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Potential involvement of kinesin-1 in the regulation of subcellular localization of Girdin

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ABSTRACT

Girdin is an actin-binding protein that has multiple functions in postnatal neural development and cancer progression. We previously showed that Girdin is a regulator of migration for neuroblasts born from neural stem cells in the subventricular zone (SVZ) and the dentate gyrus of the hippocampus in the postnatal brain. Despite a growing list of Girdin-interacting proteins, the mechanism of Girdin-mediated migration has not been fully elucidated. Girdin interacts with Disrupted-In-Schizophrenia 1 and partitioning-defective 3, both of which have been shown to interact with the kinesin microtubule motor proteins. Based on this, we have identified that Girdin also interacts with kinesin-1, a member of neuronal kinesin proteins. Although a direct interaction of Girdin and kinesin-1 has not been determined, it is of interest to find that Girdin loss-of-function mutant mice with the mutation of a basic amino acid residue-rich region (Basic mut mice) exhibit limited interaction with kinesin-1. Furthermore, expression of a kinesin-1 mutant with motor defects, leads to Girdin mislocalization. Finally, consistent with previous studies on the role of kinesin proteins in trafficking a cell–cell adhesion molecule N-cadherin, Basic mut mice showed an aberrant expression pattern of N-cadherin in migrating SVZ neuroblasts. These findings suggest a potential role of Girdin/kinesin-1 interaction in the regulation of neuroblast migration in the postnatal brain.

1. Introduction

Girdin, an actin-binding protein that we previously identified, has interesting regulatory functions during migration of neuroblasts and endothelial cells born in the postnatal period [1–4]. In pathological conditions, it is also expressed in some types of malignant tumor tissues and vascular retinas, where it regulates migration of cancer cells, cancer-associated fibroblasts, and nascent endothelial cells [5–8]. Girdin-deficient mice survive embryonic development [2], which is in contrast to a general consensus that mice deficient for most of the fundamental regulators of cell migration exhibit embryonic lethality or severe phenotypic abnormalities in embryonic growth and development.

We and others have identified several Girdin-interacting proteins, which include the Gα proteins [9,10], dynamin [11], partitioning-defective gene 3 (Par-3), which is a regulator of cell polarization [12], and Disrupted-In-Schizophrenia 1 (DISC1), which is a neuronal scaffold protein whose dysfunction is responsible for development of major mental disorders [3]. However, the mechanisms of Girdin-regulated cell migration have not yet been fully determined. Moreover, functions of Girdin-interacting proteins do not necessarily reflect cytoarchitecture alterations in the postnatal brain of Girdin-deficient mice [3,4]. For example, the mechanism of how Girdin deficiency results in defective collective migration of neuroblasts has remained elusive. In wild-type mice, neuroblasts born in the subventricular zone (SVZ) migrate collectively towards the olfactory bulb (OB) through the rostral migratory stream (RMS), forming a chain-like organization by interacting with each other (chain migration) [13]. In contrast, Girdin-deficient SVZ neuroblasts migrate randomly, sometimes oriented perpendicular to other cells, with severe defects in chain migration [4,12]. At present, it remains unclear how Girdin regulates cell–cell communication of neuroblasts that undergo collective migration.

Abbreviations: SVZ, subventricular zone; RMS, rostral migratory stream; OB, olfactory bulb.

