adhesion²⁰. However, removal of CD151 did not diminish an integrin $\beta 1$ epitope commonly associated with integrin activation.

How does CD151 affect integrin distribution and availability for clustering? One way is through the short C-terminal CD151 cytoplasmic tail, which contributes to adhesion strengthening, and integrin-dependent morphology⁵. Another possibility is that the presence or absence of CD151 could affect integrin distribution by affecting lateral molecular interactions of integrins. Indeed, CD151-integrin complexes do not exist alone, but rather occur in the context of a larger constellation of proteins known as TEMs (Tetraspanin-enriched microdomains)^{1,6}. These other proteins, including other tetraspanins and their associated partners, likely contribute to subcellular localization of integrins. For example, the cytoplasmic tails of some of these other proteins can link to ERM proteins, thereby augmenting linkage of TEMs to the cytoskeleton⁴⁴. Upon removal of CD151, $\alpha 6$ and $\alpha 3$ integrins showed diminished associations with at least five other proteins, including other tetraspanins (CD9, CD81). This major alteration in the integrin microenvironment provides numerous possibilities for explaining the functional consequences of CD151 depletion. Besides alterations in integrin diffusion, clustering, and cytoskeletal

connections, the signaling microenvironment is also likely altered. For example, loss of tetraspanin-dependent recruitment of known tetraspanin-associated signaling enzymes such as PKC and PI 4-kinase¹ could affect integrin functions.

Functional and physical collaboration between $\alpha 6$ integrins and ErbB receptors has been frequently noted^{45,46}. For example, EGF stimulation of epithelial cells disrupts hemidesmosomes, releasing $\alpha 6\beta 4$ to participate in cell motility and invasion^{45,47}. We have not observed direct physical association of $\alpha 6$ integrins with ErbB receptors. Nonetheless, three results suggest that CD151 depletion disrupts integrin collaboration with EGFR. i) Ablation of CD151 diminished EGF-dependent MCF-10A cell migration. ii) CD151 removal caused cell invasion and spreading deficits (in three different cell types) that could not be overcome by adding EGF, and in fact, iii) EGF no longer stimulated cell spreading and/or migration at all in CD151-silenced cells. This result is particularly relevant for basal-like mammary tumors, since they tend to show elevated EGFR²⁴. Phorbol ester treatment also did not overcome CD151-knockdown effects on cell invasion and spreading (not shown), despite the ability of phorbol ester (PMA) to promote integrin function by increasing integrin lateral mobility. Impaired responses to EGF and PMA, arising from CD151 silencing, again may be due to disruption of the $\alpha 6$ integrin microenvironment. In this regard, EGFR and PKC (a target of PMA) have been previously linked to tetraspanins such as CD82, CD81 and CD9^{1,48}. Hence, ablation of CD151 may lead to diminished integrin proximity for these other tetraspanins and their associated signaling molecules.

Our in vivo xenograft results establish that CD151 on MDA-MB-231 cells accelerates tumor progression, in both ectopic and orthotopic models. We suspect that the in vivo effect of CD151 depletion is mostly due to modulation of $\alpha 6\beta 4$ because: $\alpha 6\beta 4$ is a major marker of basal-like mammary tumors^{24,40}, CD151 mostly modulated $\alpha 6$ integrin functions on MDA-MB-231 cells in vitro, and other studies have shown MDA-MB-231 invasion and migration to be $\alpha 6\beta 4$ -dependent [e.g. ⁴⁹]. However, we cannot rule out contributions from $\alpha 3\beta 1$. Depletion of CD151 had no effect on mammary cell proliferation or survival in vitro. Hence, we suspect that our in vivo results are due to effects on integrin-dependent morphology, motility, adhesion, and/or invasion, rather than proliferation or survival.

While we mostly focused on basal-like mammary cells, CD151-depletion did also affect two ER-positive mammary cell lines. Murine J110 cells²⁸ and an EGF-sensitive subline of MCF-7 cells both showed diminished invasion, while the latter also showed diminished EGF responsiveness. These results generalize our findings to other types of breast cancer. In addition, we have preliminary evidence that CD151 depletion affects HER2/Neu/ErbB2 breast cancer in mice (to be published elsewhere). Interestingly, the magnitude of the in vivo delay in MDA-MB-231 tumor progression, caused by CD151 depletion, was comparable to the delay in ErbB2-driven mouse mammary tumors, caused by β 4 integrin cytoplasmic tail deletion²³.

In conclusion, we show here that CD151 may be a key contributor to breast cancer, particularly of the basal-like subtype. CD151, a master regulator of multiple laminin-binding integrins, determines their molecular organization on the cell surface, thereby affecting integrin-dependent mammary cell morphology, migration, invasion, adhesion, signaling, EGFR crosstalk, and ultimately, tumor progression in vivo. A previous study showed that CD151 could enhance tumor progression by supporting pathological angiogenesis in host mice¹¹. Now we show that tumor cell CD151 also plays a key role, thus pointing to multiple levels of CD151 contributions, and emphasizing that CD151 may be a high priority therapeutic target in certain breast cancers.

Acknowledgements

This work was funded by a grant from the National Institutes of Health (CA42368, to M.E.H), an NCI-Harvard SPORE breast cancer award, and the Breast Cancer Research Foundation (to A.R.). M. Brown received sponsored research support and is a consultant to Novartis Co.

