

An Actin-Binding Protein Girdin Regulates the Motility of Breast Cancer Cells

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Abstract

Girdin (girders of actin filaments) is a novel actin-binding Akt substrate that plays an important role in actin organization and Akt-dependent cell motility in fibroblasts. Here, we find that Girdin is expressed in a variety of cancer cell lines, including the breast cancer cell line MDA-MB-231, and is phosphorylated by the stimulation of insulin-like growth factor (IGF-I). *In vitro* migration and invasion assays showed that Girdin is required for the IGF-I-dependent cell movement of MDA-MB-231 cells. Short hairpin interfering RNA directed against Girdin markedly inhibited the metastasis of s.c. transplanted MDA-MB-231 cells in nude mice. In addition, Girdin is highly expressed in a variety of human malignant tissues, including breast, colon, lung, and uterine cervical carcinomas. These findings highlight the important role of Girdin in tumor progression in which the Akt signaling pathway is aberrantly activated. [Cancer Res 2008;68(5):1310–8]

Introduction

Alterations in intracellular signaling pathways result in increased survival, proliferation, and motility of cancer cells. As with many other cellular processes, the molecular components involved in cancer progression are being identified, but the genetic and biochemical determinants of cancer progression require further delineation. The phosphatidylinositol 3-kinase/Akt serine/threonine kinase system regulates a variety of cellular processes through the phosphorylation of a great number of downstream substrates and is now recognized as an important signaling pathway for cancer invasion and metastasis (1). A large number of studies have identified increased Akt activity and expression and even its somatic mutation in breast, prostate, ovarian, and pancreatic tumors (1–6). Akt signaling contributes to tumor growth and metastasis by activating or promoting the expression of nuclear factor- κ B (7), mammalian target of rapamycin (8), murine double minute clone 2, bcl-xL/bcl-2-associated death promoter homologue (9–11), and matrix metalloproteinases (12). In addition, Akt facilitates the growth of tumor cells through suppression or inactivation of tuberous sclerosis complex 2, p27^{Kip1}, and the FOXO subfamily of forkhead transcription factors (13–15). The role of the Akt kinase in cancer progression has been also shown *in vivo*

(16–18). In accordance with these findings, Akt1 deficiency was found to be sufficient for inhibition of tumor development that was induced by deficiency of the Pten tumor suppressor (19). It is of note that patients with tumors expressing high-protein levels of Akt develop progressive diseases with poor clinical outcome (1, 20, 21).

Although it is generally accepted that Akt facilitates the metastasis of human malignant tumors (5, 22, 23), relatively little information has been available on the role of Akt in cell migration, a key process directly linked to cancer invasion and metastasis. The Akt-binding protein Girdin (girders of actin filament), also known as Akt phosphorylation enhancer, G α -interacting vesicle-associated protein, and Hook-related protein 1, has been designated coiled-coil domain containing 88a (Ccdc88a) by the HUGO Gene Nomenclature Committee (24–28). Girdin/Ccdc88a (hereafter called Girdin) is a novel actin-binding protein expressed ubiquitously in mammalian tissues. It is a large 1870-amino acid protein with a molecular mass of 220 to 250 kDa. The protein has a predicted long coiled-coil domain and binds actin filaments via the COOH terminal domain (28). Girdin can form dimers through the NH₂ terminal domain, indicating that it may function as an actin-cross-linking protein. Akt phosphorylates Girdin at serine 1416 both *in vitro* and in fibroblasts. This phosphorylation seems to occur at the cortical actin structure (lamellipodia) at the leading edges of migrating cells, thus promoting the migration of fibroblasts (24). Considering that cancer cells often have mutations that lead to aberrant Akt activation, we speculate that Girdin may play a role in the migration, invasion, and concomitant metastasis of cancer cells.

In this report, we show that Girdin is expressed and phosphorylated under the stimulation of insulin-like growth factor (IGF-I) in the human breast cancer cell line MDA-MB-231 and that it plays an important role in IGF-I-dependent cell movement. Down-regulation of Girdin by short hairpin interfering RNA (shRNA) markedly inhibited the metastasis of s.c. transplanted tumors in nude mice. Furthermore, examination of human malignant tissues revealed that Girdin is highly expressed in a portion of many types of human cancers, including colon, breast, and cervical carcinomas. These studies suggest that Girdin plays an important role in the invasion of tumor cells under aberrant Akt activation.

Materials and Methods

Antibodies. Rabbit anti-Girdin and anti-phosphorylated Girdin polyclonal antibodies were generated as described previously (24). Other antibodies used in this study include anti-Akt polyclonal antibody (Cell Signaling Technology), anti-phosphorylated Akt polyclonal and monoclonal antibodies (Cell Signaling Technology), horseradish peroxidase-conjugated anti-rabbit/mouse IgG (Dako), and Alexa Fluor 488/594-conjugated goat anti-rabbit IgG (Invitrogen).

