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Kyoto University
The Sal-like 4 - integrin $\alpha_{6}\beta_{1}$ network promotes cell migration for metastasis via activation of focal adhesion dynamics in basal-like breast cancer cells.

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Abstract

During metastasis, cancer cell migration is enhanced. However, the mechanisms underlying this process remain elusive. Here, we addressed this issue by functionally analyzing the transcription factor Sal-like 4 (SALL4) in basal-like breast cancer cells. Loss-of-function studies of SALL4 showed that this transcription factor is required for the spindle-shaped morphology and the enhanced migration of cancer cells. SALL4 also up-regulated integrin gene expression. The impaired cell migration observed in SALL4 knockdown cells was restored by overexpression of integrin α6 and β1. In addition, we clarified that integrin α6 and β1 formed a heterodimer. At the molecular level, loss of the SALL4 - integrin α6β1 network lost focal adhesion dynamics, which impairs cell migration. Over-activation of Rho is known to inhibit focal adhesion dynamics. We observed that SALL4 knockdown cells exhibited over-activation of Rho. Aberrant Rho activation was suppressed by integrin α6β1 expression, and pharmacological inhibition of Rho activity restored cell migration in SALL4 knockdown cells. These results indicated that the SALL4 - integrin α6β1 network promotes cell migration via modulation of Rho activity. Moreover, our zebrafish metastasis assays demonstrated
that this gene network enhances cell migration *in vivo*. Our findings identify a potential new therapeutic target for the prevention of metastasis, and provide an improved understanding of cancer cell migration.

### Highlights

- SALL4 up-regulates integrin α6β1 expression at the transcription level.
- The SALL4 - integrin α6β1 network is required for a spindle-shaped morphology.
- The SALL4 - integrin α6β1 network activates focal adhesion dynamics.
- The SALL4 - integrin α6β1 network modulates Rho activation for cell migration.

### Keywords

- Breast cancer; Cell migration; Focal adhesion dynamics; Integrin; SALL4

### Abbreviations

- ECM: extracellular matrix, FA: Focal adhesion, FAK: Focal adhesion kinase, SALL4: Sal-like 4
1. Introduction

In contrast to normal cells, cancer cells display metastatic properties, including migration, invasion and anoikis resistance. During metastasis, cells must migrate to depart from the primary site and travel to other tissues and organs. However, little is known about how cancer cells change their character to enhance migratory properties. Knowledge of the underlying mechanism may help prevent metastasis, and will contribute to the understanding of cancer cells.

Transcription factors control cellular characteristics through the regulation of gene expression. Sal-like 4 (SALL4) is a zinc finger transcription factor. SALL4 has two isoforms, SALL4A and SALL4B [1, 2]; the SALL4A transcript includes the entire exon 2, while SALL4B has a truncated version. In breast cancer cells, SALL4 is positively regulated by signal transduction and activator of transcription 3 [3]. In breast cancer patients, SALL4 levels were shown to be increased in the circulating tumor cells [4]. A positive correlation between SALL4 expression and lymph node metastasis has been reported in colorectal cancer patients [5, 6]. In addition to these clinical observations, our previous study showed that loss of SALL4 function reduced cell
motility in cell lines of basal-like breast cancer, which is the most aggressive and metastatic subtype among breast cancers [7]. Therefore SALL4 appears to be involved in cancer cell migration. However, the role of SALL4 in migration has not been fully elucidated.

Basal-like breast cancer is known to be an aggressive breast cancer subtype. This cancer seems to originate from normal mammary cells that have epithelial gene expression and low migratory properties. However, in contrast to normal cells, basal-like breast cancer cells express mesenchymal genes, and possess high migratory properties. Thus, using basal-like breast cancer cell lines allowed us to analyze the acquisition of a high migratory ability. In 2-dimensional cultures, the cells are dispersed, and exhibit a polarized, spindle-shaped morphology.

A number of molecules have been shown to be involved in the polarized cell migration. Integrin is a cell-extracellular matrix (ECM) adhesion protein, and is known to promote migration [8]. The integrin α subunit and β subunit form a heterodimer on the cell membrane with an extracellular domain that binds ECM molecules [9, 10]. In
the human genome, there are 18 α and 8 β subunit genes. Ligand specificities are
different in each heterodimer, for example, integrin α6β1 binds to laminin-511 [11].

In the inside of a cell, focal adhesion (FA) is organized where integrins form a
cluster [12, 13]. FA generates actin cytoskeleton, and activation of intracellular
signaling pathways [8, 12]. FA complex contains proteins involved in cytoskeleton
anchoring, such as paxillin, vinculin and talin, and in intracellular signaling, such as
focal adhesion kinase (FAK). During FA maturation, FAK is phosphorylated at Tyr-397,
and activates downstream signaling to promote migration [14, 15].

For cell migration, the FA assembly/disassembly cycle must be active.

Therefore, in addition to FA formation and maturation, FA turn over is required [16, 17].

FAK is one of the factors for FA turn over [8]. To disassemble FA, FAK signaling
inhibits the activity of the Rho GTPase that stabilizes FA [18, 19]. Rho inhibition by
FAK results in enhancement of FA dynamics and cell migration.

In this study, we investigated the role of SALL4 in cell migration. We
demonstrated morphological change of the cells from spindle-shaped to rounded and the
loss of migration following SALL4 knockdown in basal-like breast cancer cells. We
found that SALL4 positively regulates the integrin α6 and β1 genes. Rescue experiments with integrin α6 and β1 showed restoration of cell morphology and the migratory properties in SALL4 knockdown cells. Moreover, we discovered that the SALL4 - integrin α6β1 network mediates FAK activation and Rho inhibition to promote cell migration in basal-like breast cancer. Our zebrafish metastasis assays revealed that the SALL4 - integrin α6β1 network also enhances migration in vivo. This study proposes a novel mechanism of how basal-like breast cancer cells acquire high migratory properties.

2. Material and Methods

2.1 Cell culture

SUM159 cells were obtained from Astrerand (Detroit, MI, USA), and maintained with Ham’s F-12 nutrient mixture containing 5% FBS, 5 μg/mL insulin, 1 μg/mL hydrocortisone and 10 mM HEPES. MDA-MB-231 cells were obtained from the American Type Culture Collection (Manassas, VA, USA), and maintained with RPMI-1640 containing 10% FBS. For drug selection to obtained infectants, 1 μg/mL
puromycin, 10 μg/mL blasticidin S or 250 μg/mL hygromycin B was used. Images of cultured cells were collected with an all-in-one microscope, BZ-9000 (Keyence, Osaka, Japan). Cell growth was analyzed as previously described [20].

2.2 Loss- and gain-of-function studies

For gene knockdown, a lentivector, pLKO (Addgene, 8453, Cambridge, MA, USA), was used. Double-strand DNA with shRNA sequence was inserted into the region between EcoRI and AgeI sites of the pLKO vector. The shRNA target sequences are listed in Supplementary Table S1. For the gene overexpression experiments, a pLenti vector (Life Technologies, V533-06, Carlsbad, CA, USA) was used. The EF1α promoter sequence was amplified from the pEF1α-mCherry-N1 vector (Takara, 631969, Kusatsu, Japan), and inserted between the ClaI and SpeI sites. The gene coding region was cloned into a pENTR-FLAG vector [20], and subsequently subcloned into the pLenti vector with the EF1α promoter.
Lentiviral particles were produced as described previously [7]. Lenti-X 293T cells (Takara, 632180) were used. In loss-of-function studies, infected cells were analyzed 6 days after infection.

