Supporting Information

Yun et al. 10.1073/pnas.1200817109

SI Text

Materials and Methods. ***Cell culture and differentiation.*** E14 was maintained on gelatin-coated dishes in high-glucose DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.1 mM nonessential amino acids, 0.1% β-mercaptoethanol and 100 U/ml leukemia inhibitory factor (LIF, Chemicon). For differentiation, the cells were detached from their culture plates using 0.25% trypsin with 1 mM EDTA solution (Invitrogen) and subcultured in nonadherent culture dishes in the E14 media but without LIF. Subsequently, 90% of the media was changed on a daily basis for a total of 4 d and then retinoic acid (Sigma) was added to the final concentration of 1 μM. On day six, the embryoid bodies were harvested and dissociated in 0.05% trypsin with 0.2 mM EDTA solution for 3 min at 37 °C to obtain a single cell suspension. NS5 was maintained in Euromed-N medium supplemented with 100 μg/ml Apo-transferin (Sigma), 5.2 ng/ml Sodium Selenite (Sigma), 19.8 ng/ml progesterone (Sigma), 16 μg/ml Putrescine (Sigma), 25 μg/ml insulin (Sigma), 50.25 μg/ml BSA (Gibco), 10 ng/ml bFGF (Gibco), 10 ng/ml EGF (Gibco), 100 U/ml penicillin (Gibco), 100 μg/ml streptomycin (Gibco) and 2 mM L-glutamine (Gibco). For differentiation of NS5 into astrocyte, the medium was changed to NS5 maintenance medium containing 5% FBS but without bFGF and EGF. MEF was maintained in the same media as used for E14 but without LIF. H1 was maintained in a feeder-free condition on matrigel-coated dishes in MEF-conditioned medium containing Knockout DMEM/10% serum replacement (Gibco), 0.1 mM MEM nonessential amino acids (Gibco), 1 mM L-glutamine (Gibco), 0.1 mM β-mercaptoethanol (Gibco), 8% plasmanate (NUH pharmacy), 12 ng/ml LIF, and 10 ng/ml (bFGF; Gibco). ReNcell VM (Millipore #SCC008) was maintained on laminin-coated dishes in ReNcell NSC Maintenance Medium (Millipore #SCM005) containing 20 ng/ml bFGF and 20 ng/ml EGF. For neural differentiation, ReNcell VM were seeded on PLO/Laminin-coated plates and cultured for up to 3 wks in media comprising a 1 : 1 mix of N2-DMEM/F12 and B27-Neurobasal media supplemented with 0.1 mM MEM nonessential amino acids and 1 mM L-glutamine, all obtained from Gibco/Invitrogen. For mixed primary brain cell culture, the brains of neonatal mouse pups were cut into small pieces and digested in 0.25% trypsin with 1 mM EDTA solution (Invitrogen) for 30 min at 37 °C before neutralization with FBS. After washing with PBS by centrifugation and resuspension, the tissues were triturated using a 10-ml pipette fitted with 1-ml tip and the suspension was filtered through a strainer with 40 μm nylon mesh. The obtained single cells were plated on 35 mm cell culture dishes in OptiMEM- GlutaMAX™ containing 10% FBS. Unattached cells and cell debris were removed the next day by replacing medium. One half of the medium was replaced twice a week thereafter.

Flow cytometry and FACS. The cells incubated with CDr3 were harvested by trypsin treatment, washed and resuspended in PBS. The fluorescence intensity of the cells was measured on a flow cytometry (BD™ LSR II) or collected using FACSAria™ (BD). The data were analyzed and processed using FlowJo 7 software.