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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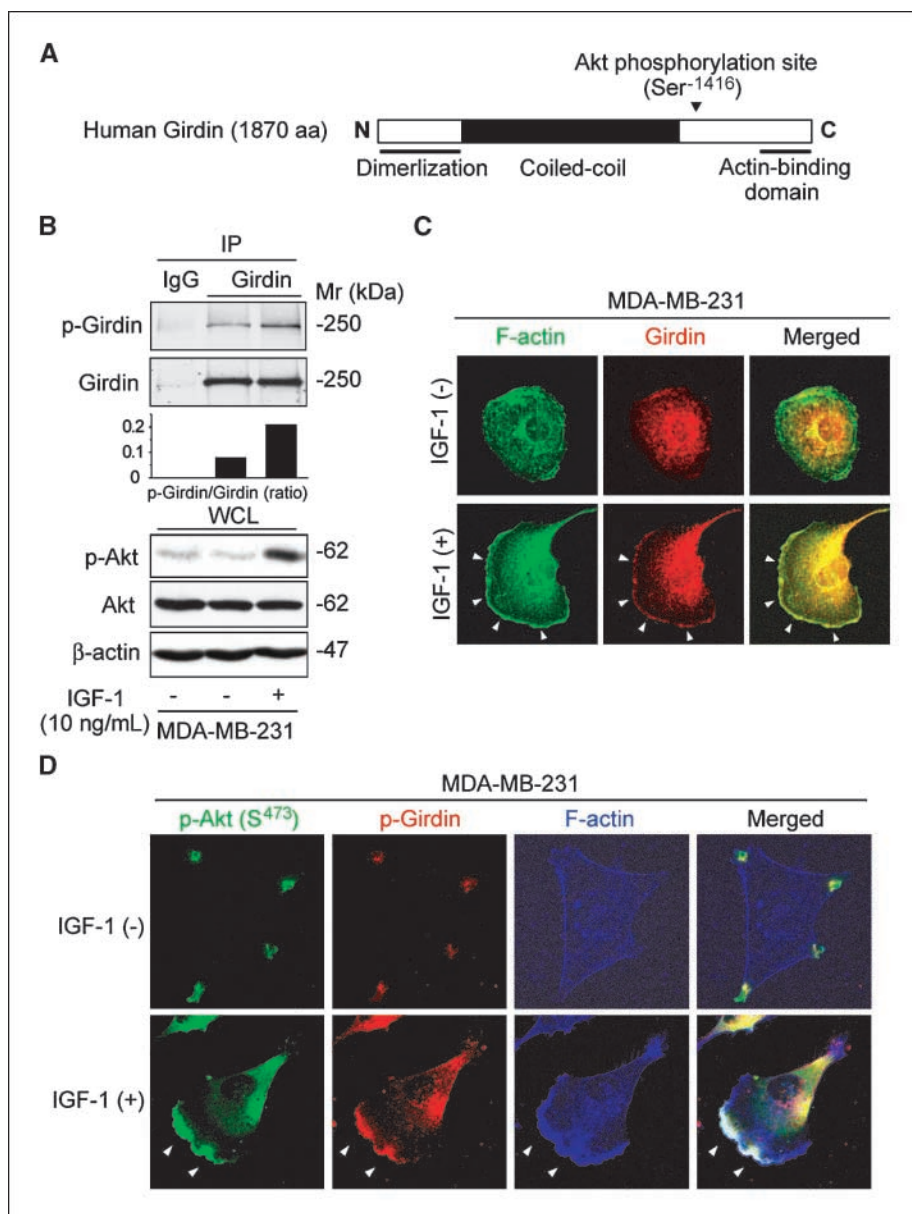
Cells. The human mammary carcinoma cell line MDA-MB-231 was maintained in RPMI with 10% fetal bovine serum (FBS). COS7, human HeLa uterine carcinoma, and human A431 squamous cell carcinoma cell lines were cultured in DMEM supplemented with 10% FBS.

Immunoprecipitation and Western blot analyses. MDA-MB-231 cells were lysed in lysis buffer [1% Triton-X, 20 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, and 0.5 mmol/L sodium orthovanadate] supplemented with Complete protease inhibitor and PhosSTOP phosphatase inhibitor cocktails (Roche Diagnostics Ltd.). Lysate samples from the cells were incubated for 16 h with 10 to 15 μ g of rabbit anti-Girdin antibody bound to 25 μ L of immobilized protein A beads (Sigma). Beads were washed five times with cold lysis buffer and suspended in Laemmli SDS sample dilution buffer [125 mmol/L Tris-HCl (pH 6.8), 2% SDS, 10 μ g/mL bromophenol blue, 80 mmol/L DTT]. In Western blot analysis, the samples were separated by SDS-PAGE. Proteins were transferred to nitrocellulose membranes, blocked in 3% skim milk in PBS and 0.05% Tween 20, incubated with primary antibodies, and detected with horseradish peroxidase-conjugated secondary antibodies (Dako).

RNA interference. The small interfering RNA (siRNA)-mediated depletion (knockdown) of Girdin was performed as previously described

(24). The 21-nucleotide synthetic duplexes were prepared by Qiagen. Cells were transfected with Girdin siRNA or a 21-nucleotide control RNA (Qiagen) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. For shRNA-mediated knockdown of Girdin, a set of single-stranded oligonucleotides encoding the Girdin target shRNA and its complement was synthesized (only the sense sequence is shown): 5'-GAAGGAGAGGCAACTGGAT-3' (nucleotides 4166–4184). The oligonucleotide was directed against a sequence, selected and provided by Dragon Genomics, that is common to both human, mouse, and rat Girdin sequences. The oligonucleotide pair was annealed, inserted into pcPURU6 β (Dragon Genomics), or the pNAMA retroviral shRNA expression vector (29). To produce retroviral supernatants, GP293 packaging cells were transfected with 24 μ g of control or Girdin shRNA-containing pNAMA vectors, 2 μ g of pVSVG, and 60 μ L of Lipofectamine 2000 reagent (Invitrogen) in 100-mm cell culture dishes in Opti-MEM medium (Invitrogen) without FBS and antibiotics. The medium was replaced 24 h later, and virus-containing supernatants were harvested 48 h post-transfection. To infect cells with retrovirus plasmids, 1×10^5 cells were mixed with 3 mL of virus-containing supernatant and seeded in 100-mm cell culture dishes. The supernatant was replaced after a 24-h incubation.

Figure 1. Expression of Girdin and its phosphorylation in breast cancer cell line MDA-MB-231. **A**, proposed primary structure of the human Girdin protein. The protein possesses an N-terminal domain through which a dimer can be formed. A large coiled-coil domain is located in the central portion followed by a COOH terminal domain that binds actin filaments. Akt phosphorylates the serine at position 1416. **B**, phosphorylation of Girdin in MDA-MB-231 cells in response to IGF-I. MDA-MB-231 cell lysates stimulated with IGF-I (10 ng/mL) for 10 min were incubated with control IgG and anti-Girdin antibody. The immunoprecipitates (IP) were analyzed by Western blot analysis using anti-Girdin and anti-phosphorylated Girdin (*p-Girdin*) antibodies. The graph shows densitometry quantification for the levels of phosphorylated Girdin using the NIH image software. The ratio of the densitometry measurement of phosphorylated Girdin to that of total Girdin. **WCL**, whole cell lysate. **C**, subcellular localization of Girdin in MDA-MB-231 cells. Quiescent cells (*top*) and IGF-I-stimulated cells (*bottom*) were fixed, followed by immunofluorescent staining using Alexa 488-conjugated phalloidin and anti-Girdin antibody. **Arrowheads**, colocalization of Girdin with actin filaments at the leading edge of migrating cells. **D**, colocalization of phosphorylated Girdin, phosphorylated Akt, and filamentous actin (*F-actin*) at the leading edge of migrating MDA-MB-231 cells. Quiescent cells (*top*) and migrating cells stimulated with IGF-I (*bottom*) were fixed, followed by immunofluorescent staining using pAkt monoclonal antibody (*green*), phosphorylated Girdin polyclonal antibody (*red*), and Alexa 350-conjugated phalloidin (*blue*). **Arrowheads**, colocalization of phosphorylated Girdin with active Akt at the lamellipodia of the leading edge.



