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DISC1-dependent switch from progenitor proliferation to migration in the developing cortex

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Abstract

Regulatory mechanisms governing the sequence from progenitor cell proliferation to neuronal migration during corticogenesis are poorly understood^{1–10}. Here we report that phosphorylation of DISC1, a major susceptibility factor for several mental disorders, acts as a molecular switch from maintaining proliferation of mitotic progenitor cells to activating migration of postmitotic neurons. Unphosphorylated DISC1 regulates canonical Wnt signaling via an interaction with GSK3 β , whereas specific phosphorylation at Serine 710 (S710) triggers the recruitment of Bardet-Biedl-Syndrome (BBS) proteins to the centrosome. In support of this model, loss of BBS1 leads to defects in migration, but not proliferation, while DISC1 knockdown leads to deficits in both. A phospho-dead mutant can only rescue proliferation, while a phospho-mimic mutant rescues exclusively migration defects. These data highlight a dual role for DISC1 in corticogenesis and suggest that phosphorylation of this protein at S710 activates a key developmental switch.

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Author Contributions

K.I. and A.S. conceived the general ideas for this study. K.I., A.K., E.C.O., N.K., and A.S. designed experiments. K.I., A.K., E.C.O., and J.F.R. performed the experiments and data analysis with assistance from H.K., S.S., H.M., A.J.D., K.K., K.F., B.H., M.Z., A.H.-T., H.O., K.N., and M.D.H. K.I., A.K., E.C.O., M.D.H., N.K., and A.S. wrote the manuscript.

Author Information

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In the developing cerebral cortex, progenitor cells exit the cell cycle in the ventricular and subventricular zone, whereafter postmitotic neurons move toward the cortical pial surface to form laminated cortical layers. Although molecules such as NDE1 and NDEL1 have been shown to regulate these processes, the molecular mechanisms that transition the cell state from proliferation to migration are largely unknown^{1–10}.

DISC1, a susceptibility factor for a wide range of mental illnesses, including schizophrenia, mood disorders, and autism, is expressed in both neuronal progenitor cells and postmitotic neurons in the developing cerebral cortex^{11–15}. We have reported previously that DISC1 plays a role in radial neuronal migration via anchoring dynein motor-related proteins to the centrosome, including NDEL1, BBS1, and BBS4, two of the proteins mutated in Bardet-Biedl-Syndrome (BBS)^{13,16}. In addition, an animal model that mimics the DISC1 mutation found in a large pedigree with familial psychosis exhibited reduced neural proliferation during cortical midneurogenesis¹⁷. More recently, DISC1 has been shown to mediate the proliferation of neuronal progenitors in the developing cortex in a Wnt/ β -catenin-dependent fashion¹². These observations suggest that DISC1 has a dual neurodevelopmental role and raise the possibility that a switch in DISC1 function might coordinate the transition from proliferation to migration during corticogenesis.

We hypothesized that post-translational modification would be a strong candidate to drive the transition between the two processes. Regulated phosphorylation is an effective, rapid functional switch^{18,19}. We therefore investigated whether DISC1 is phosphorylated *in vivo* and *in vitro*. First, we treated COS7 and SH-SY5Y cells with Okadaic acid, a phosphatase inhibitor, and observed a mobility shift (slower) of exogenously expressed human DISC1, which shifted back to the original state upon treatment with lambda phosphatase (Supplementary Fig. 1a). Moreover, mouse brain extracts treated with calf alkaline phosphatase displayed a downward mobility shift of endogenous DISC1, (Supplementary Fig. 1b), whereas Okadaic acid treatment of rat cortical primary neurons induced an upward mobility shift of endogenous DISC1. Finally, in metabolic labeling, ³²P was incorporated into DISC1, which was enhanced by Okadaic acid treatment but abolished by lambda phosphatase (Supplementary Fig. 1c). These results indicate that DISC1 is a phosphoprotein in the brain.

To identify likely phosphorylated residues in DISC1, we performed mass spectrometry on exogenously expressed human DISC1 isolated from COS7 cells, treated with or without Okadaic acid. The spectrometric profile indicated three probable phosphorylation sites in DISC1 in okadaic acid-treated cells: threonine-50 (T50), serine-58 (S58), and S713 (Supplementary Fig. 2a). Among them, only S58 and S713 are conserved in mouse. To confirm these, we performed site-directed mutagenesis of DISC1 followed by an *in vitro* phosphorylation assay; we found that both PKA and CDK5 phosphorylated a GST-tagged C-terminal fragment of human DISC1 (amino acids 598-854). A phospho-dead mutation at S713 to alanine (A713) in human C-terminal DISC1 abolished phosphorylation, as did the orthologous S710A mutation in mouse DISC1 (Supplementary Fig. 2b). Consistent with these findings, an antibody generated against a phospho-peptide at S710 for mouse DISC1 (pS710 Ab) detected the selective immunoreactivity from extracts of HEK293 cells into

which wild-type DISC1, but not phospho-dead A710-DISC1, is expressed with a catalytic subunit of PKA (Supplementary Fig. 2c). Furthermore, pS710 Ab detects phospho-mimic mutant DISC1 (E710-DISC1: with serine replaced by glutamic acid), but cannot detect either wild-type or A710-DISC1, in the absence of active PKA (Supplementary Fig. 2c). Similar experiments showed that S58 in an N-terminal human DISC1 fragment (amino acids 1-348) was phosphorylated by PKA (Supplementary Fig. 2d).

To determine how phosphorylation of DISC1 influences signaling, we examined known interactions of DISC1, including BBS1, BBS4, NDE1, and NDEL1. We observed significantly enhanced interaction of BBS1 and BBS4 with wild-type DISC1, but not with the phospho-dead mutant A710-DISC1, upon treatment with Okadaic acid in neuronal cells (Fig. 1a and Supplementary Fig. 3a). Enhanced binding of DISC1 with BBS1 was also observed by a phospho-mimic E710-DISC1, even without the presence of Okadaic acid (Fig. 1b and Supplementary Fig. 3b). This enhancement is specific to BBS proteins, but not to NDE1 or NDEL1 (Fig. 1a and Supplementary Fig. 3c). Notably, the effect on the DISC1/BBS interaction is specific to the S710 residue; a S58A mutation did not affect DISC1/BBS1 protein interaction (Supplementary Fig. 3d).

Recruitment of BBS proteins by DISC1 to the centrosome is known to underlie neuronal migration, a key mechanism of corticogenesis¹⁶. We therefore asked whether the observed phospho-regulated DISC1/BBS1 interaction affects the centrosomal recruitment of BBS1. In cortical primary neurons transfected with E710-DISC1, we found BBS1 localization to the centrosome to be increased significantly over that with wild-type and A710-DISC1 (Fig. 1c and Supplementary Fig. 4a, b), an effect not caused by changes in BBS1 levels (Supplementary Fig. 4c). To confirm this, we evaluated the subcellular distribution of BBS1 by sedimentation. Cells in which endogenous DISC1 was replaced by phospho-mimic mutant E710-DISC1 showed concentrated BBS1 protein in the γ -tubulin-enriched fractions (Supplementary Fig. 4d, e). As expected, phosphorylated DISC1 at S710 (pS710-DISC1) is also localized to the centrosome in primary neurons and PC12 cells (Supplementary Fig. 5).

