Homophilic Interactions of Tetraspanin CD151 Up-regulate Motility and Matrix Metalloproteinase-9 Expression of Human Melanoma Cells through Adhesion-dependent c-Jun Activation Signaling Pathways*

In-Kee Hong, Young-June Jin, Hee-Jung Byun, Doo-II Jeoung, Young-Myeong Kim, and Hansoo Lee

From the Vascular System Research Center and Division of Life Sciences, College of Natural Sciences, and Department of Molecular and Cellular Biochemistry, School of Medicine, Kangwon National University, Chunchon, Kangwon-do 200-701, Korea

The tetraspanin membrane protein CD151 has been suggested to regulate cancer invasion and metastasis by initiating signaling events. The CD151-mediated signaling pathways involved in this regulation remain to be revealed. In this study, we found that stable transfection of CD151 into MelJuSo human melanoma cells lacking CD151 expression significantly increased cell motility, matrix metalloproteinase-9 (MMP-9) expression, and invasiveness. The enhancement of cell motility and MMP-9 expression by CD151 over-expression was abrogated by inhibitors and small interfering RNAs targeted to focal adhesion kinase (FAK), Src, p38 MAPK, and JNK, suggesting an essential role of these signaling components in CD151 signaling pathways. Also, CD151-induced MMP-9 expression was shown to be mediated by c-Jun binding to AP-1 sites in the MMP-9 gene promoter, indicating AP-1 activation by CD151 signaling pathways. Meanwhile, CD151 was found to be associated with α5β1 and αvβ3 integrins in MelJuSo cells, and activation of associated integrins was a prerequisite for CD151-stimulated MMP-9 expression and activation of FAK, Src, p38 MAPK, JNK, and c-Jun. Furthermore, CD151 on one cell was shown to bind to neighboring cells expressing CD151, suggesting that CD151 is a homophilic interacting protein. The homophilic interactions of CD151 increased motility and MMP-9 expression of CD151-transfected MelJuSo cells, along with FAK-, Src-, p38 MAPK-, and JNK-mediated activation of c-Jun in an adhesion-dependent manner. Furthermore, C8161 melanoma cells with endogenous CD151 were also shown to respond to homophilic CD151 interactions for the induction of adhesion-dependent activation of FAK, Src, and c-Jun. These results suggest that homophilic interactions of CD151 stimulate integrin-dependent signaling to c-Jun through FAK-Src-MAPKs pathways in human melanoma cells, leading to enhanced cell motility and MMP-9 expression.

CD151 (PETA-3/SFA-1) is a member of the tetraspanins (also known as the transmembrane 4 superfamily), a group of membrane proteins that have four highly conserved transmembrane domains (1, 2). Tetraspanins have been suggested to play important roles in the regulation of various cellular functions, including cell activation, proliferation, differentiation, development, adhesion, and motility (3–5). Although the biochemical function(s) of tetraspanins remains unclear, the capability of tetraspanin proteins to associate with each other and with several other membrane proteins involved in signaling pathways suggests that tetraspanins may act as molecular adaptors facilitating the assembly of functional signaling complexes in the membrane (4, 6).

The protein CD151 is expressed in various cell types, including epidermal basal cells, epithelial cells, skeletal and cardiac muscle, endothelial cells, platelets, and Schwann cells (7). Although the physiological function of CD151 is largely unknown, in vitro functional studies showed that CD151 is involved in cell adhesion, motility, and polarity (8–10). Recently, CD151 was reported to be a positive effector of metastasis, which is contrary to the metastasis-suppressing role of other tetraspanins, such as CD9/MRP-1, CD63/ME-491, and CD82/KAI-1. High CD151 expression was found to be associated with a poor prognosis in lung, colon, and prostate cancer (11–13). Monoclonal antibodies to CD151 inhibited in vivo metastasis of human cancer cells and transfection of CD151 cDNA into different tumor cell lines resulted in enhanced cell motility and metastasis (14, 15). This implies that CD151 does not only play an important role in normal physiological processes but also in pathological events, such as tumor cell invasion and metastasis.

CD151 is predominantly localized on the cell surface in contact with basement membranes and to a lesser extent at cell–cell junctions in epithelial cells (7, 16). CD151 forms a multimeric complex with many other transmembrane proteins. CD151 has been found to form very strong complexes with the α5β1 integrin; moderately stable complexes with αvβ1, αvβ3, and αcβ1 integrins; and less stable, possibly indirect complexes with other integrins, E-cadherin, and other tetraspanins (4, 6, 8, 16–19). Since cellular processes regulated by CD151 (such as cell adhesion, migration, and spreading) are integrin-mediated adhesive events, it has been proposed that CD151 modulates integrin activity and function. It was recently demonstrated...
that CD151 association increases the binding activity of integrin α5β1 to laminin through stabilizing its activated conformation (20). It was also reported that CD151 regulates platelet function by modulating outside-in signaling events of the major platelet integrin αIIbβ3 (21). In addition to integrin association, CD151 associates with phosphatidylinositol 4-kinase and protein kinase C on the cytosolic surface, thereby linking integrins to these signaling molecules (8, 22). CD151 has also been shown to regulate expression of a protein-tyrosine phosphatase, PTPμ, and its recruitment to cell-cell junctions (19) and to inhibit adhesion-dependent activation of Ras (23). It thus appears that the intracellular signaling pathways initiated by integrin binding to the extracellular matrix could be altered by the integrin-associated tetraspanin CD151. Taken together, CD151 is thought to participate in adhesion-dependent transmembrane signaling pathways by modulating integrin activity and modifying integrin-mediated outside-in signaling pathways as well. However, the modified integrin signaling pathways by which CD151 manifests its activity have not been established.

In this report, we investigated the functional effects of CD151 expression on cellular activities related to cancer invasion and metastasis and then attempted to identify CD151-mediated signaling pathways for the induction of such cellular functions. We showed that CD151 increases motility and MMP2-9 expression on cellular activities related to cancer invasion and metastasis and then attempted to identify CD151-mediated signaling pathways. Furthermore, we established that these signaling pathways are initiated not only by the matrix binding of integrin molecules but also by homophilic interactions between CD151 proteins on the surface of neighboring cells. Finally, detailed analysis of signaling events indicated that the CD151-α5β1/αvβ3 integrin complexes increase c-Jun activity through the activation of FAK, Src, p38 MAPK, and JNK.

EXPERIMENTAL PROCEDURES

Cell Culture, Antibodies, and Reagents—C8161 and MelJuSo are amelanotic human melanoma cell lines that metastasize following intradermal, subcutaneous, or intravenous injection into athymic nude mice (24, 25). C8161 and MelJuSo cells were cultured in DMEM/F-12 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin (all obtained from Invitrogen) in 5% CO2 at 37 °C. Monoclonal antibodies against CD151, CD9, and CD63 were purchased from PharMingen (San Diego, CA). Antibodies to FAK, phospho-FAK Tyr-925, Src, phospho-Src Tyr-416, ERK1/2, phospho-ERK1/2 Tyr-202/Tyr-204, p38 MAPK, phospho-p38 MAPK Thr-180/Tyr-182, JNK, phospho-JNK Thr-183/Tyr-185, c-Jun, phospho-c-Jun Ser-63/Ser-73, and paxillin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies to MAPKAPK-2 and phospho-MAPKAPK-2 Thr-334 were from New England Biolabs (Beverly, MA). PD98059, PP1, SB203580, and SP600125 were purchased from Biomol (Plymouth Meeting, PA). All other reagents were from Sigma unless indicated otherwise.

