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Dysbindin engages in c-Jun N-terminal kinase activity and cytoskeletal organization

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ABSTRACT

A number of reports have provided genetic evidence for an association between the DTNBP1 gene (coding dysbindin) and schizophrenia. In addition, sandy mice, which harbor a deletion in the DTNBP1 gene and lack dysbindin, display behavioral abnormalities suggestive of an association with schizophrenia. However, the mechanism by which the loss of dysbindin induces schizophrenia-like behaviors remains unclear. Here, we report that small interfering RNA-mediated knockdown of dysbindin resulted in the aberrant organization of actin cytoskeleton in SH-SY5Y cells. Furthermore, we show that morphological abnormalities of the actin cytoskeleton were similarly observed in growth cones of cultured hippocampal neurons derived from sandy mice. Moreover, we report a significant correlation between dysbindin expression level and the phosphorylation level of c-Jun N-terminal kinase (JNK), which is implicated in the regulation of cytoskeletal organization. These findings suggest that dysbindin plays a key role in coordinating JNK signaling and actin cytoskeleton required for neural development.

Schizophrenia is a heritable mental disease that devastates about 1% of the population worldwide, affecting their perception, emotion, and judgment [1]. The DTNBP1 gene (coding dysbindin: dystrobrevin binding protein (1) was identified as a candidate for involvement in schizophrenia [2]. In studies of postmortem brain tissue, patients with schizophrenia had lower dysbindin expression than controls [3–5]. Moreover, long-term treatment with typical or atypical antipsychotics did not alter the mRNA expression levels or protein levels of dysbindin in the mouse frontal cortex and hippocampus [4,6]. Together these findings suggest that decreased dysbindin levels may confer susceptibility to schizophrenia. Sandy (*sdy*) mice that express no dysbindin, owing to a deletion of the DTNBP1 gene showed behavioral abnormalities such as reduced activity, heightened anxiety-like response, and deficits in social interaction, memory, and learning [7–9], which could be endophenotypes of schizophrenia. *sdy* mice also displayed lower levels of dopamine, but not glutamate, in the cerebral cortex, hippocampus, and hypothalamus [8,10].

Dysbindin is known to be widely distributed in the brain and located presynaptically and postsynaptically in the central nervous system [11]. The downregulation of endogenous dysbindin by small interfering RNA (siRNA) reportedly inhibited the release of glutamate from hippocampal cultured neurons and increased the release of dopamine from PC12 cells [12,13]. In addition, *sdy* mice reportedly exhibit defective synaptic structure and function in the hippocampal CA1 neurons [14]. However, the molecular mechanism underlying the effects of dysbindin on synaptogenesis remains elusive. On the other hand, an increasing number of studies have shown that cytoskeletal organization is essential for the dynamics of synaptogenesis [15,16]. Therefore, to examine the effects of low dysbindin levels on cytoskeletal organization, we performed an immunocytochemical analysis using SH-SY5Y cells, which have been used as an *in vitro* model to study neural development. Furthermore, to confirm the influences of dysbindin knockdown *in vivo*, we analyzed *sdy* mice similarly. In addition, we investigated whether alterations in dysbindin expression affect c-Jun N-terminal kinase (JNK) activity, which has been known to phosphorylate many cytoskeletal proteins and regulate neural development [17–20].

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Materials and methods

Mice. The *sdy* and control DBA/2J mice were originally obtained from the Jackson Laboratory and bred in the Institute of Experimental Animal Sciences, Osaka University Graduate School of Medicine. All the experiments were performed in accordance with our institutional guidelines after obtaining the permission of the Laboratory Animal Committee.

Cell culture. The SH-SY5Y cell line was obtained from the European Collection of Cell Culture (Wiltshire, UK) and maintained according to the manufacturer's protocol. Hippocampal neurons were cultured from E15 mice embryos using the Nerve-cell Culture System (SUMITOMO BAKELITE, Tokyo, Japan). Neurons (2.1×10^5) in MEM (Invitrogen) containing 10% fetal horse serum and antibiotics (50 U/ml penicillin G and 50 μg/ml streptomycin) were plated onto 3.5 cm poly-L-lysine-coated dish. After 4 h of incubation, medium was changed to Neurobasal Medium containing 2% B27 (Invitrogen) and antibiotics.

Plasmid. FLAG-human *dysbindin* (AF394226) was cloned into pcDNA 3.1/Zeo (+) vector (Invitrogen). Transfection into cells was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

RNA interference. We used 5′-AAGUGACAAGUCAAGAGAA-3′ siRNA, the sequence of which is corresponding to nucleotides 175–197 of human dysbindin mRNA. Scrambled siRNA 5′-UUCUCUUGACUUGUCACUU-3′ was used as a negative control. Both sense and antisense strands with two base overhangs were synthesized by NIPPON-EGT (Toyama, Japan) in desalted form. siRNA transfection was performed with Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's protocol.

Immunocytochemistry. Plasmid-transfected cells were fixed with cold 95% ethanol for 7 min at −20 °C and subjected to blocking in 2% BSA/PBS for 10 min. After incubating with an anti-FLAG antibody (1:150, Sigma–Aldrich, St. Louis, USA) overnight, cells were incubated with an Alexa 488 conjugated anti-rabbit secondary antibody (1:500, Invitrogen) for 2 h. siRNA-transfected cells were fixed with cold methanol for 10 min at −20 °C and incubated in PBS containing 5% BSA and 0.3% Triton X-100 for 30 min. The cells were then incubated with an anti-β-tubulin antibody (1:500, Sigma–Aldrich) at 4 °C overnight followed by an Alexa 568 conjugated anti-mouse secondary antibody (1:500, Invitrogen) for 2 h. For detection of actin filament, cells were fixed with 2.5% paraformaldehyde/PBS for 20 min, subjected to permeabilization with 0.1% Triton X-100/PBS for 3 min and then incubated with the Alexa Fluor 568 phalloidin staining solution (5 U/ml, Invitrogen) in PBS containing 1% BSA for 20 min. The coverslips were mounted onto the slides using VECTASHIELD Mounting Medium with DAPI (Vector Laboratories, Peterborough, England). Fluorescence images were acquired using a digital camera DP70 connected with a stereomicroscope (Carl Zeiss, Oberkochen, Germany). Hippocampal cultured neurons at stage 3 were similarly subjected to immunocytochemistry. Fluorescence images were acquired using a confocal laser scanning microscope (LSM-510 UV/META, Carl Zeiss).

Western blot analysis. siRNA-transfected cells were lysed in RIPA buffer containing 1 mM Na_3VO_4, 1 mM NaF and Protease Inhibitor Cocktail (Roche Diagnostics, Basel, Switzerland), incubated for 20 min at 4 °C and centrifuged at 17,000g for 20 min at 4 °C. Proteins (3 μg) were separated on SDS–PAGE and electrotransferred onto Immobilon-P Transfer Membranes (MILLIPORE, Billerica, USA). Membranes were incubated in PBS containing 5% skim milk and 0.05% Tween 20 for 1 h and blotted with primary antibodies at 4 °C overnight. An anti-dysbindin antibody (1:1000), anti-phospho-JNK antibody (1:1000, Cell Signaling Technology, Danvers, USA), anti-JNK antibody (1:1000, Cell Signaling Technology) and anti-GAPDH antibody (1:5000, Abcam, Cambridge, USA) were used as primary antibodies. The membranes were incubated with an anti-mouse or anti-rabbit HRP-linked secondary antibody (1:2000, Cell Signaling Technology) for 1 h. Mouse monoclonal anti-dysbindin antibody was produced using the GST fused human dysbindin as antigen. High titer clones to dysbindin were selected by ELISA using the dysbindin protein and the immunoreactivity of the clones were checked by Western blot analysis. For analyses of mice, hemisphere of E16 embryo was homogenized in RIPA buffer and similarly subjected to Western blot analysis.

Results

Portions of dysbindin were localized to the tips of protrusions, and dysbindin knockdown influenced the organization of actin cytoskeleton in SH-SY5Y cells

Dysbindin was previously shown to exist in axon terminals in the hippocampus and to decline in patients with schizophrenia [4,11]. However, dysbindin's function in axon terminals in the hippocampus remains unclear. Thus, to explore the role of dysbindin in neurite formation, we attempted to downregulate it in differentiating SH-SY5Y cells. To clarify how dysbindin operates at the tip of a protrusion, we used an RNA interference method to investigate whether or not alterations in dysbindin expression could influence the morphology of the terminal region of protrusions. Retinoic acid treatment gives rise to the differentiation of SH-SY5Y cells and induces neurite outgrowth [21,22]. Control or dysbindin siRNA-transfected SH-SY5Y cells were incubated with retinoic acid to differentiate and then were analyzed in cytoskeletal organization by visualizing actin filament with rhodamine-phalloidin. Interestingly, the organization of actin cytoskeleton at the tips of neurites of differentiating SH-SY5Y cells was dramatically disrupted by the dysbindin knockdown (Fig. 1A). In addition, immunocytochemical analysis with anti-β-tubulin antibody was performed to compare the lengths of neurites of dysbindin knockdown cells to those of controls. As shown in Fig. 1B, the β-tubulin-positive neurites of dysbindin knockdown cells were apparently shorter than those of the controls. We confirmed that dysbindin with FLAG-tag was expressed in the cell body as well as at the tips of protrusions of SH-SY5Y cells (Fig. 1C). These results suggest that dysbindin knockdown significantly affects the organization of actin cytoskeleton, bringing about the inhibition of neurite outgrowth in differentiating SH-SY5Y cells.