The canonical Wnt pathway is a key regulator of progenitor cell proliferation in the developing cortex²⁰. Moreover, several studies have shown that the centrosome/basal body in postmitotic cells acts as a negative regulator of canonical Wnt signaling, since suppression of BBS1 and BBS4 leads to the aberrant activation of β -catenin signaling^{21,22}. We therefore hypothesized that the phosphorylation-enhanced DISC1/BBS1 binding might titrate DISC1 away from a Wnt/ β -catenin activity and thus contribute to the switch from neuronal progenitor proliferation to neuronal migration. To test this, we examined whether phosphorylation of DISC1 at S710 influenced canonical Wnt signaling by using the established Wnt reporter cell line, HEK 293T Super8XTOPFlash^{23,24}. Knockdown of DISC1 suppressed Wnt/ β -catenin transcriptional activity upon stimulation by Wnt3a (Fig. 2a). Importantly, the rescue of these phenotypes was dependent on the phosphorylation status of DISC1: co-expression of the phospho-dead mutant A710-DISC1 resulted in efficient rescue, whereas the phospho-mimic mutant E710-DISC1 failed completely (Fig. 2a). We also evaluated the expression of cyclin D1, a known β -catenin transcriptional target. Our data were consistent with the reporter assays: cyclin D1 levels, which were suppressed

by DISC1 RNAi, were rescued by wild-type and A710-DISC1, but not by E710-DISC1 (Fig. 2b).

We next assessed the impact of DISC1 RNAi on Wnt signaling *in vivo* by *in utero* gene transfer. Consistent with our *in vitro* data, knockdown of DISC1 by RNAi co-injected with Super8xTOPFlash and pRL SV40 expression constructs at embryonic day 13 (E13) induced a significant reduction of Wnt/ β -catenin signaling activity at E15, which was rescued by wild-type and A710-DISC1 but not by E710-DISC1 (Fig. 2c). When we compared the effects of overexpression of wild-type and mutant DISC1 on β -catenin transcriptional activity by using a second reporter construct which expresses a destabilized GFP variant under the control of a β -catenin responsive promoter and constitutive CAG promoter driven mCherry in tandem (TOPdGFP-CAG mCherry)^{25,26}, we also observed significant upregulation of active β -catenin, as indicated by enhanced GFP expression with A710-DISC1, but not with E710-DISC1 (Fig. 2d). In parallel, protein binding of GSK3 β with DISC1 was augmented by expression of A710-DISC1, but not by E710-DISC1 (Fig. 2e), which was also supported by an *in vitro* binding assay (Supplementary Fig. 6).

To explore the physiological relevance of these data during cortical development, we assessed the levels of pS710-DISC1 in the developing cortex at E14 and E18 with our pS710 antibody (Supplementary Fig. 2c and Supplementary Fig. 5a). As expected, the levels of pS710-DISC1 were greater at E18 (when neuronal migration is prominent) compared with those at E14 (when progenitor proliferation is prominent) (Fig. 3a), while immunohistochemistry with pS710 antibody indicated selective staining in the cortical plate/intermediate zone, but not in the ventricular/subventricular zone (Fig. 3b and Supplementary Fig. 7). We then quantified the relative protein binding of DISC1 with interactors in mouse brain lysates at E14 and E18 by co-immunoprecipitation. We found that the affinity between DISC1 and BBS1 increased from E14 to E18, whereas the DISC1/GSK3 β affinity decreased proportionally during this period (Fig. 3c). Moreover, we observed negligible binding of GSK3 β and pS710-DISC1, whereas protein binding of BBS1 and pS710-DISC1 was augmented significantly at E18 compared to E14 (Fig. 3c). We propose the following model: during mid-embryonic stages when progenitor cell proliferation is prominent (including E14), unphosphorylated DISC1 at S710 binds with GSK3 β more tightly and regulates cell proliferation; in contrast, during later embryonic stages when cell cycle exit of progenitors and the following neuronal migration become predominant, pS710-DISC1 dissociates from GSK3 β and switches its role to the recruitment of BBS1 to the centrosome, activating neuronal migration. To test this model directly, we flow-sorted homogeneous populations of mitotic progenitor cells and post-mitotic neuronal cells from the developing cortex of transgenic mice expressing a Nestin promoter-driven Kusabira-Orange and DCX promoter-driven EGFP27. We observed an increased abundance of pS710-DISC1 and a reduced affinity for GSK3 β with a concomitant increase in the binding for BBS1 both for total DISC1 and pS710-DISC1 in the DCX-positive post-mitotic cells compared with Nestin-positive progenitor cells (Fig. 3d and Supplementary Fig. 8).

Another prediction would be that loss of BBS1 should lead to aberrant migration, but not proliferation. In *Bbs1* knockout mice²⁸, BrdU labeling and scoring at E15 blind to genotype showed that proliferation and cell cycle exit were indistinguishable between nulls and wild-

type littermates (Fig. 3e). By contrast, both Cux1 (marking layers II-IV)- and Ctip2 (marking layer V)-positive cells showed aberrant positioning in *Bbs1*^{-/-} mice (Fig. 3f); a migration defect in superficial layers was also observed, when BrdU or GFP was injected at E15 and the final positioning of the late-born superficial layer neurons was analyzed at P0 and E19 (Supplementary Fig. 9).

To obtain further *in vivo* evidence, we examined how the phosphorylation status at S710 of DISC1 differentially regulates proliferation and neuronal migration in mid- (E13-15) and later- (E15-19) embryonic stages, respectively, by *in utero* gene transfer (Fig. 4 and Supplementary Figs. 10–16). Consistent with previous reports¹², DISC1 knockdown at E13 leads to several deficits and altered cell fate associated with the progenitor proliferation (Fig. 4a and Supplementary Figs. 11, 12a–c). Of note, DISC1 knockdown did not induce marked differences in N-cadherin staining (Supplementary Fig. 12d). In contrast, several groups have reported that suppression of DISC1 at a later time point, such as E15, led to delayed neuronal migration^{13,16}, a phenotype which, if our model is correct, should be dependent on pS710-DISC1. Consistently, we efficiently rescued the DISC1 RNAi-induced migration phenotype either with wild-type DISC1, or with the phospho-mimic mutant E710-DISC1, but not with the phospho-dead mutant A710-DISC1 (Fig. 4b and Supplementary Fig. 13), which is in sharp contrast to the effects of E710-DISC1 and A710-DISC1 on progenitor cell proliferation at earlier time points (Fig. 4a and Supplementary Fig. 11). It is unlikely that the migratory defects are caused by disturbed radial scaffold formation, as we observed no significant effect of DISC1 suppression in radial fiber elongation (Supplementary Fig. 14). Finally, expression of a dominant-negative CDK529 could induce migration defects that phenocopy the DISC1 RNAi phenotypes and are ameliorated by co-expression of E710-DISC1, but not by A710-DISC1 or by wild-type DISC1 (Fig. 4c).

The main finding of our study is that DISC1 is a dynamic protein that acts as a molecular switch between two key stages of cortical development, cell proliferation and neuronal migration. Consistent with our model, *Bbs1*^{-/-} mice display intact proliferation but abnormal migration that was not rescued by wild-type DISC1 (Supplementary Fig. 15). Also, the migration deficit in *Bbs1* knockout mice is more modest compared to that of DISC1 suppression *in utero*, suggesting the presence of other mediators downstream of DISC1.

We cannot exclude the possibility that abnormal positioning in radial migration might be influenced by the observed proliferation deficit. However, DCX promoter-driven wild-type and E710-DISC1 expressed only in the post-mitotic period can also rescue the migration deficit, suggesting that the abnormal neuronal positioning caused by DISC1 RNAi is not a result of impaired proliferation (Supplementary Fig. 16). Two further lines of evidence support this: first, when we injected DISC1 RNAi into E15 brains and analyzed them at E19, cell proliferation had mostly ended⁴; second, depletion of BBS1 is sufficient for abnormal neuronal positioning *in vivo* (Fig. 3f and Supplementary Fig. 9).