Transfection of CD151 cDNA and Selection of Stable Clones—Full-length CD151 cDNA was subcloned into the EcoRI/KpnI sites of a pcDNA3 vector (Invitrogen), downstream of a cytomegalovirus promoter. The CD151 cDNA expression construct was transfected into MelJuSo human melanoma cells by using Lipofectamine (Invitrogen) according to the manufacturer’s instructions. pcDNA3 vector only was also transfected as a control. Neomycin-resistant clones were isolated by growing the cells in DMEM/F-12 containing 10% fetal bovine serum and 0.5 mg/ml G418 (Invitrogen). Stable transfectant clones were characterized by immunoblotting and flow cytometric analyses for their expression levels of CD151 protein.

Transfection of Small Interfering RNA (siRNA)—siRNAs for FAK, src, p38 MAPK, and JNK were designed and synthesized using the software and SilencerTM siRNA construction kit from Ambion (Austin, TX) according to the manufacturer’s instructions. Specific oligonucleotide sequences for each target gene were as follows: 5′-GAGAAGGCCUAGCAAGAGdTdT-3′ (sense) and 5′-CUUCUGUGAGCUCCCUDdTdT-3′ (antisense) targeting FAK; 5′-GUGCAUAAGAAGACGCGCdCdTdT-3′ (sense) and 5′-GGGCUGUGCAGUUAAUGCCAGCdCdTdT-3′ (antisense) targeting src; 5′-AGCAGGGAGCUUUAGTdTdT-3′ (sense) and 5′-CUUAAGGAGGUGCUCAGCdCdTdT-3′ (antisense) targeting p38 MAPK; 5′-UGUCUGCUAAGAUCCUCUdTdT-3′ (sense) and 5′-AGAAGAGAGAAAGACAGCdCdTdT-3′ (antisense) targeting JNK; 5′-CAUCCUUAGGAGCAAdCdTdT-3′ (sense) and 5′-UUGAGGAUCCAAAGGUAGdTdT-3′ (antisense) targeting MMP-9. The siRNA control was 5′-UCUCCGAACGUACGUGCdCdTdT-3′ (sense) and 5′-ACGGUGAGCUGUUCCGAGAdCdTdT-3′ (antisense), which bears no homology with relevant human genes (26). For siRNA transfection, cells (5 × 105) were seeded in 6-well plates and grown for 24 h to reach 60–70% confluence. The different amounts of siRNA and the Lipofectamine reagent (5 µl) were diluted in 200 µl of DMEM/F-12 medium. The diluted siRNA-liposome complex was added to cells in DMEM/F-12 medium (800 µl). Following a 6-h incubation, cells were rinsed with fresh medium and grown for 24 h in normal growth medium containing fetal bovine serum before analysis.

Reverse Transcription-PCR Analysis—Total cellular RNA was purified from the cultured cells using Trizol reagent (Invitrogen) according to the manufacturer’s protocol. First strand cDNA synthesis was performed with 1 µg of total RNA using a cDNA synthesis kit (Promega, Madison, WI). For PCR amplification, 5′-aaggtaccaggatgggtgagttcaacgag-3′ was used as the sense primer, and 5′-gatatcgccgcgctcgtcgtcgac-3′ was used as the antisense primer. This primer pair amplifies a 760-bp fragment of CD151 cDNA. The reaction mixture was subjected to 25 PCR amplification cycles of 60 s at 94 °C, 90 s at 55 °C, and 90 s at 72 °C. β-Actin amplification was used as an internal PCR control (27) with 5′-gatatcgccgcgctcgtcgtcgac-3′ as the sense primer and 5′-gatagctgctgctgtcgcg-3′ as the antisense primer. The PCR products were visualized using ethidium bromide in 1% agarose gel.

2 The abbreviations used are: MMP, matrix metalloproteinase; FAK, focal adhesion kinase; MAPK, mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase; MAPKAPK-2, MAPK-activated protein kinase 2; Ab, antibody; mAb, monoclonal antibody; siRNA, small interfering RNA; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered protein; PIPES, 1,4-piperazinediethanesulfonic acid.
**Immunoblotting Analysis**—Cells were harvested, washed, and lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 1 mM phenylmethysulfonyl fluoride, 10 μg/ml aprotinin, 20 μg/ml leupeptin, and 2 mM benzimidone) on ice for 10 min. For phosphoprotein analysis, cell lysis buffer was supplemented with phosphatase inhibitors (1 mM sodium orthovanadate, 1 mM NaF, and 10 mM β-glycerophosphate). After centrifugation at 15,000 × g for 10 min, the supernatants were collected and quantified for protein concentration by Bradford assay. Equal amounts of protein per lane were separated onto 10% SDS-polyacrylamide gel and transferred to an Immobilon-P (Millipore Corp., Bedford, MA) membrane. The membrane was blocked in 5% skim milk for 2 h and then incubated with a specific antibody for 2 h. After washing, the membrane was incubated with a secondary antibody conjugated with horseradish peroxidase. After final washes, the membrane was developed using enhanced chemiluminescence reagents (Amersham Biosciences).

**Flow Cytometric Analysis**—Cells were incubated with 10 μg/ml anti-CD151 monoclonal antibody (mAb) for 30 min, washed with cold PBS, and then incubated with saturating concentrations of fluorescein isothiocyanate-conjugated goat anti-mouse IgG (PharMingen) for 30 min at 4 °C. After washing with PBS, the cells were fixed with 2% formaldehyde in PBS. Cell surface immunofluorescence was analyzed by flow cytometry performed on a FACScan (BD Biosciences).

**Immunoprecipitation**—Cells were lysed in immunoprecipitation buffer (25 mM Hepes, pH 7.5, 150 mM NaCl, 5 mM MgCl₂) supplemented with 1 mM phenylmethysulfonyl fluoride, 10 μg/ml aprotinin, 20 μg/ml leupeptin, and 1% Brij 98 or 1% Triton X-100 for 2 h at 37 °C. The lysate was centrifuged (16,000 × g, 15 min), and the supernatant was precleared with a combination of protein A- and protein G-agarose (Amersham Biosciences) precoated with normal mouse IgG for 2 h at 4 °C. After preclearing, the lysate was incubated with a specific antibody conjugated to the protein A/G-agarose beads for 2 h at 4 °C. Immune complexes collected on the beads were then washed four times with immunoprecipitation buffer and resolved by SDS-PAGE. Proteins were detected by immunoblotting analysis using specific antibodies.

**Invasion Assay into Matrigel**—24-well Transwell chamber inserts (Corning Costar, Cambridge, MA) with 8-μm porosity polycarbonate filters were precoated with 80 μg of base ment membrane Matrigel (BD Biosciences) onto the upper surface and with 20 μg of gelatin onto the lower surface. Culture supernatant of NIH3T3 fibroblasts in DMEM supplemented with 10% fetal bovine serum was placed in the lower well. MelySo cells suspended in DMEM/F-12 medium containing 0.1% fetal bovine serum were added to the upper chambers (2 × 10⁴ cells/well) and incubated for 24 h at 37 °C in 5% CO₂. Cells were fixed and stained with hematoxylin and eosin. Noninvading cells on the upper surface of the filter were removed by wiping out with a cotton swab, and the filter was excised and mounted on a microscope slide. Invasiveness was quantified by counting cells on the lower surface of the filter.

