Supplementary Information

Full Methods

Generation of conditional knock-out of murine Pqbp1

To generate the targeting vector, three Pqbp1 genomic fragments were PCR amplified from a murine bacterial artificial chromosome (BAC) library (ID: RP23-404N15). A 3.6-kb 5’ fragment containing exons 1 and 2 was inserted upstream of a neomycin resistance cassette flanked by Flp recognition target (FRT) sites. A 3.9-kb fragment containing exons 3 to 7 was inserted between two LoxP sites and a 4.1-kb non-coding fragment was added 3’, with the diphtheria toxin A gene (DTA) to prevent random insertion. After electroporation into ES cells (C57BL/6), and G418 selection (Sigma - 200 mg/ml), clones were analysed by genomic DNA PCR using the following primers: fwd, 5’-AATCTTGGAGTTAGTAATGGTGCTT-3’, and rev, 5’-AATCTCATGTAATTGACGAGACAGAG-3’. Selected ES clones were corroborated by Southern blot analyses of genomic DNA digested with EcoRI or EcoRV. Probes for Southern blot analyses were prepared by PCR from the BAC clone using the following primers (locations given in Supplementary Fig 2): 5’ probe (408bp) fwd: AAAGTGAACCTGCATTAGAGGAAC rev: TCAGTGAGATACTCTGACTTCCACA; 3’ probe (462bp) fwd: GTCAATAAGCATTCAAGGACTCACT rev: TCAGAATACTCTTGGAACTCCCTTA. Chimeric mice were generated by injecting the recombinant ES cells into C57BL/6 blastocysts, subsequently crossed with C57BL/6 mice to generate the targeted allele. The neomycin resistance cassette was removed by crossing with CAG-FLPe recombinase transgenic mice (71). The resultant Pqbp1-floxed heterozygous female mice were further crossed with Nestin-Cre transgenic male mice (B6.Cg-Tg (Nes-cre) 1Kln/J; The Jackson Laboratory, Bar Harbor, ME) and Synapsin1-Cre transgenic male mice (B6.Cg-Tg (Syn1-Cre)671Jxm/J; The Jackson Laboratory, Bar Harbor, ME) to generate the Pqbp1 conditional knockout.

Immunohistochemistry of cKO mice

Mouse embryonic brains at E15 were fixed with 4% paraformaldehyde in 0.1 M PBS, cryoprotected in 30% sucrose, and embedded in optical cutting temperature (OCT) compound. Brain sections (10 μm) were cut by cryostat and stored at –80°C. For paraffin sections, brains were paraffin embedded and sectioned as 5μm thick slices.

Sections were subject to dual immunostains by overnight incubation at 4 °C with anti-PQBP1 goat antibody (1:500, C-20, Santa Cruz Biotechnology), anti-PQBP1 rabbit antibody (1:400, FL-265, Santa Cruz Biotechnology), anti-RC2 I mouse IgM antibody (1:200, Developmental Studies Hybridoma Bank, DSHB), anti-Sox2 rabbit antibody (1:200, Santa Cruz Biotechnology), anti-Tuj-1 mouse antibody (1:500, Sigma), mouse anti-MAP2 antibody (1:500, Sigma), anti-PAX6 mouse antibody (1:100, Developmental Studies Hybridoma Bank, DSHB), anti-Tbr2 rabbit antibody (1:500, Millipore), rabbit anti-Foxp1 antibody (1:200, abcam), anti-Cux1 rabbit antibody (1:200, abcam) or anti-GAD67 mouse antibody (1:50, Millipore). Secondary staining was with biotin anti-goat IgG (1:500, Molecular Probes) combined with tyramide signal amplification (TSA Biotin System, PerkinElmer Life and Analytical Sciences) and Alexafluor-488 or Cy3 labeled secondary antibodies (1:500, Invitrogen). Sections were counterstained with DAPI (0.2μg/ml in PBS) and mounted for microscopy (Olympus IX70, Tokyo Japan). Fluorescent images were acquired at step sizes of 0.3 μm using an Olympus FluoView800 confocal microscope (Olympus, Tokyo Japan).

Evaluation of thickness of cortical layers

Mouse brains prepared at E15 or 2 months were fixed with 4% paraformaldehyde in 0.1M PBS, and paraffin embedded. Sagittal sections were cut at 5 mm intervals from E15 mouse embryos. The plane 0.30 mm lateral from the midline was used for quantification of the cortical plate + intermediate zone (CP+IZ) or ventricular zone (VZ) thickness. Coronal sections were also at 5mm intervals from adult mice (2 months). The slice at -1.82 mm from the bregma was

used for quantification of layer thickness with anatomical structures used for further positional information.

Analysis of cleavage plane angle

The mitotic orientation and cleavage plane of neuroepithelial cells in the dorsal telencephalon was analyzed after double staining for Sox2 (with DAPI co-stain). Pqbp1-Floxed/Nestin-Cre conditional knock-out and Nestin-Cre control mice were used according to the method described previously (72). The cleavage plane was defined as the perpendicular axis to the mitotic orientation (73); its angle relative to the apical surface was calculated for each genotype.

Analysis of migration in PQBP1-cKO

For *in utero* electroporation, plasmids were prepared using the Endo-Free plasmid purification kit (Qiagen). *In utero* electroporation were performed as described previously (74) with minor modifications. In brief, pCX-EGFP (72), a generous gift from Dr. J. Miyazaki (Osaka University, Japan), was injected into the ventricular zone of Pqbp1-cKO or littermate controls (E14 embryos) with simultaneous electroporation. Forty-eight hours after electroporation, the brains of E16 embryos were fixed, cryoprotected and cryosectioned as previously described. Frozen sections were at 10 mm intervals.

Analysis of apoptosis in Pqbp1-cKO mice

ApopTag Red *In Situ* Apoptosis Detection (Chemicon) was used to detect apoptosis in tissue sections based on transferase dUTP nick end labeling (TUNEL) technology. Quantitative analyses were performed using 12-16 brain sections from six embryos for each test group.

Immunocytochemistry

NSPCs differentiated on polyethyleneimine- or poly-L-lysine-coated dishes (Corning) or undifferentiated NSPCs grown on pre-coated dishes in complete culture media were washed with 0.1 M phosphate buffer pH 7.4, and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4. The cells were incubated overnight at 4°C with rabbit anti-GFAP antiserum (1:1000, Sigma),

mouse anti-MAP2 antibody (1:400, Sigma) or rabbit polyclonal anti-cyclin B1 antibody (1:100, Santa Cruz Biotechnology) as indicated. Cells were counterstained with DAPI and staining visualized with AlexaFluor-488 or -568 conjugated secondary antibodies (1:500, Molecular Probes) using a fluorescent microscope (Olympus IX-FLA).

