Supporting Information

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SI Materials and Methods

DNA Constructs and Reagents. The Flag-tagged full-length cyclin-dependent kinase-like 5 (CDKL5) and its truncated derivatives were generated by cloning the corresponding cDNA into pCAG-tag2B (derived from Stratagene pCMV-tag2B vector with the CMV promoter replaced by a CAG promoter). Point mutations for CDKL5 were generated by QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies). For GST-CDKL5ΔN construction, the cDNA corresponding to amino acids 297–934 of rat CDKL5 was amplified by PCR and cloned into pGEX-4T-1 (GE Healthcare). The generation of GFP-CDKL5 was described previously (1). The truncated derivatives of GFP-CDKL5 were constructed by replacing full-length CDKL5 with cDNAs corresponding to the truncated proteins. For construction of Flag-tagged postsynaptic density (PSD)-95, the cDNA for rat WT PSD-95 or PSD-95 [mutation of cysteine to serine at positions 3 and 5 (C3,5S)] was cloned into pcDNA3 (Invitrogen) with a Flag tag fused to the C terminus. GFP-tagged PSD-95 (PSD-95-GFP) and its derivatives were constructed by cloning the corresponding cDNA into pEGFP-N1 (Clontech). PSD-93-GFP, synapse-associated protein (SAP) 97-GFP, and SAP102-GFP were gifts from Eunjoon Kim (Korea Advanced Institute of Science and Technology, Daejeon, South Korea). All plasmid-based RNAi constructs were generated using the pSuper vector (Oligoengine). The CDKL5 shRNA was described previously (1). The target sequence is GGAGCCTATGGAGTTG-TAC. The target sequence for PSD-95 is GCCTTCGACAGAG CCACGA, and was reported previously (2). All constructs were verified by sequencing. Palmitate and 2-bromopalmitate were purchased from Sigma. Lentiviruses used for delivering shRNAs were described previously (1) and were produced by NeuronBiotech.

Cell Cultures and Transfection. Cultures of cortical neurons were prepared from E18 Sprague-Dawley (SD) rat as previously described (1). Cultures of hippocampal neurons were prepared from P0 SD rat as described (3). Neurons were transfected using Lipofectamine 2000 following the manufacturer's instruction (Invitrogen). HEK293T cells and COS-7 cells were grown in DMEM supplemented with 10% (vol/vol) FBS and were transfected using Lipofectamine 2000 (Invitrogen).

Antibodies, Immunoblotting, and Immunocytochemistry. CDKL5 polyclonal antibody was generated by immunizing rabbits with a recombinant CDKL5 protein and affinity-purified by protein A agarose. CDKL5 rat monoclonal antibody was produced by Abmart Inc. The following antibodies were purchased from commercial sources: PSD-95 (Millipore); SynapsinI and Ras (Cell Signaling); GAP43, PSD-95 (p-S295), and PSD-95 (p-S418) (Epitomics); GluR1 N terminus (Calbiochem); GFP monoclonal antibody (Santa Cruz); GFP polyclonal antibody (Invitrogen); and Flag (Sigma).

For immunoblotting of CDKL5 in rat brains at different developmental stages, rat cortices were homogenized in radioimmunoprecipitation assay (RIPA) buffer followed by low-speed centrifugation ($1,000 \times g$) to clear the lysate. The supernatant was saved and used for immunoblotting. The expression pattern of CDKL5 showed in this study (Fig. S2*A*) looks different from our previous observation (1). This may be due to the different spinning speed used for clearing samples in the two studies: low-speed centrifugation in the present study and high-speed centrifugation ($14,000 \times g$) in the previous study (1). We have found that sample clearance by high-speed centrifugation may cause loss of CDKL5 proteins, probably because CDKL5 is incorporated into large protein complexes such as PSD. We used different CDKL5 antibodies in the two studies: purchased from Abcam in the previous study (1) and made by ourselves in the present study.

For immunostaining, neurons were fixed either in 4% paraformaldehyde/4% sucrose/PBS for 10 min (for morphological analysis) or in methanol at −20 °C for 5 min (for immunostaining of endogenous synaptic proteins). COS-7 cells were fixed in 4% paraformaldehyde/4% sucrose/PBS for 10 min at room temperature (RT). Fixed cells were permeabilized with 0.2% Triton X-100/PBS for 5 min (for cells fixed with paraformaldehyde), and blocked in 3% BSA/PBS for 1 h. Primary antibodies were incubated in blocking buffer overnight at 4 °C and visualized with Alexa Flour–conjugated secondary antibodies.