References

1. Hemler, M. E. Tetraspanin proteins mediate cellular penetration, invasion and fusion events, and define a novel type of membrane microdomain. *Ann.Rev.Cell Dev.Biol.*, 19: 397-422, 2003.
2. Fitter, S., Sincock, P. M., Jolliffe, C. N., and Ashman, L. K. Transmembrane 4 superfamily protein CD151 (PETA-3) associates with beta 1 and alpha IIb beta 3 integrins in haemopoietic cell lines and modulates cell-cell adhesion. *Biochem J*, 338 (Pt 1): 61-70, 1999.
3. Sincock, P. M., Fitter, S., Parton, R. G., Berndt, M. C., Gamble, J. R., and Ashman, L. K. PETA-3/CD151, a member of the transmembrane 4 superfamily, is localised to the plasma membrane and endocytic system of endothelial cells, associates with multiple integrins and modulates cell function. *J Cell Sci*, 112: 833-844, 1999.
4. Yáñez-Mó, M., Alfranca, A., Cabañas, C., Marazuela, M., Tejedor, R., Ursa, M. A., Ashman, L. K., De Landázuri, M. O., and Sánchez-Madrid, F. Regulation of endothelial cell motility by complexes of tetraspan molecules CD81/TAPA-1 and CD151/PETA-3 with $\alpha 3\beta 1$ integrin localized at endothelial lateral junctions. *J.Cell Biol.*, 141: 791-804, 1998.
5. Lammerding, J., Kazarov, A. R., Huang, H., Lee, R. T., and Hemler, M. E. Tetraspanin CD151 regulates alpha6beta1 integrin adhesion strengthening. *Proc Natl Acad Sci U S A*, 100: 7616-7621, 2003.
6. Nydegger, S., Khurana, S., Kremmentsov, D. N., Foti, M., and Thali, M. Mapping of tetraspanin-enriched microdomains that can function as gateways for HIV-1. *J Cell Biol*, 173: 795-807, 2006.
7. Belkin, A. M. and Stepp, M. A. Integrins as receptors for laminins. *Microsc Res Tech*, 51: 280-301, 2000.
8. Karamatic Crew, V., Burton, N., Kagan, A., Green, C. A., Levene, C., Flinter, F., Brady, L. R., Daniels, G., and Anstee, D. J. CD151, the first member of the tetraspanin (TM4) superfamily detected on erythrocytes, is essential for the correct assembly of human basement membranes in kidney and skin. *Blood*, 2004.
9. Wright, M. D., Geary, S. M., Fitter, S., Moseley, G. W., Lau, L. M., Sheng, K. C., Apostolopoulos, V., Stanley, E. G., Jackson, D. E., and Ashman, L. K. Characterization of mice lacking the tetraspanin superfamily member CD151. *Mol Cell Biol*, 24: 5978-5988, 2004.
10. Cowin, A. J., Adams, D., Geary, S. M., Wright, M. D., Jones, J. C., and Ashman, L. K. Wound Healing Is Defective in Mice Lacking Tetraspanin CD151. *J Invest Dermatol.*, 126: 680-689, 2006.

11. Takeda, Y., Kazarov, A. R., Butterfield, C. E., Hopkins, B. D., Benjamin, L. E., Kaipainen, A., and Hemler, M. E. Deletion of tetraspanin Cd151 results in decreased pathologic angiogenesis in vivo and in vitro. *Blood*, 109: 1524-1532, 2007.
12. Lau, L. M., Wee, J. L., Wright, M. D., Moseley, G. W., Hogarth, P. M., Ashman, L. K., and Jackson, D. E. The tetraspanin superfamily member, CD151 regulates outside-in integrin α IIb β 3 signalling and platelet function. *Blood*, 2004.
13. Wright, M. D., Moseley, G. W., and van Spriel, A. B. Tetraspanin microdomains in immune cell signalling and malignant disease. *Tissue Antigens*, 64: 533-542, 2004.
14. Testa, J. E., Brooks, P. C., Lin, J. M., and Quigley, J. P. Eukaryotic expression cloning with an antimetastatic monoclonal antibody identifies a tetraspanin (PETA-3/CD151) as an effector of human tumor cell migration and metastasis. *Cancer Res*, 59: 3812-3820, 1999.
15. Zavadil, J., Bitzer, M., Liang, D., Yang, Y. C., Massimi, A., Kneitz, S., Piek, E., and Bottinger, E. P. Genetic programs of epithelial cell plasticity directed by transforming growth factor-beta. *Proc Natl Acad Sci U S A*, 98: 6686-6691, 2001.
16. Ang, J., Lijovic, M., Ashman, L. K., Kan, K., and Frauman, A. G. CD151 protein expression predicts the clinical outcome of low-grade primary prostate cancer better than histologic grading: a new prognostic indicator? *Cancer Epidemiol. Biomarkers Prev.*, 13: 1717-1721, 2004.
17. Zheng, Z. Z. and Liu, Z. X. Activation of the phosphatidylinositol 3-kinase/protein kinase Akt pathway mediates CD151-induced endothelial cell proliferation and cell migration. *Int.J Biochem. Cell Biol*, 2006.
18. Garcia-Lopez, M. A., Barreiro, O., Garcia-Diez, A., Sanchez-Madrid, F., and Penas, P. F. Role of tetraspanins CD9 and CD151 in primary melanocyte motility. *J Invest Dermatol.*, 125: 1001-1009, 2005.
19. Winterwood, N. E., Varzavand, A., Meland, M. N., Ashman, L. K., and Stipp, C. S. A Critical Role for Tetraspanin CD151 in α 3 β 1 and α 6 β 4 Integrin-dependent Tumor Cell Functions on Laminin-5. *Mol Biol Cell*, 17: 2707-2721, 2006.
20. Nishiuchi, R., Sanzen, N., Nada, S., Sumida, Y., Wada, Y., Okada, M., Takagi, J., Hasegawa, H., and Sekiguchi, K. Potentiation of the ligand-binding activity of integrin α 3 β 1 via association with tetraspanin CD151. *Proc. Natl. Acad. Sci. U.S.A.*, 102: 1939-1944, 2005.
21. Shaw, L. M., Rabinovitz, I., Wang, H. H., Toker, A., and Mercurio, A. M. Activation of phosphoinositide 3-OH kinase by the α 6 β 4 integrin promotes carcinoma invasion. *Cell*, 91: 949-960, 1997.