For the analysis of RNA splicing proteins, cells were incubated overnight at 4°C with mouse anti-sm antibody (1:200, NeoMarkers), mouse anti-hnRNP A1 antibody (1:500, Abcam), and mouse anti-U2AF^{65} antibody (1:200, Sigma), as indicated.

Plasmid construction

Construction of p*Apc4*-IRES2-EGFP is described in "*In vivo* rescue experiment of Apc4"

Two shRNA expression plasmids for murine Pqbp1 silencing, shRNA-ZsGreen and shRNA-DsRed, were derived from the RNAi-Ready pSIREN-RetroQ-ZsGreen and the RNAi-Ready pSIREN-DNR-DsRed-Express (Clontech) shRNA expression vectors, respectively. Double-strand DNA oligonucleotides encoding shRNAs against *Pqbp1* comprised a sense strand, a 9-nucleotide hairpin loop, the complementary anti-sense strand, a poly(A) termination signal and an Xho I site. Four sense-strands for shRNAs (shRNA 1 - 4) were designed using *Pqbp1* specific siRNAs (Qiagen): CAAGCCCTTGTTGTTTAATAA (siRNA1: SI01387099), AGCCGCAAAGATGAAGAATTA (siRNA2: SI01387106), CCCTTTAGTGTTGTTTGTAAA (siRNA3: SI01387113), and GAGGAAGAGATTATTGCTGAA (siRNA4: SI01387120). Oligonucleotides containing the sense and anti-sense sequences (shRNA1:5'-GGATCCGCAAGCCCTTGTTGTTTAATAATTCAAGAGATTATTAAA CAACAAGGGCTTGTTTTTTCTCGAGGAATTC-3'; shRNA2: 5'-GGATCCGAGCCGCAAAGATGAAGAATTATTCAAGAGATAATTCTTCATCTT TGCGGCTCTTTTTTCTCGAGGAATTC-3'; shRNA3: 5'-GGATCCG CCCTTTAGTGTTGTTTGTAAATTCAAGAGATTTACAAACAACACTAAAGGGCT

TTTTTCTCGAGGAATTC-3’; shRNA4: 5’-GGATCCGGAGGAAGAGATTATTGCTGAATTCAAGAGATTCAGCAATAATCT CTTCCTCCTTTTTTCTCGAGGAATTC-3’) were inserted as BamHI / EcoRI fragments into shRNA vectors. A control non-silencing shRNA was also synthesized; (5’-GGATCCGTGCGTTGCTAGTACCAACTTCAAGAGATTTTTTACGGCGTGAA TTC-3’) .

The *U5-15kD*-shRNA-ZsGreen plasmid was constructed as described above. Design of the shRNA sense-strand was guided by a murine *U5-15kD*-specific siRNA (Qiagen): TTGGAGTAGAATCATCAAGTA (SI00968002). The corresponding *U5-15kD*-shRNA sequence (5’-GGATCCGTTGGAGTAGAATCATCAAGTATTCAAGAGATACTTGATGATTCT ACTC CAACTTTTTTCTCGAGG-3’) was synthesized and subcloned as a BamHI / EcoRI insert into RNAi-Ready pSIREN-RetroQ-ZsGreen. The *U5-15kD*-EGFP expression vector was derived from pEGFP-N1 (Clontech).

The *Upf1*-shRNA-ZsGreen expression plasmid was constructed using a sense-strand shRNA sequence designed from the murine *Upf1*-specific siRNAs (Qiagen): CGCCAACGCAATGGAACTTAA (SI00228795), and CAGGATGAGTGTGGAGGCGTA (SI00228802). *Upf1*-shRNA (#1: 5’-GGATCCG CGCCAACGCAATGGAACTTAATTCAAGAGATTAAGTTCCATTGCGTTGGCCT CGAGG-3’ and #2: 5’-GGATCCGCAGGATGAGTGTGGAGGCGTATT CAAGAGATACGCCTCCACACTCATCCTG-3’) containing the sense and anti-sense *Upf1*-siRNA sequences were synthesized and subcloned as BamHI / EcoRI fragments into RNAi-Ready pSIREN-DNR-DsRed-express.

To construct pEGFP-α-tubulin, total RNA was extracted from murine NSPCs using TRIzol Reagent (Invitrogen) and reverse transcribed using AMV reverse transcriptase XL (TakaRa). Full-length murine α-tubulin (Genbank accession number NM_11653) cDNAs were obtained by PCR with fwd-5’-CCGCTGCAGTGATGCGTGAGTGCATCTCCA-3’ and rev-5’-CCGGGATCCGTTAGTATTCCTCTCCTTCTT-3. The PCR product was purified and subcloned as a PstI / BamHI fragment in pEGFP-C1 (Clontech).

Lentival vector construction

Lentiviral *Pqbp1* RNAi was delivered using the BLOCK-iT inducible H1 Lentiviral RNAi System (Invitrogen). The sense strand of the lentiviral *Pqbp1* shRNA was derived from the *Pqbp1* specific siRNA4 (GAGGAAGAGATTATTGCTGAA SI01387120, Qiagen) as described above. shRNA oligonucleotides were annealed and inserted into the H1 promoter-based shRNA vector (pENTER/H1/TO, Invitrogen). Gateway recombination cloning reactions using pENTER/H1/TO entry constructs and the promoterless pLenti4/BLOCK-iT-DEST destination vector (Invitrogen) were used to generate pLenti4/BLOCK-iT - *Pqbp1* shRNA4 and a non-silencing shRNA. Recombinant lentiviruses were generated in 293FT cells (Invitrogen) by co-transfection of ViraPower Packaging Mix (Invitrogen) with titration performed on Blasticidin-selection plates using crystal violet staining; MOI for the following experiments in NSPCs was 10. The lentiviral vector was used to confirm inhibition of NSPC proliferation by *Pqbp1*-KD (data not shown).

