Supplemental Data

Disrupted in Schizophrenia 1 Regulates Neuronal Progenitor Proliferation via Modulation of GSK3β/β-Catenin Signaling

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SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Mice. Swiss Webster pregnant female mice were purchased from Taconic. Male C57/B6J mice were obtained from the Jackson Laboratory.

Cell culture. AHPs were kindly provided by Dr. Gage and cultured under conditions described previously (Palmer et al., 1997). Primary neural progenitors were isolated from E14 mouse embryos and cultured as described previously (Sanada and Tsai, 2005). N2a and 293T cells were cultured in DMEM medium containing 10 % FBS, L-glutamine, and penicillin/streptomycin. Wnt3a expressing L cells and untransfected L cells were obtained from ATCC. Wnt3a conditional medium was produced according to the ATCC protocol. To induce AHP differentiation, FGF2 was removed from the culture medium and 1 μM retinoic acid/1% FBS was added to the medium for 4 days.

Reagents. Recombinant Wnt3a protein was obtained from Chemicon. FGF2 was obtained from PeproTech. Recombinant GST-GSK3β was obtained from Active Motif. Recombinant GST-β-catenin and GST-axin were obtained from Millipore. The GSK3β inhibitor, SB-216763, and His-GSK3β, were obtained from Sigma-Aldrich. L803-mts was obtained from Calbiochem. GST-AKT1 was obtained from Cell Signaling. GST-CASK-1(CASK fragment from 316-415aa), GST-CASK-2 (CASK fragment from 777-974aa), and GST-p25 proteins were kindly provided by Dr. B.A. Samuels. Mouse DISC1 peptide-neg (40-77aa: GYMRSTAGSG IGFLSPAVGMPHPSSAG LTGQQSQHSQS) and Disctide1 (195-238aa, PADIASLPGFQDTFTSSFSFIQLSLGAA GERGEAEGCLPSREAE) were synthesized by Chi Scientific. CACNA1B peptide (2007-2018aa: QPAPNASPMKRSC) was synthesized in Tufts University. The GSK3β inhibitor CHIR-99021 was synthesized by the Broad Institute using published protocols. 15-mer peptides were synthesized using standard Fmoc solid-phase automated peptide synthesis from its C-terminus to N-terminus by GenScript. Correct synthesis of all peptidess was confirmed by matching molecular or fragment peaks using electrospray ionization mass spectrometry and purity checked by high performance liquid chromatography.

Antibodies. The primary antibodies used in this study were the following: rabbit anti-DISC1 C-terminal antibody, mouse and rabbit anti-GFP antibodies (Invitrogen); affinity purified rabbit anti-DISC1 antibody (Novus) for immunohistochemistry; mouse anti-FLAG, mouse anti-GFAP antibody, and mouse anti-Actin antibody (Sigma-Aldrich); mouse anti-GSK3β antibody, mouse anti-β-catenin antibody, mouse anti-pY216 GSK3β and total GSK3β antibodies, and mouse anti-nestin antibody (BD Biosciences); rabbit anti-pS33/S37/T41 β-catenin antibody, rabbit anti-pT222/226 C/EBPα and C/EBPα antibodies, rabbit anti-pS9 GSK3β antibody, and mouse anti-cyclin D1 antibody (Cell Signaling); mouse anti-HA antibody, mouse anti-GST antibody, mouse anti-ubiquitin antibody, goat anti-Sox2 antibody, mouse anti-axin2 antibody, rabbit anti-Cux2 antibody, rabbit anti-Ki67 antibody, mouse anti-NeuroN antibody, and rabbit anti-β-catenin antibody (Santa Cruz); mouse Tuj-1 antibody (BABCO); mouse anti-BrdU antibody (DakoCytomation); guinea pig anti-doublecortin antibody (Chemicon); chicken anti-GFP antibody (Aves Labs); rabbit anti-pS10 Histone H3 antibody (Upstate). Rabbit pS231/S234-Ngn2 antibody and Ngn2 antibody were described previously (Ma et al., 2008).

Constructs. The sequences for shRNAs targeting DISC1 are as follows: control shRNA: 5'-CGGCTGAAACAAGAGTTGG-3'; DISC1 shRNA-1: 5'-GGCAAACACTGTGA AGT GC-3'(Kamiya et al., 2005) ; DISC1 shRNA-2: 5'-GCAGGAGGTCAGCAAG GCCTTG-3'. DISC1 shRNA-1 recognizes both rat and mouse DISC1. DISC1 shRNA-2 targets human, rat, and mouse DISC1. shRNA oligoribonucleotides were cloned into the pLentiLox 3.7 vector (Rubinson et al., 2003). β-catenin shRNAs were obtained from the Broad Institute RNA interference platform and the sequences are as follows: β-catenin shRNA-1: 5'-CTGATATTGACGGGCAGTAT-3', β-catenin shRNA-2: 5'-CCCAA GCCTTAGTAAACATAA-3'. The full length mouse DISC1 cDNA was generously provided by Dr. Cris Bragg. Four different fragments (1-220aa, 221-355aa, 356-595aa, and 596-852aa) from mouse DISC1 cDNA were amplified by PCR and subcloned into pGEX-4T2 to generate GST fusion constructs. The full length human DISC1 cDNA was generously provided by Dr. A. Sawa (John Hopkins University, MD). The full length human DISC1 was amplified by PCR and subcloned into the pOZ-N retroviral vector or pEGFP-C1 to generate a HA-FLAG-tagged or GFP-tagged WT-DISC1 expressing vector. HA-FLAG-tagged WT-DISC1 was subcloned upstream of the IRES and GFP in the lentiviral vector, FUGW(Scott and Lois, 2005), provided by Dr. C. Lois (MIT, MA). Concentrated lentivirus was produced as previously described (Rubinson et al., 2003). Viral titers, as determined by GFP expression in 293T cells, were $5X10^8$-$1X10^9$ transducing units per ml. Super 8XTOPFLASH (which contains 8 copies of the TCF/LEF binding site), a gift from Dr. R. Moon (University of Washington, WA) and a Renilla-Luc-TK reporter (pRL-TK, Promega) were used for testing TCF transcriptional activity. FLAG-β-catenin (WT and S33A) and HA-GSK3β were provided by Dr. X. He (Harvard Medical School, MA). pCAGIG-Venus was provided by Dr. Zhigang Xie (Boston University, MA).

Luciferase assays. $5X10^5$ transduced AHPs, embryonic progenitors, or 293T cells were seeded into 24-well plates and transfected with 0.8 μg of Super8XTOPFLASH or Super8XFOPFLASH and 0.1 μg of pRL-TK using Lipofectamine 2000 (Invitrogen). 24

hours after transfection, transfected cells were stimulated with Wnt3a-conditioned medium (Wnt3a CM) for 14 hours and TCF reporter activity was measured using the Dual-Luciferase Assay System (Promega). For the rescue experiment, 1.6 μg of vector, WT-β-catenin, or SA-β-catenin was cotransfected with 0.4 μg of Super8XTOPFLASH and 0.1 μg of pRL-TK using Lipofectamine 2000, and transfected cells were treated with Wnt3a (200 ng/ml). For all luciferase assays, we normalized the level of different overexpressed proteins. For the *in utero* TCF reporter assay, expression vector, Super 8XTOPFLASH and pRL-TK constructs were electroporated into embryonic brains at a 5:1:0.3 ratio. For the knockdown experiments, we electroporated mice at E13 and measured TCF activity at E15. For overexpression experiments, we electroporated mice at E14 and measured TCF activity at E15. All firefly luciferase activities were normalized with Renilla luciferase activity.

Immunoblot and immunoprecipitation. Immunoblots and immunoprecipitations were performed as described previously (Mao and Lee, 2005).