Affinity Purification of CDKL5-Interacting Proteins. Rat synaptosomes were purified according to the procedures described previously (4), with minor modification. All purification steps were performed at 4 °C. The brain of 1-mo-old SD rat was rapidly decapitated, and forebrain was isolated and homogenized in ice-cold Buffer A [0.32 M sucrose, 1 mM $NaHCO_3$, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, 1× PhosSTOP (Roche), 0.1 mM PMSF (Sigma), and 1× Protease inhibitor mixture (Sigma)) with Teflon homogenizer (12 strokes)]. Homogenized brain extract (H) was centrifuged at $1400 \times g$ for 10 min. Supernatant (S1) was saved and pellet (P1) was homogenized again with Teflon homogenizer (three strokes). After centrifugation at $710 \times g$, the supernatant (S1′) was saved and pooled with S1. Pooled S1 and S1′ was centrifuged at $13,800 \times g$ for 10 min to collect the pellet (P2). P2 was resuspended in Buffer B (0.32 M sucrose, 1 mM $NaHCO_3$, 1× PhosSTOP, 0.1 mM PMSF, and 1× Protease inhibitor mixture) with Teflon homogenizer (five strokes). The P2 suspension was loaded onto a discontinuous sucrose gradient (0.85 M/1 M/1.2 M sucrose solution), followed by centrifugation for 2 h at $82,500 \times g$ in a SW-41 rotor. The synaptosome fraction between 1 M and 1.2 M sucrose was collected and solubilized with lysis buffer A (50 mM Tris·HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1× PhosSTOP, 1× protease inhibitor mixture). The lysates were centrifuged at $14,000 \times g$ for 25 min at 4 °C. The supernatant (S) was saved and the pellet was resuspended with lysis buffer A containing 1% SDS, and centrifuged again at $20,000 \times g$ for 25 min at 4 °C. The supernatant (S′) was pooled with S and diluted to reduce the concentration of SDS to less than 0.1%. The diluted lysates were passed through columns charged with GST-CDKL5 or GST affinity resin. The columns were washed extensively with lysis buffer A and the bound proteins were eluted using lysis buffer A containing 1 M NaCl. The eluates were subjected to SDS/PAGE and stained with Coomassie Brilliant Blue. The protein bands were identified by mass spectrum as described previously (5).

GST Pull-Down and Coimmunoprecipitation. For GST pull-down assay, cells were lysed in lysis buffer A and the lysates were cleared by centrifugation at $14,000 \times g$ for 20 min. The supernatant was incubated with GST-fusion protein or GST affinity beads for 4 h at 4 °C with end-over-end mixing. The bound proteins were eluted by 2× reducing SDS loading buffer, resolved by SDS/PAGE, and immunoblotted with indicated antibodies. For coimmunoprecipitation in 293T cells, cells were lysed in lysis buffer A plus protease inhibitor mixture (Sigma). The lysates were cleared by centrifugation at $14,000 \times g$ for 20 min. Antibodies or antibody-coupled resins were incubated with the cleared lysates overnight at 4 °C. For coimmunoprecipitation in neurons, cells were lysed in RIPA buffer (50 mM Tris·HCl, pH 7.4, 150 mM NaCl, 1% TritonX-100, 0.1%

SDS, 0.5% Na-deoxycholate, 1× PhosSTOP, 1× protease inhibitor mixture), sonicated briefly, and incubated on ice for 1 h. Cell debris was removed by centrifugation at 14,000 × *g* for 20 min. A CDKL5 polyclonal antibody was added to the cleared lysates (1:100 dilution), mixed, and rotated end over end at 4 °C overnight. The next day, protein A was added to lysates and incubated with rotation at 4 °C for 4 h. The resins were washed four times by RIPA. The bound proteins were eluted with 2× reducing SDS loading buffer, resolved by SDS/PAGE, and immunoblotted with indicated antibodies.

Surface Labeling of AMPA Receptors. For surface GluR1 staining, live neurons were incubated at 37 °C for 15 min with an antibody directed against the N-terminal domain of GluR1 (Calbiochem) and fixed with 4% paraformaldehyde/4% sucrose for 10 min at RT. Surface receptors were visualized by saturating levels of anti-rabbit Alexa 568–conjugated secondary antibodies (Invitrogen).