22. Zahir, N., Lakins, J. N., Russell, A., Ming, W., Chatterjee, C., Rozenberg, G. I., Marinkovich, M. P., and Weaver, V. M. Autocrine laminin-5 ligates $\alpha 6 \beta 4$ integrin and activates RAC and NF κ B to mediate anchorage-independent survival of mammary tumors. *J Cell Biol*, 163: 1397-1407, 2003.
23. Guo, W., Pylayeva, Y., Pepe, A., Yoshioka, T., Muller, W. J., Inghirami, G., and Giancotti, F. G. Beta 4 integrin amplifies ErbB2 signaling to promote mammary tumorigenesis. *Cell*, 126: 489-502, 2006.
24. Perou, C. M., Sorlie, T., Eisen, M. B., van de, R. M., Jeffrey, S. S., Rees, C. A., Pollack, J. R., Ross, D. T., Johnsen, H., Akslen, L. A., Fluge, O., Pergamenschikov, A., Williams, C., Zhu, S. X., Lonning, P. E., Borresen-Dale, A. L., Brown, P. O., and Botstein, D. Molecular portraits of human breast tumours. *Nature*, 406: 747-752, 2000.
25. Diaz, L. K., Cristofanilli, M., Zhou, X., Welch, K. L., Smith, T. L., Yang, Y., Sneige, N., Sahin, A. A., and Gilcrease, M. Z. Beta4 integrin subunit gene expression correlates with tumor size and nuclear grade in early breast cancer. *Mod.Pathol.*, 18: 1165-1175, 2005.
26. Friedrichs, K., Ruiz, P., Franke, F., Gille, I., Terpe, H.-J., and Imhof, B. A. High expression level of $\alpha 6$ integrin in human breast carcinoma is correlated with reduced survival. *Cancer Res.*, 55: 901-906, 1995.
27. Neve, R. M., Chin, K., Fridlyand, J., Yeh, J., Baehner, F. L., Fevr, T., Clark, L., Bayani, N., Coppe, J. P., Tong, F., Speed, T., Spellman, P. T., DeVries, S., Lapuk, A., Wang, N. J., Kuo, W. L., Stilwell, J. L., Pinkel, D., Albertson, D. G., Waldman, F. M., McCormick, F., Dickson, R. B., Johnson, M. D., Lippman, M., Ethier, S., Gazdar, A., and Gray, J. W. A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. *Cancer Cell*, 10: 515-527, 2006.
28. Torres-Arzuayus, M. I., Yuan, J., DellaGatta, J. L., Lane, H., Kung, A. L., and Brown, M. Targeting the AIB1 oncogene through mammalian target of rapamycin inhibition in the mammary gland. *Cancer Res*, 66: 11381-11388, 2006.
29. Schlaepfer, D. D., Mitra, S. K., and Ilic, D. Control of motile and invasive cell phenotypes by focal adhesion kinase. *Biochim.Biophys.Acta*, 1692: 77-102, 2004.
30. Mitra, S. K., Hanson, D. A., and Schlaepfer, D. D. Focal adhesion kinase: in command and control of cell motility. *Nat.Rev.Mol Cell Biol*, 6: 56-68, 2005.
31. Chakraborty, G., Rangaswami, H., Jain, S., and Kundu, G. C. Hypoxia regulates cross-talk between Syk and Lck leading to breast cancer progression and angiogenesis. *J Biol Chem.*, 281: 11322-11331, 2006.
32. Ridley, A. J., Allen, W. E., Peppelenbosch, M., and Jones, G. E. Rho family proteins and cell migration. *Biochem.Soc.Symp.*, 65: 111-123, 1999.

