The Sal-like 4 - integrin α6β1 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

65 motility in cell lines of basal-like breast cancer, which is the most aggressive and
66 metastatic subtype among breast cancers [7]. Therefore SALL4 appears to be involved
67 in cancer cell migration. However, the role of SALL4 in migration has not been fully
68 elucidated.

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

76 A number of molecules have been shown to be involved in the polarized cell
77 migration. Integrin is a cell-extracellular matrix (ECM) adhesion protein, and is known
78 to promote migration [8]. The integrin α subunit and β subunit form a heterodimer on
79 the cell membrane with an extracellular domain that binds ECM molecules [9, 10]. In

80 the human genome, there are 18 α and 8 β subunit genes. Ligand specificities are
81 different in each heterodimer, for example, integrin α6β1 binds to laminin-511 [11].
82 In the inside of a cell, focal adhesion (FA) is organized where integrins form a
83 cluster [12, 13]. FA generates actin cytoskeleton, and activation of intracellular
84 signaling pathways [8, 12]. FA complex contains proteins involved in cytoskeleton
85 anchoring, such as paxillin, vinculin and talin, and in intracellular signaling, such as
86 focal adhesion kinase (FAK). During FA maturation, FAK is phosphorylated at Tyr-397,
87 and activates downstream signaling to promote migration [14, 15].
88 For cell migration, the FA assembly/disassembly cycle must be active.
89 Therefore, in addition to FA formation and maturation, FA turn over is required [16, 17].
90 FAK is one of the factors for FA turn over [8]. To disassemble FA, FAK signaling
91 inhibits the activity of the Rho GTPase that stabilizes FA [18, 19]. Rho inhibition by
92 FAK results in enhancement of FA dynamics and cell migration.
93 In this study, we investigated the role of SALL4 in cell migration. We
94 demonstrated morphological change of the cells from spindle-shaped to rounded and the
95 loss of migration following SALL4 knockdown in basal-like breast cancer cells. We

96 found that SALL4 positively regulates the integrin α6 and β1 genes. Rescue
97 experiments with integrin α6 and β1 showed restoration of cell morphology and the
98 migratory properties in SALL4 knockdown cells. Moreover, we discovered that the
99 SALL4 - integrin α6β1 network mediates FAK activation and Rho inhibition to
100 promote cell migration in basal-like breast cancer. Our zebrafish metastasis assays
101 revealed that the SALL4 - integrin α6β1 network also enhances migration *in vivo*. This
102 study proposes a novel mechanism of how basal-like breast cancer cells acquire high
103 migratory properties.
104

105 **2. Material and Methods**

106 *2.1 Cell culture*

107 SUM159 cells were obtained from Astrerand (Detroit, MI, USA), and maintained with
108 Ham's F-12 nutrient mixture containing 5% FBS, 5 μg/mL insulin, 1 μg/mL
109 hydrocortisone and 10 mM HEPES. MDA-MB-231 cells were obtained from the
110 American Type Culture Collection (Manassas, VA, USA), and maintained with
111 RPMI-1640 containing 10% FBS. For drug selection to obtained infectants, 1 μg/mL

112 puromycin, 10 μg/mL blasticidin S or 250 μg/mL hygromycin B was used. Images of

113 cultured cells were collected with an all-in-one microscope, BZ-9000 (Keyence, Osaka,

114 Japan). Cell growth was analyzed as previously described [20].

115

116 *2.2 Loss- and gain-of-function studies*

117 For gene knockdown, a lentivector, pLKO (Addgene, 8453, Cambridge, MA, USA),

118 was used. Double-strand DNA with shRNA sequence was inserted into the region

119 between *Eco*RI and *Age*I sites of the pLKO vector. The shRNA target sequences are

120 listed in Supplementary Table S1. For the gene overexpression experiments, a pLenti

121 vector (Life Technologies, V533-06, Carlsbad, CA, USA) was used. The EF1α

122 promoter sequence was amplified from the pEF1α-mCherry-N1 vector (Takara, 631969,

123 Kusatsu, Japan), and inserted between the *Cla*I and *Spe*I sites. The gene coding region

124 was cloned into a pENTR-FLAG vector [20], and subsequently subcloned into the

125 pLenti vector with the EF1α promoter.

126 Lentiviral particles were produced as described previously [7]. Lenti-X 293T cells

127 (Takara, 632180) were used. In loss-of-function studies, infected cells were analyzed 6

128 days after infection.

129

130 *2.3 Boyden chamber assays*

131 Cells were suspended in serum-free medium, placed into the upper component of the

132 chamber at a density of 400 cells/mm^2, and incubated for 1h at 37 °C. Then, medium

133 containing 5% serum was added to the lower component. After 5h of incubation at

134 37 °C, the cells were fixed with 4% paraformaldehyde in PBS for 15 min, and washed

135 with PBS. Cells at the upper side were wiped out. Migrated cells were stained with

136 Hoechst 33342 (Dojindo, 346-07951, Kamimashiki, Japan, 1:500 dilution). The number

137 of migrated cells was analyzed in a 300 mm^2 region in the central area of a chamber.

138 The cells were counted manually.

139

140 *2.4 Immunostaining*

141 Cells were plated on a glass bottom chamber slide (Matsunami glass, SCS-008, Osaka,
142 Japan), and cultured for 2 days. To suppress the activity of Rho signaling, the cells were
143 treated with 1 μg/mL C3 transferase (Cytoskeleton, CT04, Denver, CO, USA) for 2h, or
144 10 μM Y-27632 (Wako, 257-00511, Osaka, Japan) for 4h. Half of the medium was
145 removed, and an equal amount of 4% paraformaldehyde in PBS was added (final
146 concentration was 2%) for fixation. Cells were fixed for 15-20 min at room temperature,
147 and washed with PBS containing 0.05% Tween 20. Permeabilization was performed
148 with 0.5% Triton X-100 in PBS for 15 min at room temperature. The blocking solution
149 contained 5% goat serum and 1% BSA in PBS. The primary antibodies were
150 anti-phospho-paxillin antibody (Cell Signaling Technology, 2541, Danvers, MA, USA,
151 1:20 dilution), anti-GM130 antibody (Cell Signaling Technology, 12480, 1:3200
152 dilution), anti-integrin α6 antibody (Acris Antibodies GmbH, Herford, Germany, 1:100
153 dilution) and anti-integrin β1 antibody (GeneTex, GTX23167, Alton Pkwy Irvine, CA,
154 USA, 1:10 dilution). The Secondary antibodies were goat anti-mouse IgG antibody
155 conjugated to Alexa Fluor 647 (Life Technologies, A21235, 1/1000 dilution), goat
156 anti-rat IgG antibody conjugated to Alexa Fluor 488 (Cell Signaling Technology, 4416,

157 1/1000 dilution) and goat anti-rabbit IgG antibody conjugated to Alexa Fluor 488 (Life
158 Technologies, A11008, 1/1000 dilution). Hoechst 33342 was used for nuclear staining.
159 Images of immunostained samples were collected with a BZ-9000 microscope
160 (Keyence). Optical sections were obtained with a confocal platform, TCS SP8 (Leica,
161 Tokyo, Japan).
162
163 *2.5 Messenger RNA expression analyses*
164 Total RNA samples were extracted with TRIzol reagent (Life Technologies, 15596026).
165 The RNA-seq analysis was previously described [20]. Complementary DNA samples
166 were synthesized from 1 μg of total RNA with SuperScript III (Life Technologies,
167 18080044), and quantitative reverse transcription-polymerase chain reaction
168 (qRT-PCR) was performed with a reagent, FastStart Universal SYBR Green Master
169 (Roche, 04913850001, Mannheim, Germany). Primers for qRT-PCR are listed in
170 Supplementary Table S2.
171
172 *2.6 Promoter activity analyses*

173 The SALL4A and SALL4B coding regions were cloned downstream of the CAG
174 promoter obtained from pCAG-mGFP (Addgene, 14757). To assess the promoter
175 activities, 1kbp regions upstream of the transcription start sites of the *ITGA6* and *ITGB1*
176 genes were linked to the minimal promoter in the pGL4.30 vector that also carries a
177 firefly luciferase2 gene (Promega, E8481, Madison, WI, USA). The promoter reporter
178 was co-transfected into Lenti-X 293T cells with the pGL4.73 vector that has the SV40
179 promoter and a Renilla luciferase gene (Promega, E6911), and with the SALL4
180 expression vector. A transfection reagent, FuGENE HD (Promega, E2311) was used.
181 One day after transfection, luciferase activity was measured using the Dual-Glo
182 Luciferase Assay System (Promega, E2920).
183
184 *2.7 Chromatin immunoprecipitation*
185 The SALL4B coding sequence was cloned into the pENTR-FLAG vector, and
186 subsequently subcloned into the pLenti vector. Cells expressing FLAG or
187 SALL4B-FLAG were cultured and used for chromatin immunoprecipitation. The
188 procedure was performed as previously described [20], and an anti-FLAG M2 antibody

189 (Sigma, F1804, St. Louis, MO, USA) was used. Immunoprecipitated DNA fragments

190 were used for regular PCR. The primers for *ITGA6* promoter were as follows: forward

191 5'-GCATCACCTGCACTTCTCTTTAT-3' and reverse

192 5'-CTGTGGACAGAATTGTGGTTG-3'. The primers for *ITGB1* promoter were as

193 follows: forward 5'-GGAGTCGCGGAACAGCAG-3' and reverse

194 5'-CCGGCGGCTTTAAGTGCT-3'. PCR products were electrophoresed with a 6%

195 acrylamide gel.

196

197 *2.8 Immunoblotting*

198 The primary antibodies were anti-phospho-FAK antibody (Cell Signaling Technology,

199 8556, 1:1000 dilution), anti-FAK antibody (Cell Signaling Technology, 3285, 1:1000

200 dilution), and anti-integrin β1 antibody (Cell Signaling Technology, 9699, 1:1000

201 dilution). Detection of the immunoreactions was described previously [20]. The

202 intensity of each band was analyzed using ImageJ.

203 For analysis of phosphorylated FAK levels, cells were starved with medium

204 containing 0.5% FBS for 18 h. Then, cells were trypsinized and suspended with

205 medium containing 0.1% BSA. The suspended cells were incubated for 1 h at 37 °C.

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

207 harvested for analyses.

208 To obtain immunoprecipitated samples of integrin α6 complex,

209 co-immunoprecipitation was performed with approximately 0.5 g of cells. A Dynabeads

210 Co-Immunoprecipitation Kit (Veritas, DB14321, Tokyo, Japan) and the anti-FLAG M2

211 antibody were used. Additionally, 0.5% NP-40 was added to the extraction buffer from

212 the kit.

213 Rho signals were detected with rabbit anti-RhoA antibody (Cell Signaling

214 Technology, 8789, 1:667 dilution). RhoA activity was measured with RhoA G-LISA

215 Activation Assay Kit (Cytoskeleton, BK124).

216

217 *2.9 Laminin binding assay*

218 For the laminin binding assays, 400 μL of PBS containing 1 μg of recombinant

219 laminin-511 E8 fragment (Nippi, 892013, Tokyo, Japan) was added to the wells of a

220 24-well plate and incubated for 1 h at 37 °C. The wells were washed, blocked with 1%

221 BSA solution for 1 h at 37 °C, and washed again. Then, 10,000 cells were plated, and

222 incubated for 20 min at 37 °C. The wells were washed, and the number of bound cells

223 was analyzed. The cells were counted manually.

224

225 *2.10 Time-lapse imaging*

226 The EGFP gene was cloned into the pENTR-FLAG vector. The paxillin coding

227 sequence was inserted the *Nco*I site of the pENTR-EGFP-FLAG vector. The

228 paxillin-EGFP fusion construct was subcloned into the pLenti vector (Life Technologies,

229 V533-06). Cells labeled with paxillin-EGFP were plated on a TC-coated glass bottom

230 dish (Greiner, 627975, Frickenhausen, Germany). Time-lapse images were collected

231 using a BZ-9000 microscope with a CO_2 chamber (Keyence).

232

233 *2.11 Transplantation assays*

234 For mouse xenograft assay, 1 x 10^5 MDA-MB-231 cells were suspended in 80 uL of

235 serum-free medium with 50% Matrigel (Corning, 356237, Bedford, MA, USA), and

236 transplanted into the mammary fat pad of five-week-old nude mouse. In the same

237 mouse, shGFP (control) and shSALL4 cells were injected at the right and the left sides,

238 respectively.

