Girdin/GIV Regulates Collective Cancer Cell Migration by Controlling Cell Adhesion and Cytoskeletal Organization

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Abstract

Pathological observations show that cancer cells frequently invade the surrounding stroma in collective groups rather than through single cell migration. Here, we studied the role of the actin-binding protein Girdin, a specific regulator of collective migration of neuroblasts in the brain, in collective cancer cell migration. We found that Girdin was essential for the collective migration of the skin cancer cell line A431 on collagen gels as well as their fibroblast-led collective invasion in an organotypic culture model. We provide evidence that Girdin binds to β-catenin that plays important roles in the Wnt signaling pathway and in E-cadherin-mediated cell-cell adhesion. Girdin-depleted cells displayed scattering and impaired E-cadherin-specific cell-cell adhesion. Importantly, Girdin depletion led to impaired cytoskeletal association of the β-catenin complex, which was accompanied by changes in the supracellular actin cytoskeletal organization of cancer cell cohorts on collagen gels. Although the underlying mechanism is unclear, this observation is consistent with the established role of the actin cytoskeletal system and cell-cell adhesion in the collective behavior of cells. Finally, we showed the correlation of the expression of Girdin with that of the components of the E-cadherin complex and the differentiation of human skin cancer. Collectively, our results suggest that Girdin is an important modulator of the collective behavior of cancer cells.

Keywords: collective invasion, collective migration, actin cytoskeleton, cell adhesion, Girdin

1. Introduction

Years of study have identified the general mechanisms of cancer invasion and metastasis. These processes are mostly mediated by genomic and epigenetic alterations and dysregulated cell signaling that activates cytoskeletal organization.[1] A classical view of cancer invasion is that cancer cells undergo a phenotypic change called the epithelial-mesenchymal transition (EMT) to downregulate cell-cell adhesion molecules such as E-cadherin and gain mesenchymal morphology and motility.[2-4] Indeed, many studies that used single cells in culture have extended our knowledge of the mechanisms of cancer invasion, including those that regulate the EMT and changes in cytoskeletal reorganization.[5]

In contrast, pathological observations of tissue sections from cancer patients have long suggested that most of the cancer cells from malignant epithelial tumors form variable sized groups that collectively invade surrounding tissues.[6-8] This is most evident in squamous cell carcinomas but holds as well in adenocarcinomas and non-epithelial malignancies such as melanoma.[7] Pancreatic ductal adenocarcinoma, which is one of the most devastating invasive cancers, also keeps a moderately differentiated morphology to form glands and groups of cells that invade the stroma.[9] Notably, cancer cells that invade lymphatic vessels also form groups.[8] These observations question whether the EMT program is always required for the intravasation of cancer cells into those vessels and subsequent metastasis to distant sites.[7, 8]

Recent studies have begun to reveal the mechanisms of collective invasion of cancer by using *in vitro* culture models as well as studies of collective migration of cells during embryonic development.[6, 7, 10, 11] These include the contact inhibition of locomotion (CIL) that keeps the integrity of cell-cell contact and spatially regulated trafficking of cell adhesion proteins such as N-cadherin.[12-17] We previously reported that

the collective movement of cancer cell groups requires the expression of integrin β1 by the leading cells but not the following cells.[18] Despite this progress, our knowledge of the mechanisms of collective invasion of cancer is far from complete. The primary problems are due to the difficulty of understanding the regulators of "supracellular" cytoskeletal organization in cell groups, the complex interactions between cancer cells and the stroma and the lack of identification of proteins that are specifically involved in the collective behavior of cells.[6, 19]

Since the identification of the actin-binding hub protein Girdin (also known as Gα-interacting vesicle-associated protein; GIV), we have been particularly interested in its function in neural development, angiogenesis, and cancer progression.[20-26] We reported that conventional Girdin knockout mice exhibited severe deficiency in the collective movement (termed "chain migration") of neuroblasts born in the subventricular zone (SVZ) of the lateral ventricle (LV) of postnatal and adult brains.[8, 27, 28] The data indicated specific involvement of Girdin in the collective behavior of cells, which is partly explained by the regulation of actin remodeling and cell polarity by Girdin.[20, 29, 30] However, the precise manner in which Girdin fine-tunes and maintains the integrity of cell-cell adhesion to allow for cell rearrangements that drive collective cell migration is unclear. A recent report on *Drosophila* embryogenesis showed that a fly orthologue of Girdin coordinates collective epithelial migration by promoting the anchorage of the cadherin-catenin complex to the cytoskeleton, suggesting a conserved role of Girdin between species.[31]

We and others have previously shown that Girdin is expressed by multiple types of cancers, where its expression correlated with cancer progression.[22, 32-35] In this study, we showed a role of Girdin in the collective invasion of skin cancer cells, where it interacts with β-catenin, a component of the E-cadherin complex.[36] Our data showed that Girdin is indispensable for stable cell-cell interaction, supracellular cytoskeletal organization,

and the collective migration of cancer. Finally, we also examined the clinical relevance of Girdin expression in the progression of human skin cancer.

2. Materials and Methods

2.1 Cell culture and time-lapse imaging

The human cancer cell lines A431 and HeLa, the Madin-Darby canine kidney epithelial cell line MDCK and the human embryonic kidney epithelial cell line 293FT were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, San Jose, CA, USA). The human colorectal cancer cell line DLD1 was cultured in RPMI 1640 (Thermo Fisher Scientific) supplemented with 10% FBS. Cell line authentication was assessed using a short tandem repeat (STR) DNA profiling method (BEX Co., Ltd, Japan), and Mycoplasma contamination was tested by staining with DAPI (4', 6-diamidino-2-phenylindole) every 3 months. Immortalized CAF that were established from primary CAF of human vulvar cancer and A431 cells stably expressing E-cadherin-Ruby were provided by Takuya Kato (The Francis Crick Institute, UK) and maintained in DMEM supplemented with 10% FBS. For time-lapse imaging of cells, we prepared a 1.6 mg/mL collagen type I gel with Cellmatrix type I-P (Nitta Gelatin Inc. Osaka, Japan) in a 6-well plate as described previously,[37, 38] followed by seeding A431 cells and time-lapse imaging with an IncuCyte Zoom microscope (x20 objective lens, Essen Bioscience, Ann Arbor, MI, USA).

For the analysis of the trajectories of cells, single solitary cells (**Figure 1A**) or single leading cells of the clusters (≥ 5 cells) of control and Girdin-depleted cells (**Figure 1C-G**) were randomly chosen by an investigator blinded to the groups and manually tracked using Manual Tracking plugin for ImageJ 1.52a software (US National Institutes of Health, Bethesda, MD, USA). The data were exported to a Microsoft Excel (Microsoft Corporation, Redmond, WA) spreadsheet for analysis. Cell

clusters that became less than 5 cells by cell detachment during the observational period were excluded from the analysis.

2.2 Plasmids, antibodies and Western blot analysis

The cDNAs for human β-catenin and α-catenin were generously provided by Frank Costantini (Columbia University, NY, USA) and Takashi Watanabe (Nagoya University, Nagoya, Japan), respectively. The isolation of human Girdin as well as the subcloning of Girdin domains was described previously.[24] The construction of plasmids encoding GFP-Girdin fragments has been described.[24] cDNA fragments encoding the fragments of β-catenin were inserted into the pEF-GST-BOS vector. For the production and purification of recombinant proteins in the *E. coli* expression system, α- and β-catenin cDNAs were inserted into the pGEX-4T-2 vector.

The following antibodies were used for Western blotting and immunofluorescent studies: green fluorescent protein (GFP) (598, MBL, Nagoya, Japan); GST (sc-459, Santa Cruz Biotechnology, Santa Cruz, CA, USA); β-actin (A5316, Cell Signaling Technology, Beverly, MA, USA); Girdin (AF5345, R&D Systems, Minneapolis, MN, USA); β−catenin (610153) and E−cadherin (610181) (BD Biosciences, San Jose, CA, USA); α-catenin (ALX-804-101-C100, Enzo Life Sciences, Farmingdale, NY, USA); EGFR (Ab-5, clone H11, Thermo Fisher Scientific); c-jun (9156, Cell Signaling Technology); vimentin (M0725, clone V9, Dako); HSP70 (sc-24, Santa Cruz Biotechnology).

For Western blot analysis, cells were treated with lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.2% NP40 supplemented with Complete Protease Inhibitor and PhosSTOP Phosphatase Inhibitor cocktails (Roche, Mannheim, Germany).

Lysates were clarified by centrifugation at 12,000 x g for 10 min at 4ºC, followed by the addition of sodium dodecyl sulfate (SDS) sample buffer (10 mM Tris-HCl, 2% SDS, 2 mM EDTA, 0.02% bromophenol blue, 6% glycerol; pH 6.8) and separation by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to nitrocellulose membranes, blocked in 5% milk in PBS containing 0.05% Tween 20, incubated with primary antibodies, and detected by horseradish peroxidase (HRP)-conjugated secondary antibodies (Dako, Denmark). All of the experiments were replicated at least twice, including preliminary experiments. We showed representative data from the repeated experiments.

2.3 RNA interference

Target sequences for shRNA-mediated depletion of Girdin were described.[24] A set of single-stranded oligonucleotides encoding the Girdin target sequences and their complements were synthesized as follows (only the sense sequence is shown): human Girdin shRNA (1), 5'-GGAACAAACAAGATTAGAA-3' (nucleotides 3837–3855); human Girdin shRNA (7), 5'-GAAGGAGAGGCAACTGGAT-3' (nucleotides 4166–4184). The oligonucleotide pair was annealed and inserted into the pSIREN-RetroQ retroviral shRNA expression vector (Clontech, Palo Alto, CA, USA). To produce retroviral supernatants, GP2-293 packaging cells were transfected with the pVSV-G (vesicular stomatitis virus G protein) vector and either control or Girdin shRNA-containing pSIREN-RetroQ vector using Lipofectamine 2000 reagent (Thermo Fisher Scientific). The medium was replaced 24 h later, and virus-containing supernatants were harvested 48 h post-transfection and used for infection of HeLa and A431 cells.

The small interfering RNA (siRNA)-mediated depletion of Girdin was performed as previously described.[20] siRNAs for E-cadherin and α- and β-catenins were purchased

from Qiagen (Hilden, Germany). The targeted sequences that effectively mediated the silencing of the expressions of the indicated genes are as follows (only sense sequences are shown): Girdin-1, 5'-AAGAAGGCTTAGGCAGGCAGGAATT-3'; Girdin-2, 5'-AACCAGGTCATGCTCCAAATT-3'; E-cadherin, 5'-GAATCTATCATTTTGAAGCCA-3'; ⊠catenin, 5'-AAGTGGATAAGCTGAACATTA-3'; ⊠catenin, 5'-CTCGGGATGTTCACAACCGAA-3'. Negative control siRNA (AllStars Negative Control siRNA) was purchased from Qiagen (Hilden, Germany).

2.4 Cell dissociation assay

We followed a standard protocol previously described that measures the strength of cell-cell adhesion.[39, 40] Confluent cultured HeLa or MDCK cells (4 x 10^6 cells per 6-cm dish) were treated with 0.01% trypsin in HCMF (10 mM Hepes, 140 mM NaCl, 5 mM NaOH, 5 mM KCl, 3.5 mM $Na_2HPO_4 \cdot 7H_2O$, 5.55 mM glucose; pH 7.4) supplemented with either 0.1 mM $CaCl_2$ (TC treatment) or HCMF supplemented with 1 mM EDTA (pH 7.5) (TE treatment) for 30 min at 37°C, followed by dissociation by pipetting 10 times. The numbers of cell particles that included cell clusters and single cells were counted after dissociating A431 and HeLa cells in the TC and TE treatment conditions. The extent of cell dissociation was represented by the index TC/TE, where TC and TE are the total particle numbers after the TC and TE treatment, respectively.