In Utero Electroporation. SD rats were used for the in utero electroporation experiment as described previously (1). Saline solution (1–2 μL) containing the expressing vector for GFP or GFP-CDKL5Δ1–670 (1 μg/μL) under the control of a CAG promoter was injected into the lateral ventricle through the uterus wall of E15.5 embryonic brains. The embryo's head was electroporated with five electrical pulses (50 V, 50-ms duration at 100-ms intervals) generated by an ElectroSquirePortator T830 (BTX). The pups were killed at P14. Brains were processed and sections were stained with a GFP antibody to visualize neuronal morphology.

Electrophysiological Recordings. Whole-cell patch clamp recordings of cultured hippocampal neurons [days in vitro (DIV) 14–17, 4–7 d after transfection] were performed at ∼25 °C in bath solution containing (in mM): 140 NaCl; 2.4 KCl; 10 Hepes; 10 glucose; 2 $CaCl_2$; 1 $MgCl_2$, pH 7.4; and tetrodotoxin (1 μM). (+)-MK801 (10 μM) and (−)-bicuculline methobromide (20 μM) were added during miniature excitatory postsynaptic current (mEPSC) recordings. Cells were held at −70 mV and recorded for roughly 10 min to obtain at least 200 events per cell. Pipettes were filled with an internal solution containing (in mM): 130 K-gluconate, 10 Hepes, 10 KCl, 0.5 EGTA, 2 $MgSO_4$, 4 ATP·Na_2, 0.3 GTP, and 5 sucrose, titrated to pH 7.4 with KOH. All solutions are adjusted the osmolarity to 290–300 mOsm. Voltage-clamp mode was achieved using a multiclamp 700B amplifier controlled by pCLAMP 10 acquisition software (Axon Instrument). Events were filtered at 4 kHz and sampled at 20 kHz using a Digidata 1440A (Axon Instrument). Patch pipettes were pulled from borosilicate glass capillaries (WPI) and had resistances of 4–5 MΩ. Access resistance was checked every 10 min throughout the experiment, and data were discarded when >10% change occurred. Miniature synaptic currents were analyzed off-line using SpAcAn, a custom-made, threshold-detection algorithm (www.neuralwiki.org/), based on the Igor PRO analysis environment (WaveMetrics). Electrophysiological results were reported as mean ± SEM. Student *t* test was used to assess differences between sample groups. For all tests, the number of asterisks in the figures corresponds to level of significance: $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Tetrodotoxin and (−)-bicuculline methobromide were purchased from Tocris Bioscience; (+)-MK801 was obtained from Sigma.

Confocal Imaging and Data Analysis. Images were acquired on a Nikon A1 laser scanning microscope with a 60×/1.4NA oil immersion objective. A z-stack was collected at 1,024 × 1,024 pixel resolution, averaged four times, taken at 0.4-μm intervals. For analysis of dendritic spine morphologies, spines were traced manually and analyzed using ImageJ (National Institutes of Health) in a blind manner. Student unpaired *t* tests were used to determine the statistical significance of differences between two groups. Cumulative plots were analyzed using Kolmogorov–Smimov test (K–S test). For the analysis of synaptic protein levels, fields of dendritic branches including primary and secondary arborization were randomly selected. Signal intensity of puncta not overlapping the dendritic shaft in the selected areas was measured using ImageJ.

ABE Method. The acyl–biotinyl exchange (ABE) method was performed as previously described (6). Briefly, after treated with the indicated chemicals or shRNAs, cortical neurons (DIV 16–19) were lysed with lysis buffer (LB) (150 mM NaCl, 50 mM Tris, 5 mM EDTA, pH 7.4) containing 2% Triton X-100, 10 mM *N*-ethylmaleimide (NEM), 2× protease inhibitor mixture, and 2 mM PMSF. Extracts were rotated at 4 °C for 1 h and spun at 250 × *g* for 5 min to remove particulates. Chloroform-methanol (CM) precipitation was applied to the supernatant to denature the proteins. The pellet of CM precipitation was dissolved with 4% SDS buffer (4% SDS, 50 mM Tris, 5 mM EDTA, pH 7.4) and diluted with LB containing 1 mM NEM, 1× protease inhibitor mixture, 1 mM PMSF, and 0.2% Triton X-100 and incubated overnight at 4 °C with rotation. NEM was removed from samples by three sequential CM precipitations. The pellet of CM precipitation was dissolved with 4% SDS buffer and diluted with + HAM buffer (0.7 M hydroxylamine, 1 mM Biotin-HPDP (N-[6-(Biotinamido)hexyl]-3′-(2′-pyridyldithio)-propionamide), 0.2% Triton X-100, 1 mM PMSF, 1× protease inhibitor mixture, pH 7.4) or control buffer (−HAM buffer; 50 mM Tris, 1 mM HPDP–biotin , 0.2% Triton X-100, 1 mM PMSF, 1× protease inhibitor mixture, pH 7.4) and incubated at RT for 1 h with rotation. Hydroxylamine was removed from samples by three sequential CM precipitations. The pellet of CM precipitation was dissolved with 2% SDS buffer and diluted SDS concentration to 0.1% with LB containing 1× protease inhibitor mixture, 1 mM PMSF, and 0.2% Triton X-100. After incubating at RT for 30 min with rotation, extracts were centrifuged at 15,000 × *g* for 1 min to remove particulates. Supernatants were transferred to Streptavidin-agarose that was prewashed with LB containing 0.1% SDS and 0.2% Triton X-100 twice and incubated at RT for 90 min with rotation. Unbound proteins were removed by four sequential washes with LB containing 0.1% SDS and 0.2% Triton X-100, and samples were analyzed by immunoblotting.