The derangement of cytoskeletal organization was observed in growth cones of hippocampal cultured neurons derived from sdy mice

To confirm the effects of dysbindin knockdown on actin cytoskeleton, we analyzed hippocampal cultured neurons derived from *sdy* mice, which lack dysbindin. The growth cone consists mainly of actin-based structures and can be divided into three distinct regions: the peripheral domain, the central domain, and the transition zone [15]. The peripheral domain has linear actin bundles comprising filopodia and mesh-like gels comprising lamellipodia. The central domain, which is rich in microtubules, has hardly any actin superstructures. In the transition zone located between the peripheral domain and the central domain, transverse bundles of actin filaments are observed. To explore the effects of dysbindin knockdown on actin cytoskeleton in growth cones, hippocampal neurons prepared from E15 mice embryos were cultured, fixed at stage 3, and stained with rhodamine-phalloidin. In neurons derived from wild-type mice, most of the growth cones had a characteristic shape (Fig. 2A). In contrast, the growth cones of neurons derived from *sdy* mice showed significant changes in actin-based

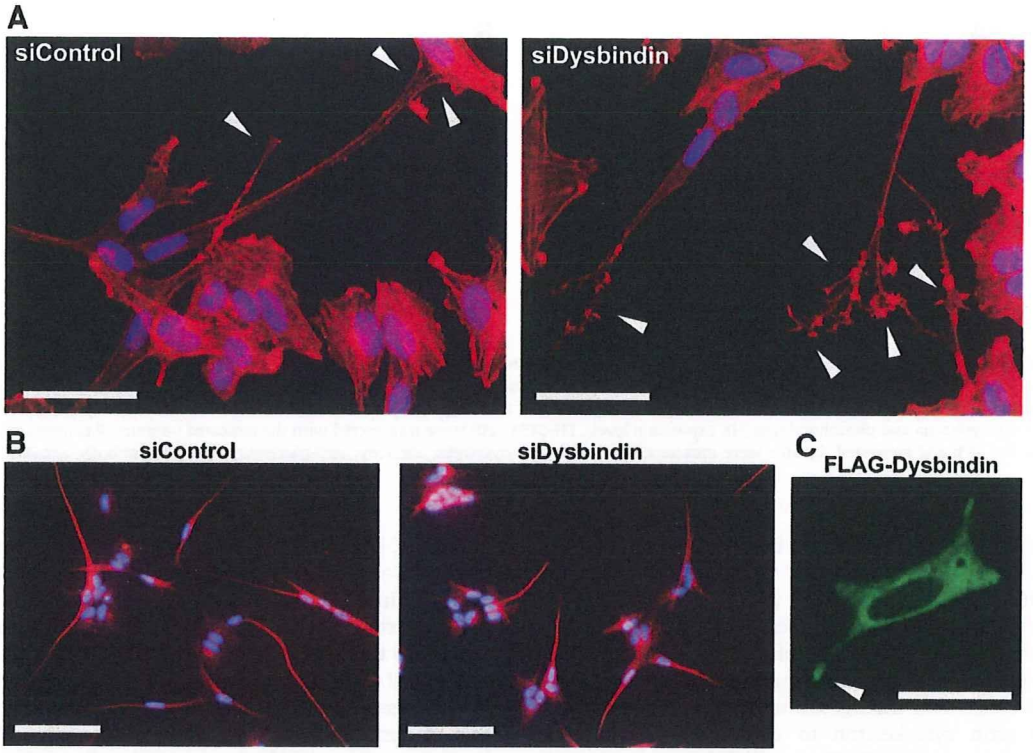

Fig. 1. Dysbindin is involved in neurite morphogenesis. (A) The effects of dysbindin knockdown on actin cytoskeleton. SH-SY5Y cells were transfected with control (siControl) or dysbindin siRNA (siDysbindin), followed by incubation with retinoic acid for 60 h. Actin filament was visualized by rhodamine-phalloidin (red) and DAPI (blue). Bar = 50 μm. (B) The effects of dysbindin knockdown on neurite length. SH-SY5Y cells were transfected with control (siControl) or dysbindin (siDysbindin) siRNA, followed by incubation with retinoic acid for 60 h. The cells were then immunostained using anti-β-tubulin antibody (red) followed by Alexa 568-labeled secondary antibody and DAPI (blue). Bar = 100 μm. (C) Localization of dysbindin in cell body and at the tips of protrusions. SH-SY5Y cells were transfected with FLAG-tagged dysbindin. They were cultured for 24 h and stained with anti-FLAG antibody, followed by Alexa 488-labeled secondary antibody. Bar = 25 μm. (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

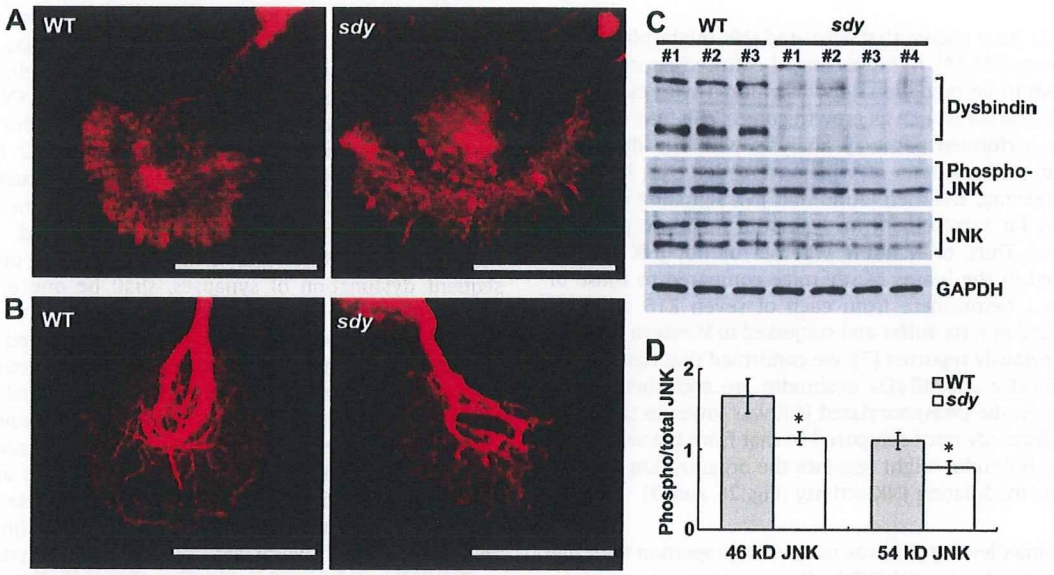

Fig. 2. Dysbindin is involved in the regulation of growth cone morphology. (A) Actin cytoskeleton within the growth cone. Embryonic hippocampi were dissociated from E15 wild-type (WT) or *sdy* mice (*sdy*) and cultured. They were stained with rhodamine-phalloidin to visualize actin filament at stage 3. Bar = 20 μm. (B) Microtubule cytoskeleton within the growth cone. Embryonic hippocampal neurons derived from E15 wild-type (WT) or *sdy* mice (*sdy*) were cultured. The neurons were stained with anti-β-tubulin antibody at stage 3, followed by Alexa 568-labeled secondary antibody. Bar = 20 μm. (C) JNK activity in the brains of wild-type (WT) or *sdy* mice (*sdy*). Lysates homogenized from the hemisphere of E16 embryos were immunoblotted with anti-dysbindin antibody, anti-phosphorylated JNK antibody, anti-JNK antibody, and anti-GAPDH antibody. (D) Quantitated data. Relative ratios of 46 kDa phospho/total JNK and 54 kDa phospho/total JNK in the brains of wild-type (WT) or *sdy* mice (*sdy*) were analyzed using NIH ImageJ software and represented graphically. Statistical comparisons were performed using the unpaired Student's *t*-test. Data represent means ± SD. $^{*}P < 0.05$ versus control.

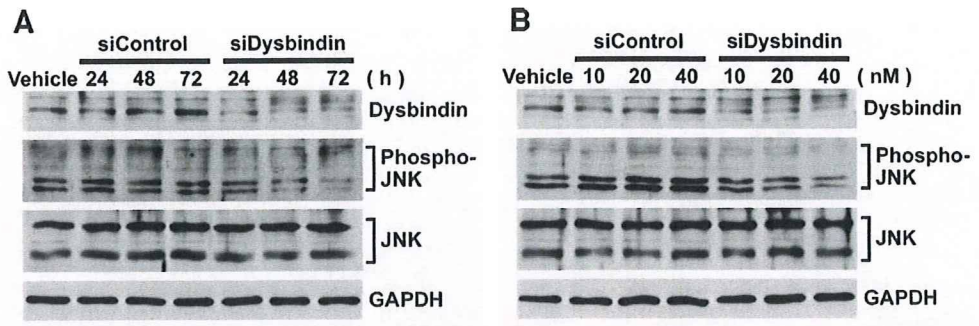

Fig. 3. The JNK phosphorylation level was susceptible to the dysbindin expression level. (A) Time-course dependent effects of dysbindin siRNA on the expression level of dysbindin and phosphorylated JNK. SH-SY5Y cells were transfected with control (siControl) or dysbindin siRNA (siDysbindin), incubated for the indicated times, and subjected to immunoblotting with anti-dysbindin antibody, anti-phosphorylated JNK antibody, anti-JNK antibody, and anti-GAPDH antibody. (B) Dose-dependent effects of dysbindin siRNA on the dysbindin and phosphorylated JNK expression levels. SH-SY5Y cells were transfected with the indicated volumes of control (siControl) or dysbindin siRNA (siDysbindin) for 48 h and harvested. Lysates were immunoblotted with anti-dysbindin antibody, anti-phosphorylated JNK antibody, anti-JNK antibody, and anti-GAPDH antibody.

structures. In those neurons, transverse bundles of actin filament in the transition zone disappeared, and then the central domain became difficult to discern. Additionally, in the peripheral domain, the palm-like shapes consisting of filopodia and lamellipodia were perturbed. On the other hand, microtubules are known to organize into bundles in the neurites, whereas upon entering the central domain of growth cones, they diverge from each other and collaborate with the actin cytoskeleton to contribute growth cone motility and axon elongation [23]. Then, we analyzed microtubule stabilization by staining β-tubulin with a specific antibody. The results revealed that microtubules in the growth cones of neurons derived from *sdy* mice were affected slightly, whereas the controls were unaffected (Fig. 2B). Our findings suggest that hippocampal neurons in *sdy* mice may tend to show the morphological disorder of growth cones.

JNK activity was suggested to be attenuated in the brains of sdy mice embryos

Recent works have shown that activated JNK might play a role in axon formation [20,24]. In hippocampal cultured neurons, JNK has been known to be predominately distributed with cytoskeleton-associated structures such as growth cones [25]. Interestingly, we previously performed pathway analysis with the dysbindin binding proteins, interactions of which were detected by yeast two-hybrid screening, and then found that JNK signaling is a candidate pathway for involvement in dysbindin function (personal communication). Thus, to examine whether or not JNK activity could be altered in the brains of *sdy* mice compared to those of the wild type, a hemisphere from each of seven E16 embryos was homogenized in lysis buffer and subjected to Western blotting analysis. As previously reported [7], we confirmed that the expression of both 50 kDa and 40 kDa dysbindin are abolished in *sdy* mice. Intriguingly, the phosphorylated JNK was lower in the brain lysate derived from *sdy* mice compared to that from the wild type, indicating that dysbindin might regulate the organization of actin cytoskeleton via modulating JNK activity (Fig. 2C and D).