FACS cell cycle analysis. N2a cells were transfected with control or DISC1 shRNAs. 48 hours after transfection, cells were fixed with 2% paraformaldehyde and permeabilized with 75% ethanol. FACS analysis was conducted as described previously (Koso et al., 2006).

***In vitro* binding and kinase assay.** GST pull-down assays were conducted as described previously (Mao et al., 2004). Briefly, 5 μg of purified GST-DISC1 fragments were incubated with glutathione–Sepharose beads (GE) for one hour. Beads were then washed with 1XPBS, 1% Triton X-100, 5 mM EDTA, 1 mM DTT, and 0.1 mM PMSF four times. 5 μg of recombinant His-GSK3β protein was then incubated with beads for one hour and nonspecific proteins were washed off 4 times with RIPA buffer and twice with lysis buffer (150 mM NaCl, 0.1% NP40, 50 mM Tris, pH7.5, 5 mM EDTA). Associated proteins were assessed by Western blot with an anti-GSK3β antibody. To determine whether DISC1 inhibits GSK3β through direct association, we have taken efforts to optimize the GSK3β kinase assay using β-catenin as a substrate. At molar ratios (β-catenin:GSK3β) of 0.5 and 1, the phosphorylation of β-catenin reached saturation after 15 minutes. Based on this, we performed kinase assays at a ratio of 2 (β-catenin :GSK3β=0.14 μM : 0.07 μM). For the length of the reaction (at a ratio of 2), phosphorylation of β-catenin reached saturation at 30 minutes (data not shown). Therefore, we performed kinase assays within the linear range with a ratio of 2 and a duration of 15 minutes. For the GSK3β *in vitro* kinase assay, 1 μM of purified DISC1 protein was incubated with 5 ng/μl GST-GSK3β in kinase buffer (2 mM MOPS, pH7.4, 0.05 mM EDTA, 2.5 mM MgAcetate, 100 μM ATP, 10 μCi of ^{32}P-ATP, 10 mM $MgCl_2$) and 18.75 ng/μl GST-axin or GST-β-catenin for 15 minutes at 30 °C. Kinase activity was measured using the anti-pY216 GSK3β antibody (BD) or radiography. For AKT kinase assay, 5 μM of purified DISC1 protein was incubated with 10 ng/μl GST-AKT1 (Cell Signaling) in kinase buffer (5 mM MOPS, pH 7.2, 2.5 mM β-glycerophosphate, 1 mM EGTA, 0.4 mM EDTA, 5 mM $MgCl_2$, 0.05 mM DTT, 50 μM ATP, 1 μCi ^{32}P-ATP) at

room temperature for 30 min. Autophosphorylation of AKT1 was detected by radiography.

***In utero* BrdU labeling.** Control or DISC1 shRNA constructs were injected into E13 embryonic brains with an enhanced GFP (EGFP)-expressing plasmid (final concentration, 2 μg/μl; pCAGIG-Venus) at a 2:1 ratio. In the rescue experiments, the concentration of human WT-DISC1 or SA-β-catenin plasmid was 2 fold higher than the shRNA constructs. In the chemical rescue experiment, SB216763 (2mg/kg) was i.p. injected into the mother mice 24 hour after electroporation for two days (E14-E15). BrdU (100 mg/kg) was i.p. injected into mice 48 hours after electroporation. After 2 hours, brains were processed and sections (20-μm thick) were co-stained with anti-GFP and anti-BrdU or anti-pH3 antibodies. In the rescue experiments, brains were harvested 24 hours after BrdU injection. Brain sections were then co-stained with anti-GFP and anti-BrdU antibodies. The distribution of GFP positive cells was determined by dividing the number of GFP positive cells in each layer by the total number of GFP-positive cells in the entire section.

Surface plasmon resonance. The GSK3β surface plasmon resonance assay was conducted on a Biacore S51 instrument using Biacore CM5 sensor chips. Ethanolamine, EDC, NHS, and P-20 surfactant were all obtained from GE Lifesciences. An anti-GST antibody (GST capture kit, GE LifeSciences) was directly immobilized through primary amines using standard EDC/NHS chemistry according to the manufacturer's instructions. Either GST alone or GST-GSK3β (BD Biosciences) was captured to generate the GSK3β sensor chip**.** GST-GSK3β was thawed immediately before use and kept at 4 °C during sample preparation. Activity of GSK3β was monitored using FRATtide and CHIR-99021 binding. Binding assays were performed at 25 °C. The characterization of Peptide-neg, DISCtide2, L-803-mts, and the 15 mer peptide from the N-type calcium channel was carried out in 10 mM Tris, 150 mM NaCl, 0.005% P-20 surfactant (TBS-P20). GST-GSK3β was diluted to 3.75 ng/μl in PBS with 0.005% P-20 surfactant (PBS-P20) and captured on the sensor surface on *Spot* 1. Between 472.1 and 520.5 response units (RU) of protein was immobilized in each assay. GST was similarly captured at a level of 443.2 RU on *Spot 2* and used as a reference surface. Compounds were injected at a flow rate of 30 μl/min into the flow cell for 120 s followed by 120 s of buffer without compound. For assays involving binding or 15mer Disc1 peptide to GST-GSK3β, peptides were stored in DMSO and diluted into TBS-P20 with 2% DMSO for binding assays. 15mer Disc1 peptide identities were verified by LCMS. GST-GSK3β was diluted to 3.75 ng/μl in PBS with 0.005% P-20 surfactant (PBS-P20) and 2034.7 RUs were captured on the sensor surface on *Spot* 1. GST was similarly captured at a level of 1489.1 RU on *Spot 2* and used as a reference surface. Compounds were injected at a flow rate of 30 μl/min into the flow cell for 120s followed by 120s of buffer without compound. Compound characterizations of L803-mts, DISCtide2, DISCtide2 reverse, mDISC1-pep89 were carried out in TBS-P20 with 2% DMSO. DISCtide2, DISCtide2 reverse, mDisc1-pep89 were >95% pure and verified by LC-MS. Sensorgram data was analyzed using the Scrubber 2 software (BioLogic Software Pty Ltd). Data was GST-reference subtracted and corrected for protein capture, DMSO concentration, and analyte molecular weight.

Disc1 peptides used in this study

peptide	peptide sequence	mDisc1 residues
38	PLASPALNG**PADIAS**	186 - 200
39	**ALNGPADIASLPGFQ**	191 - 205
40	**ADIASLPGFQDTFTS**	196 - 210
41	**LPGFQDTFTSSFSFI**	201 - 215
43(DISCtide2)	**SFSFIQLSLGAAGER**	211 - 225
44	**QLSLGAAGERGEAEG**	216 - 230
45	**AAGERGEAEGCLPSR**	221 - 235
46	**GEAEGCLPSREAEPL**	226 - 240
47	**CLPSREAE**PLHQRPQ	231 - 245
48	EAEPLHQRPQEMAAE	236 – 250
89	AQDSLPASITRRDWL	441 - 455
DISCtide2 reverse	REGAAGLSLQIFSFS	225 - 211

Sequences in bold and blue are residues from mDISCtide1.