33. Yoeli-Lerner, M. and Toker, A. Akt/PKB signaling in cancer: a function in cell motility and invasion. *Cell Cycle*, 5: 603-605, 2006.
34. Yoon, S. O., Shin, S., and Mercurio, A. M. Hypoxia stimulates carcinoma invasion by stabilizing microtubules and promoting the Rab11 trafficking of the alpha6beta4 integrin. *Cancer Res.*, 65: 2761-2769, 2005.
35. Nilsson, S., Kaniowska, D., Brakebusch, C., Fassler, R., and Johansson, S. Threonine 788 in integrin subunit beta1 regulates integrin activation. *Exp. Cell Res.*, 312: 844-853, 2006.
36. Stahl, S., Weitzman, S., and Jones, J. C. The role of laminin-5 and its receptors in mammary epithelial cell branching morphogenesis. *J Cell Sci*, 110 (Pt 1): 55-63, 1997.
37. Hemler, M. E. Tetraspanin functions and associated microdomains. *Nat. Rev. Mol Cell Biol*, 6: 801-811, 2005.
38. Berditchevski, F. Complexes of tetraspanins with integrins: more than meets the eye. *J Cell Sci*, 114: 4143-4151, 2001.
39. Chung, J. and Mercurio, A. M. Contributions of the alpha6 integrins to breast carcinoma survival and progression. *Mol Cells*, 17: 203-209, 2004.
40. Yehiely, F., Moyano, J. V., Evans, J. R., Nielsen, T. O., and Cryns, V. L. Deconstructing the molecular portrait of basal-like breast cancer. *Trends Mol Med.*, 12: 537-544, 2006.
41. Tokuhara, T., Hasegawa, H., Hattori, N., Ishida, H., Taki, T., Tachibana, S., Sasaki, S., and Miyake, M. Clinical significance of CD151 gene expression in non-small cell lung cancer. *Clin Cancer Res*, 7: 4109-4114, 2001.
42. Goldmann, W. H. p56(lck) Controls phosphorylation of filamin (ABP-280) and regulates focal adhesion kinase (pp125(FAK)). *Cell Biol Int*, 26: 567-571, 2002.
43. Sterk, L. M., Geuijen, C. A., Oomen, L. C., Calafat, J., Janssen, H., and Sonnenberg, A. The tetraspan molecule CD151, a novel constituent of hemidesmosomes, associates with the integrin alpha6beta4 and may regulate the spatial organization of hemidesmosomes. *J Cell Biol*, 149: 969-982, 2000.
44. Sala-Valdes, M., Ursa, A., Charrin, S., Rubinstein, E., Hemler, M. E., Sanchez-Madrid, F., and Yanez-Mo, M. EWI-2 and EWI-F link the tetraspanin web to the actin cytoskeleton through their direct association with ezrin-radixin-moesin proteins. *J Biol Chem.*, 281: 19665-19675, 2006.
45. Mariotti, A., Kedeshian, P. A., Dans, M., Curatola, A. M., Gagnoux-Palacios, L., and Giancotti, F. G. EGF-R signaling through Fyn kinase disrupts the function of integrin alpha6beta4 at hemidesmosomes: role in epithelial cell migration and carcinoma invasion. *J Cell Biol*, 155: 447-458, 2001.

46. Falcioni, R., Antonini, A., Nistico, P., Di Stefano, S., Crescenzi, M., Natali, P. G., and Sacchi, A. Alpha 6 beta 4 and alpha 6 beta 1 integrins associate with ErbB-2 in human carcinoma cell lines. *Exp Cell Res*, 236: 76-85, 1997.
47. Rabinovitz, I., Tsomo, L., and Mercurio, A. M. Protein kinase C-alpha phosphorylation of specific serines in the connecting segment of the beta 4 integrin regulates the dynamics of type II hemidesmosomes. *Mol Cell Biol*, 24: 4351-4360, 2004.
48. Odintsova, E., Voortman, J., Gilbert, E., and Berditchevski, F. Tetraspanin CD82 regulates compartmentalisation and ligand-induced dimerization of EGFR. *J Cell Sci.*, 116: 4557-4566, 2003.
49. Rabinovitz, I. and Mercurio, A. M. The integrin alpha6beta4 functions in carcinoma cell migration on laminin-1 by mediating the formation and stabilization of actin-containing motility structures. *J Cell Biol*, 139: 1873-1884, 1997.
50. Goldfinger, L. E., Hopkinson, S. B., deHart, G. W., Collawn, S., Couchman, J. R., and Jones, J. C. The alpha3 laminin subunit, alpha6beta4 and alpha3beta1 integrin coordinately regulate wound healing in cultured epithelial cells and in the skin. *J Cell Sci*, 112: 2615-2629, 1999.

Figure Legends

Figure 1. CD151 protein expression in human breast carcinoma. *A*, Immunohistochemistry for CD151 was performed on paraffin sections of tissue microarrays containing samples of normal breast tissue (a,b) and invasive breast tumors (c-h). (a) normal breast duct, 40X; (b) normal breast lobule, 100X, arrows indicate representative CD151-positive cells in the basal/myoepithelial layer. (c-d) Human tumors with absent and low degree of CD151 immunostaining, respectively, 40X. (e-f) Human tumors with moderate 2+ overexpression of CD151 predominantly located at membrane or in cytoplasm, respectively, 40X. (g-h) Human tumors with marked 3+ overexpression of CD151 at membrane or in cytoplasm, respectively, 40X. *B*, Human breast cancer tissue microarray samples (a total of 124 patients) were subdivided according modified Bloom-Richardson grade and estrogen receptor protein expression. The percent of each subgroup expressing high CD151 (3+ or 2+) is indicated. For further details, see Supplemental Table 1.