The phosphorylation level of JNK was reduced in proportion with the dysbindin expression level in SH-SY5Y cells

To determine whether or not a reduction in JNK activity was due specifically to the loss of dysbindin, we compared the JNK phosphorylation level with the dysbindin expression level in control or dysbindin siRNA-treated SH-SY5Y cells by Western blot analysis. Though two splice variants of dysbindin exist in DBA/2J mice (Fig. 2C), SH-SY5Y cells predominantly express 50 kDa dysbindin in our experiments. The downregulation of dysbindin was observed in a time-dependent manner in dysbindin siRNA-treated cells, and then phosphorylated JNK was similarly decreased in proportion to the dysbindin expression level (Fig. 3A). In addition, we performed dose–response analysis with each siRNA and found that the expression level of phosphorylated JNK is highly sensitive to that of dysbindin (Fig. 3B). Our data unequivocally demonstrate that there is a significant correlation between dysbindin level and JNK activity.

Discussion

Recent studies have suggested that dysfunction in neurodevelopment and neurotransmission is important for the etiology of schizophrenia [26,27]. For example, DISC1, a candidate gene for susceptibility to schizophrenia, has been known to be part of the NUDEL/LIS1/14-3-3ε complex and to regulate the transport of the protein complex into axons, leading to neuronal migration and axon elongation [28,29]. On the other hand, it has been reported that dysbindin might influence the exocytotic glutamate release and the dopaminergic system via modulation of SNAP25 and synapsin 1 expression [12,13]. Additionally, dysbindin has been suggested to regulate cell surface levels of DRD2 (dopamine D2 receptor) and the strength of the DRD2-mediated G_i signaling pathway [30]. Although a common molecular mechanism under these observations has long been unexplained, morphological abnormalities in developing neurons, which may bring about subsequent dysfunction of synapses, shall be one of the probable causes of susceptibility to schizophrenia.

In this study, we show that dysbindin is required for the normal arrangement of actin cytoskeleton, especially at neurite tips, in differentiating SH-SY5Y cells. Furthermore, we found that the morphological abnormalities are observed in growth cones of cultured hippocampal neurons derived from *sdy* mice, which lack dysbindin. In developing neurons, growth cones are involved in axon elongation and migration [31]. Therefore, the morphological dysfunction of growth cones by a loss of dysbindin may result in an insufficiency of neural circuit formation and synaptogenesis.

Recently, an increasing number of reports have strongly suggested that JNK is relevant to cytoskeletal function [25]. Moreover, it has been demonstrated that phosphorylated JNK was enriched in axons and necessary for proper axon development [20,25]. The JNK family consists of three isoforms: JNK1, JNK2, and JNK3 [32]. Mice devoid of both JNK1 and JNK2 suffer from multiple abnormalities during development of the central nervous system [33]. In addi-

tion, the mutation of JNK3 gene reportedly results in the severe encephalopathy phenotype in children [34]. Interestingly, the present study revealed that the phosphorylation level of JNK is altered by the expression level of dysbindin, raising the possibility that dysbindin functions as a mediator of the JNK signaling pathway, at least at neurite ends, where dysbindin is colocalized with JNK.

Hence, we speculate that a loss of dysbindin results in the failure of normal axon guidance by the disruption of growth cones during the embryonic stage and evokes aberrations in the neurosecretory system in adulthood. However, it remains unclear how dysbindin regulates JNK phosphorylation and how the morphological changes of growth cones in developing neurons contribute to the pathogenic mechanism of schizophrenia. Further analyses are needed to obtain the precise molecular function of dysbindin.

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RESEARCH ARTICLE

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Association Studies and Gene Expression Analyses of the DISC1-Interacting Molecules, Pericentrin 2 (*PCNT2*) and DISC1-Binding Zinc Finger Protein (*DBZ*), With Schizophrenia and With Bipolar Disorder

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Disrupted-in-Schizophrenia 1 (DISC1) and its molecular cascade have been implicated in the pathophysiology of major psychoses. Previously, we identified pericentrin 2 (PCNT2) and DISC1-binding zinc finger protein (DBZ) as binding partners of DISC1; further, we observed elevated expression of *PCNT2* in the postmortem brains and in the lymphocytes of bipolar disorder patients, compared to controls. Here, we examined the association of *PCNT2* with schizophrenia in a case–control study of Japanese cohorts. We also examined the association of *DBZ* with schizophrenia and with bipolar disorder, and compared the mRNA levels of *DBZ* in the postmortem brains of schizophrenia, bipolar and control samples. DNA from 180 schizophrenia patients 201 controls were used for the association study of *PCNT2* and *DBZ* with schizophrenia. Association of *DBZ*

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with bipolar disorder was examined in DNA from 238 bipolar patients and 240 age- and gender-matched controls. We observed significant allelic and genotypic associations of the *PCNT2* SNPs, rs2249057, rs2268524, and rs2073380 (Ser/Arg) with schizophrenia; the association of rs2249057 ($P = 0.002$) withstand multiple testing correction. Several two SNP- and three SNP-haplotypes showed significant associations; the associations of haplotypes involving rs2249057 withstand multiple testing correction. No associations were observed for *DBZ* with schizophrenia or with bipolar disorder; further, there was no significant difference between the *DBZ* mRNA levels of control, schizophrenia and bipolar postmortem brains. We suggest a possible role of PCNT2 in the pathogenesis of schizophrenia. Abnormalities of PCNT2, the centrosomal protein essential for microtubule organization, may be suggested to lead to neurodevelopmental abnormalities.

Key words: DISC1; PCNT2; DBZ; schizophrenia; bipolar disorder

INTRODUCTION

Several genetic studies including genome-wide linkage scans and association studies have implicated a potential susceptibility region for psychiatric disorders on chromosome 1q, especially involving the Disrupted-In-Schizophrenia 1 (*DISC1*) gene [Blackwood et al., 2001; Ekelund et al., 2001; Hennah et al., 2003]. *DISC1* has been identified as a disrupted gene by a balanced translocation (1; 11)(q42.1; q14.3) that cosegregated with major psychiatric disorders in a large Scottish kindred [St Clair et al., 1990; Millar et al., 2000; Blackwood et al., 2001; Millar et al., 2001].

DISC1 variations have been implicated in the positive symptoms of schizophrenia [DeRosse et al., 2007; Szeszko et al., 2008], and have been reported to influence the prefrontal function [Prata et al., 2008; Szeszko et al., 2008]. *DISC1*-transgenic mice have been found to exhibit brain abnormalities [Kvajo et al., 2008; Pletnikov et al., 2008] and behavioral phenotypes [Clapcote et al., 2007; Hikida et al., 2007; Li et al., 2007; Pletnikov et al., 2008] reminiscent of schizophrenia. Recent studies report the association of *DISC1* polymorphisms with schizophrenia [Hennah et al., 2003; Kockelkorn et al., 2004; Callicott et al., 2005; Sachs et al., 2005; Zhang et al., 2006; Chen et al., 2007; Qu et al., 2007; Saetre et al., 2008] and with bipolar disorder [Hodgkinson et al., 2004; Thomson et al., 2005].

DISC1 is a multifunctional protein capable of interacting with several cytoskeletal and centrosomal proteins via distinct functional domains [Millar et al., 2003; Miyoshi et al., 2003; Morris et al., 2003; Ozeki et al., 2003; Brandon et al., 2004]. Through these interactions, DISC1 functions as a component of the intracellular machinery that integrates multiple functions including intracellular transport, neuronal cell signaling, and neuronal migration and architecture [Hennah et al., 2006; Ishizuka et al., 2006; Porteous et al., 2006]. The impact of DISC1 across several psychiatric diagnostic categories, thus implicates a complex interaction among loci both within the gene itself, and between DISC1 and its multitude of binding partners.

Previously, we reported the fasciculation and elongation protein-zeta 1 [FEZ1; Miyoshi et al., 2003], pericentrin 2 [PCNT2; Miyoshi et al., 2004], and DISC1-binding zinc finger protein [DBZ, also known as zinc finger protein 365 (ZNF365); Hattori et al., 2007] as interacting partners of DISC1. FEZ1, PCNT2, and DBZ bind to overlapping regions of DISC1; in addition, the domain of DISC1 interacting with DBZ is close to the translocation breakpoint in *DISC1* [Hattori et al., 2007]. *FEZ1* has been found to be associated with schizophrenia in Japanese cohorts [Yamada et al., 2004]. Lipska et al. [2006] reported reduced mRNA levels of *FEZ1* in the postmortem brain tissues of schizophrenia patients. We observed elevated expression of *PCNT2* in the postmortem brains and in the peripheral blood lymphocytes (PBL) of bipolar disorder patients, compared to healthy controls; however, there was no significant association of *PCNT2* with bipolar disorder [Anitha et al., 2008]. So far, there are no reports on the association of *DBZ* with any psychiatric disorders.

Here, we examined the genetic association of *PCNT2* with schizophrenia in a case–control study of Japanese cohorts. We also examined the genetic association of *DBZ* with schizophrenia and with bipolar disorder. In addition, we compared the mRNA levels of *DBZ* in the postmortem prefrontal cortices of schizophrenia, bipolar disorder and control groups.

MATERIALS AND METHODS

Association Study

Subjects. This study was approved by the Ethics Committee of Hamamatsu University School of Medicine; patient confidentiality was maintained at all times, and written informed consent was obtained from all the participants. For the association study of *PCNT2* and *DBZ* with schizophrenia, we collected blood samples from 180 schizophrenia patients [age 53.58 ± 12 years (mean $\pm$ SD); male/female 99:81] and 201 healthy control subjects (age 40.54 ± 13 years; male/female 98:103); there was no significant difference in sex distribution between the case and control groups ($\chi^2 = 1.48$; df $= 1$; $P = 0.223$). We examined the association of *DBZ* with bipolar disorder, in blood samples collected from 238 bipolar disorder patients (age 51.28 ± 13.18 years; male/female 131:107) and 240 age- and gender-matched healthy controls (age 51.49 ± 10.73 years, male/female 120:120); the controls samples were independent of the samples used in the association study of *PCNT2* and *DBZ* with schizophrenia. All the subjects were recruited from a geographical area located in central Japan. Best-estimate lifetime diagnoses of patients were made by direct interview with experienced psychiatrists, according to DSM-IV criteria [American Psychiatric Association, 1994]. Control subjects were recruited from hospital staff and company employees documented to be free from any psychiatric problems.

Genomic DNA was extracted from whole blood using QIAamp DNA blood kit (QIAGEN, Maryland, MD).

Marker selection. The genomic structures of *PCNT2* (chromosomal location 21q22.3) and *DBZ* (10q21.2) are based on the UCSC March 2006 draft assembly of human genome (http://www.genome.ucsc.edu). SNPs for association study were selected using the Applied Biosystems (ABI; Foster City, CA) software SNPbrowser 1.0.19; SNPs reported in databases like National Centre for Biotechnology information (NCBI dbSNP: http://www.ncbi.nlm.nih.gov/SNP) and Japanese Single Nucleotide Polymorphisms (JSNP: http://snp.ims.u-tokyo.ac.jp) were also

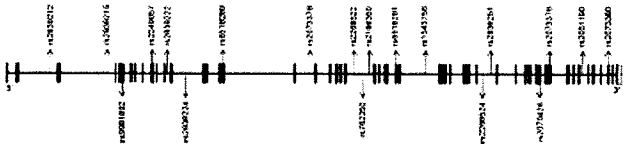

FIG. 1. Genomic structure of *PCNT2* and locations of SNPs. Exons are indicated by boxes, with translated regions in closed boxes and untranslated regions in open boxes; SNP positions are denoted by arrows.