Statistical Analysis. All values in figures and text refer to mean ± SEM unless otherwise indicated. Two-tailed Student *t* test was used to measure significance of differences between two groups. Statistical significance was defined as $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

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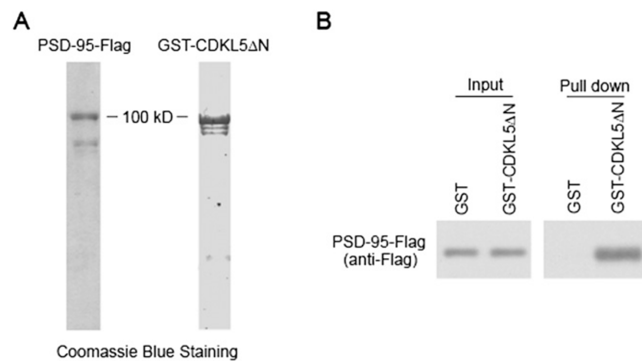

Fig. S1. Direct interaction between CDKL5 and PSD-95. (*A*) Purity of recombinant PSD-95 and CDKL5. Flag-tagged PSD-95, purified by immunoprecipitation from 293T cells, and GST-CDKL5ΔN, purified from *Escherichia coli*, were subjected to SDS/PAGE and Coomassie Blue staining. (*B*) A GST pull-down assay showing the direct interaction between PSD-95 and CDKL5.

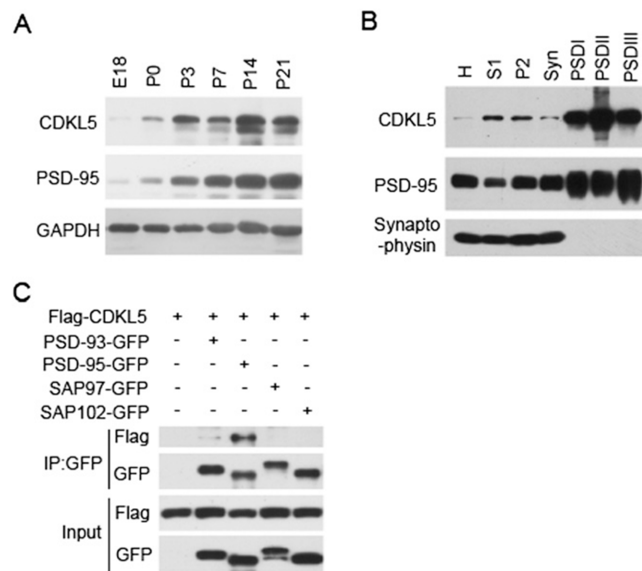

Fig. S2. Expression profile of CDKL5 and its interaction with PSD-95. (*A*) Expression of CDKL5 and PSD-95 in rat brains at different developmental stages. (*B*) Immunoblots showing the enrichment of CDKL5 and PSD-95 in the purified PSD fractions. Rat brain was fractionated to isolate the PSD. Samples from each fraction (20 μg for non-PSD fractions; 5 μg for PSD fractions) were immunoblotted with antibodies against CDKL5, PSD-95, and synaptophysin. (*C*) CDKL5 specifically interacts with PSD-95, but not other members of membrane-associated guanylate kinase (MAGU.K). Coimmunoprecipitation was performed using a GFP antibody in 293T cells coexpressing Flag-tagged CDKL5 and one of the MAGUK proteins tagged with GFP.