Our data raise a number of new questions. For instance, it is unclear why the BBS proteins have a more selective role for the switch to neuronal migration. Recent data have suggested that several BBS proteins may be crucial for the interpretation of planar cell polarity

pathway signaling, while antagonizing canonical Wnt/ β -catenin signaling²¹. Moreover, biochemical kinetics of DISC1-GSK3 β -BBS proteins in association with this allosteric regulation by S710 phosphorylation may also be important.

Finally, it is crucial to consider how disturbances in the DISC1-dependent switch mechanism might have a clinical impact. We speculate that disturbance of this switch mechanism may contribute to hypertrophic and disturbed corticogenesis observed in brains of patients with autism. Although schizophrenia has an onset in young adulthood, the initial pathological insults occur during neurodevelopment¹⁴. It is possible that disturbances of this molecular switch might also underlie this pathology.

Methods summary

Mice

C57/BL6 pregnant female mice were purchased from Charles River for *in utero* gene transfer. BBS 1 knockout mice were backcrossed 4 generations into the C57/BL6 background and characterized elsewhere²⁸.

In utero electroporation and immunohistochemistry

In utero electroporation was performed as described^{12,13,16,30–32}. The RNAi plasmids that had been fully characterized in publications from other groups and ours^{12,13,16,31–33}, were electroporated at E13 or E15. Rescue experiments were conducted by a combination of RNAi plasmid (0.1 $\mu\text{g}/\mu\text{l}$ in 1 μl) with wild-type or mutant DISC1 expression plasmid (2.5 $\mu\text{g}/\mu\text{l}$ in 1 μl). Coronal slices of developing cerebral cortex were prepared as described^{13,30}. Briefly, the brains were fixed with 4% paraformaldehyde and sectioned with a cryostat at 20 μm on E14, E15 and E19, respectively. Nuclei were labeled with DAPI (Invitrogen). Slice images were acquired with a confocal microscope (Zeiss LSM510 Meta).

Methods

Mice

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Plasmids

We used the RNAi constructs to DISC1 that have been established in the previous publications from other groups and ours^{12,13,16,31–33}. A scrambled sequence without homology to any known mRNA was used as control RNAi. The rescue constructs contained three nucleotide alterations in the RNAi target sequence to avoid silencing by RNAi. DCX promoter-driven DISC1 expression constructs (DCX-DISC1) were made by replacing the EGFP-expressing cDNA sequence in DCX-EGFP³⁴ with wt or mutant DISC1-expressing cDNA sequences.

Antibodies

A polyclonal human DISC1 antibody (hExon2 Ab) was raised against amino acids 285-298 in human DISC1. hExon2 Ab detects signals of exogenously expressed hDISC1 in COS7 cell and endogenous DISC1 in SH-SY5Y cell lysates, which were abolished by preabsorption with original antigen. A polyclonal antibody against the phosphopeptide C-LQLQEAGSpSPHAEDE (amino acids 702-716 of mouse DISC1) (pS710 Ab) was generated. These signals were abolished by preabsorption with the original phosphopeptide. An antibody against BBS135 and antibodies against DISC111,36 have been described. The following commercial antibodies were also used: mouse monoclonal antibodies against γ -tubulin (Sigma), HA-tag (Covance), GST-tag (Covance), myc-tag (Santa Cruz), GSK3 α/β (Santa Cruz), GAPDH (Santa Cruz), Pax6 (Iowa Hybridoma Bank), Cyclin D1 (Cell Signaling), N-Cadherin (Invitrogen), BrdU (Chemicon), and RC2 antibody (Iowa Hybridoma Bank); mouse polyclonal antibody against BBS1 (Novus); rat monoclonal antibody against HA-tag (Roche); goat polyclonal antibodies against ER81 (Santa Cruz), and Doublecortin (Santa Cruz); rabbit polyclonal antibodies against TBR2 (Abcam) and γ -tubulin (Sigma); and rabbit monoclonal antibody against Ki67 (NeoMarkers).

Cells and transfection

COS7, SH-SY5Y, and HT22 cells were cultured in DMEM containing 10% fetal bovine serum. PC12 cells were maintained in DMEM containing 10% fetal bovine serum and 5% horse serum. Fugene6 (Roche Applied Sciences) was used for transfection to COS7 cells. SH-SY5Y, HT22, and PC12 cells were transfected with Lipofectamine 2000 (Invitrogen). When used, the phosphatase inhibitor Okadaic Acid (0.5 μ M; Calbiochem) was added 2 h before cells were harvested. Dissociated cortical neuron cultures were prepared as described previously³³.

Immunoprecipitation

Cells or tissue were lysed in lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1% Triton X-100) containing protease inhibitor mixture (Roche Applied Sciences) and phosphatase inhibitor cocktail (Sigma). Lysates were sonicated, cell debris was cleared by centrifugation, and the soluble fraction was subjected to immunoprecipitation as described previously¹³.

Immunofluorescent staining

When transfected, 1 to 2 days after transfection, primary cortical neurons, PC12, or HT22 cells were fixed with ice-cold methanol at -20°C for 15 min. After blocking with 0.1% Triton X-100 and 2% normal goat serum in PBS, cells were incubated with primary antibodies at 4°C overnight, followed by reaction with secondary antibodies conjugated to Rhodamine Red-X, Cy5 (Jackson Immuno Research), and Alexa488 (Molecular Probe) for 1 h. DAPI (Invitrogen) was used to visualize nuclei. Confocal microscopy (Zeiss LSM 510 Meta) was used for epifluorescent image collection. The distribution of BBS1 at the centrosome in cells was quantified as described¹⁶. Briefly, a circle with 3- μ m diameter was drawn centering on the γ -tubulin and defined as the area including the centrosome. Whole cell area was determined by distribution of overexpressed DISC1-HA with HA staining. The

intensity of BBS1 staining in the whole cell area versus that in the centrosome area was quantified with Metamorph (Molecular Devices) for all experimental groups. The intensity ratio of the signal of more than 30 cells per group was analyzed in three independent experiments in a blinded manner.

In vitro β -catenin activity assays with luciferase reporter system

Luciferase reporter system assays were carried out as described²¹. HEK293T cells stably expressing pTOPFlash reporter were seeded in 24-well plates at a density of 10^4 cells per well. After 18–24 h, reporter plasmid and/or *Renilla* luciferase cDNA in an SV40 vector and the plasmid of interest were transfected in six wells by using the Fugene 6 (Roche) optimized transfection protocol. pRL SV40 (*Renilla* luciferase) was used as an internal control. When applicable, after 24 h we treated three wells from each plate with Wnt3a-enriched medium that had been aspirated from Wnt3a/L cells and sterile filtered before being applied to the luciferase assay. Cells were lysed and luciferase activity was measured 48 h after start of stimulation by using the Promega Dual Luciferase Reporter Assay System (E1910) and a FluoStar Luminometer (BMG Technologies). Each assay was repeated at least twice to ensure reproducibility of the results.

Purification of mitotic progenitors and post-mitotic neurons

Progenitor cells and post-mitotic neurons were purified by FACS from the brains of transgenic mice expressing Nestin promoter-driven Kusabira-Orange and DCX promoter-driven EGFP, respectively²⁷.

