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Girdin is phosphorylated on tyrosine 1798 when associated with structures required for migration

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ABSTRACT

The mammalian protein Girdin interacts with several key molecules such as actin, and it functions as a regulator of the cytoskeleton. Silencing of Girdin mRNA results in defective migration in a variety of cultured cells. Moreover, knockout of Girdin causes phenotypes related to defective migration, including hypoplasia of olfactory bulbs and a widened rostral migratory stream (RMS) in mice. To elucidate the molecular basis underlying cellular migration, we generated site- and phosphorylation state-specific antibodies against human Girdin peptides carrying four putative phosphorylation sites (serine1386 [S1386], S1416, tyrosine1764 [Y1764] and Y1798) that had been identified by mutagenesis analyses or mass spectrometric studies. We found that these residues were phosphorylated in an epidermal growth factor (EGF)-dependent manner. Among the four antibodies we developed, the antibody that targeted Girdin when phosphorylated at Y1798 (pY1798) worked well for immunohistochemistry of paraffin-embedded tissues as well as for cultured cells. Immunocytochemistry of HEK293FT cells transfected with an EGF receptor expression plasmid exhibited punctate signals with pY1798. These signals colocalized with those of endocytosed EGF receptors after EGF stimulation. Signals from pY1798 were also observed on lamellipodia, filopodia, focal adhesion and stress fibers in NIH3T3 cells under conventional culture conditions. Immunohistochemistry of paraffin-embedded mouse brain at P14 using anti-pY1798 antibody displayed signals at the hilum-side (internal side) of the dentate gyrus of the hippocampus, the RMS, the accessory olfactory bulb and the olfactory bulb in which Girdin expression was detected. Primary culture of RMS neurons showed punctate signals of pY1798 at the tips of leading processes as well as in the cytoplasm, whereas no signals were observed when neurons were treated with Src inhibitor, PP2. Our data revealed the changes in the phosphorylation status of Y1798 in Girdin when it associated with migration-related structures *in vitro* and *in vivo*.

1. Introduction

Girdin (also known as ccdc88a/GIV) was first identified in 2005 and was shown to interact with Akt/PKB, actin, microtubules, and Gα members [1–4]. In 2005, our group revealed possible involvement of Girdin in cell migration and stress fiber formation by silencing Girdin in Vero cells [1]. Involvement of Girdin in migratory ability was also supported by studies using human umbilical vein endothelial cells (HUVEC) [5] and cancer cells [6]. Despite the differences of methodology between individual experiments, common observations *in vitro* were that Girdin contributed to actin reorganization, leading to cell migration. In addition, Girdin-deficient mice demonstrated specific phenotypes, including a widened rostral migratory stream (RMS) and hypoplasia of the olfactory bulb [7,8]. These findings suggested that Girdin played a role in cell migration *in vivo*, at least in subpopulations of neurons.

Abbreviations: EGF, epidermal growth factor; RMS, rostral migratory stream..

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Not only the expression levels of Girdin, but also its post-translational modifications may also modulate cell migration. In fact, phosphorylation of Girdin at serine 1416 (S1416) was observed at the lamellipodia of migrating Vero cells, and mutation of S1416 in Girdin significantly impaired cell migration *in vitro* [1]. However, mice bearing serine/alanine substitution at 1416 (SA mutant mice) had normal viability and no gross anatomical change [7]. In contrast, Girdin-deficient mice exhibited complete pre-weaning lethality with severe anomalies. The lack of macroscopic abnormalities in SA mutant mice implied that ablation of S1416 phosphorylation was insufficient to disrupt whole functions of Girdin. Thus, we expanded the range of investigation of Girdin phosphorylation. Serine 1386 (S1386), tyrosine 1764 (Y1764), and Y1798 were chosen for further analyses. S1386 was identified by mass-spectrometry [9] and was selected from top-scoring results in a phosphorylation site search engine, PhosphositePlus (Cell Signaling Technology, Danvers, MA, http://www.phosphosite.org). Y1764 and Y1798 were identified as phosphorylation sites by EGF receptor and Src kinase [10].

In this study, we generated new rabbit polyclonal site- and phosphorylation state-specific antibodies that recognized phosphorylated S1386, Y1764, or Y1798, and we investigated the roles of Girdin phosphorylation in cell migration.

2. Materials and methods

2.1. Generation of anti-Girdin antibodies

Anti-phospho-S1416 Girdin antibodies were previously described [1]. To develop new anti-site and phosphorylation state-specific Girdin antibodies at S1386, Y1764, and Y1798, peptide sequences (Fig. 1A human sequences) were designed and both phosphorylated-peptides and nonphosphorylated-peptides were synthesized. Cysteine residues were added to the N-terminus of the synthesized phosphorylated peptides. A carrier protein (bovine thyroglobulin) was subsequently added to the cysteine residue using N-[e-maleimidocaproyloxy]-succinimide ester (EMCS) (Dojindo, Kumamoto, Japan) as a cross-linking agent. The resultant immunogen solution (0.5 mL) was mixed with adjuvant solution (0.5 mL) and subcutaneously administered to each rabbit ($n \geq 3$ per each peptide sequence).