239 For zebrafish metastasis assay, cancer cell lines were labeled with mCherry

240 via lentiviral infection. Two-day-old *fli1-EGFP* fish embryos were dechorionated and

241 embedded into 1.0% low-melt agarose. Approximately 30 cells were injected into the

242 abdominal cavity. Transplanted embryos were maintained at 32 °C for 3 days, and

243 observed with a BZ-9000 microscope (Keyence).

244 The animal experiments in this study were approved by the Ethics Review

245 Board for Animal Experiments of Kyoto University. All animals were maintained

246 according to the Guide for the Care and Use of Laboratory Animals (National Institute

247 of Health Publication).

248

249 *2.12 Statistical analyses*

250 The number of migrated cell, the ratio of the FA-rich region, mRNA levels, luciferase

251 activity, phosphorylated FAK levels, the cell number bound to the laminin-511 E8

252 fragment, the active Rho level, and cell growth were analyzed using Student's *t*-test.

253 The ratio of polarized cells and the metastasis rate in the zebrafish assay were analyzed

254 using Fisher's exact test. For TCGA data, *SALL4* expression levels among breast cancer

255 subtypes were analyzed using a Kruskal-Wallis H test, and *ITGA6* and *ITGB1*

256 expression levels were analyzed using a Mann-Whitney *U* test. $P < 0.05$ was considered

257 statistically significant.

258

259 **3. Results**

260 *3.1 SALL4 is required for cell migration and normal FA pattern.*

261 To investigate the role of SALL4 in cell migration, we conducted loss-of-function

262 studies. We used a previously established shRNA system that targets the common

263 sequence of the SALL4 isoforms (shSALL4) [7]. shRNA for the GFP gene (shGFP)

264 was used as a control. We introduced shRNA expression into basal-like breast cancer

265 cell lines, SUM159 and MDA-MB-231, which are both highly migratory, and have a

266 spindle-shaped morphology. We found that the SALL4 knockdown cells were rounded,

267 while there was no morphological change in the shGFP control cells (Fig. 1A). Because

268 rounded morphology is indicative of low migratory potential, we explored changes in

269 the migratory properties of SALL4 knockdown cells. Using Boyden chamber assays,
270 we observed a loss of migration following SALL4 knockdown in SUM159 and
271 MDA-MB-231 cells (Fig. 1B,C), indicating that SALL4 positively regulates cell
272 migration.

273 In a polarized migrating cell, FAs are observed in restricted areas of the cell
274 periphery, whereas a low migratory cell has expanded FA-rich peripheral regions [21,
275 22]. To analyze the FA pattern, we immunostained cells with an antibody for
276 phosphorylated paxillin, which is a marker of FAs. In the control cells, FAs were
277 localized to the restricted areas, while the FA-positive area was expanded in SALL4
278 knockdown cells (Fig. 1D). To quantify this difference, we measured the length of the
279 FA-rich region, and divided it by the perimeter (Supplementary Fig. S1). In SALL4
280 knockdown cells, the ratio of the FA-rich region to the perimeter was significantly
281 increased (Fig. 1E). This result indicates that SALL4 modulates the FA pattern, which
282 may result in a spindle-shaped morphology and the high migratory properties.

283 A migrating cell has front-rear polarity, and loss of polarity results in a
284 rounded morphology and reduction in migration. Therefore SALL4 knockdown cells

285 may have lost this polarity. GM130 is a Golgi marker, and the Golgi apparatus is
286 localized near the front of the nucleus in a polarized cell. If the polarity is lost, the
287 GM130 signal is observed around the nucleus. We analyzed GM130 signals, and
288 observed no difference in the percentages of polarized cells between the controls and
289 SALL4 knockdown cells (Fig. 1F,G). This result suggests that SALL4 is not involved in
290 the regulation of cell polarity.
291
292 *3.2 SALL4 up-regulates integrin genes.*
293 Since SALL4 is a transcription factor, we hypothesized that it regulates the expression
294 of genes involved in cell migration. To identify SALL4-regulated genes, we obtained
295 gene expression data by RNA-seq, and compared the data from the shGFP and
296 shSALL4 groups (deposited in the DNA Data Bank of Japan, Sequence Read Archive
297 as DRA004721 and DRA004722, respectively) (Fig. 2A, Supplementary Table S3). We
298 found that the expression of several integrin genes was reduced by SALL4 knockdown.
299 Because integrin is known to promote cell migration, we focused on integrin family
300 genes. To validate the result of RNA-seq analysis, we performed qRT-PCR (Fig. 2B).

301 We identified candidate genes, namely *ITGA3*, *ITGA6*, *ITGA10*, *ITGB1* and *ITGB4*, the

302 expression levels of which were reduced to less than half of that of the controls by

303 SALL4 knockdown in both SUM159 and MDA-MB-231 cells.

304 To determine which integrin gene is involved in basal-like breast cancer cell

305 migration under the control of SALL4, we performed shRNA-mediated knockdown for

306 each gene. Morphological analysis showed that knockdowns of integrin α6 (encoded by

307 *ITGA6*) and β1 (encoded by *ITGB1*) induced a rounded morphology, similar to the

308 SALL4 knockdown (Fig. 2C), while the others showed no notable changes

309 (Supplementary Fig. S2). Next, we analyzed the migratory properties of these cells, and

310 observed significant reductions in migration (Fig. 2D). Although knockdown of each

311 candidate gene impaired cell migration, only the integrin α6 and β1 knockdowns

312 reduced the migration to levels similar to that of SALL4 knockdown (Fig. 2D). These

313 results suggest that reduced expression of integrin α6 and β1 is involved in the rounded

314 morphology and the reduced migration of SALL4 knockdown cells.

315 To study the regulation of integrin α6 and β1 expression by SALL4, we

316 performed reporter assays with the promoter regions of the *ITGA6* and *ITGB1* genes.

317 We linked each promoter region to the minimal promoter (miniP) and the luciferase2
318 reporter gene. We then prepared expression vectors carrying each SALL4 isoform.
319 Co-transfection with the integrin promoter reporter and the SALL4 expression vector
320 resulted in up-regulation of reporter gene expression, while no increase was observed in
321 the miniP control (Fig. 2E), indicating that both SALL4 isoforms activate the promoters
322 of the *ITGA6* and *ITGB1* genes. To determine whether SALL4 binds to these promoters,
323 we introduced FLAG-tagged SALL4B expression vectors to MDA-MB-231 cells, and
324 performed chromatin immunoprecipitation assays with an anti-FLAG M2 antibody. The
325 results showed an enrichment of the *ITGA6* and *ITGB1* promoter regions in the sample
326 of SALL4B-FLAG cells (Fig. 2F). These observations suggest that SALL4 directly
327 up-regulates integrin α6 and β1 at the transcriptional level.

328 The Cancer Genome Atlas (TCGA) network has published the gene
329 expression data of a number of cancer patients [23]. We analyzed the expression levels
330 of SALL4 in breast cancer patients, and found that it was up-regulated in the cancer
331 tissues of basal-like breast cancer patients (Supplementary Fig. S3A). To assess the
332 correlation with integrin gene expression, we classified the basal-like breast cancer

333 patients to two groups, SALL4 high and SALL4 low. The SALL4 high group had
334 higher SALL4 expression in the cancer tissues than in the normal tissues of the same
335 patient. We observed higher integrin α6 and β1 expression in the SALL4 high group
336 than in the SALL4 low group (Supplementary Fig. S3B). These results suggest that
337 SALL4 up-regulates integrin α6 and β1 expression in basal-like breast cancer.
338
339 *3.3 SALL4-regulated integrin α6 and β1 promote migration.*
340 To determine whether SALL4 promotes cell migration via up-regulation of integrin α6
341 and β1, we performed rescue experiments by overexpressing integrin α6 and β1 in
342 SALL4 knockdown cells. We constructed overexpression vectors for the integrin α6
343 variants, α6v1 and α6v2 (also known as α6B and α6A, respectively), and β1
344 (Supplementary Fig. S4A). The two transcriptional variants of integrin α6 share an
345 extracellular domain and a transmembrane region, but have different cytoplasmic tails
346 [24]. We introduced these expression vectors into MDA-MB-231 cells, and obtained
347 stably expressing cells (Supplementary Fig. S4B). The results of the migration assays
348 showed that cell migration was restored when the cells overexpressed both integrin α6

349 and β1, but integrin α6 alone and β1 alone were not able to rescue the migratory ability
350 of SALL4 knockdown cells (Fig. 3A). In addition to cell migration, the rounded
351 morphology caused by SALL4 knockdown was restored in the cells overexpressing
352 both integrin α6 and β1 (Fig. 3B). These results indicate that SALL4-regulated integrin
353 α6 and β1 are required for the spindle-shaped morphology and cell migration in
354 basal-like breast cancer cells.

355 Because integrin mediates FAs, we analyzed the FA pattern. In SALL4
356 knockdown cells, the FA-positive area was expanded compared to that of the control,
357 but this change was not observed in the cells overexpressing integrin α6 and β1 (Fig.
358 3C). Additionally, the FA-rich region was significantly expanded by SALL4
359 knockdown, and no change was observed in the cells overexpressing integrin α6 and β1
360 (Fig. 3D).

361 In integrin-mediated FAs, FAK is phosphorylated at Tyr-397 [14]. We
362 analyzed the levels of phosphorylated FAK in the cells overexpressing integrin α6 and
363 β1 (Fig. 3E). In the control cells, reduced FAK phosphorylation was observed following
364 SALL4 knockdown (Fig. 3F). However, overexpression of both integrin α6 and β1

365 restored the phosphorylated FAK levels (Fig. 3F), suggesting that integrin α6 and β1

366 are required for FAK activation. Our observations indicate that SALL4 modulates FAs

367 via up-regulation of integrin α6 and β1.

368

369 *3.4 Integrin α6 and β1 form a heterodimer in basal-like breast cancer.*

370 The integrin α and β subunits form a heterodimer [9, 10]. Integrin α6 and β1 can bind

371 to each other [25], but it is unclear whether they form a heterodimer in basal-like breast

372 cancer cells. To clarify this, we conducted a co-immunoprecipitation assay in

373 MDA-MB-231 cells. We precipitated protein complexes from the lysates of cells

374 overexpressing FLAG-tagged integrin α6 using an anti-FLAG M2 antibody. We

375 detected integrin β1 in the protein complexes of integrin α6v1 and α6v2 (Fig. 4A),

376 indicating that integrin α6 and β1 form a heterodimer. Furthermore, we

377 double-immunostained SUM159 and MDA-MB-231 cells with antibodies for integrin

378 α6 and β1, and performed optical sectioning with a confocal microscope (Fig. 4B). We

379 observed overlapping signals, supporting that there are integrin α6β1 heterodimers in

380 basal-like breast cancer cells.

381 The integrin α6β1 heterodimer can bind to laminin-511 [11]. Because SALL4
382 up-regulates integrin α6 and β1 expression, and integrin α6 and β1 form a heterodimer,
383 we hypothesized that SALL4 knockdown cells lose the ability to bind to laminin-511. If
384 integrin α6 and β1 do not form a heterodimer in basal-like breast cancer cells, there will
385 be no change in the laminin-511 binding ability in SALL4 knockdown cells. To address
386 this hypothesis, we performed a binding assay with a recombinant laminin-511 E8
387 fragment to which integrin binds [26]. We used a BSA solution for blocking. A small
388 number of cells bound to the BSA-coated dish, and there was no difference in the
389 number of bound cells between the control and SALL4 knockdown cells (Fig. 4C).
390 When we coated a dish with the laminin-511 E8 fragment in combination with the BSA
391 blocking solution, the number of bound SUM159 and MDA-MB-231 cells increased.
392 Additionally, we observed that the number of bound SALL4 knockdown cells was
393 approximately half that of the controls (Fig. 4D), indicating that SALL4 promotes the
394 binding to laminin-511. Furthermore, we performed rescue experiments by
395 overexpressing integrin α6 and β1, and the binding to laminin-511 was restored (Fig.

396 4E,F). These results showed that the SALL4-regulated integrin α6 and β1 function by

397 forming an α6β1 heterodimer.