In utero electroporation and immunohistochemistry

In utero electroporation was performed as described^{12,13,16,30–32}. The RNAi plasmids that had been fully characterized in publications from other groups and ours^{12,13,16,31–33}, were electroporated at E13 or E15. Rescue experiments were conducted by a combination of RNAi plasmid (0.1 $\mu\text{g}/\mu\text{l}$ in 1 μl) with wild-type or mutant DISC1 expression plasmid (2.5 $\mu\text{g}/\mu\text{l}$ in 1 μl). Coronal slices of developing cerebral cortex were prepared as described^{13,30}. Briefly, the brains were fixed with 4% paraformaldehyde and sectioned with a cryostat at 20 μm on E14, E15 and E19, respectively. Nuclei were labeled with DAPI (Invitrogen). Slice images were acquired with a confocal microscope (Zeiss LSM510 Meta).

In vivo β -catenin activity assays with luciferase reporter system

Super8xTOPFlash and pRL SV40 together with expression constructs and/or RNAi constructs were electroporated at E13 and luciferase activity was measured at E15.

In vivo β -catenin activity assays with TOPdGFP-CAG mCherry

A dual reporter construct expressing a destabilized GFP variant under the control of a β -catenin responsive promoter and constitutive CAG promoter-driven mCherry in tandem (TOPdGFP-CAG mCherry)^{25,26} together with expression constructs and/or RNAi constructs were electroporated at E13. After 24 h, the brains were extracted and assessed for mCherry and GFP expression.

BrdU incorporation assay

BrdU (50 mg/kg) was injected i.p. into pregnant mice 48 h after electroporation. After 2 h, brains were processed and sections were stained with anti-BrdU antibodies.

Cell cycle exit assay

BrdU (50 mg/kg) was injected i.p. into pregnant mice 24 h after electroporation. Twenty-four hours later, brains were processed and sections were stained with anti-BrdU and anti-Ki67 antibodies.

Definition of the ventricular zone (VZ), subventricular zone (SVZ), and intermediate zone (IZ) for assessment of progenitor proliferation at E15

The VZ/SVZ boundary was defined by the segregation of Pax6- and Tbr2-positive cells. IZ was determined as Tbr2-negative and DCX-positive area. In addition, morphological characteristics were used as indicators of VZ/SVZ and SVZ/IZ boundaries in this study: VZ and SVZ were separated by existence of multipolar cells (arrowhead), and SVZ and IZ were divided by cell density detected by DAPI staining³⁷.

Quantitative bin analysis for assessment of migration at E19 or P0

To quantify the pattern of migration in E19 or P0 brains, the numbers of GFP-positive cells in the developmental cerebral cortex were counted from three independent sections. We quantified the RNAi effect on neuronal migration status by bin analysis, in which the developing cerebral cortex was divided into 10 equal spaces (10 bins) and the percentage of GFP-positive cells in each bin was determined^{13,16}. The numbers of neurons in each category from more than three independent experiments were counted in a blinded manner. Migration distance was defined as the relative distance of each cell migration (from the surface of the ventricle) to the radial thickness of the cerebral cortex where the cells were located¹⁶. The cells reaching the superficial layers of the cortex (bins 9 and 10) were examined as migrated cells.

Phosphatase treatment

Soluble proteins obtained from the cells overexpressing myc-tagged wild-type human DISC1 (hDISC1) were incubated with an antibody against myc-tag (Santa Cruz) and Protein G Plus/Protein A agarose beads (Calbiochem) at 4 °C overnight. Beads were washed in 20 mM Tris-HCl, pH 7.6, three times and in lambda phosphatase buffer (New England Biolabs) once, and phosphatase reactions were performed directly on the beads at 30°C for 2 h with lambda phosphatase (New England Biolabs) as per manufacturer's protocol. Immune complexes were then washed three times in lysis buffer, separated on SDS-PAGE, and analyzed by Western blotting. Mouse brains were homogenized in lysis buffer. Fifty micrograms of soluble proteins from each sample in 20 µl of dephosphorylation buffer (Roche Applied Sciences) were incubated with or without 2 µl of calf alkaline phosphatase (Roche Applied Sciences) at 37°C for 1 h. Reactions were stopped with SDS sample buffer and the samples were subjected to Western blotting.

Metabolic Labeling

COS7 cells expressing myc-tagged wild-type human DISC1 (hDISC1) were metabolically labeled for 4 h at 37 °C with 0.5 mCi/ml [³²P]orthophosphate (PerkinElmer Life Sciences) in phosphate- and serum-free media with or without Okadaic acid (0.5 μM; Calbiochem). Immunoprecipitation and lambda phosphatase treatment were carried out essentially as described above. Immune complexes were separated on SDS-PAGE, transferred to PVDF membrane, and analyzed by autoradiography and immunoblotting.

Mass spectrometry

COS7 cells expressing myc-tagged wild-type human DISC1 were treated with or without Okadaic acid (0.5 μM; Calbiochem). Cells were lysed in RIPA buffer (150 mM NaCl, 50 mM Tris HCl, pH 7.5, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate) containing protease inhibitor mixture (Roche Applied Sciences) and phosphatase inhibitor cocktail (Sigma). The solubilized proteins were immunoprecipitated by using an anti-myc antibody (Santa Cruz). The precipitated proteins were separated by SDS-PAGE under non-reducing conditions and visualized by colloidal Coomassie staining. Gel-purified DISC1 protein was digested with trypsin and sequence analysis was performed by using microcapillary reverse-phase HPLC nano-electrospray tandem mass spectrometry on a Finnigan LTQ quadrupole ion trap mass spectrometer in the mass spectrometry facility at JHU. Identified sequences were confirmed by manually inspecting CID spectra. Protein identifications were considered significant if at least two individual peptide Mascot scores were above the Mascot calculated threshold.

In vitro kinase assays

GST-tagged human C-terminal (amino acids 598-854), human N-terminal (amino acids 1-348), mouse C-terminal (amino acids 594-853), and their site-directed mutants were generated in *E. coli*, and purified. These recombinant proteins were incubated with purified recombinant PKA (New England Biolabs) or CDK5/p35 (Sigma) for 30 min at 30°C. Reactions were supplemented with 10 μCi/ml of [γ -³²P]ATP (PerkinElmer Life Sciences) and 1 mM MgATP. The phosphorylation reactions were terminated with SDS sample buffer, and samples were analyzed by SDS-PAGE followed by autoradiography and immunoblotting.

Fractionation by sucrose gradient

HEK293T cells 3 days after transfection with hDISC1 RNAi constructs together with A710- or E710-DISC1 expression construct were harvested and fractionated by a discontinuous sucrose gradient, as described by Moudjou and Bornens³⁸. In brief, cells were treated with 0.2 μM nocodazole for 1 h. Cells were washed in Tris buffered saline (TBS), then trypsinized and homogenized in 0.1 × TBS/8% sucrose. After centrifugation at 1000 × g for 5 min, cells were resuspended in 0.1 × TBS/8% sucrose followed by addition of lysis buffer [1 mM HEPES, pH 7.2, 0.5% Nonidet P-40, 0.5 mM MgCl₂, 0.1% β-mercaptoethanol, and protease inhibitor mixture (Roche Applied Sciences)]. The lysate was centrifuged at 2,500 × g for 10 min to remove swollen nuclei, chromatin aggregates, and unlysed cells. To the resulting supernatant fraction HEPES buffer and DNaseI were added to a final concentration

of 10 μM and 1 $\mu\text{g/ml}$, respectively, and incubated on ice for 30 min. The mixture was loaded onto a discontinuous sucrose gradient consisting of 70, 50, and 40% solutions from the bottom, respectively, and centrifuged at $40,000 \times g$ for 1 h. Fractions were collected from the bottom and stored at -80°C for further analysis.