2.5 Cell fractionation

The isolation of nuclear, membrane and cytoskeletal fractions of A431 cells was done using an S-PEK cell fractionation kit (539790, Merck, Kenilworth, NJ, USA), which is

based on the different solubility of subcellular compartments in proprietary detergents, following the manufacturer's instructions.

2.6 Organotypic culture model

The organotypic cultivation of A431 and CAF was carried out as described elsewhere.[41] Immortalized CAF derived from a human vulvar cancer (5 x 10^5) were embedded in a mixture of type I collagen and Matrigel (BD Biosciences, San Jose, CA, USA), yielding a final collagen concentration of 4 mg/mL and a final Matrigel concentration of 2 mg/mL. The gel was incubated at 37°C for 30 min in 24-well plates, on top of which A431 cells (5 x 10^5) were plated in a serum-free medium for 24 h. Gels were then mounted on 6-well chambers and fed from underneath with a complete medium. After 10 days, the cultures were fixed with 4% paraformaldehyde plus 0.25% glutaraldehyde in PBS, followed by paraffin embedding, sectioning, and hematoxylin and eosin (H&E) staining or immunohistochemistry.

2.7 Immunohistochemical staining of mouse and human tissues

We followed a standard protocol for immunohistochemical staining of mouse brain and human skin cancer tissues. Antigen retrieval was performed by microwave treatment in antigen retrieval buffer (Dako, pH 6 or pH 9) at 95ºC for 10 min. Human skin cancer tissues were obtained with informed patient consent at the time of surgery in Nagoya University Hospital. We also used tissue arrays of human skin cancers and matched normal adjacent tissues that were purchased from US Biomax (SK802b, Rockville, MD, USA). To evaluate the expression levels of Girdin and the components of the E-cadherin/catenin complex, cases with total scores (the sum of intensity and proportion

scores) of more than 3 were considered positive (see **Figure 6E**). The study was conducted in accordance with the Helsinki Declaration for Human Research and approved by the Ethics Committee of Nagoya University Graduate School of Medicine (protocol number 2017-0127).

2.8 Data analysis

All statistical analysis was performed using GraphPad Prism 6 software (GraphPad, San Diego, CA, USA). Data are presented as the means ± S.D. Statistical significance was evaluated with Student's t test. The χ^2-test was used to analyze correlations between Girdin expression and clinicopathological parameters. P values < 0.05 were considered statistically significant.

3. Results

3.1 The significance of Girdin in the collective migration of cancer cells on collagen gels

To address the involvement of Girdin in the collective migration of cancer cells, we first conducted experiments to visualize collective migration *in vitro*. Our previous studies reported that a highly invasive skin cancer cell line (A431) seeded on a collagen gel initiated collective migration that was different from that observed when the cell line was plated on plastic dishes.[37, 38, 42] We reproducibly observed that the behavior of A431 single cells and cell groups on plastic dishes was significantly different from that on collagen gels **(Figures 1A, B**). We found that the distance between start and end points (d), but not the actual trajectory (D), of single A431 cells on collagen gels was statistically longer than those plated on plastic dishes. Furthermore, the directionality (persistency) of migrating single cells, which was calculated as the ratio of d to D (d/D), was much higher in cells on collagen gels than those on plastic dishes (**Figure 1A**). This was also the case when we observed the behavior of A431 cancer cell cohorts (**Figure 1B, Movies S1, 2**). In contrast to cell groups on plastic dishes that proliferated in place, cells on the collagen gel dynamically moved with directionality.

We next depleted Girdin with shRNA to determine the impact on the collective behavior of A431 cells seeded on collagen gels (**Figures 1C-G**). We focused on leading cells located at the front of the collectively migrating cell groups (≥ 5 cells) and calculated directionality indices (**Figure 1C**). The results showed that, in contrast to control cell groups collectively migrating with high persistency, Girdin-depleted cells were not migratory and tended to stay in place (**Figures 1D-G, Movies S3-5**). We found that d and d/D, rather than D, were affected by Girdin depletion in the collective migration of A431 cell groups (**Figure 1G**). It was noted that the number of cell groups was affected by Girdin depletion over the early part of the observation period but later it did not depend on Girdin (**Figures S1A, B**). Girdin depletion also had a modest effect

on cell proliferation of A431 cells (**Figure S1C**). Taken together, although not conclusive, these data suggested that Girdin plays an essential role in collective cancer cell migration.

3.2 Identification of β-catenin as a Girdin-interacting protein

To examine the mechanism by which Girdin mediated collective cell migration, we exploited a mass spectrometric shotgun approach to identify Girdin immunocomplexes isolated by tandem affinity purification (TAP) from the lysate of HeLa cervical cancer cells **(Figure S2A).** Among the identified proteins, we focused on β-catenin, a critical regulator of the Wnt signaling pathway and cell-cell adhesion that was reportedly involved in the function of Girdin in *Drosophila*.[31, 36]

Immunoprecipitation (IP) experiments showed that β-catenin was co-immunoprecipitated by Girdin antibody in several types of cancer cells or immortalized epithelial cells including A431, HeLa, DLD1 (colon cancer), and MDCK (renal epithelium) cells (**Figures 2A, S2B-D**). α-catenin was also co-immunoprecipitated with Girdin, leading to the speculation that Girdin, α-catenin, β-catenin, and E-cadherin might form a complex in these cells. The interaction was also confirmed by a reciprocal IP test that showed that β-catenin immunoprecipitates contained Girdin, α-catenin, and E-cadherin in A431 cells (**Figure 2B**).

Immunofluorescent staining on A431 and MDCK cells clearly showed the colocalization of Girdin and β- and α-catenins at cell-cell adhesion sites on either plastic dishes or collagen gels, suggesting that Girdin plays a role in the regulation of intercellular adhesion (**Figures 2C, D, S2E**). Of note, Girdin/β-catenin interaction was not obvious in 293FT cells by IP tests or by immunofluorescent staining (**Figures S2F,**

G). Given that 293FT cells are neither migratory nor invasive by nature, these data suggested that Girdin/β-catenin interaction may be vital for cell motility.

3.3 The Girdin carboxyl-terminal domain interacts with β-catenin

We and others have shown that Girdin's amino- (NT) and carboxyl-terminal (CT) domains flank the central coiled-coil domain and interact with multiple proteins including actin filaments, the subunits of the tripartite G proteins, the cell polarity regulator Par-3 and Dynamin guanosine triphosphatase (GTPase) (**Figure 2E**).[20, 25, 29, 43, 44] To address which of Girdin's domains is responsible for β-catenin interaction, we expressed the domains of Girdin and β-catenin in 293FT cells, and mapped interacting domains by IP tests (**Figures 2F, G**). The data showed that Girdin's CT domain interacts with the N-terminal domain of β-catenin. An *in vitro* binding assay using purified recombinant GST-fused β-catenin and the His-fused Girdin CT domain showed their direct interaction (**Figures 2H, I**). Interestingly, the Girdin CT domain also bound to recombinant α-catenin, suggesting the possibility that the Girdin CT domain possessed multiple interfaces to bind to multiple proteins.

3.4 Involvement of Girdin in the strength of cell-cell adhesion

It was important to determine how Girdin was involved in E-cadherin-mediated adhesion between cells. We found that Girdin localization at cell-cell contact sites was more prominent in high density cultured A431 cells that formed mature cell-cell contacts than in low density cultured cells with immature contacts (**Figure 3A**). We used siRNA to deplete the endogenous levels of Girdin in HeLa cells. This treatment induced HeLa cells to scatter such that the cells could not adhere to each other tightly or

regularly (**Figures 3B, C**). This observation was consistent with our previous finding that the Girdin-depleted SH-SY5Y neuroblastoma cell line showed a similar response.[28] Together, these data indicated that Girdin played an essential role in cell-cell adhesion.

Mammalian cells express many types of adhesion molecules and a number of cell surface proteins that mediate cell-cell adhesion. Thus, we next examined the role of Girdin in calcium-dependent, E-cadherin-mediated adhesion between cells as determined by a cell dissociation assay (**Figures 3D-H**).[39, 40] Thus, we assessed the numbers of cell particles that included cell clusters and single cells after dissociating A431 and HeLa cells in the presence of 0.1 mM calcium (TC treatment) or 1 mM EDTA (TE treatment) (see Materials and Methods; **Figure S3A**). We found that the depletion of Girdin as well as E-cadherin, β- and α-catenins significantly increased the number of cell particles after the TC treatment, indicating that these molecules are involved in the strength of cell-cell adhesion mediated by E-cadherin (**Figures 3D-H**). These results were reproduced when MDCK cells were stably depleted of Girdin and examined in the cell dissociation assay (**Figures S3B, C**). These data suggested the involvement of Girdin in E-cadherin-mediated cell-cell adhesion.

3.5 Girdin regulates the association between the E-cadherin complex and the cytoskeleton and supracellular cytoskeletal organization

Many studies showed that E- and N-cadherin and their complexes undergo remodeling during cell migration.[15, 45, 46] Those observations are supported by our experiment in which E-cadherin interaction with β-catenin was attenuated by inducing collective cell migration by scratching a monolayer of confluent A431 cells (**Figure S4A**). We investigated the effect of Girdin depletion on the formation of the E-cadherin/catenin complex (**Figure S4B**). The results showed that there was no obvious difference

between control and Girdin-depleted cells, suggesting that Girdin had limited, if any, impact on the assembly of this core adhesion complex. We also examined the localization of E-cadherin by time-lapse imaging in A431 cells stably expressing E-cadherin-Ruby on collagen gels (**Figure S4C**). Again, we did not find any obvious changes in E-cadherin localization or its intracellular trafficking even in control cells. All of these data showed that the mechanism of Girdin-mediated collective migration cannot be explained by dysregulated formation or dynamics of the E-cadherin/catenin complex, which may not be the same as those reported on other cell types and experimental systems.

Another possible mechanism supporting collective migration involves the dynamic regulation of supracellular cytoskeletal organization and its link to cell-cell adhesion.[6, 47] Interestingly, a fractionation experiment showed that the amounts of β- and α-catenins and E-cadherin that reside in the cytoskeletal fraction were decreased, whereas those in a membrane fraction were increased by Girdin depletion, suggesting a role of Girdin in cytoskeletal association of the catenin protein complex (**Figures 4A, B**). Another difference between control and Girdin-depleted cells was revealed by the staining of the actin filaments by phalloidin (**Figure 4C**). Under these conditions, we found obvious differences of the rearrangement of the actin cytoskeleton during collective migration of A431 cells on collagen gels (**Figure 4C**). Specifically, filopodia formation at the edges and cell-cell adhesion sites at the basal, but not apical, plane of the cell groups was significantly attenuated in Girdin-depleted cells. Given the importance of supracellular cytoskeletal organization, we speculate that Girdin regulates collective migration by facilitating the cytoskeletal association of β-catenin and related cytoskeletal reorganization (**Figure 4D**). Furthermore, the overexpression of the NT domain of β-catenin, which is responsible for binding with Girdin, led to a competitive disruption of endogenous Girdin/β-catenin interaction (**Figure 5A**) and directional

migration of A431 cell groups on collagen gels (**Figures 5B-D, Movies S6, 7**), supporting the physiological role of Girdin/β-catenin interaction in collective cell migration.