2.2. Dot blot assay

Peptide samples (non-phospho-S1386, pS1386, non-phospho-Y1764, pY1764, non-phospho-Y1798, and pY1798) were reconstituted with distilled water to make 1 μg/μL peptide solutions. Five microliter of methanol was pipetted and placed on dry polyvinylidene difluoride (PVDF) membranes (Immobilon-P, IPVH00010, Millipore, Billerica MA), and 2 μL of peptide solution (2 μg peptide) was immediately added onto the methanol drop. The peptide-bound membrane was treated with 1:50 diluted rabbit antisera, and incubated at 4 °C overnight. Membrane-bound rabbit antibodies were detected with 1:5000 diluted polyclonal swine anti-rabbit immunoglobulins/HRP (P0399, Dako, Glostrup, Demark) and Amersham ECL Western Blotting Detection Reagents (RPN2106, GE Healthcare, Little Chalfont, UK).

2.3. Generation of Girdin phosphorylation-deficient constructs

Mutagenesis to generate full-length Girdin phosphorylation-deficient mutants was performed following the PCR-based mega-primer method using V5-tagged full-length wild-type human Girdin coding sequence on a pCAGGS plasmid (10403 bp) as a template, and using PrimeSTAR Max (Takara Bio, Otsu, Japan) as the DNA polymerase. Targeted serine residues (S1386 or S1416) were replaced with alanine residues and that targeted tyrosine residues (Y1764 or Y1798) were replaced with phenylalanine residues using following primers (Gothic letters represent altered codons).

Cloning sense; 5′-TACATCTTGGCTGGGAACTGGAACA-3′

Cloning antisense; 5′-AGCCAGAAGTCAGATGCTCAAGGGGCT-3′

S1386A sense; 5′-GACCCA **GCG** CCTCCTAGAAGGAGAGGC-3′

S1386A antisense; 5′-AGGAGG **CGC** TGGGTCATAAAATTTGTAT TGATCC-3′

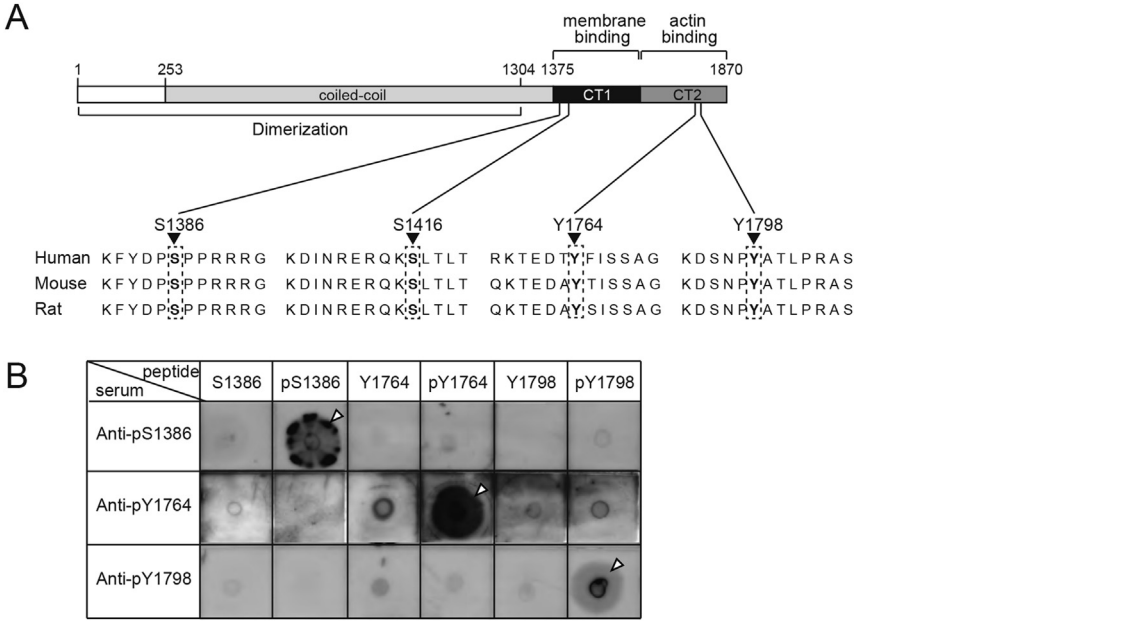

Fig. 1. Generation of site- and phosphorylation state-specific antibodies against four putative phosphorylation sites of human Girdin. **A**. Domain structures of Girdin, and positions of putative phosphorylation sites. Amino acid sequences of human, mouse and rat Girdin are aligned. **B**. Specificities of three newly generated antibodies (anti-pS1386, anti-pY1764, and anti-pY1798) were assessed using dot-blot assay. Peptides including S1386, Y1764, and Y1798 with or without phosphorylation were placed on PVDF membranes and reacted with each antibody. Note that each antibody reacted with the corresponding site-specific phosphorylated peptide (open arrow heads).