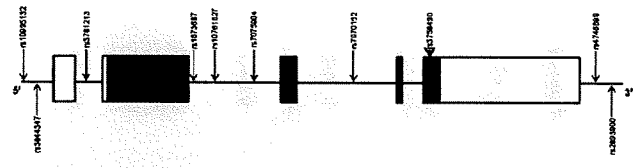

FIG. 2. Genomic structure of *DBZ* and locations of SNPs. Exons are indicated by boxes, with translated regions in closed boxes and untranslated regions in open boxes; SNP positions are denoted by arrows.

referred to. On the basis of their genomic locations and minor allele frequencies (MAF > 0.1) in Japanese population, 19 SNPs of *PCNT2* (Fig. 1) and 10 SNPs of *DBZ* (Fig. 2), were chosen for the association study, aiming at an average spacing of one common SNP at every 3–5 kb.

Genotyping. Assay-on-Demand™ or Assay-by-Design SNP genotyping products (ABI) were used to score SNPs, based on the TaqMan assay method [Ranade et al., 2001]. Genotypes were determined in ABI PRISM 7900 Sequence Detection System (SDS), and analyzed using SDS v2.0 (ABI).

Statistical analysis. All the genotyping results were tested for Hardy–Weinberg Equilibrium (HWE). The statistical significance of variations in allelic and genotypic distributions between the schizophrenia and control groups was evaluated using Fisher's exact test. Haplotype associations were examined using the COCAPHASE program of UNPHASED v2.403 [Dudbridge, 2003; http://www.litbio.org]. Expectation maximization (EM) algorithm was used to resolve uncertain haplotypes, infer missing genotypes and provide maximum-likelihood estimation of frequencies. LD parameters were estimated using the ldmax option of GOLD v1.1.0 [Excoffier and Slatkin, 1995; Abecasis and Cookson, 2000; http://www.well.ox.ac.uk/asthma/GOLD/]. LD strength was estimated in terms of the squared correlation coefficient [r^2; Devlin and Risch, 1995].

Gene Expression Analysis

Brain RNA. RNA from dorsolateral prefrontal cortex (DLPFC; Brodmann's area 46) was donated by The Stanley Medical Research Institute [SMRI; http://www.stanleyresearch.org/programs/brain_collection.asp; Torrey et al., 2000]. RNA from 31 schizophrenia patients, 33 bipolar disorder patients, and 32 control subjects were used in the study; the demographic details of each group are shown in Table I. Since the RNA samples were coded, the diagnoses of the subjects were masked, while the assays were performed.

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). Real-time qRT-PCR analysis was performed using the ABI PRISM 7900 SDS. TaqMan primer/probes for *DBZ* and for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), which served as the endogenous reference, were purchased (Assay-on-Demand) from ABI; TaqMan assays were performed in duplicate according to the manufacturer's protocol. A comparative threshold cycle (C_T) method validation experiment was done to check whether the efficiencies of target and reference amplifications were approximately equal (the slope of the log input amount vs. $\Delta C_T < 0.1$). One sample was randomly chosen as the calibrator, and was amplified in each plate, to correct for experimental differences among consecutive PCR runs. The amounts of *DBZ* mRNAs were normalized to the endogenous reference, and expressed relative to the calibrator as $2^{-\Delta\Delta C_T}$ (comparative C_T method).

Statistical analysis. Statistical calculations were performed using the SPSS statistical package, version 11.0.1 (SPSS Co. Ltd., Tokyo, Japan). One-way analysis of variance (ANOVA) was used to check the variability in the distribution of demographic variables and the variability in *DBZ* expression across groups. Any effect of various demographic or clinical variables on *DBZ* expression was examined by Pearson's correlation coefficient.

TABLE I. Demographic Characteristics of Control, Schizophrenia and Bipolar Disorder Brain Samples

Variables	Control (N = 32)	Schizophrenia (N = 31)	Bipolar disorder (N = 33)	*P*-value
Age (years) (mean ± SD)[a]	43.5 ± 7.39	42.64 ± 8.90	45.41 ± 10.70	0.457
Postmortem interval (hr)[a]	29.87 ± 13.30	31.23 ± 16.50	36.97 ± 17.70	0.164
Brain pH[a]	6.63 ± 0.25	6.47 ± 0.24	6.42 ± 0.30	***0.007***
Male/female[b]	23:9	23:8	16:7	***0.039***
Lifetime dose of antipsychotics[c,d]	—	89,360 ± 105,375	10,339 ± 23,181	***<0.001***

[a]One-way ANOVA.
[b]χ^2 test.
[c]Fluphenazine equivalents.
[d]*t*-test.
Bold values indicate significant *P*-values.

TABLE II. Allelic and Genotypic Distributions of *PCNT2* SNPs in Schizophrenia and Control Groups

Marker	SNP	Samples	Allele[a]		*P*-value[b]	Genotype[a]			*P*-value[b]
SNP01	rs2839212		C	T		C/C	C/T	T/T	
	Intron 2	SC (180)	261 (0.73)	99 (0.27)	0.111	96 (0.53)	69 (0.38)	15 (0.09)	0.251
		CT (201)	312 (0.78)	90 (0.22)		121 (0.60)	70 (0.35)	10 (0.05)	
SNP02	rs2839215		G	A		G/G	G/A	A/A	
	Intron 3	SC (180)	260 (0.72)	100 (0.28)	0.156	94 (0.52)	72 (0.40)	14 (0.08)	0.327
		CT (201)	309 (0.77)	93 (0.23)		120 (0.60)	69 (0.34)	12 (0.06)	
SNP03	rs9981892		G	A		G/G	G/A	A/A	
	Intron 5	SC (178)	254 (0.71)	102 (0.29)	0.135	90 (0.51)	74 (0.42)	14 (0.07)	0.308
		CT (200)	305 (0.76)	95 (0.24)		116 (0.58)	73 (0.37)	11 (0.05)	
SNP04	rs2249057		C	A		C/C	C/A	A/A	
	Exon 10 (silent)	SC (180)	230 (0.64)	130 (0.36)	***0.002***	81 (0.45)	68 (0.38)	31 (0.17)	***0.003***
		CT (201)	211 (0.52)	191 (0.48)		56 (0.28)	99 (0.49)	46 (0.23)	
SNP05	rs2839222		A	G		A/A	A/G	G/G	
	Intron 12	SC (178)	258 (0.72)	98 (0.28)	0.075	93 (0.52)	72 (0.40)	13 (0.08)	0.18
		CT (200)	313 (0.78)	87 (0.22)		122 (0.61)	69 (0.35)	9 (0.04)	
SNP06	rs2839224		G	A		G/G	G/A	A/A	
	Intron 13	SC (180)	260 (0.72)	100 (0.28)	0.052	94 (0.52)	72 (0.40)	14 (0.08)	0.138
		CT (200)	314 (0.79)	86 (0.21)		123 (0.62)	68 (0.34)	9 (0.04)	
SNP07	rs6518289		C	T		C/C	C/T	T/T	
	Exon 15 (missense)	SC (180)	258 (0.72)	102 (0.28)	0.114	94 (0.52)	70 (0.39)	16 (0.09)	0.227
		CT (201)	309 (0.77)	93 (0.23)		118 (0.59)	73 (0.36)	10 (0.05)	
SNP08	rs2073378		C	G		C/C	C/G	G/G	
	Intron 16	SC (176)	258 (0.73)	94 (0.27)	0.175	94 (0.53)	70 (0.40)	12 (0.07)	0.379
		CT (201)	312 (0.78)	90 (0.22)		120 (0.60)	72 (0.36)	9 (0.04)	
SNP09	rs2268522		G	A		G/G	G/A	A/A	
	Intron 21	SC (180)	245 (0.68)	115 (0.32)	0.204	83 (0.46)	79 (0.44)	18 (0.10)	0.57
		CT (201)	291 (0.72)	111 (0.28)		103 (0.51)	82 (0.41)	16 (0.08)	
SNP10	rs762250		G	C		G/G	G/C	C/C	
	Intron 21	SC (180)	245 (0.68)	115 (0.32)	0.204	83 (0.46)	79 (0.44)	18 (0.10)	0.417
		CT (201)	291 (0.72)	111 (0.28)		106 (0.53)	79 (0.39)	16 (0.08)	
SNP11	rs2186350		A	G		A/A	A/G	G/G	
	Intron 21	SC (179)	243 (0.68)	115 (0.32)	0.202	82 (0.46)	79 (0.44)	18 (0.10)	0.392
		CT (199)	288 (0.72)	110 (0.28)		105 (0.53)	78 (0.39)	16 (0.08)	
SNP12	rs6518291		A	G		A/A	A/G	G/G	
	Exon 26 (missense)	SC (180)	262 (0.73)	98 (0.27)	0.051	95 (0.53)	72 (0.40)	13 (0.07)	0.149
		CT (201)	317 (0.79)	85 (0.21)		125 (0.62)	67 (0.33)	9 (0.05)	
SNP13	rs1543756		G	A		G/G	G/A	A/A	
	Intron 27	SC (180)	262 (0.73)	98 (0.27)	0.051	95 (0.53)	72 (0.40)	13 (0.07)	0.149
		CT (201)	317 (0.79)	85 (0.21)		125 (0.62)	67 (0.33)	9 (0.05)	
SNP14	rs2268524		C	T		C/C	C/T	T/T	
	Intron 31	SC (178)	211 (0.59)	145 (0.41)	***0.016***	63 (0.35)	85 (0.48)	30 (0.17)	***0.045***
		CT (201)	203 (0.50)	199 (0.50)		49 (0.24)	105 (0.52)	47 (0.24)	

SNP15	rs2839251		C	T		C/C	C/T	T/T			
	Intron 31	SC (179)	260 (0.73)	98 (0.27)	0.211	94 (0.53)	72 (0.40)	13 (0.07)	0.434		
		CT (201)	308 (0.77)	94 (0.23)		117 (0.58)	74 (0.37)	10 (0.05)			
SNP16	rs2070426		C	G		C/C	C/G	G/G			
	Exon	SC (179)	243 (0.68)	115 (0.32)	0.176	82 (0.46)	79 (0.44)	18 (0.10)	0.364		
	37 (missense)										
		CT (200)	290 (0.73)	110 (0.27)		105 (0.53)	80 (0.40)	15 (0.07)			
SNP17	rs2073376		G	A		G/G	G/A	A/A			
	Exon	SC (179)	243 (0.68)	115 (0.32)	0.176	82 (0.46)	79 (0.44)	18 (0.10)	0.364		
	38 (missense)										
		CT (200)	290 (0.73)	110 (0.27)		105 (0.53)	80 (0.40)	15 (0.07)			
SNP18	rs2051190		T	C		T/T	T/C	C/C			
	Intron 41	SC (180)	245 (0.68)	115 (0.32)	0.177	83 (0.46)	79 (0.44)	18 (0.10)	0.374		
		CT (201)	292 (0.73)	110 (0.27)		106 (0.53)	80 (0.40)	15 (0.07)			
SNP19	rs2073380		A	C		A/A	A/C	C/C			
	Exon	SC (180)	216 (0.60)	144 (0.40)	***0.009***	66 (0.37)	84 (0.47)	30 (0.16)	***0.025***		
	45 (missense)										
		CT (200)	202 (0.50)	198 (0.50)		49 (0.25)	104 (0.52)	47 (0.23)			

SC, schizophrenia; CT, control; the number of genotyped individuals are given in parentheses.
[a]Number followed by frequency in parentheses.
[b]Fisher's Exact test; significant *P*-values are indicated in bold italics.