NSPC and P19 cell culture and transfection

NSPCs were isolated from Pqbp1-cKO and littermate Nestin-Cre transgenic mice embryos (E14) as described previously (75). Embryos were washed with ice-cold phosphate-buffered saline. The cerebral cortex was dissected with scalpel blades in a dish containing ice-cold DMEM/F12 medium (GIBCO). The cerebral cortex was cut into 1~2 mm^3 cubes and digested at 37 °C with 0.0625% trypsin (GIBCO) and 100 μg/ml DNAse (Roche) to dissociate cells. Tissue was treated with 350 μg/ml Ovomucoid (Sigma) to inhibit trypsin activity and passed through a 70 μm cell strainer (BD). NSPCs were cultured in complete culture media comprising DMEM/F12 medium (GIBCO), basic fibroblast growth factor (bFGF; 20 ng/ml, Promega), epidermal growth factor (EGF; 20 ng/ml, Promega) and B27 supplement (GIBCO). Cells were plated at a clonal density of 1.33 cells/μl (2000 cells per 35mm well) in 6 well plates (corning). A sphere derived from a single NSPC was picked as a clone, and passaged every 3-4 days. Cells at passage 3-5 were used to analyze cell growth and cell cycle. Expression of neural stem cell markers was confirmed by immunocytochemistry and a

Nestin+/Sox2+/Pax6+/Tbr2- status confirmed for all clones used. Four clones/genotype were used for cell growth assays. NSPCs were dissociated as described above and 10^6 cells (per 100 mm dish) were transfected with the indicated plasmids using Effectene Transfection Reagent (Qiagen). To differentiate NSPCs, they were dispersed mechanically into fresh DMEM/F12 medium (GIBCO) with B27 supplement (GIBCO) and plated on dishes pre-coated with 0.05% polyethyleneimine (Sigma) in 0.15 M borate buffer (pH 8.4) or 10 μg/ml poly-L-lysine (Sigma). P19 cells were cultured in alpha-modified Eagle's medium (α-MEM) supplemented with 10% (v/v) fetal bovine serum (FBS, GIBCO) and 50 μg/ml kanamycin (MP Biomedicals, Inc.). Cells were maintained at 37°C in 5% CO_2. Transfection of shRNAs was performed using Lipofectamine 2000 (Invitrogen).

Cell proliferation assays

Cells were transfected or infected as described, and collected at defined time-points. Viable cell numbers were determined by 0.4% Trypan Blue dye exclusion. Growth curves were expressed as the average ± SD of viable cell number from at least three separate experiments. Statistical analyses were performed with ANOVA or Student's *t* test as indicated.

***In vitro* rescue experiments**

Full-length murine *Apc4* cDNA (Genbank accession number NM_024213) was cloned by RT-PCR using fwd (5'-GGGGGATCCCCCATGCTGCGCTTTCCGACC-3') and rev (5'-GGGGCGGCCGCAGTTTCTCCACTTTGATGACTACGTCCGGG-3').

Subcloning was as a BamHI / NotI fragment into pIRES-hrGFPII (Stratagene) to generate *pApc4*-IRES-hrGFPII. For the Apc rescue experiments, p*Apc4*-IRES-hrGFPII was co-transfected with PQBP1-shRNA4-DsRed or non-silencing shRNA-DsRed with pIRES-hrGFP II as a negative control. A FLAG tag was included at the 3'-terminus of the insert, upstream of the IRES in pIRES-hrGFP II.

For the PQBP1 rescue experiments, pEGFP-huPQBP1 containing full-length human PQBP1 was co-transfected with PQBP1-shRNA4-DsRed or

non-silencing shRNA-DsRed. pEGFP-N1 (Clontech) was used as a negative control.

Western blot analyses

Cells were lysed in 62.5 mM Tris-HCl (pH 6.8), 2% (w/v) sodium dodecyl sulfate (SDS), 2.5% (v/v) 2-mercaptoethanol, 5% (v/v) glycerin, and 0.0025% (w/v) bromophenol blue, and denatured at 100ºC for 5 min. Samples were resolved by 12% SDS-PAGE gels, transferred onto PVDF membranes (Millipore) and incubated with goat polyclonal anti-PQBP1 primary antibody (1:1000, C-20, Santa Cruz Biotechnology) overnight at 4ºC. Horseradish peroxidase (HRP)-conjugated anti-goat IgG antibody (1:3000, Dako) was added for 1 h at room temperature and antibody binding was visualized using the ECL-PLUS Western Blotting Detection System (Amersham Biosciences). Anti-U5-15kD (1μg/ml, Abnova), anti-Flag (1:3000, Sigma), anti-hrGFP (1:2000, Stratagene), anti-cyclin B1 (1:100, Santa Cruz Biotechnology), and anti-APC4 (1:200, Santa Cruz Biotechnology) antibodies were used.

FACS analyses

Third passage neurospheres (48h after plating) from Pqbp1-cKO mice embryo and littermate Nestin-cre transgenics were used for FACS. Cells were harvested and fixed with 70% ethanol in PBS at -20°C overnight. Cells were washed and labeled with propidium iodide (Sigma) at 40 μg/ml for 30 min at 37°C. For each analysis, 10,000 gated cells were collected by FACSCalibur system and analyzed with CELLQuest (Becton Dickinson).

For intracellular staining of M-phase cyclins, fixed NSPCs were washed and incubated with a rabbit polyclonal anti-cyclin A or B1 antibody (1:100, Santa Cruz Biotechnology) in PBS containing 0.3% Saponin (Sigma) and 0.1% BSA (Sigma) overnight at 4°C. Cells were washed with 0.1% Saponin in PBS and then incubated with a phycoerythrin (PE)-conjugated anti-rabbit IgG antibody (1:200, Santa Cruz Biotechnology) at room temperature for 30 min. In parallel, normal rabbit IgG (1:200, Santa Cruz Biotechnology) was used as a negative control.

Cumulative labeling

The method for analyzing cell cycle parameters in the neuroepithelium (45) was employed with minor modifications. BrdU (Sigma; 100 mg/kg of body weight) was injected intraperitoneally into pregnant mice at E14. Cumulative labeling was performed by repeated injections (at 3-hour intervals) into pregnant mice which were sacrificed 1, 1.5, 2, 3.5, 6.5, 15.5 and 24.5 hours after the first BrdU injection. Embryonic brains were fixed with 4% paraformaldehyde and paraffin embedded. Sections were made at 3 mm intervals, deparaffinized, rehydrated and then microwaved in 10 mM of citrate buffer, pH 6.0, for 15 min. Antibody incubations were with the mouse anti-BrdU antibody (1:200, BD Biosciences) and rabbit anti phospho-histone H3 (pH3) antibody, a marker for M-phase cells, (1:500, Millipore) at 4˚C overnight. Secondary antibody incubations were with Alexafluor-488 or Cy3 conjugates (1:500, Invitrogen). The ratio of BrdU/pH3-double positive cells to pH3-positive cells in the ventricular zone was calculated at 1, 1.5 and 2 hours after a single injection of BrdU to determine the length of the G2/M phase. A straight line graph of the labeling index values (LIs) at 1, 1.5, 2, 3.5 and 6.5 hours allowed us to extrapolate to a y-axis intercept (the LI at 0 hour) and calculate the slope. Since the growth fraction (the ratio of proliferating cells) is nearly 1.0 in the ventricular zone of wild mice, the LI at 0 hour and slope represent the ratio of S-phase to total cell cycle (Ts/Tc) and the reciprocal of total cell cycle (1/Tc), respectively. Ts and Tc denote the length of the S-phase and total cell cycle, respectively. From these values (Ts/Tc and 1/Tc), Ts and Tc were calculated.