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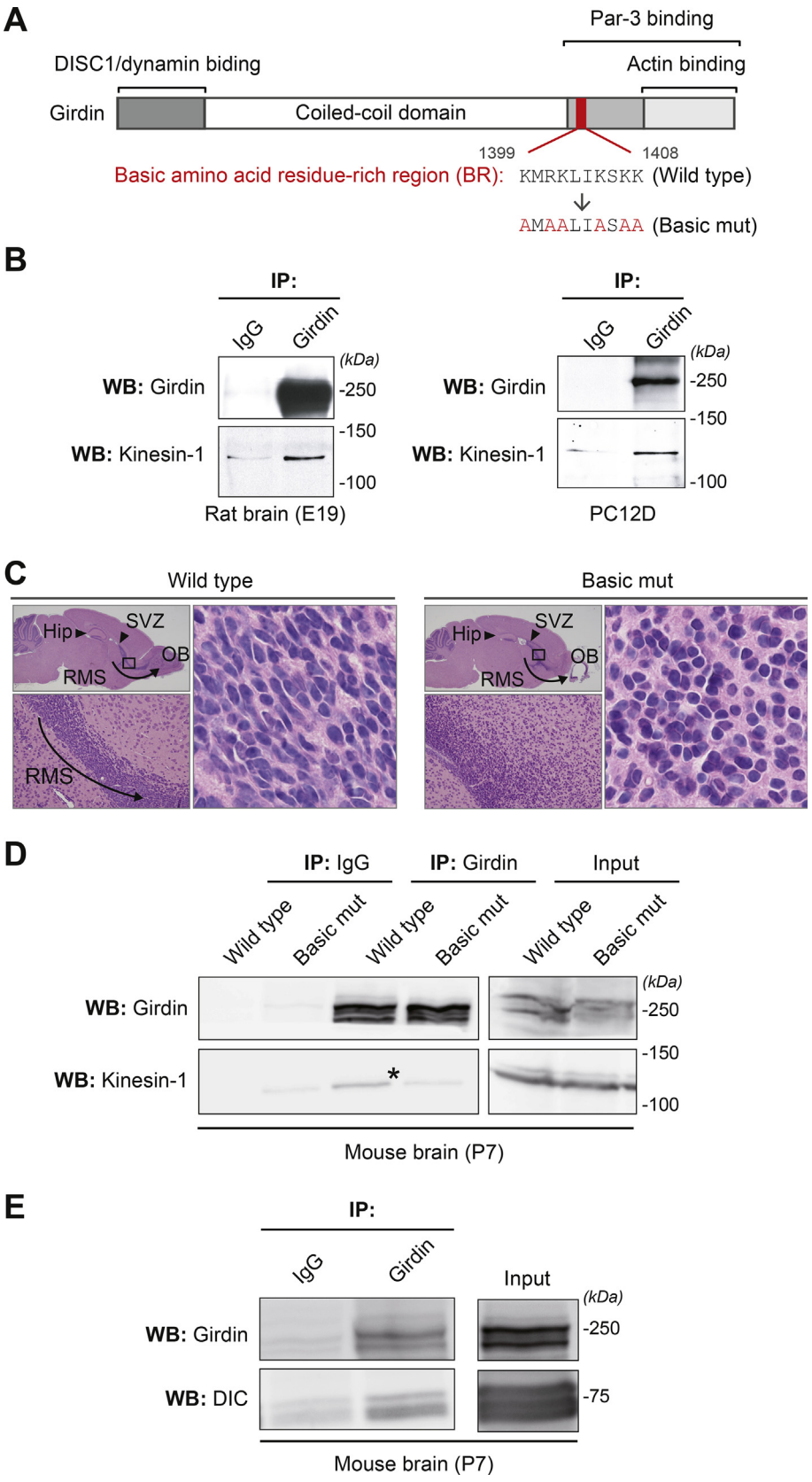

Fig. 1. Interaction of Girdin and kinesin-1 in the brain and cultured cells. (A) Domain structures and interacting proteins of human Girdin. Basic residues in the BR region (red box) that are replaced with alanines are also shown. (B) Interaction between Girdin and kinesin-1. Lysates from E19 rat brain and PC12D cells were immunoprecipitated (IP) with Girdin, followed by western blot analysis (WB) with the indicated antibodies. kDa, kilodaltons. (C) Cytoarchitecture (hematoxylin and eosin stain) of the brains of wild-type (left) and Basic mut (right) mice. The box areas in the upper left panels are magnified in the lower panels. High-power images of SVZ neuroblasts are also shown. Arrows indicate the stream of

Given that Girdin interacts with Par-3 and DISC1, both of which are involved in protein complexes comprising kinesin microtubule motor proteins [14,15], it is of interest to know whether Girdin also interacts with kinesin proteins to participate in the intracellular traffic system. In the present study, we have identified kinesin-1, one of the kinesin proteins expressing in the nervous system [16], as a novel Girdin-interacting protein. We biochemically determined the domains of Girdin and KIF5A (kinesin family member 5A), one of the isoforms of kinesin-1, for their reciprocal interaction, followed by cell biological analyses, which show that expression of a KIF5A mutant leads to Girdin mislocalization. Next, consistent with previous reports showing that kinesin proteins are involved in the traffic of N-cadherin [16,17], we found that mice expressing a loss-of-function Girdin mutant (Basic mut) show deficiency in the expression and localization of N-cadherin in the SVZ neuroblasts. Thus, the present study provides a clue to understanding the role of Girdin in collective migration of SVZ neuroblasts.