RESULTS

Association Study of PCNT2 With Schizophrenia

The genotypic distributions of all the SNPs were found to be in HWE in the control group; however, in the schizophrenia group, SNP04 showed a deviation from HWE ($P = 0.015$). The allelic and genotypic frequencies of the 19 SNPs of *PCNT2* in the schizophrenia and control groups are summarized in Table II. SNP04 ($P = 0.002$; OR = 1.6), SNP14 ($P = 0.016$; OR = 1.43), and SNP19 ($P = 0.009$; OR = 1.47) showed significant allelic associations with schizophrenia; the association of SNP04 withstands multiple testing correction. The frequencies of C allele of SNP04 (rs2249057; Exon 10; Ser/Ser), C allele of SNP 14 (rs2268524; Intron 31) and A allele of SNP19 (rs2073380; Exon 45; Ser/Arg) were higher in the schizophrenia group compared to controls. Further, SNP04 ($P = 0.003$), SNP14 ($P = 0.045$), and SNP19 ($P = 0.025$) showed significant variations in the distributions of genotypic frequencies between schizophrenia and control groups.

The *P*-values of haplotypic distributions between schizophrenia and control groups, involving groups of two SNPs (with one SNP overlap) and groups of three SNPs (with two SNP overlap) are given in Table III. Several two-SNP haplotypes showed significant associations; among these, the association of SNP03–SNP04 ($P = 0.001$) reached Bonferroni-corrected significance [0.0027 (α/number of comparisons = 0.05:18)]. Several three-SNP haplotypes also showed significant associations, among which, the association of SNP03–SNP04–SNP05 ($P = 0.002$) reached Bonferroni-corrected significance [0.0029 (α/number of comparisons = 0.05:17)].

A graphic representation of the LD strength (r^2 values) between markers in the schizophrenia group is shown in Figure S1; strong LD ($r^2 = 0.920$) was observed between SNPs 14 and 19, which showed

TABLE III. Two- and Three-SNP Haplotype Analysis of *PCNT2*

Two-SNP	*P*-value[a]	Three-SNP	*P*-value[a]
1-2	0.182	1-2-3	0.057
2-3	***0.049***	2-3-4	***0.019***
3-4	***0.001***	3-4-5	***0.002***
4-5	***0.006***	4-5-6	***0.004***
5-6	***0.043***	5-6-7	0.086
6-7	0.091	6-7-8	0.297
7-8	0.199	7-8-9	0.129
8-9	0.066	8-9-10	***0.044***
9-10	0.241	9-10-11	0.225
10-11	0.178	10-11-12	***0.008***
11-12	***0.008***	11-12-13	0.055
12-13	0.055	12-13-14	***0.047***
13-14	***0.039***	13-14-15	***0.004***
14-15	0.061	14-15-16	0.246
15-16	***0.038***	15-16-17	***0.03***
16-17	0.162	16-17-18	0.162
17-18	0.164	17-18-19	***0.017***
18-19	***0.023***		

[a]Significant *P*-values are indicated in bold italics.

associations with schizophrenia. The LD pattern was found to be similar in the schizophrenia and control (data not shown) groups.

Association Study of DBZ With Schizophrenia

The allelic and genotypic frequencies of the 10 SNPs of *DBZ* are given in Table IV. Genotypic distributions of all the SNPs were found to be in HWE, in the schizophrenia and control groups. None of the SNPs showed any significant associations with schizophrenia. No significant haplotype associations were observed in the two-SNP or three-SNP haplotype analyses.

Association Study of DBZ With Bipolar Disorder

The allelic and genotypic frequencies of the 10 SNPs of *DBZ* are shown in Table V. The genotypic distributions of all the SNPs were found to be in HWE in the control group; however, SNP09 (rs4746698) showed a marginal deviation from HWE, in the bipolar group ($P = 0.033$). None of the SNPs showed any significant associations with bipolar disorder. No significant haplotype associations were observed in the two-SNP or three-SNP haplotype analyses.

DBZ Gene Expression Analysis

The variability in the distribution of demographic variables across the control, schizophrenia and bipolar disorder groups are summarized in Table I. There was no significant difference ($F = 0.34$; df = 2.93; $P = 0.713$) in *DBZ* expression across the three groups (Fig. S2). No significant correlations were observed between *DBZ* expression and any of the clinical features of schizophrenia or bipolar groups.

DISCUSSION

In this study, we observed SNP- and haplotype- associations of the DISC1-interacting molecule *PCNT2* with schizophrenia. SNP04, SNP14, and SNP19 showed significant allelic and genotypic associations. The allelic association of SNP04 and the haplotypic associations involving SNP04 withstand multiple testing correc-

TABLE IV. Allelic and Genotypic Distributions of *DBZ* SNPs in Schizophrenia and Control Groups

Marker	SNP	Samples	Allele[a]		*P*-value[b]	Genotype[a]			*P*-value[b]
SNP01	rs10995132		G	A		G/G	G/A	A/A	
	5′	SC (179)	311 (0.87)	47 (0.13)	0.660	135 (0.75)	41 (0.23)	3 (0.02)	0.794
		CT (199)	345 (0.86)	47 (0.14)		148 (0.74)	49 (0.24)	2 (0.02)	
SNP02	rs3844347		A	G		A/A	A/G	G/G	
	5′	SC (180)	288 (0.80)	72 (0.20)	0.426	116 (0.65)	56 (0.31)	8 (0.04)	0.541
		CT (201)	311 (0.77)	91 (0.23)		119 (0.59)	73 (0.36)	9 (0.05)	
SNP03	rs3781213		G	C		G/G	G/C	C/C	
	Intron 1	SC (180)	287 (0.79)	73 (0.21)	0.428	115 (0.64)	57 (0.31)	8 (0.05)	0.561
		CT (201)	310 (0.77)	92 (0.23)		118 (0.59)	74 (0.36)	9 (0.05)	
SNP04	rs1873687		T	C		T/T	T/C	C/C	
	Intron 2	SC (180)	285 (0.79)	75 (0.21)	0.659	114 (0.63)	57 (0.32)	9 (0.05)	0.560
		CT (200)	311 (0.78)	89 (0.22)		119 (0.60)	73 (0.36)	8 (0.04)	
SNP05	rs10761627		T	C		T/T	T/C	C/C	
	Intron 2	SC (180)	285 (0.79)	75 (0.21)	0.539	114 (0.63)	57 (0.32)	9 (0.05)	0.595
		CT (200)	309 (0.77)	91 (0.23)		118 (0.59)	73 (0.37)	9 (0.04)	
SNP06	rs7075904		C	G		C/C	C/G	G/G	
	Intron 2	SC (180)	309 (0.86)	51 (0.14)	0.917	133 (0.74)	43 (0.24)	4 (0.02)	1.000
		CT (200)	345 (0.86)	55 (0.14)		149 (0.74)	47 (0.24)	4 (0.02)	
SNP07	rs7070152		A	G		A/A	A/G	G/G	
	Intron 3	SC (180)	286 (0.79)	74 (0.21)	0.857	116 (0.64)	54 (0.30)	10 (0.06)	0.544
		CT (201)	322 (0.80)	80 (0.20)		128 (0.64)	66 (0.33)	7 (0.03)	
SNP08	rs3758490		T	G		T/T	T/G	G/G	
	Exon 5 (Ser/Ala)	SC (176)	221 (0.63)	131 (0.37)	0.063	66 (0.37)	89 (0.51)	21 (0.12)	0.143
		CT (199)	276 (0.69)	122 (0.31)		93 (0.47)	90 (0.45)	16 (0.08)	
SNP09	rs4746698		A	G		A/A	A/G	G/G	
	3′	SC (179)	246 (0.69)	112 (0.31)	0.641	87 (0.49)	72 (0.40)	20 (0.11)	0.301
		CT (200)	268 (0.67)	132 (0.33)		86 (0.43)	96 (0.48)	18 (0.09)	
SNP10	rs2893900		C	T		C/C	C/T	T/T	
	3′	SC (179)	337 (0.94)	21 (0.06)	0.207	159 (0.89)	19 (0.10)	1 (0.01)	0.249
		CT (201)	368 (0.91)	34 (0.09)		168 (0.83)	32 (0.16)	1 (0.01)	

SC, schizophrenia; CT, control; the number of genotyped individuals are given in parentheses.
[a]Number followed by frequency in parentheses.
[b]Fisher's Exact test.