S1416A sense; 5′-GCCAGAAA **GCG** CTAACATTAACACCCACCCG-3′
S1416A antisense; 5′-AATGTTAG **CGC** TTTCTGGCGTTCCCGATT-3′
Y1764F sense; 5′-GAAGATACC **TTC** TTCATTAGTTCTGCGGGAA AAC-3′
Y1764F antisense; 5′-ACTAATGAA **GAA** GGTATCTTCAGTTTTTC GAGG-3′
Y1798F sense; 5′-GTAACCCT **TTC** GCAACTTTACCTCGTGCA-3′
Y1798F antisense; 5′-AAAGTTGC **GAA** AGGGTTACTATCTTTTG ATTGTCG-3′

2.4. Immunoprecipitation and immunoblotting

HEK293FT cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 8% FBS and penicillin/streptomycin. Cells (1.5 million) were seeded on 10-cm plates (AGC Techno glass, Tokyo, Japan) and were incubated overnight. After growth medium was refreshed, 5 μg human GirdinV5 pCAGGS or mutant plasmids, 5 μg of human EGF receptor expression vector (pTJNeo-ErbB1) were transfected with 10 μL of X-treamGENE HP DNA Transfection Reagent (Roche Applied Science, Penzberg, Germany). After 48 h of incubation, the growth medium was replaced with serum-free DMEM. After overnight serum starvation, EGF (final concentration, 100 ng/mL, PHG0311, Invitrogen) was added to each dish, and incubated for 10 min at 37 °C. Cells were lysed with 500 μL of IP lysis buffer (for 20 mL; 16.2 mL deionized water, 1 mL 1 M Tris pH 7.6, 0.2 mL 0.5 M EDTA pH 8.0, 0.6 mL 5 M sodium chloride, 2 mL 10% Triton-X100, 4 μL 500 mM sodium orthovanadate, 100 μL 100 mM phenylmethylsulfonyl fluoride in isopropanol, 1 tablet COMPLETE MINI EDTA free (Roche) and 1 tablet PHOSSTOP (Roche)). Immunoprecipitation and immunoblotting were performed as previously described [1].

2.5. Animal experiments

Construction of Girdin−/− mice (LacZ knock-in mice) was previously described [5,7,8]. Briefly, one β-galactosidase (LacZ)-PGK-Neo cassette was inserted five amino acids downstream from the start codon of the mouse Girdin gene, and PGK-Neo was excised *in vivo*. Both The Animal Care and Use Committee (#26323) and The Recombinant DNA Safety Committee (#09–73) of Nagoya University Graduate School of Medicine approved this animal protocol.

2.6. Immunohistochemistry of formalin-fixed, paraffin-embedded brain sections

Brains of wild-type and Girdin−/− mice were perfused with 10% formalin (Muto Pure Chemical, Tokyo, Japan) and postfixed in the same fixative overnight. Paraffin-embedded brains were sectioned at 4 μm, and deparaffinized in xylene, and rehydrated in decreasing concentrations of ethanol. Antigen retrieval was performed in pH 7 antigen retrieval reagent (0.01M sodium citrate, pH 7) at 98 °C for 30 min. Immunohistochemisty was performed as previously described [7].

2.7. β-galactosidase single staining and β-galactosidase immunohistochemical double staining of mouse brain cryosections

β-galactosidase staining was performed by a partially modified version of a method that was previously reported [8]. For double-staining, β-galactosidase singly stained slides were blocked with Protein Block (Dako), and bound with 1:100 diluted goat anti-doublecortin antibodies (sc-8066, Santa Cruz Biotechnology, Santa Cruz, CA) in CangetSignal solution A (NKB-501, TOYOBO, Osaka, Japan) at 4 °C overnight. Color reaction was performed with DAB solution (Dako), and counter-stained with hematoxylin.

2.8. Immunofluorescent detection of phosphorylated endogenous Girdin

Immunofluoresecent staining was performed as previously described [1]. Detailed protocols were described in supplementary information.