2.3 Boyden chamber assays

Cells were suspended in serum-free medium, placed into the upper component of the chamber at a density of 400 cells/mm², and incubated for 1h at 37 °C. Then, medium containing 5% serum was added to the lower component. After 5h of incubation at 37 °C, the cells were fixed with 4% paraformaldehyde in PBS for 15 min, and washed with PBS. Cells at the upper side were wiped out. Migrated cells were stained with Hoechst 33342 (Dojindo, 346-07951, Kamimashiki, Japan, 1:500 dilution). The number of migrated cells was analyzed in a 300 mm² region in the central area of a chamber. The cells were counted manually.

2.4 Immunostaining
Cells were plated on a glass bottom chamber slide (Matsunami glass, SCS-008, Osaka, Japan), and cultured for 2 days. To suppress the activity of Rho signaling, the cells were treated with 1 µg/mL C3 transferase (Cytoskeleton, CT04, Denver, CO, USA) for 2h, or 10 µM Y-27632 (Wako, 257-00511, Osaka, Japan) for 4h. Half of the medium was removed, and an equal amount of 4% paraformaldehyde in PBS was added (final concentration was 2%) for fixation. Cells were fixed for 15-20 min at room temperature, and washed with PBS containing 0.05% Tween 20. Permeabilization was performed with 0.5% Triton X-100 in PBS for 15 min at room temperature. The blocking solution contained 5% goat serum and 1% BSA in PBS. The primary antibodies were anti-phospho-paxillin antibody (Cell Signaling Technology, 2541, Danvers, MA, USA, 1:20 dilution), anti-GM130 antibody (Cell Signaling Technology, 12480, 1:3200 dilution), anti-integrin α6 antibody (Acris Antibodies GmbH, Herford, Germany, 1:100 dilution) and anti-integrin β1 antibody (GeneTex, GTX23167, Alton Pkwy Irvine, CA, USA, 1:10 dilution). The Secondary antibodies were goat anti-mouse IgG antibody conjugated to Alexa Fluor 647 (Life Technologies, A21235, 1/1000 dilution), goat anti-rat IgG antibody conjugated to Alexa Fluor 488 (Cell Signaling Technology, 4416,
1/1000 dilution) and goat anti-rabbit IgG antibody conjugated to Alexa Fluor 488 (Life Technologies, A11008, 1/1000 dilution). Hoechst 33342 was used for nuclear staining. Images of immunostained samples were collected with a BZ-9000 microscope (Keyence). Optical sections were obtained with a confocal platform, TCS SP8 (Leica, Tokyo, Japan).

2.5 Messenger RNA expression analyses

Total RNA samples were extracted with TRIzol reagent (Life Technologies, 15596026). The RNA-seq analysis was previously described [20]. Complementary DNA samples were synthesized from 1 μg of total RNA with SuperScript III (Life Technologies, 1808044), and quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed with a reagent, FastStart Universal SYBR Green Master (Roche, 04913850001, Mannheim, Germany). Primers for qRT-PCR are listed in Supplementary Table S2.

2.6 Promoter activity analyses
The SALL4A and SALL4B coding regions were cloned downstream of the CAG promoter obtained from pCAG-mGFP (Addgene, 14757). To assess the promoter activities, 1kbp regions upstream of the transcription start sites of the *ITGA6* and *ITGB1* genes were linked to the minimal promoter in the pGL4.30 vector that also carries a firefly luciferase2 gene (Promega, E8481, Madison, WI, USA). The promoter reporter was co-transfected into Lenti-X 293T cells with the pGL4.73 vector that has the SV40 promoter and a Renilla luciferase gene (Promega, E6911), and with the SALL4 expression vector. A transfection reagent, FuGENE HD (Promega, E2311) was used. One day after transfection, luciferase activity was measured using the Dual-Glo Luciferase Assay System (Promega, E2920).

2.7 Chromatin immunoprecipitation

The SALL4B coding sequence was cloned into the pENTR-FLAG vector, and subsequently subcloned into the pLenti vector. Cells expressing FLAG or SALL4B-FLAG were cultured and used for chromatin immunoprecipitation. The procedure was performed as previously described [20], and an anti-FLAG M2 antibody
(Sigma, F1804, St. Louis, MO, USA) was used. Immunoprecipitated DNA fragments were used for regular PCR. The primers for ITGA6 promoter were as follows: forward 5'-GCATCACCTGCACCTTCTTTAT-3' and reverse 5'-CTGTGGACAGAATTGTGGTG-3'. The primers for ITGB1 promoter were as follows: forward 5'-GGAGTCGCGGAACAGCAG-3' and reverse 5'-CCGGCGGCTTTAAGTGCT-3'. PCR products were electrophoresed with a 6% acrylamide gel.

2.8 Immunoblotting

The primary antibodies were anti-phospho-FAK antibody (Cell Signaling Technology, 8556, 1:1000 dilution), anti-FAK antibody (Cell Signaling Technology, 3285, 1:1000 dilution), and anti-integrin β1 antibody (Cell Signaling Technology, 9699, 1:1000 dilution). Detection of the immunoreactions was described previously [20]. The intensity of each band was analyzed using ImageJ.

For analysis of phosphorylated FAK levels, cells were starved with medium containing 0.5% FBS for 18 h. Then, cells were trypsinized and suspended with
medium containing 0.1% BSA. The suspended cells were incubated for 1 h at 37 °C.

Cells were then plated onto a TC-coated culture dish. After a 1 h incubation, cells were harvested for analyses.

To obtain immunoprecipitated samples of integrin α6 complex, co-immunoprecipitation was performed with approximately 0.5 g of cells. A Dynabeads Co-Immunoprecipitation Kit (Veritas, DB14321, Tokyo, Japan) and the anti-FLAG M2 antibody were used. Additionally, 0.5% NP-40 was added to the extraction buffer from the kit.

Rho signals were detected with rabbit anti-RhoA antibody (Cell Signaling Technology, 8789, 1:667 dilution). RhoA activity was measured with RhoA G-LISA Activation Assay Kit (Cytoskeleton, BK124).

2.9 Laminin binding assay

For the laminin binding assays, 400 μL of PBS containing 1 μg of recombinant laminin-511 E8 fragment (Nippi, 892013, Tokyo, Japan) was added to the wells of a 24-well plate and incubated for 1 h at 37 °C. The wells were washed, blocked with 1%
BSA solution for 1 h at 37 °C, and washed again. Then, 10,000 cells were plated, and incubated for 20 min at 37 °C. The wells were washed, and the number of bound cells was analyzed. The cells were counted manually.

2.10 Time-lapse imaging

The EGFP gene was cloned into the pENTR-FLAG vector. The paxillin coding sequence was inserted the NcoI site of the pENTR-EGFP-FLAG vector. The paxillin-EGFP fusion construct was subcloned into the pLenti vector (Life Technologies, V533-06). Cells labeled with paxillin-EGFP were plated on a TC-coated glass bottom dish (Greiner, 627975, Frickenhausen, Germany). Time-lapse images were collected using a BZ-9000 microscope with a CO₂ chamber (Keyence).