**Wound-healing Migration Assay**—For the measurement of cell migration during wound healing, cells (5 × 10⁵) were seeded in individual wells of a 24-well culture plate. When the cells reached a confluent state, cells were wounded with a plastic micropipette tip having a large orifice. The medium and debris were aspirated away and replaced by 2 ml of fresh serum-free medium. Cells were photographed every 12 h after wounding by phase-contrast microscopy. For evaluation of “wound closure,” five randomly selected points along each wound were marked, and the horizontal distance of migrating cells from the initial wound was measured.

**Gelatin Zymography**—Type IV collagenase activities present in conditioned medium were visualized by electrophoresis on gelatin-containing polyacrylamide gel as previously described (28). Briefly, conditioned medium from cells cultured in serum-free medium was mixed 3:1 with substrate gel sample buffer (40% (v/v) glycerol, 0.25 M Tris-HCl, pH 6.8, and 0.1% bromphenol blue) and loaded without boiling onto 10% SDS-polyacrylamide gel containing type I gelatin (1.5 mg/ml). After electrophoresis at 4 °C, the gel was soaked in 2.5% Triton X-100 with gentle shaking for 30 min with one change of detergent solution. The gel was rinsed and incubated for 24 h at 37 °C in substrate buffer (50 mM Tris-HCl, pH 7.5, 5 mM CaCl₂, and 0.02% NaN₃). Following incubation, the gel was stained with 0.05% Coomassie Brilliant Blue G-250 and destained in 10% acetic acid and 20% methanol.

**Cell Aggregation Assay**—L cells transfected with CD151 or vector alone were washed with PBS containing 2 mM EDTA and rendered into single cell suspension by seven gentle passages through a 22-gauge needle after scraping. After washing with Puck’s saline (5 mM KCl, 140 mM NaCl, 8 mM NaHCO₃, pH 7.4), suspensions of single cells (1 × 10⁷ cells/ml) were seeded into individual wells of a 24-well culture plate and incubated in 5% CO₂ at 37 °C with agitation at 70–80 rpm using an orbital shaker. Photographs were taken every 15 min after incubation under a phase-contrast microscope on three predetermined fields, and both the total cell number (A) and the number of cells remaining as single cells (B) were counted. The results were expressed as the percentage of cells that formed aggregates as follows: (A−B)/A × 100 (%). In some experiments, the transfectants were preincubated with antibody (20 μg/ml) and then washed free of unbound antibody before incubation. In experiments to determine whether aggregation was homophilic, distinct populations of cells were prelabeled with 5- and 6-CFSE (carboxyfluorescein diacetate succinimidyl ester) (Molecular Probes, Inc., Eugene, OR) before suspension. For these experiments, phase and fluorescent images of the same field were photographed after a 30-min incubation with orbital shaking.

**Promoter Assay**—A 1305-bp DNA fragment (−1285 to +20), corresponding to the promoter of the human MMP-9 gene (29, 30), was generously provided by Dr. Seung-Taek Lee (Yonsei University, Korea) (31). For mt-AP-1 of the MMP-9 promoter, in which distal and proximal AP-1 binding sites (−533 to −527 and −79 to −73, respectively) were destroyed, 5′-TGAGTCA-3′ was changed to 5′-TGAGTG-3′ (underlined lowercase letters indicate the mutated bases) by the QuikChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA). For mt-NF-κB of the MMP-9 promoter, in which
The CD151 Signaling Pathways Inducing Melanoma Cell Invasion

FIGURE 1. CD151 expression in human melanoma cell lines and stable clones of CD151-transfected MelJuSo cells. A and B, mRNA levels of CD151 (A) and protein levels of CD151, CD9, and CD63 (B) in the parental C8161 and MelJuSo cell lines were analyzed by reverse transcription-PCR using CD151 cDNA-specific primers and immunoblotting using mAbs specific to each protein, respectively. β-Actin mRNA and actin protein from each cell line were also analyzed to control for equal amounts of mRNAs and proteins, respectively. C, the stable clones of CD151 cDNA-transfected MelJuSo cells were examined for CD151 expression by immunoblotting analysis using anti-CD151 mAb. D, cell surface expression levels of CD151 protein in CD151 transfectant clones were analyzed by flow cytometry using anti-CD151 mAb.

a NF-κB binding site (−600 to −590) was destroyed, 5′-GGAATTTCCC-3′ was mutated to 5′-GATCTGCCC-3′. After subcloning the mutant MMP-9 promoters into a promoterless luciferase expression vector, pGL3 (Promega), the corresponding mutations in the constructs were verified by DNA sequencing. The pGL3 vector containing wild-type or mutant MMP-9 promoter was transfected into MelJuSo cells by using Lipofectamine. Luciferase activity in cell lysate was measured using the Promega luciferase assay system according to the instructions of the manufacturer. To normalize luciferase activity, each of the pGL3 vectors was co-transfected with a pRL-SV40L enhancerless luciferase expression vector, pGL3 (Promega), the corresponding mutations in the constructs were verified by DNA sequencing. The pGL3 vector containing wild-type or mutant MMP-9 promoters was transfected into a parental cell line in a cell line-specific manner among human melanoma cells. The tet-

resolved on native 5% polyacrylamide gel, and the gel was dried and subjected to autoradiography. Specificity for binding of AP-1 factors and NF-κB to the corresponding sequences of the MMP-9 promoter was confirmed by using cold competitors having typical AP-1 and NF-κB binding sequences (Promega), respectively.

Detergent-free Purification of Membrane Fractions—Mock and CD151 transfectant cells were washed with ice-cold PBS and then scraped into buffer A (20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 20 μg/ml leupeptin, and 2 mM benzamidine). The cells were homogenized using a Dounce homogenizer (20 strokes). A postnuclear supernatant was obtained by centrifugation (2500 × g, 10 min, 4 °C), adjusted to 10% sucrose and loaded onto a 30% sucrose cushion in an ultracentrifuge tube. After centrifugation for 60 min at 150,000 × g in a T-1270 rotor of a tabletop ultracentrifuge (Beckman Instruments), a light-scattering band confined to the 10–30% sucrose interface was collected and stored at −70 °C until use.

In Vitro Kinase Assays—Cellular proteins (200 μg) were incubated with anti-FAK, anti-Src, or anti-paxillin Abs and immunoprecipitated using protein A/G-agarose beads. Immune complexes collected on the beads were washed three times with immunoprecipitation buffer and once with kinase buffer (20 mM PIPES, pH 7.2, 10 mM MgCl₂, 1 mM dithiothreitol) and added to an in vitro kinase reaction mixture containing 5 μg of acid-denatured enolase and 10 μCi of [γ-32P]ATP (33). The reaction was incubated at 30 °C for 30 min and then stopped by boiling with SDS-sample buffer. After electrophoresis on a 10% polyacrylamide gel, the radioactive proteins were visualized by autoradiography.