In vitro binding assays

MBP-DISC1 recombinant proteins were generated as described³³. GST-GSK3 β and GST-BBS1 were purchased from SignalChem and Abnova, respectively. Proteins and an antibody against GST were incubated in 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1% Triton X-100, 0.1 mg/ml BSA, and protease inhibitor mixture (Roche Applied Sciences) overnight at 4°C . MBP-DISC1 bound to GST-GSK3 β or GST-BBS1 was precipitated with Sepharose beads. The protein precipitates were analysed with SDS-PAGE, followed by Western blotting with an antibody against DISC1 (D27).

Statistical analyses

Optical density of immunoreactivity in Western blotting was obtained by using Image J software. For determination of the statistical significance between two groups, either the Student's t-test (equal variances) or the modified Welch t-test (unequal variances) was employed. Result of the F test was used to decide which test was appropriate. To compare three or more groups, one-way ANOVAs followed by Bonferroni post hoc for multiple comparisons was used. Probability values (p values) < 0.05 were considered to be statistically significant (* $P < 0.05$, † $P < 0.01$, # $P < 0.001$). Values depicted are means \pm SEM.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Ayala R, Shu T, Tsai LH. Trekking across the brain: the journey of neuronal migration. *Cell*. 2007; 128:29–43. [PubMed: 17218253]
2. Bielas S, Higginbotham H, Koizumi H, Tanaka T, Gleeson JG. Cortical neuronal migration mutants suggest separate but intersecting pathways. *Annu Rev Cell Dev Biol*. 2004; 20:593–618. [PubMed: 15473853]
3. Gupta A, Tsai LH, Wynshaw-Boris A. Life is a journey: a genetic look at neocortical development. *Nat Rev Genet*. 2002; 3:342–355. [PubMed: 11988760]

4. Dehay C, Kennedy H. Cell-cycle control and cortical development. *Nat Rev Neurosci.* 2007; 8:438–450. [PubMed: 17514197]
5. Gotz M, Huttner WB. The cell biology of neurogenesis. *Nat Rev Mol Cell Biol.* 2005; 6:777–788. [PubMed: 16314867]
6. Kriegstein A, Noctor S, Martinez-Cerdeno V. Patterns of neural stem and progenitor cell division may underlie evolutionary cortical expansion. *Nat Rev Neurosci.* 2006; 7:883–890. [PubMed: 17033683]
7. Mochida GH, Walsh CA. Genetic basis of developmental malformations of the cerebral cortex. *Arch Neurol.* 2004; 61:637–640. [PubMed: 15148137]
8. Leone DP, Srinivasan K, Chen B, Alcamo E, McConnell SK. The determination of projection neuron identity in the developing cerebral cortex. *Curr Opin Neurobiol.* 2008; 18:28–35. [PubMed: 18508260]
9. Shu T, et al. Ndel1 operates in a common pathway with LIS1 and cytoplasmic dynein to regulate cortical neuronal positioning. *Neuron.* 2004; 44:263–277. [PubMed: 15473966]
10. Reiner O, et al. Isolation of a Miller-Dieker lissencephaly gene containing G protein beta-subunit-like repeats. *Nature.* 1993; 364:717–721. [PubMed: 8355785]
11. Schurov IL, Handford EJ, Brandon NJ, Whiting PJ. Expression of disrupted in schizophrenia 1 (DISC1) protein in the adult and developing mouse brain indicates its role in neurodevelopment. *Mol Psychiatry.* 2004; 9:1100–1110. [PubMed: 15381924]
12. Mao Y, et al. Disrupted in schizophrenia 1 regulates neuronal progenitor proliferation via modulation of GSK3beta/beta-catenin signaling. *Cell.* 2009; 136:1017–1031. [PubMed: 19303846]
13. Kamiya A, et al. A schizophrenia-associated mutation of DISC1 perturbs cerebral cortex development. *Nat Cell Biol.* 2005; 7:1167–1178. [PubMed: 16299498]
14. Jaaro-Peled H, et al. Neurodevelopmental mechanisms of schizophrenia: understanding disturbed postnatal brain maturation through neuregulin-1-ErbB4 and DISC1. *Trends Neurosci.* 2009; 32:485–495. [PubMed: 19712980]
15. Chubb JE, Bradshaw NJ, Soares DC, Porteous DJ, Millar JK. The DISC locus in psychiatric illness. *Mol Psychiatry.* 2008; 13:36–64. [PubMed: 17912248]
16. Kamiya A, et al. Recruitment of PCM1 to the centrosome by the cooperative action of DISC1 and BBS4: a candidate for psychiatric illnesses. *Arch Gen Psychiatry.* 2008; 65:996–1006. [PubMed: 18762586]
17. Shen S, et al. Schizophrenia-related neural and behavioral phenotypes in transgenic mice expressing truncated Disc1. *J Neurosci.* 2008; 28:10893–10904. [PubMed: 18945897]
18. Greengard P, et al. The DARPP-32/protein phosphatase-1 cascade: a model for signal integration. *Brain Res Brain Res Rev.* 1998; 26:274–284. [PubMed: 9651542]
19. Xie Z, Sanada K, Samuels BA, Shih H, Tsai LH. Serine 732 phosphorylation of FAK by Cdk5 is important for microtubule organization, nuclear movement, and neuronal migration. *Cell.* 2003; 114:469–482. [PubMed: 12941275]
20. Chenn A, Walsh CA. Regulation of cerebral cortical size by control of cell cycle exit in neural precursors. *Science.* 2002; 297:365–369. [PubMed: 12130776]
21. Gerdes JM, et al. Disruption of the basal body compromises proteasomal function and perturbs intracellular Wnt response. *Nat Genet.* 2007; 39:1350–1360. [PubMed: 17906624]
22. Corbit KC, et al. Kif3a constrains beta-catenin-dependent Wnt signalling through dual ciliary and non-ciliary mechanisms. *Nat Cell Biol.* 2008; 10:70–76. [PubMed: 18084282]
23. Veeman MT, Slusarski DC, Kaykas A, Louie SH, Moon RT. Zebrafish prickle, a modulator of noncanonical Wnt/Fz signaling, regulates gastrulation movements. *Curr Biol.* 2003; 13:680–685. [PubMed: 12699626]
24. Korinek V, et al. Constitutive transcriptional activation by a beta-catenin-Tcf complex in APC-/- colon carcinoma. *Science.* 1997; 275:1784–1787. [PubMed: 9065401]
25. Yokota Y, et al. The adenomatous polyposis coli protein is an essential regulator of radial glial polarity and construction of the cerebral cortex. *Neuron.* 2009; 61:42–56. [PubMed: 19146812]