Figure 2. CD151 supports mammary epithelial cell migration and invasion. *A*, After treatment with siRNAs, MCF-10A cells, grown to confluence, were then incubated in a 24 well plate with serum-free medium containing 10 µg/ml mitomycin C at room temperature for 1 h. After gaps were scratched into cell monolayers, 0.5 ml serum-free medium containing 10 ng/ml EGF was added, and gaps were evaluated after 0 and 18 h at 37°C. Percentage of gap closure was determined by measuring the mean change in gap width at 3 representative sites, in three independent experiments (N = 3; *, p < 0.05). *B*, After treatment with siRNAs, MDA-MB-231 cells (5 x 10⁴) in serum-free medium containing 0.1% BSA were added to the top of Matrigel-coated transwell chambers. Serum-free medium (0.75 ml) containing 10 ng/ml EGF and 0.1% BSA was added to the bottom of transwell chambers. After ~18 h at 37° C, cells that had invaded through the Matrigel were fixed, stained, photographed, and the mean number of invaded cells was determined from triplicate chambers. Bar = 100 µm. *C*, After treatment with siRNAs (alone or in combination), MDA-MB-231 cells were again analyzed for invasion through Matrigel, as in *B*. *D*, A metastatic mouse mammary tumor cell line (J110) was treated with siRNA to murine CD151 (70% knockdown) or murine α6 integrin (>80% knockdown). Invasion was then analyzed as in *B*. *, p < 0.05; **, p < 0.01.

Figure 3. CD151 effects on EGF-stimulated mammary cell spreading and invasion. *A*, After siRNA treatment, MDA-MB-231 cells were suspended in serum-free medium at 37°C for 45 min, and then plated onto laminin-1 or fibronectin-coated 24 well plates with or without EGF (10 ng/ml). After 45 minutes, representative fields were photographed. *B*, Percentages spread cells were determined (N = 4). Note: No cell spreading was observed on plastic surfaces coated with BSA (not shown). *C*, Invasion was also analyzed (as in Fig. 2B), with or without EGF (10 µg/ml) added to the bottom of the invasion chamber. After tumor formation in nude mice, MDA-MB-231 cells were re-isolated (now called t-MDA-MB-231). Sublines were isolated from tumors originating from MDA-MB-231 cells that had been treated with control siRNA (C1) or CD151 siRNA (K1 and K2). *D*, A subline of MCF7 (enriched for Hoechst dye exclusion and elevated EGFR) was analyzed for invasion as in *C*.

Figure 4. Impact of CD151 ablation on integrin-mediated tyrosine phosphorylation cascade. *A*, After siRNA treatment, MDA-MB-231 cells were detached, washed and suspended in serum-free DMEM containing 0.1% BSA. Cells were then kept in suspension for 45 minutes at 37°C to remove residual growth factor effects prior to being plated on laminin-1 or fibronectin substrate. At the indicated times cells were lysed in RIPA buffer followed by blotting with 4G10 antibody (to probe tyrosine phosphorylation), phospho-specific antibody to *Src* kinase (p-Y416-*Src*), and antibody to β -tubulin (protein loading control). *B*, After immunoprecipitation of FAK, we analyzed FAK tyrosine phosphorylation (mAb 4G10) and total FAK by immunoblotting. Total cell lysates were also probed for activated Lck (p-Y505-Lck), total *Src* and tubulin. *C*, To assess activation of small GTPase rac1, cell lysates were incubated with GST-PBD beads (45 min, 4°C) to recover activated rac1 (Rac1-GTP form). Beads were washed, boiled, and released proteins were blotted with anti-Rac1 antibody (upper panel). Total rac1 protein in lysates was also blotted to serve as a control (lower panel). Activated Akt (p-Akt), and total Akt were also blotted, using antibodies to phospho-Akt (p-S473) and total Akt.

Figure 5. CD151 affects molecular organization of integrins. *A*, MCF-10A cells were initially seeded onto coverslips and treated with siRNAs for 5 days. Live MCF-10A cells were incubated with primary anti-integrin antibodies for 1 h at 4°C, and then stained with Alexa Fluor-594-

conjugated secondary antibody. After washing, cells were further stained with FITC-conjugated CD151 antibody. After staining, cells were fixed in 2% paraformaldehyde (20 min, 4°C) and mounted on slides using Prolong anti-fade solution (Molecular Probes, Eugene, OR) and then ventral sections were visualized by confocal microscopy. Antibodies used were mAb GoH3 (integrin α 6, panels a, d), mAb X8 (integrin α 3, panels g, j), mAb IIE10 (integrin α 2 (panels m, n), and mAb 5C11 (CD151, panels c, f, i, l). Merged green and red staining is shown in the right column. Ventral sections of cells were visualized by confocal microscopy. Panels a-c, g-h and m are treated with control siRNA; panels d-f, j-l and n are treated with CD151 siRNA. Bar = 50 μ m. *B*, MCF-10A cells were treated with siRNAs, and then labeled with [3 H]-palmitate and lysed in 1% Brij 96 buffer. Immunoprecipitations of α 2, α 3, α 6 integrins, and CD151 were carried out using mAbs IIE10, X8, GoH3 and 5C11. Note that the integrin β 1 subunit does not appear, since it does not undergo palmitoylation. Lower panels show immunoblots for α 3, α 6, and CD9, present in the immunoprecipitated complexes.

Figure 6. CD151 accelerates tumor formation in vivo. *A*, MDA-MB-231 cells were treated with either control or CD151 siRNA, and then injected s.c. into nude mice, and tumor formation was monitored. *B*, MDA-MB-231 cells, treated with siRNA, were injected into mammary fat pads of nude mice. Mice were terminated when they became moribund, or tumors reached 2 cm (in any dimension). Statistical significance was analyzed using the log rank test. *C*, After tumor formation in nude mice, MDA-MB-231 cells were re-isolated and cultured in vitro. From these sublines (C1, C2 from control-siRNA treated cells; K1, K2, K3 from CD151-knockdown cells), cell lysates were prepared and blotted for CD151 (using mAb 1A5), and integrin α 3 (using rabbit polyclonal antibody).