3. Results

3.1. Generation of site- and phosphorylation state-specific antibodies against four putative phosphorylation sites of human Girdin

To elucidate the roles of the phosphorylation sites on human Girdin, four sites on the protein were selected. Of these, S1386 phosphorylation (pS1386) was suggested to exist by mass spectrometric studies [9] and by an internet-based protein phosphorylation browser, PhosphoSitePlus (Cell Signaling Technology). Moreover, pS1416 was identified by Enomoto et al., in 2005 [1], and pY1764 and pY1798 were identified as sites phosphorylated by the EGF receptor and by Src kinase [10]. All four of the selected sites are located in the C-terminal domain of Girdin (Fig. 1A). Since anti-pS1416 antibody had previously been developed, antibodies against the remaining three phosphopeptides carrying pS1386, pY1764 or pY1798 were newly generated. As immunogens to sensitize rabbits, 12 − 14 amino acid peptides of human Girdin were selected (Fig. 1A). Amino acid sequences around S1386, S1416 and Y1798 were highly conserved among species, while fluctuation among them was observed for the amino acid sequence around Y1764 (Fig. 1A). The titers and specificities of the developed antisera were estimated with dot blot assays using membrane-bound peptides (phosphorylated or nonphosphorylated). Dot blot assays showed that all tested anti-serum samples detected peptides in a site- and phosphorylation state-specific manner (Fig. 1B).

3.2. Characterization of the site- and phosphorylation state-specific antibodies against human Girdin

To verify the specificity of four different site- and phosphorylation state-specific antibodies against human Girdin, HEK293FT cells were transfected with a human EGF receptor expression vector plus the expression vectors for V5-tagged (C-terminus) human Girdin wild-type or phosphorylation-deficient mutants (S1386A, S1416A, Y1974F, Y1798F, S1386A/S1416A, Y1764F/Y1798F and all four mutations), and treated with EGF. The resulting cell lysates were immunoprecipitated using V5-specific antibodies, followed by immunoblotting with four different site- and phosphorylation state-specific antibodies (anti-pS1386, anti-pS1416, anti-pY1764 and anti-pY1798). Expression of Girdin protein (220 kDa) stayed constant in each transfectant (Fig. 2A), and the specificity of each antibody was confirmed using EGF-stimulated lysates from mutant Girdin-expressing cells (Fig. 2A). These results showed that the four antibodies were site- and phosphorylation-specific as designed.

Time course analyses of phosphorylation revealed that phosphorylation at the four sites increased 30 s after EGF stimulation and reached maximal levels around 5 min (Fig. 2B), indicating that all four sites (S1386, S1416, Y1764, Y1798) underwent phosphorylation in an EGF-dependent manner.

3.3. Subcellular localization of phosphorylated Girdin in cultured cells

To detect the subcellular localization of endogenous phosphorylated Girdin, immunofluorescence was performed using all four antibodies. HEK293FT cells were transfected with EGF receptor

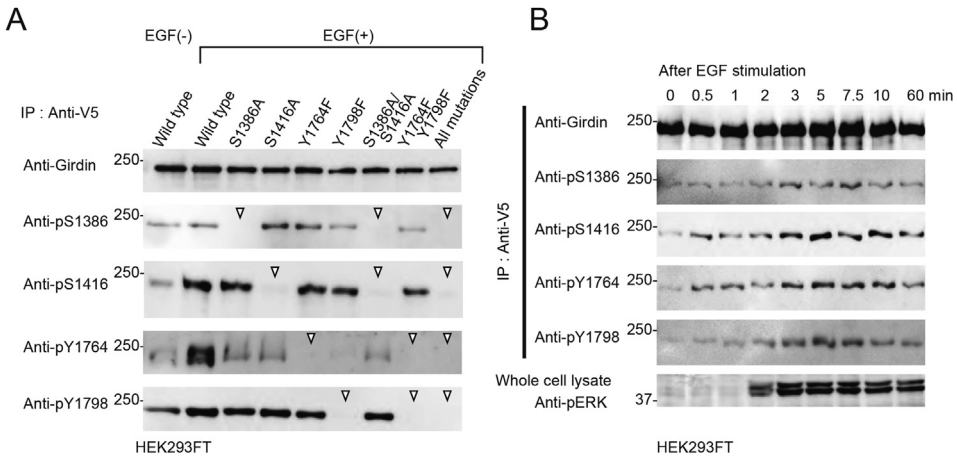

Fig. 2. Specificities of site- and phosphorylation state-specific antibodies determined by immunoblotting. **A**. Single or multiple amino acid substitutions at phosphorylation sites were introduced into the V5-tagged full-length human Girdin plasmid. Serines and tyrosines were substituted with alanine (S1386A, S1416A) and phenylalanine (Y1764F, Y1798F), respectively. HEK293FT cells expressing each of the mutants were treated with EGF for 10 min, and the resulting cell lysates were immunoprecipitated with V5-antibodies, followed by immunoblotting with the indicated antibodies. Absence of phosphorylation signals in mutant sites (open arrowheads) validated specificities of antibodies. **B**. HEK293FT cells were transfected with V5-tagged full-length wild-type human Girdin expression plasmid plus human EGF receptor expression plasmid. Cells were treated with EGF for the indicated times, and the resulting cell lysates were immunoprecipitated with V5-antibodies, followed by immunoblotting with the indicated antibodies. The lysates were separately submitted to immunoblot analysis with anti-phosphorylated ERK antibodies. (anti-pERK).