26. Zhang J, et al. Cortical neural precursors inhibit their own differentiation via N-cadherin maintenance of beta-catenin signaling. *Dev Cell*. 2010; 18:472–479. [PubMed: 20230753]
27. Kanki H, Shimabukuro MK, Miyawaki A, Okano H. "Color Timer" mice: visualization of neuronal differentiation with fluorescent proteins. *Mol Brain*. 2010; 3:5. [PubMed: 20205849]
28. Kulaga HM, et al. Loss of BBS proteins causes anosmia in humans and defects in olfactory cilia structure and function in the mouse. *Nat Genet*. 2004; 36:994–998. [PubMed: 15322545]
29. Ohshima T, et al. Cdk5 is required for multipolar-to-bipolar transition during radial neuronal migration and proper dendrite development of pyramidal neurons in the cerebral cortex. *Development*. 2007; 134:2273–2282. [PubMed: 17507397]
30. Tabata H, Nakajima K. Efficient in utero gene transfer system to the developing mouse brain using electroporation: visualization of neuronal migration in the developing cortex. *Neuroscience*. 2001; 103:865–872. [PubMed: 11301197]
31. Duan X, et al. Disrupted-In-Schizophrenia 1 regulates integration of newly generated neurons in the adult brain. *Cell*. 2007; 130:1146–1158. [PubMed: 17825401]
32. Niwa M, et al. Knockdown of DISC1 by in utero gene transfer disturbs postnatal dopaminergic maturation in the frontal cortex and leads to adult behavioral deficits. *Neuron*. 2010; 65:480–489. [PubMed: 20188653]
33. Hayashi-Takagi A, et al. Disrupted-in-Schizophrenia 1 (DISC1) regulates spines of the glutamate synapse via Rac1. *Nat Neurosci*. 2010; 13:327–332. [PubMed: 20139976]
34. Wang X, Qiu R, Tsark W, Lu Q. Rapid promoter analysis in developing mouse brain and genetic labeling of young neurons by doublecortin-DsRed-express. *J Neurosci Res*. 2007; 85:3567–3573. [PubMed: 17671991]
35. Tan PL, et al. Loss of Bardet Biedl syndrome proteins causes defects in peripheral sensory innervation and function. *Proc Natl Acad Sci U S A*. 2007; 104:17524–17529. [PubMed: 17959775]
36. Ishizuka K, et al. Evidence that many of the DISC1 isoforms in C57BL/6J mice are also expressed in 129S6/SvEv mice. *Mol Psychiatry*. 2007; 12:897–899. [PubMed: 17895924]
37. Regad T, Bellodi C, Nicotera P, Salomoni P. The tumor suppressor Pml regulates cell fate in the developing neocortex. *Nat Neurosci*. 2009; 12:132–140. [PubMed: 19136970]
38. Moudjou M, Bordes N, Paintrand M, Bornens M. gamma-Tubulin in mammalian cells: the centrosomal and the cytosolic forms. *J Cell Sci*. 1996; 109(Pt 4):875–887. [PubMed: 8718679]

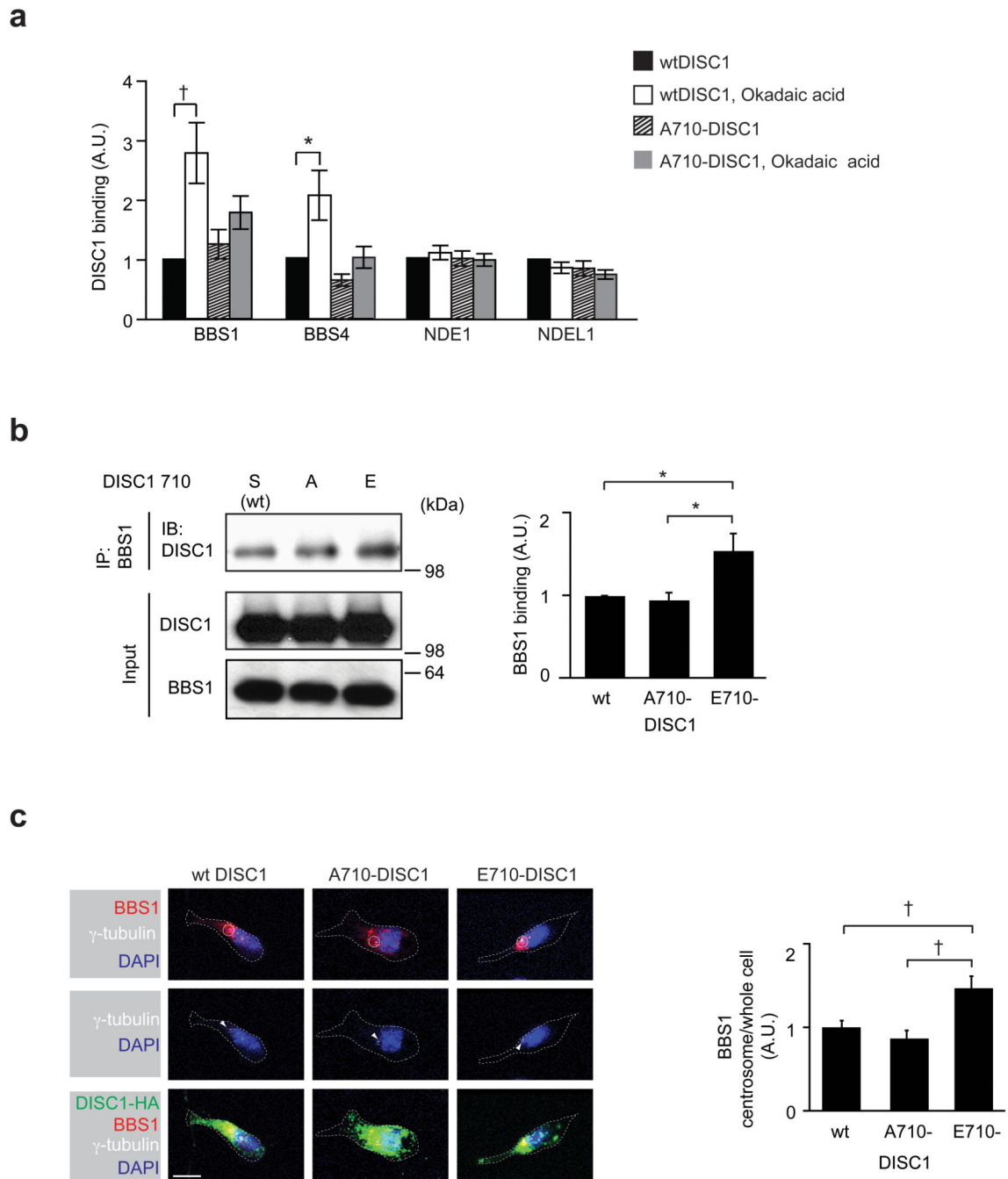


Figure 1. Phosphorylation of DISC1 at S710 selectively increases binding of DISC1 with BBS proteins, resulting in enhanced BBS1 accumulation at the centrosome

a, HA-tagged wild-type (wt) or phospho-dead mutant A710-DISC1 with myc-tagged BBS1, BBS4, NDEL1, or NDE1 were co-transfected into HT22 cells treated with or without Okadaic acid. Protein binding was examined by co-immunoprecipitation. Okadaic acid treatment increased the affinity of wt, but not that of A710-DISC1, with BBS1 and BBS4. No change of DISC1 binding with NDEL1 and NDE1 was observed by Okadaic acid treatment and A710 mutation.

b, In HT22 cells without Okadaic acid treatment, phospho-mimic E710-DISC1 showed increased binding to BBS1, compared with wt and A710-DISC1.

c, Mouse cortical neurons were transfected with wt, A710-, or E710-DISC1. E710-DISC1 induced significantly greater accumulation of BBS1 into the centrosome compared to that induced by wt and A710-DISC1. Scale bar, 20 μ m.

Error bars indicate SEM. * $P < 0.05$, † $P < 0.01$.