398

399 *3.5 SALL4 is required for FA dynamics.*

400 Impaired FA assembly/disassembly dynamics causes changes in the FA pattern and loss

401 of migration. To analyze the FA dynamics, we monitored the localization of paxillin, a

402 component of the FA complex, using a paxillin-EGFP fusion construct. We obtained

403 images at 0 and 10 min, and observed the FA assembly/disassembly (Fig. 5A). FA

404 signals appeared as punctate dots, while the free paxillin-EGFP molecules localized

405 near the nucleus and appeared as diffuse signals. In these experiments, we used an

406 shRNA with a scrambled sequence (shScr) as the control. In the control cells, assembled

407 and disassembled FAs were observed (Fig. 5A, arrowheads). However, FAs in SALL4

408 knockdown cells were unchanged, suggesting that FAs were stabilized by SALL4

409 knockdown.

410 We quantified the ratios of assembled and disassembled FAs in each cell (Fig.

411 5B,C). The ratio of assembled FAs was calculated by dividing the number of newly

412 formed FAs in 10 min by the total number of FAs at 10 min. The ratio of disassembled

413 FAs was calculated by dividing the number FAs lost in 10 min by the total number of

414 FAs at 0 min. The results showed that both the ratios of assembled and disassembled

415 FAs were reduced by SALL4 knockdown, and this reduction was restored by

416 overexpression of integrin α6β1, suggesting that FA stabilization in SALL4 knockdown

417 cells is due to the loss of integrin α6β1. These observations indicate that the SALL4 -

418 integrin α6β1 network activates the FA dynamics.

419

420 *3.6 The SALL4 - integrin α6β1 network prevents aberrant Rho activation in cell*

421 *migration.*

422 Loss of FAK causes over-activation of Rho [18]. Rho over-activation stabilizes FAs,

423 and reduces cell migration [8, 22, 27]. In the SALL4 knockdown cells, FAK

424 phosphorylation levels and migration were reduced, and these reductions were restored

425 by integrin α6β1 expression (Fig. 3). We therefore hypothesized that the SALL4 -

426 integrin α6β1 network is involved in regulating Rho activity in cell migration. Thus, we

427 measured Rho activity, and observed that SALL4 knockdown did not change the

428 protein level of Rho, but increased RhoA activity (Fig. 6A,B). In SALL4 knockdown
429 cells, Rho over-activation was suppressed by integrin α6β1 (Fig. 6B). These results
430 indicate that the SALL4 - integrin α6β1 network prevents Rho over-activation in
431 basal-like breast cancer cells.

432 To determine whether over-activation of Rho is the responsible for the
433 rounded morphology and expansion of the FA-rich region in SALL4 knockdown cells,
434 we treated SALL4 knockdown cells with Rho signaling inhibitors, C3 transferase and
435 Y-27632. C3 transferase ADP-ribosylates Asn-41 on the Rho family of small GTPases,
436 Rho A, B and C [28]. We analyzed cell morphology following C3 transferase treatment
437 in SALL4 knockdown cells, and observed that the treated cells exhibited a
438 spindle-shaped morphology and a reduced FA-rich region (Supplementary Fig. S5).
439 Rho A activates Rho-associated coiled-coil forming protein serine/threonine kinase
440 (ROCK). Appropriate activation and suppression of the Rho-ROCK pathway is required
441 for cell migration [8, 22, 27]. We used a chemical ROCK inhibitor, Y-27632 [29], and
442 observed that the rounded morphology of the SALL4 knockdown cells converted to a
443 spindle-shaped morphology (Fig. 6C). In addition, expansion of the FA-rich region was

444 not observed in Y-27632-treated cells (Fig. 6D). These results indicate that SALL4

445 regulates cell morphology and the FA pattern through modulation of Rho activity.

446 Long-term treatment with C3 transferase causes cellular stress, and increases

447 the difficulty in analyzing cell migration. Y-27632 is milder than C3 transferase in

448 basal-like breast cancer cells, and it is easier to assess cell migration following Y-27632

449 treatment. Using Boyden chamber assays, we analyzed the number of cells that

450 migrated in 5 hours, and found that the reduced cell migration was recovered by ROCK

451 inhibition in SALL4 knockdown cells (Fig. 6E). Taken together, the SALL4 - integrin

452 α6β1 network promotes cell migration through modulation of the Rho-ROCK pathway

453 (Supplementary Fig. S6).

454

455 *3.7 The SALL4 - integrin α6β1 network is required for* in vivo *cell migration.*

456 SALL4 knockdown reduces cell growth [7, 30], and the reduced cell growth was not

457 rescued by integrin α6β1 expression (Supplementary Fig. S7A). This indicates that the

458 mouse transplantation model can not be used to analyze the *in vivo* migratory properties

459 of SALL4 knockdown cells, because the tumor size of the SALL4 knockdown cells can

460 not be compared to that of the controls at both the transplanted focus and the metastatic
461 foci, and evaluation using the number of foci and/or the size of metastatic tumors is not
462 accurately show a difference in migration. In support of this notion, our mouse
463 xenograft experiments showed the reduced tumor growth in SALL4 knockdown cells
464 (Supplementary Fig. S7B). Therefore, to analyze *in vivo* cell migration, we used
465 zebrafish metastasis assays. These assays require 2 or 3 days to assess migration, and do
466 not require cell growth and tumor formation [31].

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

475 with control cancers underwent metastasis, while few fish with SALL4 knockdown
476 cells showed metastasis.

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

485 We next determined whether integrin α6β1 expression restores *in vivo*
486 migration in SALL4 knockdown cells. We transplanted cells overexpressing integrin
487 α6β1 and observed similar migration rates as that of the controls in SALL4 knockdown
488 cells (Fig. 7C). These results indicate that the SALL4 - integrin α6β1 network is
489 required for enhanced migration *in vivo*.

490

491 *3.8 Integrin α6β1 expression is an indicator of poor metastasis-free survival.*

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

503

504 **4. Discussion**

505 One of the crucial steps in cancer metastasis is the acquisition of the high migratory
506 properties. In this study, we elucidated the role of the SALL4 - integrin α6β1 network

507 in cell migration in basal-like breast cancer cells. SALL4 knockdown cells showed
508 reduced migration and a rounded morphology with expansion of the FA-rich region.
509 Gene expression analyses revealed that SALL4 positively regulates the expression of
510 the integrin α6 and β1 genes. Cells with loss of integrin α6 and β1 showed identical
511 phenotypes to that of SALL4 knockdown cells, suggesting that they are the causative
512 factors of SALL4-regulated cell migration. Overexpression of both integrin α6 and β1
513 restored cell migration and reversed the morphology in SALL4 knockdown cells,
514 further supporting this notion. Moreover, we observed that SALL4 knockdown cells
515 have aberrant Rho activation, and integrin α6β1 expression suppressed this phenotype.
516 Pharmacological inhibition of Rho over-activation restored cell migration in SALL4
517 knockdown cells. These results suggest that the SALL4 - integrin α6β1 network is
518 required for cell migration via modulation of Rho activity. In addition, we performed
519 zebrafish metastasis assays, and showed that exogenous integrin α6β1 expression
520 restored cell migration in SALL4 knockdown cells, indicating that the SALL4 - integrin
521 α6β1 network promotes cell migration *in vivo*. Our findings identify a novel mechanism
522 underlying the high migratory properties of cancer cells.

523 SALL4 is known to be a factor in stem cell maintenance and proliferation [2,
524 40]. In cancer cells, SALL4 up-regulates genes involved in cell proliferation, such as
525 *BMI1* and *CCND1* [7, 41, 42]. However, the role of SALL4 in cell migration has not
526 been fully elucidated, because SALL4-depleted cells display impaired cell proliferation,
527 which hinders the analysis of other biological features. In this study, we used an
528 shRNA-mediated SALL4 knockdown system, and prepared fresh SALL4 knockdown
529 cells in each experiment. We performed SALL4 loss-of-function studies in the
530 basal-like breast cancer cell lines, SUM159 and MDA-MB-231. The cells have a
531 spindle-shaped morphology and high migratory properties, and these characteristics can
532 be altered by genetic manipulation and pharmacological treatment, which allowed us to
533 easily analyze the role of SALL4 in cell migration. Using this procedure, we elucidated
534 the role of SALL4 in cell migration.

535 Although our previous study found that SALL4 is involved in the
536 spindle-shaped morphology of basal-like breast cancer cells [7], the causative factors
537 and the detailed mechanism underlying this characteristic remained elusive. In this
538 study, we discovered that the SALL4 - integrin α6β1 network regulates the

539 spindle-shaped morphology through the maintenance of FA dynamics. We observed an
540 expansion of the FA-rich region in SALL4 knockdown cells. However, SALL4
541 knockdown cells did not show a reduction in FA number, although SALL4 knockdown
542 reduces the mRNA levels of several integrin genes. No reduction in FA number may be
543 due to the function of the remaining integrins. SALL4 knockdown reduced expression
544 levels of its downstream genes, including integrins, but it did not completely deplete
545 their expression. In addition, cells express other integrins that are not regulated by
546 SALL4. Therefore SALL4 knockdown cells had reduced integrin function, which
547 appeared to be sufficient to form FAs, but not for activation of FA dynamics.

548 Although knockdown of each SALL4-regulated integrin gene showed reduced
549 cell migration, this study focused on the integrin α6 and β1 genes, because the
550 morphological change and the degree of reduction in the migration following their
551 knockdowns were identical to those of the SALL4 knockdown. The integrin α6β1
552 heterodimer was found to be the main factor in SALL4-regulated cell migration. The
553 human genome has 18 integrin α and 8 integrin β subunit genes, and various integrin
554 genes are expressed in basal-like breast cancer. Although integrins appear to be

555 involved in cancer metastasis, the role of each integrin has not been fully characterized
556 in metastatic process, such as cell migration, invasion, and anoikis resistance. In this
557 study, through analyses of SALL4 function, we showed that integrin α6β1 is required
558 for migration during basal-like breast cancer metastasis. Additionally, analyses of the
559 data from several cohort studies showed that breast cancer patients with high integrin
560 α6 and β1 expression had poorer metastasis-free survival than that of the patients with
561 low integrin α6 and β1 expression. These results suggest that a therapy targeting
562 integrin α6β1 will inhibit metastasis by suppressing cell migration.

563 Integrin α6 has two variants. The functions of the integrin α6 variants differ
564 with respect to stemness [43, 44]. However, we observed that the variants have
565 comparable role in cell migration. Integrin β1 cytoplasmic tail binds to FAK to form
566 focal adhesion, and to activate FAK signaling for cell migration [45]. Because the
567 integrin α6 extracellular and transmembrane domains are shared by the variants, and
568 overexpression of each variant restored the reduced cell migration of SALL4
569 knockdown cells in combination with integrin β1 overexpression, integrin α6 may
570 function to support integrin β1 in SALL4-regulated cell migration.

571 In SALL4 knockdown cells, the FAs were stabilized, and the FA dynamics
572 were restored by inhibition of Rho signaling. Aberrant Rho activity was normalized by
573 exogenous expression of integrin α6β1. We thought that the SALL4 - integrin α6β1
574 network modulates Rho activity to promote cell migration. Although Rho activation is
575 required for FA maturation, its over-activation stabilizes FA, which in turn inhibits the
576 FA assembly/disassembly cycle. Our results showed that the SALL4 - integrin α6β1
577 network modulates Rho activity, but we did not identify the Rho activation factor.
578 There are a number of factors involved in Rho activation [19], and they might function
579 in FA maturation in basal-like breast cancer cells.

580 This study showed that the SALL4 up-regulates integrin signaling, and
581 modulates Rho activity. However there is a possibility that SALL4 regulates genes
582 involved in recruitment of FA molecules, such as *PAG3* (paxillin-associated protein
583 with ADP-rebosylation factor [ARF] GTPase-activating protein [GAP] activity, number
584 3) (also known as ASAP2/KIAA0400) [46]. PAG3 recruits paxillin molecules to FA,
585 and PAG3 overexpression impairs cell migration in leukemic monocyte lymphoma cell
586 line, U937, and kidney cell line, COS-7 [46]. We therefore analyzed the relation

587 between SALL4 and PAG3. However, in our RNA-seq analysis, SALL4 does not
588 regulate *PAG3* expression. We did not observed enrichment of *PAG3* upstream region
589 in ChIP assay. In our unpublished observation, although the morphology of basal-like
590 breast cancer cells overexpressing PAG3 was slightly rounded, it is still spindle-shaped.
591 These suggest that PAG3 is not involved in the function of SALL4 with respect to cell
592 migration.