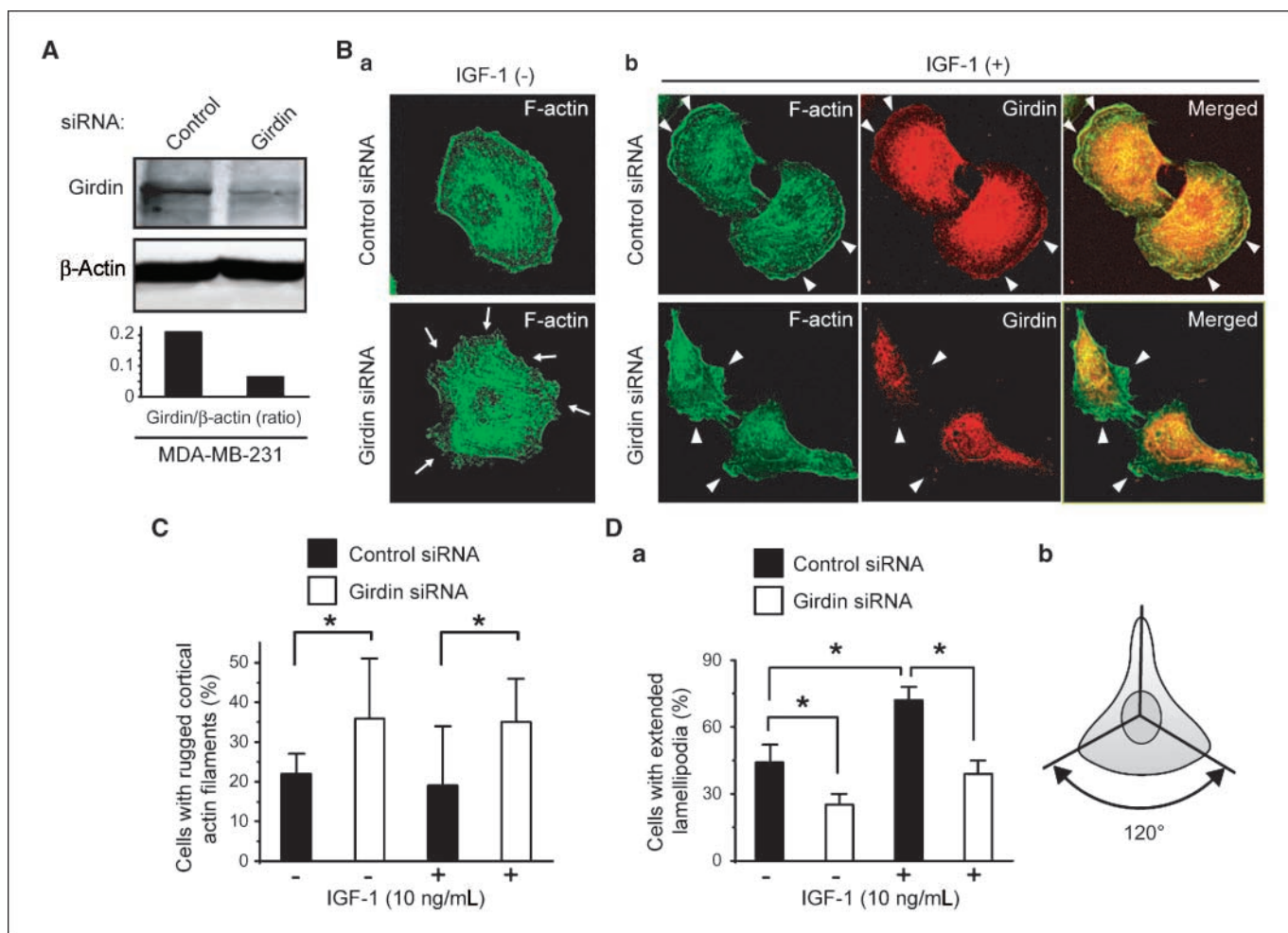


Figure 2. Girdin is required for the formation of extended lamellipodia in IGF-I-stimulated MDA-MB-231 cells. **A**, MDA-MB-231 cells were transfected with control or Girdin siRNAs. The cells were incubated for 48 h, and the cell lysates were analyzed by Western blot analyses using anti-Girdin (top) and anti- β -actin (bottom) antibodies. The graph shows densitometry quantification for Girdin expression using the NIH image software. Ratio of the densitometry measurement to that of β -actin. **B-D**, the effect of Girdin depletion on actin reorganization in breast cancer cells. MDA-MB-231 cells transfected with control (top) and Girdin (bottom) siRNAs were incubated for 30 min in the absence (a) or presence (b) of IGF-I (10 ng/mL), fixed, and stained by Alexa 488-conjugated phalloidin and anti-Girdin antibody. Arrows signify the ruffled cortical actin filaments in Girdin siRNA-transfected cells. Arrowheads, localization of Girdin at the leading edge of IGF-I-stimulated cells, which do not appear in Girdin siRNA-transfected cells. **C**, the percentage of cells with ruffled cortical actin filaments was quantitatively analyzed. Columns, means; bars, SD. Asterisks, statistical significance (Student's *t* test; $P < 0.05$). **D**, a, the percentage of cells with extended lamellipodia was quantitatively analyzed. Columns, means; bars, SD. Asterisks, statistical significance (Student's *t* test; $P < 0.05$). b, schematic illustration of the scoring principle: cells with lamellipodia formation along more than one third of the cell periphery (large lamellipodia) were counted. Five hundred cells in each group were counted to quantify the percentage of cells with morphologic changes. All cells in each field were counted.

Clones in which the expression of Girdin was effectively suppressed were selected and used for further study.

In vitro cell migration assay. MDA-MB-231 cells seeded in 24-well plates were cotransfected with either control or Girdin siRNA (20 nmol) along with pEGFP N1 (Clontech) as a fill (0.8 μ g; to identify transfected cells), and cell migration assays were performed using Transwell plates (pore size of 8 μ m; HTS FluoroBlok Insert, Becton Dickinson). The undersurface of the membrane was coated with 10 μ g/mL collagen (Upstate) diluted in PBS at 37°C overnight. Tumor cells were seeded in the upper chamber (2.5×10^4 per well) in 200 μ L of RPMI with 0.1% bovine serum albumin. RPMI supplemented with IGF-I (10 ng/mL) was added into the lower chamber. Cells were allowed to migrate for 6 h, after which nonmigrated or migrated EGFP-positive cells were counted using a confocal laser-scanning microscope (Fluoview FV500, Olympus Optical Co.). The experiment was performed thrice with each sample in triplicate, and cell counting was performed under high-power magnification in five randomly selected fields. The percentage of migrated cells ranged from 15% to 50%. The ratio of migrated cells to total (migrated + nonmigrated) cells was

calculated. At least 500 EGFP-positive cells were counted in each group of each experiment. To remove intersubject variability, results were normalized and expressed as a migration index, in which the lowest ratio in each experiment was designated as one.