2.11 Transplantation assays

For mouse xenograft assay, 1 x 10⁵ MDA-MB-231 cells were suspended in 80 uL of serum-free medium with 50% Matrigel (Corning, 356237, Bedford, MA, USA), and transplanted into the mammary fat pad of five-week-old nude mouse. In the same
mouse, shGFP (control) and shSALL4 cells were injected at the right and the left sides, respectively.

For zebrafish metastasis assay, cancer cell lines were labeled with mCherry via lentiviral infection. Two-day-old *fli1-EGFP* fish embryos were dechorionated and embedded into 1.0% low-melt agarose. Approximately 30 cells were injected into the abdominal cavity. Transplanted embryos were maintained at 32 °C for 3 days, and observed with a BZ-9000 microscope (Keyence).

The animal experiments in this study were approved by the Ethics Review Board for Animal Experiments of Kyoto University. All animals were maintained according to the Guide for the Care and Use of Laboratory Animals (National Institute of Health Publication).

2.12 Statistical analyses

The number of migrated cell, the ratio of the FA-rich region, mRNA levels, luciferase activity, phosphorylated FAK levels, the cell number bound to the laminin-511 E8 fragment, the active Rho level, and cell growth were analyzed using Student’s *t*-test.
The ratio of polarized cells and the metastasis rate in the zebrafish assay were analyzed using Fisher’s exact test. For TCGA data, SALL4 expression levels among breast cancer subtypes were analyzed using a Kruskal-Wallis H test, and ITGA6 and ITGB1 expression levels were analyzed using a Mann-Whitney U test. $P < 0.05$ was considered statistically significant.

3. Results

3.1 SALL4 is required for cell migration and normal FA pattern.

To investigate the role of SALL4 in cell migration, we conducted loss-of-function studies. We used a previously established shRNA system that targets the common sequence of the SALL4 isoforms (shSALL4) [7]. shRNA for the GFP gene (shGFP) was used as a control. We introduced shRNA expression into basal-like breast cancer cell lines, SUM159 and MDA-MB-231, which are both highly migratory, and have a spindle-shaped morphology. We found that the SALL4 knockdown cells were rounded, while there was no morphological change in the shGFP control cells (Fig. 1A). Because rounded morphology is indicative of low migratory potential, we explored changes in
the migratory properties of SALL4 knockdown cells. Using Boyden chamber assays, we observed a loss of migration following SALL4 knockdown in SUM159 and MDA-MB-231 cells (Fig. 1B,C), indicating that SALL4 positively regulates cell migration.

In a polarized migrating cell, FAs are observed in restricted areas of the cell periphery, whereas a low migratory cell has expanded FA-rich peripheral regions [21, 22]. To analyze the FA pattern, we immunostained cells with an antibody for phosphorylated paxillin, which is a marker of FAs. In the control cells, FAs were localized to the restricted areas, while the FA-positive area was expanded in SALL4 knockdown cells (Fig. 1D). To quantify this difference, we measured the length of the FA-rich region, and divided it by the perimeter (Supplementary Fig. S1). In SALL4 knockdown cells, the ratio of the FA-rich region to the perimeter was significantly increased (Fig. 1E). This result indicates that SALL4 modulates the FA pattern, which may result in a spindle-shaped morphology and the high migratory properties.

A migrating cell has front-rear polarity, and loss of polarity results in a rounded morphology and reduction in migration. Therefore SALL4 knockdown cells
may have lost this polarity. GM130 is a Golgi marker, and the Golgi apparatus is localized near the front of the nucleus in a polarized cell. If the polarity is lost, the GM130 signal is observed around the nucleus. We analyzed GM130 signals, and observed no difference in the percentages of polarized cells between the controls and SALL4 knockdown cells (Fig. 1F,G). This result suggests that SALL4 is not involved in the regulation of cell polarity.

3.2 SALL4 up-regulates integrin genes.

Since SALL4 is a transcription factor, we hypothesized that it regulates the expression of genes involved in cell migration. To identify SALL4-regulated genes, we obtained gene expression data by RNA-seq, and compared the data from the shGFP and shSALL4 groups (deposited in the DNA Data Bank of Japan, Sequence Read Archive as DRA004721 and DRA004722, respectively) (Fig. 2A, Supplementary Table S3). We found that the expression of several integrin genes was reduced by SALL4 knockdown. Because integrin is known to promote cell migration, we focused on integrin family genes. To validate the result of RNA-seq analysis, we performed qRT-PCR (Fig. 2B).
We identified candidate genes, namely \textit{ITGA3}, \textit{ITGA6}, \textit{ITGA10}, \textit{ITGB1} and \textit{ITGB4}, the expression levels of which were reduced to less than half of that of the controls by SALL4 knockdown in both SUM159 and MDA-MB-231 cells.

To determine which integrin gene is involved in basal-like breast cancer cell migration under the control of SALL4, we performed shRNA-mediated knockdown for each gene. Morphological analysis showed that knockdowns of integrin α6 (encoded by \textit{ITGA6}) and β1 (encoded by \textit{ITGB1}) induced a rounded morphology, similar to the SALL4 knockdown (Fig. 2C), while the others showed no notable changes (Supplementary Fig. S2). Next, we analyzed the migratory properties of these cells, and observed significant reductions in migration (Fig. 2D). Although knockdown of each candidate gene impaired cell migration, only the integrin α6 and β1 knockdowns reduced the migration to levels similar to that of SALL4 knockdown (Fig. 2D). These results suggest that reduced expression of integrin α6 and β1 is involved in the rounded morphology and the reduced migration of SALL4 knockdown cells.

To study the regulation of integrin α6 and β1 expression by SALL4, we performed reporter assays with the promoter regions of the \textit{ITGA6} and \textit{ITGB1} genes.
We linked each promoter region to the minimal promoter (miniP) and the luciferase reporter gene. We then prepared expression vectors carrying each SALL4 isoform. Co-transfection with the integrin promoter reporter and the SALL4 expression vector resulted in up-regulation of reporter gene expression, while no increase was observed in the miniP control (Fig. 2E), indicating that both SALL4 isoforms activate the promoters of the ITGA6 and ITGB1 genes. To determine whether SALL4 binds to these promoters, we introduced FLAG-tagged SALL4B expression vectors to MDA-MB-231 cells, and performed chromatin immunoprecipitation assays with an anti-FLAG M2 antibody. The results showed an enrichment of the ITGA6 and ITGB1 promoter regions in the sample of SALL4B-FLAG cells (Fig. 2F). These observations suggest that SALL4 directly up-regulates integrin α6 and β1 at the transcriptional level.

The Cancer Genome Atlas (TCGA) network has published the gene expression data of a number of cancer patients [23]. We analyzed the expression levels of SALL4 in breast cancer patients, and found that it was up-regulated in the cancer tissues of basal-like breast cancer patients (Supplementary Fig. S3A). To assess the correlation with integrin gene expression, we classified the basal-like breast cancer
patients to two groups, SALL4 high and SALL4 low. The SALL4 high group had higher SALL4 expression in the cancer tissues than in the normal tissues of the same patient. We observed higher integrin α6 and β1 expression in the SALL4 high group than in the SALL4 low group (Supplementary Fig. S3B). These results suggest that SALL4 up-regulates integrin α6 and β1 expression in basal-like breast cancer.