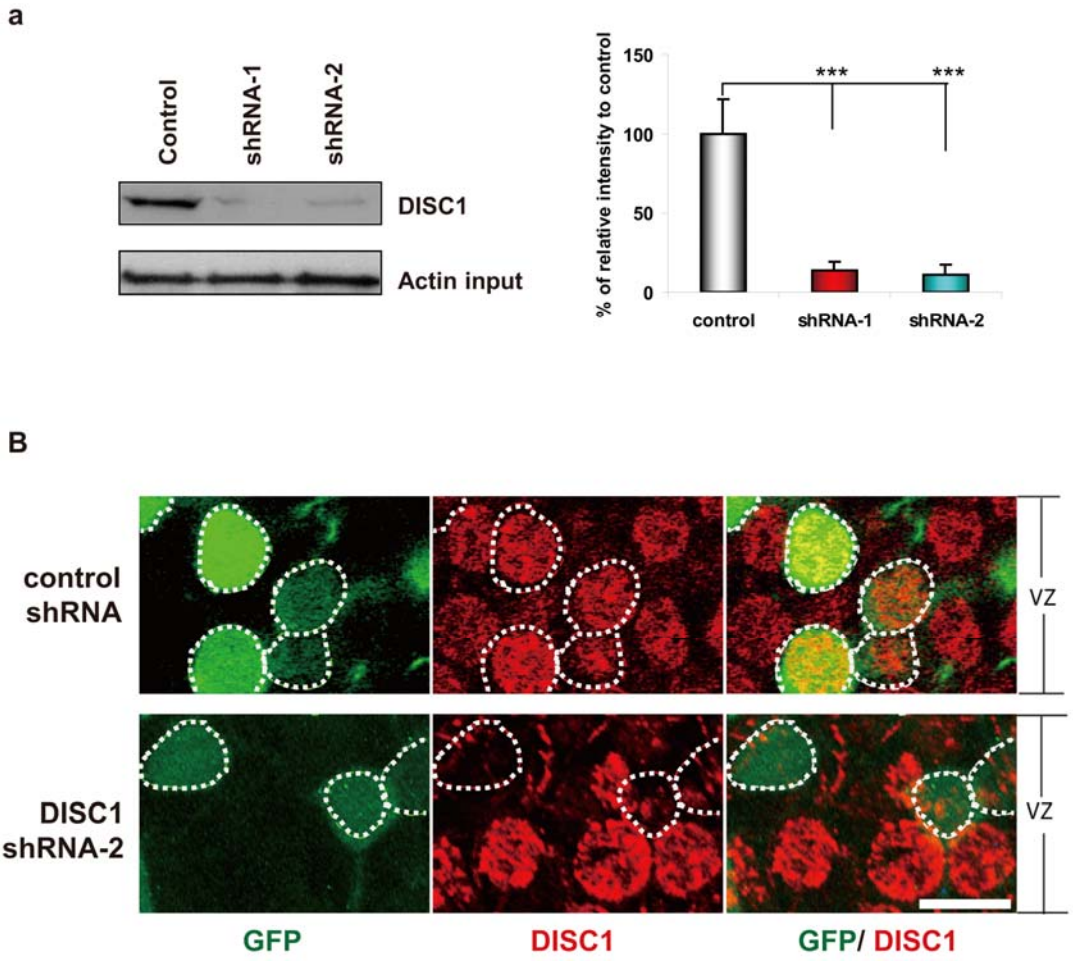

Figure S1. (A) Suppression of DISC1 expression by RNA interference. N2a cells were transduced with control or DISC1 shRNA lentivirus. Endogenous DISC1 protein was immunoprecipitated and blotted with an anti-DISC1 antibody (n=3, $p<0.005$, mean ± SEM). **(B)** DISC1 expression is knocked down *in vivo*. Electroporated embryonic brain sections were stained with anti-DISC1. Circled cells are GFP-positive cells. Scale Bar=10 μm.

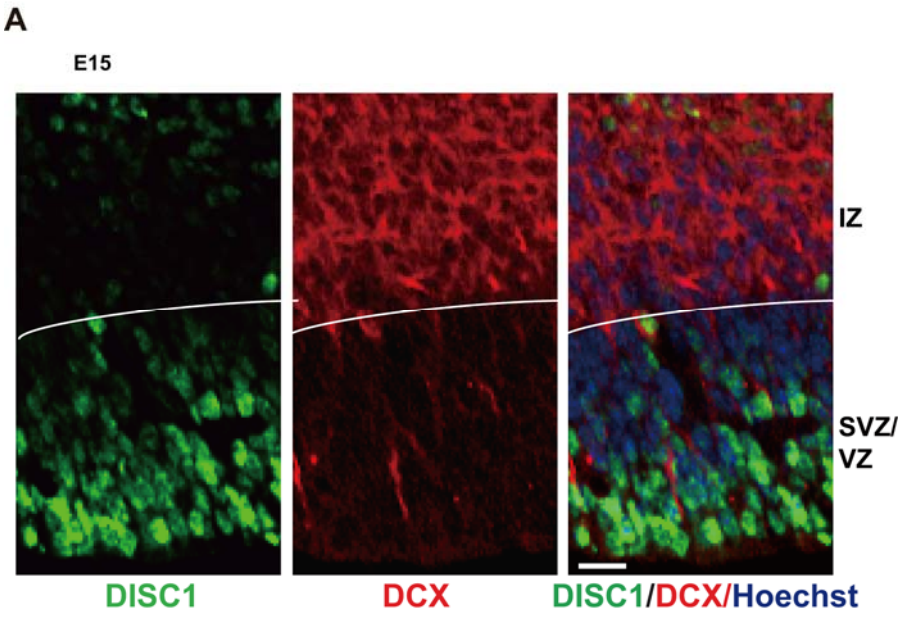

Figure S2. (A) E15 embryonic brains were sectioned and co-stained with anti-DISC1 and anti-DCX antibodies. Scale bar=20 μm.