In vitro invasion assay. MDA-MB-231 cells (3×10^4) seeded in 24-well plates were cotransfected with either control or Girdin siRNA and pEGFP N1 and loaded in Matrigel invasion chambers (24 wells; BD Biocoat Matrigel invasion chamber, BD Biosciences) according to the manufacturer's instructions. Cells were allowed to invade for 40 h before invasive EGFP-positive cells were counted with a confocal laser-scanning microscope. The experiment was performed thrice with each sample in triplicate. The percentage of invaded cells ranged from 5% to 20%. The ratio of invaded MDA-MB-231 cells to total (invaded + noninvaded) cells was calculated. At least 500 EGFP-positive cells were counted in each experiment. The results were normalized and expressed as an invasion index, in which the lowest ratio in each experiment was designated as 1.

Wound healing assay. Directional cell migration of MDA-MB-231 cells was stimulated in a monolayer using an *in vitro* scratch-wound assay (24).

Cells were seeded on fibronectin-precoated 35-mm glass base dishes and transfected with the indicated siRNAs. Forty-eight hours after transfection, the confluent cells were scratched with a 200- μ L disposable plastic pipette tip and allowed to migrate toward the wound. Images were taken with an Olympus inverted microscope coupled to a CCD-SPOT digital camera (Olympus DP11).

Rescue experiments. To perform rescue experiments, the V5 epitope-tagged siRNA-resistant (siRNA-r) versions of Girdin harboring silent mutations were constructed as described previously (24). MDA-MB-231 cells were cotransfected with GFP (0.5 μ g; as a transfection marker), siRNAs (20 pmol), and siRNA-r Girdin constructs (2.5 μ g), incubated for 48 h, and subjected to Matrigel invasion assays. Cell motility was quantified using a fluorescence microscope to count the GFP-positive cells that invaded through the Matrigel. To validate the resistance of siRNA-r Girdin to Girdin siRNA, COS7 cells were cotransfected with expression constructs for siRNA-r Girdin and siRNAs, and total cell lysates were subjected to Western blot analyses with anti-V5 antibody.

Immunofluorescent staining. Cells were plated on collagen I-coated glass base dishes, fixed, and stained with the indicated antibodies. Fluorescence was examined using a confocal laser-scanning microscope (Fluoview FV500, Olympus).

Immunohistochemistry. Tissue arrays of human malignant tumors and matched normal adjacent tissues were purchased from Folio Biosciences, Inc. Immunohistochemistry was performed using standard techniques. Antigen retrieval was performed with microwave treatment in a 0.01 mol/L citrate buffer (pH 6.0) at 95°C for 10 min. For fluorescence staining of the breast carcinoma tissue array, the section was stained with anti-Girdin antibody, followed with Alexa 594-conjugated secondary antibody. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; Invitrogen) and observed with a confocal laser-scanning microscope.

Quantification of immunohistochemistry. We quantified the intensity of Girdin immunostaining using the Win ROOF software (Mitani Corporation) and compared the expression levels of Girdin in cancer cells

with those in normal epithelial cells. For each staining, individual cells were traced, and the mean intensity of at least 100 cancer cells was measured and compared with that of normal epithelial cells. The expression of Girdin was defined as low (L; relative intensity, 1–2), moderate (M; relative intensity, 2–3), or high (H; relative intensity, ≥ 3).

Tumor metastasis assay in nude mice. Tumor cells (3×10^6) were s.c. injected into 7-week-old female nude mice. Over a period of 10 weeks, the nude mice were kept in sterilized, filtered cages in a 12-h light/dark cycle under standardized environmental conditions throughout the experiments. Lung metastasis of the tumor was analyzed after dissection at the end of the experiments. This study was approved by the Animal Care and Use Committee of Nagoya University Graduate School of Medicine.

Data analysis. Data are presented as the mean \pm SD. Statistical significance was evaluated with Student's *t* test.

Results

Girdin expression and IGF-I-dependent phosphorylation in a breast cancer cell line. Girdin is an actin-binding protein and Akt substrate (Fig. 1A; ref. 24). Western blotting analysis of Girdin expression in cell lines derived from human malignant tumors was used to define the role of Girdin in cancer progression. Girdin was widely expressed in a variety of cancer cell lines, including MDA-MB-231 (estrogen-independent breast carcinoma), A431 (skin squamous cell carcinoma), HeLa (uterine cervical carcinoma), HT-1080 (fibrosarcoma), and TGW (neuroblastoma; data not shown). Because MDA-MB-231 cells are highly invasive and metastasize extensively (30–32), this line was chosen for the study of cancer invasion and metastasis.

IGF-I is a potent mitogen for most breast cancer cell lines. To determine if Girdin is phosphorylated in response to IGF-I,

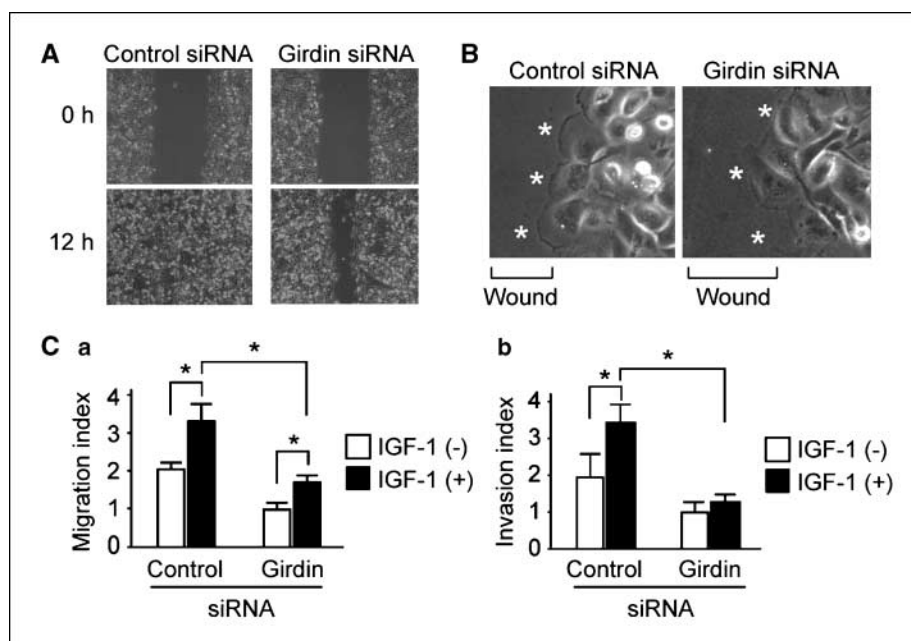


Figure 3. Girdin is required for directional migration and invasion of MDA-MB-231 cells *in vitro*. **A**, directional cell migration of MDA-MB-231 cells was stimulated in a monolayer using an *in vitro* scratch-wound assay. Cells were seeded on fibronectin-precoated 35-mm glass base dishes and transfected with the indicated siRNAs. Forty-eight hours after transfection, the confluent cells were scratched with a 200- μ L disposable plastic pipette tip and were allowed to migrate toward the wound for 12 h. Phase contrast images were taken with an inverted microscope. **B**, Girdin is required for the formation of extended lamellipodia at the leading edge. In wound healing assays described in **A**, images of migrating cells facing the wound were taken with an inverted microscope. Asterisks, lamellipodia of migrating cells. **C**, **a**, *in vitro* cell migration assays (Boyden chamber assays). MDA-MB-231 cells were transfected with either control or Girdin siRNAs, incubated for 48 h, and subjected to Boyden chamber assays with or without IGF-I (10 ng/mL) in the lower chamber. Columns, means; bars, SD. Asterisks, statistical significance (Student's *t* test; $P < 0.05$). **b**, *in vitro* Matrigel invasion assays. Control or Girdin siRNA-transfected cells were allowed to invade into Matrigel for 16 h with or without IGF-I (10 ng/mL). Columns, means; bars, SD. Asterisks, statistical significance (Student's *t* test; $P < 0.05$).