TABLE V. Allelic and Genotypic Distributions of *DBZ* SNPs in Bipolar Disorder and Control Groups

Marker	SNP	Samples[a]	Allele[b]		*P*-value[c]	Genotype[b]			*P*-value[c]
SNP01	rs10995132		G	A		G/G	G/A	A/A	
	5′	BD (237)	407 (0.86)	67 (0.14)	0.783	174 (0.73)	59 (0.25)	4 (0.02)	0.801
		CT (240)	409 (0.85)	71 (0.15)		172 (0.72)	65 (0.27)	3 (0.01)	
SNP02	rs3844347		A	G		A/A	A/G	G/G	
	5′	BD (238)	372 (0.78)	104 (0.22)	0.489	146 (0.61)	80 (0.34)	12 (0.05)	0.565
		CT (240)	366 (0.76)	114 (0.24)		137 (0.57)	92 (0.38)	11 (0.05)	
SNP03	rs3781213		G	C		G/G	G/C	C/C	
	Intron 1	BD (238)	367 (0.77)	109 (0.23)	0.818	143 (0.60)	81 (0.34)	14 (0.06)	0.443
		CT (239)	365 (0.76)	113 (0.24)		136 (0.57)	93 (0.39)	10 (0.04)	
SNP04	rs1873687		T	C		T/T	T/C	C/C	
	Intron 2	BD (236)	368 (0.78)	104 (0.22)	0.489	145 (0.62)	78 (0.33)	13 (0.05)	0.419
		CT (240)	365 (0.76)	115 (0.24)		136 (56.67)	93 (38.75)	11 (4.58)	
SNP05	rs10761627		T	C		T/T	T/C	C/C	
	Intron 2	BD (237)	368 (0.78)	106 (0.22)	0.591	144 (0.61)	80 (0.34)	13 (0.05)	0.540
		CT (239)	364 (0.76)	114 (0.24)		136 (0.57)	92 (0.38)	11 (0.05)	
SNP06	rs7075904		C	G		C/C	C/G	G/G	
	Intron 2	BD (237)	405 (0.85)	69 (0.15)	0.927	173 (0.73)	59 (0.25)	5 (0.02)	0.676
		CT (240)	409 (0.85)	71 (0.15)		172 (0.72)	65 (0.27)	3 (0.01)	
SNP07	rs7070152		A	G		A/A	A/G	G/G	
	Intron 3	BD (237)	385 (0.81)	89 (0.19)	0.333	159 (0.67)	67 (0.28)	11 (0.05)	0.156
		CT (240)	377 (0.78)	103 (0.22)		145 (0.61)	87 (0.36)	8 (0.03)	
SNP08	rs3758490		T	G		T/T	T/G	G/G	
	Exon 5 (Ser/Ala)	BD (236)	323 (0.68)	149 (0.32)	0.445	115 (0.49)	93 (0.39)	28 (0.12)	0.242
		CT (240)	317 (0.66)	163 (0.34)		102 (0.43)	113 (0.47)	25 (0.10)	
SNP09	rs4746698		A	G		A/A	A/G	G/G	
	3′	BD (235)	323 (0.69)	147 (0.31)	0.333	118 (0.50)	87 (0.37)	30 (0.13)	0.086
		CT (240)	315 (0.66)	165 (0.34)		101 (0.42)	113 (0.47)	26 (0.11)	
SNP10	rs2893900		C	T		C/C	C/T	T/T	
	3′	BD (238)	438 (0.92)	38 (0.08)	0.364	202 (0.85)	34 (0.14)	2 (0.01)	0.633
		CT (240)	433 (0.90)	47 (0.10)		196 (0.82)	41 (0.17)	3 (0.01)	

[a]BD: Bipolar disorder; CT: Control; the number of genotyped individuals are given in parentheses.
[b]Number followed by frequency in parentheses.
[c]Fisher's Exact test.

tion. Deviation from HWE was observed for SNP04 in schizophrenia patients; heterozygotes of patients were lower than the expected values based on HWE. Since SNP04 showed association, which withstands correction for multiple testing, the deviation from HWE in patients, but not in controls, may be viewed as an additional evidence of association. We observed a strong LD between SNPs 14 and 19. SNP19, located in exon 45, is a non-synonymous SNP with Ser/Arg substitution. Since Ser is a neutral amino acid and Arg is a strong basic amino acid, the substitution may be suggested to exert an influence on the protein structure of PCNT2. To the best of our knowledge, this is the first report of a genetic association study of *PCNT2* with schizophrenia.

Abnormalities of brain morphogenesis, especially cortical dysplasia, have been observed in individuals with partial deletion of chromosome 21q22.3 harboring the *PCNT2* gene [Yao et al., 2006]. PCNT2, an integral component of the pericentriolar material, interacts with pericentriolar material 1 (PCM1), another member of the DISC1 interactome [Kamiya et al., 2008], for its centrosomal localization, and to accomplish its function in microtubule organization [Balczon et al., 1994; Doxsey et al., 1994; Kubo et al., 1999; Dammermann and Merdes, 2002]. Recently, *PCM1* has been implicated in susceptibility to schizophrenia [Gurling et al., 2006]. In the developing cerebral cortex, suppression of PCM1 has been reported to lead to neuronal migration defects [Kamiya et al., 2008]. Thus, centrosomal proteins such as PCNT2 may be suggested to have an important role in cortical development, and therefore, in the pathogenesis of neurodevelopmental disorders like schizophrenia. The PCNT2-binding region of DISC1 overlaps with the region interacting with FEZ1 [Miyoshi et al., 2004], a schizophrenia susceptibility gene that plays a vital role in axonal outgrowth and fasciculation [Yamada et al., 2004].

In our previous study, elevated expression of *PCNT2* was observed in the brain and in the PBL of bipolar disorder patients, compared to controls; however, there was no significant difference in *PCNT2* expression between control and schizophrenia groups [Anitha et al., 2008]. We suggested that a possible apoptotic process in the brain, resulting from over-expression of *PCNT2*, may underlie the pathogenesis of bipolar disorder. However, in the subsequent association study, none of the SNPs showed association with bipolar disorder.

Considering the results from the genetic analyses of *PCNT2* in bipolar disorder and schizophrenia, it might be suggested that DISC1 and its interacting molecules are involved in psychiatric symptoms that cross diagnostic boundaries. The translocation in the original Scottish family [St Clair et al., 1990] demonstrated that the disruption of DISC1 gene, although sufficient to predispose an individual to psychiatric disorder, was, in itself, insufficient to predict any particular disorder. Although the family members showed predominantly schizophrenic symptoms, they also manifested a wide spectrum of other psychiatric phenotypes. In addition to schizophrenia, DISC1 has also been found to be associated with other neuropsychiatric disorders including bipolar disorder [Hodgkinson et al., 2004; Thomson et al., 2005], major depression [Hashimoto et al., 2006], and autism [Kilpinen et al., 2008]. Accumulating evidence show that malfunction of DISC1 lead to brain structural and functional abnormalities [Faulkner et al., 2008; Kvajo et al., 2008; Pletnikov et al., 2008; Prata et al., 2008; Szeszko et al., 2008]. Thus, it may be suggested that the basis of the shared risk for major neurodevelopmental disorders could be centered on DISC1, while its interaction with other members of the interactome lead to different phenotypic outcomes.

Several other DISC1-interacting molecules, including phosphodiesterase 4B [PDE4B; Numata et al., 2008], 14-3-3ε [YWHAE; Ikeda et al., 2008], nudE nuclear distribution gene E homolog 1 [NDE1; Hennah et al., 2007], nudE nuclear distribution gene E homolog like 1 [NDEL1; Burdick et al., 2008], and activating transcription factor 4 [*ATF4*; Qu et al., 2008], have been suggested as potential susceptibility genes for schizophrenia. The DISC1 interactome is expanding with the discovery of novel interacting proteins, and this information could be useful in the identification of potential pathways involving DISC1, in the pathology of major psychoses.

In this study, no significant genetic associations were observed for *DBZ*, the other DISC1-interacting molecule, with schizophrenia or with bipolar disorder; in addition, there was no significant difference between the *DBZ* mRNA levels of control, schizophrenia and bipolar postmortem brains.

In conclusion, we suggest a possible role of PCNT2 in the pathogenesis of schizophrenia. Abnormalities of PCNT2 may lead to defects in microtubule function; during the development of the central nervous system, this dysfunction might result in alterations in neuronal migration, axonal extension, and neurite outgrowth, subsequently leading to impaired neurodevelopment. Our study is limited by small sample size; therefore, the results should be treated with caution, until replication using a larger sample size. It would also be interesting to study the role of PCNT2 in neurodevelopment.

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DISC1–kendrin interaction is involved in centrosomal microtubule network formation

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ABSTRACT

Disrupted-In-Schizophrenia 1 (*DISC1*) was identified as a novel gene disrupted by a (1;11)(q42.1;q14.3) translocation segregating with schizophrenia, bipolar disorder and other major mental illnesses in a Scottish family. We previously identified 446–533 amino acids of DISC1 as the kendrin-binding region by means of a directed yeast two-hybrid interaction assay and showed that the DISC1–kendrin interaction is indispensable for the centrosomal localization of DISC1. In this study, to confirm the DISC1–kendrin interaction, we examined the interaction between deletion mutants of DISC1 and kendrin. Then, we demonstrated that the carboxy-terminus of DISC1 is indispensable for the interaction with kendrin. Furthermore, the immunocytochemistry revealed that the carboxy-terminus of DISC1 is also required for the centrosomal targeting of DISC1. Overexpression of the DISC1-binding region of kendrin or the DISC1 deletion mutant lacking the kendrin-binding region impairs the microtubule organization. These findings suggest that the DISC1–kendrin interaction plays a key role in the microtubule dynamics.

Schizophrenia is a severe mental disorder affecting about 1% of the population worldwide. The pathogenic mechanism of schizophrenia is thought to involve the combined effects of multiple genetic components. And although genetic linkage and association studies have identified potential susceptibility genes, the pathogenic molecular mechanism of schizophrenia has yet to be elucidated.

In a large Scottish family, a balanced (1;11)(q42.1;q14.3) translocation segregating with schizophrenia and affective disorders was identified [1,2]. The *Disrupted-In-Schizophrenia 1* (*DISC1*) gene on chromosome 1 was originally identified as the gene disrupted by this translocation [2,3]. The open reading frame in DISC1 encodes a protein of 854 amino acids that has no significant homology to other known proteins. The amino-terminal region (amino acids 1–347) consists of one or more globular domains, while the helical carboxy-terminal region (amino acids 348–854) contains the translocation breakpoint and three stretches with coiled-coils that have the potential to interact with other proteins [2]. In a previous study, we identified two DISC1-binding partners, the fasciculation and elongation protein zeta-1 (FEZ1) and DISC1-binding zinc-finger protein (DBZ). Both these proteins are involved in neurite outgrowth [4,5]. In addition, several groups have identified DISC1 interaction partners such as NudE-like (NUDEL) [6–8] and lissenchphaly-1 (LIS1) [9]. NUDEL and LIS1 regulate the motor protein dynein, and the NUDEL/LIS1/dynein complex plays a key role in neuronal migration [10–13]. DISC1 has been reported to maintain the NUDEL/LIS1/dynein complex at the centrosome and to regulate neural migration [14]. On the other hand, DISC1 is also involved in the transport of either NUDEL/LIS1/14-3-3ϵ complex or growth factor receptor bound protein 2 (Grb2) to the axonal tips through its interaction with kinesin-1 [15,16]. Therefore, DISC1 plays crucial roles in brain development—including roles in neuronal migration, neurite outgrowth and neural maturation—through its interaction with several cytoskeletal proteins.