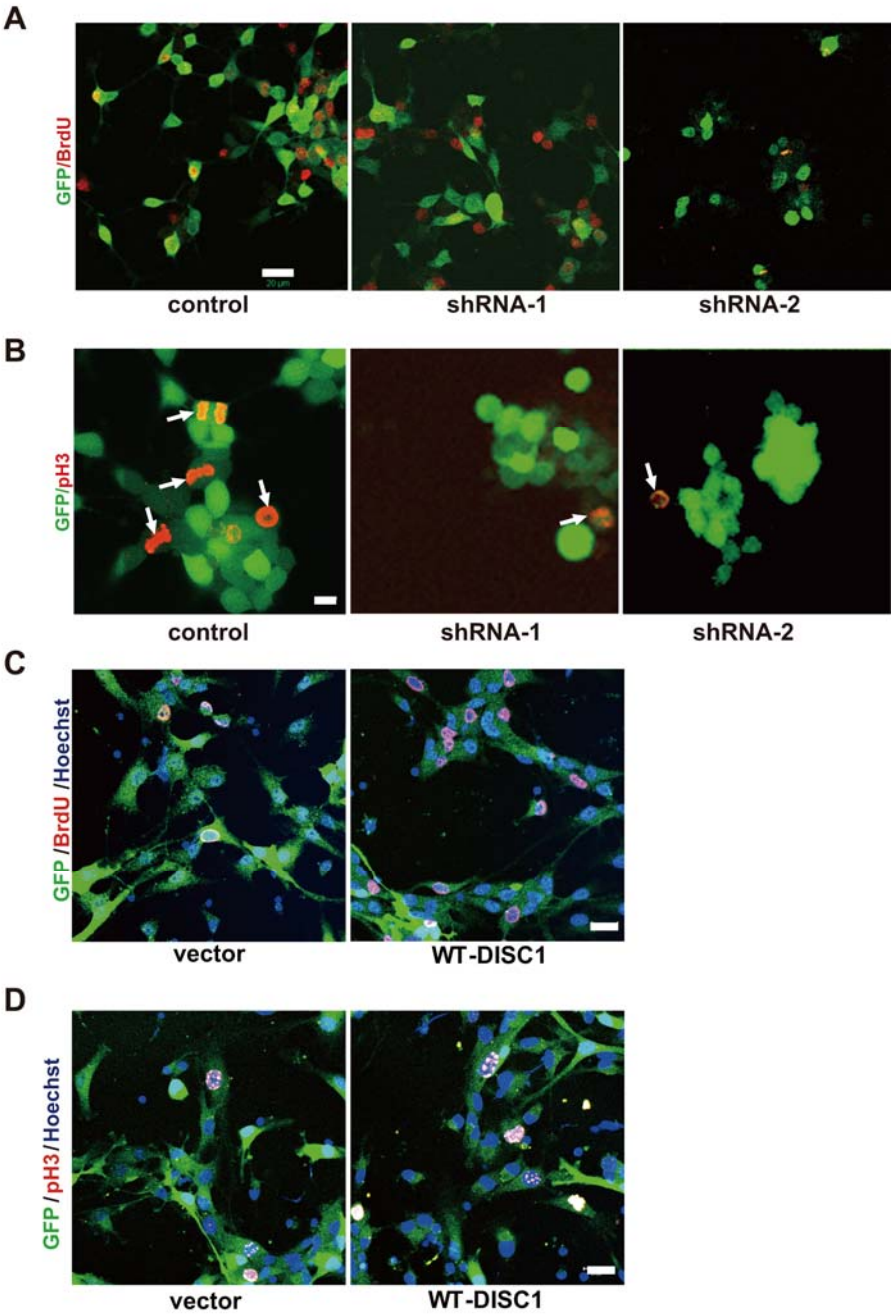

Figure S3. (A) BrdU incorporation is reduced in DISC1 knockdown cells. AHPs infected with either control or DISC1 shRNA lentivirus were pulse labeled with 10 μM of BrdU and stained with BrdU antibody. Scale bar=20 μm. **(B)** Mitotic index is decreased in DISC1-silenced AHPs. AHPs infected with either control or DISC1 shRNA lentivirus were stained with anti-phospho-Histone H3 (pH3) antibody. Scale bar=10 μm. **(C)** BrdU incorporation is increased in DISC1 overexpressing AHPs. AHPs infected with either control or WT-DISC1 lentivirus were labeled with 10 μM of BrdU for an hour and stained with anti-BrdU antibody. Scale bar=20 μm. **(D)** Mitotic index is increased in DISC1 overexpressing AHPs. AHPs infected with either control or WT-DISC1 lentivirus were stained with the pH3 antibody. Scale bar=20 μm.

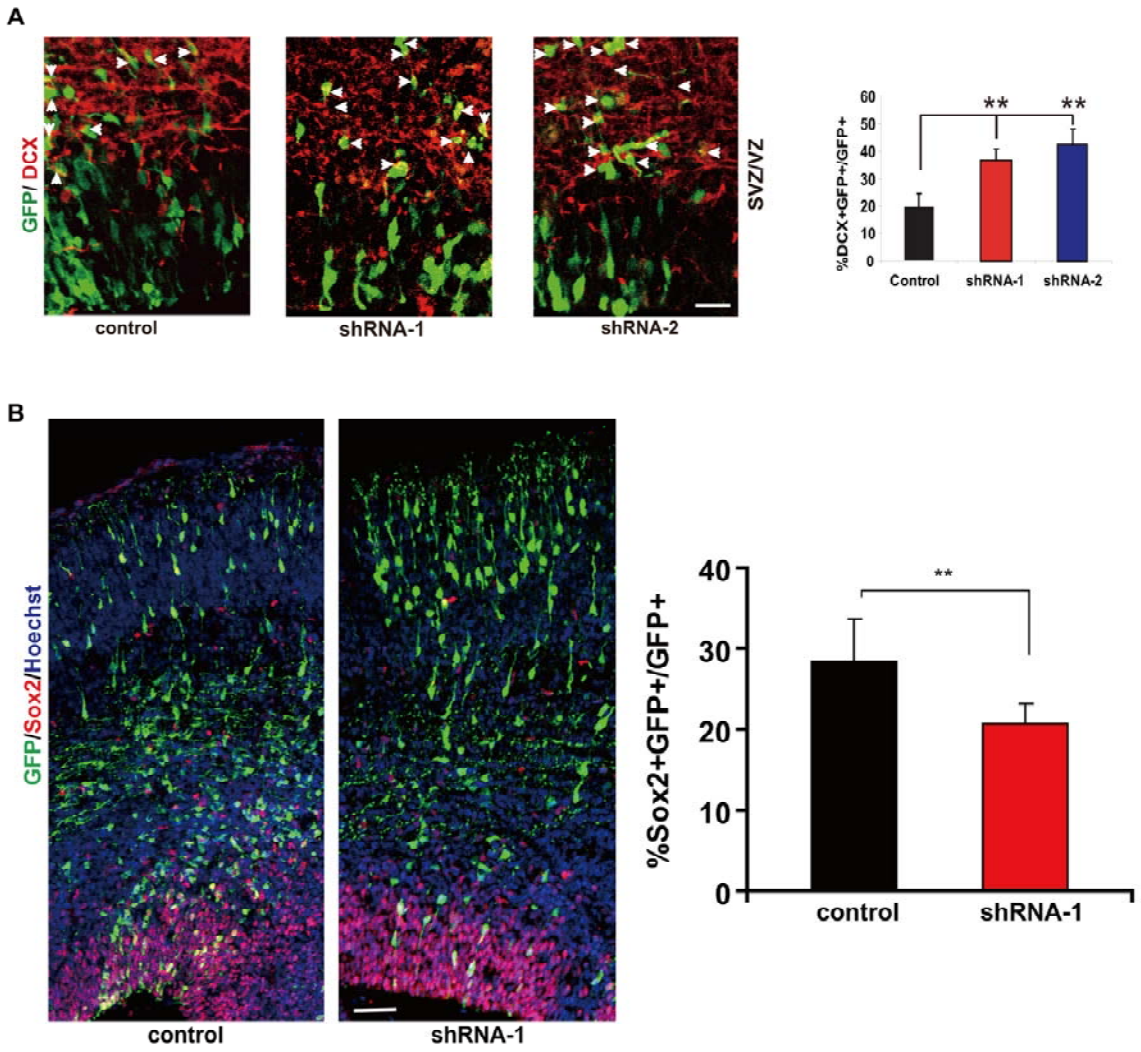

Figure S4. (A) Neuronal differentiation of DISC1 knockdown cells *in utero.* Control or DISC1 shRNA constructs were electroporated into E13 embryonic brains and mice were sacrificed at E16. Brain sections were stained with anti-GFP antibody and anti-DCX antibody. Shown is the percentage of GFP and DCX double positive cells in the SVZ (n=3, $p<0.01$, mean ± SEM). Scale bar=10 μm. **(B)** Reduced neural progenitors of DISC1 knockdown *in utero.* Control or DISC1 shRNA constructs were electroporated into E13 embryonic brains and mice were sacrificed at E16. Brain sections were stained with anti-GFP antibody and anti-Sox2 antibody. Shown is the percentage of GFP and Sox2 double positive cells in the brain (n=3, $p<0.01$, mean ± SEM). Scale bar=20 μm.