RESULTS

CD151 Increases Motility, MMP-9 Expression, and Invasiveness of Human Melanoma Cells—First, we examined CD151 expression levels in two human metastatic melanoma cell lines, C8161 and MelJuSo, among which C8161 was illustrated to have a higher metastatic ability than MelJuSo (24, 25). A 760-bp PCR product and a 29-kDa protein band were detected in C8161, but not in MelJuSo cells, by reverse transcription-PCR analysis using CD151 cDNA-specific primers and immunoblotting analysis using anti-CD151 mAb, respectively (Fig. 1, A and B), indicating that CD151 is differentially expressed in a cell line-specific manner among human melanoma cells. The tet-

Electrophoretic Mobility Shift Assay—Cells were incubated with serum-free medium for 4 h, and nuclear extracts were prepared as previously described (32). Double-stranded oligonucleotide probes corresponding to the putative AP-1 binding site (−86 to −66; 5′-TGACCCCTGAGTCACTTG-3′; the AP-1 recognition sequence is underlined) and the putative NF-κB site (−607 to −582; 5′-GCCCCGTTGGAATTTCCCCC- AAATCCTG-3′; the NF-κB recognition sequence is underlined) in the proximal MMP-9 promoter sequences were labeled with [γ-32P]ATP using T4 polynucleotide kinase and purified by a G-50 Sephadex column. The 32P-labeled probes (~40,000 cpm) were then incubated with nuclear extracts (10 μg of protein) for 20 min at room temperature. Samples were
The CD151 Signaling Pathways Inducing Melanoma Cell Invasion

raspains CD9 and CD63, which were previously reported to suppress melanoma metastasis (28, 34–36), exhibited similar protein amounts between these two human melanoma cell lines (Fig. 1B). Since a positive role of CD151 in cancer metastasis has been shown in some other cancer cell types (14, 15), we hypothesized that the higher metastatic ability of C8161 cells, as compared with MelJuSo cells, may be attributed to the up-regulation of CD151 rather than the down-regulation of CD9 and CD63. To determine whether CD151 expression indeed elevates the metastatic potential of melanoma cells, MelJuSo cells devoid of endogenous CD151 expression were transfected with a CD151 cDNA expression vector. Among several transfectant clones displaying high CD151 protein expression, two clones, CD151/M-76 and CD151/ M-77, were selected for further functional analyses (Fig. 1, C and D). To investigate the functional effect of CD151 expression on cellular activities related to the cancer metastasis process, the in vitro invasive efficacy of each transfectant clone was determined by Boyden chamber assay using Matrigel. CD151 transfectant clones, CD151/M-76 and CD151/ M-77, showed a 3-fold higher invasiveness than the mock transfectant (Fig. 2A), suggesting that CD151 expression increases the invasive ability of melanoma cells. We further examined the migrating ability of CD151 transfectant clones into wounded spaces on culture plates. Both CD151 transfectant clones exhibited a 3-fold higher migrating ability than the mock transfectant (Fig. 2B). The basement membrane-degrading ability of the transfectant clones was also examined by measuring gelatinase activity in the culture supernatant. Among the two types of gelatinases, MMP-2 and MMP-9, the enzyme activity and protein level of MMP-9 were much higher in CD151 transfectant clones than in the mock transfectant, whereas the activity and expression of MMP-2 were not affected by CD151 expression (Fig. 2, C and D). To examine whether increased MMP-9 activity by CD151 affects cell motility, siRNA targeted to pro-MMP-9 was transfected into both CD151 transfectant clones. As a result, knockdown of MMP-9 suppressed the stimulating effect of CD151 on the motility of MelJuSo cells, indicating that cell motility induced by CD151 involves MMP-9 activity (Fig. 2, E and F). Since cell migration and gelatinase secretion are essential for the invasion process, it seems likely that the CD151-induced invasiveness is in part due to the positive effect of CD151 expression on motility and MMP-9 expression of melanoma cells.

Functional Involvement of FAK, Src, p38 MAPK, and JNK in CD151-stimulated Motility and MMP-9 Expression—To identify the signaling molecules involved in CD151-induced cell motility and MMP-9 expression, CD151 transfectants were analyzed in the presence of several inhibitors of signal transduction mediators. Among the inhibitors tested, the inhibitors...
The CD151 Signaling Pathways Inducing Melanoma Cell Invasion

including PP1 (a Src kinase family inhibitor), SB203580 (a p38 MAPK inhibitor), and SP600125 (a JNK inhibitor) suppressed the motility and MMP-9 expression of CD151 transfectant clones close to the levels of the mock transfectant (data not shown). To verify the participation of FAK, Src, p38 MAPK, and JNK in CD151 signaling pathway(s) for the induction of cell motility and MMP-9 expression, siRNAs targeted to FAK, Src, p38 MAPK, and JNK were transfected into CD151 transfectant cells. Protein levels of FAK, Src, p38 MAPK, and JNK were effectively knocked down by each specific siRNA (Fig. 3A, D, G, and J). All four siRNA types employed inhibited the migrating ability of CD151 transfectant cells in a dose-dependent manner (Fig. 3B, E, H, and K). Also, all of the FAK, Src, p38 MAPK, and JNK siRNA-transfected cells exhibited significantly decreased activities and expression levels of MMP-9 compared with control siRNA-transfected cells retaining endogenous levels of these signaling molecules (Fig. 3C, F, I, and L). It thus appears that FAK, Src, p38 MAPK, and JNK are functionally involved in CD151 signaling pathway(s) leading to increased motility and MMP-9 expression of MelJuSo melanoma cells.

Induction of MMP-9 Expression by CD151 Is Mediated by Activation of AP-1 Factors—Since MMP-9 appeared to be a target gene up-regulated by CD151 signaling pathway(s) in MelJuSo melanoma cells, we investigated the transcriptional regulation mode of the MMP-9 gene by using several mutants of its 5’-proximal promoter region. When a reporter vector containing a wild-type promoter of the MMP-9 gene was transiently transfected into MelJuSo cells, the CD151 transfectant cells showed about a 20-fold higher luciferase activity than the mock transfectant cells (Fig. 4A). In contrast to the wild-type promoter, the promoters having mutations at the AP-1 binding sites (mt-5’-AP-1 and mt-3’-AP-1) did not respond to CD151 for their activities for reporter gene expression. However, mutation of the NF-κB binding site did not abolish the stimulating effect of CD151 on MMP-9 promoter activity. To determine whether CD151 expression increases DNA binding activity of AP-1 transcriptional factors, we compared the binding of nuclear proteins to a putative AP-1 binding site (−79 to −73) of the MMP-9 promoter between mock and CD151 transfectant cells. As shown in Fig. 4B, DNA binding activity of AP-1 factors in CD151 transfectant cells was more significant than that in mock transfectant cells. Moreover, incubation with anti-c-Jun antibody resulted in a partial supershift of the AP-1/DNA complex with the gel shift assay, indicating that c-Jun participates in the formation of the AP-1/DNA complex. Thus, these data indicate that CD151 increases MMP-9 gene transcription by activating AP-1 transcription factors, including c-Jun.

CD151 Signaling Pathway(s) Depends on Activation of the Associated α6β1 and αvβ3 Integrins—CD151 has been reported to associate with various types of integrins and, in particular, forms stable complexes with α6β1 and αvβ3 integrins in many types of cells (4, 6, 8, 17, 18). To determine whether α6β1 and αvβ3 integrins are also associated with CD151 in MelJuSo melanoma cells, the CD151-transfected MelJuSo cells were lysed with the nonionic detergent Brij 98, a mild lysis condition preserving tetraspanin-integrin interactions, and the cell lysates were immunoprecipitated with anti-CD151 antibody. As a result, αv, αv, and β3 integrin subunits were detected in the CD151 immunoprecipitate of CD151 transfectant cells but not in mock transfectant cells (Fig. 5A), although the protein level of each integrin subunit was not different between CD151 and mock transfectant cells (Fig. 5B). This result indicates that CD151 can form complexes with αvβ3 and αvβ3 integrins in MelJuSo cells. Since αvβ3 and αvβ3 integrins are known to be receptors of laminin/fibronectin and laminin, respectively, we examined whether CD151-stimulated MMP-9 expression is dependent on cell adhesion to