2. Materials and methods

2.1. Antibodies, reagents, and cells

Antibodies used in this study include anti-Girdin (R&D Systems, Minneapolis, MN), anti-Girdin (IBL, Gumma, Japan), anti-kinesin-1 (clone H2, Millipore, Billerica, MA), anti-dynein intermediate chain (DIC) (clone 74.1, Millipore), anti-c-myc and β-actin (Sigma, St Louis, MO), anti-green fluorescent protein (GFP) (MBL, Nagoya, Japan), anti-GM130 (BD Biosciences, Lexington, KY), anti-N-cadherin (clone 32/N-cadherin, BD Biosciences), and anti-poly-sialylated neural cell adhesion molecule (PSA-NCAM) (Millipore) antibodies. The construction of human Girdin and KIF5A expression vectors was described previously [1,15]. COS7 and N1E-115 cells, and 293FT cells were purchased from American Type Culture Collection (Rockville, MD), and Invitrogen (Carlsbad, CA), respectively. K. Kaibuchi (Nagoya University) and M. Katsuno (Nagoya University) generously provided PC12D and SH-SY5Y cells, respectively.

2.2. Immunoprecipitation, western blot analysis, immunofluorescence, and retrovirus-mediated RNA interference

For immunoprecipitation (IP), embryonal day (E) 19 rat or postnatal day (P) 7 mouse brain was homogenized on ice in chilled buffer containing 20 mM Tris–HCl (pH 7.4), 120 mM NaCl, 1 mM EDTA, 1 mM DTT, 1% NP-40, 0.1% SDS, 0.1% sodium deoxycholate supplemented with Complete protease inhibitor cocktail (Roche, Indianapolis, IN) and PhosSTOP phosphatase inhibitor cocktail (Roche) at a ratio of 3:1 (v/w) by ten strokes with a pestle in a glass homogenizer. The lysates were sonicated and centrifuged at 100,000× *g* for 30 min prior to conventional IP using the indicated antibodies and western blot analyses. In some IP experiments, RNase (10 μg/ml, Sigma) was added to the lysis buffer to reduce non-specific binding of proteins to the protein A/G beads (Sigma). For IP experiments on cultured cells, cells were lysed in the buffer containing 20 mM Tris–HCl (pH 7.4), 120 mM NaCl, 1 mM EDTA, 1% NP-40 supplemented with the Complete and PhosSTOP cocktails. Lysates were cleared by centrifugation at 12,000× *g* for 10 min, followed by IP using the indicated antibodies and western blot analyses. The primary antibodies were diluted with Can-Get-Signal Solution 1 (Toyobo, Osaka, Japan) to enhance antibody-antigen binding.

For immunofluorescence (IF) studies on cultured cells, COS7 cell transfected with indicated constructs (GFP-KIF5A) on glass base dishes were fixed with 3.7% formalin in PBS for 15 min, immersed in −20 °C methanol for 15 min, rehydrated in PBS for 10 min at room temperature (RT), and incubated with primary antibodies diluted in PBS overnight at 4 °C. After washes with PBS, cells were incubated with Alexa Fluor-conjugated secondary antibodies (Invitrogen), followed by observation with a confocal microscope LSM 700 (Carl Zeiss, Oberkochen, Germany). For IF studies on brain tissues, brains were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer, postfixed in the same fixative overnight, cut into 50–60 μm sections on a microslicer (VT1200S, Leica, Heidelberg, Germany), and immunolabeled by a conventional procedure.

Target sequences for short hairpin RNA (shRNA)-mediated knock down of Girdin were described previously [12]. 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 no. 1, 5′-GGAACAAACAAGATTAGAA-3′ (nucleotides 3837–3855); human Girdin shRNA no. 7, 5′- GAAGGAGAGGCAACTGGAT -3′ (nucleotides 4166–4184). The generation of shRNA-expressing retroviruses and their transduction into SH-SY5Y cell were performed as described previously [12].

2.3. Generation of the Girdin basic mut mice

Conventional gene-targeting techniques were used to generate the knock-in mice, in which six basic lysine or arginine residues in the basic amino acid residue-rich region (BR) of Girdin were replaced with alanines, as described in our previous study [4].

3. Results

3.1. Girdin interacts with kinesin-1 in the brain and PC12D cells

Given our previous findings that Girdin interacts with DISC1 and Par-3, both of which also interact with kinesin proteins (Fig. 1A) [3,12,14,15], we hypothesized that Girdin also functions in protein complexes comprising the kinesin proteins. IP experiments on lysates prepared from E19 rat brain as well as a pheochromocytoma cell line PC12D revealed that kinesin-1, one of the kinesin superfamily enriched in neural tissues [16], interacts with endogenous Girdin (Fig. 1B).