Figure 1

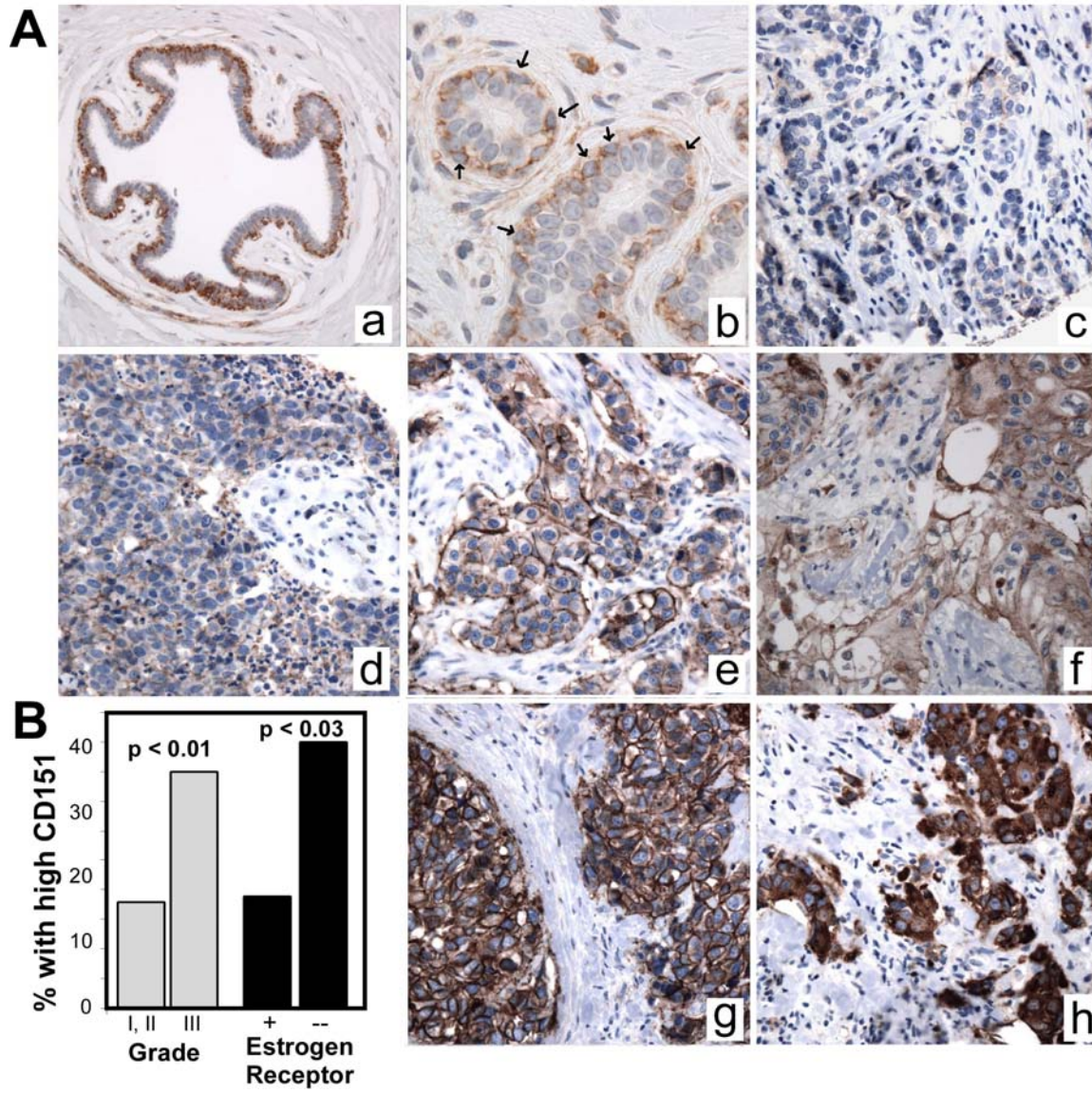


Figure 2

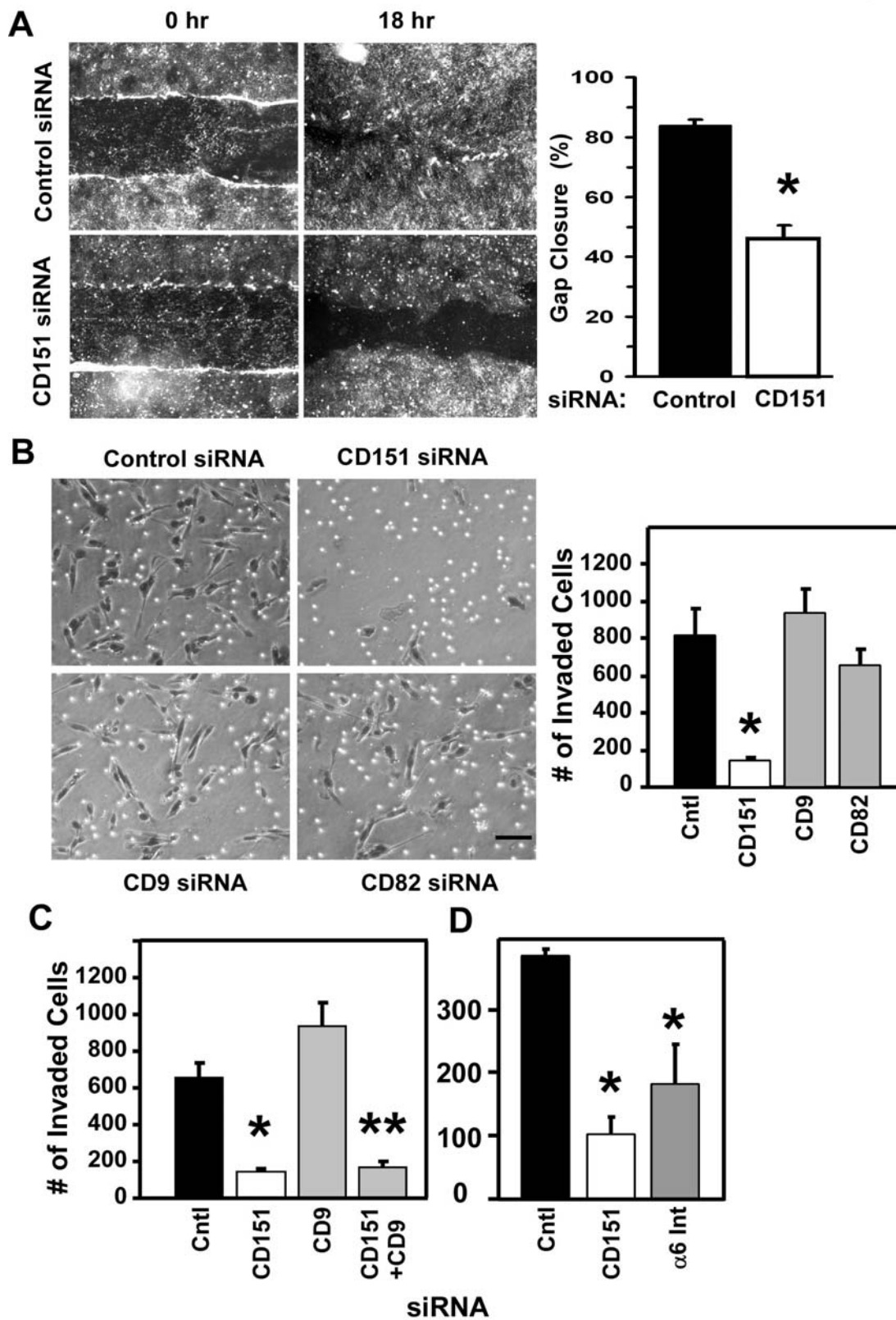