3.3 SALL4-regulated integrin α6 and β1 promote migration.

To determine whether SALL4 promotes cell migration via up-regulation of integrin α6 and β1, we performed rescue experiments by overexpressing integrin α6 and β1 in SALL4 knockdown cells. We constructed overexpression vectors for the integrin α6 variants, α6v1 and α6v2 (also known as α6B and α6A, respectively), and β1 (Supplementary Fig. S4A). The two transcriptional variants of integrin α6 share an extracellular domain and a transmembrane region, but have different cytoplasmic tails [24]. We introduced these expression vectors into MDA-MB-231 cells, and obtained stably expressing cells (Supplementary Fig. S4B). The results of the migration assays showed that cell migration was restored when the cells overexpressed both integrin α6
and β1, but integrin α6 alone and β1 alone were not able to rescue the migratory ability
of SALL4 knockdown cells (Fig. 3A). In addition to cell migration, the rounded
morphology caused by SALL4 knockdown was restored in the cells overexpressing
both integrin α6 and β1 (Fig. 3B). These results indicate that SALL4-regulated integrin
α6 and β1 are required for the spindle-shaped morphology and cell migration in
basal-like breast cancer cells.

Because integrin mediates FAs, we analyzed the FA pattern. In SALL4
knockdown cells, the FA-positive area was expanded compared to that of the control,
but this change was not observed in the cells overexpressing integrin α6 and β1 (Fig.
3C). Additionally, the FA-rich region was significantly expanded by SALL4
knockdown, and no change was observed in the cells overexpressing integrin α6 and β1
(Fig. 3D).

In integrin-mediated FAs, FAK is phosphorylated at Tyr-397 [14]. We
analyzed the levels of phosphorylated FAK in the cells overexpressing integrin α6 and
β1 (Fig. 3E). In the control cells, reduced FAK phosphorylation was observed following
SALL4 knockdown (Fig. 3F). However, overexpression of both integrin α6 and β1
restored the phosphorylated FAK levels (Fig. 3F), suggesting that integrin α6 and β1 are required for FAK activation. Our observations indicate that SALL4 modulates FAs via up-regulation of integrin α6 and β1.

3.4 Integrin α6 and β1 form a heterodimer in basal-like breast cancer.

The integrin α and β subunits form a heterodimer [9, 10]. Integrin α6 and β1 can bind to each other [25], but it is unclear whether they form a heterodimer in basal-like breast cancer cells. To clarify this, we conducted a co-immunoprecipitation assay in MDA-MB-231 cells. We precipitated protein complexes from the lysates of cells overexpressing FLAG-tagged integrin α6 using an anti-FLAG M2 antibody. We detected integrin β1 in the protein complexes of integrin α6v1 and α6v2 (Fig. 4A), indicating that integrin α6 and β1 form a heterodimer. Furthermore, we double-immunostained SUM159 and MDA-MB-231 cells with antibodies for integrin α6 and β1, and performed optical sectioning with a confocal microscope (Fig. 4B). We observed overlapping signals, supporting that there are integrin α6β1 heterodimers in basal-like breast cancer cells.
The integrin α6β1 heterodimer can bind to laminin-511 [11]. Because SALL4 up-regulates integrin α6 and β1 expression, and integrin α6 and β1 form a heterodimer, we hypothesized that SALL4 knockdown cells lose the ability to bind to laminin-511. If integrin α6 and β1 do not form a heterodimer in basal-like breast cancer cells, there will be no change in the laminin-511 binding ability in SALL4 knockdown cells. To address this hypothesis, we performed a binding assay with a recombinant laminin-511 E8 fragment to which integrin binds [26]. We used a BSA solution for blocking. A small number of cells bound to the BSA-coated dish, and there was no difference in the number of bound cells between the control and SALL4 knockdown cells (Fig. 4C).

When we coated a dish with the laminin-511 E8 fragment in combination with the BSA blocking solution, the number of bound SUM159 and MDA-MB-231 cells increased. Additionally, we observed that the number of bound SALL4 knockdown cells was approximately half that of the controls (Fig. 4D), indicating that SALL4 promotes the binding to laminin-511. Furthermore, we performed rescue experiments by overexpressing integrin α6 and β1, and the binding to laminin-511 was restored (Fig. 4D).
These results showed that the SALL4-regulated integrin α6 and β1 function by forming an α6β1 heterodimer.

3.5 SALL4 is required for FA dynamics.

Impaired FA assembly/disassembly dynamics causes changes in the FA pattern and loss of migration. To analyze the FA dynamics, we monitored the localization of paxillin, a component of the FA complex, using a paxillin-EGFP fusion construct. We obtained images at 0 and 10 min, and observed the FA assembly/disassembly (Fig. 5A). FA signals appeared as punctate dots, while the free paxillin-EGFP molecules localized near the nucleus and appeared as diffuse signals. In these experiments, we used an shRNA with a scrambled sequence (shScr) as the control. In the control cells, assembled and disassembled FAs were observed (Fig. 5A, arrowheads). However, FAs in SALL4 knockdown cells were unchanged, suggesting that FAs were stabilized by SALL4 knockdown.

We quantified the ratios of assembled and disassembled FAs in each cell (Fig. 5B,C). The ratio of assembled FAs was calculated by dividing the number of newly
formed FAs in 10 min by the total number of FAs at 10 min. The ratio of disassembled FAs was calculated by dividing the number FAs lost in 10 min by the total number of FAs at 0 min. The results showed that both the ratios of assembled and disassembled FAs were reduced by SALL4 knockdown, and this reduction was restored by overexpression of integrin α6β1, suggesting that FA stabilization in SALL4 knockdown cells is due to the loss of integrin α6β1. These observations indicate that the SALL4 - integrin α6β1 network activates the FA dynamics.

3.6 The SALL4 - integrin α6β1 network prevents aberrant Rho activation in cell migration. Loss of FAK causes over-activation of Rho [18]. Rho over-activation stabilizes FAs, and reduces cell migration [8, 22, 27]. In the SALL4 knockdown cells, FAK phosphorylation levels and migration were reduced, and these reductions were restored by integrin α6β1 expression (Fig. 3). We therefore hypothesized that the SALL4 - integrin α6β1 network is involved in regulating Rho activity in cell migration. Thus, we measured Rho activity, and observed that SALL4 knockdown did not change the
protein level of Rho, but increased RhoA activity (Fig. 6A,B). In SALL4 knockdown cells, Rho over-activation was suppressed by integrin α6β1 (Fig. 6B). These results indicate that the SALL4 - integrin α6β1 network prevents Rho over-activation in basal-like breast cancer cells.

To determine whether over-activation of Rho is the responsible for the rounded morphology and expansion of the FA-rich region in SALL4 knockdown cells, we treated SALL4 knockdown cells with Rho signaling inhibitors, C3 transferase and Y-27632. C3 transferase ADP-ribosylates Asn-41 on the Rho family of small GTPases, Rho A, B and C [28]. We analyzed cell morphology following C3 transferase treatment in SALL4 knockdown cells, and observed that the treated cells exhibited a spindle-shaped morphology and a reduced FA-rich region (Supplementary Fig. S5). Rho A activates Rho-associated coiled-coil forming protein serine/threonine kinase (ROCK). Appropriate activation and suppression of the Rho-ROCK pathway is required for cell migration [8, 22, 27]. We used a chemical ROCK inhibitor, Y-27632 [29], and observed that the rounded morphology of the SALL4 knockdown cells converted to a spindle-shaped morphology (Fig. 6C). In addition, expansion of the FA-rich region was
not observed in Y-27632-treated cells (Fig. 6D). These results indicate that SALL4 regulates cell morphology and the FA pattern through modulation of Rho activity.