593 In addition to cell migration, integrins are involved in cell growth [47].
594 However, overexpression of integrin α6β1 did not restore the cell growth in SALL4
595 knockdown cells, implying that integrin α6β1 is not involved in SALL4-regulated cell
596 growth. Due to the impaired cell growth of SALL4 knockdown cells, we are not able to
597 compare tumor sizes at the transplanted focus and metastatic foci between control and
598 SALL4 knockdown cells in mouse transplantation assay. Therefore, instead of mouse
599 transplantation assays, we utilized zebrafish metastasis assays that allowed us to analyze
600 the migration of single cells in 2-3 days without cell growth [31]. In the zebrafish assay,
601 we observed reduced cell migration in SALL4 knockdown cells, and restoration of
602 migration by integrin α6β1 expression, consistent with the results of the *in vitro*

603 experiments. Our results demonstrated that the zebrafish metastasis assay is a useful
604 tool to analyze cell migration *in vivo*, especially if cells show reduced growth. Although
605 the conditions, such as temperature, osmotic pressure and nutrients, are different
606 between human and zebrafish bodies, and the results do not strictly reflect the cancer
607 cell behavior in the human body, we can use zebrafish to evaluate the *in vivo* migration
608 potential.

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

619

620 **5. Conclusions**

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

626

627 **Conflict of interest**

628 JI is an employee of Kyoto University's Sponsored Research Program funded by Taiho
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643

644 **Appendices**

645 Supplementary materials

646 Supplementary Table S3

647

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830

831 **Figure legends**

832 **Fig. 1.** SALL4 is required for the spindle-shaped morphology and cell migration. (A)
833 Controls and SALL4 knockdowns of SUM159 and MDA-MB-231 cells are shown. (B)
834 Images of migrated cells are shown. Cell nuclei were stained with Hoechst 33342.
835 Arrowheads indicate the signals of migrated cells. For simplicity, not all signals are
836 pointed. (C) Graphs show the number of migrated cells (n = 4). The vertical axis
837 indicates the number of migrated cells. (D) Cells were stained with an
838 anti-phosphorylated paxillin antibody. Arrowheads indicate the immunoreactions. For
839 simplicity, not all signals are pointed. (E) Graphs show the ratio of the FA-rich region.
840 The vertical axis indicates the value obtained by dividing the length of the FA-rich
841 region by the perimeter. The cell numbers were 31 in SUM159;shGFP, 36 in
842 SUM159;shSALL4, 40 in MDA-MB-231;shGFP and 41 in MDA-MB-231;shSALL4
843 cells. (F) Images of immunostaining with anti-GM130 are shown. (G) Ratios of
844 polarized cells are shown in the graph. The vertical axis indicates the percentage of
845 polarized cells. The cell numbers were 195 in SUM159;shGFP, 249 in
846 SUM159;shSALL4, 163 in MDA-MB-231;shGFP and 162 in MDA-MB-231;shSALL4

847 cells. Scale bars indicate 100 μm in A,B, and 20 μm in D,F. Student's *t*-test was used in

848 C,E. Fisher's exact test was used in G. n.s.: not significant, **: $P < 0.01$. Error bars

849 represent the standard deviation.

850

851 **Fig. 2.** SALL4 up-regulates integrin α6 and β1 expression. (A) The scatter plot shows

852 the gene expressions in the control and SALL4 knockdown SUM159 cells. Integrin

853 genes reduced by SALL4 knockdown are indicated. The accession numbers of the

854 shGFP and shSALL4 data are DRA004721 and DRA004722, respectively. (B) Relative

855 expression levels of integrin family genes are shown (n = 3). In each gene, mean

856 expression level of the shGFP cells was defined as 1. Arrowheads point the genes that

857 had expression reduced to less than half of that of the controls by SALL4 knockdown in

858 both SUM159 and MDA-MB-231 cells. (C) Images show cells with integrin α6 and β1

859 knockdown. (D) The results of the Boyden chamber assays are shown (n = 4).

860 MDA-MB-231 cells were used. The vertical axis indicates the number of migrated cells.

861 (E) Reporter assays using the integrin α6 and β1 gene promoters were performed (n =

862 4). The vertical axis indicates the relative light units of luciferase. The mean value of

863 the FLAG control was defined as 1. (F) Enrichment of the integrin α6 and β1 promoter

864 region is shown. MDA-MB-231 cells were used. Chromatin immunoprecipitation

865 samples were used for PCR amplification with primers for the integrin α6 and β1

866 promoters. ChIP: chromatin immunoprecipitation. Scale bars indicate 100 μm.

867 Student's *t*-test was used for statistical analyses. *: $P < 0.05$, **: $P < 0.01$. Error bars

868 represent the standard deviation.

869

870 **Fig. 3.** SALL4-regulated integrin α6 and β1 expression is required for spindle-shaped

871 morphology and cell migration in basal-like breast cancer cells. (A) Boyden chamber

872 assays were performed with cells overexpressing integrin α6 and β1 ($n = 4$ in control, n

873 $= 5$ in the α6 and β1 overexpressing groups). The vertical axis indicates the number of

874 migrated cells. (B) Cells overexpressing integrin α6 and β1 are shown. (C) Images of

875 immunostaining with anti-phosphorylated paxillin are shown. Arrowheads indicate the

876 immunoreaction signals. For simplicity, not all signals are pointed. (D) Graphs show the

877 ratio of the FA-rich region. The cell numbers were 33 in control;shGFP, 36 in

878 control;shSALL4, 45 in α6v1,β1;shGFP, 37 in α6v1,β1;shSALL4, 39 in

879 α6v2,β1;shGFP and 33 in α6v2,β1;shSALL4. (E) Immunoblotting for FAK was

880 performed. (F) The intensity of phosphorylated FAK and the total FAK bands were

881 measured. Relative FAK phosphorylation levels to shGFP are graphed (n = 3). Scale

882 bars indicate 100 μm in B, and 20 μm in C. Student's t-test was used. *: $P < 0.05$, **: P

883 < 0.01. Error bars represent the standard deviation.

884

885 **Fig. 4.** Integrin α6 and β1 form a heterodimer. (A) Co-immunoprecipitation samples

886 were used for immunoblotting with an anti-integrin β1 antibody. IP:

887 immunoprecipitation. IB: immunoblotting. (B) Confocal images of immunostained cells

888 are shown. Antibodies for integrin α6 and β1 were used for double-immunostaining.

889 (C-F) Binding assays were performed (n = 4). The number of cells bound to the BSA

890 blocking solution and the recombinant laminin-511 E8 fragment are shown in C,E and

891 D,F, respectively. The vertical axis indicates the number of bound cells. SUM159 and

892 MDA-MB-231 cells were used in C,D. MDA-MB-231 cells overexpressing integrin α6

893 and β1 were used in E,F. Scale bars indicate 20 μm. Student's t-test was used. n.s.: not

894 significant, **: $P < 0.01$. Error bars represent the standard deviation.

895

896 **Fig. 5.** SALL4 activates the FA dynamics. (A) Cells with a paxillin-EGFP fusion were

897 used to monitor the FA dynamics. Images of shScr (control) and shSALL4 cells are

898 shown. Yellow and blue arrowheads indicate the FA signals assembled and

899 disassembled in 10 min, respectively. For simplicity, not all signals are pointed. (B) The

900 ratio of assembled FAs was calculated by dividing the number of newly formed FA in

901 10 min by the total FA number at 10 min. (C) The ratio of disassembled FA was

902 calculated by dividing the number of FAs lost in 10 min by the total FA number at 0

903 min. The cell numbers analyzed in B,C were 33 in the control;shGFP, 36 in the

904 control;shSALL4, 31 in α6v1,β1;shGFP, 37 in α6v1,β1;shSALL4, 41 in

905 α6v2,β1;shGFP and 47 in α6v2,β1;shSALL4 cells. Scale bars indicate 20 μm.

906 Student's *t*-test was used. **: $P < 0.01$. Error bars represent the standard deviation.

907

908 **Fig. 6.** The SALL4 - integrin α6β1 network modulates Rho activity to promote cell

909 migration. (A) RhoA protein level was not changed by SALL4 knockdown in

910 MDA-MB-231 cells. (B) RhoA activity was measured with RhoA G-LISA Activation

911 Assay Kit. Relative RhoA activity to shGFP was calculated (n = 3). (C)

912 Immunofluorescence images of phosphorylated paxillin are shown. A ROCK inhibitor,

913 Y-27632, was used. Arrowheads indicate the signals. For simplicity, not all signals are

914 pointed. (D) The FA-rich region was analyzed. The cell numbers analyzed were 43 in

915 SUM159;shGFP, 35 in SUM159;shSALL4 with water, 43 in SUM159;shSALL4 with

916 the ROCK inhibitor, 41 in MDA-MB-231;shGFP, 36 in MDA-MB-231;shSALL4 with

917 water and 31 in MDA-MB-231;shSALL4 with the ROCK inhibitor. (E) Boyden

918 chamber assays were performed with the ROCK inhibitor (n = 4). The vertical axis

919 indicates the number of migrated cells. Scale bars indicate 20 μm. Student's t-test was

920 used. **: $P < 0.01$. Error bars represent the standard deviation.

921

922 **Fig. 7.** The SALL4 - integrin α6β1 network is required for *in vivo* cell migration. (A)

923 Metastasis tracking assay of transplanted SUM159 cells in zebrafish larvae. In the

924 control condition (shGFP), the SUM159 cells (magenta) moved to the anterior (yellow

925 square) and posterior (blue square) from the injected site at day 3. The metastasized

926 cells (arrowheads) were localized outside of the vasculatures (green, *fli1-EGFP*). On the

927 other hand, the SALL4 knockdown cells did not migrate out from the abdominal cavity.

928 Scale bars indicate 500 μm. (B,C) Statistical data for the metastasis assay. The ratio of

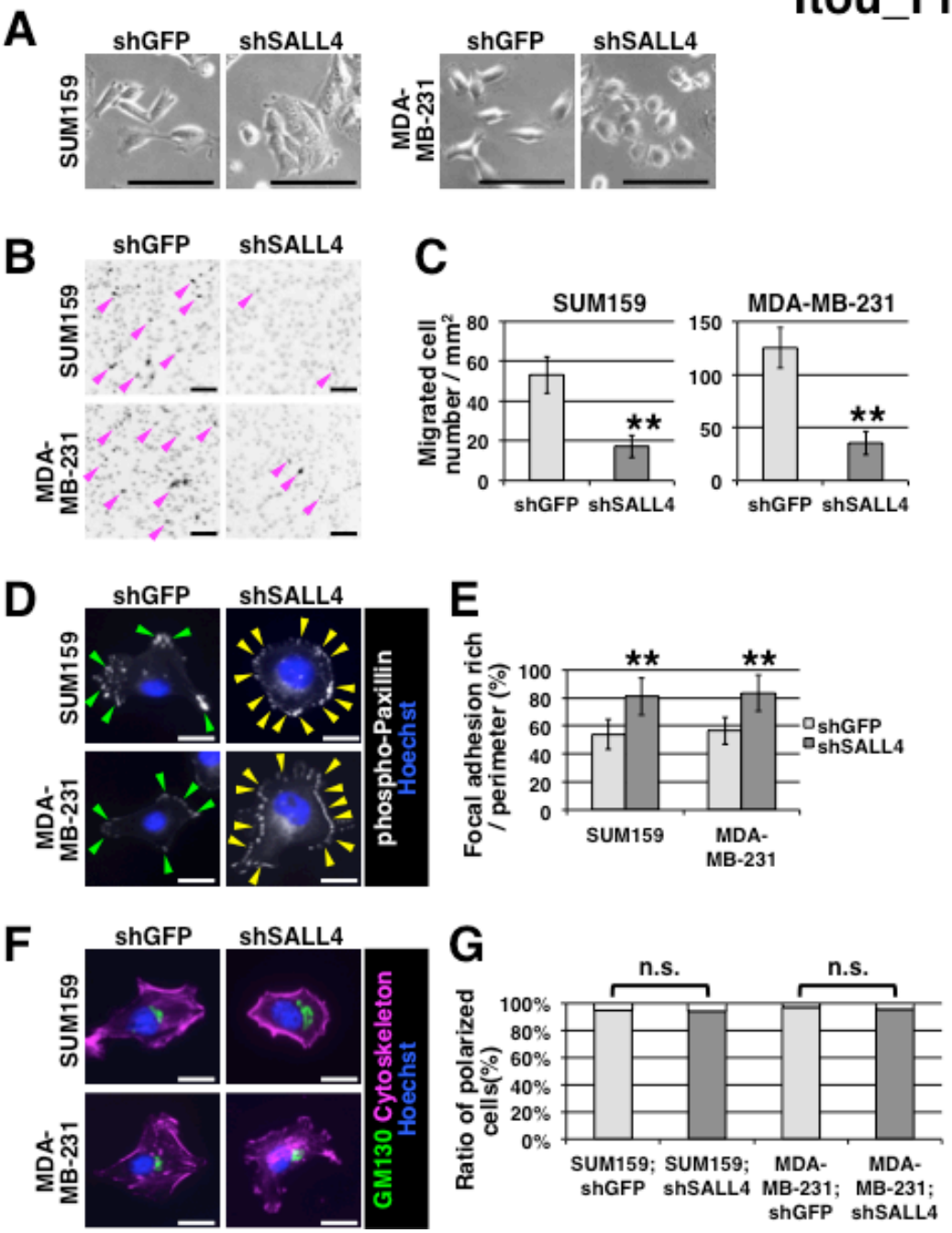
929 fish with the metastasis was decreased under the shSALL4 condition in the both of

930 SUM159 and MDA-MB-231 cells (B). The shSALL4-induced phenotype was rescued

931 by overexpression of integrin α6 and β1 (C). Fisher's exact test was used. **: $P < 0.01$.