3.6 The role of Girdin in three-dimensional collective invasion of cancer cells

Our data on A431 cells cultured on collagen gels suggested the importance of Girdin in their collective invasion. To support this hypothesis, we adopted an organotypic culture model in which we prepared Matrigel containing cancer-associated fibroblasts (CAFs) on which we seeded A431 cells, rendering them air-exposed (**Figure 6A**).[18, 41] In the model, CAFs remodel the extracellular matrix in Matrigel to make a path for the cancer cells, thus allowing us to observe their collective invasion. We found that the depletion of Girdin, as well as that of the other components of the E-cadherin/catenin complex, attenuated the number of collective cell groups (clusters) invading into Matrigel, but not cell number in each group, further confirming a role of Girdin in collective invasion of cancer cells (**Figures 6B, C**). The depth of invasion was regulated by Girdin and E-cadherin, but not β- and α-catenins (**Figure 6D**), suggesting that Girdin and E-cadherin promote collective invasion with high persistency, but not elucidating the role of the E-cadherin protein complex.

3.7 Clinical relevance of Girdin expression in the progression of skin cancer

Finally, we investigated the significance of Girdin expression in the progression of squamous cell carcinoma of the skin that is known to collectively invade the stroma. To do this, we developed a scoring system by which the expression levels were evaluated by both intensity scores (IS) and proportion scores (PS) (**Figure 6E**). Statistical analysis

of 76 cases of skin cancer showed that the expression level of Girdin was upregulated in most cases of squamous cell carcinoma of the skin (**Figure 6F, Table 1**), suggesting a role of Girdin in cancer progression, as already shown for other types of cancer including breast, colon, and brain tumors.[22, 33-35] We also found that Girdin expression level correlated well with the differentiation of the cancer (**Table 1**) and the expression of β- and α-catenins as well as E-cadherin (**Table 2**), suggesting that Girdin synergizes with its partner proteins in the progression of skin cancer. One unresolved issue, however, was that Girdin expression level had a tendency to correlate with the depth of tumor infiltration, but not with statistically significant difference (P value, 0.1824; **Table 1**). We also did not find a correlation between Girdin expression and the presence of isolated cell clusters comprised of 1 – 4 cancer cells (tumor budding) at the invasive front of tumors (**Table S1**). Thus, the present study did not show the significance of Girdin in the tumor budding of human skin cancer. This may partially be attributed to the involvement of various microenvironmental factors or synergistic effects of Girdin with other intrinsic factors that could influence the invasion of the cancer cells.

4. Discussion

In the present study, which was based on the defective collective migration of neuroblasts in mice that lacked the actin-binding hub protein Girdin,[27, 28] we dissected the role of Girdin in collective migration and the invasion of cancer cells. We showed that Girdin is involved in the function of the E-cadherin/catenin complex, where it controls the stability of cell-cell adhesion, but at the same time regulates supracellular cytoskeletal organization.

An intriguing finding from this study is that the role of Girdin may be shared by both developing neuroblasts and cancer cells. The role of Girdin in chain migration of SVZ neuroblasts has been shown in whole mice, but not in tissue- or cell-specific conditional Girdin knockouts, indicating the specificity of Girdin's function in neuroblasts' collective migration.[8, 27] We and others reported the aberrant expression of Girdin in several types of human cancers, findings that suggest similar roles for Girdin in neuroblasts and cancer cells.[22, 33-35] Given the identification of β-catenin as a Girdin-interacting protein in this study, we examined β-catenin expression in migrating neuroblasts. Interestingly, β-catenin and N-cadherin (with which it interacts) were robustly expressed in the neuroblasts in control animals, whereas the expression of those proteins was apparently downregulated or dysregulated in Girdin knockout mice (**Figure S5**). These data suggested that Girdin might cooperate with β-catenin in migratory processes of both the neuroblasts and cancer cells.

One should consider a previous study that showed retrograde flow of N-cadherin along the cell-cell contact sites during collective migration of primary cultured astrocytes.[15] Our observation using A431 cells expressing E-cadherin-Ruby, however, did not reveal any significant movement along the cell-cell junctions or intracellular trafficking of E-cadherin during collective migration (**Figure S4C**). Those results suggest that the mechanism underlying cadherin dynamics in cancer cells might be

distinct from that in neural cells. Further studies are needed to clarify the cell- or tissue-specific mechanisms for collective migration.

At present, the precise mechanism by which Girdin controls cell-cell adhesion together with actin remodeling cannot be explained solely by Girdin/β-catenin interaction. We speculate that other binding partner(s) of Girdin are also involved in this process. One of those may be the large GTPase Dynamin that we previously reported binds to the Girdin NT domain.[44] The binding of Girdin to Dynamin activates its GTPase activity to pinch off clathrin-coated vesicles and stimulate E-cadherin-specific endocytosis.[44] It will be interesting if Girdin/Dynamin interaction and its regulation of E-cadherin endocytosis are also involved in Girdin-mediated collective migration, which will be a subject for future research (**Figure 4D**).

Another limitation of the study is that we could not clearly show whether Girdin forms a four-protein complex containing α- and β-catenins and E-cadherin at cell-cell adhesion sites. We showed their colocalization by immunofluorescent staining. However, those data did not necessarily show evidence of a four-protein complex or its biological significance. Also, there are no Girdin mutants that do not specifically interact with β-catenin, making it difficult to solve the specific importance of Girdin/β-catenin interaction. A future challenge is to identify upstream regulators of Girdin/β-catenin interaction, preferably kinases. However, as far as we tested, neither Akt, nor cyclin-dependent kinase 5 (Cdk5) nor Src, all of which are known to phosphorylate the Girdin CT domain,[20, 48, 49] had any effects on Girdin/β-catenin interaction. Further investigations of the mechanisms of Girdin/β-catenin interaction and its coupling to Girdin-mediated Dynamin GTPase activity will clarify the mechanisms of collective cell migration.

Collective cell migration has been studied for years and several mechanisms have been proposed. However, it remains unclear how proteins involved in collective migration differ from those involved in single cell migration. One attractive model for collective migration argued the importance of supracellular cytoskeletal organization, where the dynamics of cytoskeleton is shared between multiple cells to jointly generate force for migration and polarization.[6, 11] Our finding that Girdin is involved in actin remodeling at the cell-cell interface in A431 cancer cell groups (**Figure 4C**) supports the model, but it is unknown at present whether it was a direct or indirect effect of Girdin depletion. Another intriguing mechanism for collective migration is that cells maintain contact inhibition of locomotion (CIL) to avoid interfering with their neighbors.[14, 16, 17] We previously showed that the Girdin CT domain also binds the cell polarity regulator Par-3 (partitioning-defective gene 3) that is known to be critical for collective migration and CIL by downregulating actomyosin contractility at cell-cell contact (**Figure 2E**).[29, 50] Further sophisticated experimental approaches and imaging methods may reveal the involvement of Girdin in CIL in the future.

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Disclosure

No potential conflicts of interest were disclosed.

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Figure Legends

Figure 1. Girdin regulates the collective migration of A431 cells on collagen gels

(A) The behavior of single cells on collagen gels is different from that on conventional plastic dishes. The directionality of migrating single cells was calculated as the ratio (d/D), i.e., the distance between the starting and ending points (d) divided by the actual trajectory (D). Ten and five dishes were evaluated for the collagen gel group and plastic dish group, respectively, and 25 cells in each dish were manually tracked, followed by quantification.

(B) Representative images of A431 cell groups cultured on plastic dishes (upper panel) and a collagen gel (lower panel). Note that the cell groups (dotted circles) undergo proliferation without movement on plastic dishes, whereas those seeded on the collagen gel collectively migrate with directionality. Arrows indicate the direction of the movement of the cell group. See also **Movies S1 and S2**.

(C) A schematic illustration showing the measurement of collective migration of A431 cells cultured on a collagen gel. We focused on one single cell on the edge of migrating cell groups and tracked its trajectory by tracing the nuclear centroid.

(D) shRNA-mediated depletion of Girdin in A431 cells. MW, molecular weight.

(E-G) Representative images of control and Girdin-depleted A431 cell groups cultured on a collagen gel. Time interval between each panel is 2 h. Note that the control cell group underwent collective directional migration, whereas the Girdin-depleted cells tended to remain in place. Shown in **(F)** are the representative paths of the migration of control shRNA- (red) and Girdin shRNA (1) (green)-transduced cell groups, as determined by tracing the nuclear centroid over a period of 5 h (n = 25 for each group). The directionality of migrating cell groups was quantified and shown in **(G)** (n = 50 for each group). See also **Movies S3-5**.

Figure 2. Interaction of Girdin with the β-catenin complex

(A, B) IP with anti-Girdin antibody showed that Girdin interacts with β- and α-catenins in A431 cells **(A)**. Interaction between Girdin and catenins was also shown by reciprocal IP with anti-β-catenin antibody **(B)**. TCL, total cell lysates.

(C, D) Immunofluorescence staining showed the colocalization of Girdin (green) with α- and β-catenins (red) in A431 cells seeded on plastic dishes **(C)** and collagen gels **(D)**.

(E) Domain structures and interacting proteins of human Girdin and β-catenin. Fragments and domains of Girdin and β-catenin used in the study are shown. The domains responsible for the interaction are shown in red.

(F) Mapping of interacting domains of Girdin and β-catenin. β-catenin-binding site maps to the CT domain of Girdin. Lysates from 293FT cells transfected with the Girdin fragments fused with GFP were immunoprecipitated with anti-GFP antibody, followed by Western blot analyses using β- and α-catenin antibodies. The bound catenins are indicated by asterisks.

(G) Girdin-binding site maps to the NT domain of β-catenin. Lysates from 293FT cells transfected with the Flag tag-fused Girdin CT domain and the β-catenin fragments fused with GST were precipitated with glutathione-Sepharose beads, followed by Western blot analyses using anti-Flag antibody. The bound Flag-Girdin CT is indicated by asterisks.

(H, I) Direct interaction of Girdin and β-catenin. Coomassie brilliant blue staining showing recombinant GST, GST-fused β- and α-catenins that were expressed and purified from the *E. coli* expression system **(H)**. Purified recombinant Girdin CT domain was incubated with the recombinant catenins fused with GST (60 - 90 pmol) for

1 h at 4ºC, followed by precipitation with glutathione-Sepharose beads and Western blot analysis **(I)**. Girdin CT domain that bound to GST-catenins is indicated by asterisks.

Figure 3. Girdin controls the strength of cell-cell adhesion

(A) Girdin localized at cell-cell contacts. Its colocalization with β-catenin was not evident in immature cell-cell adhesion of A431 cells sparsely plated on dishes (left), whereas it was clearly observed in confluent cells with mature cell-cell adhesion (right).

(B, C) HeLa cells depleted of Girdin exhibited morphology of scattered cells.

(D) Efficiency of siRNA-mediated knockdown of Girdin, E-cadherin, α- and β-catenins in A431 cells was shown by Western blot analyses using the indicated antibodies.

(E, F) A431 cells transfected with the indicated siRNA were treated with trypsin for 30 min in the presence of either 0.1 mM Ca^{2+} (TC) or 1 mM EDTA (TE) at 37ºC. The cells were dissociated by pipetting, and the number of particles was counted. Cells of each group were seeded in three 6-cm dishes, followed by counting the numbers of all particles in the dishes and quantification. Representative images are shown in **(E)**. The extent of cell dissociation was represented by the index TC/TE, where TC and TE are the total particle numbers after the TC and TE treatment, respectively **(F)**. The TC/TE ratio represents the inverse strength of cadherin activity.

(G, H) shRNA-mediated Girdin depletion decreased cadherin activity in HeLa cells, which is shown by high TC/TE value in the cell dissociation assay.

Figure 4. Girdin participates in the cytoskeletal association of the E-cadherin/catenin complex and cytoskeletal organization

(A) A representative data showing successful fractionation of various subcellular fractions of A431 cells.

(B) The cytosolic, cytoskeletal, and membrane fractions from A431 cells transfected with control or Girdin siRNA were examined by Western blot analyses using the indicated antibodies. In the lower panel, the expression levels of the proteins in each fraction were quantified and presented as arbitrary unit (AU).