Long-term treatment with C3 transferase causes cellular stress, and increases the difficulty in analyzing cell migration. Y-27632 is milder than C3 transferase in basal-like breast cancer cells, and it is easier to assess cell migration following Y-27632 treatment. Using Boyden chamber assays, we analyzed the number of cells that migrated in 5 hours, and found that the reduced cell migration was recovered by ROCK inhibition in SALL4 knockdown cells (Fig. 6E). Taken together, the SALL4 - integrin α6β1 network promotes cell migration through modulation of the Rho-ROCK pathway (Supplementary Fig. S6).

3.7 The SALL4 - integrin α6β1 network is required for in vivo cell migration.

SALL4 knockdown reduces cell growth [7, 30], and the reduced cell growth was not rescued by integrin α6β1 expression (Supplementary Fig. S7A). This indicates that the mouse transplantation model can not be used to analyze the in vivo migratory properties of SALL4 knockdown cells, because the tumor size of the SALL4 knockdown cells can
not be compared to that of the controls at both the transplanted focus and the metastatic foci, and evaluation using the number of foci and/or the size of metastatic tumors is not accurately show a difference in migration. In support of this notion, our mouse xenograft experiments showed the reduced tumor growth in SALL4 knockdown cells (Supplementary Fig. S7B). Therefore, to analyze in vivo cell migration, we used zebrafish metastasis assays. These assays require 2 or 3 days to assess migration, and do not require cell growth and tumor formation [31].

We labeled basal-like breast cancer cells with a red fluorescent protein, mCherry, and the cells were transplanted to the abdominal cavity of 2-day-old embryos of fli1-EGFP fish that expressed green fluorescent protein in the vascular endothelial cells [32]. When we transplanted metastatic cancer cells, they moved into the blood vessels, subsequently migrated out to other organs, such as the brain and trunk. We monitored the migration of transplanted cells and observed metastasis (Fig. 7A). Circulating cancer cells were observed as overlapping signals of EGFP and mCherry. Metastasized cells were observed as separate signals (Fig. 7A, arrowheads). Most fish
with control cancers underwent metastasis, while few fish with SALL4 knockdown
cells showed metastasis.

During the zebrafish metastasis assay, we noted that several cancer cells
circulated from end to end of the body in the blood stream in a few seconds, which
suggested that analysis of migratory distance is inadequate to analyze the migratory
properties. Therefore we analyzed the metastatic rate using methods from previous
studies [31, 33]. We counted the number of fish with or without metastasis, and
calculated the ratio of fish with metastasis (Fig. 7B). The results showed that the
metastatic rate was significantly reduced by SALL4 knockdown, indicating that SALL4
positively regulates the migratory ability \textit{in vivo} as well as \textit{in vitro}.

We next determined whether integrin $\alpha_6\beta_1$ expression restores \textit{in vivo}
migration in SALL4 knockdown cells. We transplanted cells overexpressing integrin
$\alpha_6\beta_1$ and observed similar migration rates as that of the controls in SALL4 knockdown
cells (Fig. 7C). These results indicate that the SALL4 - integrin $\alpha_6\beta_1$ network is
required for enhanced migration \textit{in vivo}.
3.8 Integrin α6β1 expression is an indicator of poor metastasis-free survival.

The integrin α6β1-regulated cell migration suggests that integrin α6β1 expression promotes metastasis in breast cancer patients. To investigate the relationship between integrin α6β1 expression and metastasis, we analyzed publically available clinical data using the SurvExpress platform [34]. Six clinical studies from five cohorts have published data on mRNA expression and metastasis-free survival [35-39]. The data from three studies showed that the breast cancer patients with high integrin α6β1 expression had a lower metastasis-free survival rate than that of the patients with low integrin α6β1 expression (Supplementary Fig. S8A-C). However, data from the other 3 studies showed no significant difference between the integrin α6β1 high and low groups (Supplementary Fig. S8D-F). These results suggest that integrin α6β1 expression may promote metastasis in some cases.

4. Discussion

One of the crucial steps in cancer metastasis is the acquisition of the high migratory properties. In this study, we elucidated the role of the SALL4 - integrin α6β1 network
in cell migration in basal-like breast cancer cells. SALL4 knockdown cells showed reduced migration and a rounded morphology with expansion of the FA-rich region. Gene expression analyses revealed that SALL4 positively regulates the expression of the integrin α6 and β1 genes. Cells with loss of integrin α6 and β1 showed identical phenotypes to that of SALL4 knockdown cells, suggesting that they are the causative factors of SALL4-regulated cell migration. Overexpression of both integrin α6 and β1 restored cell migration and reversed the morphology in SALL4 knockdown cells, further supporting this notion. Moreover, we observed that SALL4 knockdown cells have aberrant Rho activation, and integrin α6β1 expression suppressed this phenotype. Pharmacological inhibition of Rho over-activation restored cell migration in SALL4 knockdown cells. These results suggest that the SALL4 - integrin α6β1 network is required for cell migration via modulation of Rho activity. In addition, we performed zebrafish metastasis assays, and showed that exogenous integrin α6β1 expression restored cell migration in SALL4 knockdown cells, indicating that the SALL4 - integrin α6β1 network promotes cell migration in vivo. Our findings identify a novel mechanism underlying the high migratory properties of cancer cells.
SALL4 is known to be a factor in stem cell maintenance and proliferation [2, 40]. In cancer cells, SALL4 up-regulates genes involved in cell proliferation, such as *BMI1* and *CCND1* [7, 41, 42]. However, the role of SALL4 in cell migration has not been fully elucidated, because SALL4-depleted cells display impaired cell proliferation, which hinders the analysis of other biological features. In this study, we used an shRNA-mediated SALL4 knockdown system, and prepared fresh SALL4 knockdown cells in each experiment. We performed SALL4 loss-of-function studies in the basal-like breast cancer cell lines, SUM159 and MDA-MB-231. The cells have a spindle-shaped morphology and high migratory properties, and these characteristics can be altered by genetic manipulation and pharmacological treatment, which allowed us to easily analyze the role of SALL4 in cell migration. Using this procedure, we elucidated the role of SALL4 in cell migration.

Although our previous study found that SALL4 is involved in the spindle-shaped morphology of basal-like breast cancer cells [7], the causative factors and the detailed mechanism underlying this characteristic remained elusive. In this study, we discovered that the SALL4-integrin α6β1 network regulates the
spindle-shaped morphology through the maintenance of FA dynamics. We observed an expansion of the FA-rich region in SALL4 knockdown cells. However, SALL4 knockdown cells did not show a reduction in FA number, although SALL4 knockdown reduces the mRNA levels of several integrin genes. No reduction in FA number may be due to the function of the remaining integrins. SALL4 knockdown reduced expression levels of its downstream genes, including integrins, but it did not completely deplete their expression. In addition, cells express other integrins that are not regulated by SALL4. Therefore SALL4 knockdown cells had reduced integrin function, which appeared to be sufficient to form FAs, but not for activation of FA dynamics.

