

Disrupted-in-schizophrenia 1 (DISC1) Regulates Dysbindin Function by Enhancing Its Stability*

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Seol-Ae Lee[‡], Seong-Mo Kim[‡], Bo Kyoung Suh[‡], Hwa-Young Sun[‡], Young-Un Park[‡], Ji-Ho Hong[‡], Cana Park[‡], Minh Dang Nguyen[§], Koh-ichi Nagata[¶], Joo-Yeon Yoo[‡], and Sang Ki Park^{‡1}

From the [‡]Department of Life Sciences, Pohang University of Science and Technology, Pohang 790-784, Republic of Korea, the [§]Hotchkiss Brain Institute, Departments of Clinical Neurosciences, Cell Biology and Anatomy, and Biochemistry and Molecular Biology, University of Calgary, Calgary T2N 4N1, Canada, and the [¶]Department of Molecular Neurobiology, Institute for Developmental Research, Aichi Human Service Center, 713-8 Kamiya, Kasugai 480-0392, Japan

Background: Dysbindin and Disrupted-in-schizophrenia 1 (DISC1) are major schizophrenia susceptibility factors.

Results: DISC1 enhances stability of dysbindin, which is critical for neurite outgrowth.

Conclusion: Dysbindin and DISC1 form a physiologically functional complex that is essential for normal neurite outgrowth.

Significance: Our findings indicate the existence of a protein complex composed of multiple schizophrenia susceptibility factors functioning in a pathway for neurite outgrowth.

Dysbindin and DISC1 are schizophrenia susceptibility factors playing roles in neuronal development. Here we show that the physical interaction between dysbindin and DISC1 is critical for the stability of dysbindin and for the process of neurite outgrowth. We found that DISC1 forms a complex with dysbindin and increases its stability in association with a reduction in ubiquitylation. Furthermore, knockdown of DISC1 or expression of a deletion mutant, DISC1 lacking amino acid residues 403–504 of DISC1 (DISC1^{Δ403–504}), effectively decreased levels of endogenous dysbindin. Finally, the neurite outgrowth defect induced by knockdown of DISC1 was partially reversed by coexpression of dysbindin. Taken together, these results indicate that dysbindin and DISC1 form a physiologically functional complex that is essential for normal neurite outgrowth.

Neurite outgrowth is a critical process in neuronal development. Abnormalities in this process have been linked to the biological basis of various mental illnesses (1–3). Especially, defects in neurite outgrowth have been proposed to underlie the reduced neuropil hypothesis stating that schizophrenia is involved in a decrease in axons and dendrites (4–8). In support of this hypothesis, the multiple schizophrenia susceptibility factors, including Disrupted-in-schizophrenia 1 (DISC1) and dystrobrevin-binding protein 1 (dysbindin, DTNBP1) (9–11), are involved in neurite outgrowth (12, 13).

Dysbindin was initially identified as an interactor of dystrobrevin in muscle and brain (14). Subsequent genetic studies

have associated dysbindin with schizophrenia (10, 15–18). Consistent with these findings, the sandy (*sdy*) mouse strain, which lacks functional dysbindin, displays significant schizophrenia-like behaviors, including deficits in social interaction and working memory (19–22). Dysbindin mRNA and protein are reduced significantly in schizophrenic patients (23, 24). Notably, dysbindin is also involved in several neuronal developmental processes, including neurite outgrowth. Knockdown of dysbindin results in disorganization of the actin cytoskeleton and shortening of neurites, and sandy mice show defective neurite outgrowth and abnormal growth cone morphology (12, 13, 25). Moreover, a recent report shows that dendritic spine morphogenesis depends on the interaction of dysbindin with WAVE2 and Abi-1 (26), underscoring the essential function of dysbindin.

DISC1 has emerged as a susceptibility factor in mental disorders on the basis of the cosegregation of a balanced chromosomal translocation (t1;11) that disrupts the *DISC1* gene in multiple psychiatric conditions, including schizophrenia (10). Subsequently, mice that are defective in DISC1 display neuroanatomical and behavioral abnormalities relevant to schizophrenia (27, 28). Furthermore, DISC1 plays multiple roles during neurodevelopment, including neuronal proliferation (29), neurite outgrowth (3, 30–32), neuronal migration (29, 33, 34), and synapse formation (35), all implicated in the pathophysiology of schizophrenia.

A considerable number of studies have characterized interacting partners of dysbindin and DISC1. Interestingly, several reports show that the protein interaction networks associated with dysbindin and DISC1 share common components, implying a common physiological pathway for these two major schizophrenia susceptibility factors (36, 37). Consistent with this, dysbindin and DISC1 physically associate *in vitro*, although the biological relevance of this has not been investigated (38). In this study, we demonstrate the functional collaboration of dysbindin and DISC1 in the neurite outgrowth process which involves fine-tuning of dysbindin

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¹ To whom correspondence should be addressed: Dept. of Life Sciences, Pohang University of Science and Technology, Pohang 790-784, Republic of Korea. Tel.: 82-54-279-2349; Fax: 82-54-279-2199; E-mail: skpark@postech.ac.kr.

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degradation by DISC1, and provide evidence of a convergent pathway where dysbindin and DISC1, two schizophrenia-associated proteins, cooperate.

EXPERIMENTAL PROCEDURES

Plasmids—For yeast two-hybrid assays, pPC97-DISC1 and pPC86-NDEL1 were prepared as described previously (39). The human dysbindin coding sequence was amplified by PCR from a human dysbindin cDNA clone (clone ID 4139934, Open Biosystems, GenBank accession number BC011912) and inserted into the pPC86 vector (Invitrogen) using the *Sall* and *NotI* sites. Full-length human and mouse DISC1 constructs were prepared as described previously (39). For constructs of DISC1 fragments, corresponding regions of human DISC1 (hDISC1) were amplified by PCR and inserted into the pEGFP-C3 (Clontech) and pFLAG-CMV2 (Sigma). For mouse dysbindin constructs, the full-length dysbindin coding sequence was amplified using reverse transcription products generated from CAD² mouse neuroblastoma cells, subcloned into pEGFP-N1 (Clontech), pUB-GFP (Addgene), pFLAG-CMV2 (Sigma), and pCDNA3.1 Myc-His (Invitrogen). To construct the deletion mutant of dysbindin, a region of human dysbindin corresponding to the designated codon was amplified by PCR using EGFP-dysbindin as a template and inserted into pEGFP-N1. Sequences were verified by DNA sequencing. For shRNA constructs, mouse DISC1 shRNA was prepared following a previous description (39). The oligonucleotide sequences used for the constructs were GTGAT-AAGTCAAGA GAAGCTTCAAGAGAGCTTCTCTTGACTT-ACACTTTTTT and TCGAGAAAAAAGTGATAA GTCAAG-AGAAGCTCTCTTGAAGCTTCTCTTGACTTAT CACA for mouse dysbindin. These oligonucleotides were annealed and ligated into the pLentiLox3.7 vector using the *HpaI* and *XhoI* sites. To generate the shRNA-resistant GFP-mouse DISC1 and mouse dysbindin constructs by inserting silent mutations in the shRNA target sequence in pUB-GFP (Addgene), site-directed mutagenesis of DISC1 and dysbindin was performed using PCR-based methods. The PCR primer sequences used were as follows: 5'-AAG ACT TTA AGT GAC AAA TCA AGG GAG GCA AAAGTGAAG-3' for dysbindin and 5'-CGCACAATGAAGGC-CAATACCGTCAAATGTATGGAAGTGTG-3' for DISC1.