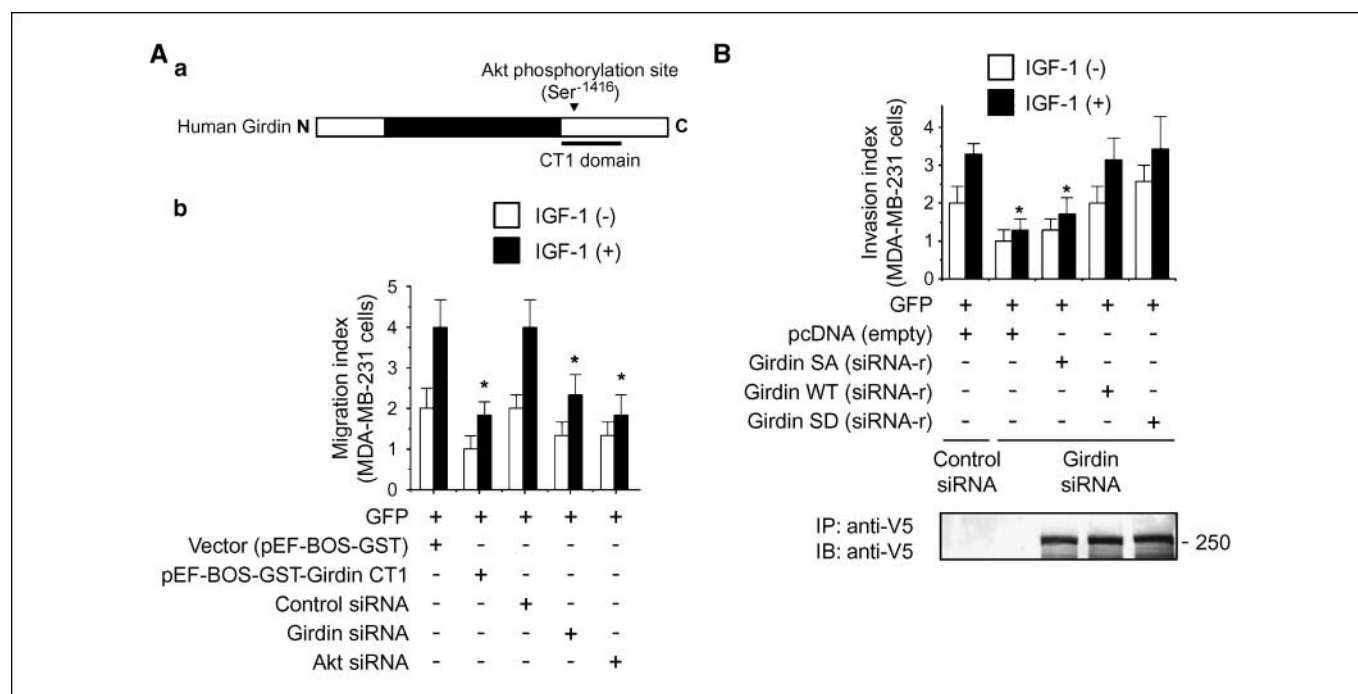


Figure 4. Akt-dependent phosphorylation of Girdin is required for the migration and invasion of MDA-MB-231 cells *in vitro*. **A**, inhibition of the interaction between Akt and Girdin impairs directional migration of MDA-MB-231 cells. **A**, **a**, the CT1 domain and Akt-phosphorylation site in Girdin. **b**, cells were cotransfected with GFP (1 μ g) as a fill (a transfection marker) and either indicated siRNAs (20 pmol) or constructs (2 μ g), incubated for 48 h, and subjected to Boyden chamber assays with or without IGF-I (10 ng/mL) in the lower chamber. *Columns*, means; *bars*, SD. *Asterisks*, statistical significance compared with control (GFP + vector; Student's *t* test, $P < 0.05$). **B**, rescue of the migratory defect in Girdin-depleted cells by the transfection of siRNA-resistant Girdin. MDA-MB-231 cells were cotransfected with GFP (1 μ g), siRNAs (20 pmol), and the indicated siRNA-r Girdin constructs (WT, SA, or SD; 2 μ g), incubated for 48 h, and subjected to *in vitro* invasion assays with or without IGF-I (10 ng/mL) in the lower chamber. *Columns*, means; *bars*, SD. *Asterisks*, statistical significance compared with control (GFP + pcDNA; Student's *t* test, $P < 0.05$). *Bottom*, validation of the resistance of siRNA-r Girdin to Girdin siRNA. The expression of siRNA-r Girdin WT, SA, and SD was analyzed using anti-V5 antibody.

MDA-MB-231 cells were serum-starved for 12 h and stimulated with IGF-I (10 ng/mL) for 10 min. The phosphorylation of Akt and Girdin was subsequently analyzed by Western blotting (Fig. 1B). The addition of IGF-I induced elevated phosphorylation of Girdin, as well as Akt phosphorylation (Fig. 1B).

The subcellular localization of Girdin was investigated using immunocytochemistry, which showed that Girdin was localized diffusely throughout the cytoplasm in quiescent cells (Fig. 1C, *top*). In contrast to our prior study of Girdin in Vero fibroblasts (24), the colocalization of Girdin with actin stress fibers was not apparent. The difference was presumably due to a lower expression level (approximately five times lower) of Girdin and more weakly organized actin fibers in MDA-MB-231 cells compared with Vero cells. In migrating MDA-MB-231 cells stimulated with IGF-I, Girdin seemed specifically in the peripheral actin structure called lamellipodia at the leading edges of migrating cells (Fig. 1C, *bottom*). Consistent with our previous data, the phosphorylated Girdin localized predominantly to the leading edge of migrating cells, where active Akt is also localized (Fig. 1D). These data suggest that Girdin may play an important role in cell motility by reorganizing the actin cytoskeleton at the leading edge of the breast cancer cells.