Although knockdown of each SALL4-regulated integrin gene showed reduced cell migration, this study focused on the integrin α6 and β1 genes, because the morphological change and the degree of reduction in the migration following their knockdowns were identical to those of the SALL4 knockdown. The integrin α6β1 heterodimer was found to be the main factor in SALL4-regulated cell migration. The human genome has 18 integrin α and 8 integrin β subunit genes, and various integrin genes are expressed in basal-like breast cancer. Although integrins appear to be
involved in cancer metastasis, the role of each integrin has not been fully characterized in metastatic process, such as cell migration, invasion, and anoikis resistance. In this study, through analyses of SALL4 function, we showed that integrin α6β1 is required for migration during basal-like breast cancer metastasis. Additionally, analyses of the data from several cohort studies showed that breast cancer patients with high integrin α6 and β1 expression had poorer metastasis-free survival than that of the patients with low integrin α6 and β1 expression. These results suggest that a therapy targeting integrin α6β1 will inhibit metastasis by suppressing cell migration.

Integrin α6 has two variants. The functions of the integrin α6 variants differ with respect to stemness [43, 44]. However, we observed that the variants have comparable role in cell migration. Integrin β1 cytoplasmic tail binds to FAK to form focal adhesion, and to activate FAK signaling for cell migration [45]. Because the integrin α6 extracellular and transmembrane domains are shared by the variants, and overexpression of each variant restored the reduced cell migration of SALL4 knockdown cells in combination with integrin β1 overexpression, integrin α6 may function to support integrin β1 in SALL4-regulated cell migration.
In SALL4 knockdown cells, the FAs were stabilized, and the FA dynamics were restored by inhibition of Rho signaling. Aberrant Rho activity was normalized by exogenous expression of integrin α6β1. We thought that the SALL4 - integrin α6β1 network modulates Rho activity to promote cell migration. Although Rho activation is required for FA maturation, its over-activation stabilizes FA, which in turn inhibits the FA assembly/disassembly cycle. Our results showed that the SALL4 - integrin α6β1 network modulates Rho activity, but we did not identify the Rho activation factor. There are a number of factors involved in Rho activation [19], and they might function in FA maturation in basal-like breast cancer cells.

This study showed that the SALL4 up-regulates integrin signaling, and modulates Rho activity. However there is a possibility that SALL4 regulates genes involved in recruitment of FA molecules, such as PAG3 (paxillin-associated protein with ADP-rebosylation factor [ARF] GTPase-activating protein [GAP] activity, number 3) (also known as ASAP2/KIAA0400) [46]. PAG3 recruits paxillin molecules to FA, and PAG3 overexpression impairs cell migration in leukemic monocyte lymphoma cell line, U937, and kidney cell line, COS-7 [46]. We therefore analyzed the relation
between SALL4 and PAG3. However, in our RNA-seq analysis, SALL4 does not regulate PAG3 expression. We did not observe enrichment of PAG3 upstream region in ChIP assay. In our unpublished observation, although the morphology of basal-like breast cancer cells overexpressing PAG3 was slightly rounded, it is still spindle-shaped. These suggest that PAG3 is not involved in the function of SALL4 with respect to cell migration.

In addition to cell migration, integrins are involved in cell growth [47]. However, overexpression of integrin α6β1 did not restore the cell growth in SALL4 knockdown cells, implying that integrin α6β1 is not involved in SALL4-regulated cell growth. Due to the impaired cell growth of SALL4 knockdown cells, we are not able to compare tumor sizes at the transplanted focus and metastatic foci between control and SALL4 knockdown cells in mouse transplantation assay. Therefore, instead of mouse transplantation assays, we utilized zebrafish metastasis assays that allowed us to analyze the migration of single cells in 2-3 days without cell growth [31]. In the zebrafish assay, we observed reduced cell migration in SALL4 knockdown cells, and restoration of migration by integrin α6β1 expression, consistent with the results of the in vitro
experiments. Our results demonstrated that the zebrafish metastasis assay is a useful tool to analyze cell migration in vivo, especially if cells show reduced growth. Although the conditions, such as temperature, osmotic pressure and nutrients, are different between human and zebrafish bodies, and the results do not strictly reflect the cancer cell behavior in the human body, we can use zebrafish to evaluate the in vivo migration potential.

A correlation between SALL4 expression and cancer metastasis has been reported in colorectal cancer [5, 6]. Circulating tumor cells have high SALL4 levels in breast cancer patients [4]. However, it was unclear how SALL4 promotes metastasis. This study bridges the gap between observations of SALL4 expression and metastasis. In addition, we identified integrin α6β1 as a causative factor of SALL4-regulated cell migration. This study contributes to elucidating cancer cell migration, and suggests a therapeutic target to prevent metastasis. SALL4 is expressed in various cancers [48]. An anti-SALL4 peptide prevents tumor growth in a mouse transplantation model, and may be useful as a treatment for liver cancer [49]. Our findings suggest the possibility of SALL4-targeted therapies for metastasis.
5. Conclusions

This study discovered the SALL4 - integrin α6β1 network in metastatic cancer cells. Our data contributes to understanding of the regulation of metastasis. Although several factors are involved in metastasis, and the SALL4 - integrin α6β1 network is not likely to be activated in all metastatic cancers, this study may contribute to a future therapy for breast cancer patients with high SALL4 expression.

Conflict of interest

JI is an employee of Kyoto University’s Sponsored Research Program funded by Taiho Pharmaceutical Co., Ltd. ST, WL, AI, ASF and FS received no specific funding for this work. MT received research funding from Taiho Pharmaceutical Co., Ltd. The funding sources had no role in the study design, experiment, analysis, interpretation or writing the manuscript. The corresponding author had full access to the data and final responsibility for submission.
Acknowledgements

We thank Dr Kojiro Taura for sharing the time-lapse imaging system. We thank the members of departments of breast surgery, and of hepato-biliary-pancreatic surgery and transplantation for sharing laboratory equipment. We thank the Medical Research Support Center, Graduate School of Medicine, Kyoto University for technical assistance.

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Appendices

Supplementary materials

Supplementary Table S3

References


ADP-ribosylation factor GTPase-activating protein activity, is involved in paxillin recruitment to focal adhesions and cell migration, Molecular biology of the cell, 11 (2000) 1315-1327.


Figure legends

**Fig. 1.** SALL4 is required for the spindle-shaped morphology and cell migration. (A) Controls and SALL4 knockdowns of SUM159 and MDA-MB-231 cells are shown. (B) Images of migrated cells are shown. Cell nuclei were stained with Hoechst 33342. Arrowheads indicate the signals of migrated cells. For simplicity, not all signals are pointed. (C) Graphs show the number of migrated cells (n = 4). The vertical axis indicates the number of migrated cells. (D) Cells were stained with an anti-phosphorylated paxillin antibody. Arrowheads indicate the immunoreactions. For simplicity, not all signals are pointed. (E) Graphs show the ratio of the FA-rich region. The vertical axis indicates the value obtained by dividing the length of the FA-rich region by the perimeter. The cell numbers were 31 in SUM159;shGFP, 36 in SUM159;shSALL4, 40 in MDA-MB-231;shGFP and 41 in MDA-MB-231;shSALL4 cells. (F) Images of immunostaining with anti-GM130 are shown. (G) Ratios of polarized cells are shown in the graph. The vertical axis indicates the percentage of polarized cells. The cell numbers were 195 in SUM159;shGFP, 249 in SUM159;shSALL4, 163 in MDA-MB-231;shGFP and 162 in MDA-MB-231;shSALL4.
cells. Scale bars indicate 100 μm in A,B, and 20 μm in D,F. Student’s t-test was used in C.E. Fisher’s exact test was used in G. n.s.: not significant, **: P < 0.01. Error bars represent the standard deviation.