(C) Control cells (left panel) and Girdin-depleted cells (right panel) were stained by β-catenin (red) and phalloidin to visualize actin filaments (green). Yellow arrowheads denote filopodia formation found in the edges and cell-cell contact sites at the basal planes of control cell groups, whereas filopodia formation was weak or disrupted in Girdin-depleted cells (soft magenta arrowheads).

(D) A schematic illustration of the speculated function of Girdin at cell-cell adhesions. Girdin mediates the link of β-catenin and the actin cytoskeleton (left panel), which is lost by the absence of Girdin accompanied by dysregulated cytoskeletal organization (right panel). A previous study showed that Girdin interaction with Dynamin is essential for E-cadherin endocytosis, which may also be involved in collective cell migration.

Figure 5. The effect of overexpression of the β-catenin NT domain on collective migration of A431 cells

(A) Inhibition of endogenous Girdin/β-catenin interaction by expression of the β-catenin NT domain. Lysates from A431 cells transfected with the indicated combination of

expression plasmids were immunoprecipitated with anti-Girdin antibody. After washing, bound proteins were detected by Western blot analyses using the indicated antibodies. β-catenin precipitated by Girdin antibody is shown by asterisk.

(B) A431 cells were transfected with GFP (as a fill) and either GST or GST-β-catenin NT at the ratio of 1:4, and were sorted for GFP-positive cells. They were cultured on collagen gels and assessed by tracking of single cells on the edge of migrating cell groups (more than 5 or equal to cells).

(C, D) Representative images of GST and GST-β-catenin NT-transduced A431 cell groups cultured on collagen gels. Time interval between each panel is 2 h. Shown in **(D)** is the quantification of the directionality of migrating cell groups (n = 18 for each group). See also **Movies S6 and S7**.

Figure 6. Girdin is essential for collective invasion of cancer cells

(A) An illustration showing the experimental setup to recapitulate the collective invasion of cancer cells *in vitro*.

(B, C) Depletion of Girdin and the components of the E-cadherin complex impedes the collective invasion of A431 cells. Representative H&E-stained images of sections through Matrigel invaded by A431 cells. Boxed regions are magnified in lower panels, where arrows denote the groups of cells collectively invading into Matrigel. In **(C)**, the numbers of cell groups (clusters; ≥ 3 cells) invading into Matrigel and cell numbers per each cluster were counted and quantified (n = 3).

(D) The depth of invasion for each cluster found in each group was calculated and quantified (n = 3).

(E) Images of representative Girdin staining intensity for each intensity score (IS) (0-3). Cases with total scores, which represents the sum of IS and proportion scores (PS), of more than three were considered positive.

(F) Representative images of immunohistochemical staining for Girdin and the components of the E-cadherin/catenin complex in cases A and B of invasive squamous cell carcinoma of the skin. Invasive lesions of the tumors (lower panels) and the adjacent normal skin (upper panels) are shown. Note that Girdin expression in the carcinomas is more evident than in normal skin.

Legends for Supporting Figures

Figure S1. Effects of Girdin depletion on the cluster formation of A431 cells plated on collagen gels and their proliferation

(A, B) A431 cells were dissociated by trypsin, seeded on collagen gels, and cultured for 6 **(A)** and 18 **(B)** hours, followed by counting the number of cell clusters (defined as a group containing ≥ 5 cells) and quantification.

(C) Proliferation of A431 cells either transfected with the indicated siRNAs or transduced with the indicated shRNA was assessed by MTT assay.

Figure S2. Interaction of Girdin with the β-catenin complex and its involvement in cell-cell adhesion

(A) Isolation of Girdin immunocomplexes by tandem affinity purification (TAP) from the lysate of HeLa cervical cancer cells. Cells were transfected with either EGFP or Girdin carboxyl terminal (CT) domain (Girdin-CT) that are tagged with the Flag-HA (HA) epitope, followed by immunoprecipitation (IP) with Flag antibody and mass spectrometric analysis. Shown is the IP products separated by SDS-PAGE and stained with silver staining. We did the experiment only one time.

(B-D) Immunoprecipitation (IP) with anti-Girdin antibody showed that Girdin interacts with β- and α-catenins in HeLa, DLD1, and MDCK cells. TCL, total cell lysates; MW, molecular weight.

(E) Girdin localized at cell-cell contacts in MDCK cells. Confluent monolayers of MDCK cells were stained with the indicated antibodies, followed by observations by a confocal microscopy.

(F, G) No apparent interaction of Girdin and catenins in 293FT cells, as shown by IP **(F)** and immunofluorescence **(G)**.

Figure S3. Girdin controls the strength of cell-cell adhesion

(A) An illustration (left panel) and an example of analysis (right panel) showing the principle for the calculation of the index TC/TE. We defined a “cell particle” as a cell cluster (the number of cells in the cluster was not considered) or a single cell, followed by the calculation of particle numbers after dissociating A431 and HeLa cells in the presence of 0.1 mM calcium (TC condition) or 1 mM EDTA (TE condition). A high TC/TE value indicates a decrease in the strength of E-cadherin-mediated cell-cell adhesion.

(B, C) MDCK cells transduced with the indicated shRNA were treated with trypsin for 30 min in the presence of either Ca^{2+} (TC) or EDTA (TE) at 37ºC. The cells were dissociated by pipetting, and the number of particles was counted. Representative images are shown in **(B)**. The extent of cell dissociation was represented by the index TC/TE, where TC and TE are the total particle numbers after the TC and TE treatment, respectively **(C)**. The TC/TE ratio represents the inverse strength of cadherin activity.

Figure S4. E-cadherin/β-catenin complex was dynamically regulated during collective cell migration but with no apparent involvement of Girdin in that complex formation

(A) A monolayer of confluent A431 cells was extensively scratched to induce collective sheet migration, followed by IP with anti-β-catenin antibody. The data showed that the association between E-cadherin and β-catenin was attenuated by scratching the monolayer of the cells (asterisk).

(B) No apparent effect of Girdin depletion on the formation of the E-cadherin/catenin complex. Control or Girdin-depleted A431 cell lysates were immunoprecipitated with anti-β-catenin antibody, followed by Western blot analyses.

(C) No difference in the localization of E-cadherin was apparent in a comparison of control and Girdin-depleted cells that underwent collective migration on a collagen gel. A431 cells stably expressing E-cadherin-Ruby transduced with the indicated siRNA was monitored by time-lapse confocal microscopy. Representative screenshots of the cells are shown.

Figure S5. Aberrant expression of β-catenin and N-cadherin in collectively migrating neuroblasts in Girdin KO mice

Expression of β-catenin and N-cadherin in migrating neuroblasts in control (heterozygous) or Girdin-deficient (knockout) mice at postnatal (P) day 14. In the control mice, all cells that undergo chain migration express β-catenin and N-cadherin, whereas in Girdin-deficient mice, the cells are incapable of undergoing chain migration and exhibit dysregulated expression of those proteins. Boxed areas are magnified in lower panels. IHC, immunohistochemistry; LV, lateral ventricle; RMS, rostral migratory stream; OB, olfactory bulb.

<u>Legends for Supporting Movies</u>

Movie S1. The behavior of A431 cells plated on a plastic dish

A431 cells were seeded on a 6-well plastic plate, followed by time-lapse imaging with an IncuCyte Zoom microscope. The images were collected every 5 min for a period of 100 hr. Representative cell groups are indicated by arrows.

Movie S2. The behavior of A431 cells plated on a collagen gel

A431 cells were seeded on a collagen gel in a 6-well plastic plate, followed by time-lapse imaging with an IncuCyte Zoom microscope. The images were collected every 5 min for a period of 100 hr. Representative cell groups are indicated by arrows.

Movies S3-5. The effect of Girdin depletion on the behavior of A431 cells on collagen gels

A431 cells transduced with control (Movie S3), Girdin shRNA (1) (Movie S4) and Girdin shRNA (7) (Movie S5) were seeded on a collagen gel in a 6-well plastic plate, followed by time-lapse imaging with an IncuCyte Zoom microscope. The images were collected every 10 min for a period of 70-74 hr. Representative cell groups are indicated by arrows.

Movies S6-7. The effect of the overexpression of the β-catenin NT domain on the behavior of A431 cells on collagen gels

A431 cells were transfected with either control GFP (Movie S6) or GFP-β-catenin NT (Movie S7) and were sorted for GFP-positive cells, followed by culture on collagen gels and tracking of single cells on the edge of migrating cell groups (more than 3-4 cells). The images were collected every 30 min for a period of 68 hr. Representative cell groups are indicated by arrows.

Table 1. Girdin expression in human squamous cell carcinoma of the skin and its relation to clinical and pathological characteristics.

	Total number n	**Girdin positive (TS ≥ 3) n (%)**	**P value**
Age			0.2929
60 ≥	34	21 (61.7)	
> 60	42	22 (52.3)	
Sex			0.1621
Male	42	21 (50.0)	
Female	34	22 (67.6)	
WHO classification			< 0.0001
Well diff.	49	37 (75.6)	
Mod. diff.	19	6 (31.5)	
Poor. diff.	5	0 (0.0)	
Tumor invasion			0.1824
T1/T2	64	36 (56.2)	
T3/T4	11	9 (81.8)	

Mod., moderately; Poor., poorly; diff., differentiated

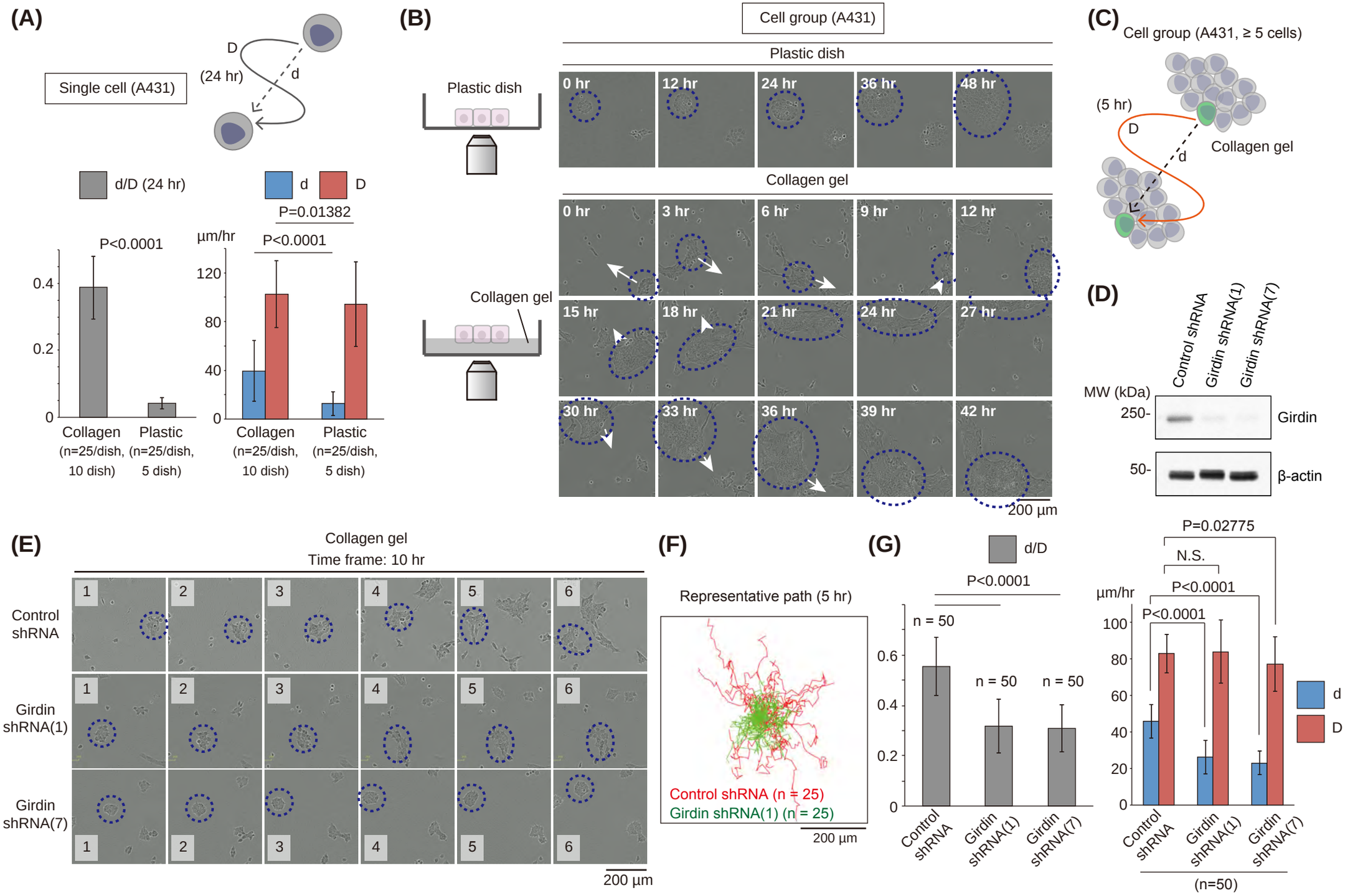
Table 2. Correlation of Girdin expression with the expression of the components of E-cadherin/catenin complex in human squamous cell carcinoma of the skin.