Cells and Transfections—HEK293T, HEK293, COS7, and CAD cells were grown in DMEM supplemented with 10% fetal bovine serum and antibiotics under 5% CO₂ at 37 °C. Cells were transfected using Lipofectamine 2000 (Invitrogen) or Viva-Magic (Vivagen) according to the instructions of the manufacturer. For neuronal cultures, developing cortices were dissected from embryonic day 15 Institute for Cancer Research (CrljBgi: CD-1) mouse embryos in Hanks' balanced salt solution (Invitrogen). Dissected tissue was dissociated by treatment with DNase I (0.1%) and Trypsin (0.25%) for 6 min at 37 °C. The dissociated cells were diluted in plating medium, Neurobasal medium (Invitrogen) containing 10 mM HEPES (pH 7.4) and 10% horse serum (Gibco), and then plated onto cover slips coated with poly-D-lysine. Plating media was replaced with cul-

ture media (Neurobasal media supplemented with 2% B27 (Invitrogen), 2 mM glutamine, and antibiotics).

Antibodies and Immunoblot Analyses—Rabbit polyclonal anti-dysbindin was used as described previously (26). Anti-mDISC1 antibodies were a gift from Dr. Kaibuchi (University of Nagoya). A rabbit polyclonal anti-GFP protein (Molecular Probes), anti-FLAG (ABR), and mouse monoclonal anti-FLAG M2 (Sigma) were used. Anti- α tubulin (catalog no. DM1A), anti-GAPDH (catalog no. 6C5), anti-GFP (catalog no. B-2), anti-DISC1 (catalog no. N-16), and anti-Myc (catalog no. 9E10) antibodies were purchased from Santa Cruz Biotechnology. For immunoblotting, cells were lysed in Nonidet P-40 lysis buffer (50 mM Tris (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 5 mM EDTA, 5 mM glycerol-2-phosphate, 2 mM sodium pyrophosphate, 5 mM NaF, 2 mM Na₃VO₄, 1 mM DTT, and EDTA-free protease inhibitor mixture (Roche)) and precleared by centrifugation for 10 min at 12,000 \times *g*. Supernatants were denatured in SDS sample buffer by boiling for 5 min and subjected to SDS-PAGE followed by immunoblotting.

Immunoprecipitation and in Vitro Binding Assay—Whole brain tissues from an Institute for Cancer Research mouse were homogenized in Nonidet P-40 lysis buffer and precleared by centrifugation for 15 min at 12,000 \times *g*. Supernatants were incubated with antibody (0.5–1 μ g) on a rocking platform for 2 h or overnight at 4 °C, and then 100 μ l of 10% protein A-agarose (GE Healthcare) resuspended in the same lysis buffer was added and incubated with gentle shaking for an additional 90 min at 4 °C. The precipitate was washed three times with lysis buffer and resuspended in 2 \times SDS sample loading buffer. For the *in vitro* binding assay, transfected HEK293T cell lysates were incubated with 1 μ g of anti-FLAG M2 (Sigma) antibody for 2 h at 4 °C, and then protein A-agarose beads (GE Healthcare) were added and incubated for an additional 90 min at 4 °C. Precipitates were washed three times with lysis buffer and incubated with 5 μ g of GST-dysbindin for 2 h at 4 °C. Beads were collected by centrifugation at 2000 \times *g* and rinsed four times with lysis buffer. Precipitates were subjected to anti-GST and anti-FLAG immunoblotting.

Immunocytochemistry—Cells were fixed for 10 min in 4% paraformaldehyde in PBS and incubated for 30 min in blocking solution (2% goat serum and 1% Triton X-100 in PBS). Cells were incubated with anti-Myc (1:200, Santa Cruz Biotechnology) and rabbit anti-GFP antibodies (1:2000, Molecular Probes) for 1.5 h at room temperature, followed by incubation with Alexa Fluor 488-conjugated goat anti-rabbit IgG and Alexa Fluor 568-conjugated goat anti-mouse IgG secondary antibodies (Molecular Probes) for 1 h at room temperature. Hoechst dye was used for nuclear staining, and cells on coverslips were rinsed three times with PBS and mounted in antifade medium (Molecular Probes). Pictures were taken with a confocal microscope (Olympus, FluoView-1000).

Ubiquitylation Analysis—HEK293 cells were transfected with HA-ubiquitin (a gift from Dr. Chin Ha Chung, Seoul National University, Seoul, Korea) along with Myc-dysbindin and EGFP-DISC1 constructs. 48 h after transfection, cells were treated with 25 μ M MG132 (Enzo) for 8 h prior to lysis. The cell lysates were prepared in Nonidet P-40 lysis buffer with 10 mM *N*-ethylmaleimide and incubated with anti-Myc anti-

²The abbreviations used are: CAD, Cath.a-differentiated; EGFP, enhanced GFP; ANOVA, analysis of variance; UPS, ubiquitin-proteasome system; FL, full-length.