Fig. 2. SALL4 up-regulates integrin α6 and β1 expression. (A) The scatter plot shows the gene expressions in the control and SALL4 knockdown SUM159 cells. Integrin genes reduced by SALL4 knockdown are indicated. The accession numbers of the shGFP and shSALL4 data are DRA004721 and DRA004722, respectively. (B) Relative expression levels of integrin family genes are shown (n = 3). In each gene, mean expression level of the shGFP cells was defined as 1. Arrowheads point the genes that had expression reduced to less than half of that of the controls by SALL4 knockdown in both SUM159 and MDA-MB-231 cells. (C) Images show cells with integrin α6 and β1 knockdown. (D) The results of the Boyden chamber assays are shown (n = 4). MDA-MB-231 cells were used. The vertical axis indicates the number of migrated cells. (E) Reporter assays using the integrin α6 and β1 gene promoters were performed (n = 4). The vertical axis indicates the relative light units of luciferase. The mean value of
the FLAG control was defined as 1. (F) Enrichment of the integrin α6 and β1 promoter region is shown. MDA-MB-231 cells were used. Chromatin immunoprecipitation samples were used for PCR amplification with primers for the integrin α6 and β1 promoters. ChIP: chromatin immunoprecipitation. Scale bars indicate 100 μm. Student’s t-test was used for statistical analyses. *: P < 0.05, **: P < 0.01. Error bars represent the standard deviation.

Fig. 3. SALL4-regulated integrin α6 and β1 expression is required for spindle-shaped morphology and cell migration in basal-like breast cancer cells. (A) Boyden chamber assays were performed with cells overexpressing integrin α6 and β1 (n = 4 in control, n = 5 in the α6 and β1 overexpressing groups). The vertical axis indicates the number of migrated cells. (B) Cells overexpressing integrin α6 and β1 are shown. (C) Images of immunostaining with anti-phosphorylated paxillin are shown. Arrowheads indicate the immunoreaction signals. For simplicity, not all signals are pointed. (D) Graphs show the ratio of the FA-rich region. The cell numbers were 33 in control;shGFP, 36 in control;shSALL4, 45 in α6v1;β1;shGFP, 37 in α6v1;β1;shSALL4, 39 in
α6v2,β1;shGFP and 33 in α6v2,β1;shSALL4. (E) Immunoblotting for FAK was performed. (F) The intensity of phosphorylated FAK and the total FAK bands were measured. Relative FAK phosphorylation levels to shGFP are graphed (n = 3). Scale bars indicate 100 μm in B, and 20 μm in C. Student’s t-test was used. *: P < 0.05, **: P < 0.01. Error bars represent the standard deviation.

Fig. 4. Integrin α6 and β1 form a heterodimer. (A) Co-immunoprecipitation samples were used for immunoblotting with an anti-integrin β1 antibody. IP: immunoprecipitation. IB: immunoblotting. (B) Confocal images of immunostained cells are shown. Antibodies for integrin α6 and β1 were used for double-immunostaining. (C-F) Binding assays were performed (n = 4). The number of cells bound to the BSA blocking solution and the recombinant laminin-511 E8 fragment are shown in C,E and D,F, respectively. The vertical axis indicates the number of bound cells. SUM159 and MDA-MB-231 cells were used in C,D. MDA-MB-231 cells overexpressing integrin α6 and β1 were used in E,F. Scale bars indicate 20 μm. Student’s t-test was used. n.s.: not significant, **: P < 0.01. Error bars represent the standard deviation.
**Fig. 5.** SALL4 activates the FA dynamics. (A) Cells with a paxillin-EGFP fusion were used to monitor the FA dynamics. Images of shScr (control) and shSALL4 cells are shown. Yellow and blue arrowheads indicate the FA signals assembled and disassembled in 10 min, respectively. For simplicity, not all signals are pointed. (B) The ratio of assembled FAs was calculated by dividing the number of newly formed FA in 10 min by the total FA number at 10 min. (C) The ratio of disassembled FA was calculated by dividing the number of FAs lost in 10 min by the total FA number at 0 min. The cell numbers analyzed in B,C were 33 in the control;shGFP, 36 in the control;shSALL4, 31 in α6v1,β1;shGFP, 37 in α6v1,β1;shSALL4, 41 in α6v2,β1;shGFP and 47 in α6v2,β1;shSALL4 cells. Scale bars indicate 20 μm. Student’s t-test was used. **: P < 0.01. Error bars represent the standard deviation.

**Fig. 6.** The SALL4 - integrin α6β1 network modulates Rho activity to promote cell migration. (A) RhoA protein level was not changed by SALL4 knockdown in MDA-MB-231 cells. (B) RhoA activity was measured with RhoA G-LISA Activation
Relative RhoA activity to shGFP was calculated ($n = 3$). (C) Immunofluorescence images of phosphorylated paxillin are shown. A ROCK inhibitor, Y-27632, was used. Arrowheads indicate the signals. For simplicity, not all signals are pointed. (D) The FA-rich region was analyzed. The cell numbers analyzed were 43 in SUM159;shGFP, 35 in SUM159;shSALL4 with water, 43 in SUM159;shSALL4 with the ROCK inhibitor, 41 in MDA-MB-231;shGFP, 36 in MDA-MB-231;shSALL4 with water and 31 in MDA-MB-231;shSALL4 with the ROCK inhibitor. (E) Boyden chamber assays were performed with the ROCK inhibitor ($n = 4$). The vertical axis indicates the number of migrated cells. Scale bars indicate 20 μm. Student’s $t$-test was used. **: $P < 0.01$. Error bars represent the standard deviation.