Confocal microscopy of PQBP1

293 or Hela cells were maintained at 37°C in 5% CO_2 in DMEM (Sigma) supplemented with 10% FBS. Transfection of EGFP-PQBP1 or EGFP-U5-15kD was performed using Lipofectamine 2000 (Invitrogen). After 2 days of transfection, cells were fixed with 4% with PFA, incubated overnight at 4 °C with rabbit anti-sc-35 antibody (1:500, Sigma) or anti-PQBP1 antibody (1:400, FL-265, Santa Cruz Biotechnology). Fluorescent images were acquired using an LSM510 Meta confocal microscope (Zeiss).

In utero electroporation of Pqbp1-KD

For *in utero* electroporation, plasmids were prepared using the Endo-Free plasmid purification kit (Qiagen). The method described previously (74) was used to estimate cell cycle length of *Pqbp1*-shRNA-electroporated cells. ZsGreen-shRNA-*Pqbp1* or ZsGreen-shRNA-ns were electroporated into embryonic cerebral cortices at E14. Twenty-four hours later, pregnant mice were intraperitoneally injected with IdU (Sigma; 50 mg/g of body weight). BrdU injection (Sigma; 50 mg/g of body weight) was performed 3 hours after the IdU injection; 5 hours after the IdU injection, the electroporated brains were fixed with 4% paraformaldehyde. Frozen sections were incubated with 2N HCl for 30 min at 37˚C. Anti-EGFP rabbit polyclonal antibody (MBL) was added for 2 hours at room temperature. A FITC-conjugated anti-rabbit secondary antibody was then added. Subsequently, sections were incubated with an anti-IdU mouse antibody (which recognizes both IdU and BrdU; BD Biosciences) and an anti-BrdU rat monoclonal antibody (Abcam) at 4˚C overnight. A Cy5-conjugated anti-mouse, and TRITC-conjugated anti-rat, antibody were used as secondary antibodies.

Single positive BrdU, or IdU/BrdU-double staining in GFP-expressing cells was quantified in the ventricular zone of each section. The ratio of IdU/BrdU/GFP-triple positive cells to GFP-positive cells in the ventricular zone represents electroporated cells in S-phase during the 5 hours between IdU injection and fixation. This ratio is designated the LI at 5 hours. Similarly, the ratio of BrdU/GFP-double positive cells to GFP-positive cells in the ventricular zone shows the LI at 2 hours. From the plots of the LIs at 2 and 5 hours, a y-axis intercept (the LI at 0 hour) and slope of the line were calculated. Ts and Tc were calculated as described in "*Cumulative labeling*".

Total RNA preparation and RT-PCR

Fluorescent NSPCs transfected with shRNA-ZsGreen were harvested 72 hours after transfection using the FACS-Vantage Flow Cytometer (Becton Dickinson). Total RNA was isolated immediately after cell sorting using TRIzol Reagent (Invitrogen) and treated with amplification grade DNase I (Invitrogen).

RT-PCR was with the RNA LA PCR Kit (TaKaRa). Total RNA was transcribed into first-strand cDNA at 42 °C for 30 min using an oligo dT-adaptor primer, followed by heat-inactivation at 99 °C for 5 min. The PCR amplification of anaphase promoting complex subunit 4 (*Apc4*) fragments was performed for 35 cycles (30 sec at 94°C, 30 sec at 44~60°C as indicated, and 30 sec at 72°C) using the following primer sets that span the indicated intron(s): Intron 1-Fwd (F1): GGTGGTGGGAGAGAAGC, Intron 1-Rev (R1): CTTGCAAGGCGATGCAGC, annealing temperature (Ta), 60°C; Introns 4-5-Fwd (F4-5): GCTGAGGATGAGTCC, Introns 4-5-Rev (R4-5): GACATCTCCCAAGAGC, Ta, 55 °C; Intron 7-Fwd (F7): CCTTGGAGGAAGCTCTG, Intron 7-Rev (R7): GGAAACCTCTGTGACCAC, Ta, 55°C; Intron 12-Fwd (F12): GCTGAACTTCAGACTC, Intron 12-Rev (R12): GATGACTCTATAGACTGGC, Ta, 50°C; Intron 14-Fwd (F14): GGCTCTGAGTCTTTACTG, Intron 14-Rev (R14): GAGGATGAAAGAACCCAC, Ta, 50°C; Intron 16-Fwd (F16): CGGTGGCTGTATGTAG, Intron 16-Rev (R16): GGAAGCACATGGTCC, Ta, 55°C; Intron 21-Fwd (F21): GGATGGAGAACGTTATTGATC, Intron 21-Rev (R21): GCTTGATTCATCGATCTTCC, Ta, 60°C; Intron 23-Fwd (F23): GGACTGTGCACGAAG, Intron23-Rev (R23): CTCCTTAAGATGCAC, Ta, 44°C; Intron 25-Fwd (F25): GCTGACAAGGTCAGAAG, Intron 25-Rev (R25): GAATCTTCACTGTTATACAC, Ta, 50°C.

RT-PCR of indicated control gene fragments was carried out using the following primer sets: *GAPDH*-Intron 6-Fwd: GCCAAGTATGATGAC, *GAPDH*-Intron 6-Rev: CCTGTTGCTGTAGCCG, Ta, 50 °C; *Ccna1*-Intron3-Fwd: GACAGAGATACCTGCTCGG, *Ccna1*-Intron 3-Rev: CTCGAAGGTAGCGATGAATC, Ta, 60°C; *Ccnb1*-Intron 1-Fwd: GCTGAGACCGAGAACTGCTC, *Ccnb1*-Intron1-Rev: CAGGTTCAGGCTCAGGC, Ta, 60°C; *β-actin* primer set (R&D Systems), Ta, 55°C; and 18S rRNA-Fwd: GCCATGCATGCTTAAGTACG, 18S rRNA-Rev: CCTCCAATGGATCCTCG, Ta, 60°C.