Neurosphere preparation and assay. E14.5 fetal mouse brains were trypsinized in 0.25% trypsin with 1 mM EDTA solution (Invitrogen) for 30 mins at 37 °C before neutralization with FBS. The tissues were triturated sequentially with a 10-ml pipette followed by a 1-ml blue tip and a 0.2-ml yellow tip attached to the 10-ml pipette until the cell suspension flows through smoothly. The tissue suspension was washed three times with PBS by repeated resuspension and centrifugation and filtered through a 40-μm strainer. The obtained single cells were plated in a DMEM/F12 medium containing 10 ng/ml bFGF, 20 ng/ml EGF and B27 without vitamin A (Invitrogen) to grow forming spheres. For cytotoxicity assay, dissociated neurosphere cells were plated in triplicate in six well culture plates at a density of 3,000 cells per well and cultured in the presence of 1 μM CDr3 or 0.1% DMSO for 6 d. After 6 d, the number of neurospheres was counted manually and the images were taken using a microscope (Eclipse Ti, Nikon) for measuring the sizes of neurospheres. For serial assay, the neurospheres were further passaged in the same condition as they were generated in. All animal experiment procedures were performed in accordance with a protocol approved by the Institutional Animal Care and Use Committee.

Cell proliferation assay. NS5 were seeded into 96 well plates (Greiner) at a density of 1,000 cells/well. The next day, DMSO and 1 mM DMSO stock of CDr3 was added into 32 wells for each to be diluted to 0.1% and 1 uM, respectively. At 6 h and 48 h time points, 1 ug/ml of Hoechst 33342 was added and incubated for 15 min for image acquisition and analysis using an ImageXpress Micro™ and MetaXpress Imaging system (Molecular Devices). Hoescsht 33342 and CDr3 signals were detected via DAPI and Texas red filters, respectively, and the images of a total of four areas were captured per well. Multi wavelength scoring analysis was then run to quantify the number of cells based on Hoechst 33342-stained nuclei image. Due to the uneven distribution of the cells in wells, the highest and lowest values from the obtained four values from a well were excluded for statistical analysis using ANOVA. For the quantification of pulse-labeled cells with BrdU, the cells were stained using FITC conjugated anti-BrdU antibody (BD Pharmingen™) according to the manufacturer's instruction. Total numbers of Hoechst 33342-stained and BrdU-labeled nuclei were counted by image based analysis using ImageJ-ITCN software.

Two-dimensional gel electrophoresis. CDr3-stained NS5 pellet was lysed in a lysis buffer (40 mM Trizma, 7 M Urea, 2 M thiourea and 4% CHAPS) premixed with 10 μl/ml Protease Inhibitor Cocktail (EDTA free, GE healthcare), 50 μg/ml DNase I and 50 μg/ml RNase A (Roche). The cell extract was homogenized by vortexing followed by ultrasonication on ice for 10 s, and incubated for 30 min at room temperature. The supernatant was collected after centrifugation at 20,000 × g for 45 min at 10 °C and protein concentration was determined by Bradford protein assay reagent (Bio-Rad). Isoelectric focusing (IEF) was performed using PROTEAN IEF Cell (Bio-Rad) with an 18 = −cm ReadyStrip pH 3–10 NL (Bio-Rad). The sample of 1 mg protein was diluted into 340 μl of rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 20 mM DTT, 0.5% IPG buffer pH 3–10 NL (GE healthcare), and loaded to each IPG strip with passive rehydration and focused for 60,000 Vhrs at 20 °C. The IEF strips were reduced in equilibration buffer I (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 2% DTT) at room temperature for 10 min and alkylated with SDS-PAGE Equilibration Buffer II (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 2.5% iodoacetamide, and a trace of bromophenol blue) at room temperature for an additional 10 min. The equilibrated IEF strips were embedded in 0.5% low melting temperature agarose in 1× Tris-glycine-SDS buffer on top of a second dimension SDS-PAGE (12%) gel. After electrophoresis for 5 h at 30 mA, the 2D fluorescence image of gels was acquired using a Typhoon 9400 scanner (GE healthcares)

at excitation/emission wavelengths of 532 nm/610 nm with PMT at 500 v and a duplicate gel was stained using PlusOne™ Silver Staining Kit (GE healthcare) according to the manufacturer's protocol. The fluorescently labelled protein spots were directly excised from the gel for in-gel trypsin digestion and peptide extraction as described previously (1).