Fig. S3. CDKL5 interacts with palmitoylated PSD-95. (*A*) Schematic diagram for domain structures of rat PSD-95 protein and its truncation mutants. FL, full length; ΔN, amino acids 65–724; ΔC, amino acids 1–64. (*B*) Coimmunoprecipitation of GFP-tagged PSD-95 and its derivatives with Flag-tagged CDKL5 in 293T cells. (*C*) A GST pull-down experiment showing that the palmitoylation-deficient PSD-95 mutant does not interact with CDKL5. (*D*) The interaction of CDKL5 and PSD-95 requires palmitoylation and a specific sequence on the N-terminal region of PSD-95. The 293T cells were transfected with indicated constructs and the lysates were immunoprecipitated with a Flag antibody. The lysates and immunoprecipitates were detected with antibodies against GFP and Flag. (*E*) CDKL5 does not interact with other palmitoylated proteins. Lysates from cultured neurons were incubated with GST or GST-CDKL5ΔN affinity beads. The precipitates were immunoblotted with antibodies against PSD-95, v-Ha-ras Harvey rat sarcoma viral oncogene homolog (HRas) and growth-associated protein-43 (GAP43).

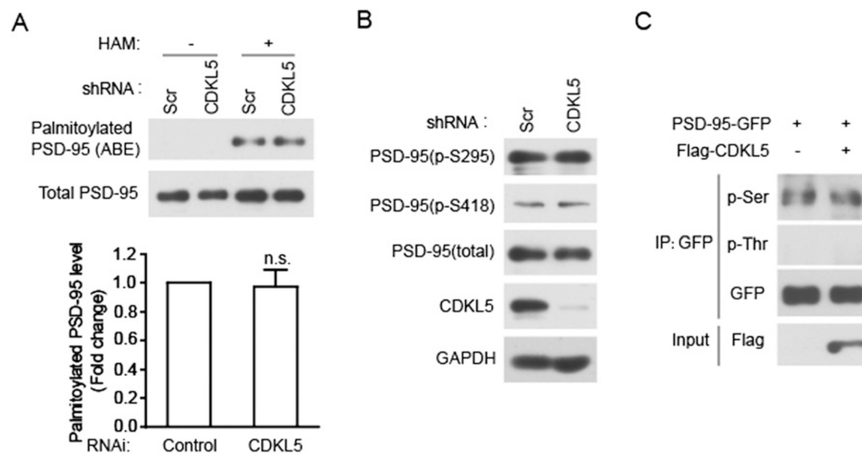

Fig. S4. Down-regulation of CDKL5 has no effect on the levels of total, palmitoylated, and phosphorylated PSD-95. (*A*) Cultured neurons were infected with lentivirus expressing scrambled shRNA or CDKL5 shRNA. Cells were harvested and the level of palmitoylated PSD-95 was examined by the acyl-biotinyl exchange method. $n = 4$; $P = 0.8339$; *t* test. (*B*) Effects of CDKL5 knockdown on the phosphorylation levels of serine-295 (S295) and serine-418 (S418). Neurons infected with lentivirus expressing scrambled or CDKL5 shRNA were harvested and the lysates were immunoblotted with antibodies against phospho-S295 (p-S295), phospho-S418 (p-S418), PSD-95, CDKL5, and GAPDH. (*C*) Overexpression of CDKL5 does not increase PSD-95 phosphorylation. PSD-95-GFP was transfected individually or together with Flag-CDKL5 into HEK293T cells and immunoprecipitated (IP) with a GFP antibody. The lysates and precipitates were immunoblotted with antibodies against phospho-serine (p-Ser), phospho-threonine (p-Thr), GFP, and Flag.

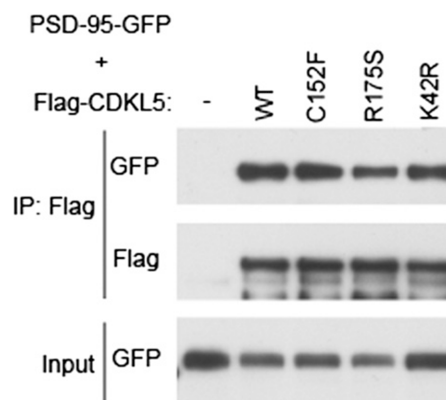

Fig. S5. Kinase activity is not required for the interaction of CDKL5 with PSD-95. Coimmunoprecipitations were performed in 293T cells coexpressing PSD-95-GFP and Flag-tagged CDKL5 (or its mutants) using Flag antibody. Immunoprecipitates and lysates (input) were immunoblotted with antibodies against GFP and Flag.