	Total number n	**Girdin positive (TS ≥ 3) n (%)**	**P value**
E-cadherin			< 0.0001
Positive (TS ≥ 3)	49	40 (81.6)	
Negative (TS < 2)	27	3 (11.1)	
β-catenin			< 0.0001
Positive (TS ≥ 3)	44	35 (79.5)	
Negative (TS < 2)	32	8 (25)	
α-catenin			< 0.0001
Positive (TS ≥ 3)	63	43 (68.2)	
Negative (TS < 2)	13	0 (0.0)	

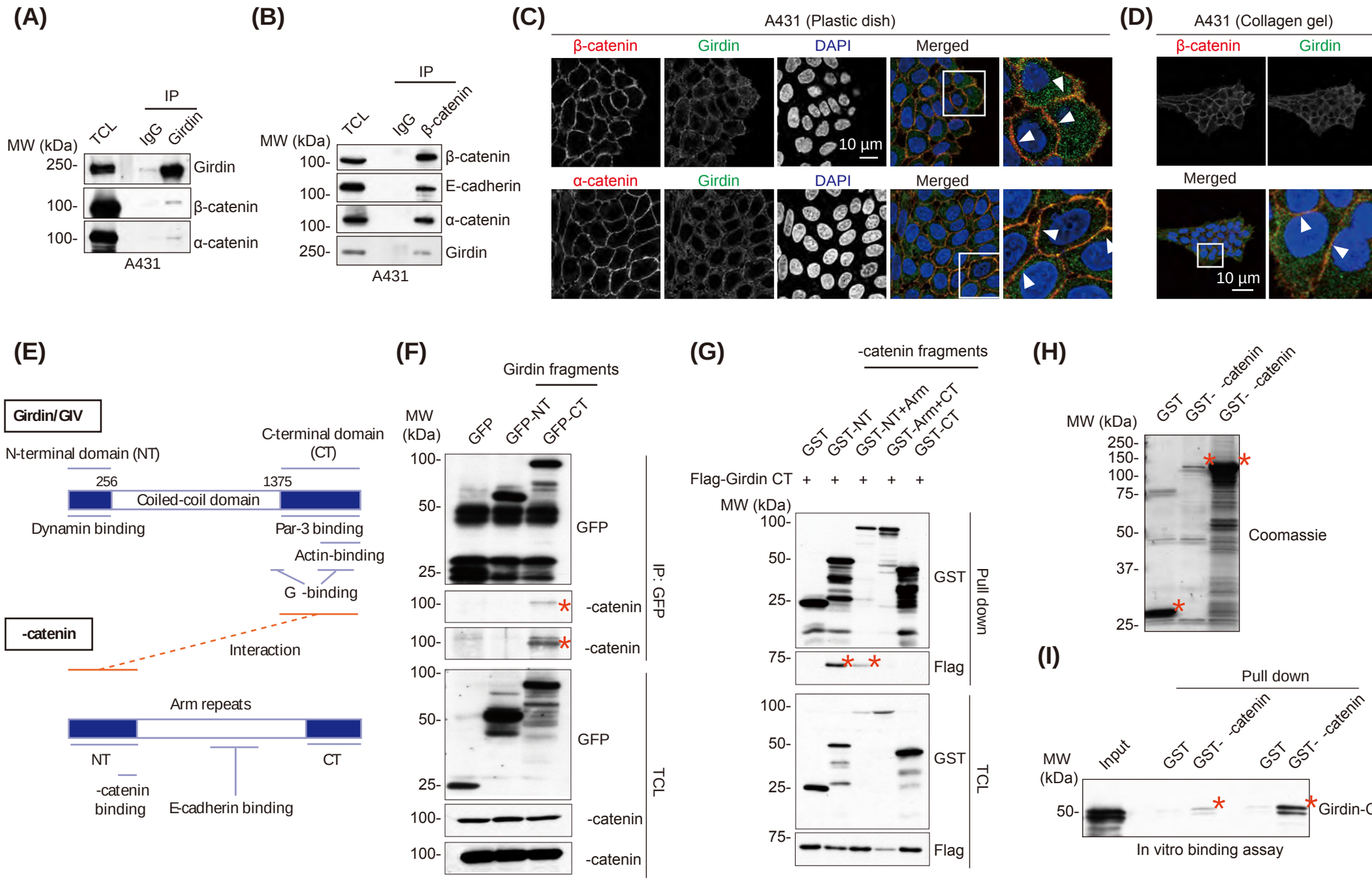
n, number; TS, total score (see **Figure 6E**)

Table S1. Relationship between Girdin expression and the presence of isolated cell clusters (1- 4 cells) at the invasive front of tumors in human squamous cell carcinoma of the skin.

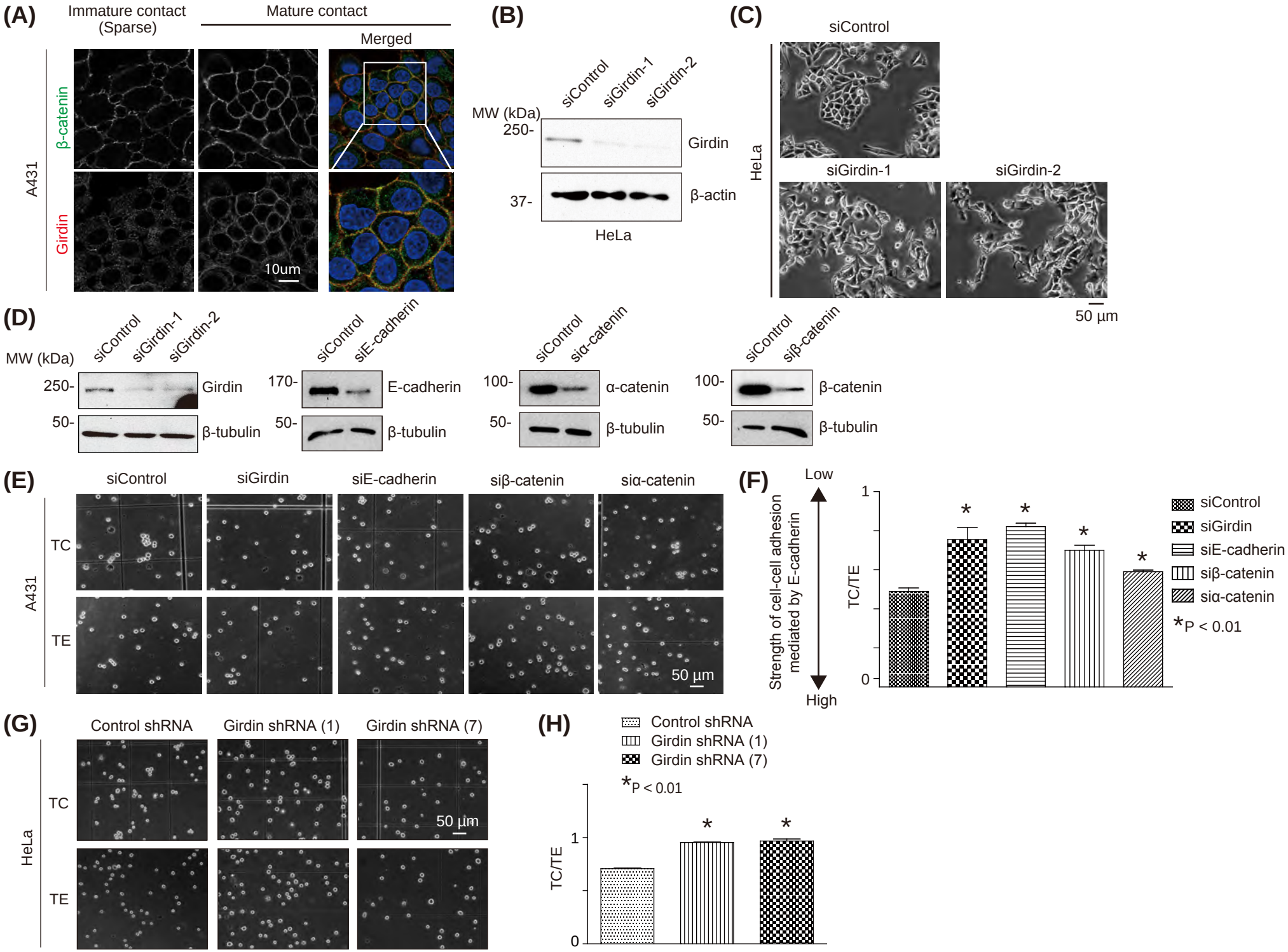
	Total number n	**Girdin positive (TS ≥ 3) n (%)**	**P value**
Cell cluster (1-4 cells) at the invasive front			0.6920
Positive	7	5 (71.4)	
Negative	69	38 (55.1)	