![Figure 3. CD151-stimulated cell motility and MMP-9 production are abrogated by siRNAs targeted to FAK, src, p38 MAPK, and JNK. A CD151 transfectant clone of MelJuSo cells, CD151/M-77, was transfected with control, FAK, src, p38 MAPK, or JNK siRNAs. A, D, G, and J, protein levels of FAK, Src, p38 MAPK, and JNK were analyzed by immunoblotting using antibodies specific to each protein at 48 h after transfection. B, E, H, and K, following siRNA transfection, cell migration was measured at 48 h after wounding in a similar fashion as in Fig. 2B. C, F, I, and L, activities and protein levels of MMP-9 in the conditioned media of the siRNA-transfected cells were assessed by gelatin zymography and immunoblotting analysis (IB), respectively. Asterisks and daggers indicate that the differences are statistically significant (*, **, and †, p < 0.01 versus control siRNA-transfected cells, Student’s t test).](image-url)
extracellular matrix components, such as laminin and fibronectin. As illustrated in Fig. 5C, the stimulating effect of CD151 on MMP-9 expression became more prominent when the cells were attached to laminin, fibronectin, and laminin-rich Matrigel. However, CD151 did not exert its inducing activity for MMP-9 expression when the cells were plated on poly-(L)-lysine, which does not activate integrins. In CD151-deficient mock transfectant cells, a slight increase in MMP-9 expression was observed by cell adhesion to laminin and fibronectin, suggesting that integrin activation alone is not sufficient to induce MMP-9 expression. Thus, CD151 appears to cooperate with associated integrins to induce MMP-9 expression in melanoma cells.

To examine how associated integrins activated by their binding to extracellular matrix modulate CD151-dependent signaling pathway(s), the activation status of the CD151 signaling mediators, which were demonstrated in Fig. 3, were compared between mock and CD151 transfectant cells a short time after plating the cells on laminin. CD151 expression significantly elevated phosphorylation-dependent activation of signaling components, such as FAK, Src, p38 MAPK, and JNK dependently of cell adhesion to laminin (Fig. 5D). CD151-mediated phosphorylation of MAPKAPK-2 and c-Jun, downstream effectors of p38 MAPK and JNK, respectively, was also found to be dependent on cell adhesion to laminin. However, adhesion events without integrin activation, such as cell binding to poly-(L)-lysine, did not increase the phosphorylation levels of these CD151 signaling components. On the other hand, phosphorylation of ERK1/2 appeared to be affected by neither integrin activation nor CD151 expression, implying that ERK1/2 does not participate in integrin-dependent CD151 signaling pathways in MelJuSo cells. Taken together, these data strongly suggest that CD151 cooperates with associated integrins to provoke outside-in signaling pathways leading to the activation of FAK, Src, p38 MAPK, JNK, MAPKAPK-2, and c-Jun.

**CD151 Is a Homophilic Interacting Protein**—Since some membrane proteins involved in cell adhesion and migration, such as E-cadherin and CD99, were found to be self-ligand molecules and their homophilic interactions regulate intracellular signaling pathways (37, 38), we tested the possibility that CD151 is a homophilic interacting membrane protein. After transfecting a CD151 expression vector into murine L-cell fibroblast cells, which do not exhibit homotypic cell-to-cell adhesion, we compared the ability of stable CD151 transfectant L cells to adhere to each other or to empty vector-transfected control L cells by spontaneous cell aggregation assay using cells in suspension. We found that control L cells did not aggregate, but CD151-transfected L cells aggregated in a time-dependent manner (Fig. 6B). The aggregation of CD151-transfected cells was reduced to half after incubation with anti-CD151 antibody, suggesting that the L cell aggregation is mediated by CD151. To confirm whether this aggregation was homophilic, we mixed CD151-transfected L cells with an equal number of fluorescently labeled control L cells. As a result, no fluorescent cells were present in the aggregates, indicating that CD151-expressing L cells did not bind to control L cells lacking CD151 (Fig. 6C). However, when fluorescently labeled CD151 transfectant cells were mixed with unlabeled control L cells, every cell in the aggregates was labeled (Fig. 6D). These data illustrate that CD151-expressing L cells bind only to the same type of L cells having CD151 but not to L cells lacking CD151. It thus appears that CD151 is a homophilic interacting cell surface protein.

**Homophilic CD151 Interactions Enhance Cell Motility and MMP-9 Expression**—To assess the effect of homophilic CD151 interactions on the motility and MMP-9 expression of MelJuSo cells, we prepared membrane fractions of MelJuSo cells, we prepared membrane fractions of MelJuSo cells transfected with either a CD151 expression vector or empty vector. As expected, CD151 was present in the membrane fraction of the CD151 transfectant but not in that of the mock transfectant (Fig. 7A). The CD151 transfectant cells treated with membrane fraction containing CD151 exhibited increased cell motility compared with untreated cells (Fig. 7B). The CD151-containing membrane fraction also increased MMP-9 expression in
CD151 transfectant cells (Fig. 7C). However, pretreatment of CD151 transfectant cells with anti-CD151 Ab blocked the inducing effect of the CD151 membrane fraction on MMP-9 expression (Fig. 7D). Meanwhile, the CD151-deficient membrane fraction obtained from mock transfectant cells did not affect the motility and MMP-9 expression of CD151 transfectant cells. In addition, mock-transfectant cells lacking CD151 did not respond to the CD151-containing membrane fraction for the induction of cell motility and MMP-9 expression. Thus, these results indicate that homophilic interactions of CD151 increase cell motility and MMP-9 expression in MelJuSo cells.

Homophilic CD151 Interactions Stimulate the c-Jun Activation Signaling Pathways in an Integrin-dependent Manner—We examined whether homophilic interactions of CD151 trigger the outside-in signaling pathway(s) leading to c-Jun activation. Treatment of CD151 transfectant cells, but not mock transfectant cells, with the CD151-containing membrane fraction increased the phosphorylation levels of CD151 signaling mediators, such as FAK, Src, and c-Jun, in a time-dependent manner (Fig. 8A). However, phosphorylation levels of these signaling molecules were not increased in CD151 transfectant cells incubated with the CD151-deficient membrane fraction. These data suggest that homophilic CD151 interactions between neighboring cells activate the signaling pathways for c-Jun activation in one another.

We next investigated the possible influence of integrin activation on signaling pathways provoked by homophilic CD151 interactions. When CD151 transfectant cells were seeded onto plates coated with poly-L-lysine, homophilic CD151 interactions resulted in a slight increase in the phosphorylation levels of Src and c-Jun, along with no increase in FAK phosphorylation (Fig. 8B). However, cell adhesion to laminin not only increased the phosphorylation level of FAK but also significantly augmented the positive effect of homophilic CD151 interactions on the phosphorylation of Src and c-Jun. Kinase activities of FAK and Src associated with paxillin in focal adhesion complexes were also found to be increased by homophilic CD151 interactions dependent on cell adhesion to laminin (Fig. 8C). These data indicate a stimulating role of integrins in CD151-mediated signaling pathways. Meanwhile, as illustrated in mock transfectant cells attached to laminin, simple activation of laminin-binding integrins without any homophilic CD151 interaction was not sufficient to induce phosphorylation of these signaling molecules. To assess the involvement of CD151-associated integrins, α1β1, and α5β1, in up-regulating CD151 signaling to c-Jun, we incubated CD151 transfectant cells with anti-β1 integrin antibody before seeding the cells on laminin-coated plates. The anti-β1 integrin antibody effectively suppressed the stimulating effect of homophilic CD151 interactions on c-Jun phosphorylation (Fig. 8D), indicating direct participation of β1-type integrins in modulating CD151 signaling for c-Jun activation. The dependence of CD151 signaling on β1-type integrins was also observed in MMP-9 expression (Fig. 8D). Taken together, these results strongly suggest that activation of the CD151-associated α1β1 and α5β1 integrins amplifies the c-Jun activation signaling pathways initiated by homophilic CD151 interactions. We next investigated the participation of MAPKs in CD151 signaling to c-Jun by using inhibitors specific for ERK, p38 MAPK, and JNK. c-Jun phosphorylation in CD151 transfectant cells was significantly blocked by the p38 MAPK inhibitor, SB203580, and the JNK inhibitor, SP600125, as well as by the Src kinase inhibitor, PP1, but not by the ERK inhibitor, PD98059 (Fig. 8E). Since previous results showed CD151-induced adhesion-dependent activation of Src, p38 MAPK, and JNK, but not ERK (Fig. 5D), it is very likely that Src-mediated activation of p38 MAPK and JNK may play an important role in transducing CD151 signals to c-Jun. We finally examined whether integrin-dependent CD151 signaling events also occur in another melanoma cell line, C8161, which possesses endogenous CD151 (Fig. 1A). Similar to CD151-transfected MelJuSo cells, C8161 cells responded to the CD151-containing membrane fraction for the
The CD151 Signaling Pathways Inducing Melanoma Cell Invasion