Immunostaining. The primary and secondary antibodies used for Western blotting and immuocytochemistry in this study are as follows: anti- beta-Actin (1:2,000, Santa Cruz), beta-Tubulin III (1:500, Sigma), B-FABP (1:1,000, Santa Cruz), BLBP (1:1,000, abcam), GFAP (1:1,000 DAKO), mouse IgG-Alexa Fluor 488 (1:300, Invitrogen), mouse IgG-HRP (1:4,000, Santa Cruz), rabbit IgG-Cy3 (1:300, Zymed) and rabbit IgG-HRP (1:4,000, Santa Cruz).

Real time RT-PCR. Total RNA was extracted from the cells using RNeasy Mini Kit (QIAGEN Inc.) according to the manufacturer's instruction. For the RNA samples isolated from E14, NS5, D-NS5 and MEF, the reverse transcription and amplification were carried out using Power SYBR® Green RNA-to-CT™ 1-Step Kit (Applied Biosystem) on a StepOne™ Real-Time PCR System (Applied Biosystem). For the RNA samples from H1, ReNcell VM and ReNcell-differentiated neurons, cDNA was synthesized from the total RNA using the High Capacity cDNA Archive kit (Applied Biosystems) according to the manufacturer's instructions. PCR reaction was conducted using Power SYBR ® Green PCR Master Mix (Applied Biosystems) on an ABI Prism 7900 machine. The relative mRNA levels of the genes of interest were normalized and compared to that of GAPDH using Q-gene relative expression software tool. The primer sequences (5′ to 3′) used in this study are as follow:

Mouse FABP7	Forward	ccagctgggagaagagtttg
Mouse FABP7	Reverse	tttctttgccatcccacttc
Mouse GAPDH	Forward	aagggctcatgaccacagtc
Mouse GAPDH	Reverse	ggatgcagggatgatgttct
Human FABP7	Forward	acagaaatgggatggcaaag
Human FABP7	Reverse	ctcatagtggcgaacagcaa
Human GAPDH	Forward	cagcctcaagatcatcagca
Human GAPDH	Reverse	tgtggtcatgagtccttcca

Mouse and human FABP7 gene cloning. cDNAs of mouse and human FABP7 genes were synthesized by RT-PCR from the total RNAs extracted from NS5 and U251 human neuroblastoma cell line, respectively. The primer sequences (5′ to 3′) for FABP7 ORF cloning are as follows:

Mouse FABP7	Forward	cccagatctccaccatggtagatgctttctgcgcaacct
Mouse FABP7	Reverse	cccaagctttgccttttcataacagcgaacagca
Human FABP7	Forward	cccagatctccaccatggtggaggctttctgtgctacct
Human FABP7	Reverse	cccaagctttgccttctcatagtggcgaacagcaa

BglII/HindIII restriction enzyme sites underlined were incorporated. Acquired PCR products were digested with BglII/HindIII and inserted into the pEGFP-N1 vector (Clontech). For bacterial expression of FABP7, ORF of human FABP7 was amplified by PCR, digested with SalI/HindIII and inserted into pQE31 vector (Qiagen). The primer sequences (5′ to 3′) for the subcloning are as follows:

Human FABP7	Forward	cccgtcgacatggtggaggctttctgtgctagc
Human FABP7	Reverse	cccaagctttgccttctcatagtggcgaacagcaa

SalI/HindIII restriction enzyme sites underlined were incorporated.