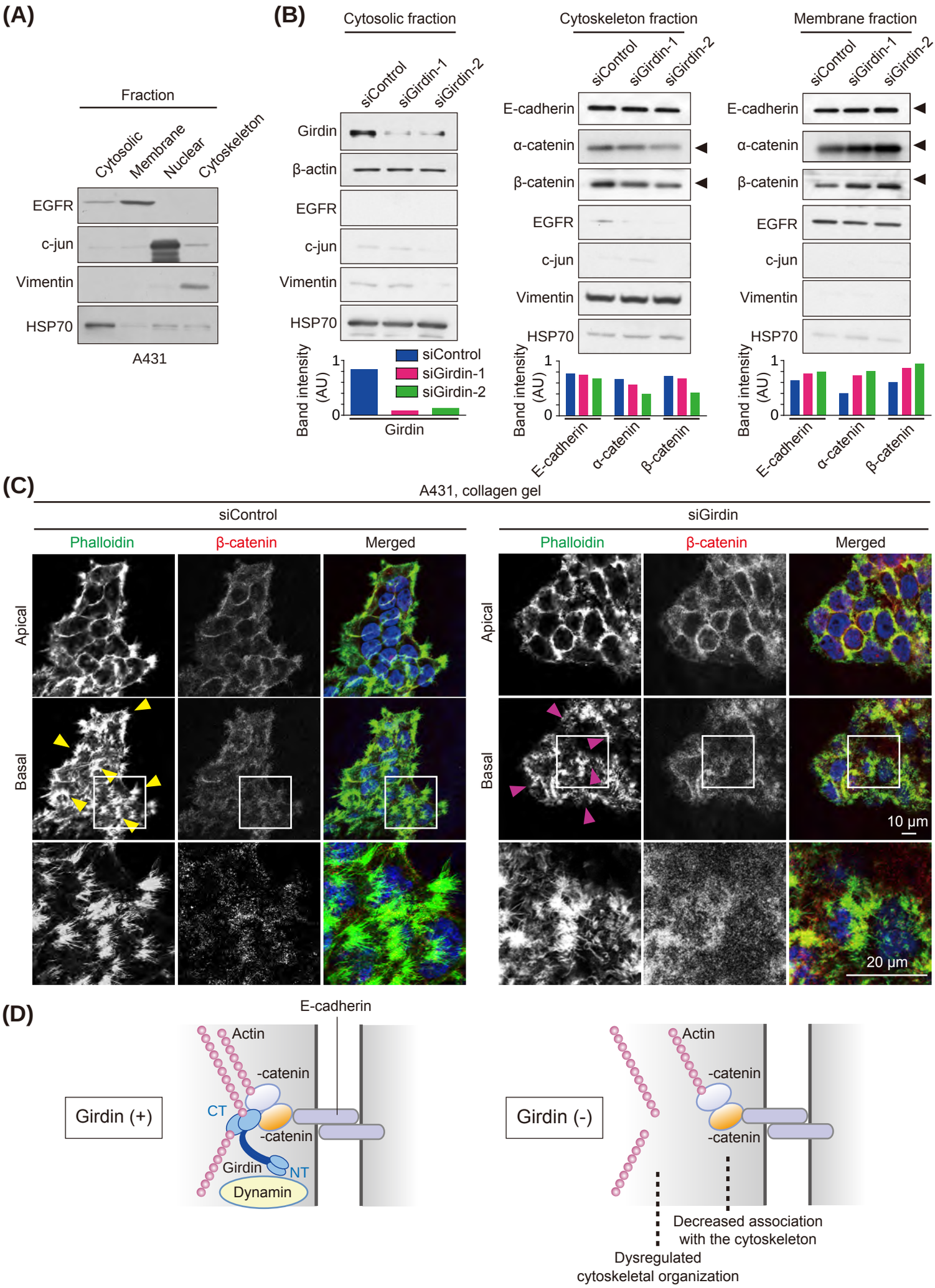
Wang et al., Figure 1



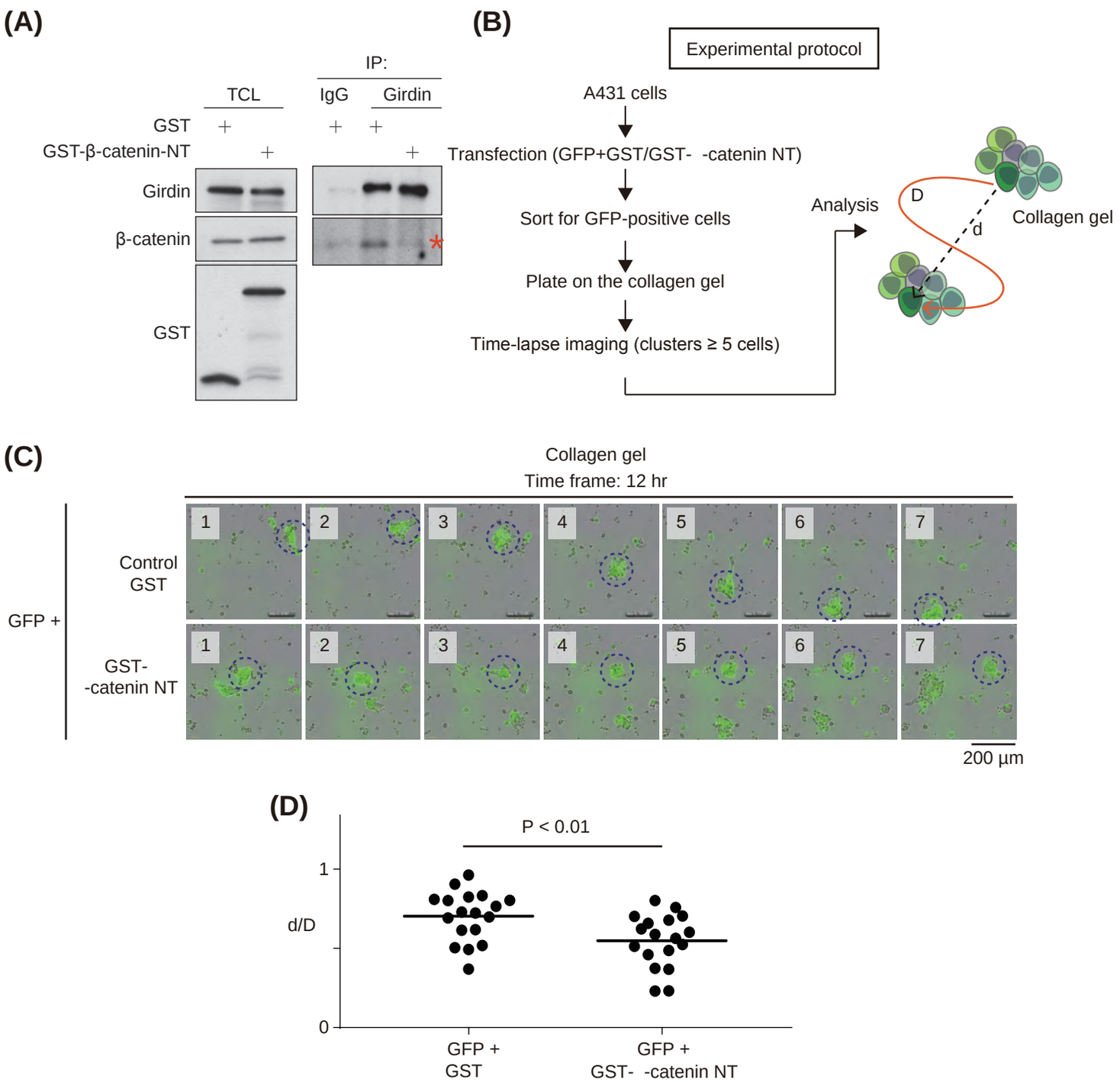
Wang et al., Figure 2



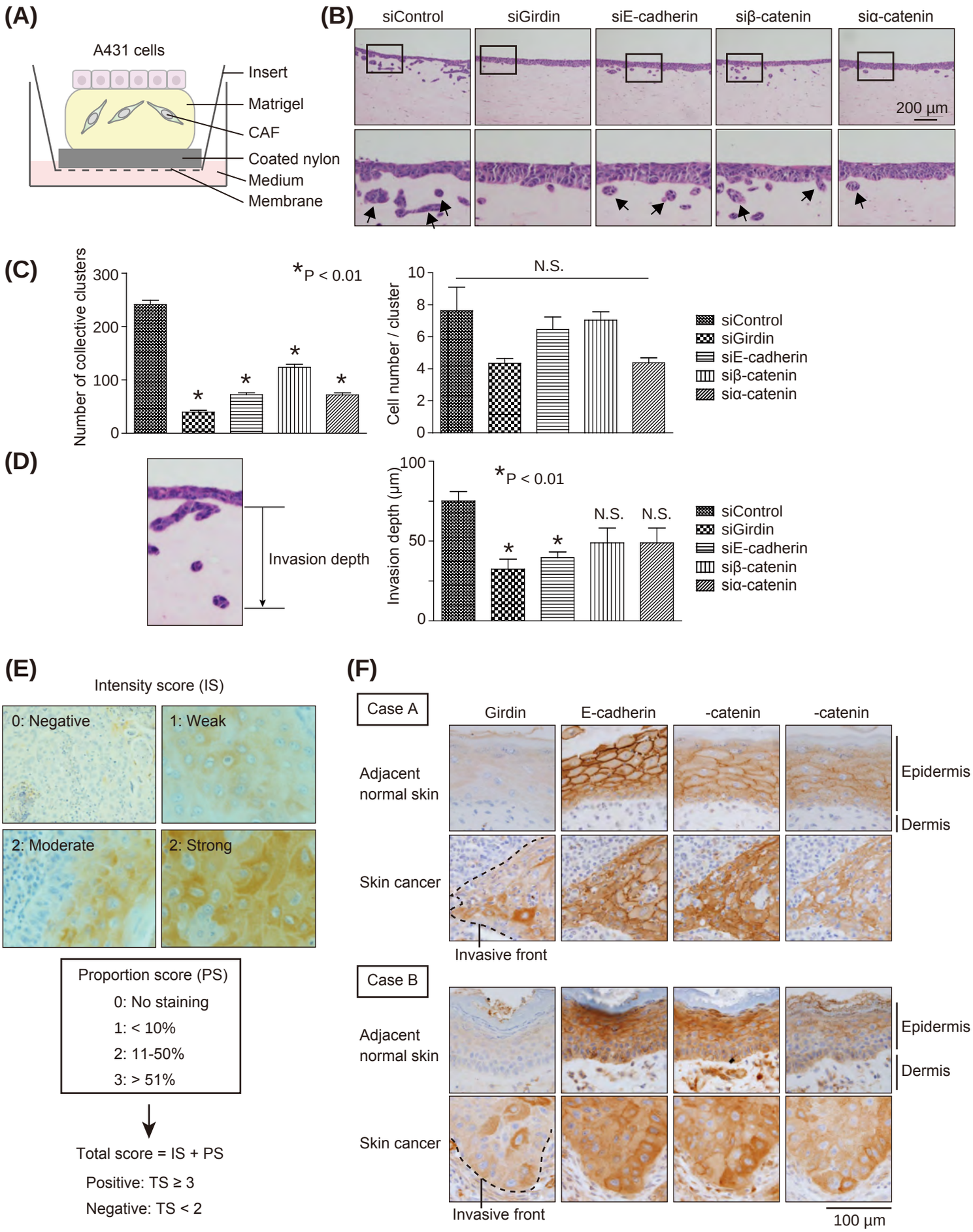
Wang et al., Figure 3



Wang et al., Fig 4



Wang et al., Figure 5



Wang et al., Figure 6

Girdin/GIV Regulates Collective Cancer Cell Migration by Controlling Cell Adhesion and Cytoskeletal Organization

Xiaoze Wang et al.

Supporting Information

1) Figures S1-5

2) Table S1

3) Supporting Materials and Methods

Supporting Materials and Methods

Immunofluorescence

For immunofluorescent staining, cells grown on glass base dishes (Iwaki, Japan) or collagen gels were fixed and permeabilized with ice-cold 100% methanol for 10 min at -20ºC, and then rinsed in ice-cold PBS for 5 min. The cells were washed with PBS 3 times and then incubated with the indicated antibodies, followed by staining with Alexa 488/594-conjugated goat anti-mouse or anti-rabbit IgG (Thermo Fisher Scientific). After washing in PBS, fluorescence was visualized with a confocal laser scanning microscope using the oil-immersion x60 magnification objective (LSM 700, Carl Zeiss). Nuclei were visualized with DAPI.

Mass spectrometry

Total cell lysates from HeLa cells expressing either EGFP or Girdin carboxyl terminal (CT) domain (Girdin-CT) tagged with the Flag-HA-epitope were immunoprecipitated with an anti-Flag antibody (Sigma), followed by elution of the protein complex including mouse Linx-SF with the Flag peptide (Sigma). The eluate was separated by sodium dodecyl-polyacrylamide gel electrophoresis (SDS-PAGE) and detected by silver staining (SilverQuest Silver Staining Kit, Invitrogen) following the manufacturer's instructions. For a shotgun approach to identify proteins in the eluate, whole eluates were digested with Trypsin Gold (Promega) for 16 h at 37°C after reduction, alkylation, demineralization, and concentration, followed by analysis on an Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific).