We previously reported the Basic mut mice, in which lysines and arginines in the basic amino acid residue-rich region (BR) in the carboxyl (C)-terminal (CT) domain of Girdin were replaced with alanines [4]. These mice showed severe deficiency in collective migration of the SVZ neuroblasts toward the OB (Fig. 1A, C) [4]. The Basic mut mice recapitulate the phenotype of Girdin-null mice, indicating that the BR region is indispensable for Girdin in exerting its function [4]. Interestingly, we found that kinesin-1 was more effectively immunoprecipitated in the brain lysate prepared from wild-type mice than that of Basic mut mice (Fig. 1D), implicating the possibility that Girdin/kinesin-1 interaction has a role in the migration of SVZ neuroblasts. It is well established that kinesin proteins are plus-end-directed microtubule motor proteins that transport their cargos toward the cell periphery [16]. Our data suggest that Girdin may be a cargo for kinesin-1 or a regulator or modulator of kinesin-1function. Intriguingly, our additional IP test showed that DIC, a component of the minus-end-directed microtubule motor dynein, also co-immunoprecipitates with Girdin,

collective migration of SVZ neuroblasts through the RMS. Hip, hippocampus. (D) Lysates from the brains of wild-type and Basic mut P7 mice were immunoprecipitated with Girdin, followed by western blot analysis. An asterisk indicates the coprecipitated kinesin-1. (E) Interaction between Girdin and DIC. Lysates from P7 mouse brain were immunoprecipitated with Girdin, followed by western blot analysis.