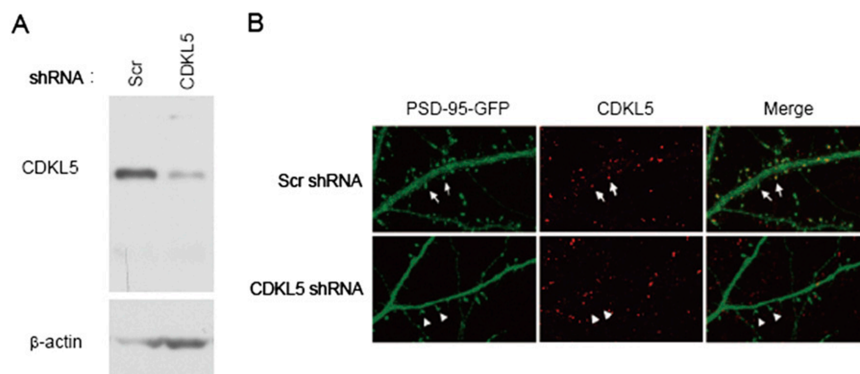

Fig. S6. Validation of CDKL5 knockdown efficiency. (*A*) Scrambled (Scr) or CDKL5 shRNA was electroporated into cultured neurons. Five days after transfection, cells were harvested and the lysates were immunoblotted with CDKL5 and β-actin antibodies. (*B*) Knockdown of CDKL5 reduces synaptic CDKL5 levels. Hippocampal neurons were transfected with scrambled or CDKL5 shRNA together with PSD-95-GFP. Five days after transfection, cells were fixed by methanol and stained for CDKL5. Arrows indicate CDKL5 clusters in control cells; arrowheads indicate CDKL5 clusters in CDKL5 knockdown cells.

Fig. S7. Down-regulation of CDKL5 suppresses dendritic spine formation and function. (*A*) Representative images of hippocampal neurons transfected at DIV 14 with GFP together with scrambled or CDKL5 shRNA. Cells were fixed at DIV 18 and stained for GFP. [Scale bars: 20 μm (*Upper*) and 10 μm (*Lower*).] (*B*) Quantification of spine density in neurons in *A*. $n = 20$ neurons for each condition; $^{***}P < 0.001$; *t* test. (*C*) Cumulative distribution of spine length plotted for the indicated conditions. $P = 0.0392$; K–S test. (*D*) Cumulative distribution of spine width plotted for the indicated conditions. $P < 0.001$; K–S test. (*E*) Representative mEPSC traces for each condition as indicated. (*F*) Quantification of mEPSC amplitude and frequency. $n = 10–15$; $^{***}P < 0.001$; *t* test. (*G*) Effects of CDKL5 knockdown on surface expression of AMPA receptors. Neurons were transfected with scrambled or CDKL5 shRNA together with a GFP expressing vector and stained for surface GluR1 five days after transfection. [Scale bar: 20 μm (*Upper*) and 5 μm (*Lower*).] (*H*) Quantification of surface GluR1 levels in neurons in *G*. $n = 8–12$ neurons for each condition; $^{***}P < 0.001$; *t* test.

Fig. S8. CDKL5-Δ1–670 competes with full-length CDKL5 for binding to PSD-95. (*A*) 293T cells were transfected as indicated. Cells were harvested and the lysates were immunoprecipitated with a Flag antibody. Total lysates and immunoprecipitates were immunoblotted with antibodies against GFP, Flag, and Myc. (*B*) CDKL5-Δ1-670 is not recognized by the CDKL5 monoclonal antibody. The 293T cells were transfected with a plasmid expressing GFP-CDKL5-Δ1-670. Cells were lysed and immunoblotted with the CDKL5 monoclonal antibody, the CDKL5 polyclonal antibody, or a GFP antibody. (*C*) Expression of CDKL5-Δ1-670 in neurons reduces synaptic clustering of endogenous CDKL5. Cultured neurons were transfected with either GFP-CDKL5-Δ1-670 or GFP-actin (control), and 4 d later were stained for endogenous CDKL5 with the CDKL5 monoclonal antibody. Bar graph shows mean ± SEM of CDKL5 cluster intensity, normalized to untransfected neurons. $n = 5–7$ neurons; ***$P < 0.001$ compared with neurons transfected with GFP-actin.

Fig. S9. Model of synaptic targeting of CDKL5 regulated by palmitoylated PSD-95 and a proposed mechanism for CDKL5 to regulate spine growth. PAT, palmitoyl acyltransferase.