expression vectors and stimulated with EGF. EGF receptor signals were detected on the cell membrane before EGF stimulation (Fig. 3A, Supplementary figure 1). Four anti-phospho-antibodies showed similar weak punctate signals, mainly in the cytoplasm of unstimulated cells. Interestingly, EGF stimulation significantly increased the frequencies and intensities of punctate signals detected with anti-pY1764 and anti-pY1798 antibodies, and they co-localized with internalized EGF receptor (Fig. 3A). In contrast, the staining patterns with anti-pS1386 or anti-pS1416 antibodies in the transfectants did not significantly change after EGF stimulation, and their staining was not colocalized with internalized EGF receptor (Supplementary figure 1).

To further evaluate Girdin's phosphorylation status in migration related-subcellular structures, NIH3T3 cells were stained with anti-pY1798 antibodies that provided the best staining performance and the highest contrast of signals following EGF stimulation of HEK293FT cells. Staining with anti-pY1798 antibodies in NIH3T3 cells exhibited signals on lamellipodia (Fig. 3C-1), as well as punctate signals in the cytoplasm (Fig. 3B). When these signals were co-stained with phalloidin to visualize F-actin-containing stress fibers, part of the punctate signals in the cytoplasm were detected on stress fibers (Figs. 3C-2, 4). The signals were also detected on the tips of filopodia (Figs. 3B, 3C-3, 4). In addition, pY1798-positive punctate signals were highly co-stained with paxillin, indicating that pY1798-positive signals were located at focal adhesions of NIH3T3 cells (Fig. 3D).

3.4. Immunostaining of mouse brain with anti-pY1798 antibodies

To determine whether Girdin phosphorylation could be detected in animal tissues, immunostaining of formalin fixed and paraffin embedded mouse brain tissues was performed using four anti-phospho-antibodies. Previous studies had already shown that Girdin mRNA and Girdin protein were strongly expressed in mouse brain, and distinctive phenotypes appeared in the brains of Girdin knockout mice [7,8,11]. While anti-S1386, anti-S1416 and anti-Y1764 antibodies did not work well for the staining of formalin-fixed mouse tissues, anti-pY1798 antibodies strongly stained the dentate gyrus of the hippocampus, the RMS and the olfactory bulb in the wild-type mouse brain at P14 (Fig. 4A, upper panels) where Girdin expression had been reported [7,8,11]. In contrast, almost no signal was observed in the corresponding regions in Girdin knockout brains (Fig. 4A, lower panels), which verified the reliability of anti-pY1798 antibodies in formalin fixed and paraffin embedded tissues.

In addition to cytoplasmic staining, reticular signals at the hilum-side of the dentate gyrus in wild-type mice were observed between granular cells at P10 (Supplementary figure 2A). Similar staining patterns were also detected in accessory olfactory bulbs and the nerve fiber layer of the olfactory bulb (data not shown). In the RMS and the internal granular layer of the olfactory bulb, the cytoplasm and protrusions of large cells with large and round nuclei (seemingly migrating neuroblasts or immature neurons with dendrites) were clearly stained (Fig. 4A).

β-galactosidase staining of the brains of heterozygous Girdin LacZ knock-in mice was carried out at P10. β-galactosidase single staining showed high endogenous Girdin promoter activity on the molecular layer side of the granular layer rather than in the hilum side of the granular layer (Supplementary figure 2B, left panels). Using double staining of β-galactosidase with anti-doublecortin antibodies, β-galactosidase signals showed that endogenous Girdin transcription was barely colocalized with doublecortin signals located in the subgranular zone (SGZ) of the dentate gyrus (Supplementary figure 2B, right panels). Thus, it seems likely that at least some of the strong pY1798 signals on the hilum-side of the dentate gyrus, which showed a reticular staining pattern, were derived from mossy fibers originating from mature granule cells on the molecular layer side of the dentate gyrus.