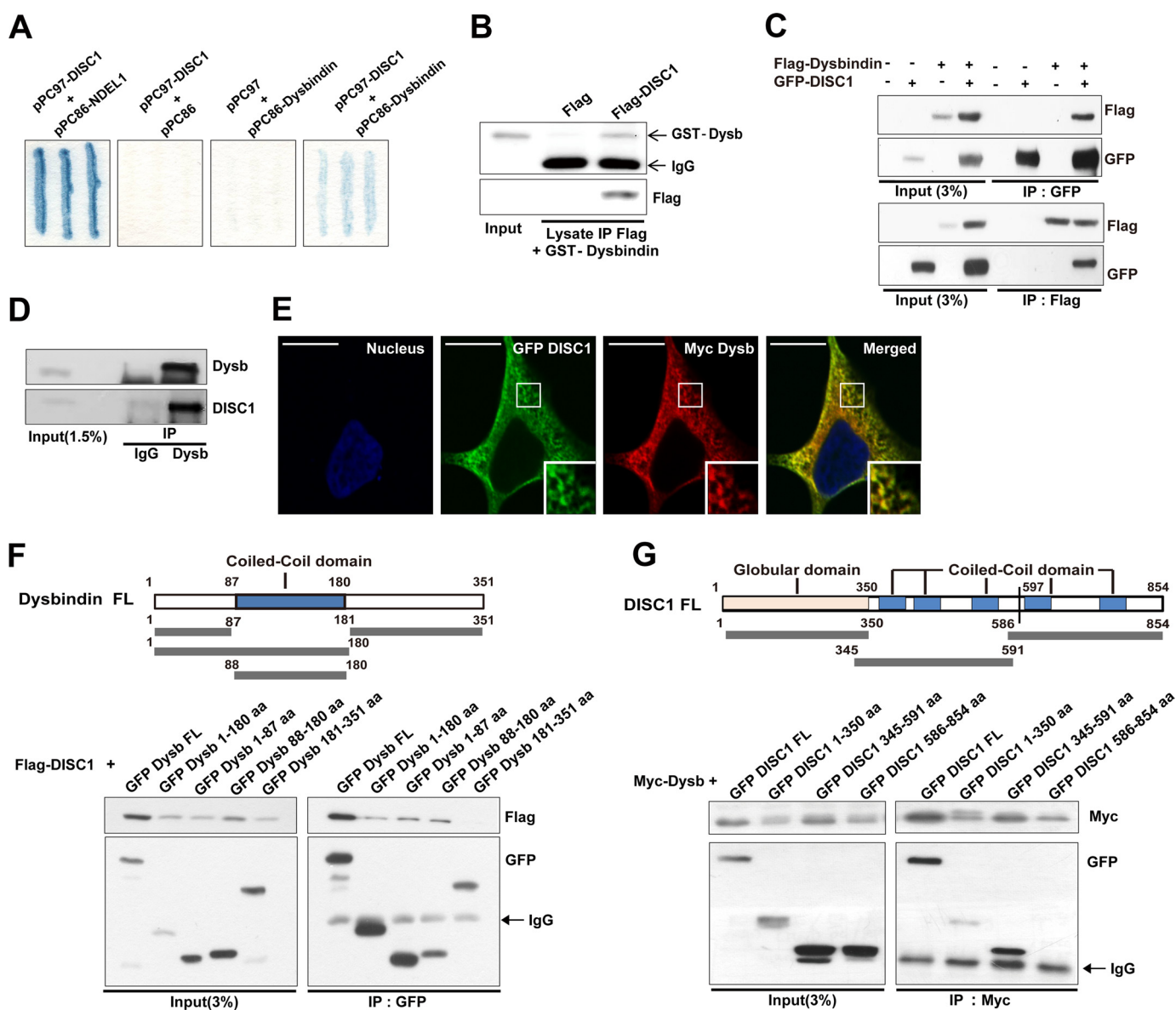


FIGURE 1. Dysbindin interacts with DISC1. *A*, yeast two-hybrid assay using a β -galactosidase reporter. Cotransformation with pPC97-DISC1 and pPC86-NDEL1 was used as a positive control. *B*, interaction of dysbindin (*Dysb*) and DISC1 in an *in vitro* binding assay. *IP*, immunoprecipitation. *C*, reciprocal coimmunoprecipitation of dysbindin with DISC1. Lysates from HEK293T cells transfected with dysbindin and DISC1 were immunoprecipitated with anti-GFP (*top panel*) or anti-Flag (*bottom panel*) antibodies. Immunoprecipitates were analyzed by immunoblotting with anti-FLAG and anti-GFP antibodies. *D*, coimmunoprecipitation of endogenous dysbindin and DISC1 from mouse brain lysates. Anti-dysbindin immunoprecipitates were analyzed by immunoblotting with anti-dysbindin and anti-DISC1 antibodies. *E*, colocalization of dysbindin and DISC1 in HEK293 cells shown by immunocytochemistry. Myc-dysbindin and EGFP-DISC1 were stained with anti-Myc (*red*) and anti-GFP (*green*) antibodies. Nuclei were stained with Hoechst dye (*blue*). Scale bars = 10 μ m. *F*, schematic of dysbindin fusion proteins used for coimmunoprecipitation analyses. *aa*, amino acids; *FL*, full-length. *G*, coimmunoprecipitation assays with DISC1 fragments and Myc-dysbindin. A schematic of DISC1 fusion proteins is also shown.

bodies for 4 h. Protein A-agarose beads were washed three times with lysis buffer, and immunoprecipitated proteins were eluted in 2 \times SDS/PAGE sampling buffer and analyzed by immunoblotting with anti-Myc, anti-GFP, and anti-HA antibodies.

Neurite Outgrowth Assay—CAD cells were cultured in DMEM containing 10% FBS and antibiotics for 1 day and then transfected with the indicated constructs using VivaMagic (Vivagen). After 48 h of transfection, cells were detached using trypsin, diluted in DMEM with antibiotics, seeded again onto 12-well plates, and cultured for 72 h. Neurites on 150–250 fluorescent cells from three independent experiments were

selected randomly, and neurites longer than the diameter of the soma were measured by ImageJ software (National Institute of Health). The length of the longest neurite and total neurite length were used for analyses. For cultured primary neurons, control red fluorescent protein (RFP) vector or RFP-expressing shRNAs were transfected into cells to visualize the neuronal morphology using Lipofectamine 2000 at days *in vitro* 0 and cultured for 3 days. Neurons were fixed, and neurite length was measured in randomly selected microscopic fields using a \times 20 objective. Neurites on 190–249 transfected neurons from three independent experiments were measured by ImageJ software and analyzed blindly.