Girdin is essential for the formation of extended lamellipodia in an IGF-I-stimulated breast cancer cells. To confirm that Girdin is important for actin remodeling at the leading edge, endogenous Girdin expression was suppressed by introducing siRNA into the cells. The Girdin protein level was effectively suppressed by the transfection of Girdin siRNA but not by the control siRNA (Fig. 2A). Transfection of Girdin siRNA resulted in an

increase in the number of cells with rugged cortical actin filaments (Fig. 2B, *a* and *C*), suggesting that Girdin may be pivotal for the remodeling of peripheral actin filaments or cell extracellular matrix adhesions. In addition, when cells were stimulated with IGF-I, the wide extension of lamellipodia at the leading edges was significantly attenuated in Girdin siRNA-transfected cells (Fig. 2B, *b* and *D*), further suggesting a role for Girdin in actin dynamics at the peripheral lamellipodia in migrating cells. Immunostaining of Girdin siRNA-transfected cells showed that Girdin staining at the leading edge almost disappeared in the cells, although some residual staining remained in the cytoplasm (Fig. 2B, *b*) that was presumably due to a silencing effect of up to ~70% (Fig. 2A).

Girdin is required for directional migration and invasion of breast cancer cells *in vitro*. The involvement of Girdin in cell motility was initially examined using two-dimensional wound-healing assays (Fig. 3A and B). MDA-MB-231 cells were transfected with either control or Girdin siRNA, starved for 12 h, scratched with a sterile pipette tip, and incubated with serum for 12 h. The control siRNA-transfected cells were evenly distributed at the wound area after the 12-h incubation (Fig. 3A, *left*), whereas the Girdin siRNA-transfected cells exhibited delayed closure of the wound (Fig. 3A, *right*). Phase contrast images taken 2 h after the scratch showed extensive lamellipodia formation at the leading edge of the control cells facing the wound area, whereas the formation of lamellipodia in the Girdin siRNA-transfected cells was significantly inhibited (Fig. 3B).

The role of Girdin in three-dimensional directed cell migration was further investigated using Boyden chamber assays (Fig. 3C, *a*)

and Matrigel invasion assays (Fig. 3C,b). The migration and invasion of MDA-MB-231 cells increased in response to IGF-I stimulation in control siRNA-transfected cells. Compared with the control cells, Girdin siRNA-transfected cells displayed a significant reduction in cell migration and invasion in response to IGF-I ($P < 0.05$). The cell migratory role of Girdin was not specific to MDA-MB-231 cells: Girdin was expressed and phosphorylated in response to growth factors in HeLa and A431 cancer cells, and its depletion by siRNA also significantly attenuated their migration and invasion ability (Supplementary Figs. S1A-D).

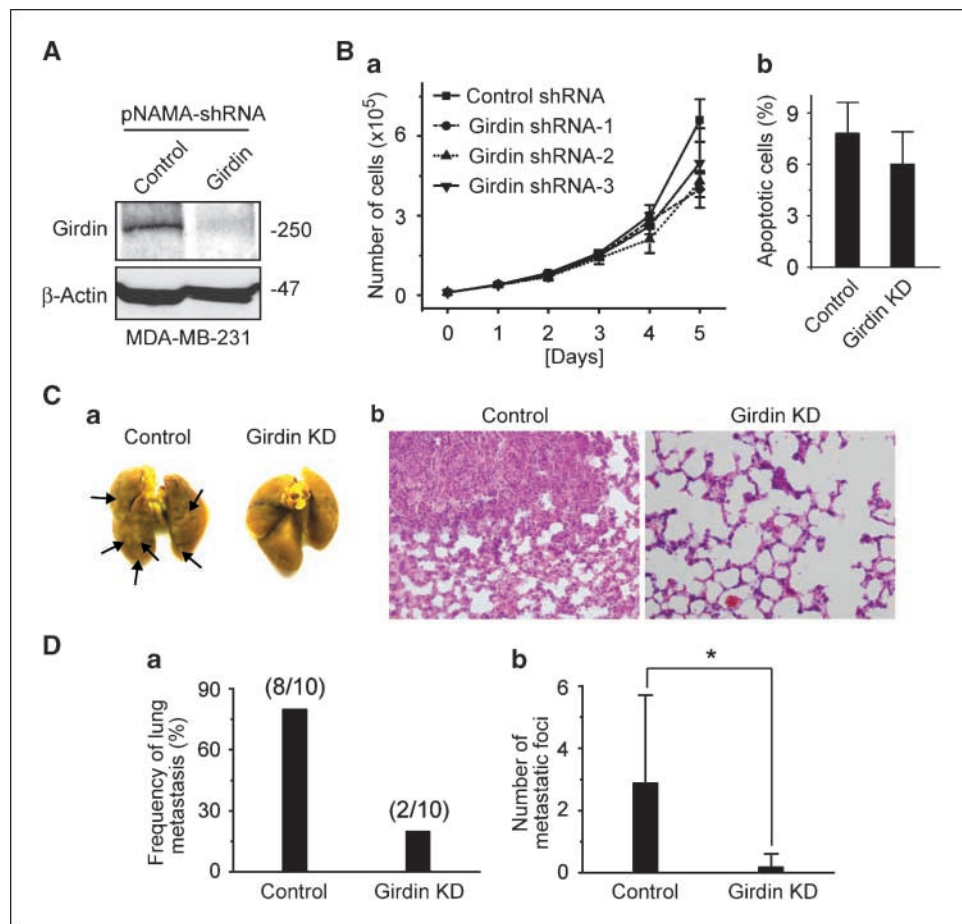
To examine the role of Akt-mediated phosphorylation of Girdin in the migration of MDA-MB-231 cells, we constructed the expression plasmid carrying the CT1 domain of Girdin which contains the Akt phosphorylation site (Fig. 4A,a). It was confirmed that the transfection of the CT1 domain inhibits the phosphorylation of endogenous Girdin in COS7 cells, indicating that exogenously expressed CT1 domain competes with endogenous Girdin for the association with Akt (Supplementary Fig. S2A). Migration assays using MDA-MB-231 cells showed that the transfection of the CT1 domain significantly impaired the cell motility (Fig. 4A,b). The most likely explanation for this result is that the exogenously expressed CT1 domain acts as an inhibitor of Akt-mediated Girdin phosphorylation, which plays a crucial role in the motility of MDA-MB-231 cells. The migration of MDA-MB-231 cells was also significantly inhibited by siRNA-mediated depletion of Akt (Fig. 4A,b) which decreased the levels of both Akt and Girdin phosphorylation (Supplementary Fig. S2B).