For discrimination of murine and human PQBP1 by RT-PCR, a common fwd

primer: was used: GGACTCCCTTACTATTGGAAT, with a mouse-specific reverse primer: GGTCTGCCTTGTCAAATG (product size: 322bp) or a human specific reverse primer: TCAGACTTGTCGTGGCCC (product size: 236bp), with a Ta of 55°C. For detection of mouse *Apc1* and *Apc6*, primers spanning *Apc1* exons 3 and 4: AATTTTCTTAGACAGA and GAGTTTACCTGAAAA (product size: 196bp) and exons 2 and 3: AGTGCTCTGTTTTGGG and GCTTGCGTGACCGGAG (133bp) were used.

Southern blot analysis of RT-PCR products

For nested-PCR amplification, the first PCR reaction were performed using the following primer sets that cover the indicated intron(s): Intron 21-Fwd (F21): GGATGGAGAACGTTATTGATC, Intron 23-Rev (R23): CTCCTTAAGATGCAC, Ta, 52°C; Intron 21-Fwd (F21): GGATGGAGAACGTTATTGATC, Intron-22-Rev (R22): CTTCGTGCACAGTCC, Ta, 55°C; Intron 12-Fwd (F12): GCTGAACTTCAGACTC, Intron 12-Rev (R12): GATGACTCTATAGACTGGC, Ta, 50°C; Intron 1-Fwd' (F1'): GCCTCAGTTTCCCCTTTGGC, Intron 1-Rev' (R1'): CCTCGGAACCAAGCATCCTC, Ta, 60°C. The second PCR reaction was performed using the following primer sets: Fwd22: CGATGAATCAAGC and Rev22: CTTCGTGCACAGTCC, 64 bp from mRNA, 144 bp from genomic DNA; Fwd21: GGATGGAGAACGTTATTGATC, Rev22: CTTCGTGCACAGTCC, 121 bp from mRNA, 201 bp from genomic DNA; Fwd12: GCTGAACTTCAGACTC, Rev12: GATGACTCTATAGACTGGC, 77 bp from mRNA, 195 bp from genomic DNA; Fwd1': GCCTCAGTTTCCCCTTTGGC, Rev1': CCTCGGAACCAAGCATCCTC, 0 bp from mRNA, 325 bp from genomic DNA. To confirm that the PCR products were derived from mRNA, the template RNAs were pre-treated with RNase A (Sigma, 1 mg/ml) at 37°C for 1 h. The amplified products were resolved by 12~15% polyacrylamide gel electrophoresis and stained with ethidium bromide.

Southern blot analyses were performed using the following APC4 intron sequences as probes: probe 1 (for intron 1): CATCTCTCCTGCCCCTACATCTCAGCATGGCTGTTAACTTTAAGAGACTGTG TAAAATACTGTGCCTTCCAGACAGGATGTGTATTC; probe 12 (for intron 12:

TAAGTGGCTGTGAACACATTTTTAATAGTGGTCTTTTTTGGGAATGACATATA GTATCATAGTTACATGCTAAATGTATT; probe 22 (for intron 22): GTAATTCTCTTTACCCATTGTAAGCTATAAGTTTAACAATGATTATAAATTTTTC TCATTTTGTTTTCTTGTTGTTTTAG. Secondary PCR products were electrophoresed in 12% polyacrylamide gels and transferred to Hybond-N membranes (Amersham Biosciences). Probes were labeled using the DNA 5'end-labeling Kit MEGALABEL (Takara), hybridized overnight at 65 °C, and washed twice at 68°C for 60 min.

In-vitro splicing assay

In-vitro splicing was performed as described previously (76). Briefly, HeLa nuclear extract was preincubated with anti-PQBP1 serum (100 μl) or control serum (100 μl) in 1 x splicing buffer (0.5 mM adenosine 5'-triphosphate(ATP), 20 mM creatine phosphate, 1.6 mM MgCl2) with 20 mM Hepes (pH7.9), 60 mM KCl, 0.2 mM EDTA at 30°C for 20 min. After preincubation, 32P-labeled pre-mRNA (20 fmol, chicken δ-crystallin or murine Apc4) was added for splicing at 30°C for 45 min followed by phenol/chloroform extraction and ethanol precipitation. RNA samples were resolved on a 6 % denaturing polyacrylamide gel. PQBP1 C-terminus and GST-PQBP1 antisera were generated as described previously (51). PQBP1 N-terminus antiserum was similarly obtained following immunization of rabbits with peptide C-MPLPVALQTRLAKRG (51). The N- and C-terminal sequences are conserved between the murine and human proteins. YAP and HDGF antisera were described previously (77, 78).

Preparation of pre-mRNA of murine *Apc4* exon 27-28

The murine (B6) *Apc* genomic region containing exons 27 and 28 was PCR amplified using fwd: 5'-CATGGATCCGCACTTTGAGAAACACTGG-3', and rev: 5' -CATGAATTCGGTCACGAGTCCAACTCAGG-3'. The PCR product was subcloned into BamH I/ EcoR I cut pcDNA3. P32-labeled pre-mRNA was in vitro transcribed using the linearized plasmid as a template with T7 polymerase. Labeled pre-mRNA was gel-purified and used for experiments.

Immunoaffinity purification of U5 snRNPs

Immunoaffinity purification of U5 snRNP was performed according to the method described previously (59) with minor modifications. As the spliceosome is formed after 10 minutes (59), we stopped the reaction with heparin added to a final concentration of 0.5mg/ml, incubated for an additional 10mins at 30°C. Subsequent procedures were performed at 4°C. The splicing reaction was diluted 10-fold with IP buffer (20mM HEPES, pH 7.9, 150mM NaCl, 1.5mM $MgCl_2$, 0.5mM DTT) containing 0.05% NP-40 and incubated for 1h with 1ml of protein-A sepharose preblocked with 0.5mg/ml of BSA and 50μg/ml of yeast tRNA. The reaction was then incubated with anti-EFTUD2/U5-116kD antibody (Abcam) at 2μg/ml and precipitated with protein-A sepharose. Precipitates were blotted using anti-PQBP1 or anti-U5-15kD antibody by the method described above.

***In vivo* rescue experiment of Apc4**

In utero electroporation and determination of Pial-to-Apical surface area experiments were performed as described previously (16). Full-length murine *Apc4* cDNA (Genbank accession number NM_024213) was obtained by RT-PCR using fwd (5'-GGGCTCGAGACCATGGGAATGCTGCGCTTTCCGACCTGTTT-3') and rev (5'-GGCGGATCCCTATTATTTGTCGTCATCATCC-3') primers. The product was inserted into XhoI / BamHI cut pIRES2-EGFP (Clontech) to generate p*Apc4*-IRES2-EGFP. For the Apc4 rescue experiments, p*APC4*-IRES2-EGFP or pIRES2-EGFP were electroporated into the ventricular zone of E13 embryos. Brain tissues of E18 embryos were fixed, embedded in 3% agarose, and sectioned on the rostral-to-caudal axis as 50-μm-thick sections using a vibratome. Brain sections were incubated with mouse anti-GFP monoclonal antibody (1:500, Millipore) at 4 °C overnight, followed by treatment with Alexafluor-488 labeled secondary antibodies. Sections were analyzed with a confocal fluorescence microscope (Olympus FV10i) and the ratio of pial-to-apical surface determined as described previously (17).