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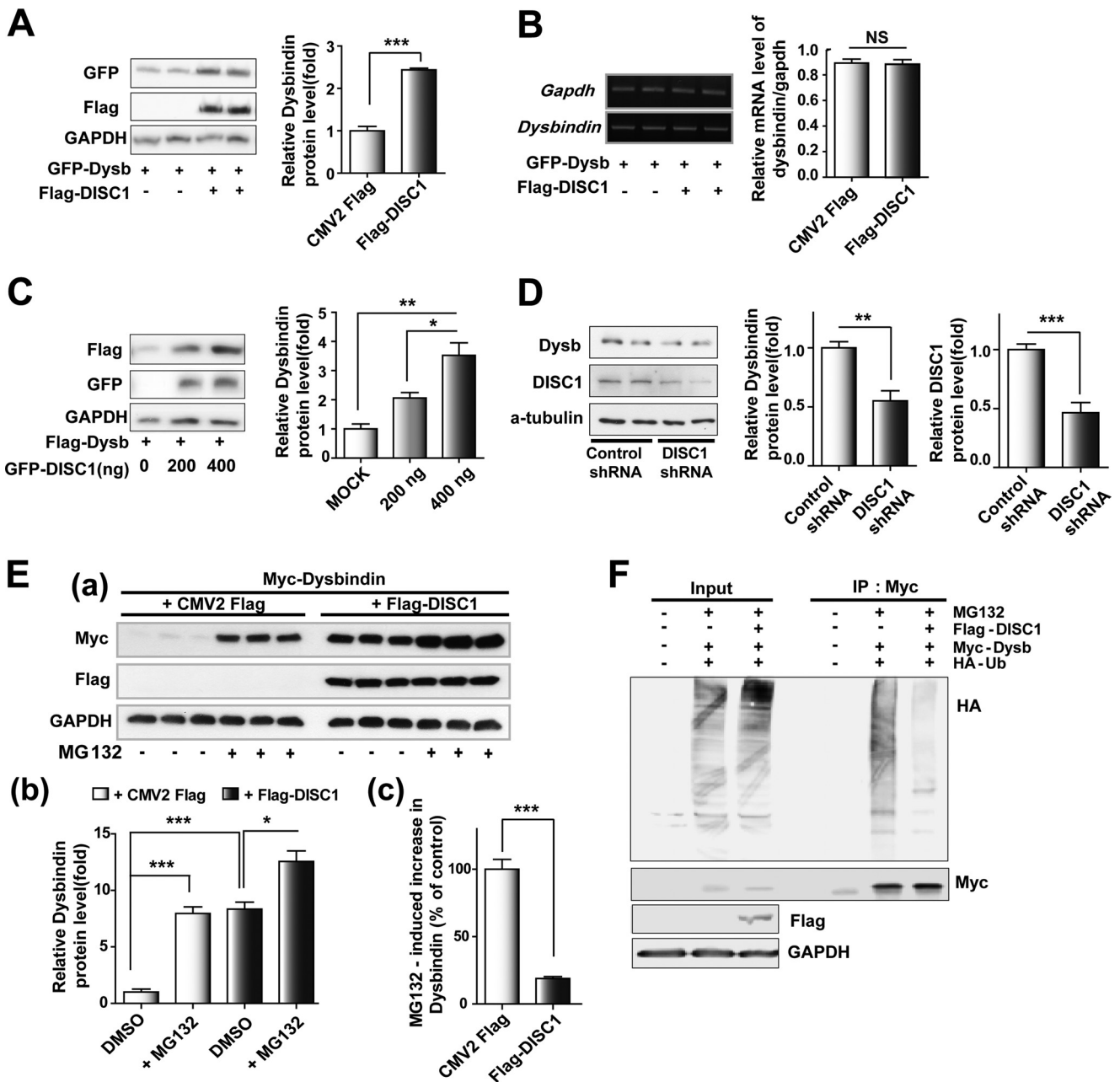


FIGURE 2. DISC1 up-regulates dysbindin. *A*, up-regulation of dysbindin (*Dysb*) protein by DISC1 coexpression. 30 h after transfection with the indicated plasmids. Cells were subjected to immunoblotting analyses. *Error bars* show mean \pm S.E. *******, $p < 0.001$ by Student's *t* test ($n = 3$). *B*, no changes in the level of dysbindin mRNA by DISC1 coexpression. Dysbindin mRNA levels were measured by RT-PCR and normalized to GAPDH mRNA. *NS*, not significant by Student's *t* test ($n = 3$). *C*, dose-dependent increases in dysbindin protein by DISC1. Dysbindin protein levels were normalized to GAPDH. *Error bars* show mean \pm S.E. *****, $p < 0.05$; ******, $p < 0.01$; *******, $p < 0.001$ by Student's *t* test ($n = 3$). *D*, down-regulation of dysbindin protein by knockdown of DISC1 in differentiated CAD cells. *Error bars* show mean \pm S.E. ******, $p < 0.01$; *******, $p < 0.001$ by Student's *t* test ($n = 6$). *E*, reduction of MG132 efficacy on dysbindin by coexpression of DISC1. 30 h after transfection, MG132 (10 μ M) was treated for 8 h in *a*. Protein levels relative to control cells are shown in *b*, and the relative increase in dysbindin upon MG132 treatment is shown in *c*. *Error bars* show mean \pm S.E. *****, $p < 0.05$; *******, $p < 0.001$; Student's *t* test ($n = 3$). *DMSO*, dimethyl sulfoxide. *F*, decreased ubiquitylation of dysbindin following DISC1 coexpression. HEK293 cells were cotransfected with the indicated plasmids, treated with MG132 for 8 h, followed by immunoprecipitation (*IP*) and immunoblotting as indicated. *Ub*, ubiquitin.

Statistical Analysis—Data were analyzed using GraphPad Prism 5 software and are presented as mean \pm S.E. Statistical significance was determined by ANOVA or Student's *t* test.

RESULTS

Dysbindin Interacts with DISC1—To examine whether dysbindin interacts with DISC1, we employed a yeast-two-hybrid assay. We found that cotransformants containing dysbindin and DISC1

showed a positive signal, indicating interaction-dependent β -galactosidase activity (Fig. 1*A*). DISC1 and NDEL1 (nude nuclear distribution gene E homolog (*A. nidulans*)-like 1) were used as positive controls in this assay. In addition, GST-dysbindin also interacted directly with FLAG-DISC1 purified by immunoprecipitation (Fig. 1*B*). Moreover, reciprocal coimmunoprecipitation of dysbindin and DISC1 was detected consistently in transfected HEK293T cell lysates (Fig. 1*C*), indicating potential protein com-