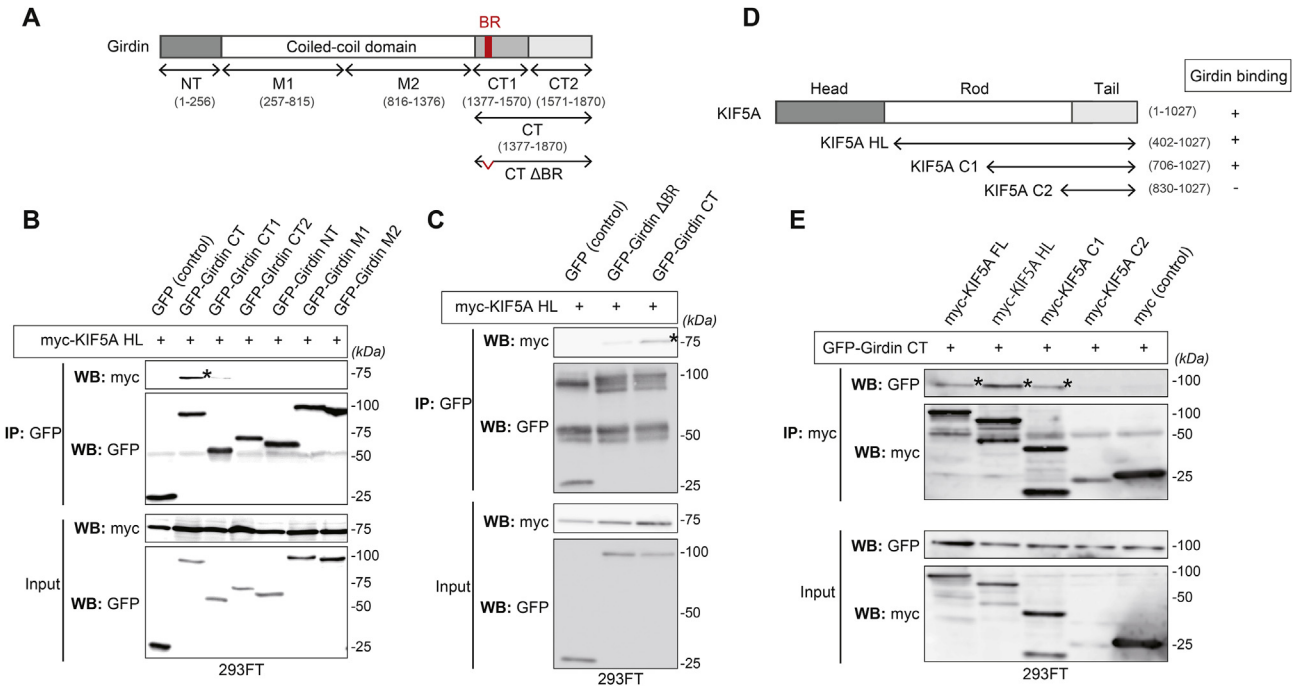

Fig. 2. Mapping of interacting domains for Girdin/kinesin-1 interaction. (A) Fragments and domains of Girdin are shown. The corresponding amino acid numbers for the fragments are given in parentheses. In the CT ΔBR construct, the BR region (red) is deleted. (B) KIF5A-binding sites map to the CT domain of Girdin. Lysates from 293FT cells transfected with the indicated Girdin fragments fused to GFP and myc-KIF5A HL were immunoprecipitated with GFP antibody. An asterisk indicates immunoprecipitated myc-KIF5A HL. (C) Requirement of the Girdin BR region for interaction with KIF5A. Lysates from 293FT cells transfected with the myc-KIF5A HL and wild-type Girdin CT domain and its mutant ΔBR were immunoprecipitated with GFP antibody. An asterisk indicates myc-KIF5A HL immunoprecipitated with GFP-Girdin CT. (D) Domain architectures and fragments of KIF5A used in the study are shown. (E) Girdin-binding sites map to the C-terminal part of the rod domain of KIF5A. Lysates from 293FT cells transfected with the indicated KIF5A fragments and GFP-Girdin CT were immunoprecipitated with myc antibody, followed by western blot analysis with indicated antibodies. Asterisks indicate immunoprecipitated GFP-Girdin CT. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

suggesting an intricate mode of Girdin involvement in the intracellular traffic system (Fig. 1E).

3.2. Mapping Girdin and kinesin-1 interacting domains

Girdin is composed of unique amino-terminal (NT) and CT domains, which flank a long coiled-coil domain. Our previous studies identified that the CT domain interacts with Par-3 as well as actin filaments, whereas the NT domain interacts with DISC1 and dynamin (Fig. 1A) [1,3,11,12]. To identify which domains of Girdin are responsible for its interaction with kinesin-1, we constructed fragments of Girdin and transfected them with KIF5A, a representative isoform of kinesin-1 (Fig. 2A, B). The results showed that Girdin CT domain is responsible for the binding with KIF5A. Our experiments with smaller constructs (CT1 and CT2) to narrow down the region of Girdin CT domain for KIF5A interaction were unsuccessful, suggesting that the entire Girdin CT domain may be necessary for KIF5A interaction (Fig. 2B). In the IP experiments, we used a headless (HL) mutant of KIF5A for the reason that it most effectively and reproducibly interacts with Girdin, as described later.

We also constructed a deletion mutant (ΔBR) of Girdin CT, in which the BR region was deleted (Fig. 2A). Consistent with IP experiments on endogenous proteins from brain lysates of Basic mut mice (Fig. 1D), Girdin ΔBR showed limited interaction with KIF5A (Fig. 2C), supporting the notion that the BR region is critical for the intact function of Girdin.

Next, we mapped the region of KIF5A that interacts with Girdin. A KIF5A mutant lacking both the head and rod domains (KIF5A C2), but not full-length KIF5A (KIF5A FL) and other deletion mutants (HL and C1), lost the capacity to bind with Girdin, suggesting that a C-terminal part of the rod domain seems to be important for the interaction with Girdin (Fig. 2D, E).

3.3. Intact function of KIF5A is necessary for proper subcellular localization of Girdin

To see the co-localization of Girdin and KIF5A, we observed COS7 cells transfected with KIF5A FL by confocal microscopy (Fig. 3A). We found that Girdin colocalized with KIF5A at the cell periphery as well as the perinuclear region that is positive for GM130, a marker for the Golgi apparatus. In a confluent monolayer of cells, both proteins localized at the intercellular adhesion sites, implicating a role of Girdin/KIF5A complex in cell–cell adhesion (Fig. 3B). Expression of the KIF5A HL mutant, which lacks the head domain that constitutes the motor domain with ATP hydrolysis and microtubule binding capacity, resulted in the mislocalization of Girdin around the perinuclear area. This suggests that intact function of KIF5A is essential for physiological localization of Girdin (Fig. 3B). Unfortunately, our attempts to observe the localization of Girdin/KIF5A complex in other neuroblastoma cell lines were unsuccessful; due to their tiny cytoplasms, we could not observe their subcellular localization at fine resolution (data not shown).

3.4. Severe defect in the expression and localization of N-cadherin in basic mut mice

Given a wide variety of functions exerted by kinesin-1, which undergo spatiotemporal regulation depending on cellular activity [18], it is difficult to determine which cellular process (es) are regulated by the Girdin/kinesin-1 complex. A previous study has shown that a member of kinesin-2, another subfamily of neural