phosphorylation of FAK, Src, and c-Jun in an adhesion-dependent manner (Fig. 8F). However, the phosphorylation levels of these signaling molecules in C8161 cells were not increased in the absence of homophilic CD151 interaction and integrin activation. These results indicate that homophilic CD151 interactions between two contacting human melanoma cells with endogenous CD151 activate the intracellular signaling pathways in one another with the cooperation of associated integrins.

**DISCUSSION**

Tetraspanin CD151 has been implicated in the regulation of cell motility, cancer invasion, and metastasis (14, 15, 39). Anti-CD151 antibody has been shown to inhibit wound-healing migration of endothelial cells, chemotactic motility of neutrophils, and phagokinetic motility of cancer cells (14, 39). CD151 overexpression enhanced invasive and metastatic abilities of several cancer cell lines, whereas treatment of cells with anti-CD151 antibody suppressed these abilities (14, 15). In this report, we also demonstrated that transfection of CD151 cDNA into a CD151-deficient melanoma cell line up-regulates MMP-9 expression, resulting in the promotion of cancer cell motility and invasiveness (Fig. 2). Among the tetraspanin proteins, CD9 and CD63 have also been associated with the invasion metastasis of melanoma, but these associations have opposing effects to that of CD151 (28, 34–36, 40–42). Transfection with CD9 resulted in suppression of cell motility and metastasis of murine melanoma cells (34, 36). CD63 expression has been shown to be inversely correlated with the malignant progression of human melanoma (40, 41), and several transfection studies have demonstrated the suppressing role of CD63 in the invasion and metastasis of melanoma cells (28, 35, 42). Thus, tetraspanins CD9, CD63, and CD151 appear to play a role in the processes of melanoma invasion and metastasis, in which CD151 acts as a positive effector opposite to the role of CD9 and CD63.

Tetraspanin proteins have been suggested to be involved in signal transduction by regulating the organization and assembly of signaling complexes in membrane microdomains, referred to as the “tetraspanin web” (5, 6, 43). Among the tetraspanins, CD151 shows strong lateral association with laminin-binding integrins, such as $\alpha_5\beta_1$, $\alpha_6\beta_1$, and $\alpha_5\beta_1$. We here also found that, in MelJuSo human melanoma cells, CD151 expressed by gene transfection became associated with $\alpha_5\beta_1$ and $\alpha_5\beta_1$ integrins (Fig. 5A). Additionally, CD151 was reported to interact with phosphatidylinositol 4-kinase and protein kinase C, thereby linking integrins to these signaling molecules (8, 22). Several studies have demonstrated that CD151 modulates integrin-dependent cellular activities, including cell adhesion, migration, spreading, and cell morphology on Matrigel (8, 20, 39, 44–46). The broad range of integrin association of CD151 and its involvement in integrin-mediated adhesive events strongly suggests a primary role of CD151 in regulating integrin activity and function. Indeed, CD151 association was found to modulate the ligand-binding activity of $\alpha_5\beta_1$ integrin through stabilizing its activated conformation (20). The strength of $\alpha_5\beta_1$ integrin-mediated adhesion to laminin was also enhanced by...
CD151 (46). Furthermore, outside-in signaling through $\alpha_6\beta_1$ integrin was markedly influenced by its lateral association with CD151 (45). The short C-terminal cytoplasmic region of CD151 was found to be particularly important for determining the outside-in signaling functions of $\alpha_6\beta_1$ integrin (45). Thus, most studies of CD151 have focused on its role in modulating the activity and function of associated integrin molecules. Therefore, participation of CD151 in signal transduction has been confined to its regulatory activity toward integrin-mediated transmembrane signaling events.

In contrast to previously identified roles for CD151 as a modulator for integrin-mediated signaling, we here demonstrated that CD151 can transduce its own signals leading to increases in MMP-9 expression, cell motility, and invasiveness. Cross-linking of tetraspanins CD81 and CD82 at the cell surface with antibodies was reported to transduce activation signals, such as tyrosine phosphorylation, calcium fluxes, and inositol turnover (47–50). Therefore, we postulated that the CD151-involved signaling pathways may be initiated by ligand binding to CD151 as well as by activation of associated integrins. As a result of searching for a ligand for CD151, we found that CD151 is a self-ligand molecule, implying that homophilic interactions of CD151 proteins take place between two neighboring cells (Fig. 6). We also found that the positive effect of CD151 expression on cell motility and MMP-9 expression is further elevated when CD151-expressing cells were treated with a CD151-containing membrane fraction but not with a CD151-deficient membrane fraction (Fig. 7). Furthermore, treatment with a CD151-containing membrane fraction activated signaling molecules, such as FAK, Src, and c-Jun, in CD151-expressing cells (Fig. 8A), suggesting that homophilic CD151 interactions between two contacting cells provoke intracellular signaling events in both cells. However, the CD151 signaling appears to be dependent on the activation of laminin-binding integrins. Adhesion-dependent activation of several signaling molecules, including FAK, Src, p38 MAPK, JNK, MAPKAPK-2, and c-Jun, became
The CD151 Signaling Pathways Inducing Melanoma Cell Invasion

The CD151 interactions generating cell-to-laminin adhesion but also provoked by homophilic CD151 interactions generating cell-to-cell adhesion.