932

Itou_Fig1



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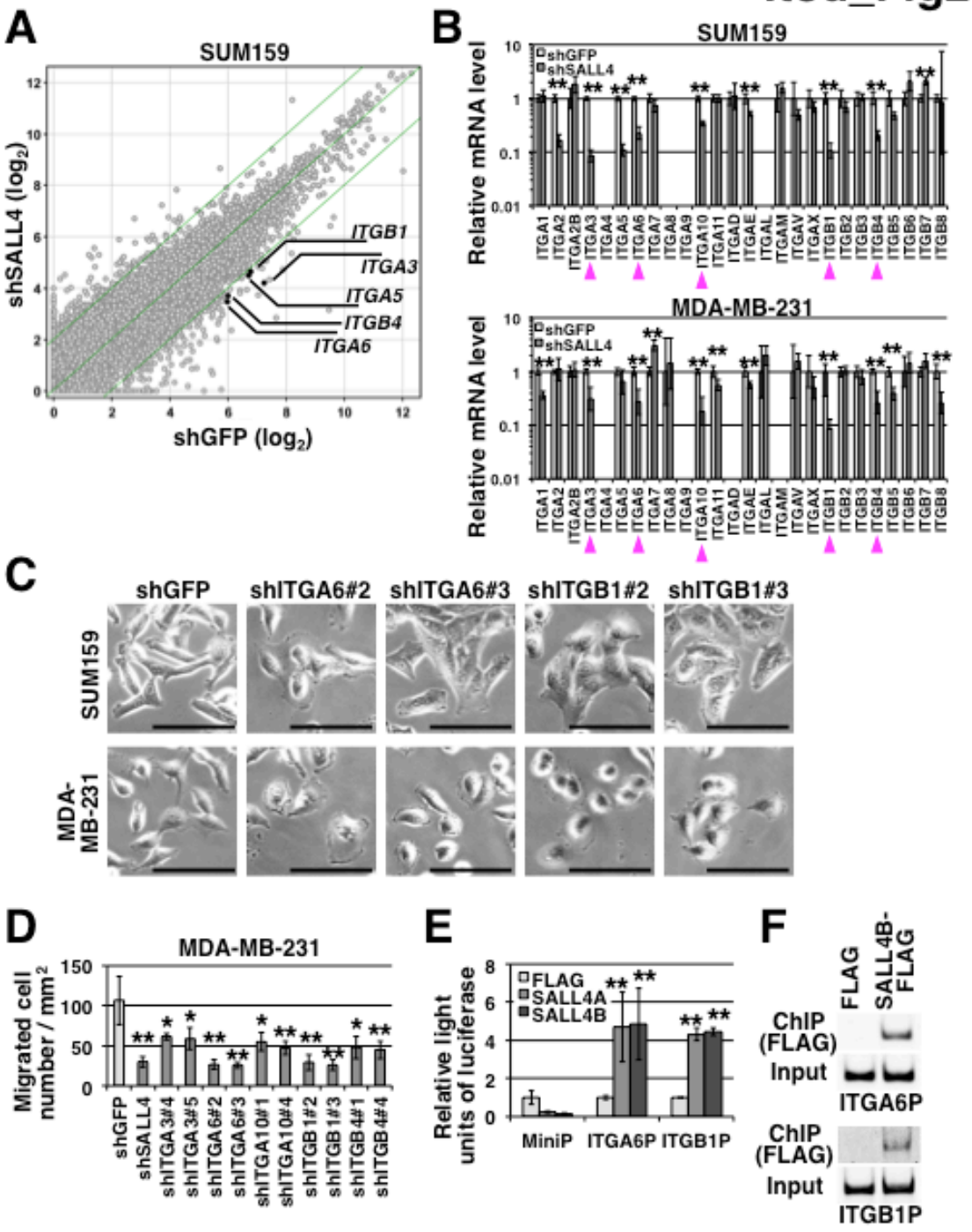

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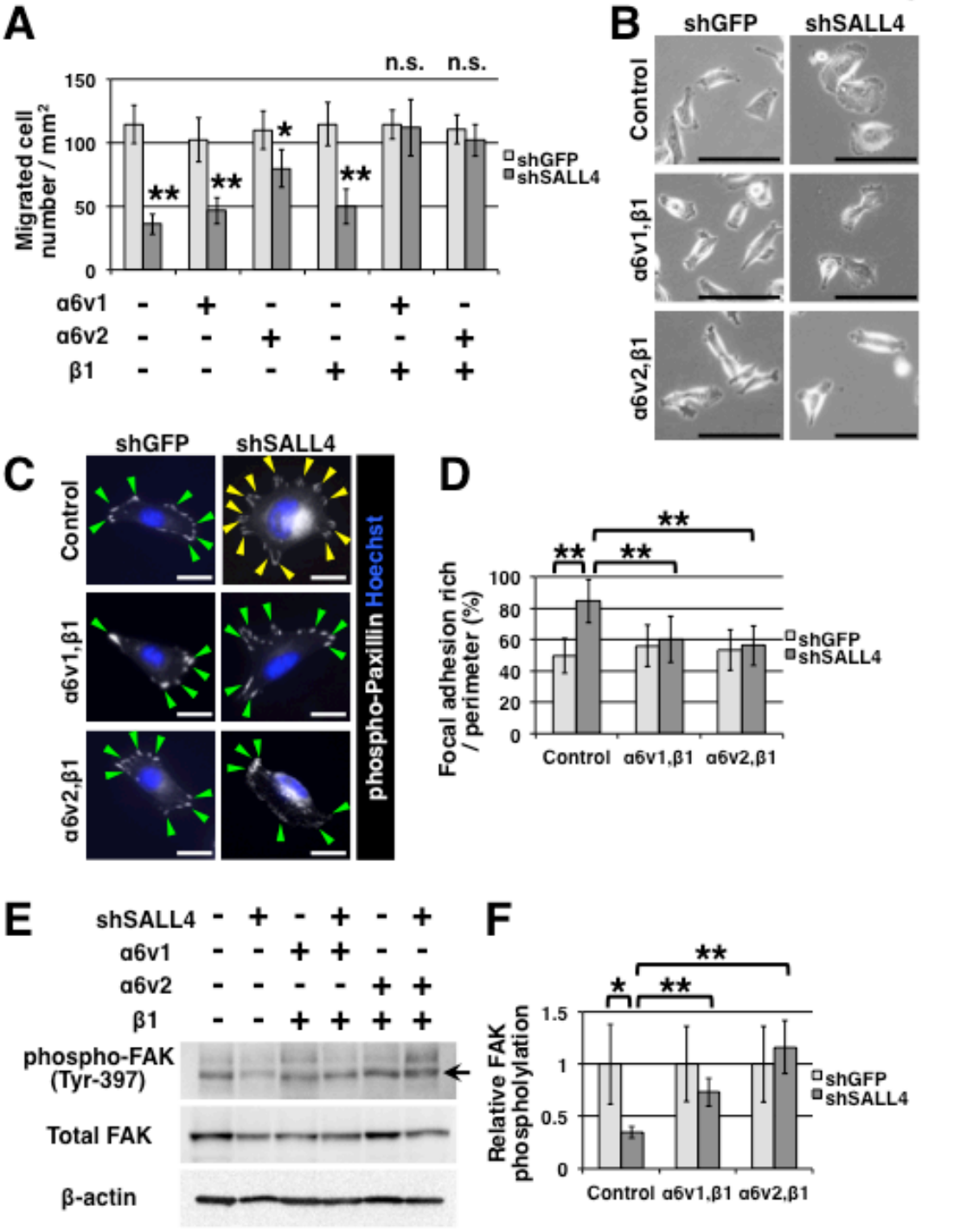

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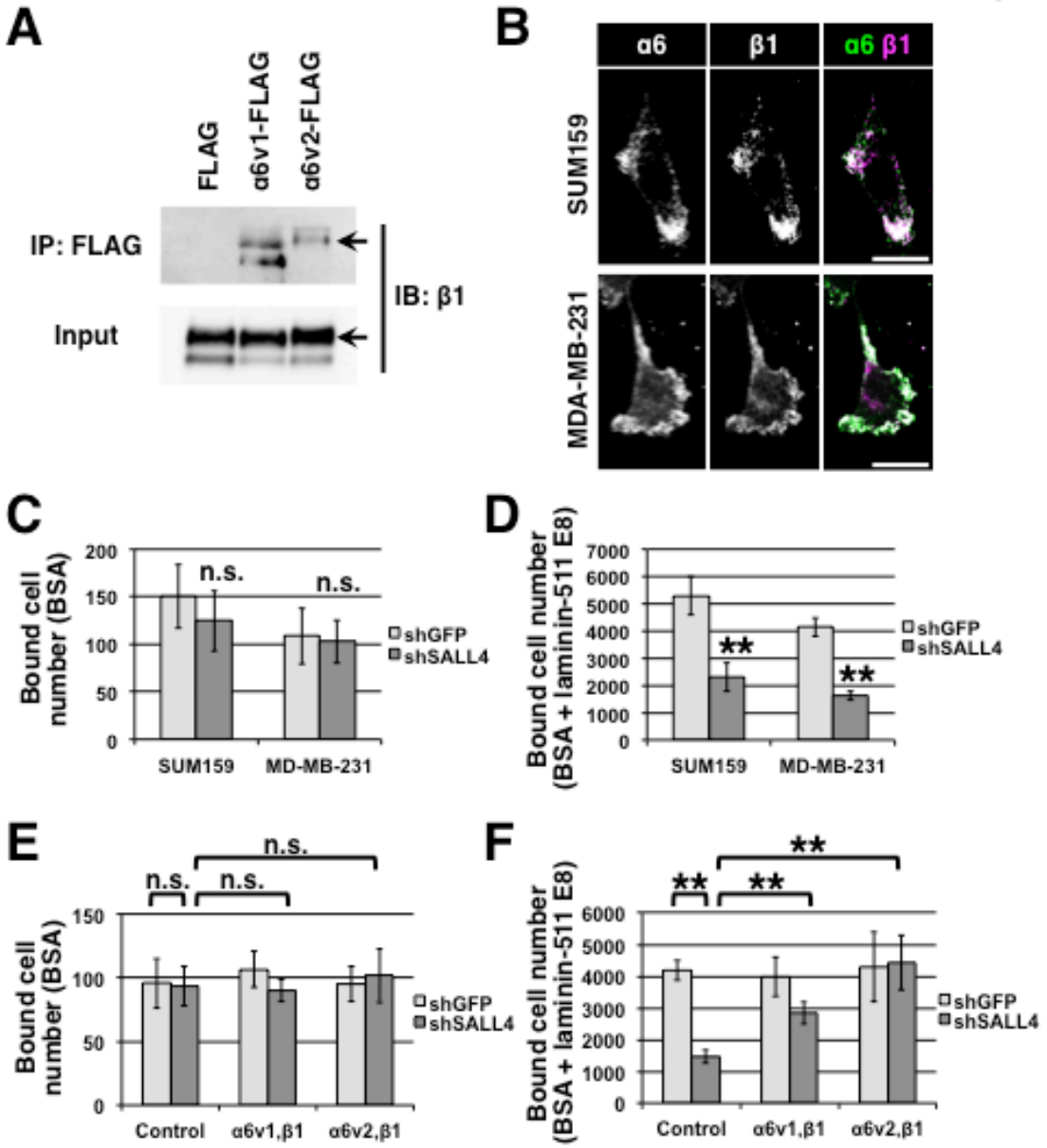