Figure 3

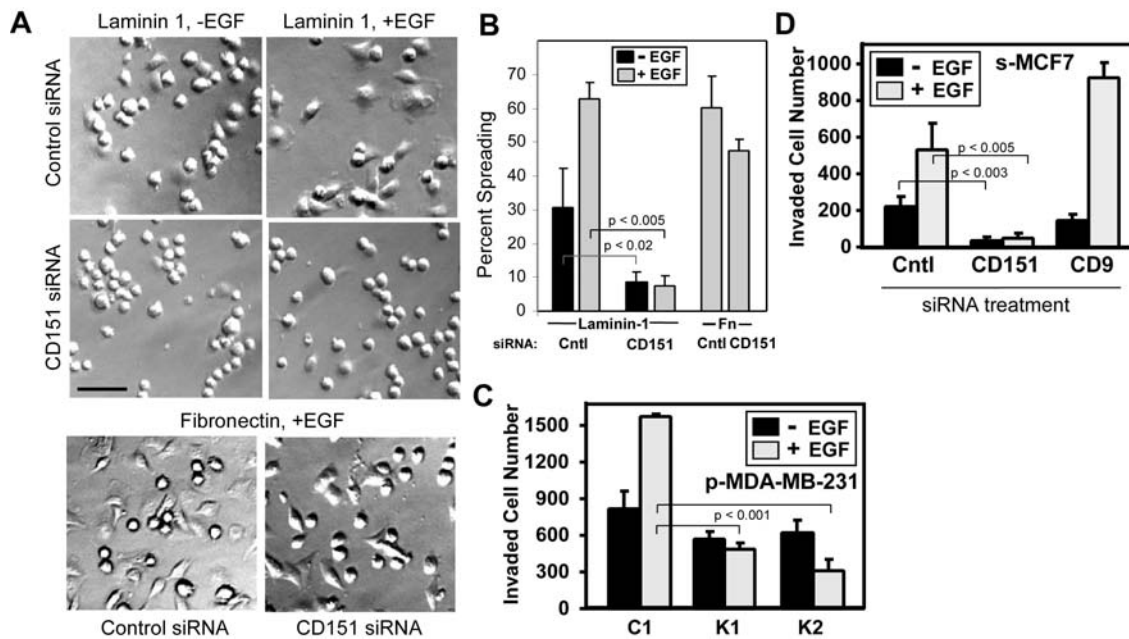
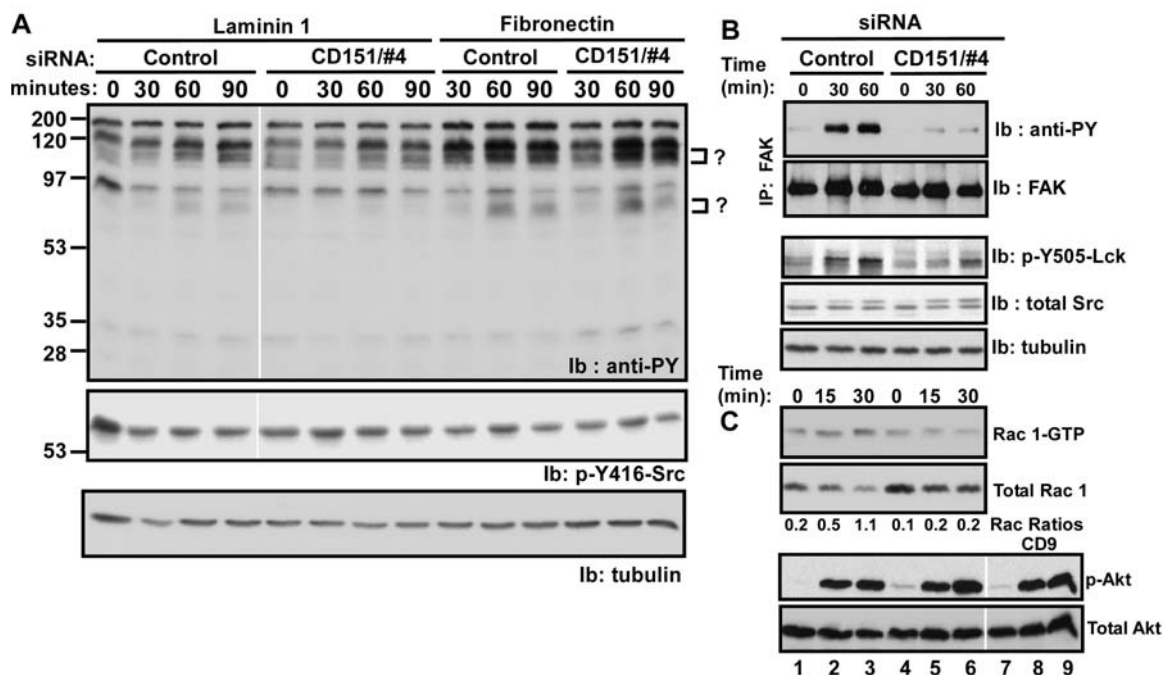