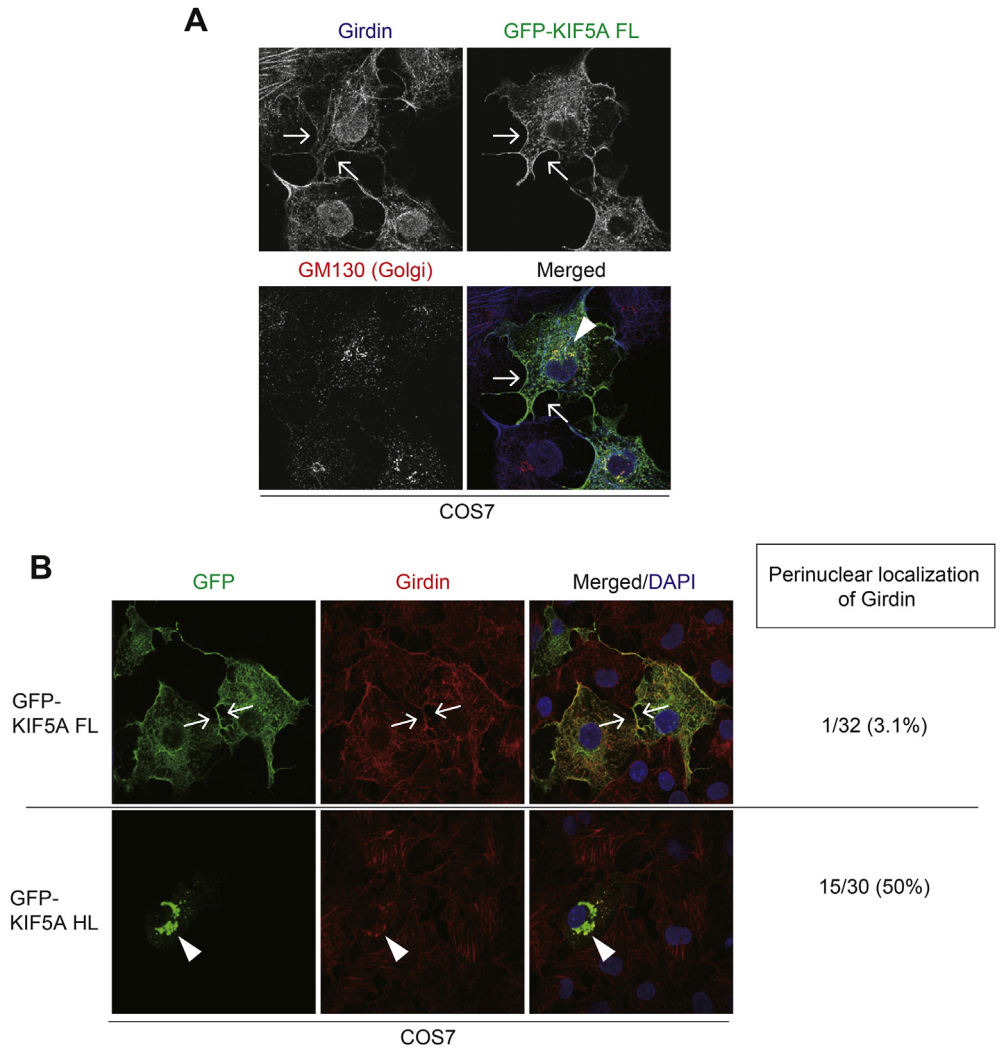

Fig. 3. Colocalization of Girdin with KIF5A at the cell periphery and perinuclear region. (A) Colocalization of Girdin with KIF5A in COS7 cells. Cells transfected with full-length KIF5A (GFP-KIF5A FL) were stained for Girdin (blue) and GM130 (red), showing the colocalization of Girdin and GFP-KIF5A at the cell periphery (arrows) and the Golgi apparatus (an arrowhead). (B) Expression of KIF5A HL results in the mislocalization of Girdin around the nucleus. COS7 cells transfected with GFP-KIF5A FL (upper) or HL (lower) were stained with Girdin (red). Nuclei were visualized with DAPI (6-diamidino-2-phenylindole) (blue). Arrows indicate the colocalization of Girdin and KIF5A FL at intercellular adhesion. Girdin accumulation around the nucleus in KIF5A HL-expressing cells was indicated by arrowheads (lower panel). In the far right panel, the number of cells with the perinuclear localization of Girdin was counted in each group.

kinesin proteins, has a role for the intracellular transport of N-cadherin [17]. Considering that N-cadherin is expressed in SVZ neuroblasts [19], we hypothesized that Girdin/kinesin-1 interaction is involved in the regulation of N-cadherin localization. Immunofluorescence studies showed that SVZ neuroblasts migrating through the RMS pathway express N-cadherin in the cytoplasm as well as at cell–cell contacts (Fig. 4A). Strikingly, in Basic mut mice, not only the localization but also the expression level of N-cadherin were severely affected (Fig. 4A). This observation was not attributable to technical issues, because the expression and localization of PSA-NCAM, an important regulator of the SVZ neuroblast migration, were comparable between wild type and Basic mut mice (Fig. 4A). This suggests that Girdin specifically functions in the intracellular dynamics and expression of N-cadherin.