To further clarify the subcellular distribution of pY1798 signals in neurons, cultured RMS explants were stained with anti-pY1798 antibodies together with anti-doublecortin antibody. The double immunofluorescence staining showed that the pY1798 signals were punctate and were detected in the cytoplasm whereas doublecortin staining was distributed throughout the cell bodies except the nuclei (Fig. 4B). Signals of pY1798 in the cytoplasm in doublecortin-positive cultured neurons were also detected at the tips of the leading processes (Fig. 4B). Z-stack analyses of confocal microscopic data revealed that the punctate signals were located at submembranous sites (Fig. 4B). When a Src inhibitor, PP2, was added to the culture medium, cell migration of doublecortin-positive neuroblasts from RMS explants was largely impaired, and punctate pY1798 signals in all regions were almost completely eliminated

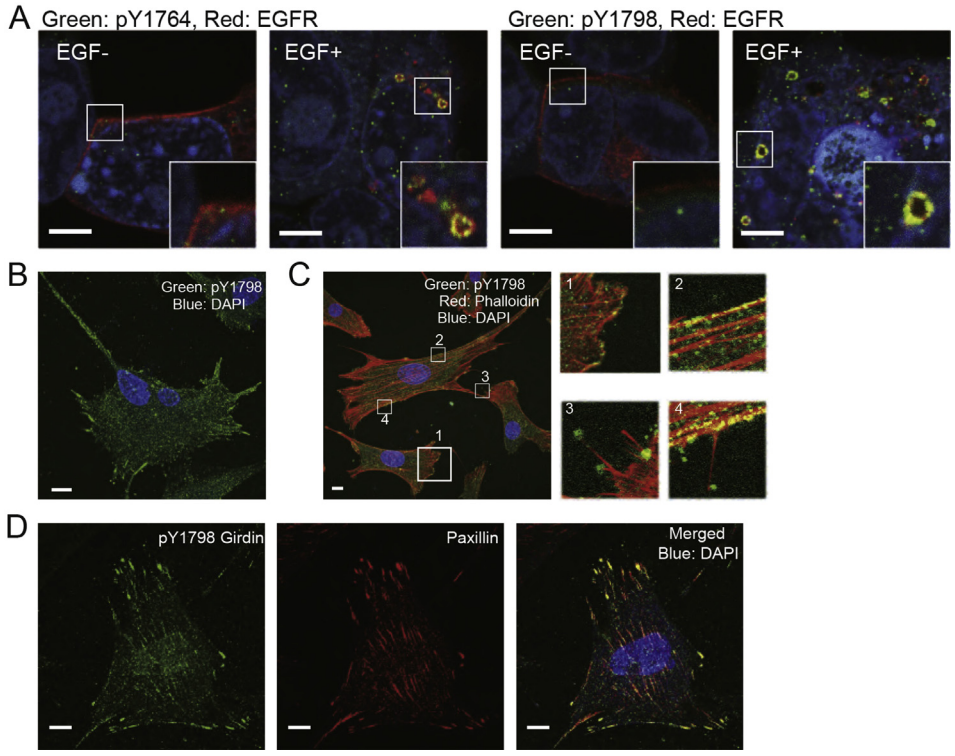

Fig. 3. Subcellular distributions of phosphorylated Girdin in cultured mammalian cells. **A**. Subcellular distribution of phosphorylated endogenous human Girdin in HEK293FT cells transfected with the EGF receptor expression plasmid. Cells were treated with EGF (100 ng/mL) for 15 min, fixed, and immunostained with the designated site- and phosphorylation state-specific Girdin antibodies (rabbit) and anti-EGF receptor antibody (mouse). Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). Co-localization of phosphorylated Girdin with internalized EGF receptor was observed using anti-pY1764 and anti-Y1798 antibodies. Scale bar, 10 μm. **B, C**. Subcellular distribution of Y1798 phosphorylated mouse Girdin in NIH3T3 cells. Punctate signals of pY1798 Girdin were seen in the cytoplasm and filopodia (**B**). To visualize F-actin bundles, cells were stained with phalloidin. Punctate signals of pY1798 Girdin colocalized with phalloidin-positive stress fibers of NIH3T3 cells (**C 2, 4**). Signals of pY1798 were also detected at lamellipodia (**C 1**) and on the tips of filopodia (**C 3, 4**). **D**. Colocalization of pY1798 with paxillin in NIH3T3. Scale bar, 10 μm.

(Fig. 4B), indicating that Y1798 was phosphorylated by Src kinase in RMS neurons.

4. Discussion

In eukaryotic cells, cytoskeletal actin filaments contribute not only to the maintenance of static cellular structures, but also to cell migration. Actin filaments enable cell migration by dynamically changing the cell shape by regulating filamentous actin (F-actin) polymerization and regulating the interaction between actin and actin-binding proteins. Girdin is one such actin-binding protein, and silencing of Girdin inhibits cell migration in certain limited contexts [1,5–7]. Girdin-deficient mice exhibit characteristic migration defects (widened RMS and hypoplasia of the olfactory bulb) in subpopulations of neuronal cells [7,8]. Thus, one can ask how the loss of Girdin leads to defective cell migration. Furthermore, how does Girdin contribute to cell migration when cells are stimulated by growth factors? We assumed that Girdin fine-tunes the tensile force or the extent of actin polymerization through its phosphorylation, as is the case with another actin-binding protein, filamin (ABP280) that reportedly regulates the functions of actin filament through its phosphorylation [12]. To test this assumption, we generated site- and phosphorylation state-specific antibodies against four putative phosphorylation sites (S1386, S1416, Y1764, and Y1798) of Girdin.