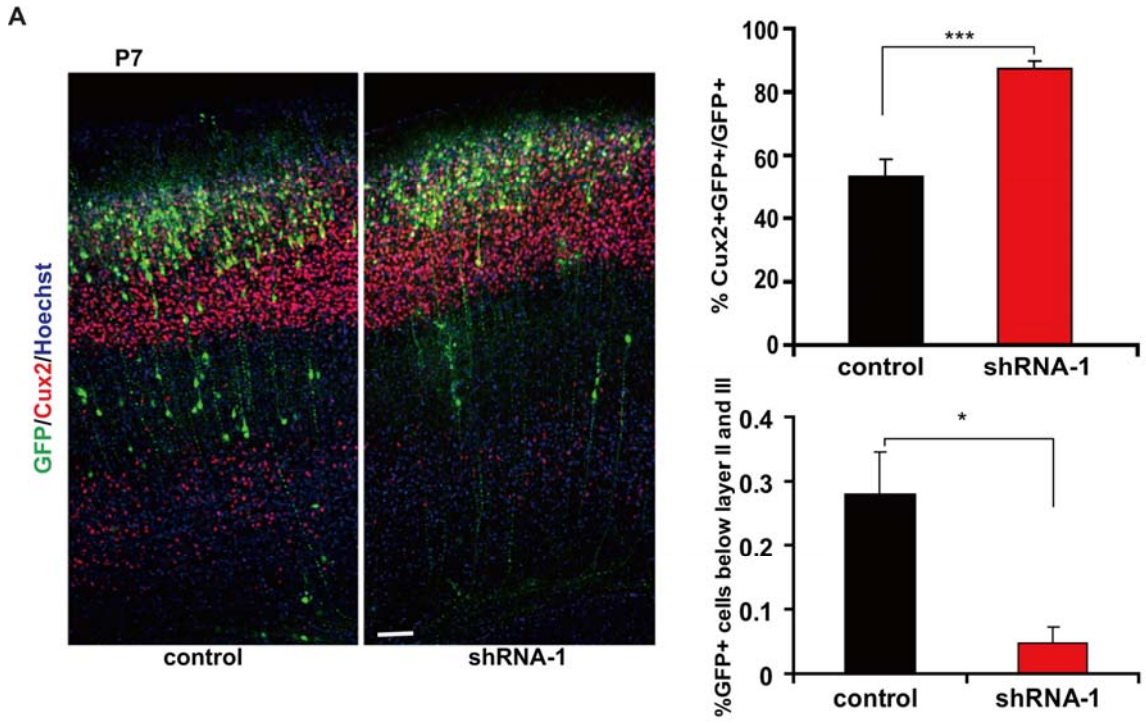

Figure S5. Increased Cux2 neuronal differentiation of DISC1 knockdown *in utero.* Control or DISC1 shRNA constructs were electroporated into E15 embryonic brains and mice were sacrificed at P7. Brain sections were stained with anti-GFP antibody and anti-Cux2 antibody. Shown are the percentage of GFP and Cux2 double positive cells in the brain and the percentage of GFP positive cells below layer II and III (n=3, *, $p<0.05$; ***, $p<0.005$, mean ± SEM). Scale bar=20 μm.

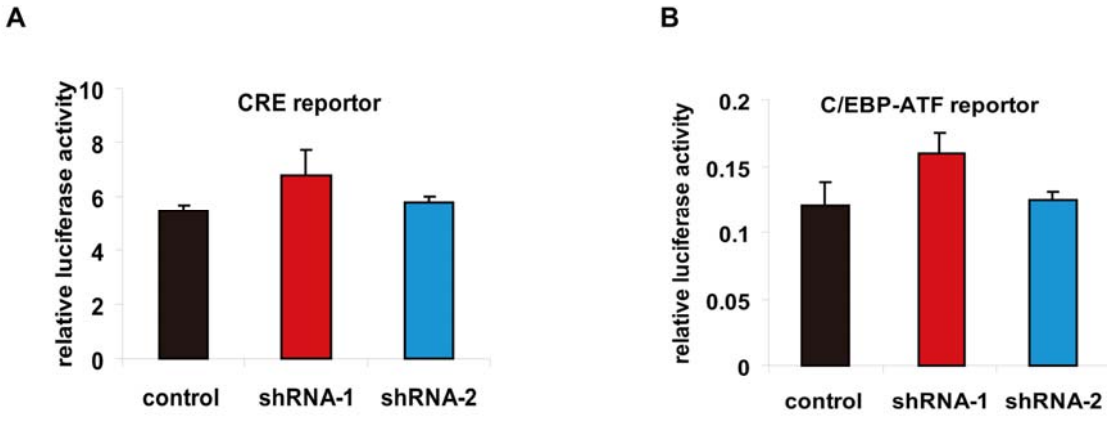

Figure S6. (A and B) DISC1 shRNAs did not affect the CRE (A) or C/EBP-ATF reporters (B). Bar graph shows the relative luciferase activity (n=3, mean ± SEM).

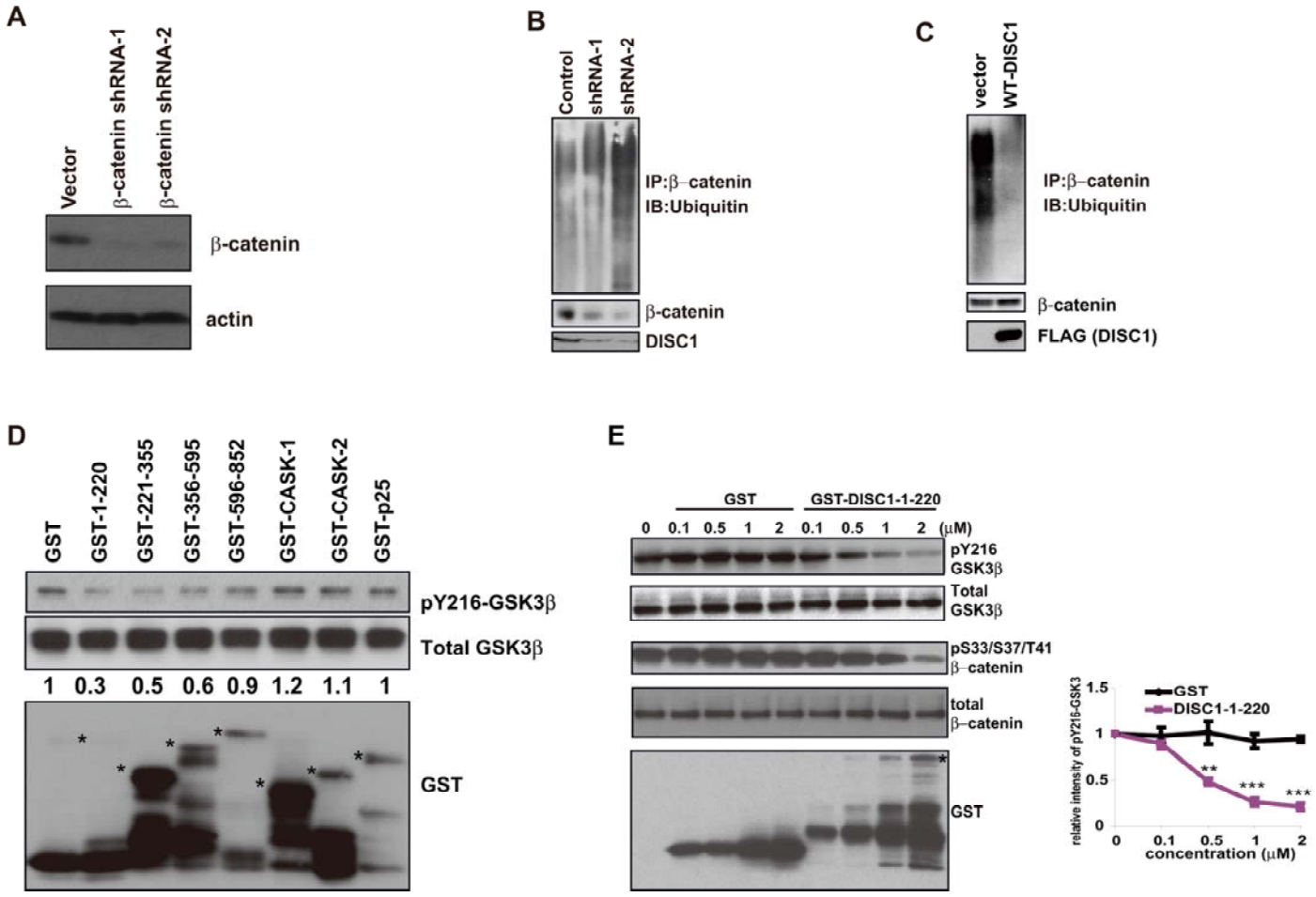

Figure S7. (A)N2a cells were transfected with control, β-catenin shRNA-1 or -2 for 48 hours. Cell lysates were probed with anti-β-catenin and actin antibodies. **(B)** Increased ubiquitination of β-catenin in AHPs expressing DISC1 shRNAs. **(C)** Overexpression of DISC1 suppresses β-catenin ubiquitination. **(D)** Phosphorylation of Y216 on GSK3β is reduced by all DISC1 fragments at 2 μM *in vitro*. Stars indicate the different intact GST fusion proteins. Numbers in the panel indicate the relative intensity of the band compared to GST (n=3). **(E)** Reduction of Y216 phosphorylation on GSK3β by DISC1 fragment 1-220 is dose-dependent. Stars indicate the different intact GST fusion proteins. The dose-response curve is shown (n=3, **, $p<0.01$;***, $p<0.005$, mean ± SEM).