Flag-tagged wild-type PQBP1 construct for human PQBP1 experiments

Human PQBP1 (GenBank accession no. NM_005710) derived from PQBP1-c-myc (2) was subcloned as an *Eco*RI / *Sal*I insert (in frame with FLAG tag) into pCMVTag2A (Stratagene).

Cell culture and transfection experiments

HEK-293T cells were maintained in DMEM medium supplemented with 10% fetal calf serum, L-glutamine and penicillin/streptomycin. $1x10^6$ cells were grown overnight in 75 cm^2 flasks prior to transfection with 5 μg of FLAG-*PQBP1* and 15 μl lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. $1x10^5$ cells were grown overnight per well in 6-well plates prior to transfection with 1.3 μg of synthetic small interfering RNA (siRNA) per well using oligofectamine (Invitrogen). After 48 or 72 hours cells were harvested and RNA and proteins isolated. HEK-293T cells were treated with 50 μg/ml of cycloheximide or DMSO for a total of 4 hours before harvesting the cells. The following siRNA duplexes were used (sense sequence shown): si-*PQBP1* 5'-CCAACTCCGTGGTTACCAA-3' and control non-silencing siRNA 5'GGCCTTTCACTACTCCTAC-3'.

Protein extraction and Western blot

HEK-293T cells were lysed in buffer containing 48% urea, 15 mM Tris pH 7.5, 8,7% glycerol, 1% SDS, 0,004% bromophenol blue, and 143 mM β-mercaptoethanol. Proteins were resolved by 12% SDS PAGE, transferred to PVDF membranes (Roche), blocked with 5% non-fat dry milk in Tris-buffered saline (TBS: 25 mM Tris-HCl, pH 7.4, 150 mM NaCl), then incubated with antibodies overnight at 4°C.

Immunoprecipitation

HEK-293T cells were resuspended in HNM buffer (20mM Hepes, 50mM NaCl, 5mM $MgCl_2$) supplemented with Complete Protease Inhibitor Cocktail (Roche) and 100U/ml RiboLock RNase inhibitors (Fermentas) and lysed using a sonicator (Bandelin). Cell debris was removed by centrifugation at 12,000g for 20min. After preclearing the lysate with 50μl protein G-agarose, supernatants were incubated with 20μl of FLAG-agarose beads or 20μl of mIgG-agarose

beads (as a negative control) with rocking for 1 hour at 4°C. Afterwards, beads were washed three times with HNM. 1/10 of the bead volume was resuspended in 1x SDS PAGE buffer (6% SDS, 25mM Tris-HCl pH 6.5, 50mM DTT, 10% glycerol and bromophenol blue), boiled for 5 min and centrifuged at 10,000*g* for 1 min. Supernatants were separated by SDS-PAGE, followed by Western blot analysis. The remainder was treated with proteinase K for 30 min at 55°C, followed by phenol/chloroform extraction, and RNA precipitation.

Antibodies

The following antibodies were used: anti-N1-PQBP1 (79) (polyclonal, rabbit WB: 1:1000), anti-FLAG M2 affinity gel (Sigma, monoclonal, mouse), anti mouse IgG agarose (Sigma), anti-FLAG M2-HRP (Sigma, monoclonal, WB: 1:10000); anti-tubulin (Abcam, polyclonal, rat, WB: 1:10000).

RNA extraction and RT-PCR experiments

Total RNA was extracted from HEK-293T cells using RNeasy Plus mini (Qiagen) according to the manufacturer's recommendations. One μg RNA was used for reverse transcription with 10 U of Superscript III (Invitrogen), and 150 μM random hexamers in the presence of RNAguard (Amersham Pharmacia). Subsequently, cDNA quality was assessed using the primer set TGGCGTCGTGATTAGTGATG and TATCCAACACTTCGTGGGT for *HPRT*, which was also used for normalization of *APC4* expression. RT-PCR of *APC4* were carried out using BIO-X-ACT Long Mix (Bioline) and primers F21_h22 5'-GGATGGAGAATATTATTGATCAGT-3', located in human exon 22 with Rtrin22_h23 5'-GGCTGCGGAATTACACCATC-3', located in human intron 23. Following initial denaturation at 95°C for 5 min, cycling conditions were: 40 cycles at 95°C, 58°C and 72°C. Each step was for 30s. Final elongation was at 72°C for 10 min. RNAs precipitated from RNA-protein immunoprecipitations were used for reverse transcription with 10 U of Superscript III (Invitrogen) and 150 μM random hexamers in the presence of RNAguard (Amersham Pharmacia). For *APC4* amplification the following primers were used: F21_h22 with R23_h25 5'-GTATGTCTCCTTAAGATGCAC-3', located in human exon 25. Following initial denaturation at 95°C for 5 min, cycling conditions were: 40 cycles at 95°C for

30s, 58°C for 30 s and 72°C for 45 s. Final elongation was at 72°C for 10 min. Following PCR, products were resolved on 1% or 3% agarose gels, and band intensities analysed using the ImageQuant program. For all primer combinations, representative bands were selected, gel fragments eluted and sequenced.

Real-time RT-PCR

Total RNA was extracted from HEK-293T cells using RNeasy Plus mini (Qiagen) according to the manufacturer's recommendations. cDNA was synthesized using the TaqMan reverse transcription kit (Applied Biosystems) with real time PCR carried out using SYBRGreen PCR master mix (Applied Biosystems). PCR was with an ABI 7900HT cycler under the following conditions: 50°C for 2 min; 95°C for 10 min; 95°C for 15 s, 60°C for 1 min for 40 cycles; and 95°C for 15 min, 60°C for 15 min, 95°C for 15 min for the dissociation stage. Amplification of the reference gene *GPBP1* was with *GPBP1*_fwd 5'-TCACTTGAGGCAGAACAC-3' and *GPBP1*_rev 5'-AGCACTGTTTCATCATT-3'; F21_h22 and R23_h25, was used for *APC4* amplification.