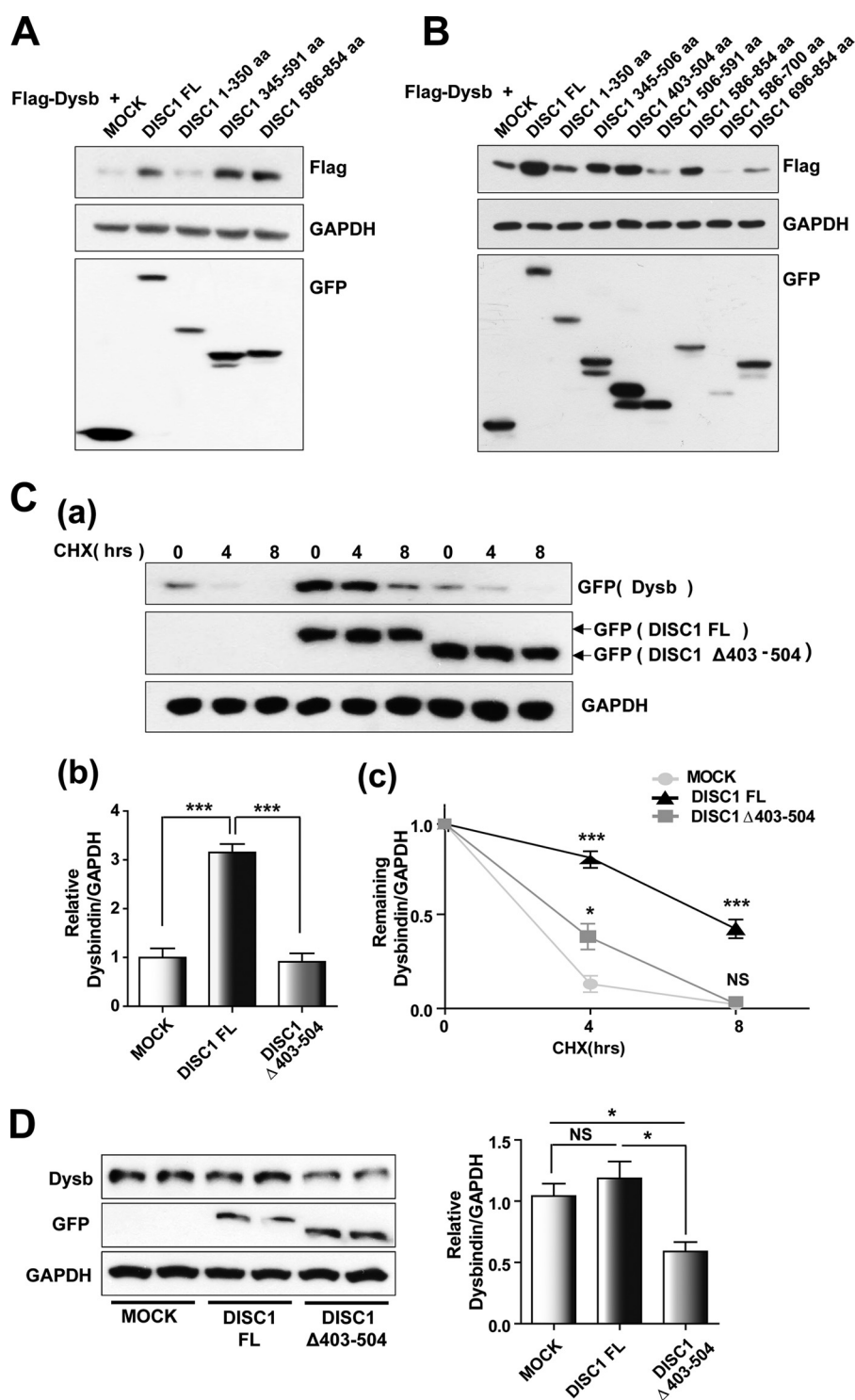


FIGURE 3. DISC1 enhances dysbindin stability. *A*, up-regulation of dysbindin is mediated by DISC1 amino acid residues 345–854. *FL*, full-length; *aa*, amino acids. *B*, increased expression of dysbindin (*Dysb*) upon coexpression with DISC1^{403–504}. 30 h after transfection with the indicated plasmids, cells were harvested for immunoblotting analyses. *C*, effect of DISC1^{Δ403–504} coexpression on dysbindin stability. HEK293T cells were treated with 100 μg/ml cycloheximide (*CHX*) 30 h after cotransfection of EGFP-dysbindin constructs with either full-length DISC1 or DISC1^{Δ403–504}. Dysbindin levels were quantified by immunoblotting. Representative data are shown in *a*, and the relative dysbindin protein level at time 0 is shown in *b*. *Error bars* show mean ± S.E. *****, *p* < 0.001 by one-way ANOVA with Bonferroni's multiple comparison test (*n* = 4); *NS*, not significant. *c*, the dynamics indicating the remaining protein levels of dysbindin were measured as a ratio of the value at time 0 in the same group. *Error bars* show mean ± S.E. ***, *p* < 0.05; *****, *p* < 0.001 versus EGFP-dysbindin with empty vector (*MOCK*) by one-way ANOVA with Bonferroni's multiple comparison test (*n* = 4). *D*, down-regulation of dysbindin protein by overexpression of DISC1^{Δ403–504}. COS7 cells were transfected with either EGFP-DISC1 or EGFP-DISC1^{Δ403–504} and subjected to immunoblotting. *Error bars* show mean ± S.E. ***, *p* < 0.05 by one-way ANOVA with Bonferroni's multiple comparison test (*n* = 3).

plex formation in the cellular context. Consistently, DISC1 and dysbindin were coimmunoprecipitated from mouse brain lysates (Fig. 1*D*). We also examined the subcellular localization of these

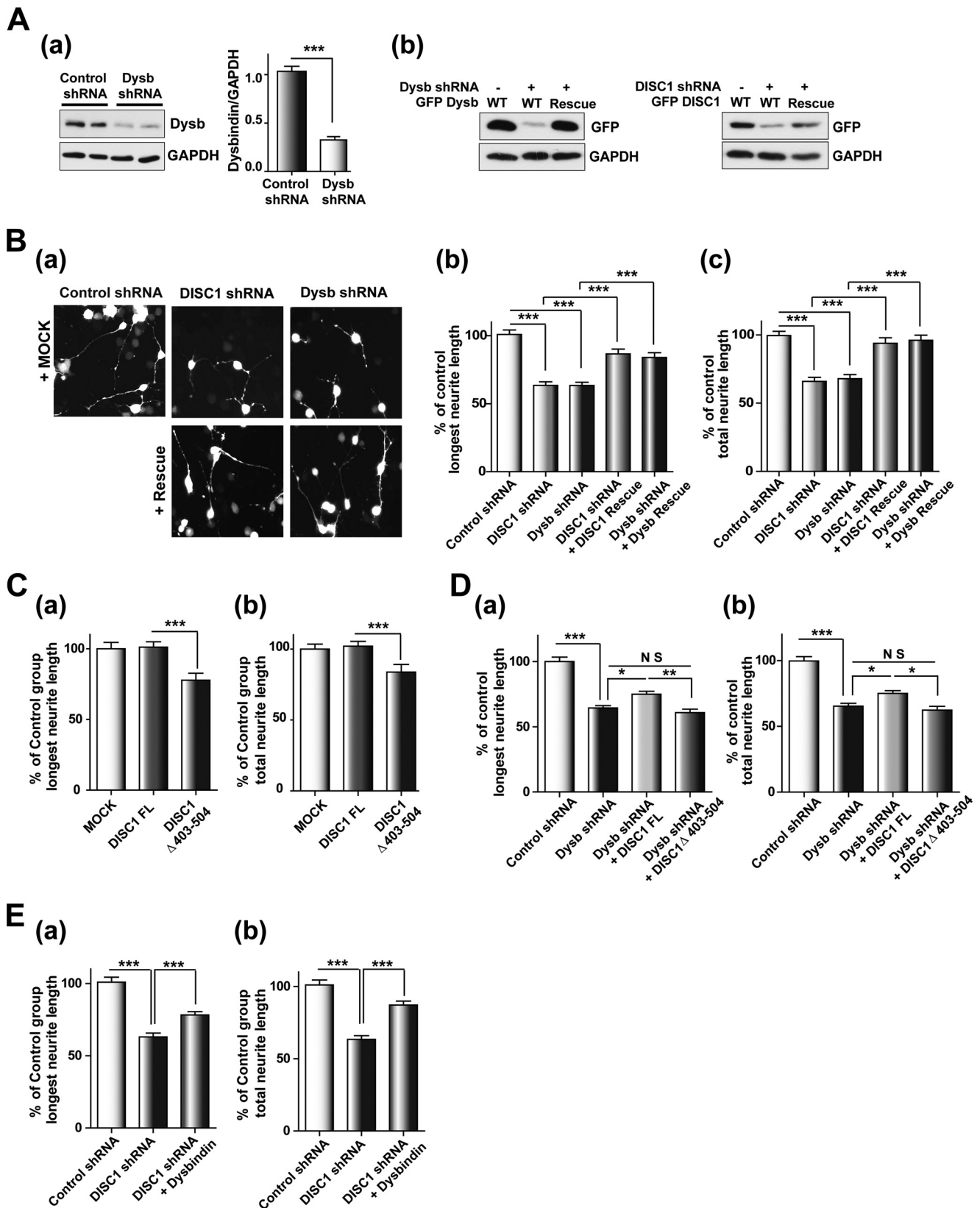
two proteins using immunocytochemistry in HEK293 cells. Confocal microscopy images showed significant colocalization between Myc-dysbindin and EGFP-DISC1 in the cytoplasm (Fig.