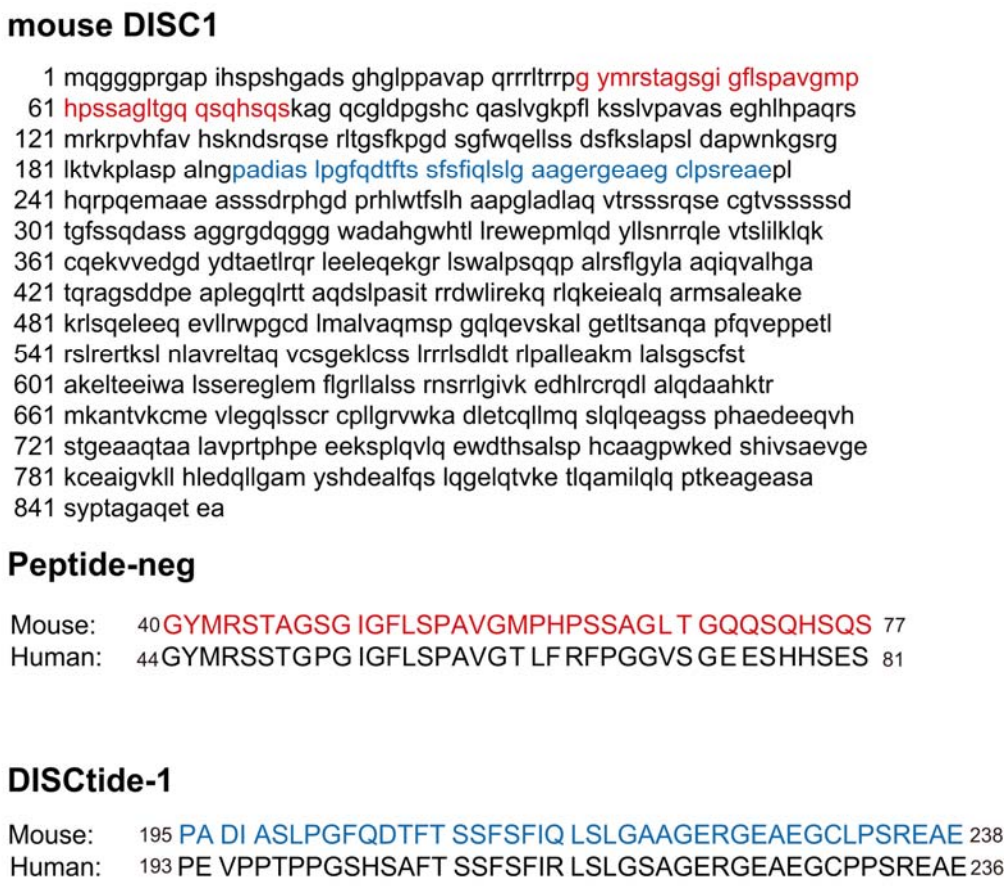

Figure S8. DISC1 peptides are conserved from mouse to human. Peptide-neg (red) spans amino acids 40 to 77 and Disctide1 (blue) spans amino acids 195 to 238 of mouse DISC1.

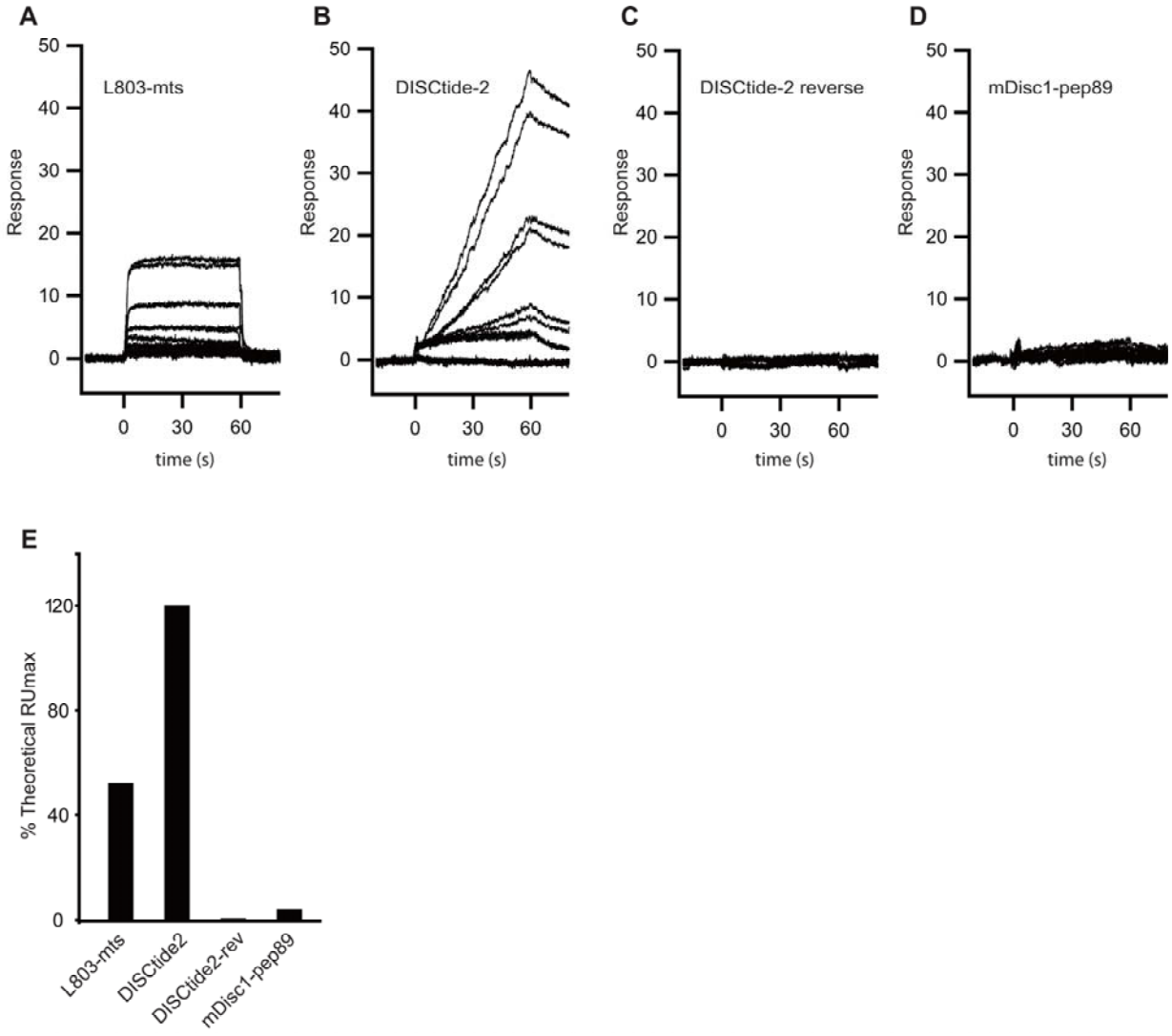

Figure S9. Surface plasmon resonance (SPR) characterization of the DISCtide2 GSK3β interaction. Binding of (A) L803-mts (B) Disctide2 (C) DISCtide2 reverse and (D) a non-binding DISC1 15 mer peptide, mDISC1-pep89 from 0.195 – 50 μM to GSK3β. (E) The percent theoretical maximal response for SPR binding assays at 50 μM for panels A – D is summarized.