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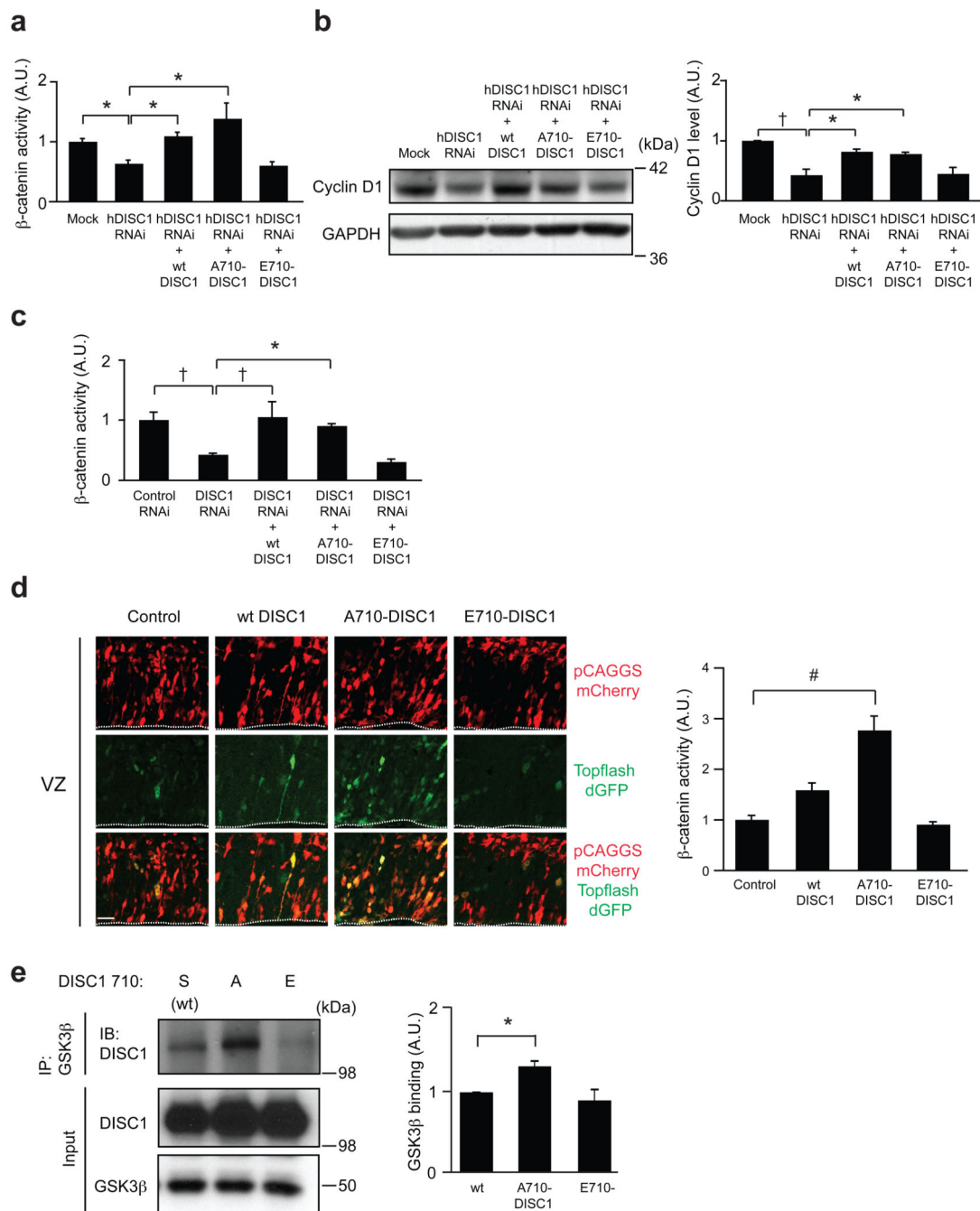


Figure 2. Non-phosphorylated DISC1 at S710 activates β -catenin signaling via its interaction with GSK3 β

a, *In vitro* luciferase assay showed that DISC1 knockdown suppressed β -catenin-dependent activity, which was rescued by wt and phospho-dead A710-DISC1, but not by phospho-mimic E710-DISC1.

b, Expression of Cyclin D1 was downregulated by DISC1 RNAi, which was normalized by wt and A710-DISC1, but not by E710-DISC1.

c, Super8XTOPFlash and pRL SV40 plasmids together with various constructs were injected *in utero* at E13 brain and were analyzed at E15. Knockdown of DISC1 suppressed β -catenin-dependent activity, which was rescued by wt and A710-DISC1, but not by E710-DISC1.

d, *In utero* assay to monitor activation of the β -catenin pathway with the TOPdGFP-CAG mCherry assay system. Brains were analyzed at E14. Relative β -catenin activity was assessed by the ratio of the number of GFP-positive cells to the number of mCherry-positive cells; significant upregulation of β -catenin signaling activity was evident in the brain injected with A710-DISC1. Scale bar, 20 μ m.

e, Increased binding of GSK3 β with A710-DISC1 compared to wt DISC1 in HT22 cells by co-immunoprecipitation.

Error bars indicate SEM. * $P < 0.05$, † $P < 0.01$, # $P < 0.001$.

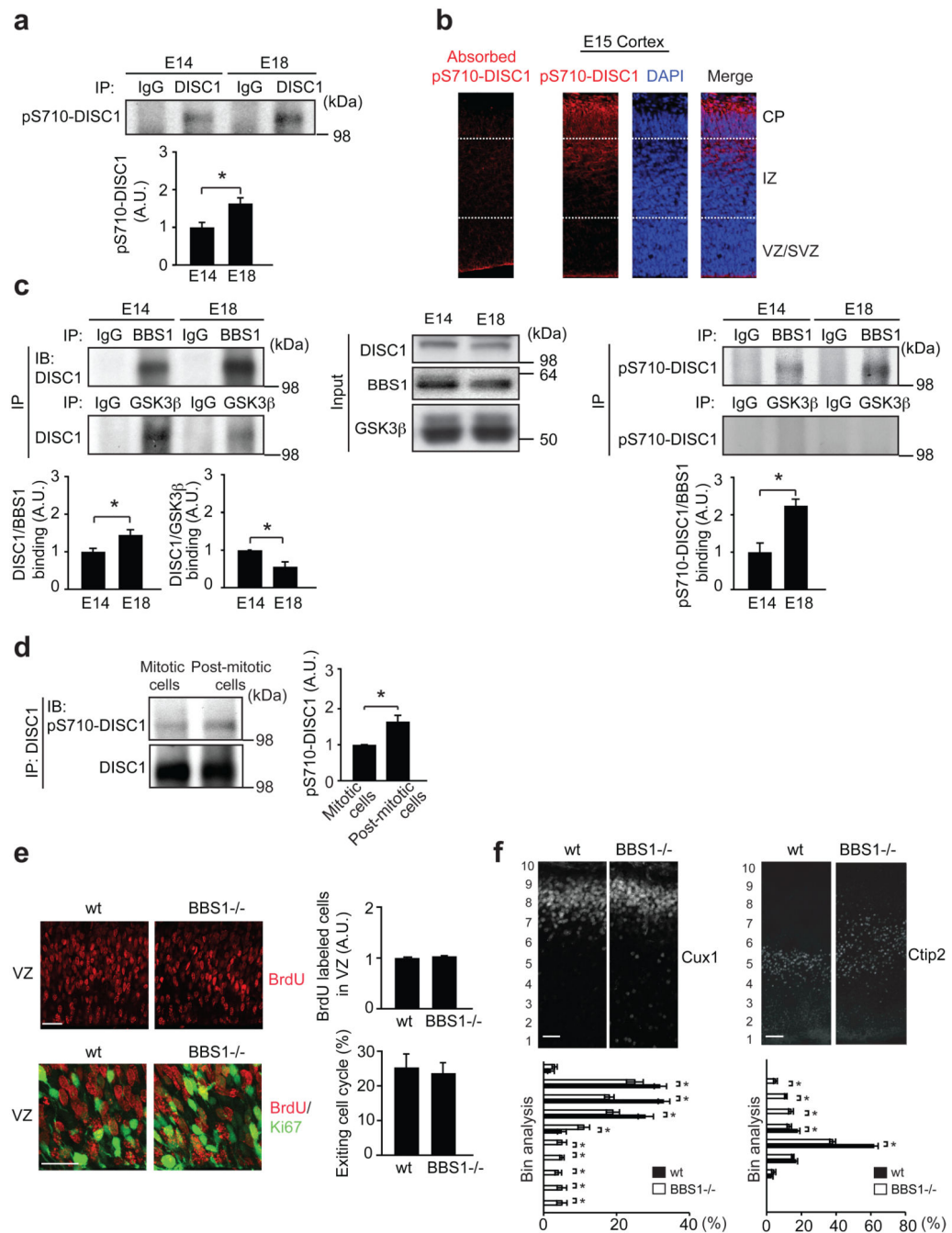


Figure 3. Binding affinity of DISC1/GSK3 β to DISC1/BBS1 depends on developmental stage: roles of BBS1 in corticogenesis

a. Levels of phosphorylated DISC1 at S710 (pS710-DISC1) at E14 and E18.