Expression profiling

Total RNA from NSPCs of Pqbp1-cKO mice and their littermates were prepared using RNeasy (QIAGEN). SurePrint G3 Mouse Gene Expression 8x60K (agilent) was used for microarray analysis. Briefly, Cyanine-3 (Cy3) labeled cRNA was prepared from 0.1 μg Total RNA using the Low input Quick Amp Labeling Kit (Agilent) according to the manufacturer's instructions, followed by RNeasy column purification (QIAGEN, Valencia, CA). Dye incorporation and cRNA yield were checked with the NanoDrop ND-1000 Spectrophotometer. 0.6 μg of Cy3-labelled cRNA was fragmented at 60°C for 30 minutes in a reaction volume of 55 μl containing 1x Agilent fragmentation buffer and 2x Agilent blocking agent following the manufacturer's instructions. On completion of the fragmentation reaction, 55 μl of 2x Agilent hybridization buffer was added to the fragmentation mixture and hybridized to Agilent SurePrint G3 Human (Mouse) GE 8x60K for 17 hours at 65°C in a rotating Agilent hybridization oven. After hybridization, microarrays were washed 1 minute at room temperature with GE Wash Buffer 1 (Agilent) and 1 minute with 37°C GE Wash buffer 2 (Agilent), then dried

immediately by brief centrifugation.Slides were scanned immediately after washing on the Agilent DNA Microarray Scanner (G2565CA) using one color scan setting for 8x60k array slides (Scan Area 61x21.6 mm, Scan resolution 3um, Dye channel is set to Green PMT is set to 100%).The scanned images were analyzed with Feature Extraction Software 10.10.1.1 (Agilent) using default parameters to obtain background subtracted and spatially detrended Processed Signal intensities.

GSEA

We used Gene Set Enrichment Analysis (46) to test which gene sets are significantly repressed, i.e., possibly dysfunction, in NSPCs of Pqbp1-cKO mice. For GSEA, since three samples are available for both Pqbp1-cKO and control mice, we set the order of genes by p-values computed from the one-side t-test and the fold-change between the mean values of Pqbp1-cKO and control samples. We also used the fold-changes between all possible pairs of Pqbp1-cKO and control samples to consider the variation of each sample; this achieved nine comparisons. False Discovery Rate (FDR) q value less than 10% was judged significant following previous research (46).

PPI analysis

A molecular network was obtained by using relationships compiling in STRING 9 database (http://string-db.org/). A sub-network was then extracted so that it contains genes within two paths from Pqbp1 and belonging to the seven significantly repressed gene sets, “G2/M transition”, “APCDC20 mediated degradation of cyclin B”, “Conversion from APC CDC20 to CDH1 in late anaphase”, “Phosphorylation of the APC”, “CDC20phospho APC mediated degradation of cyclin A”, “G2/M checkpoints” and “Mitotic prometaphase”. A path in STRING includes the following 7 types: Neighborhood, Fusion, Occurrence, Coexpression, Experiments, Database, Textmining. From the obtained sub-network, we excluded genes whose log fold-change is less than or equal to -0.2. Together with the gene exclusion, we deleted the edges connecting to the excluded genes. Finally, we obtained the molecular network using Cytoscape (http://www.cytoscape.org/). We divided the genes in the final network into three

groups as follows: genes belonging to S phase in cell cycle, these are categorized into “S group”, genes having the functions of protein degradation and cell maintenance are in “U group” and genes relating to G2/M phase, splicing, transcription, centrosome and cytoskeleton are in “G2/M group”.

Two photon microscopy

Two-photon imaging of dendritic spines was performed using a laser-scanning microscope system FV1000MPE2 (Olympus, Japan) equipped with an upright microscope (BX61WI, Olympus, Japan), a water-immersion objective lens (XLPlanN25xW; numerical aperture, 1.05), and a pulsed laser (MaiTai HP DeepSee, Spectra Physics, USA). EGFP was excited at 890 nm and scanned at 500–550 nm. The scanning area used for three-dimensional imaging was 100 × 100 μm (1 μm Z steps, 1,024 × 1,024 pixels). Two weeks before imaging, adeno-associated virus 1 (AAV1)-EGFP with the synapsin I promoter (titer 1 × 10^{10} vector genomes/mL, 1 μL) was injected into the retrosplenial cortex (anteroposterior, –2.0 mm and mediolateral, 0.6 mm from bregma; depth, 1 mm) of mice under anesthesia with 2.5% isoflurane. Dendrites and cell bodies were imaged through a thinned-skull window 36 h after infection, as described previously (80). Reconstruction of images and quantitative analyses of dendrite length and total cell volume were performed by IMARIS 7.2.2 (Bitplane, Switzerland).

Gene therapy with Pqbp1-AAV vector

The AAV vector plasmids contained an expression cassette, consisting of a human cytomegalovirus immediate-early promoter (CMV promoter); followed by cDNA encoding human PQBP1 or human PQBP1-EGFP; and a simian virus 40 polyadenylation signal sequence (SV40 poly (A)) between the inverted terminal repeats of AAV 3 genome. The recombinant AAV vectors were produced by transient transfection of HEK293 cells using the vector plasmid, an AAV2 *rep* and AAV1 *vp* expression plasmid, and an adenoviral helper plasmid, pHelper (Agilent Technologies). The recombinant viruses were purified by isolation form 2 sequential CsCl gradients, and the viral titers were determined by qRT-PCR. For *in vivo* administration of AAV vectors, C57BL/6J pregnant mice (E10) were

injected with AAV-PQBP1 vector (2.0×10^{11} genome copies) by intraperitoneal administration.

Mouse behavioral tests

Open-field test

Mice were placed in the center of an open-field space (50 cm × 50 cm × 40 cm [H]) and allowed to explore for 15 min. Light intensity was 70 lux in the center of the field. Distance traveled (cm) and % duration of staying at the center area of the field (36% of the field) were adopted as the indices.

Light–dark exploration test

Light box was made of white plastic (20 cm × 20 cm × 20 cm [H]) and illuminated by LEDs (250 lux at the center of the box); a CCD camera was equipped on the ceiling. Dark box was made of black plastic (20 cm × 20 cm × 20 cm [H]) and an infrared camera was equipped on the ceiling. There was a tunnel for transition on the center panel between the light box and dark box (3 cm × 5 cm) with a sliding door. In the L–D box test, mice were individually introduced into the light box, and the door of the tunnel opened 3 s after the introduction of a mouse. Then mouse was allowed to move freely in the L–D box for 10 min. Total distance traveled, distance traveled in the light box, duration staying in the light box, numbers of the transition between light and dark box and the first latency to enter the dark box were measured as indices.