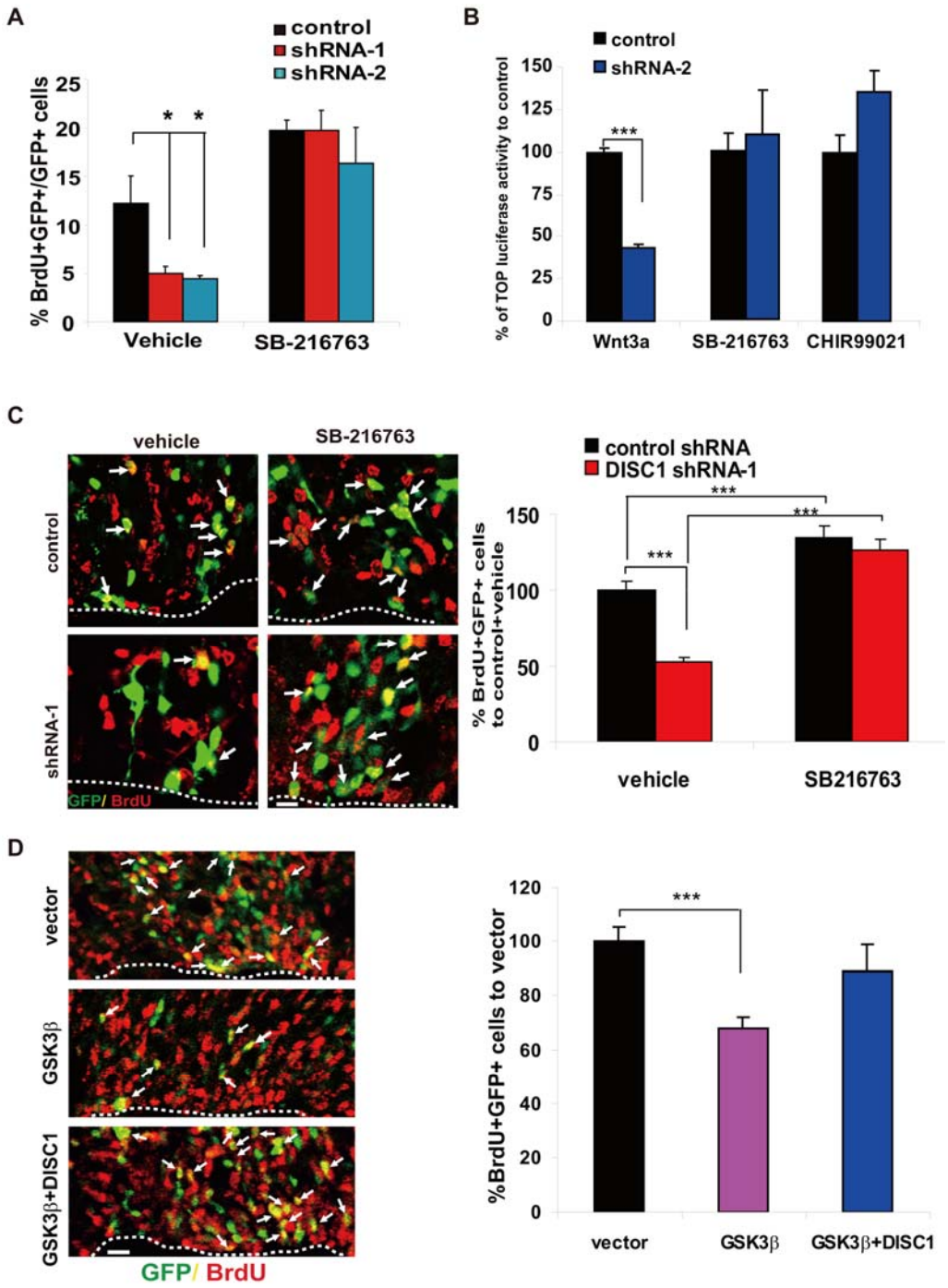

Figure S10. **(A)** The GSK3β inhibitor, SB-216763, can rescue DISC-1 knockdown-induced proliferation defects. Primary neural stem cells from E14 brains were infected with control or DISC1 shRNA lentivirus. 48 hours after transduction, cells were treated with vehicle or SB-216763 (5 μM) for 16 hours and then pulsed with 10μM BrdU for 2 hours. Shown is percentage of GFP and BrdU double positive cells to total GFP positive cells (n=3, $p<0.05$, mean ± SEM). **(B)** The GSK3β inibitor, SB-216763 or CHIR-99021, rescues the TCF activation defect caused by DISC1 knockdown in 293T cells. 0.5 μg

control or DISC1 shRNA-2 was cotransfected with 0.1 μg TCF reporter and 10 ng pRL-TK vector into 293T cells. 48 hours after transfection, Wnt3a conditional medium with vehicle, 5 μM of SB-216763 or CHIR-99021 was added for another 16 hours. The bar graph shows the percentage of relative TCF reporter activity compared to the corresponding controls (n=3, $p<0.0001$, mean ± SEM). **(C)** The GSK3β inibitor, SB-216763, rescues the proliferation defect caused by DISC1 knockdown *in utero*. Control or DISC1 shRNA constructs were electroporated into E13 embryonic brains and pregnant mice were i.p. injected with SB-216763 (2mg/kg) at E14 and E15. BrdU (100mg/kg) was given 2 hours before sacrifice at E16. Brain sections were stained with anti-GFP antibody and anti-BrdU antibody. Shown is the percentage of GFP and BrdU double positive cells in the SVZ (n=4, $p<0.001$, mean ± SEM) to control+vehicle. Scale bar=10 μm. **(D)** DISC1 overexpression can suppress the effect of GSK3β on neural progenitor proliferation *in utero*. Shown is the percentage of GFP and BrdU double positive cells in the SVZ to control (n=3, $p<0.001$, mean ± SEM). Scale bar=10 μm.