Transformation and transfection. The constructs were transformed into E. coli DH5α for amplification and verified by sequencing. The transfection of 293HEK cells with the plasmid constructs were carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Recombinant protein expression and purification. pQE31-hFABP7 plasmid was transformed into an E. Coli strain SG13009(Qiagen), which allows stringent control of IPTG inducible promoter. Transformed SG13009 was cultured in SOB medium and the recombinant hFABP7 was induced by 0.5 mM IPTG for 8 h at 30 °C. Bacterial pellet was harvested by centrifugation and the His-tagged hFABP7 was purified using a Ni-NTA column of QiaExpress expression kit (Qiagen) according to the manufacturer's instruction. The protein was delipidated by incubation with an equal volume of Lipidex-1000 at 37 °C for 10 min and elution. The buffer was exchanged to 10 mM potassium phosphate buffer (pH = 7.4) by five times repeated concentration and dilution using Amicon-Ultra 3K (Millipore) and the protein was finally concentrated to the concentration of 1.05 mg/ml.

In vitro binding assay. To determine dissociation constant CDr3 (10 μM) was incubated with different concentrations of FABP7 (0–1 mg/ml) in 10 mM potassium phosphate buffer (pH = 7.4) at 37 °C for 10 min and the fluorescence intensities were recorded on a SpectraMax M2 plate reader (excitation: 530 nm; emission: 590 nm). The dissociation constant was obtained from a fitting curve using GraphPad Prism 5.0 software. For stoichiometry determination of CDr3-FABP7 complex, a total concentration of CDr3 (final concentrations: 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 μM) and FABP7 (final concentrations: 20, 18, 16, 14, 12, 10, 8, 6, 4, 2, 0 μM) mixtures were incubated in 10 mM potassium phosphate buffer (pH = 7.4) containing 1% DMSO at 37 °C for 10 min, and the fluorescence intensities of each mixture were recorded on a SpectraMax M2 plate reader (excitation: 530 nm; emission: 590 nm) for Job plot analysis.

1. Bi X, et al. (2006) Proteomic analysis of colorectal cancer reveals alterations in metabolic pathways: Mechanism of tumorigenesis. *Mol Cell Proteomics* 5:1119–1130.

2. Malan SF, et al. (2004) Fluorescent ligands for the histamine H2 receptor: synthesis and preliminary characterization. *Bioorg Med Chem* 12:6495–6503.