To further determine if Akt phosphorylation of Girdin is requisite for cell migration, a rescue experiment was performed using the Matrigel assays as described in Materials and Methods (Fig. 4B). siRNA-resistant versions of wild-type (termed siRNA-r WT) Girdin and its mutant (siRNA-r SA; ref. 24), where the Akt phosphorylation site (serine 1416) was mutated, were added to Girdin-depleted MDA-MB-231 cells. The cell invasion assay showed that transfection of siRNA-r Girdin WT significantly restored the invasive capacity of Girdin-depleted cells ($P < 0.05$; Fig. 4B). In contrast, the transfection of siRNA-r Girdin SA resulted in limited rescue of cell mobility (Fig. 4B). The results suggest that an adequate amount of Girdin and its phosphorylation are necessary for motility of MDA-MB-231 cells. In addition, we mutated the serine 1416 to aspartic acid (termed SD) to mimic its phosphorylated state. We found that the transfection of the siRNA-resistant SD mutant of Girdin (siRNA-r Girdin SD) could restore the defects in the invasive capacity of Girdin-depleted MDA-MB-231 cells even without the stimulation by IGF-I (Fig. 4B). These data support our hypothesis that Akt-mediated phosphorylation of Girdin has a role in the invasion of MDA-MB-231 cells.

Girdin suppression decreases the metastasis of tumor cells *in vivo*. The s.c. injection of MDA-MB-231 cells has been established as *in vivo* model system for the study of tumor metastasis (32, 33). Therefore, this model was used to examine the role of Girdin in migration and metastasis *in vivo*. MDA-MB-231 cells were infected with a retrovirus harboring either control or Girdin shRNAs, and several independent stable clones were

Figure 5. Girdin is important for the metastasis of tumors *in vivo*.

A, MDA-MB-231 cells were infected with either control or Girdin shRNA-harboring retrovirus, followed by the isolation of clones that stably expressed the shRNAs. Cell lysates were analyzed by Western blot analysis using anti-Girdin and anti- β -actin antibodies. B, a, the effect of Girdin depletion on the proliferation of MDA-MB-231 cells. Columns, means; bars, SD. Cell proliferation was weakly attenuated by the depletion of Girdin but no significant difference was observed. b, no difference in the number of apoptotic cells between control and Girdin-depleted cells. Apoptosis in MDA-MB-231 cells infected with control and Girdin shRNA-harboring retroviruses were analyzed by TUNEL staining. Columns, means; bars, SD. C, a, tumor metastasis was inhibited by the knockdown of Girdin. An equal number of control or Girdin-depleted MDA-MB-231 cells (3×10^6) were s.c. injected into nude mice. Ten weeks after the injection, lung metastatic tumors were grossly observed, followed by fixation. Arrows, metastatic tumor in the lung. b, paraffinized lung sections were stained with H&E. D, a, frequency of lung metastasis was analyzed quantitatively (10 mice for each group). Lung metastasis was observed in eight mice of the control group and two mice of the Girdin knockdown group. b, the number of metastatic lung tumors per section was counted. Columns, means; bars, SD ($n = 10$ for each group). Asterisk, statistical significance (Student's *t* test, $P < 0.05$).