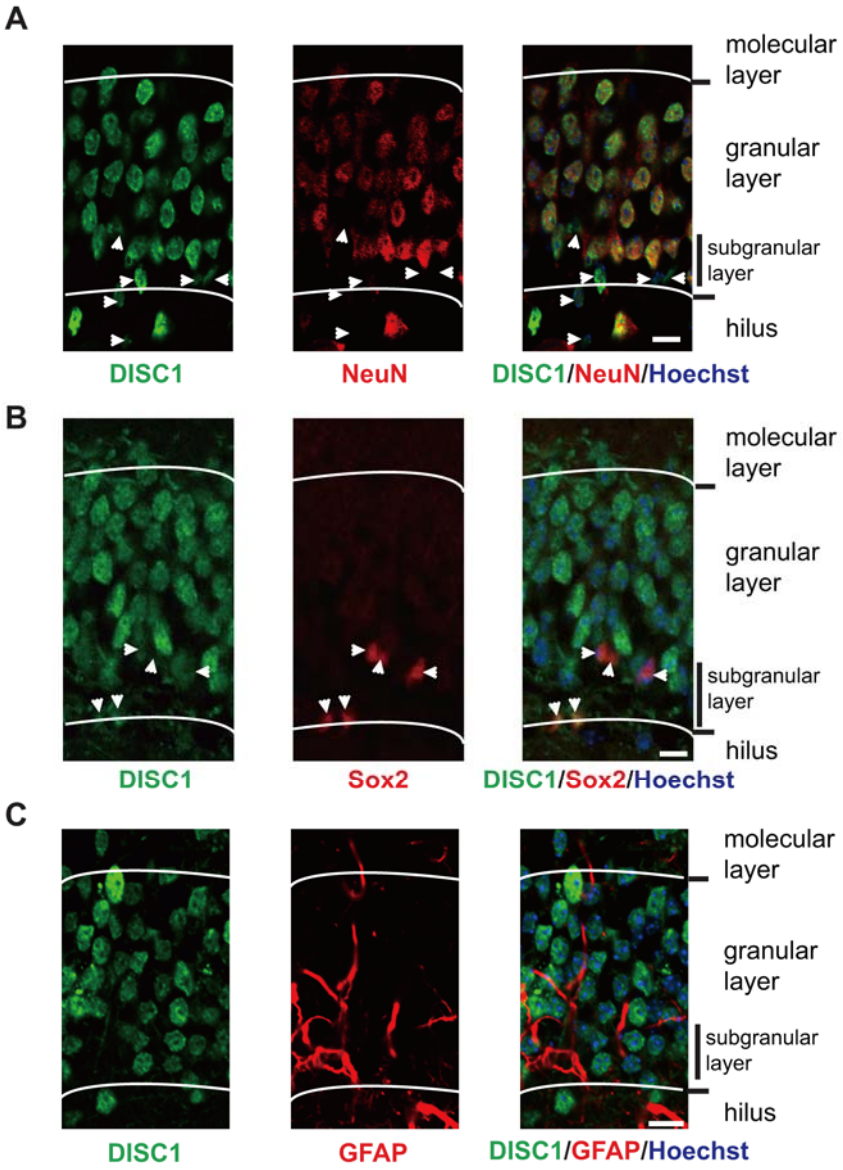

Figure S11. (A-C) DISC1 was co-stained with NeuN (A), Sox2 (B), or GFAP (C) in adult dentate gyrus. Arrows in (A) indicate cells that are DISC1 positive but NeuN negative. Arrows in (B) indicate cells that are DISC1 positive and Sox2 positive. Scale Bar= 10 μm.

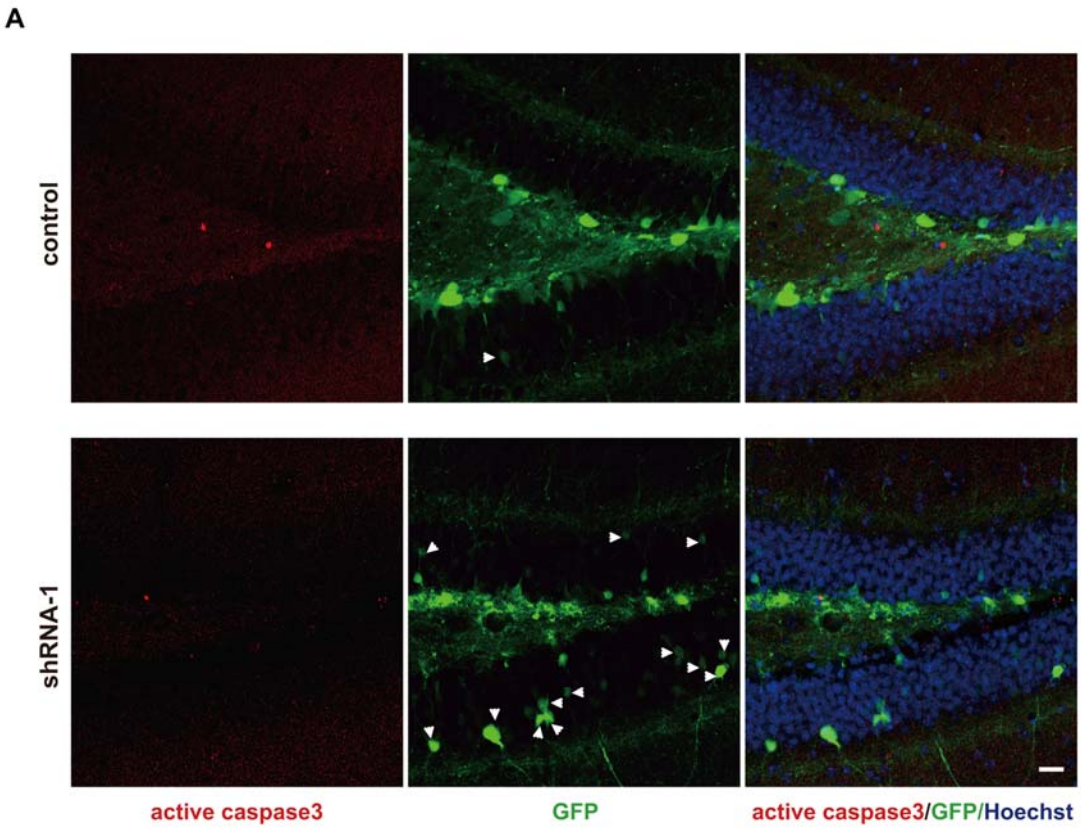

Figure S12. Apoptosis is not elevated by DISC1 silencing in the dentate gyrus. Control or DISC1 shRNA-1 lentivirus was injected into the adult dentate gyrus. Brain sections were stained with an anti-active caspase 3 antibody. Arrowheads indicate GFP positive cells distributed in the middle and outer granular layers. Scale Bar= 20 μm.

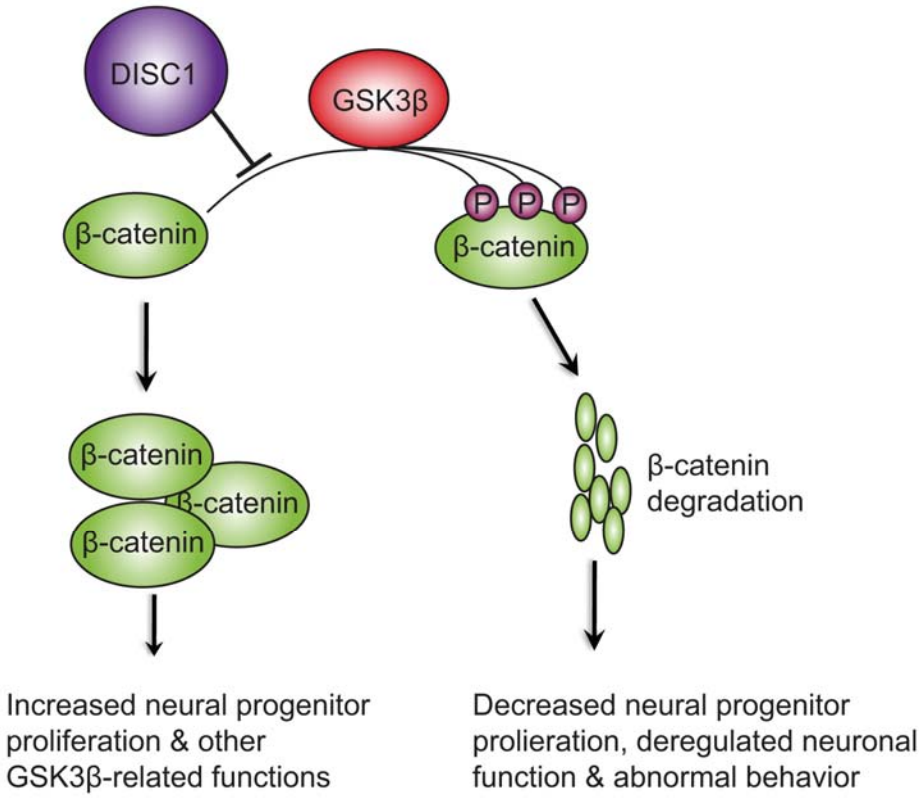

Figure S13. Model
Model depicting a role for DISC1 in regulation of neural progenitor proliferation. By regulating GSK3β activity, DISC1 modulates β-catenin stability and neural progenitor cell proliferation. DISC1 loss-of-function results in neural progenitor proliferation defects and abnormal behavior.

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