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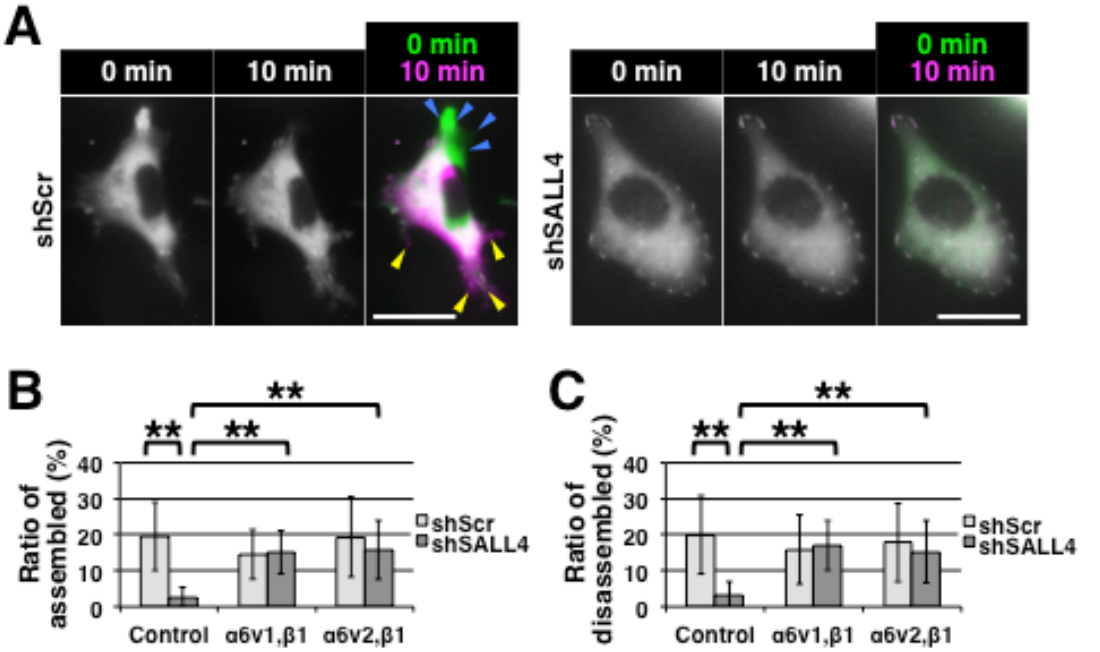

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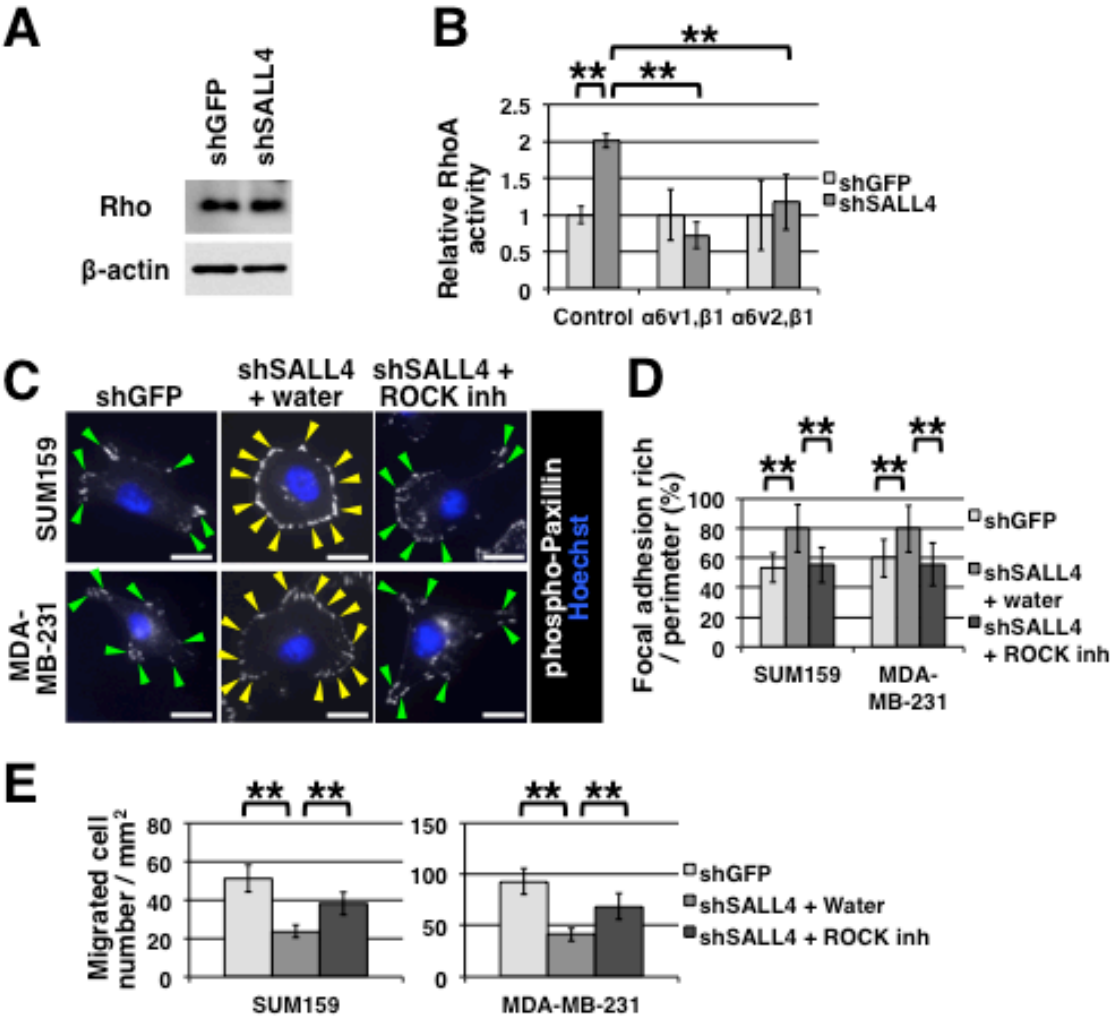

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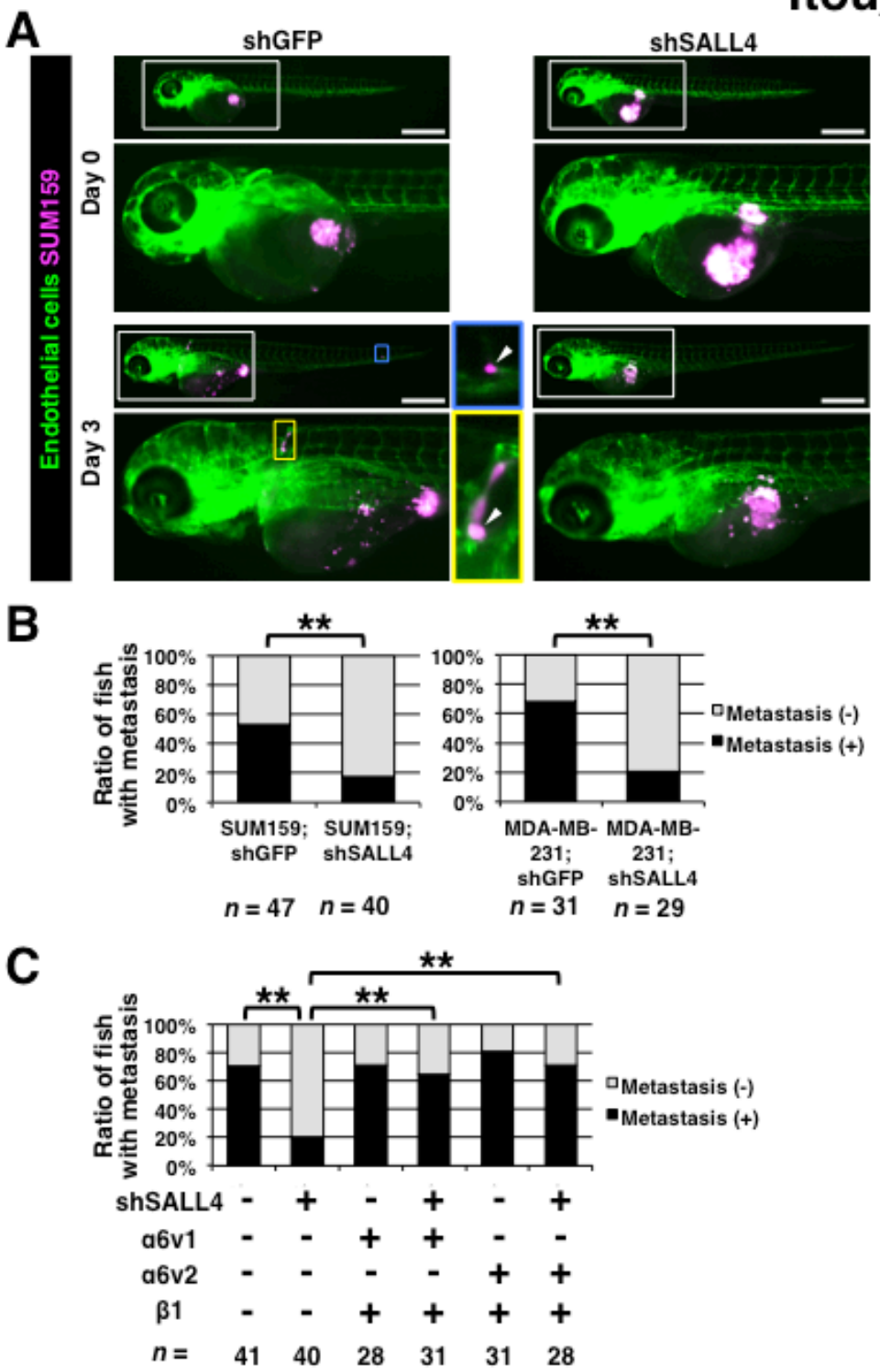


The Sal-like 4 - integrin α6β1 network promotes cell migration for metastasis via activation of focal adhesion dynamics in basal-like breast cancer cells.

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.

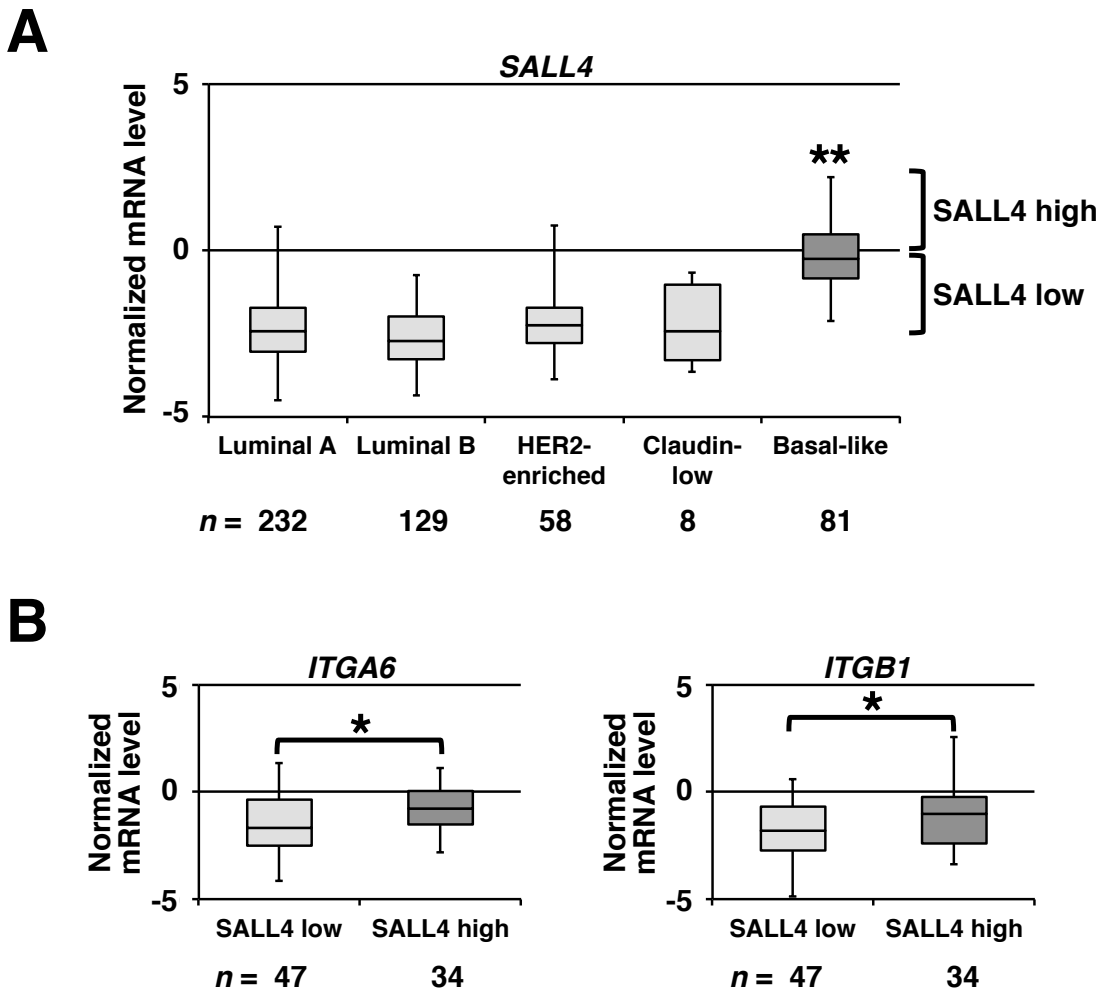
Supplementary Fig. S6. The SALL4 - integrin α6β1 network augments migration through modulation of Rho activity.