We previously identified the large coiled-coil centrosomal protein kendrin (the human orthologue of mouse pericentrin B) as one of the DISC1-binding partners by yeast two-hybrid screening. In the same study, we showed that DISC1 localizes to the centrosome by binding to kendrin and that the residues 446–533 of DISC1 are critical for the interaction with kendrin [17]. Kendrin mediates the nucleation of microtubules by anchoring the gamma-tubulin ring complex, which initiates the assembly of the mitotic spindle apparatus [18–21]. Furthermore, our recent study revealed that *kendrin* is associated with bipolar affective disorder [22]. And mutations in

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kendrin cause Seckel syndrome, which is characterized by microcephaly [23]. Thus, the physiological role of the DISC1–kendrin interaction at the centrosome should be involved in brain development. At present, however, little is know about this role of the DISC1–kendrin interaction.

In this paper, we present the key sequence of DISC1 responsible for the interaction with kendrin and the centrosomal targeting, and provide evidence that the DISC1–kendrin interaction plays a role in the microtubule network formation.

Materials and methods

Plasmids. Plasmids expressing full-length human *DISC1* cDNA were described previously [5]. Full-length human *DISC1* was tagged with a FLAG or HA sequence at the 3′ end and cloned into pcDNA3.1(+) (Invitrogen, Carlsbad, CA). The deletion mutants of DISC1 were constructed: DISC1 ΔKBR (amino acids 446–533 were deleted), GDBP (amino acids 348–597), BPC (amino acids 598–854), BR (amino acids 446–633), KBR (amino acids 446–533) and KBRC (amino acids 446–854). These fragments were tagged with the FLAG or HA sequence at the 3′ end and cloned into pcDNA3.1(+). The deletion mutants of kendrin were constructed by polymerase chain reaction (PCR) using human cDNA as a template and tagged with FLAG at the 5′ end cloned into pcDNA3.1(+): DBR-PACT (amino acids 2913–3232) and PACT (amino acids 3105–3232). These fragments were tagged with the FLAG sequence at the 5′ end and cloned into pcDNA3.1(+).

Antibodies. Polyclonal anti-kendrin antibody was used for the Western blot analysis and immunocytochemistry, and was the kind gift of M. Takahashi and Y. Ono, Kobe University. Polyclonal anti-pericentrin antibody (Covance, Richmond, CA) was used for immunoprecipitation in HEK293T cells. The following antibodies were also used: monoclonal and polyclonal anti-FLAG, monoclonal anti-HA, and monoclonal anti-acetylated tubulin (Sigma–Aldrich, St. Louis, MO) antibodies. The secondary antibodies used were Alexa Fluor 546-conjugated goat anti-mouse or rabbit IgG, Alexa Fluor 488-conjugated goat anti-mouse or rabbit IgG antibody (Molecular Probes Inc., Eugene, OR), and horseradish peroxidase (HRP)-conjugated horse anti-mouse or rabbit IgG antibody (Cell Signaling Tech., Beverly, MA).

Cell culture and transfection. HEK293T and COS-7 cells were cultured in DMEM containing 10% fetal bovine serum (FBS). SK-N-SH cells were cultured in alpha MEM containing 10% FBS. For transient transfection of cells, Lipofectamine 2000 (Invitrogen, Carlsbad, CA) was used for HEK293T, SK-N-SH, and COS-7 according to the manufacturer's instructions.

Western blot analysis and immunoprecipitation. These experiments were performed as described previously [17].

Immunocytochemistry. Cells were fixed with 4% paraformaldehyde for 20 min at 4 °C or ice cold methanol for 10 min at −20 °C, followed by permeabilization with 0.3% Triton X-100 for 15 min. After blocking with 5% bovine serum albumin, the fixed cells were incubated at 4 °C overnight with the primary antibodies: anti-kendrin (1:500), anti-FLAG (1:1000), and anti-acetylated tubulin (1:500). The secondary antibodies labeled with Alexa Fluor 488 and 546 were then applied for 1 h at room temperature. Cells were observed using a Zeiss LSM510 microscope (Carl Zeiss, Oberkochen, Germany).

Microtubule aster formation assay. COS-7 cells were transfected with the FLAG-tagged DBR-PACT, FLAG-tagged PACT and mock vector, individually. After transfection, cells were incubated on ice for 30 min to depolymerize microtubules. The cold medium was removed and prewarmed medium was added for 1 min. Cells were washed with PBS and then incubated with microtubule stabilizer buffer (80 mM PIPES-KOH pH 6.8, 1 mM EGTA, 1 mM $MgCl_2$ and 0.1% TritonX-100) for 1 min. Cells were fixed with cold methanol for 10 min at −20 °C and immunostained.

Results

The carboxy-terminal region of DISC1 is essential for the DISC1–kendrin interaction

We have previously reported an interaction between overexpressed DISC1 and endogenous kendrin by immunoprecipitation assay in mammalian cells, and further suggested that a short fragment of amino acids 446–533 of DISC1 was essential for the interaction with kendrin by means of a directed yeast two-hybrid

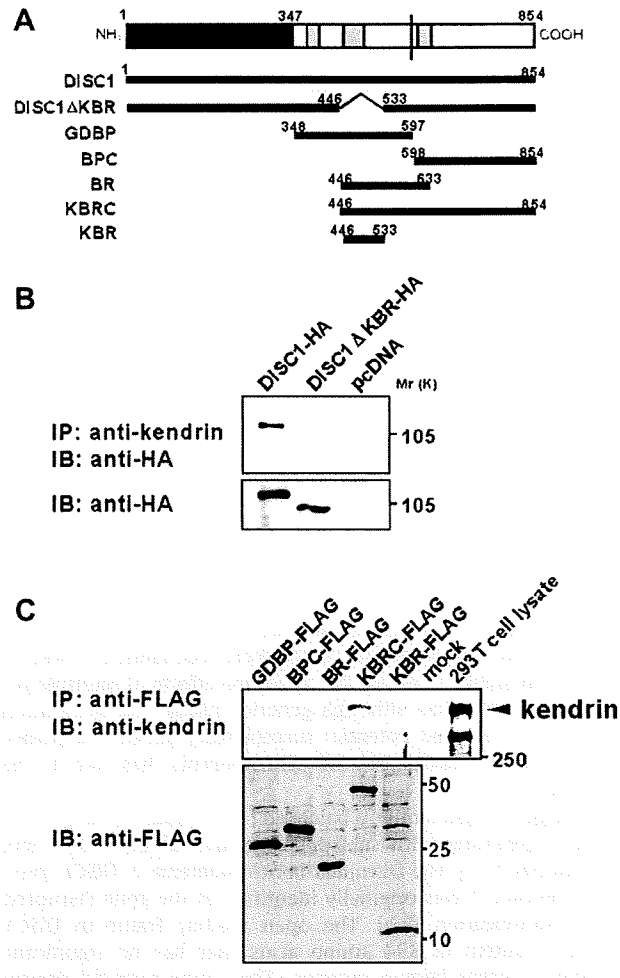

Fig. 1. The carboxy-terminal region of DISC1 is essential for the interaction with kendrin. (A) Structure of deletion mutants of DISC1. The predicted structure of the DISC1 protein is shown in the upper panel. An N-terminal globular region, a carboxy-terminal helical region and three stretches with coiled-coil-forming potential are indicated by black, open and grey rectangles, respectively. The position of the translocation breakpoint is indicated by a vertical line. Various fragments of DISC1 used in this study are shown by closed bars. The numbers indicate the positions of the respective amino acids. (B) HEK 293 cells were transfected with full-length DISC1 (DISC1-HA), a deleted DISC1 that lacks the kendrin-binding region (DISC1 ΔKBR-HA), and empty vector, individually. Immunoprecipitates by anti-kendrin antibody were subjected to Western blot analysis using anti-HA antibody. (C) HEK 293 cells were transfected with various FLAG-tagged fragments of DISC1 shown in (A): GDBP-FLAG, BPC-FLAG, BR-FLAG, KBRC-FLAG and KBR-FLAG. Immunoprecipitates by anti-FLAG antibody were subjected to Western blot analysis using anti-kendrin antibody. KBRC-FLAG interacts with kendrin.

interaction assay [17]. Consistent with the results of our previous study using the yeast two-hybrid assay, the present study confirmed that KBR is critical for the interaction with kendrin by immunoprecipitation assay in HEK293 cells in which kendrin was endogenously expressed. The cells were transiently transfected with the expression vectors for HA-tagged full-length DISC1 (DISC1-HA) and the HA-tagged deletion mutant DISC1 that lacks KBR (DISC1 ΔKBR-HA), individually (Fig. 1A). Cell lysates were prepared, and immunoprecipitates using anti-kendrin antibody were subjected to Western blotting and detected with anti-HA antibody. Endogenous kendrin was coimmunoprecipitated with DISC1-HA, but not with DISC1 ΔKBR-HA (Fig. 1B). These findings indicate that KBR is the binding region of DISC1 to kendrin. Thus, we next examined whether KBR itself could bind to kendrin using an immunoprecipitation assay. For this experiment, we constructed several DISC1 deletion mutants: GDBP-FLAG (amino acids 348–597), BPC-FLAG (amino acids 598–854), BR-FLAG (amino acids 446–633), KBR-FLAG (amino acids 446–533) and KBRC-FLAG (amino acids 446–854) (Fig. 1A). HEK293 cells were transfected with the expression vectors for each deletion mutant. Cell lysates were prepared, and immunoprecipitates using anti-FLAG antibody were subjected to Western blotting and detected with anti-kendrin antibody. Surprisingly, in the immunoprecipitates from cells transfected with KBRC-FLAG, endogenous kendrin was detected. On the other hand, endogenous kendrin was hardly detected in the immunoprecipitates from cells transfected with GDBP-FLAG, BPC-FLAG, BR-FLAG or KBR-FLAG (Fig. 1C). These findings show that KBR is the binding region of DISC1 to kendrin, but this binding to kendrin is enhanced remarkably in the presence of the carboxy-terminal region downstream of KBR. Thus, these results suggest that KBR is required but not sufficient for the interaction, and the carboxy-terminal region of DISC1 is also indispensable for the binding to kendrin.