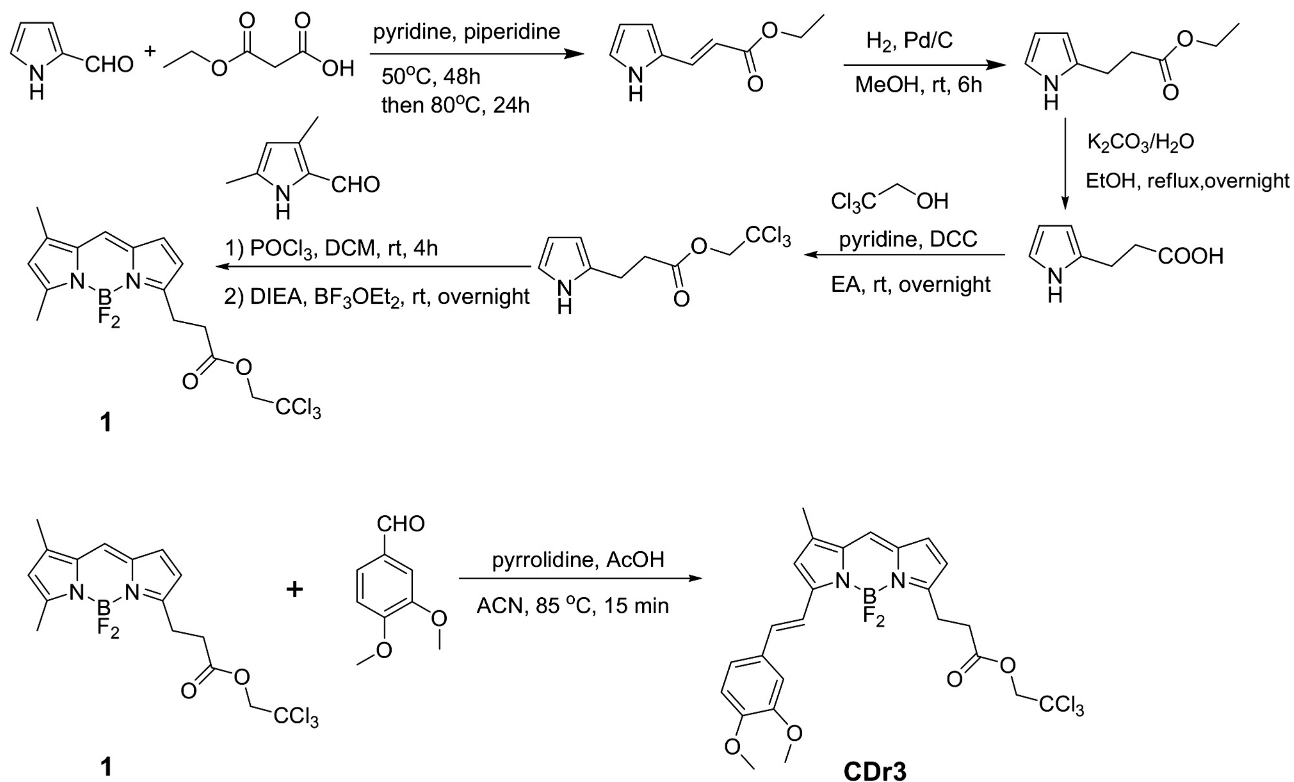

Fig. S1. *Synthesis of CDr3*. The intermediate 1 was synthesized as reported earlier (2). 1, (20 mg, 0.047 mM) and 3,4-dimethoxybenzaldehyde (16 mg, 0.094 mM) were dissolved in acetonitrile (4 ml), followed by the addition of the mixture of pyrrolidine (23.6 μl, 0.282 mM) and acetic acid (16.1 μl, 0.282 mM). The reaction was heated at 85 °C for 15 min and then cooled down to room temperature. The resulting crude mixture was concentrated under vacuum and purified by normal-phase column chromatography (eluting system: hexane/ethyl acetate (6:1) to render CDr3 as purple solid (15 mg, 56% yield).

Fig. S2. *HPLC-MS characterization of CDr3. Upper*, HPLC chromatogram: (descending order) at 254, 350, 500 and 560 nm. HPLC conditions: *A*: H_2O-HCOOH: 99.9 : 0.1. *B*: ACN-HCOOH: 99.9 : 0.1; gradient 30% B to 100% B (5 min). Reverse-phase Agilent C_{18} Zorbax column (2.1×30 mm^2) 3.5 μm, flow rate: 1 ml/ min; *Lower*, ESI-MS spectra: (*left*) ESI-positive, (*right*) ESI-negative spectra.