Fig. 7. The SALL4-integrin $\alpha6\beta1$ network is required for in vivo cell migration. (A) Metastasis tracking assay of transplanted SUM159 cells in zebrafish larvae. In the control condition (shGFP), the SUM159 cells (magenta) moved to the anterior (yellow square) and posterior (blue square) from the injected site at day 3. The metastasized cells (arrowheads) were localized outside of the vasculatures (green, $fli1$-EGFP). On the
other hand, the SALL4 knockdown cells did not migrate out from the abdominal cavity. Scale bars indicate 500 μm. (B,C) Statistical data for the metastasis assay. The ratio of fish with the metastasis was decreased under the shSALL4 condition in the both of SUM159 and MDA-MB-231 cells (B). The shSALL4-induced phenotype was rescued by overexpression of integrin α6 and β1 (C). Fisher’s exact test was used. **: $P < 0.01$. 
A  shGFP  shSALL4
  SUM159  MDA-MB-231

B  shGFP  shSALL4
  SUM159  MDA-MB-231

C

SUM159  MDA-MB-231

D  shGFP  shSALL4
  SUM159  MDA-MB-231

E

F  shGFP  shSALL4
  SUM159  MDA-MB-231

G

n.s.
Itou_Fig2

A

SUM159

shSALL4 (log2) vs. shGFP (log2)

ITGB1
ITGA3
ITGA5
ITGB4
ITGA6

B

SUM159

Relative mRNA level

MDA-MB-231

Relative mRNA level

C

shGFP  shITGA6#2  shITGA6#3  shITGB1#2  shITGB1#3

SUM159

MDA-MB-231

D

MDA-MB-231

Migrated cell number / mm²

shGFP  shSALL4  shSALL4A  shSALL4B  shITGA6#2  shITGA6#3  shITGA6#4  shITGA6#5  shITGA6#6  shITGA6#7  shITGA6#8  shITGA6#9  shITGB1#2  shITGB1#3  shITGB1#4

E

Relative light units of luciferase

MiniP  ITGA6P  ITGB1P

F

ChIP

FLAG  SALL4B-FLAG

Input

ITGB1P
**Itou_Fig5**

A

![Images showing cell morphology changes over time with shScr and shSALL4](image)

B

- Ratio of assembled (%)

C

- Ratio of disassembled (%)

**Itou_Fig6**

A

- shGFP
- shSALL4

- Rho
- β-actin

B

- Relative RhoA activity

C

- shGFP + water
- shSALL4 + ROCK inh

D

- Focal adhesion rich / perimeter (%)

E

- Migrated cell number / mm²

**Graphs showing statistical significance**
Itou_Fig7

A

Endothelial cells SUM159

Day 0

shGFP

Day 3

shSALL4

B

Ratio of fish with metastasis

SUM159; shGFP
SUM159; shSALL4
MDA-MB-231; shGFP
MDA-MB-231; shSALL4

n = 47  n = 40
n = 31  n = 29

C

Ratio of fish with metastasis

shSALL4
α6v1
α6v2
β1

-  -  -  -  -  -
-  +  -  +  -  +
-  -  -  -  +  +
-  -  -  +  +  +

n = 41  40  28  31  31  28
The Sal-like 4 - integrin α6β1 network promotes cell migration for metastasis via activation of focal adhesion dynamics in basal-like breast cancer cells.

Junji Itou, Sunao Tanaka, Wenzhao Li, Atsuo Iida, Atsuko Sehara-Fujisawa, Fumiaki Sato, Masakazu Toi

**Supplementary Fig. S1.** The FA-rich region and the perimeter were measured.

**Supplementary Fig. S2.** Knockdown of integrin α3, α10 and β4 causes no remarkable morphological change.

**Supplementary Fig. S3.** Breast cancer patients with high SALL4 expression have high integrin α6 and β1 expression.

**Supplementary Fig. S4.** Integrin α6 and β1 overexpression constructs were introduced into MDA-MB-231 cells.

**Supplementary Fig. S5.** Rho inhibition reverses cell morphology of the SALL4 knockdown cells.

**Supplementary Fig. S6.** The SALL4 - integrin α6β1 network augments migration through modulation of Rho activity.

**Supplementary Fig. S7.** SALL4 knockdown impairs cell growth and tumor formation.

**Supplementary Fig. S8.** Co-expression of integrin α6 and β1 genes causes poor metastasis-free survival in some cases.

**Supplementary Table S1.** List of shRNA sequences

**Supplementary Table S2.** Primer sequences for qRT-PCR
Supplementary Fig. S3. Breast cancer patients with high SALL4 expression have high integrin α6 and β1 expression. (A) SALL4 expression levels in breast cancer subtypes are graphed. (B) The expression level of the integrin α6 and β1 genes is shown. A Kruskal-Wallis H test was used in A. A Mann-Whitney U test was used in B. *: $P < 0.05$, **: $P < 0.01$. In the box plots, values of 25%, the median and 75% are indicated. Error bars represent the range between the minimum and maximum values.
**Supplementary Fig. S1.** The FA-rich region and the perimeter were measured. Phosphorylated paxillin immunostaining is shown. Yellow and blue lines indicate the FA-rich region and the perimeter, respectively. Scale bars indicate 20 μm.

**Supplementary Fig. S2.** Knockdown of integrin α3, α10 and β4 causes no remarkable morphological change. Images of the knockdown cells are shown. SUM159 and MDA-MB-231 cells were used. Scale bars indicate 100 μm.
Supplementary Fig. S4. Integrin α6 and β1 overexpression constructs were introduced into MDA-MB-231 cells. (A) Constructs for overexpressing α6v1, α6v2 and β1 are depicted. (B) Immunoblotting was performed to confirm expression from the constructs. An anti-FLAG antibody was used.
Supplementary Fig. S5. Rho inhibition reverses cell morphology of the SALL4 knockdown cells. (A) Images show immunostaining with an anti-phosphorylated paxillin antibody. A Rho inhibitor, C3 transferase, was used. Arrowheads indicate the signals. For simplicity, not all signals are pointed. (B) The FA-rich region was analyzed. The cell numbers analyzed were 38 in SUM159;shGFP, 35 in SUM159;shSALL4 with water, 37 in SUM159;shSALL4 with the Rho inhibitor, 34 in MDA-MB-231;shGFP, 38 in MDA-MB-231;shSALL4 with water and 38 in MDA-MB-231;shSALL4 with the Rho inhibitor. Scale bars indicate 20 μm. Student’s t-test was used. **: P < 0.01. Error bars represent the standard deviation.
Supplementary Fig. S6. The SALL4 - integrin α6β1 network augments migration through modulation of Rho activity. In the nucleus, SALL4 binds to the promoters of the integrin α6 and β1 genes, and up-regulates their expression. Integrin α6 and β1 form a heterodimer, and modulate Rho activity. This network promotes cell migration in basal-like breast cancer cells.
Supplementary Fig. S7. SALL4 knockdown impairs cell growth and tumor formation.

(A) The cell number was counted on days 1, 3 and 5. Statistical analyses were performed on day 5 between the shGFP and shSALL4 groups in control, α6β1, and α6β2 cells. Student’s t-test was used. **: P < 0.01. Error bars represent the standard deviation. (B) Typical image of mouse xenograft experiments with control and SALL4 knockdown cells. The transplanted site of SALL4 knockdown cells showed no visible tumor formation at 1 month-post-transplantation (filled arrowhead), whereas a tumor was observed in the site where control cells were injected (open arrowhead).
Supplementary Fig. S8. Co-expression of integrin α6 and β1 genes causes poor metastasis-free survival in some cases. (A-F) The correlation between integrin α6 and β1 expression and metastasis-free survival was analyzed using the SurvExpress platform. Red and green lines indicate groups with high and low integrin α6 and β1 expression, respectively. The log-rank test was used. The database names are Chin Gray Breast E-TAB-158 (A), Kao Huang Breast GSE20685 (B), Wang Foekens Minn Massague Breast GSE5327 (C), van’t Veer - Van De Vijver Nature 2002 (D), Vant Veer Breast Cancer (E), and Vincent Darbon Breast GSE9893 (F).
### Supplementary Table S1. List of shRNA sequences

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**Supplementary Table S2. Primer sequences for qRT-PCR**

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