Our results using HEK293FT cell lysates clearly showed that phosphorylation at all four sites of Girdin was induced in an EGF-dependent manner, although it remains unknown whether EGF receptor-mediated phosphorylation is direct or indirect. Observation of punctate signals in the cytoplasm is consistent with a previous report [3] that showed the localization of Girdin on intracellular vesicles. The presence of Girdin on intracellular vesicles suggests a high affinity of Girdin for the membrane. In our results with HEK293FT cells, vesicles with phosphorylated Girdin at Y1764 or Y1798 were located within an area contiguous with internalized EGF receptors (Fig. 3A), suggesting that activated- and internalized-EGF receptors directly phosphorylated Girdin.

Anti-pY1798 antibodies also stained multiple subcellular structures, including F-actin-rich leading edges (lamellipodia), filopodia, stress fibers and focal adhesions of NIH3T3 mouse fibroblasts. Recently, Lin et al. reported that phosphorylation of Y1764 and Y1798 in Girdin was induced by the EGF receptor or Src that in turn activated cell migration via actin remodeling [10]. Our results obtained with NIH3T3 cells seem basically compatible with their model. Considering the lack of endogenous EGF receptor in NIH3T3 cells, it seems likely that certain kinases other than EGF receptor phosphorylate Y1798 of Girdin. Signals at focal adhesions of NIH3T3 cells imply that Girdin is phosphorylated by kinases present in focal adhesion complexes via outside-in integrin signaling from the extracellular matrix [13].

Besides subcellular structures in cultured cells, the histological distribution of Girdin phosphorylated at Y1798 was concordant with the distribution of migrating neurons in adult mouse brain, including the olfactory bulb, the RMS and the hippocampal dentate gyrus (Fig. 4A). Girdin knockout mice have exhibited abnormalities in these brain regions [7,11]. Lack of pY1798 signals at the corresponding regions of Girdin knockout mice have verified the presence of Girdin phosphorylation in wild-type mice under physiological conditions. Staining of RMS explants with anti-pY1798 Girdin showed punctate signals mostly in the soma and