The carboxy-terminal region of DISC1 is required for the localization of DISC1 to the centrosome

In our previous study, we showed that DISC1 colocalizes with kendrin at the centrosome [17]. As shown in Fig. 1, the KBRC region of DISC1 is essential for the interaction with kendrin. Therefore, we determined which part of DISC1 is indispensable for colocalization of DISC1 with kendrin at the centrosome. For this experiment, human neuroblastoma SK-N-SH cells were transfected with several DISC1 deletion mutants individually: KBRC-FLAG, KBR-FLAG, BR-FLAG, GDBP-FLAG and BPC-FLAG (Fig. 2). Localization of KBRC-FLAG was a diffuse pattern in the cytoplasm but clearly revealed a strong "dot" pattern in the perinucleus (Fig. 2A). The merged image of KBRC-FLAG and kendrin showed that they were colocalized at the centrosome (Fig. 2A). On the other hand, localization of KBR-FLAG showed a diffuse distribution pattern in the nucleus and cytoplasm without strong staining at the centrosome (Fig. 2B). Localization of BR-FLAG and GDBP-FLAG was characterized by a small punctate distribution pattern (Fig. 2C and D). Furthermore, BPC-FLAG exhibited a diffuse distribution in the cytoplasm (Fig. 2E). However, it should be noted that no accumulation of BPC-FLAG was seen at the centrosome. Taken together, these

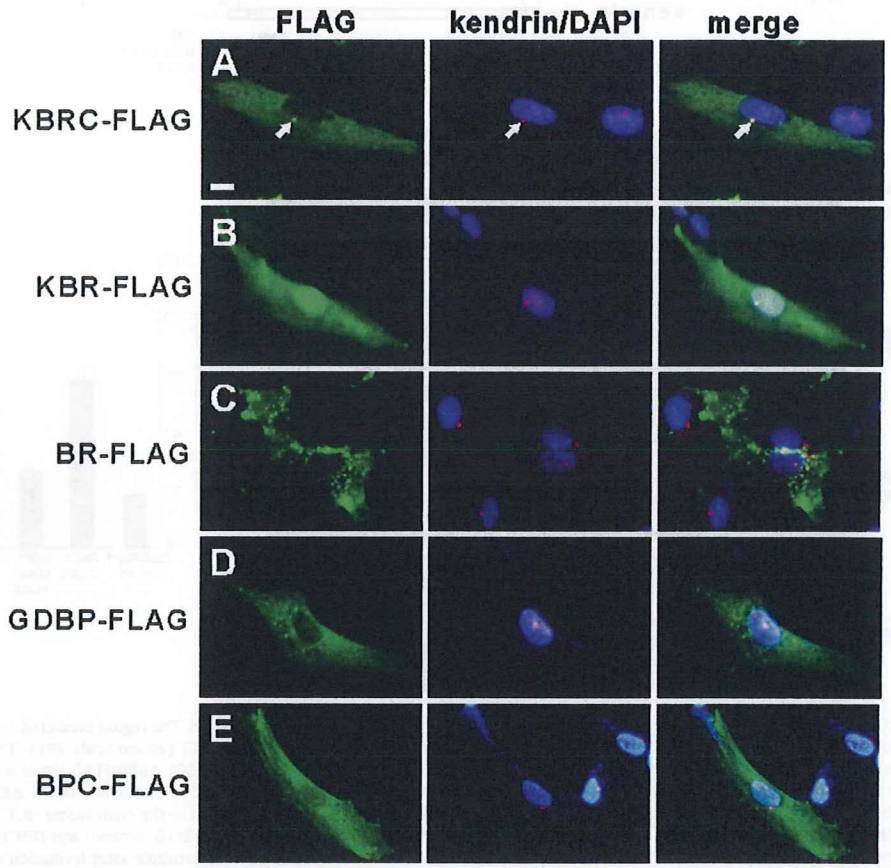

Fig. 2. The carboxy-terminus of DISC1 mediates centrosomal localization. (A–E) Localization of various fragments of FLAG-tagged DISC1. SK-N-SH cells were transiently transfected with KBRC-FLAG (A), KBR-FLAG (B), BR-FLAG (C), GDBP-FLAG (D) and BPC-FLAG (E), respectively. Cells were stained by anti-FLAG antibody (left panels) and anti-kendrin antibody (middle panels). Nuclei were stained blue by DAPI. Merged figures are shown in the right panels. Arrows indicate the colocalization of KBRC-FLAG and kendrin at the centrosome (A). Scale bar, 10 μm.

findings demonstrate that the carboxy-terminal half of the DISC1 protein containing KBR and the downstream region of KBR is necessary and sufficient to target the DISC1 protein to the centrosome, suggesting that this centrosomal localization of DISC1 is mediated by the interaction with kendrin.

Inhibition of DISC1–kendrin interaction perturbs the microtubule network formation

As mentioned above, DISC1 interacts with kendrin at the centrosome. In addition, kendrin has a key role in the microtubule nucleation at the centrosome [20]. Therefore, we analyzed whether the interaction between DISC1 and kendrin might have an influence on the microtubule network formation. To confirm that the DISC1–kendrin interaction is associated with the microtubule organization, we overexpressed the DISC1-binding region of kendrin (DBR) in COS-7 cells. We previously identified amino acids 2918–3035 of kendrin as the DISC1-binding region by yeast two-hybrid screening [17] (Fig. 3A). In addition, to inhibit the DISC1–kendrin interaction specifically at the centrosome, we prepared a FLAG-tagged DBR-PACT (DBR-PACT-FLAG) that includes DBR and the PACT domain (amino acids 3105–3232 of kendrin), a conserved centrosomal targeting motif in CG-NAP and pericentrin [24] (Fig. 3A). We observed the microtubule aster formation in COS cells transfected with either the DBR-PACT-FLAG or PACT-FLAG expression vector (Fig. 3B and C). Mock-transfected cells revealed the microtubule aster formation at the centrosome (Fig. 3E). However, overexpression of DBR-PACT-FLAG resulted in a significant decrease in the percentages of the cells containing the microtubule aster as compared with PACT-FLAG (Fig. 3F). Although previous studies have reported the displacement of endogenous kendrin from centrosomes by overexpression of the PACT domain [25], both DBR-PACT-FLAG and PACT-FLAG were localized at the centrosome without delocalization of endogenous kendrin from the centrosome (data not shown). Furthermore, we analyzed the effect of DISC1 ΔKBR-FLAG, which could not bind to kendrin, on the microtubule aster formation (Fig. 3D). Overexpression of DISC1 ΔKBR-FLAG resulted in a significant decrease in the percentage of cells containing the microtubule aster as compared with mock-transfected cells (Fig. 3F). These results suggest that the DISC1–kendrin interaction is involved in the microtubule organization.

Discussion

The carboxy-terminal region of DISC1 is indispensable for the interaction with kendrin

In the present study, we attempted to confirm the interaction between DISC1 and kendrin by immunoprecipitation assay before analyzing the physiological functions of the DISC1–kendrin interaction at the centrosome. Previously, we suggested that amino acid residues 446–533 of the DISC1 protein would be essential for the

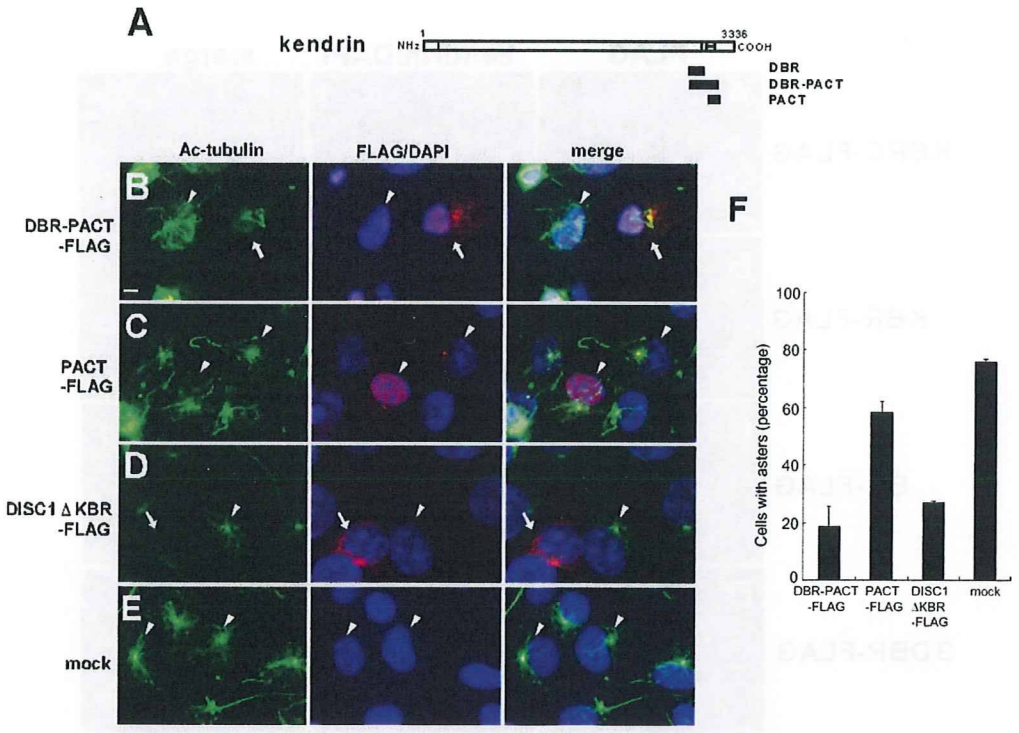

Fig. 3. DISC1–kendrin interaction regulates the microtubule network formation. (A) The structure of the kendrin protein. The region predicted to be a coiled-coil is shown in grey, and various fragments of kendrin, i.e., DBR (DISC1-binding region of kendrin, amino acids 2913–3041), DBR-PACT (amino acids 2913–3232) and PACT (amino acids 3105–3232) are shown with closed bars. (B–E) COS cells were transfected with DBR-PACT-FLAG (B), PACT-FLAG (C), DISC1 ΔKBR-FLAG (D) or mock vector (E). Twenty-four hours after transfection, aster formation was visualized by anti-acetylated tubulin antibody (left panels; green). DBR-PACT, PACT and DISC1 ΔKBR were visualized by anti-FLAG antibody (middle panels; red). Merged images are shown in the right panels. DBR-PACT and PACT were localized to the centrosome (B, C). Normal microtubule aster formation was observed in the PACT-overexpressing cells (C; arrowheads), but not in the DBR-PACT-overexpressing cells (B; arrows) and DISC1 ΔKBR-overexpressing cells (D; arrows). Nuclei were stained blue by DAPI. Scale bar, 10 μm. (F) The percentages of COS cells showing normal microtubule aster formation in DBR-PACT-, PACT-, DISC1 ΔKBR- or mock-overexpressing cells. 58.5% of the PACT-overexpressing cells showed the normal microtubule aster formation. On the other hand, 18.6% of DBR-PACT- and 27.0% of DISC1 ΔKBR-overexpressing cells exhibited the normal microtubule aster formation. At least 50 cells were assessed in each experiment, and columns and vertical bars denote the means ± SEM (triplicate independent experiments). DBR-PACT- and DISC1 ΔKBR-overexpressing cells demonstrated a significant reduction of the normal microtubule aster formation compared with PACT-overexpressing cells ($P < 0.001$, Student's t-test). (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)