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1E). Taken together, these results suggest that dysbindin and DISC1 form a physiologically functional complex.

We next attempted to identify the protein subregions mediating the interaction between dysbindin and DISC1. A series of

deletion mutants of dysbindin were generated on the basis of domain predictions, and their interactions with DISC1 were assayed by coimmunoprecipitation in HEK293T cells. As shown in Fig. 1F, the unstructured N-terminal and coiled-coil



domains of dysbindin (residues 1–87 (dysbindin^{1–87}) and 88–180 (dysbindin^{88–180}), respectively) were coimmunoprecipitated with full-length DISC1, whereas the dysbindin C terminus (residues 181–351, dysbindin^{181–351}) was not. Conversely, we also analyzed the interaction of full-length dysbindin with EGFP-tagged fragments of DISC1 (residues 1–350, 345–591, and 586–854). DISC1^{1–350} and DISC1^{345–591} were both coimmunoprecipitated with full-length dysbindin, whereas DISC1^{586–854} was not (Fig. 1G). Therefore, the interaction between dysbindin and DISC1 involves a rather broad interface encompassing residues 1–180 of dysbindin and residues 1–591 of DISC1.

DISC1 Protects Dysbindin from the Ubiquitin-Proteasome System—The reduction of dysbindin protein in schizophrenic patients has been shown previously (23, 24), implying that the abundance of dysbindin can be associated with the disease. It has been reported recently that TRIM32, a ubiquitin E3 ligase, ubiquitylates dysbindin and facilitates its degradation (40). Moreover, some reports hint at the possibility that DISC1 affects the ubiquitylation and stability of multiple DISC1-interacting proteins (39, 41). In this line, we examined whether DISC1 regulates stability of dysbindin protein by monitoring dysbindin level transiently transfected in the presence or absence of DISC1 coexpression. The results showed a remarkable increase in dysbindin following transfection with DISC1 (Fig. 2A) without alteration of the dysbindin mRNA level (Fig. 2B). In addition, the degree of increase in dysbindin showed a strong correlation with the DISC1 expression level (Fig. 2C). To ascertain whether DISC1 is essential for maintaining the endogenous dysbindin protein level, we introduced shRNA constructs targeting DISC1 into Cath.a-differentiated (CAD) mouse neuroblastoma cells. The level of dysbindin protein was reduced significantly following knockdown of DISC1 (Fig. 2D).

Dysbindin is degraded by the ubiquitin-proteasome system (UPS) (40). Therefore, we used the proteasome inhibitor MG132 to test whether DISC1 affects dysbindin stability via the UPS. As shown in Fig. 2E, dysbindin was up-regulated dramatically following MG132 treatment (Fig. 2E, *a* and *b*). Indeed, this up-regulation was decreased under coexpression of DISC1 (Fig. 2E, *c*). Moreover, a significant decrease in dysbindin ubiquitylation was consistently detected upon coexpression of DISC1 (Fig. 2F). In sum, these results indicate that DISC1 enhances the stability of dysbindin, at least in part, by protecting dysbindin from ubiquitylation-dependent proteasomal degradation.

Amino Acid Residues 403–504 of DISC1 Are Involved in the Regulation of Dysbindin Stability—We next investigated which regions of DISC1 were important for regulating dysbindin. Interestingly, the level of dysbindin protein was up-regulated upon coexpression of DISC1^{345–591} and DISC1^{586–854}, comparable with the effect of full-length DISC1 coexpression (Fig. 3A), indicating that the effect of DISC1 on dysbindin stability was dependent on amino acid residues 345–854. Although the C-terminal part of DISC1 is not responsible for interaction with dysbindin, amino acid residues 345–591 are involved in the physical interaction and have the capacity to protect dysbindin from degradation. Therefore, we attempted to further narrow down the region of DISC1 responsible for regulating dysbindin stability. We separated the region into five fragments, amino acid residues 345–506, 403–504, 506–591, 586–700, and 696–854 (DISC1^{345–506}, DISC1^{403–504}, DISC1^{506–591}, DISC1^{586–700}, and DISC1^{696–854}, respectively) without disrupting the predicted coiled-coil structures and tested the impact on dysbindin stability upon coexpression. Although both fragments of C-terminal DISC1 (DISC1^{586–700} and DISC1^{696–854}) did not result in a noticeable effect on dysbindin, DISC1^{345–506} and DISC1^{403–504} caused an up-regulation of dysbindin levels (Fig. 3B). This result may reflect that DISC1^{403–504} can mediate the enhancement of dysbindin stability by direct interaction, which is consistent with a recent report showing that incubation of purified dysbindin with a recombinant DISC1 fragment containing amino acid residues 316–854 with a deletion of amino acid residues 403–504 did not result in an additional higher molecular peak in size exclusion chromatography (supplemental data in Ref. 38). On the basis of this information, we introduced DISC1 lacking this region, DISC1^{Δ403–504}, into the analysis. We observed a consistent decrease in dysbindin protein upon coexpression of DISC1^{Δ403–504} compared with full-length DISC1, suggesting that amino acid residues 403–504 of DISC1 are critical for preventing dysbindin from degradation (Fig. 3C, *a* and *b*). To further confirm this, we compared the stability of dysbindin upon coexpression of either full-length DISC1 or DISC1^{Δ403–504} in cycloheximide chase experiments. In these experiments, the dysbindin level declined more rapidly upon coexpression of DISC1^{Δ403–504} compared with full-length DISC1 (Fig. 3C, *c*). Furthermore, expression of DISC1^{Δ403–504} caused a reduction in endogenous dysbindin (Fig. 3D), further supporting the notion that amino acid residues 403–504 of DISC1 are important for protecting dysbindin from proteasome-mediated degradation.