Elevated plus maze test

The elevated plus maze consisted of four 5 × 25 cm orthogonal arms connected by a 5 × 5 cm central square. The floor of each arm was made of gray plastic and the wall of closed arms (15 cm) and ridge of open arms (0.3 cm) were made of clear plastic. Closed arms and open arms were set 60 cm above the floor. Light intensity was 70 lux at the central square of the maze. In the elevated plus maze test, mice were individually put on the central square facing to an open arm, and then mice were allowed to move freely in the maze for 10 min. To test for memory retention, mice were placed back on the same maze one day after the first trial. Duration staying in the open and closed arms, duration staying in the central square, times of entry into the open or closed arm, and total distance of traveling were measured as indices.

Rotarod test

Mice were placed on a rotating rod (diameter: 3 cm), and the rotating speed was linearly increased from 3.5 rpm to 35 rpm in 300 sec and continued at 35 rpm until 600 sec. Mice received nine trials (three trials per day for 3 consecutive days) with a 10 min rest interval between trials. The amount of time for each mouse to fall from the rod was recorded for each trial. The mean latency to fall off the rotarod was recorded and used in subsequent analysis.

Morris water maze test

The Morris water maze test was conducted in a circular pool (diameter: 1 m) and filled with water at 23-25°C. Water was colored by white painting in order that mice could not see the platform (20 cm high, 10 cm diameter; 1 cm below the surface of water) or other cues under the water. There were some extra-maze landmark cues that were visible to the mice in the maze. Mice received four trials per day for 5 consecutive days. Each acquisition trial was initiated by placing an individual mouse into the water facing the outer edge of the maze at one of four designated starting point quasi-randomly, but the submerged platform remained constant for each mouse throughout testing. A trial was terminated when the mouse reached the platform, and the latency and distance swam were measured. Cut-off time of the trial was 60 sec, and mice that did not reach the platform within 60 sec were removed from the water and placed on the platform for 30 sec before being toweled off and placed back into their home cage. The inter-trial interval was 30 sec. After the 5 days' training, a probe test was conducted. In the probe test, the platform was taken away, and each mouse was placed into the water at the point of the opposite position of the target platform, and allowed to swim in the maze for 60 sec. The swimming distance, the times of crossing over the position of target and other three platforms, and time staying in the quadrants of the four platforms were measured.

Fear conditioning test

This test consisted of two parts: a conditioning trial and a test trial. Fear conditioning was carried out on a clear plastic chamber equipped with a stainless steel grid floor (34 cm × 26 cm × 30 cm [H]). A CCD camera was equipped on the ceiling of the chamber and was connected to a video monitor and computer. The grid floor was wired to a shock generator. White noise (65

dB) was supplied from a loudspeaker as an auditory cue (the conditioned stimulus, CS). A continuous 0.4 mA foot shock (the unconditioned stimulus, US) for 2 sec was administered at the end of the 30 sec CS period. The conditioning trial consisted of a 2 min exploration period followed by three CS–US pairings separated by 30 sec each. A test was performed in the same conditioning chamber for 5 min in the absence of the foot shock at 24 h after the conditioning trial. Rate of freezing response of mice was measured as an index of fear memory.

Exon Array-based analysis of alternative splicing

We analyzed wild type (WT) and PQBP1 conditional knock-out (cKO) mouse brain samples using Affymetrix GeneChip Mouse Exon 1.0 ST array (exon array) [http://www.affymetrix.com] to find significantly changed genes in terms of potential alternative splicing.

The exon array contains over five million probes representing about 1.4 million probesets that are designed based on the genomic regions of known genes and exons to measure both gene-level and exon-level expression in samples. The sequences of the probes and the probesets were downloaded at the Affymetrix website [http://www.affymetrix.com/support/technical/whitepapers/exon_probeset_trans_clust_whitepaper.pdf].

To summarize the probes into the exon-level probesets, the PLIER algorithm [http://www.affymetrix.com/analysis/index.affx] was applied to the probe signals of our samples.

Two types of analysis based on statistical hypothesis testing, exon-exon and variance analyses, were performed on the exon-level probesets to detect changes in alternative splicing. Before these tests, the signals of each exon-level probeset in one gene were normalized by the total signals of them to eliminate the effects of different numbers of exons among compared genes.

The exon-exon analysis was performed to compare each exon's signals between WT and cKO mice to find the significant change in exon-level that could be induced by the change of alternatively splicing or transcription. The difference

was examined by Student's t-test based on the null hypothesis that the means of the two samples are the same. The variance analysis was performed on exon-level probesets in each gene of WT and cKO mice to detect changes in relative expression levels among exons by an F-test for the null hypothesis that these two samples have the same pattern and the variance.
The smallest p-value was selected as the significance level of the gene to find alternatively spliced exons and it was significant with $p\text{-value} < 0.05$.

Genes showing significantly different expression between WT and cKO mice in exon-exon and/or variance analysis were listed to apply to PANTHER analysis [http://www.pantherdb.org/]. In Panther analysis, statistical overrepresentation test was performed to determine whether the genes were enriched or deprived for specific biological processes.
Three groups of significant changed genes, (A) Nestin-cKO-NSC specific genes, (B) co-occurrence genes between NSC and cortex of Nestin-cKO mice and (C) co-occurrence genes of the three types of cKO samples, were tested individually by PANTHER analysis to be compared with all the genes detected in the assay and then pie-charts of protein function classification were formed.

Immunohistochemistry of the primary cilium on ventricular surface

Immunohistochemistry analyses of the primary cilia on ventricular surface of mouse cortex were performed as described with modifications (81). E15 Brains were fixed with 4% paraformaldehyde. Cortex was dissected to expose the ventricular surface, blocked for 24 hours with blocking solution (10% goat serum, 0.5% Triton X-100, and PBS), and incubated for 24 hours with rabbit anti-AC3 (1:500, Santa Cruz) and mouse anti-bcatenin (1:1000, BD) in the blocking solution. After three washes with PBS, tissue was incubated with secondary antibodies (Alexa 488-conjugated goat anti-mouse IgG and Alexa 568-conjugated goat anti-rabbit IgG, 1:500, Invitrogen) for four hours. Cortex was mounted with Fluoromount-G (SouthernBiotech) and images of the ventricular surface cilia were collected with a Nikon Ti-E microscope with a 60x/1.45NA objective lens and a spinning disk confocal unit CSU22 (Yokogawa), using Volocity software (Perkin Elmer).

Ethics Statement

This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, and had been approved by the Committees on Human Ethics and Animal Experiments of the Tokyo Medical and Dental University (Number: 2011-22-3 and 0130225).

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