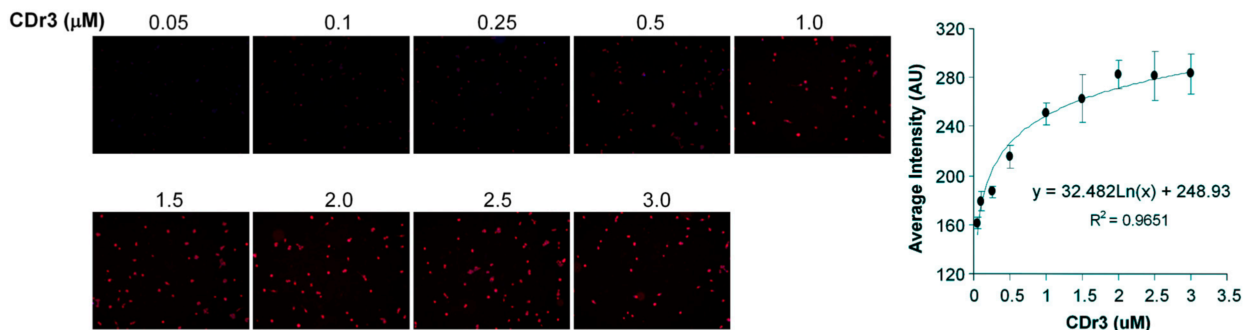

Fig. S3. *Image-based CDr3 Titration for Neural Stem Cell Staining*. NS5 cells were incubated with different concentrations of CDr3 for 1 h and washed out with fresh medium for 1 h. The cell images (12 images for each concentration) were acquired using ImageXpress Micro™ and the fluorescence intensity of the stained cells was analyzed using MetaXpress® image processing software. Mean ± SD values of the average intensity were used to draw the graph with a trend line added. The half maximal staining intensity was obtained at 0.39 μM.

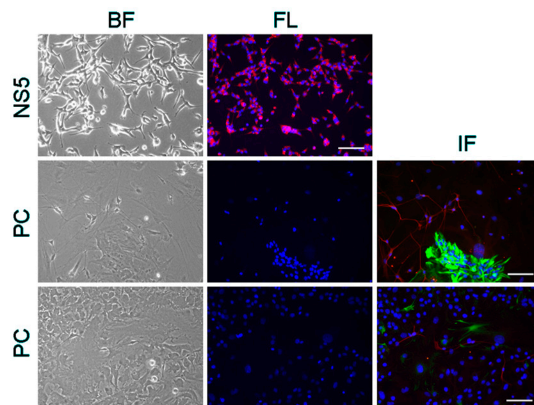

Fig. S4. *Differentiated Primary Neural Cell Staining*: Mixed primary mouse brain cells (PC) cultured for 2 wks in vitro were incubated with CDr3 and Hoechst 33342. The images of live cells are shown in phase contrast bright-field (BF) and fluorescence (FL) panels. The images of the same cells were acquired after immunofluorescence staining (IF) with antibodies to neuron-specific class III β-tubulin (Tuj1, red) and astrocyte-specific glial fibrillary acidic protein (GFAP, green). Scale bar, 100 μm.