Our IP tests on the lysates from mouse brain and a neuroblastoma cell line N1E-115 showed a marginal but insignificant interaction between Girdin and N-cadherin, suggesting that Girdin may be involved in a protein complex including N-cadherin but there is no direct interaction (Fig. 4B). To clarify the mechanism of how Girdin regulates N-cadherin localization and expression, we generated stable Girdin knock down N1E-115 cell lines by the transduction of specific shRNAs. However, Girdin depletion had no apparent effects on the expression of both kinesin-1 and N-cadherin (Fig. 4C). Girdin depletion also had no effect on either cell morphology or the subcellular localization of N-cadherin (Fig. 4D, E). Taken together, it seemed difficult to reproduce changes of N-cadherin expression and localization found in Basic mut mice by using cultured cells, which leaves the biological significance of the Girdin/kinesin-1 interaction an unresolved question.

4. Discussion

In the present study, we have identified kinesin-1, a member of neuronal kinesins, as a new interacting partner for Girdin, which may help us understand the function of Girdin and the mechanisms of SVZ neuroblast migration in the postnatal brain. Although we showed distinct biochemical interaction between Girdin and kinesin-1, the biological significance of that interaction remains unclear. Given the importance of kinesin proteins in N-cadherin trafficking [16,17] and the deregulation of N-cadherin in mice