Many genes that participate in tumor cell invasion and migration have been identified, including adhesion molecules, small GTPases, cytoskeletal components, and matrix metalloproteinases (51). However, there is little consensus on what controls the expression of these genes and how a program of gene expression is coordinated to manifest an invasive phenotype. In the present study, we demonstrated that CD151 functions as a positive regulator in the adhesion-dependent activation of c-Jun, a component of the AP-1 transcription complex. An increase in the phosphorylation level of c-Jun by cell adhesion to laminin was observed in CD151 transfectant cells but not in mock transfectant cells (Fig. 5D). Homophilic interactions of CD151 further increased c-Jun phosphorylation in an adhesion-dependent manner (Fig. 5, A, B, and F), demonstrating the marked effect of CD151 signaling on the activation of c-Jun upon integrin activation. Increased expression of AP-1 component proteins and AP-1 activity has been shown to enhance invasion and motility in various model systems (52). Overexpression of c-Jun induces the invasiveness of chick embryo fibroblasts and MCF-7 breast cancer cells (53, 54). In contrast, expression of a c-Jun mutant in which Ser-63 and Ser-73 are mutated to alanine residues, so that the protein cannot be phosphorylated by JNK, inhibits the migration of fibroblasts (55). A dominant negative mutant of c-fos, one of the Jun subfamily partners in AP-1 dimers, inhibits the motility of fibrosarcoma cells, along with growth arrest at the G1 phase of the cell cycle (56). Another Fos subfamily member, Fra-1 also modulates the invasiveness and motility of MCF-7 cells (57). We here showed that the stimulating effect of CD151 on cell motility was abolished when JNK was knocked down by siRNA (Fig. 5K). The functional involvement of AP-1 activity in invasion is more evident in the regulation of MMP-9 gene expression. Two AP-1 binding sites exist in the 5′-proximal promoter region of the MMP-9 gene (29), and both sites were found to be essential for CD151-induced MMP-9 gene transcription (Fig. 4A). Results from a gel mobility shift assay indicated that CD151 overexpression in MelJuSo cells increased the binding of nuclear proteins, such as c-Jun, to oligonucleotides containing AP-1 consensus sequences (Fig. 5, A, B, and F).
FIGURE 8. Homophilic CD151 interactions trigger the integrin-dependent c-Jun activation signaling pathways. A, mock and CD151 transfectant cells (4 × 10⁶) were seeded into 6-well plates for 24 h and starved for serum for 60 min. Suspension of the membrane fractions of each transfectant (50 μg of protein) were added to the cell cultures for the indicated time periods. Phosphorylation levels of FAK, Src, and c-Jun in the cell lysates were compared by immunoblotting analyses using specific antibodies for phospho-FAK Tyr-925, phospho-Src Tyr-416, and phospho-c-Jun Ser-63/Ser-73, respectively. The protein levels of FAK, Src, and c-Jun were also determined in the same blots by immunoblotting analyses using antibodies for each protein. B, cells were seeded onto plates precoated with laminin (LN) or poly-(L)-lysine (p-Lys) for 24 h. Following 60 min of serum starvation, the cells were treated with membrane fractions of CD151 transfectant cells for the indicated time periods. Immunoblotting analyses were performed in the same fashion as in A. C, FAK, Src, and paxillin were immunoprecipitated from the lysates of mock and CD151 transfectant cells treated with CD151-containing membrane fragments for 60 min, using antibodies specific to each protein. The FAK, Src, and paxillin immune complexes were incubated with acid-denatured enolase in kinase buffer containing [γ-³²P]ATP for 30 min. All kinase reaction samples were subjected to SDS-PAGE and autoradiography. D, the CD151 transfectant cells were suspended into serum-free medium containing normal mouse IgG or anti-β₁ integrin Ab (0.1 mg/ml) and kept in suspension for 60 min. 18 h after seeding onto laminin- or poly-(L)-lysine-coated plates, the cells were incubated with membrane fractions of mock or CD151 transfectant cells for 60 min. The cell lysates were subjected to immunoblotting analyses using anti-phospho-c-Jun, anti-c-Jun, and anti-MMP-9 Abs; E, the CD151 transfectant cells were serum-starved for 60 min and treated with PP1 (0.25 μM), PD98059 (PD; 30 μM), SB203580 (SB; 20 μM), SP600125 (SP; 20 μM), or MeSO (for vehicle control) for 2 h. After incubating the cells with serum-free medium containing the membrane fractions of CD151 transfectant cells for 60 min, c-Jun phosphorylation levels in the cell lysates were examined by immunoblotting analysis. F, C8161 melanoma cells possessing endogenous CD151 were seeded onto plates precoated with laminin or poly-(L)-lysine for 24 h. Following 60 min of serum starvation, the cells were treated with mock or CD151 transfectant membrane fractions of MeJuSo cells for the indicated time periods. Immunoblotting analyses were performed in the same fashion as in A.
The CD151 Signaling Pathways Inducing Melanoma Cell Invasion

REFERENCES

1. Fitter, S., Tetaz, T. J., Berndt, M. C., and Ashman, L. K. (1995) Blood 86, 1348–1355
2. Hasegawa, H., Utsunomiya, Y., Kishimoto, K., Yanagisawa, K., and Fujita, S. (1996) J. Virol. 70, 3258–3263
3. Maecker, H. T., Todd, S. C., and Levy, S. (1997) FASEB J. 11, 428–442
4. Boucheix, C., and Rubinstein, E. (2001) Cell Mol. Life Sci. 58, 1189–1205
5. Yanez-Mo, M., Mittelbrunn, M., and Sanchez-Madrid, F. (2001) Microcirculation 8, 153–168
6. Hemler, M. E. (2001) J. Cell Biol. 155, 1103–1107
7. Sincoc, P. M., Mayrhofer, G., and Ashman, L. K. (1997) J. Histochem. Cytochem. 45, 515–525
8. Yauch, R. L., Berditchevski, F., Harler, M. B., Reichner, J., and Hemler, M. E. (1998) Mol. Biol. Cell 9, 2751–2765
9. Penas, P. F., Garcia-Diez, A., Sanchez-Madrid, F., and Yanez-Mo, M. (2000) J. Invest. Dermatol. 114, 1126–1135
10. Hemler, M. E. (2001) J. Cell Sci. 114, 577–587
11. Tokuhara, H., Hasegawa, H., Hattori, N., Ishida, H., Taki, T., Tachibana, S., Sasaki, S., and Miyake, M. (2001) Clin. Cancer Res. 7, 4109–4114
12. Hashida, H., Takabayashi, A., Tokuhara, T., Hattori, N., Taki, T., Hasegawa, H., Sato, S., Kobayashi, N., Yamaoka, Y., and Miyake, M. (2003) Br. J. Cancer 89, 158–167
13. Ang, J., Ljovic, M., Ashman, L. K., Kan, K., and Frauman, A. G. (2004) Cancer Epidemiol. Biomarkers Prev. 13, 1717–1721
14. Testa, J. E., Brooks, P. C., Lin, J. M., and Quigley, J. P. (1999) Cancer Res. 59, 3812–3820
15. Kohno, M., Hasegawa, H., Miyake, M., Yamamoto, T., and Fujita, S. (2002) Int. J. Cancer 97, 336–343
16. Sterk, L. M., Guijjen, C. A., Oomen, L. C., Calafat, J., Janssen, H., and Sonnenberg, A. (2000) J. Cell Biol. 149, 969–982
17. Stipp, C. S., Kolesnikova, T. V., and Hemler, M. E. (2003) Trends Biochem. Sci. 28, 106–112
18. Sterk, L. M., Guijjen, C. A., van den Berg, J. G., Claessen, N., Weening, J. J., and Sonnenberg, A. (2002) J. Cell Sci. 115, 1161–1173
19. Chattopadhay, N., Wang, Z., Ashman, L. K., Brady-Kalnay, S. M., and Kreidberg, J. A. (2003) J. Cell Biol. 163, 1351–1362
20. Nishiuchi, R., Sanzen, N., Nada, S., Sumida, Y., Wada, Y., Okada, M., Takagi, J., Hasegawa, H., and Sekiguchi, K. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 1939–1944
21. Lau, L. M., Wei, J. L., Wright, M. D., Moseley, G. W., Hogarth, P. M., Ashman, L. K., and Jackson, D. E. (2004) Blood 104, 2368–2375
22. Zhang, X. A., Bontrager, A. L., and Hemler, M. E. (2001) J. Biol. Chem. 276, 25005–25013
23. Sawada, S., Yoshimoto, M., Odintsova, E., Hotchin, N. A., and Berditchevski, F. (2003) J. Biol. Chem. 278, 26323–26326
24. Welch, D. R., Bisi, J. E., Miller, B. E., Conaway, D., Seftor, E. A., Yokem, K. H., Gilmore, L. B., Seftor, R. E., Nakajima, M., and Hendrix, M. J. (1991) Int. J. Cancer 47, 227–237
25. Miele, M. E., Robertson, G., Lee, J. H., Coleman, A., McGary, C. T., Fisher, P. B., Lugo, T. G., and Welch, D. R. (1996) Mol. Carcinog. 15, 284–299
26. Duxbury, M. S., Ito, H., Zinner, M. J., Fisher, P. B., Lugo, T. G., and Welch, D. R. (1996) Mol. Carcinog. 15, 284–299
27. Nakajima-Iijima, S., Hamada, H., Reddy, P., and Kakunaga, T. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 6133–6137
28. Jiang, H. I., and Lee, H. (2003) Exp. Mol. Med. 35, 317–323
29. Sato, H., and Seiki, M. (1993) Oncogene 8, 395–405
30. Eberhardt, W., Schulze, M., Engels, C., Klasmeier, E., and Pfeilschifter, J.
The CD151 Signaling Pathways Inducing Melanoma Cell Invasion