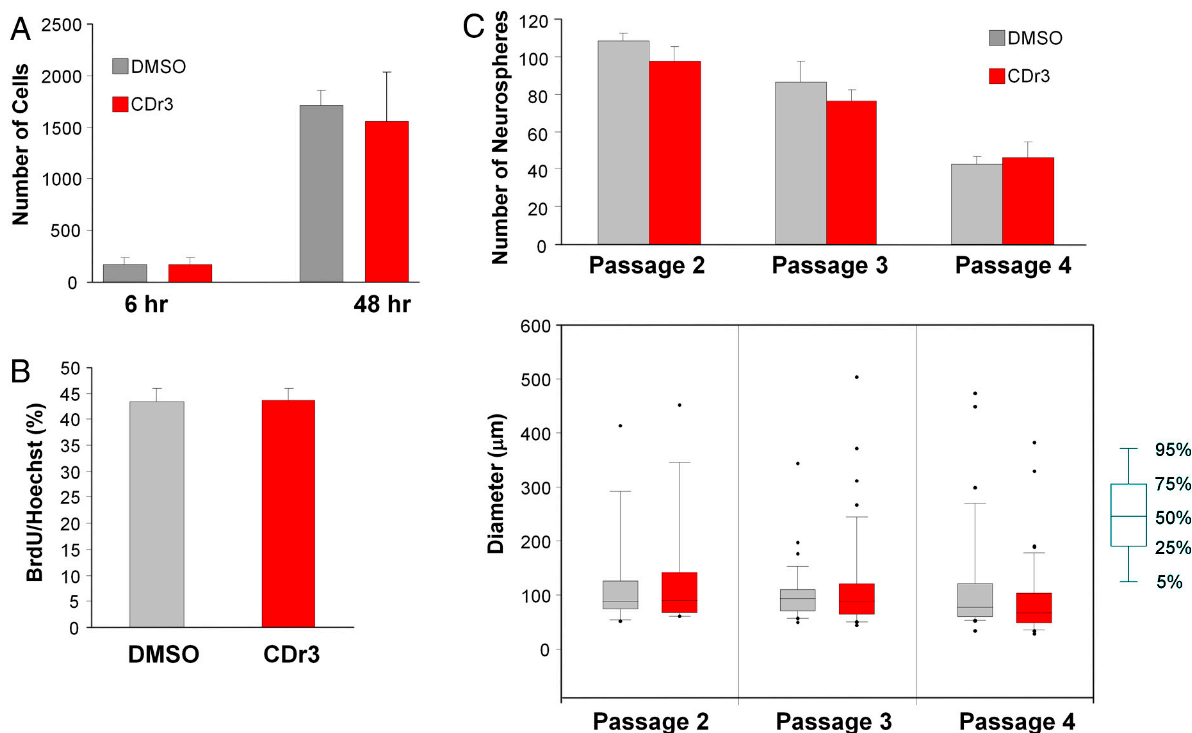

Fig. S5. *Cell Proliferation Assay.* (*A*) NS5 cells were cultured in the presence of 1 μM CDr3 or 0.1% DMSO for 6 h and 48 h. Nuclei were stained with Hoechst33342 for image acquisition and cell counting using MetaXpress® image processing software (n = 32). (*B*) NS5 cells pulse-labeled with BrdU for 6 h were visualized using FITC-conjugated anti-BrdU antibody. The percentage of BrdU-positive cells was determined by counting both Hoechst 33342 stained nuclei and BrdU positive nuclei in the same images (n = 8). (*C*) Same numbers of neurosphere cells were serially plated and cultured in six well plates in the presence of 1 μM CDr3 or 0.1% DMSO. The numbers (*Upper*) and sizes (*Lower*) of neurospheres were determined every 6 d (n = 3).

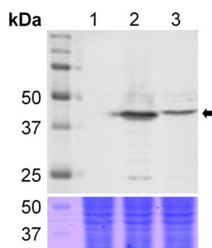

Fig. S6. *Western Blot Detection of FABP7 in Transfected HEK293 Cell Lysates*. The EGFP (27 kDa) + FABP7 (14 kDa) fusion protein of 41 kDa was detected by an anti-mouse FABP7 antibody, which crossreacts to human protein. Lane1, pEGFP-N1 vector only; lane2, pEGFP-mouse FABP7; lane3, pEGFP-human FABP7. A duplicate gel was stained with Coomassie blue (*Lower*).

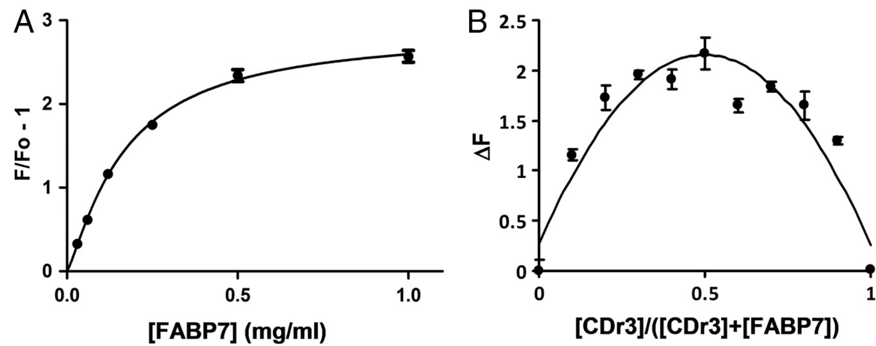

Fig. S7. *Binding Kinetics*. (*A*) The best fitting curve was obtained by plotting CDr3 fluorescence fold increase (F/F_0-1) against various concentrations of human FABP7. The data are presented as mean ± SD of 3 independent experiments. (*B*) Job plot for the complexation of CDr3 with human FABP7. ΔF [fluorescence fold increase × CDr3 ratio] was plotted against CDr3 ratio in a total concentration of 20 μM.