Immunoprecipitation (IP)

Cells were lysed in a buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.2% NP40, 10% glycerol supplemented with Complete protease inhibitor and PhosSTOP phosphatase inhibitor cocktails (Roche). Lysates were cleared by centrifugation at 12,000 x g for 10 minutes, followed by IP using the indicated antibodies. Samples were separated by SDS-PAGE and analysed by Western blot analyses.

Protein expression and purification

For the purification of glutathione S-transferase (GST) fusion and histidine (His)-tagged proteins, BL21 competent cells (Stratagene, Santa Clara, CA) transformed with GST-α- and β-catenins plasmids were cultured in 2× YT medium containing ampicillin (100 μg/mL) at 37°C until the absorbance at 600 nm (A600) reached 0.6–0.8. Protein expression was induced by adding 100 μM isopropyl beta-D-thiogalactoside (IPTG), followed by culture at 25°C for an additional 4 h. The cell pellets were suspended in homogenizing buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM DTT, 10% sucrose) supplemented with complete protease inhibitor cocktail (Roche) and sonicated extensively. The lysates were cleared via centrifugation at 37,000 rpm for 1 h and applied to a column of Glutathione Sepharose 4B beads with a 1-mL bed volume (GE Healthcare) equilibrated with 20 mL of TED buffer (20 mM Tris-HCl, 1 mM EDTA, 1 mM DTT, pH 8.0). The column was washed extensively with 10 mL of TED buffer and the GST fusion protein was eluted using elution buffer (10 mM glutathione in TED buffer), followed by dialysis with TED buffer.

Girdin knockout mice

Generation of Girdin knockout mice and analysis of its phenotype were described previously.[23, 24, 27] All animal protocols were approved by the Animal Care and Use Committee of Nagoya University Graduate School of Medicine. All in vivo experiments were performed in compliance with Nagoya University's Animal Facility regulations.

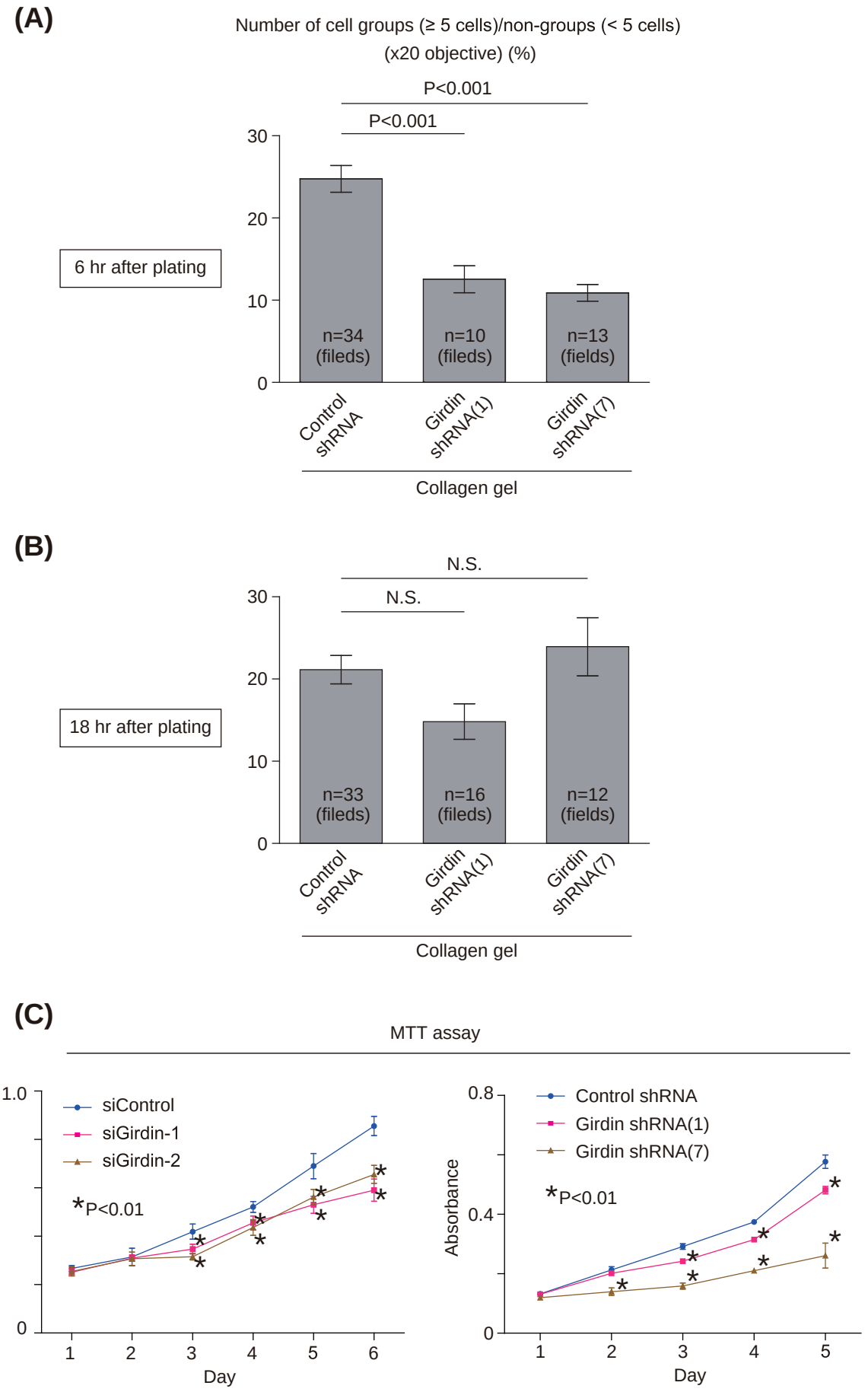

(A)
Number of cell groups (≥ 5 cells)/non-groups (< 5 cells)
(x20 objective) (%)
P<0.001
P<0.001
6 hr after plating
n=34
(fileds)
n=10
(fileds)
n=13
(fields)
Control
shRNA
Girdin
shRNA(1)
Girdin
shRNA(7)
Collagen gel
(B)
N.S.
N.S.
18 hr after plating
n=33
(fileds)
n=16
(fileds)
n=12
(fields)
Control
shRNA
Girdin
shRNA(1)
Girdin
shRNA(7)
Collagen gel
(C)
MTT assay
siControl
siGirdin-1
siGirdin-2
*P<0.01
Absorbance
Day
Control shRNA
Girdin shRNA(1)
Girdin shRNA(7)
*P<0.01
Absorbance
Day

Figure S1

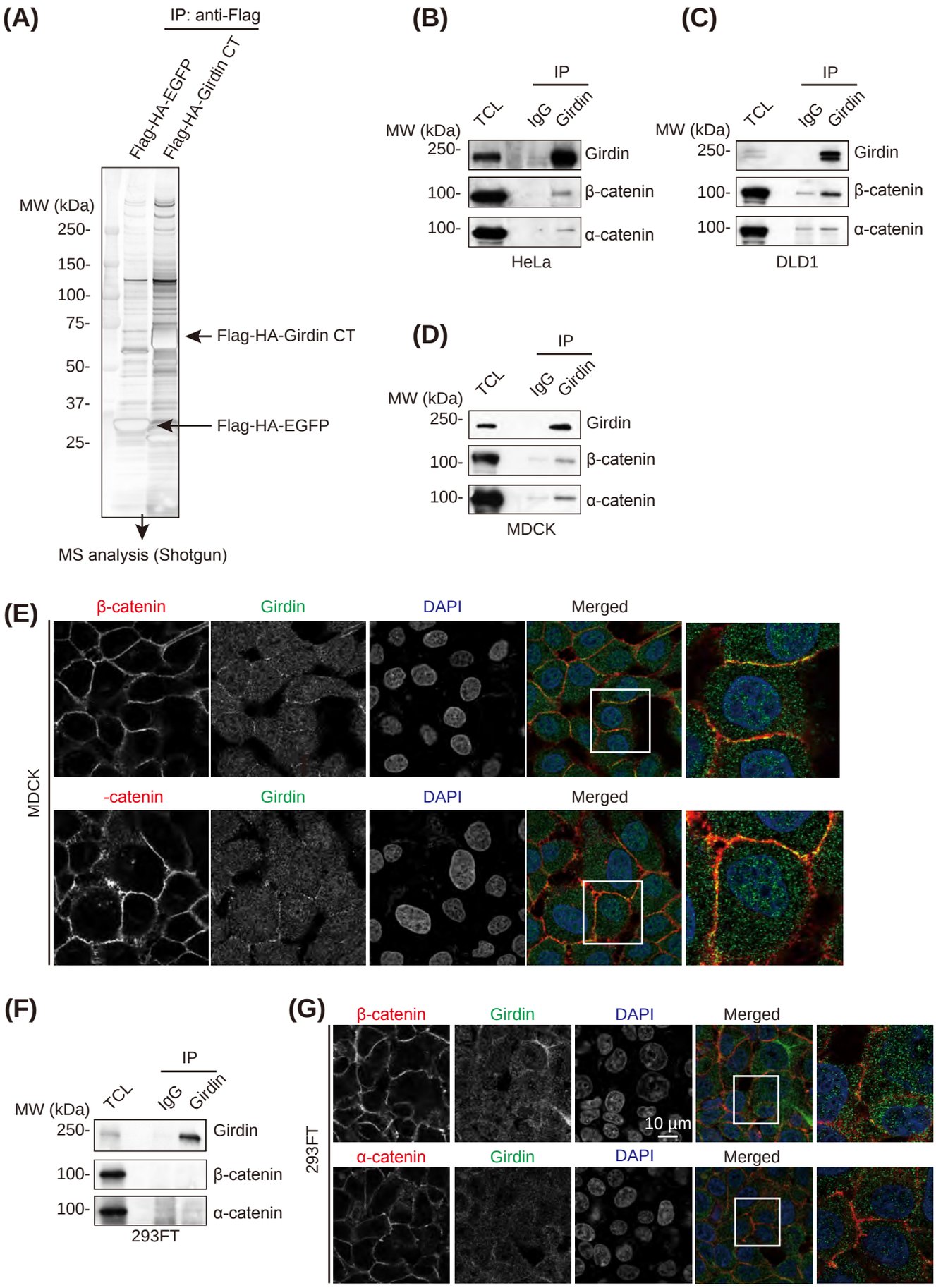

(A)
IP: anti-Flag
Flag-HA-EGFP
Flag-HA-Girdin CT
MW (kDa)
250-
150-
100-
75-
50-
37-
25-
Flag-HA-Girdin CT
Flag-HA-EGFP
MS analysis (Shotgun)
(B)
IP
TCL
IgG
Girdin
MW (kDa)
250-
100-
100-
Girdin
β-catenin
α-catenin
HeLa
(C)
IP
TCL
IgG
Girdin
MW (kDa)
250-
100-
100-
Girdin
β-catenin
α-catenin
DLD1
(D)
IP
TCL
IgG
Girdin
MW (kDa)
250-
100-
100-
Girdin
β-catenin
α-catenin
MDCK
(E)
β-catenin
Girdin
DAPI
Merged
MDCK
-catenin
Girdin
DAPI
Merged
(F)
IP
TCL
IgG
Girdin
MW (kDa)
250-
100-
100-
Girdin
β-catenin
α-catenin
293FT
(G)
β-catenin
Girdin
DAPI
Merged
293FT
10 μm
α-catenin
Girdin
DAPI
Merged