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

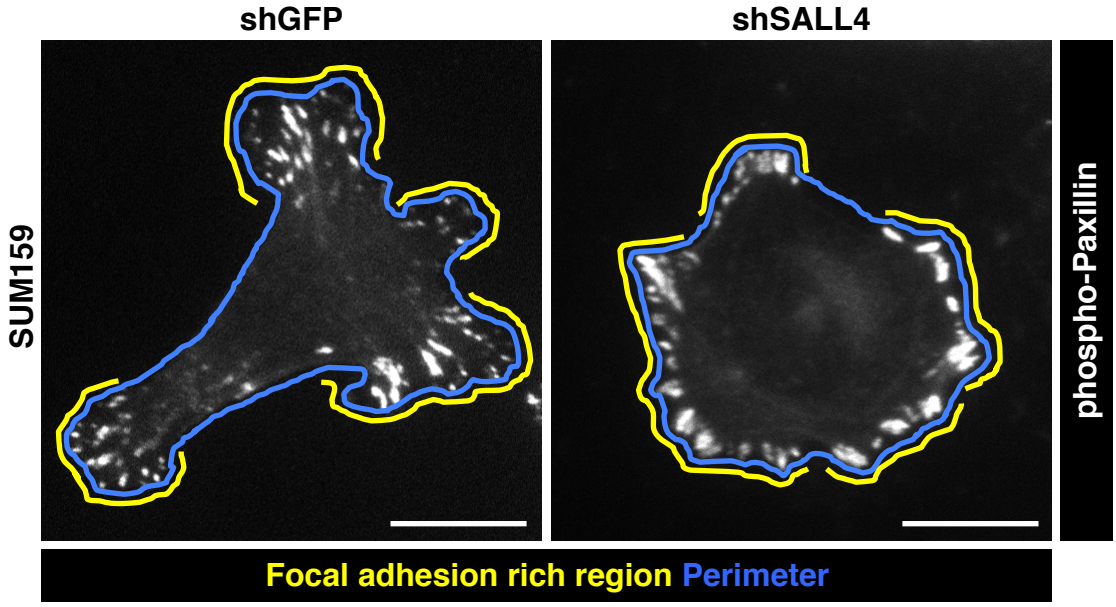
Supplementary Fig. S8. Co-expression of integrin α6 and β1 genes causes poor metastasis-free survival in some cases.

Supplementary Table S1. List of shRNA sequences

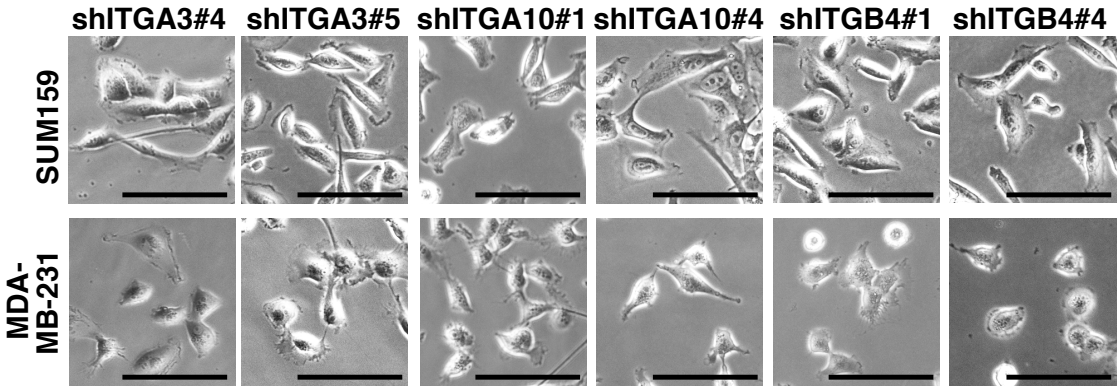
Supplementary Table S2. Primer sequences for qRT-PCR



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.

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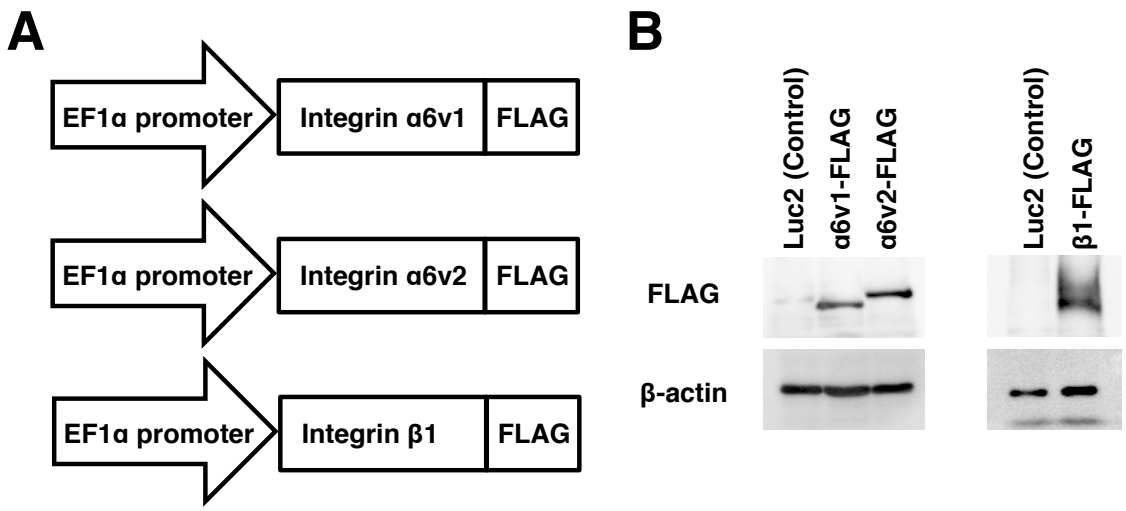
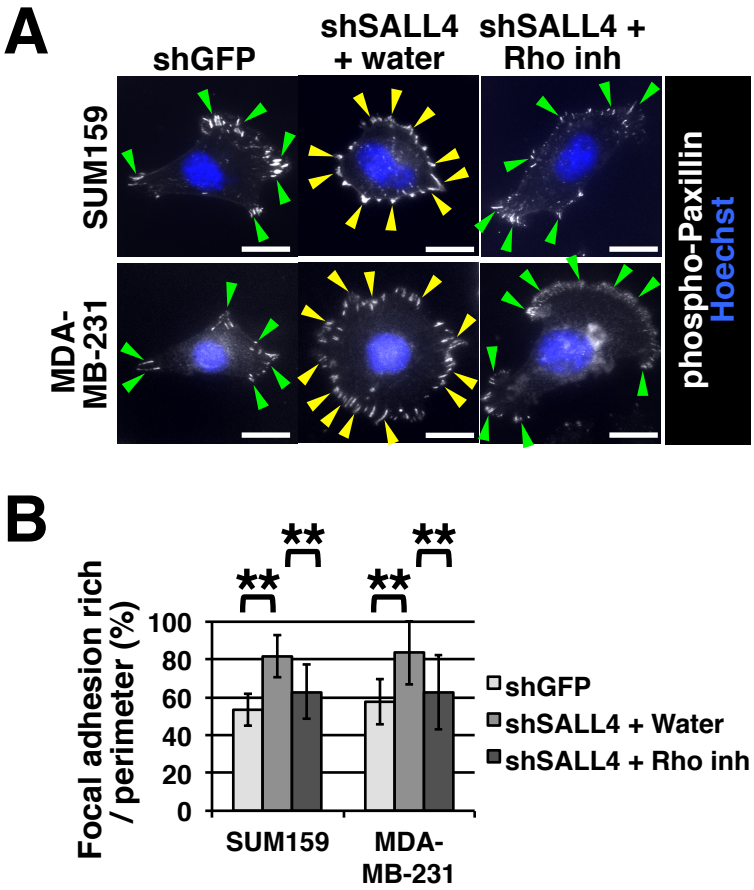
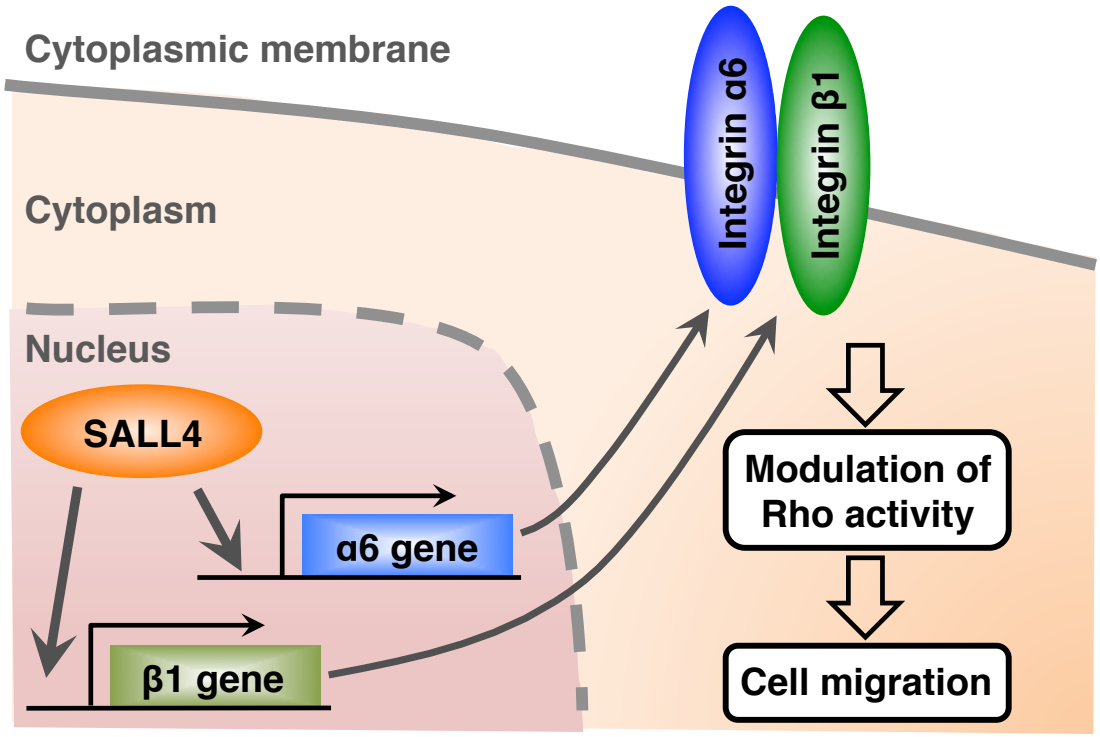