FIGURE 4. DISC1 affects the dysbindin-mediated neurite outgrowth process. *A*, characterization of shRNA constructs. *a*, knockdown of endogenous dysbindin (*Dysb*) by dysbindin shRNA in differentiated CAD cells ($n = 3$). Error bars show mean \pm S.E. ***, $p < 0.001$ by Student's *t* test. *b*, expression of shRNA-resistant DISC1 and dysbindin. *B*, neurite outgrowth defects in DISC1 and dysbindin knockdown CAD cells. Shown are representative images of differentiated CAD cells transfected with shRNA constructs alone or in combination with rescue constructs of DISC1 and dysbindin (*a*). The longest neurite length (*b*) and total neurite length (*c*) were measured using ImageJ software and normalized to the control group ($n = 243$ for control shRNA, $n = 237$ for DISC1 shRNA, $n = 245$ for dysbindin shRNA, $n = 215$ for DISC1 shRNA + DISC1 rescue, and $n = 217$ for dysbindin shRNA + dysbindin rescue). Error bars show mean \pm S.E. ***, $p < 0.001$ by one-way ANOVA with Bonferroni's multiple comparison test. *C*, inhibition of neurite outgrowth by coexpression of DISC1^{Δ403–504}. The longest neurite length (*a*) and total neurite length (*b*) were analyzed in CAD cells differentiated 3 days ($n = 193$ for MOCK, $n = 237$ for DISC1 FL, and $n = 223$ for DISC1^{Δ403–504}). Error bars show mean \pm S.E. ***, $p < 0.001$ by one-way ANOVA with Bonferroni's multiple comparison test. *D*, partial rescue of the dysbindin knockdown phenotype by coexpression of DISC1. The longest neurite length (*a*) and total neurite length (*b*) are shown ($n = 211$ for control shRNA, $n = 210$ for dysbindin shRNA, $n = 193$ for dysbindin shRNA + DISC1 FL, and $n = 106$ for dysbindin shRNA + DISC1^{Δ403–504}). Error bars show mean \pm S.E. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; NS, not significant by one-way ANOVA with Bonferroni's multiple comparison test. *E*, reversal of the neurite outgrowth defects by dysbindin coexpression in DISC1 knockdown cells. The longest neurite length (*a*) and total neurite length (*b*) are shown ($n = 192$ for control shRNA, $n = 219$ for DISC1 shRNA, and $n = 219$ for DISC1 shRNA + dysbindin). Error bars show mean \pm S.E. ***, $p < 0.001$ by one-way ANOVA with Bonferroni's multiple comparison test.

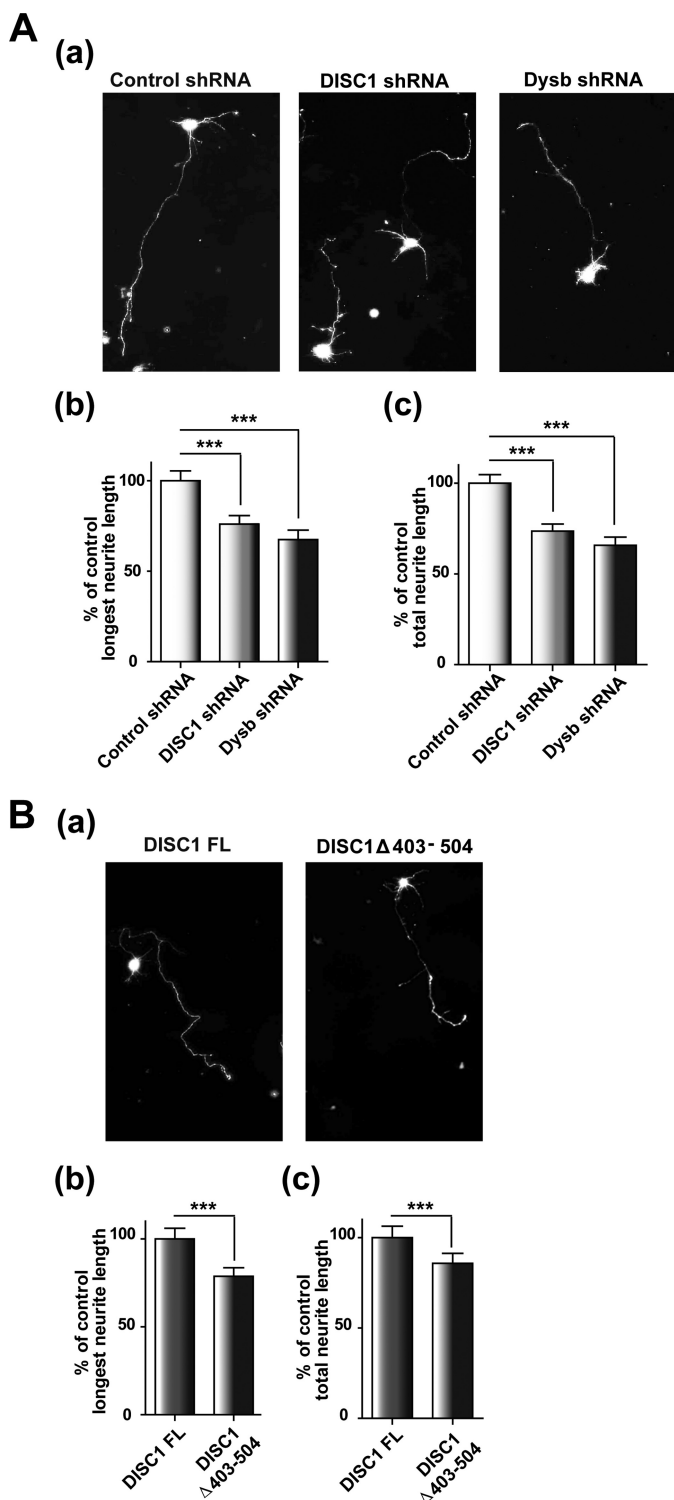


FIGURE 5. Compromised neurite outgrowth by deficiencies in DISC1 and dysbindin in primary cortical neurons. *A*, neurite outgrowth defects in DISC1- and dysbindin (*Dysb*) shRNA-transfected cortical neurons. Shown are representative images of cortical neurons transfected with indicated shRNA constructs (*a*). The longest neurite length (*b*) and total neurite length (*c*) were analyzed. Neurites on ~249 transfected neurons from three independent experiments were measured and normalized to control cells ($n = 214$ for control shRNA, $n = 249$ for DISC1 shRNA, and $n = 192$ for dysbindin shRNA). Error bars show mean \pm S.E. *******, $p < 0.001$ by Student's *t* test. *B*, inhibition of neurite outgrowth by DISC1 Δ 403-504 coexpression. Shown are representative images of cortical neurons transfected with full-length DISC1 or DISC1 Δ 403-504 (*a*). The longest neurite length (*b*) and total neurite length (*c*) were analyzed ($n = 193$ for DISC1 FL and $n = 223$ for DISC1 Δ 403-504). Neurons were