Immunoprecipitation with a pan-DISC1 antibody was conducted, and immunoprecipitates were analyzed with pS710 phospho-specific antibody or another pan-DISC1 antibody. Total levels of DISC1 were unchanged (see Fig. 3c), whereas levels of pS710-DISC1 were significantly higher at E18 compared to E14.

- b**, pS710-DISC1 was prominent only in the cortical plate/intermediate zone but not in ventricular/subventricular zone at E15, suggesting that phosphorylation may occur preferentially in post-mitotic neurons, but not in progenitors. Scale bar, 20 μ m.
- c**, Binding of DISC1/BBS1 and DISC1/GSK3 β was assessed in E14 and E18 mouse brains by co-immunoprecipitation. DISC1/BBS1 binding was increased during this period, whereas DISC1/GSK3 β binding was decreased. Furthermore, pS710-DISC1/BBS1 binding was significantly greater at E18 compared with E14; minimal binding of pS710-DISC1 with GSK3 β was observed.
- d**, Levels of pS710-DISC1 in mitotic progenitors and post-mitotic neurons were assessed by immunoprecipitation. Total levels of DISC1 were unchanged, whereas levels of pS710-DISC1 were significantly higher in post-mitotic neurons compared to mitotic progenitors.
- e**, No appreciable differences in the progenitor cell proliferation of the cortex between *Bbs1*^{-/-} mice and wild-type littermates, as determined by BrdU incorporation and cell cycle exit index. Scale bar, 20 μ m.
- f**, Aberrant radial migration in *Bbs1*^{-/-} mice compared to wt littermates, assayed by bin analysis with the brains at postnatal day 0 (P0). Cux1 (layers II-IV) and Ctip2 (layer V) were used as indicators. Scale bar, 50 μ m.
- Error bars indicate SEM. **P* < 0.05.

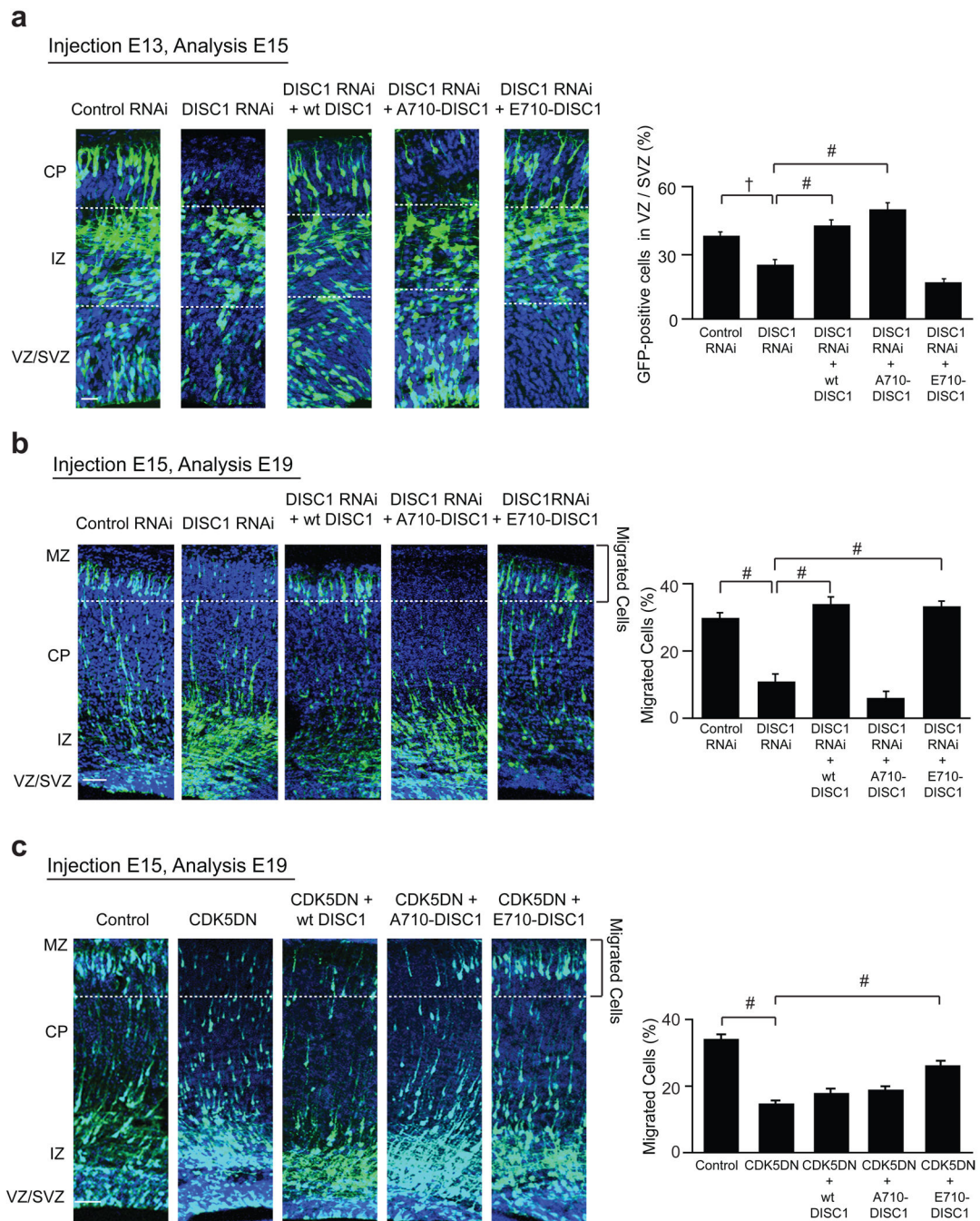


Figure 4. Suppression of DISC1 leads to phospho-dependent defects in cell proliferation and neuronal migration: implication of CDK5

a, Various constructs were injected *in utero* at E13 and analyzed at E15. In brains with DISC1 RNAi, the percentage of GFP-positive cells in the ventricular and subventricular zones (VZ/SVZ) was lower compared to brains with control RNAi, which were rescued by wt DISC1 and phospho-dead A710-DISC1 but not by phospho-mimic E710-DISC1. CP, cortical plates; IZ, intermediate zone. Scale bar, 20 μ m.

b, *In utero* gene transfer at E15, analyzed at E19. The percentage of GFP-positive cells reaching the superficial layers of the cortex (bins 9 and 10; migrated cells) was examined. Silencing of DISC1 induced delayed neuronal migration, rescued by wt and E710-DISC1 but not by A710-DISC1. Scale bar, 50 μm .

c, Wt, A710-, or E710-DISC1 constructs, together with a dominant-negative CDK5 construct (CDK5 DN) were electroporated at E15 and their effects on migration were analyzed at E19. Migration defects elicited by CDK5 DN were rescued significantly by co-expression with E710-DISC1, but not with A710-DISC1 or with wt DISC1. Scale bar, 50 μm .

Error bars indicate SEM. † $P < 0.01$, # $P < 0.001$.