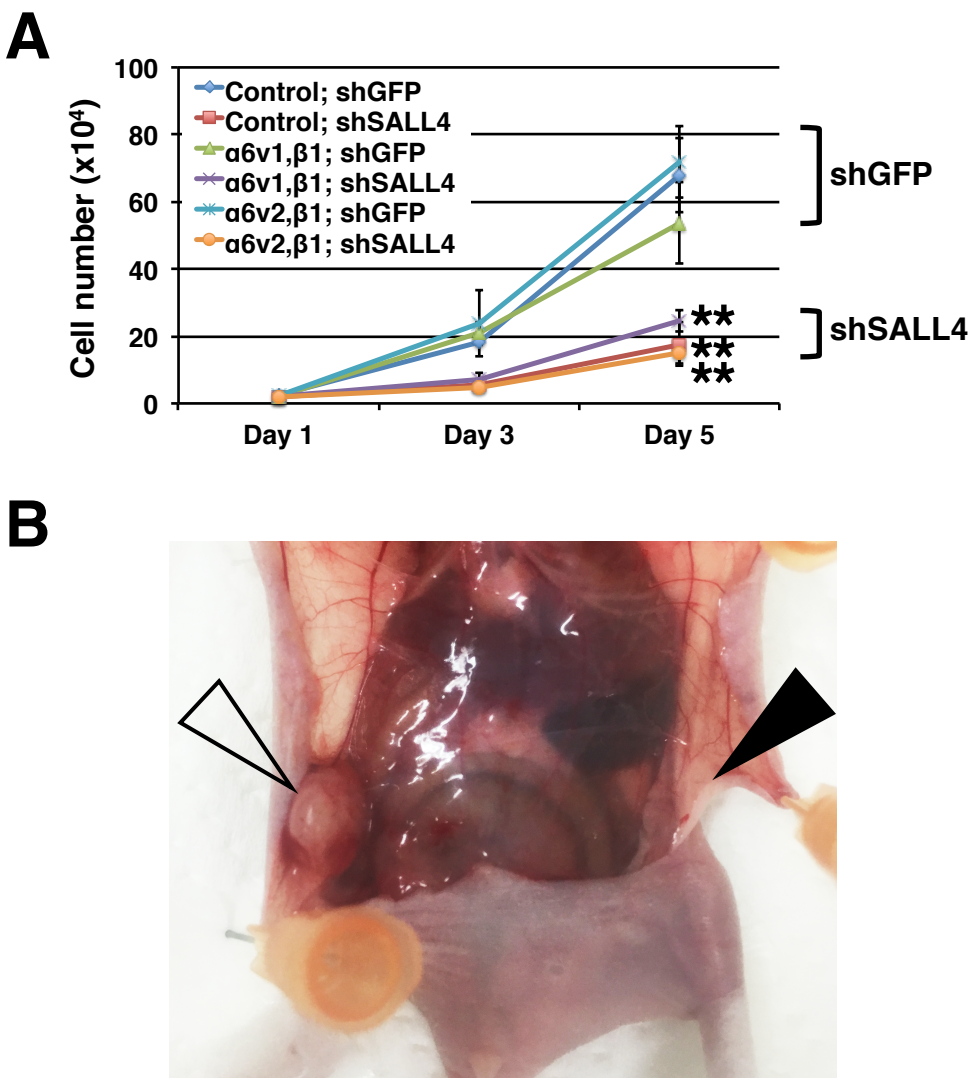
1035 **Supplementary Fig. S4.** Integrin α6 and β1 overexpression constructs were introduced
1036 into MDA-MB-231 cells. (A) Constructs for overexpressing α6v1, α6v2 and β1 are
1037 depicted. (B) Immunoblotting was performed to confirm expression from the constructs.
1038 An anti-FLAG antibody was used.
1039



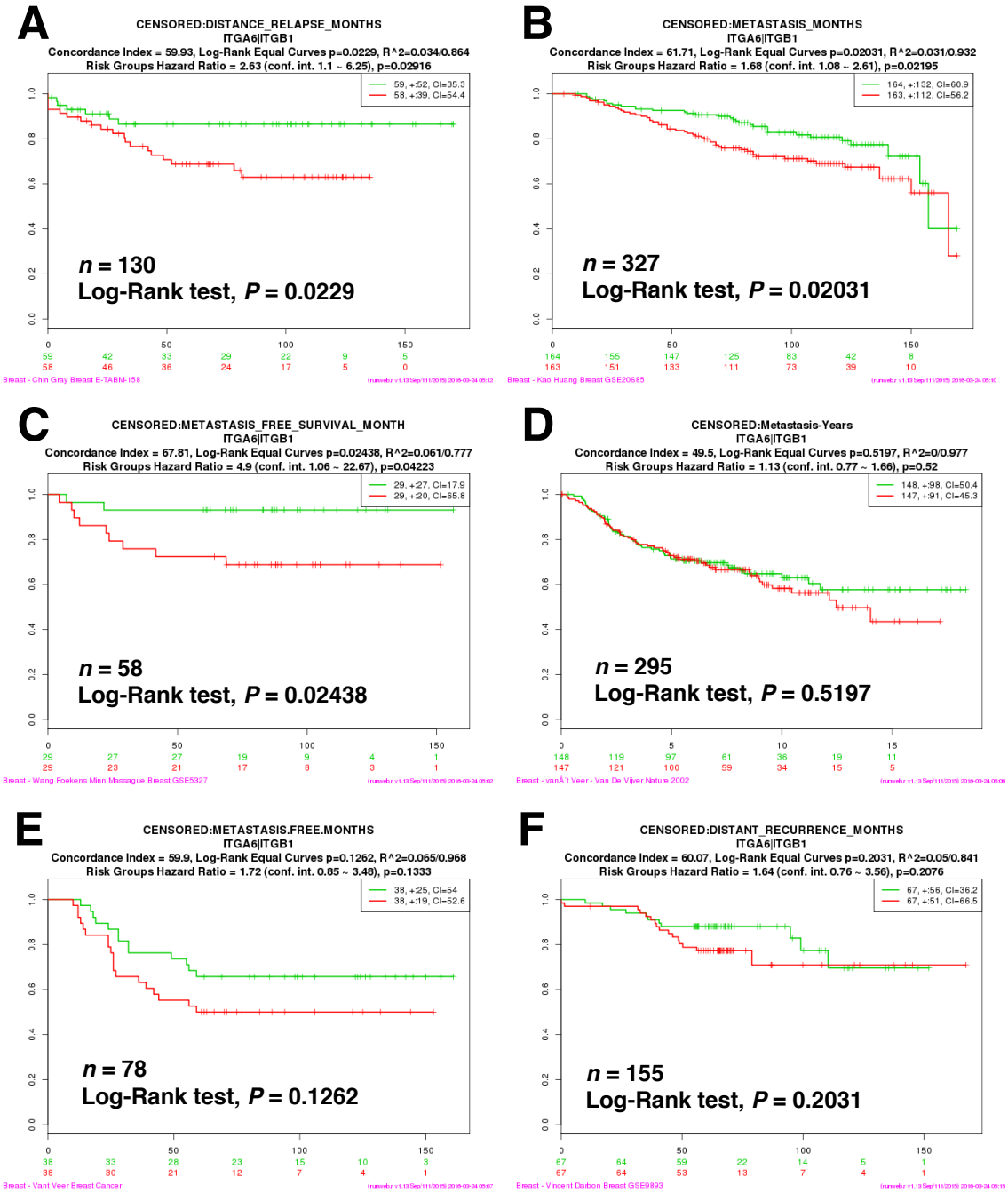
Supplementary Fig. S5. Rho inhibition reverses cell morphology of the SALL4 knockdown cells. (A) Images show immunostaining with an anti-phosphorylated paxillin antibody. A Rho inhibitor, C3 transferase, was used. Arrowheads indicate the signals. For simplicity, not all signals are pointed. (B) The FA-rich 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, α6v1,β1, and α6v2,β1 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).

1155 **Supplementary Table S1. List of shRNA sequences**

Name	Target sequence (5' → 3')
shGFP	GCACGACTTCTTCAAGTCCGC
shScr	CCTAAGGTTAAGTCGCCCTCG
shSALL4	GTGAGGATGAAGCCACAGTAA
shITGA3#4	CCAGGATGGATTTCAGGATAT
shITGA3#5	CATCGAGGATTACAGAGACTT
shITGA6#2	CGAGAAGGAAATCAAGACAAA
shITGA6#3	CGGATCGAGTTTGATAACGAT
shITGA10#1	CCTGAGAGAAATTAGAACTAT
shITGA10#4	CGGCTAAAGGATGGGATTCTT
shITGB1#2	CCTTGCATTACTGCTGATAT
shITGB1#3	GCACGATGTGATGATTTAGAA
shITGB4#1	CTCCTCAGCTACTCCATCCTT
shITGB4#4	GAGGGTGTCATCACCATTGAA

1156

1157

1158 **Supplementary Table S2. Primer sequences for qRT-PCR**

Name	Direction	Sequence (5' → 3')
ITGA1_F	Forward	CAGGTCATTATCTACAGGATGGAAG
ITGA1_R	Reverse	AGAATCCTTGTCAATGTCAGTTG
ITGA2_F	Forward	CAGAATTTGGAACGGGACTTTCG
ITGA2_R	Reverse	CTCAGGGTTATAGGTGTTGATTTC
ITGA2B_F	Forward	CGGTGCTGGCCTTCCTGTGG
ITGA2B_R	Reverse	GGAGGACACGTTGAACCATGCG
ITGA3_F	Forward	CTGATCATCCTCCTGCTGTGG
ITGA3_R	Reverse	GCCTTCTGCCTCTTAGCTTCATAC
ITGA4_F	Forward	CAATAGTATGGCTCCCAATGTTAG
ITGA4_R	Reverse	AGTGGCATTCTCCAGTAGTAG
ITGA5_F	Forward	TGCACCAACAAGAGAGCCAAAG
ITGA5_R	Reverse	CTCACACTGCAGGCTAAATGG
ITGA6_F	Forward	GGACAGCAAGGCGTCTCTTATT
ITGA6_R	Reverse	CGGCAGCAGCAGTCACATCAA
ITGA7_F	Forward	CTCTGGAACAGCACCTTTCTGG
ITGA7_R	Reverse	CTCGGAGCATCAAGTTCTTTATGG
ITGA8_F	Forward	ACTCCCAGAAGGAAGCATAGTA
ITGA8_R	Reverse	TGGCGAGAACCAACAATCCAAG
ITGA9_F	Forward	CTCATGGGAACCCAGAAGAGG
ITGA9_R	Reverse	GAGGATTCCCACCAACAAACTG
ITGA10_F	Forward	GAGCTGGGAACCGAAGAGGG
ITGA10_R	Reverse	CCACAGGGAGATGAGGATAG
ITGA11_F	Forward	GCCCCTTCATCTTCCGTGAGGAG
ITGA11_R	Reverse	GGTGCTGCCTACAATGATCC
ITGAD_F	Forward	CCCTGTGTTTCAGAGAGAAAACC
ITGAD_R	Reverse	GCAGTCAGCAATGGAGCAGTC
ITGAE_F	Forward	GCAGAGAACCACAGAACTAAG
ITGAE_R	Reverse	CTTGAACAGGATGACCAGAATCACG
ITGAL_F	Forward	CTCCACACTCTATGTCAGTTTC
ITGAL_R	Reverse	GTGGGTATGTTGTGGTCGTGGATG

ITGAM_F	Forward	TTCAATGCTACCCTCAAAGGC
ITGAM_R	Reverse	GAACACGGAATCGTTAAACAAG
ITGAV_F	Forward	GAGATTAGACAGAGGAAAGAGTG
ITGAV_R	Reverse	AAGCAGACGACTTCAGAGAATAG
ITGAX_F	Forward	GCAGAATCAACCACCTCATCTTCCG
ITGAX_R	Reverse	CTCACATTGGCTGTCAGAAG
ITGB1_F	Forward	CTTGTCCAGAAACTGAGTGAA
ITGB1_R	Reverse	GCTGACTTAGGGATCAAGTTT
ITGB2_F	Forward	GACCGCTACCTCATCTATGTGG
ITGB2_R	Reverse	GAATGCCGATCAGCACGATG
ITGB3_F	Forward	TCCATCCTGTATGTGGTAGAAG
ITGB3_R	Reverse	CAAGGCCAATGAGCAGAATG
ITGB4_F	Forward	AGGGCATCATCACCATAGAG
ITGB4_R	Reverse	GATGCTGCTGTACTCGCTTTG
ITGB5_F	Forward	GGGGAGATGTGTGAGAAGTGC
ITGB5_R	Reverse	GGTCTGGTTGTCAGGTTTCCC
ITGB6_F	Forward	ACCATCATTCACAGCATCAA
ITGB6_R	Reverse	GAGAAGAATAGCCAGGGAAAC
ITGB7_F	Forward	GTCGTGCTCAGAGTGAGACCC
ITGB7_R	Reverse	AGCCGGTAAGCCAGGACCAG
ITGB8_F	Forward	TCTCATGGAACAACAGCATTATG
ITGB8_R	Reverse	GACTTTAAGCAACCCAATCAAG