DISC1 Regulates Dysbindin-mediated Neurite Outgrowth—Dysbindin is involved in neurite outgrowth in differentiated neuroblastoma cells and primary neurons (12, 13, 25). Comparable neurite outgrowth defects are caused by deficiencies in DISC1 function (3, 30–32). Therefore, we attempted to test whether DISC1 and dysbindin are involved in neurite outgrowth through a common pathway. For this, shRNA against the mouse form of dysbindin (Fig. 4*A*, *a*), shRNA-resistant mouse dysbindin, and DISC1 constructs (Fig. 4*A*, *b*) were generated and utilized for neurite outgrowth assays in CAD cells. Consistent with previous observations, the longest neurite and total neurite length were both reduced significantly by shRNA-mediated knockdown of either dysbindin or DISC1. A remarkable restoration of neurite outgrowth by coexpression with shRNA-resistant DISC1 and dysbindin in knockdown cells was also observed, verifying the target specificity of knockdown constructs (Fig. 4*B*). Notably, although the full-length DISC1 did not influence neurite length, small but significant reductions of the longest neurite and total neurite length were observed upon overexpression of DISC1 Δ 403-504 (Fig. 4*C*). Although the stability of dysbindin in HEK293 cells was increased upon coexpression of full-length DISC1, the neurite outgrowth in CAD cells was not affected, implying that the endogenous level of DISC1 in CAD cells appears sufficient to ensure maximal neurite outgrowth. Moreover, we tested whether DISC1 can affect neurite outgrowth in CAD cells by stabilization of dysbindin protein held at low levels in dysbindin knockdown cells. Indeed, the defects in neurite outgrowth in dysbindin knockdown cells were partially rescued by coexpression of full-length DISC1 but not by DISC1 Δ 403-504, supporting the hypothesis that DISC1 affects the availability of dysbindin protein in the cell (Fig. 4*D*). To characterize the cooperation of DISC1 and dysbindin, we examined whether the DISC1 knockdown phenotype can be restored by dysbindin coexpression. Indeed, the neurite outgrowth defects caused by DISC1 knockdown were significantly restored by dysbindin coexpression (Fig. 4*E*). These results collectively indicate that DISC1 and dysbindin collaborate in the regulation of neurite outgrowth.

We also examined neurite outgrowth in primary cultured mouse cortical neurons. The longest neurite and total neurite length were both reduced significantly upon knockdown of either dysbindin or DISC1 (Fig. 5*A*). Moreover, significantly shorter neurite lengths in the longest neurite and total neurite length were observed upon overexpression of DISC1 Δ 403-504 compared with the overexpression of full-length DISC1 (Fig. 5*B*). These results are largely consistent with the CAD cell results and support the notion that DISC1 and dysbindin not only play roles but also cooperate in the regulation of neurite outgrowth.

DISCUSSION

In this study, we describe the physical and functional interaction between DISC1 and dysbindin, two major schizophrenia susceptibility factors. We found that DISC1 physically interacts with dys-

transfected with constructs for GFP-DISC1 FL or GFP-DISC1 Δ 403-504 in combination with the red fluorescent protein (*RFP*) construct (ratio 3:1) at days *in vitro* 0 to visualize neuronal morphology. Error bars show mean \pm S.E. *******, $p < 0.001$ by Student's *t* test.

bindin and stabilizes the protein by decreasing its ubiquitylation. Although the interaction was mediated by a broad region of DISC1, its capacity to up-regulate dysbindin appeared to be restricted to a relatively specific region (amino acid residues 403–504). Finally, we demonstrated that DISC1 and dysbindin cooperate in the neurite outgrowth process. The potential functional interaction between DISC1 and dysbindin has been suggested on the basis of their close proximity in the protein-protein interaction network of schizophrenia susceptibility factors (36, 37). Moreover, a recent report showed a colocalization of DISC1 and dysbindin in cellular aggresomes and the association of purified recombinant dysbindin and DISC1 (38). However, it was unclear whether DISC1 and dysbindin interact under more physiological conditions and what its functional relevance might be. In this line, our results provide additional mechanistic insights, supporting the idea that multiple schizophrenia susceptibility factors form a common biological pathway associated with the etiology of schizophrenia and related mental illnesses.

We provided evidence indicating that DISC1 affects dysbindin functions by enhancing its stability. Studies have revealed extended roles for the UPS in neuronal growth and development, synaptic function and plasticity, and pathway-specific modulators (42–44). However, only few UPS-associated players have been studied in neurons so far, and many components and functions of the UPS in the neuronal system have yet to be identified. Interestingly, in addition to the DISC1 region responsible for the interaction with dysbindin, the C-terminal region of DISC1 (amino acid residues 586–854) appears to be involved in the dysbindin level in an interaction-independent manner. This is consistent with previous reports showing that the C-terminal region of DISC1 participates in controlling the stability of the counterpart-interacting proteins by affecting their ubiquitylation levels (39, 41). These results raise the possibility that there might be a common ubiquitin-associated mechanism involving the C-terminal region of DISC1. The ubiquitylation state of a protein is determined by the antagonistic activities of the ubiquitin-conjugating enzyme (E2)-ubiquitin ligase (E3) complex and deubiquitylating enzymes (45, 46). Therefore, it is intriguing to speculate that DISC1 might affect the function of either the ubiquitin-conjugating enzyme complex or deubiquitylating enzymes to alter the stability of its binding partners.

Although dysbindin was initially identified as a component of the dystrophin-dystroglycan complex, subsequent studies showed that dysbindin in the brain exists as a stable component of biogenesis of lysosome-related organelles complex 1 (BLOC-1), a multiprotein complex containing at least eight components (dysbindin (dystrobrevin-binding protein 1, DTNBP1), muted (MUTED), pallidin (PLDN), cappuccino (CNO), snapin (SNARE-associated protein, SNAPAP), and biogenesis of lysosome-related organelles complex 1, subunit 1–3 (BLOC1S1–3)) with functions in organelle biogenesis and intracellular membrane trafficking (12, 47–49). It is notable that other BLOC-1 subunits, BLOC1S2, BLOC1S3, and MUTED, have been reported to be potential susceptibility factors of schizophrenia (2, 50, 51) and that a deficiency in BLOC-1 induces neurite outgrowth defects in rodent hippocampal neurons (12). Interestingly, destabilization of dysbindin protein has been observed consistently in muted (*mu*) (51, 52) and pallid (*pa*)

mice (12, 52), implying the potential importance of decreased dysbindin stability as a common cellular correlative associated with schizophrenia. Taken together, the findings in this study suggest that DISC1 may regulate the integrity of BLOC-1 and that dysbindin mediates this interplay among multiple schizophrenia susceptibility factors.

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