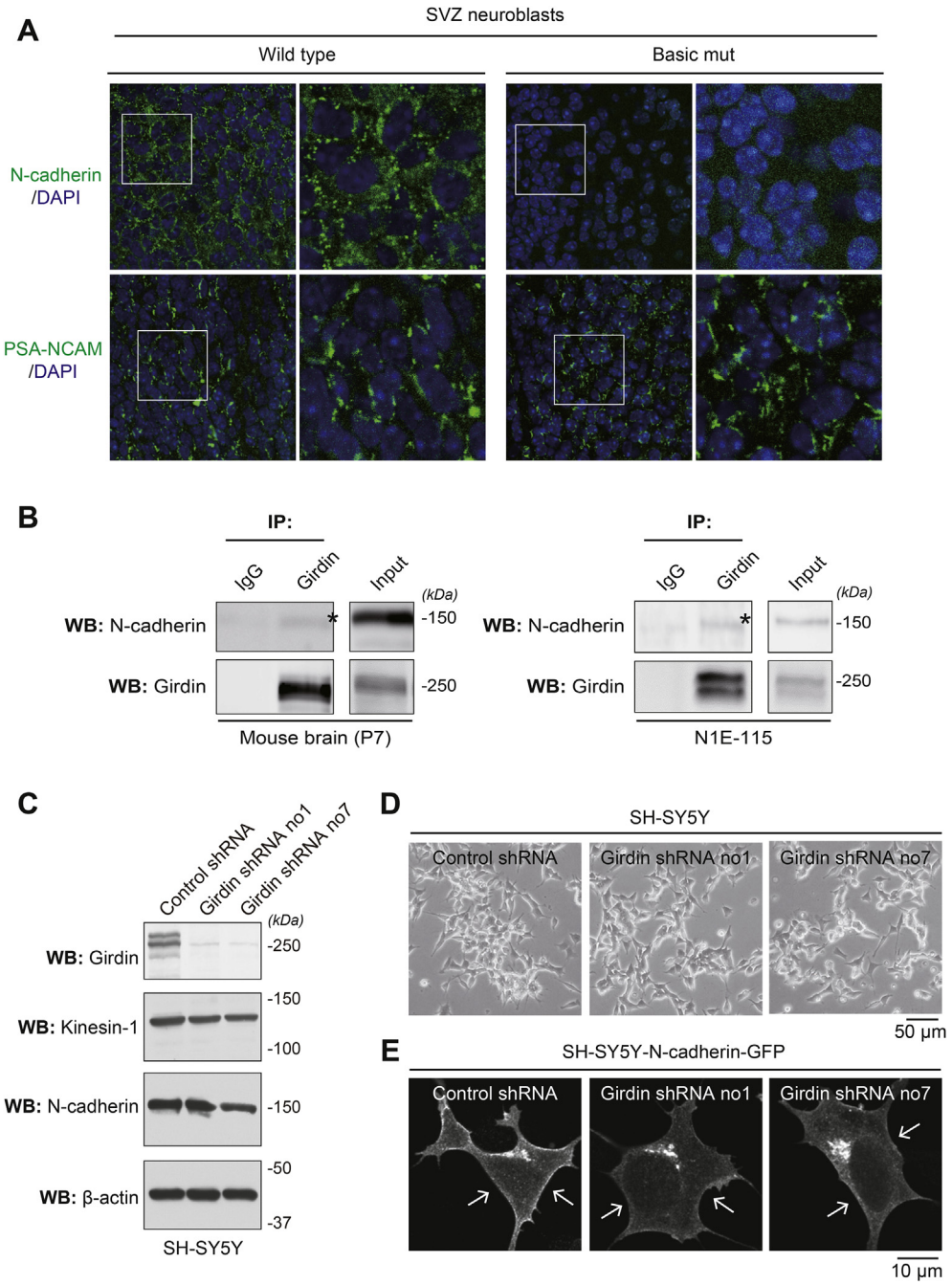

Fig. 4. Basic mut mice exhibited aberrant expression and localization of N-cadherin, which was not reproduced in cultured cells. (A) Defects in N-cadherin expression of SVZ neuroblasts in the Basic mut mice. Sagittal sections through the RMS of wild-type (left) and Basic mut (right) P17 mice were stained for N-cadherin (upper panel) and PSA-NCAM (lower panel). White box areas are magnified in adjacent panels. (B) A marginal but reproducible interaction of Girdin with N-cadherin. Lysates from P7 mouse brain (left) and N1E-115 cells (right) were immunoprecipitated with Girdin, followed by western blot analysis using the indicated antibodies. (C) Retrovirus-delivered shRNAs in silencing endogenous Girdin in SH-SY5Y neuroblastoma cells. Cell lysates were collected seven days post-infection of shRNA-expressing retroviruses and analysed by western blot analysis. (D) No apparent effects of Girdin depletion on cell morphology in SH-SY5Y cells. (E) No apparent effects of Girdin depletion on N-cadherin localization. Control and Girdin shRNAs-expressing SH-SY5Y cells were further transduced with a retrovirus encoding N-cadherin-GFP and observed by confocal microscopy. Arrows indicate the localization of N-cadherin-GFP at cell periphery.

expressing a loss-of-function Girdin mutant (Fig. 4A), it would be plausible to speculate that the Girdin/kinesin-1 complex has a role in N-cadherin dynamics in the SVZ neuroblasts. Further studies are required to fully address the function of the Girdin/kinesin-1 complex.

One issue that has not been addressed in the present study is the mode of interaction between Girdin and kinesin-1. Direct interaction between the two proteins was not shown, leaving the possibility that Girdin indirectly interacts with kinesin-1. Previous studies have shown that DISC1 and Par-3, which interact with Girdin, directly bind to the members of the kinesin family [14,15]. Thus, it is likely that Girdin indirectly interacts with kinesin-1 through DISC1, Par-3, or other unidentified proteins.

Another issue of the study is the discrepancy of the phenotype observed in Basic mut mice and Girdin-depleted cultured cells (Fig. 4). The expression of N-cadherin was almost undetectable in

the neuroblasts of Basic mut mice (Fig. 4A), whereas shRNA-mediated Girdin knock down had no effects in cultured neuroblastoma cells (Fig. 4C–E). This suggests that the cultured cells lack component(s) that are critical for N-cadherin dynamics, which are essential for the SVZ neuroblasts undergoing collective migration. Also, the reason why expression of N-cadherin was downregulated in the Basic mut mice has also remained a mystery. An intriguing speculation is that N-cadherin undergoes degradation pathways due to its mislocalization in Basic mut mice. Further analysis should be conducted by more sophisticated techniques, such as the culture of tissue slices freshly prepared from animals.

As we previously reported, Girdin is a critical regulator of collective migration of SVZ neuroblasts found in the RMS in the postnatal brain (Fig. 1C) [4]. Although many investigators have been engaged in studying the mechanism of the chain migration of SVZ neuroblasts, a complete picture of how they form a chain-like organization is far from complete [20,21]. A recent report has suggested that the retrograde flow of N-cadherin along the intercellular adhesion sites and its recycling are critical for the collective migration of astrocytes [22]. Our immunofluorescence study showed that Girdin colocalized with kinesin-1 at cell–cell adhesion sites in a confluent monolayer of cells (Fig. 3B). Therefore, it would be interesting to study whether Girdin/kinesin-1 interaction is involved in such dynamics of N-cadherin in SVZ neuroblasts. Finally, Girdin expression was also detected in cancer tissues including breast carcinoma and glioblastoma [5,6]. Given recent consensus that most cancer cells undergo collective invasion rather than single cell invasion into the stroma [23,24], it would be interesting to investigate whether Girdin is involved in the common pathway of collective migration of cancer cells and neuroblasts.

Financial interests

The authors declare no competing financial interests.

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