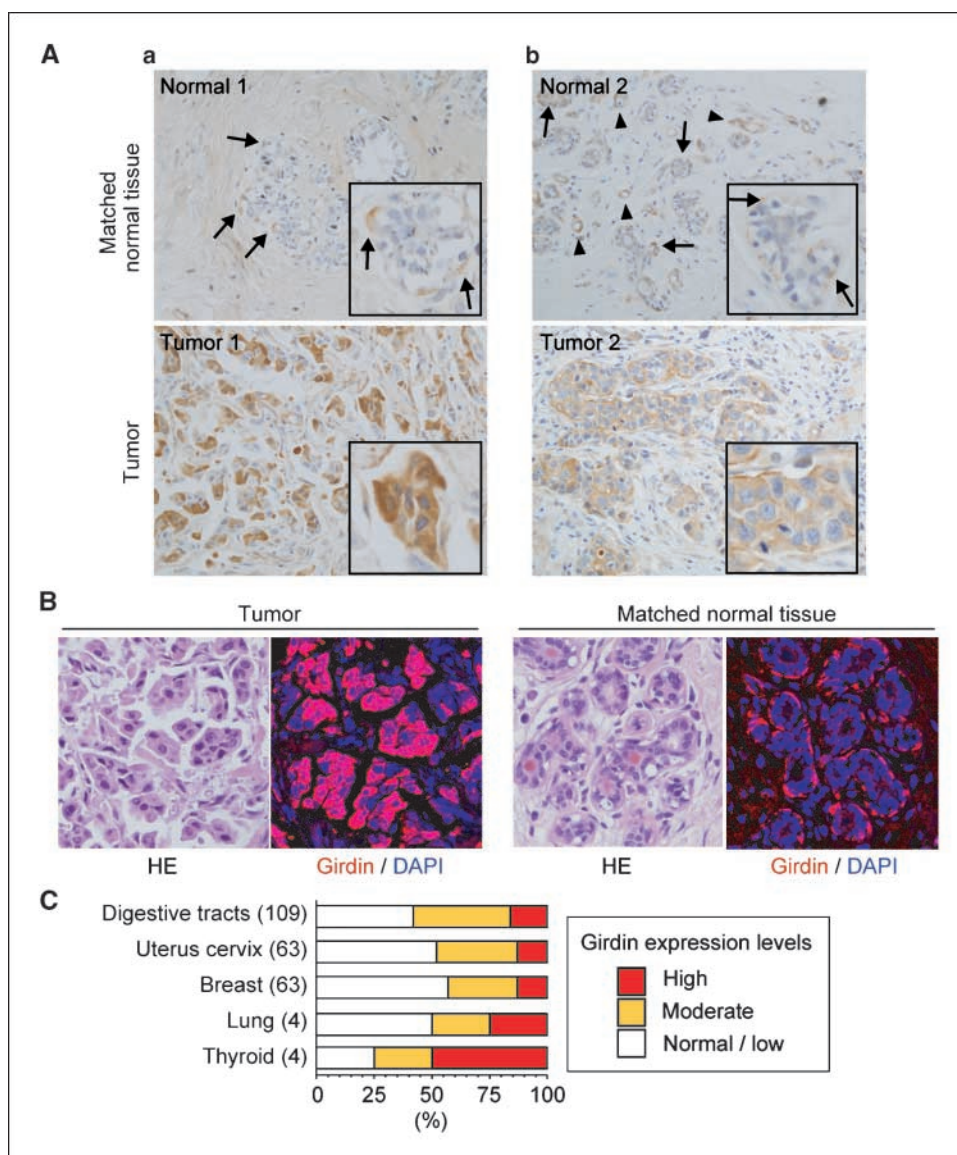


Figure 6. Girdin is highly expressed in some human breast carcinoma tissues. **A,** Girdin immunohistochemistry on representative examples of paired normal (*Normal 1* and *Normal 2*) and invasive ductal carcinoma (*Tumor 1* and *Tumor 2*) tissues from a breast cancer tissue array. Positive immunoreactive protein (*brown*) was found at high and moderate levels in the tumor cells from *Tumor 1* and *Tumor 2*, respectively. Note that Girdin is also moderately expressed in myoepithelial cells (*arrows*) and microvessels (*arrowheads*) in normal breast tissues, with no or low-level expression in normal luminal cells. **B,** Girdin immunofluorescence on representative examples of paired normal and breast carcinoma tissues. *Tumor (left)* and matched normal (*right*) samples were stained with anti-Girdin antibody followed by Alexa 594-conjugated secondary antibody. Nuclei were stained with DAPI. Girdin is expressed in tumor cells and normal myoepithelial cells. **C,** Girdin is expressed in some malignant tumors derived from various types of tissues. The scoring of Girdin expression levels in tumor tissues was determined as described in Materials and Methods and Supplementary Fig. S3. Tumor tissues with the same 3,3'-diaminobenzidine staining intensity compared with normal tissue were designated as normal, those with apparently higher than normal staining were designated as moderate, and those with much higher staining than normal were designated as high.

isolated. Girdin was effectively depleted in Girdin shRNA-introduced cells (Fig. 5A), and cell proliferation was weakly attenuated by Girdin depletion with no statistical significance (Fig. 5B,a). The decrease in cell number was not due to apoptosis as was shown by terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) staining (Fig. 5B,b). Because all of the independent Girdin-depleted clones showed the same phenotype *in vitro* (Fig. 5B,a), one clone was chosen for further *in vivo* tumor metastasis assays.

S.c. injection of MDA-MB-231 cells into immunocompromised nude mice resulted in metastasis into the lung which could be observed macroscopically 10 weeks after injection. The metastatic ability of the injected cells in nude mice was statistically assessed by histochemical analysis of the lung metastasis. Multiple metastatic tumors were observed in the lungs of mice injected with control cells but not in those of mice injected with Girdin-depleted cells (Fig. 5C,a). Small metastatic tumors were detected by staining lung sections with H&E (Fig. 5C,b), which showed that the frequency and number of lung metastasis were significantly attenuated by Girdin depletion (Fig. 5D,a and b).

Girdin is expressed in human breast carcinoma tissues.

Finally, immunohistochemical staining and scoring analyses (see Materials and Methods) were performed to ascertain if Girdin is expressed in human breast malignant tissues (Fig. 6 and Supplementary Fig. S3). The staining of breast carcinoma and matched normal adjacent tissue microarrays showed that Girdin is highly (Fig. 6A,a) and moderately (Fig. 6A,b) expressed in ~10% and 35% of invasive ductal carcinomas, respectively. We found that the apparent expression of Girdin is limited in the myoepithelial cells of normal mammary glands (Fig. 6A, *arrows* and B, *right*).

In addition, to the mammary tissues, a total number of 180 human cancer specimens, including carcinomas of the digestive tract, uterine cervix, lung, and thyroid, were analyzed (Fig. 6C and Supplementary Fig. S4A and B). High expression of Girdin was also detected in 10% to 50% of these carcinomas (Fig. 6C).

Discussion

We recently discovered Girdin as a novel actin-binding Akt substrate and found that it is pivotal for the directed cell migration

of fibroblasts (24). The present study was directed at understanding the potential role of Girdin in the behavior of malignant breast cancer cells. RNA interference-mediated depletion of Girdin attenuated actin reorganization, leading to a concomitant impairment of cancer cell migration and invasion *in vitro* and metastasis *in vivo*. In addition, high or moderate expression of Girdin was observed in some human malignant tumors, including breast carcinomas. These findings suggested that Girdin plays a crucial role in cancer invasion and metastasis.

Akt has been intensely studied for its roles in the processes of cancer progression. Kim et al. showed that Akt regulates invasion/metastasis of fibrosarcoma cells in a manner that is highly dependent on its kinase activity and membrane-translocating ability (12). Because then, increasing evidence has established that the Akt signaling pathway plays an important role in the motility of cancer cells. Therefore, Akt and its downstream substrates have become attractive targets for the development of therapies for malignant tumors. Our previous report revealed that Akt phosphorylates a serine at position 1416 in Girdin, both *in vitro* and in cells. In this report, we found that, in MDA-MB-231, A431, and HeLa human cancer cell lines, Girdin was phosphorylated under the stimulation of growth factors, which is required for directed cell migration.

To elucidate the role of Akt-mediated phosphorylation of Girdin in the motility of MDA-MB-231 cells, we performed a rescue experiment using the Matrigel invasion assay. When siRNA-resistant versions of wild-type Girdin and its SA or SD mutant (siRNA-r WT, siRNA-r SA, or siRNA-r SD) were added to Girdin-depleted MDA-MB-231 cells, siRNA-r WT, and siRNA-r SD, but not siRNA-r SA, could restore their invasive capacity. In addition, the transfection of the Girdin CT1 domain, which contains the Akt phosphorylation site, impaired the migration of MDA-MB-231 cells. These findings strongly suggested that Akt-mediated phosphorylation of Girdin increases the motility of cancer cells.

Phosphorylated Girdin colocalized with active Akt at the leading edge of the migrating MDA-MB-231 cells, suggesting that the phosphorylated form of the protein may regulate actin reorganization at the leading edge. However, the significance of Girdin phosphorylation on actin reorganization should be explored further.

A complex interaction among many proteins and signaling pathways contributes to cell migration, invasion, and metastasis.

Indeed, the involvement of Girdin in a multitude of cellular processes has been suggested by its binding proteins, which include actin filaments, Akt, heterotrimeric G proteins, and dynamin GTPase (24–27). Although the significance of these interactions has not been fully elucidated, Girdin may be involved in various cellular processes including signaling from G protein-coupled receptors or membrane trafficking, such as endocytosis and exocytosis. Several lines of evidence suggest the presence of endocytosis at the leading edge of migrating cells and its roles in promoting cell migration by participating in lamellipodia extension (34). Thus, much work will be required to arrive at a comprehensive picture of the function of Girdin at the interface of the membrane and cytoskeleton.

Previously, Anai et al. reported that the depletion of Girdin resulted in a reduction of DNA synthesis, leading to an increase in apoptosis (25). However, our current data suggest that Girdin is not involved in the induction of apoptosis in MDA-MB-231 cells. Considering that Girdin binds to actin filaments, it is possible that it is involved in a wide variety of cellular functions that lead to cell growth and cell division.

Collectively, this study shows that Girdin is highly expressed in some human malignant tumors. As an Akt substrate and an actin-binding protein, Girdin is an integral component for cell motility and may participate in Akt-mediated cancer progression. Further studies are required to delineate whether Girdin and/or any of its regulators represent novel targets for therapeutic interventions in human cancer.

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