Figure 4



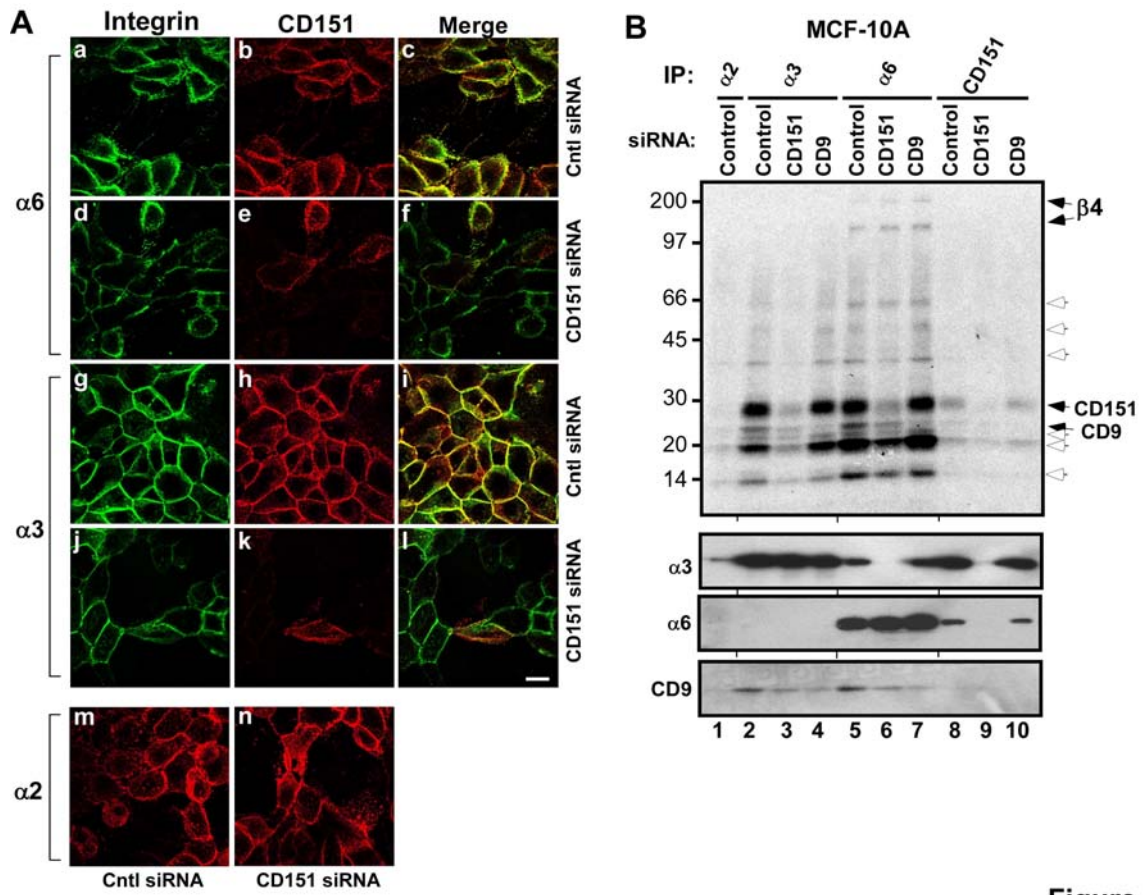


Figure 5

Figure 6