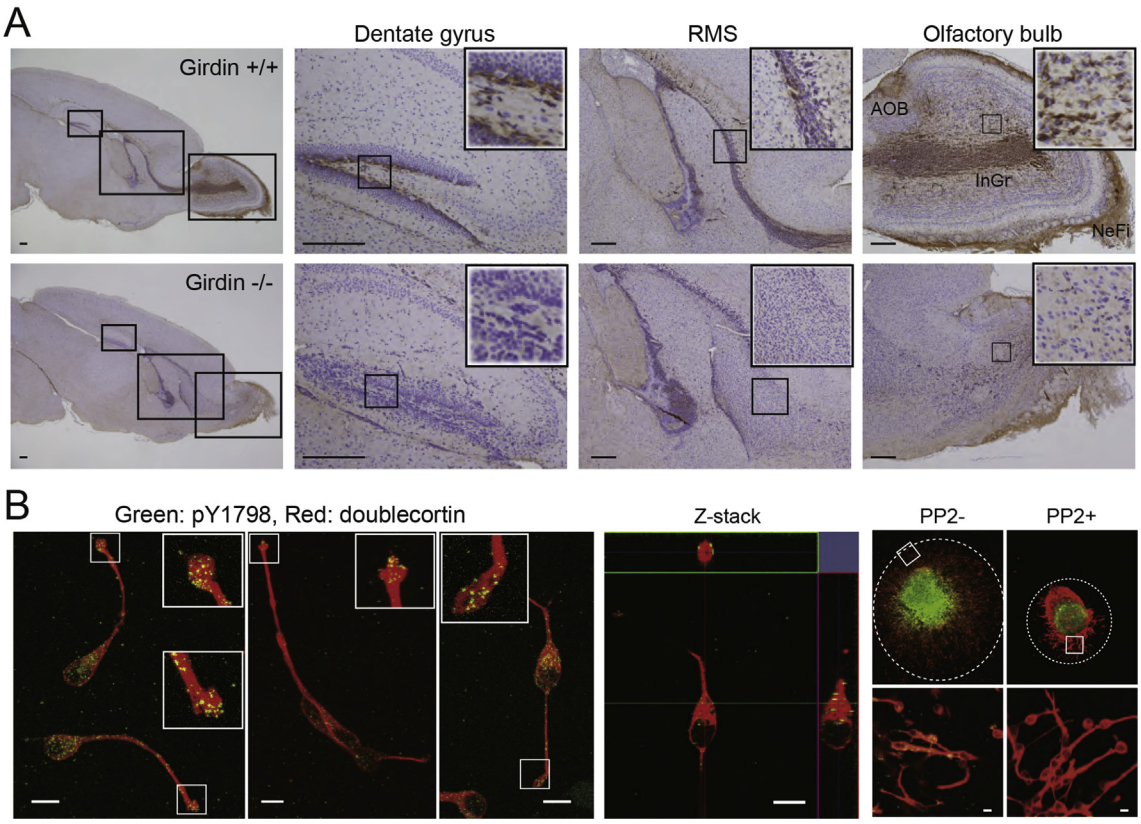

Fig. 4. Distribution of pY1798 Girdin in mouse brain. **A**. Paraffin-embedded brain tissues from P14 Girdin wild-type mouse (Girdin +/+) or Girdin LacZ knock-in mouse (Girdin −/−) were reacted with pY1798 Girdin antibodies, visualized with 3,3'-dianimobenzidine (DAB) and counterstained with hematoxylin. Panels at the far left show sagittal images under low magnification. Boxed regions were individually magnified on the right. Signals (brown color) were observed at the dentate gyrus of the hippocampus, the RMS, and the olfactory bulb of wild-type Girdin mice (Girdin +/+), whereas signals were barely visible in Girdin LacZ knock-in mice (Girdin −/−). Signals in the dentate gyrus were located at the hilum-side of the granule cell layer. Signals along the RMS were derived from migrating neuroblasts. The majority of signals in the olfactory bulb were seen at internal granular layers (InGr), nerve fiber layers (NeFi) and the accessory olfactory bulb (AOB). Scale bars, 200 μm. **B**. Immunofluorescence of the explants from mouse RMS using doublecortin (red) and pY1798 Girdin antibodies (yellow). Z-stack images are also shown. The RMS explants were treated with or without a Src inhibitor, PP2, and stained with anti-doublecortin and anti-pY1798 antibodies (far right). Note that PP2 largely inhibited both cell migration and pY1798 Girdin signals. Dotted lines show migrating area of neuroblasts. Scale bars, 10 μm.

at the tips of leading processes of cultured neurons. Treatment with a Src inhibitor, PP2, inhibited the migration of neurons from RMS explants and Girdin Y1798 phosphorylation. This result also suggests a relationship between cell migration and Girdin phosphorylation.

There remain unanswered questions over the phenotypes of Girdin knockout mice, one of which is differences in migratory phenotype between the dentate gyrus and RMS. While widened RMS and hypoplasia of the olfactory bulb of Girdin knockout mouse indicate migration-defective neuroblasts, why do granule cells in the dentate gyrus of the hippocampus show, seemingly, 'over-migration' during development? In this study, we succeeded in segregating signals of Girdin in two different layers of the dentate gyrus using direct detection of phosphorylated Girdin together with the detection of Girdin promoter activity with LacZ staining. Phosphorylated Y1798 of Girdin was observed at the innermost part (hilum-side) of the granular layer, whereas high levels of endogenous Girdin promoter activity were observed at the outermost part (molecular layer-side) of the granular layer at P10 when both early-born granule cells and late-born granule cells are easily viewable [14]. Combined with the fact that Girdin LacZ-positive cells and doublecortin-positive cells were mutually exclusive in the granular layer, signals of phosphorylated Girdin at the innermost part of the granular layer appeared to be partly derived from the proximal part of mossy fibers of LacZ-positive cells. As we previously reported, granule cell dispersion in the dentate gyrus is associated with mesial-temporal lobe epilepsy (MTLE), and an MTLE-like phenotype was actually observed in mutant Girdin mice (data not shown and [8]). All these facts suggest that the phenotype of granule cells may be interpreted as a non-cell autonomous effect of mature neurons by an epilepsy-related change of microenvironment [15], rather than simple 'over-migration'.

In summary, we developed and verified site- and phosphorylation state-specific rabbit polyclonal antibodies against four sites of Girdin (S1386, S1416, Y1764, and Y1798). Using these antibodies, we identified simultaneous changes of phosphorylation state at multiple sites of Girdin, and particularly pY1798 signals in migration-related structures. These observations suggest a possible role for phosphorylation of Y1798 in Girdin in modulating cell migration via reorganization of the actin cytoskeleton. Further *in vivo* analyses are necessary to elucidate the physiological significance of Girdin phosphorylation.

Conflict of interest

None.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this chapter can be found at http://dx.doi.org/10.1016/j.bbrc.2015.02.065.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.02.065.

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