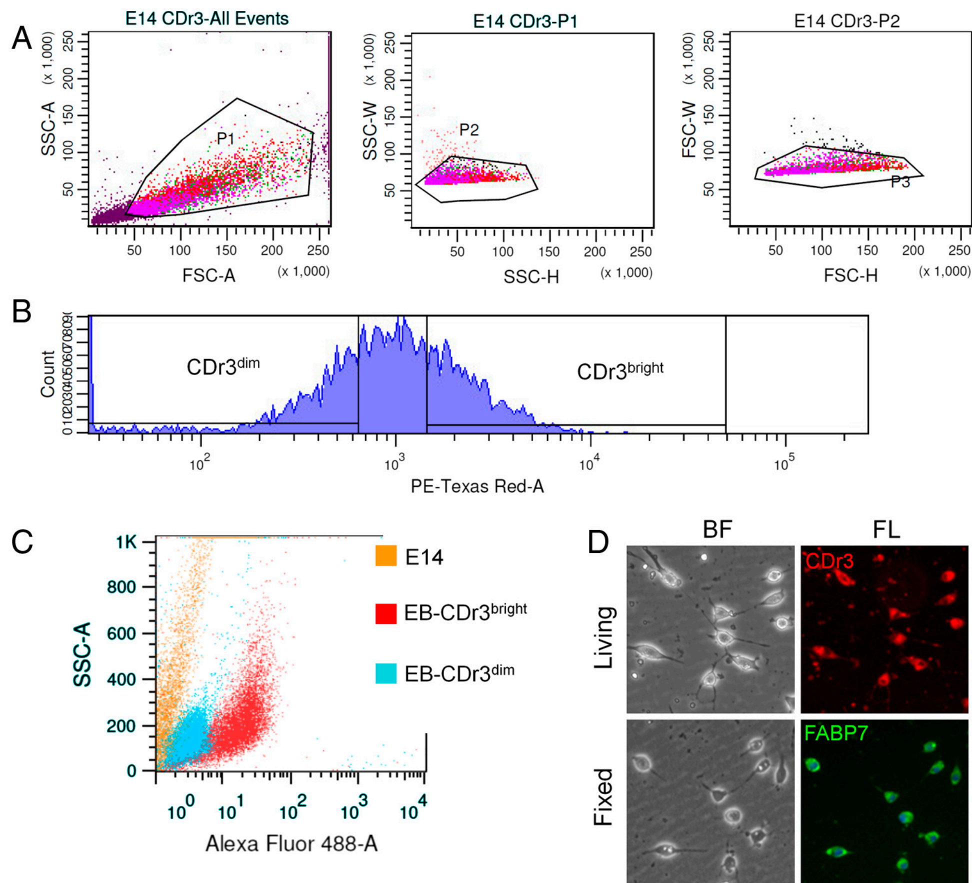

Fig. S8. *Differentiated mESC FACS Using CDr3*. (*A*) The CDr3-stained embryoid body cells were gated by forward and side scattering to remove cell debris. (*B*) The gated cells were separated into $CDr3^{bright}$ and $CDr3^{dim}$ populations. (*C*) The expression of FABP7 in $CDr3^{bright}$ and $CDr3^{dim}$ cells was determined by immunocytochemistry followed by flow cytometry. (*D*) $CDr3^{bright}$ cells were cultured and stained by CDr3. Subsequent fixation and immunofluorescence staining with anti-FABP7 antibody showed the expression of FABP7 in the $CDr3^{bright}$ cells. Scale bar, 50 μm.