Figure S2

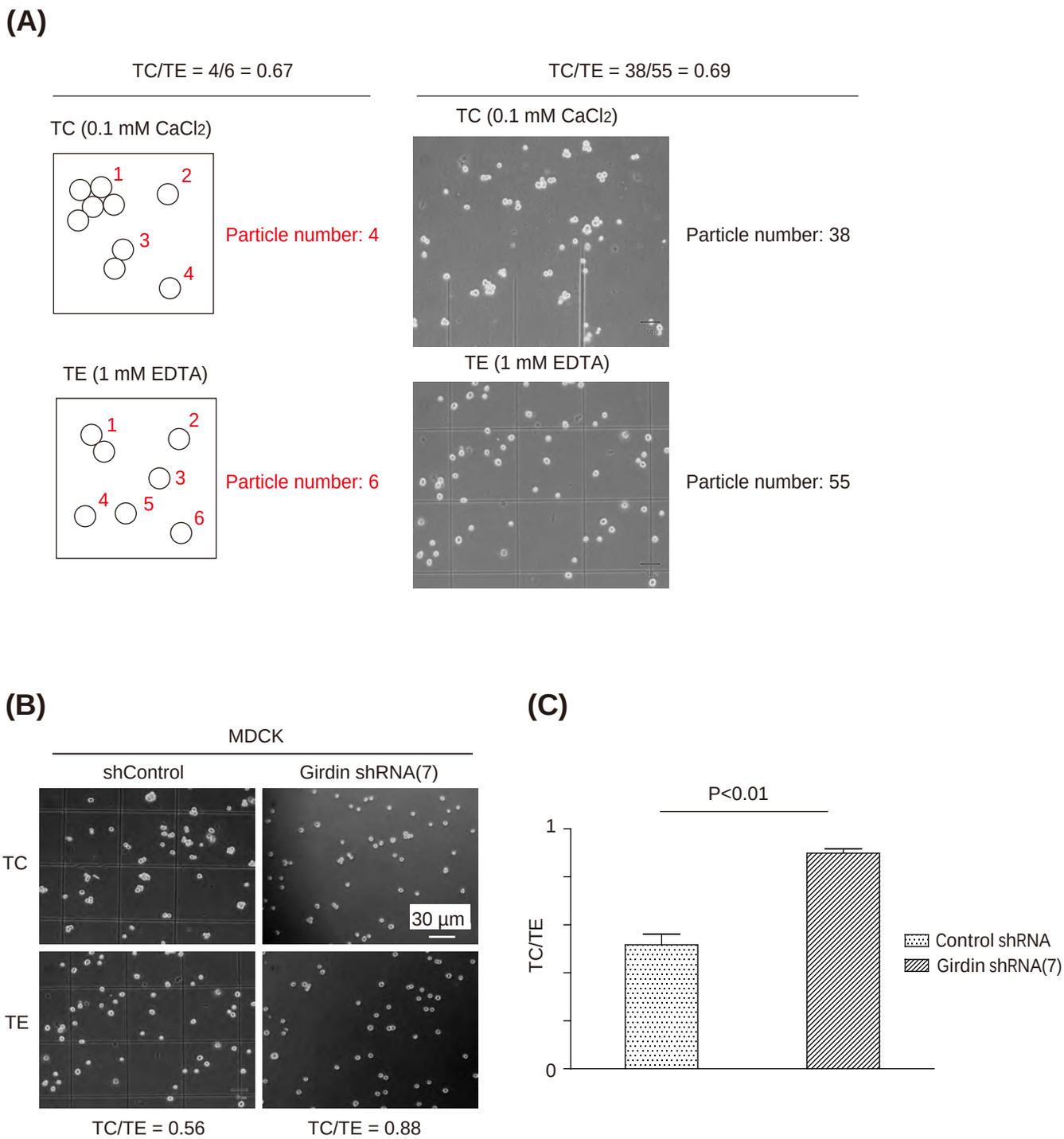

(A)
TC/TE = 4/6 = 0.67
TC/TE = 38/55 = 0.69
TC (0.1 mM CaCl2)
1
2
3
4
Particle number: 4
TC (0.1 mM CaCl2)
Particle number: 38
TE (1 mM EDTA)
1
2
3
4
5
6
Particle number: 6
TE (1 mM EDTA)
Particle number: 55
(B)
MDCK
shControl
Girdin shRNA(7)
TC
TE
30 µm
TC/TE = 0.56
TC/TE = 0.88
(C)
P<0.01
1
0
TC/TE
Control shRNA
Girdin shRNA(7)

Figure S3

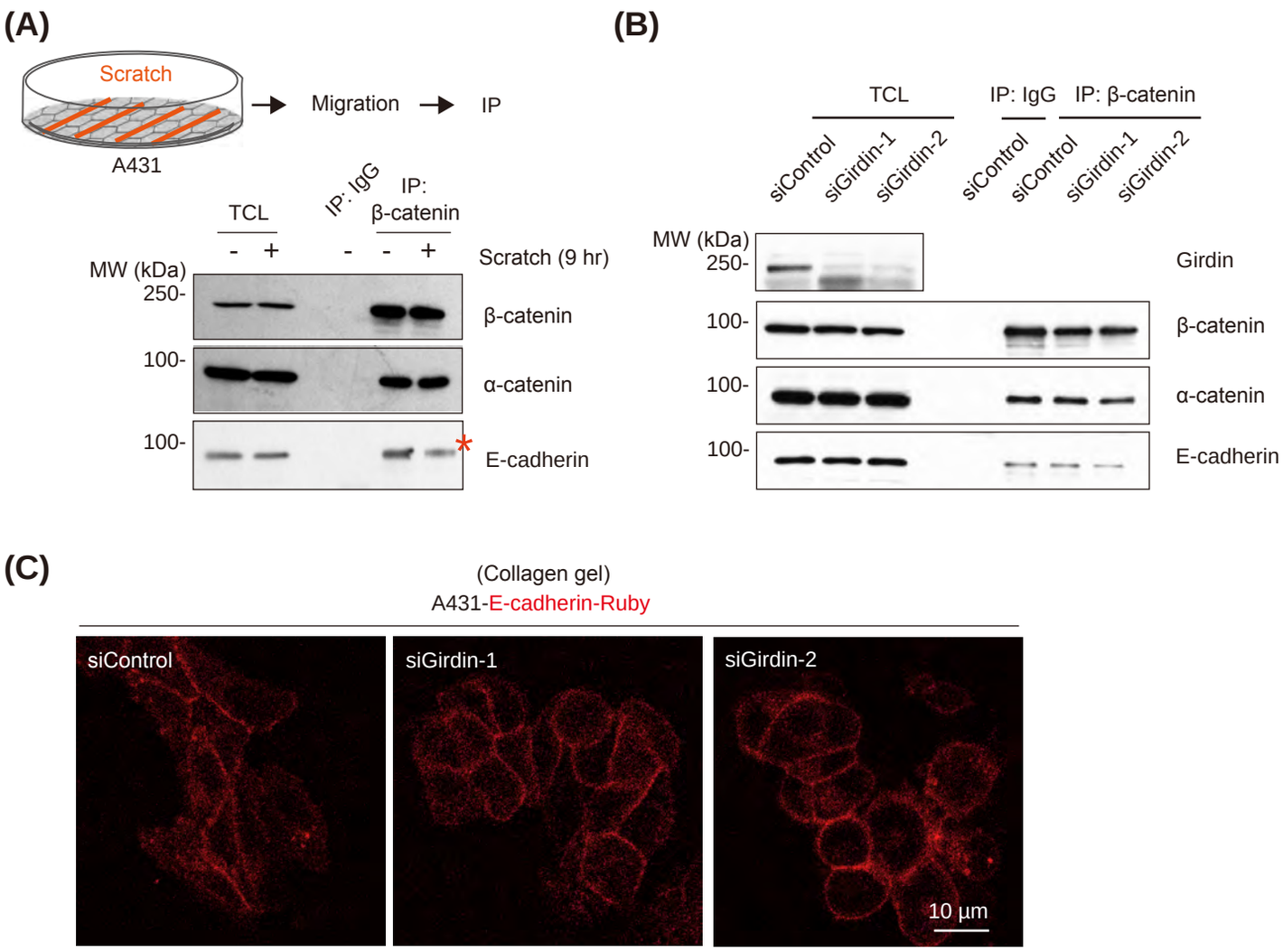

(A)
Scratch
Migration
IP
A431
TCL
IP: IgG
IP: β-catenin
- +
- - +
Scratch (9 hr)
MW (kDa)
250-
β-catenin
100-
α-catenin
100-
E-cadherin
*
(B)
TCL
IP: IgG
IP: β-catenin
siControl
siGirdin-1
siGirdin-2
siControl
siControl
siGirdin-1
siGirdin-2
MW (kDa)
250-
Girdin
100-
β-catenin
100-
α-catenin
100-
E-cadherin
(C)
(Collagen gel)
A431-E-cadherin-Ruby
siControl
siGirdin-1
siGirdin-2
10 µm

Figure S4

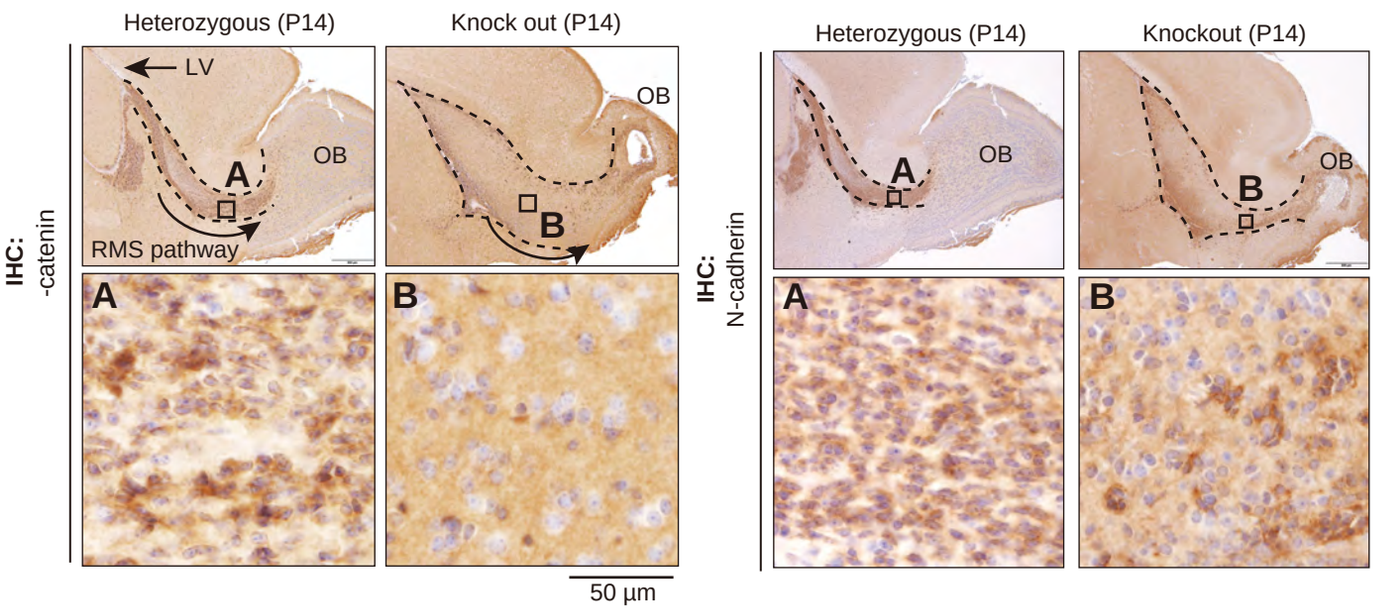

Heterozygous (P14)
Knock out (P14)
LV
OB
A
RMS pathway
B
IHC:
-catenin
50 µm
Heterozygous (P14)
Knockout (P14)
IHC:
N-cadherin

Figure S5