(2002) Mol. Endocrinol. 16, 1752–1766
31. Hah, N., and Lee, S. T. (2003) Biochem. Biophys. Res. Commun. 305, 428–433
32. Na, H. J., Lee, S. I., Kang, Y. C., Cho, Y. L., Nam, W. D., Kim, P. K., Ha, K. S., Chung, H. T., Lee, H., Kwon, Y. G., Koh, J. S., and Kim, Y. M. (2004) J. Immunol. 173, 1276–1283
33. Gabarra-Niecko, V., Keely, P. J., and Schaller, M. D. (2002) Biochem. J. 365, 591–603
34. Ikeyama, S., Koyama, M., Yamaoko, M., Sasada, R., and Miyake, M. (1993) J. Exp. Med. 177, 1231–1237
35. Radford, K. J., Mallesch, J., and Hersey, P. (1995) Int. J. Cancer 62, 631–635
36. Miyake, M., Inufusa, H., Adachi, M., Ishida, H., Hashida, H., Tokuhara, T., and Kakehi, Y. (2000) J. Cell. Biol. 141, 791–804
37. Nelson, W. J., and Nusse, R. (2004) Science 303, 1483–1487
38. Schenkel, A. R., Mamdouh, Z., Chen, X., Liebman, R. M., and Muller, W. A. (2002) Nat. Immunol. 3, 143–150
39. Yaney-Mo, M., Alfranca, A., Cabanas, C., Marazuela, M., Tejedor, R., Ursa, M. A., Ashman, L. K., de Landazuri, M. O., and Sanchez-Madrid, F. (1999) J. Cell Biol. 141, 791–804
40. Atkinson, B., Ernst, C. S., Ghrist, B. F., Herlyn, D., Steplewski, Z., and Koprowski, H. (1984) Cancer Res. 44, 2577–2581
41. Kondoh, M., Ueda, M., Ichihashi, M., and Mishima, Y. (1993) Melanoma Res. 3, 241–245
42. Radford, K. J., Thorne, R. F., and Hersey, P. (1997) J. Immunol. 158, 3353–3358
43. Berditchevski, F. (2001) J. Cell Sci. 114, 4143–4151
44. Stipp, C. S., and Hemler, M. E. (2000) J. Cell Sci. 113, 1871–1882
45. Zhang, X. A., Kazarov, A. R., Yang, X., Bontrager, A. L., Stipp, C. S., and Hemler, M. E. (2002) Mol. Biol. Cell 13, 1–11
46. Lammerding, J., Kazarov, A. R., Huang, H., Lee, R. T., and Hemler, M. E. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 7616–7621
47. Gil, M. L., Vita, N., Lebel-Binay, S., Miloux, B., Chalon, P., Kaghad, M., Marchiol-Fournigault, C., Conjeaud, H., Caput, D., Ferrara, P., Fradelizi, D., and Quillet-Mary, A. (1992) J. Immunol. 148, 2826–2833
48. Schick, M. R., Nguyen, V. Q., and Levy, S. (1993) J. Immunol. 151, 1918–1925
49. Lebel-Binay, S., Lagaudriere, C., Fradelizi, D., and Conjeaud, H. (1995) J. Leukocyte Biol. 57, 956–963
50. Lee, B., Jin, K., Hahn, J. H., Song, H. G., and Lee, H. (2003) Exp. Mol. Med. 35, 30–37
51. Liotta, L. A., Steeg, P. S., and Stetler-Stevenson, W. G. (1995) Cell 64, 327–336
52. Ozanne, B. W., McGarry, L., Spence, H. I., Johnston, L., Winnie, I., Meagher, L., and Stapleton, G. (2000) Eur. J. Cancer 36, 1640–1648
53. Bos, T. J., Margiotta, P., Bush, L., and Wasielenko, W. (1999) Int. J. Cancer 81, 404–410
54. Rinehart-Kim, J., Johnston, M., Birrer, M., and Bos, T. (2000) Int. J. Cancer 88, 180–190
55. Javelaud, D., Laboureau, J., Gabilson, E., Verrecchia, F., and Mauvial, A. (2003) J. Biol. Chem. 278, 24624–24628
56. Bahassi, E. M., Karyala, S., Tominson, C. R., Sartor, M. A., Medvedovic, M., and Hennigian, R. F. (2004) Clin. Exp. Metastasis 21, 293–304
57. Belguise, K., Kersual, N., Galter, F., and Chalbos, D. (2005) Oncogene 24, 1434–1444
58. Minden, A., and Karin, M. (1997) Biochim. Biophys. Acta 1333, F85–F104
59. Leppa, S., Safir-rich, R., Ansorge, W., and Bohmann, D. (1998) EMBO J. 17, 4404–4413
60. Dunn, C., Wiltshire, C., MacLaren, A., and Gillespie, D. A. (2002) Cell. Signal. 14, 585–593
61. Johnson, G. L., and Lapadat, R. (2002) Science 298, 1911–1912
62. Khosravi-Far, R., Campbell, S., Rossman, K. L., and Der, C. J. (1998) Adv. Cancer Res. 72, 57–107
63. Xiao, Y. Q., Malcolm, K., Worthen, G. S., Gardai, S., Schiemann, W. P., Fadok, V. A., Bratton, D. L., and Henson, P. M. (2002) J. Biol. Chem. 277, 14884–14893
64. Rangaswami, H., Bulbule, A., and Kundu, G. C. (2005) J. Biol. Chem. 280, 19381–19392
65. Waetzig, V., and Herdegen, T. (2005) Mol. Cell Neurosci. 30, 67–78
66. Hoepfner, D., van den Berg, M., Philippsen, P., Tabak, H. F., and Hettema, E. H. (2001) J. Cell Biol. 155, 979–990
67. Stokes, K. D., and Osteryoung, K. W. (2003) Gene (Amst.) 320, 97–108
68. Pörschke, S., Froehlich, J. E., Koksharova, O., Pyke, K. A., van Erp, H., and Osteryoung, K. W. (2003) Plant Cell 15, 1918–1933
69. Simon, C., Simon, M., Vucelic, G., Hicks, M. J., Pilkert, P. K., Koitschev, A., and Zenner, H. P. (2001) Exp. Cell Res. 271, 344–355
70. Hong, S., Park, K. K., Magae, J., Ando, K., Lee, T. S., Kwon, T. K., Kwik, J. Y., Kim, C. H., and Chang, Y. C. (2005) J. Biol. Chem. 280, 25202–25209