Supporting Information

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SI Materials and Methods

Antibodies. The following primary antibodies were used for immunocytochemistry: monoclonal mouse anti-gephyrin (mAb7a, Connex, 1:3,000), polyclonal guinea-pig anti-VIAAT (vesicular inhibitory amino acid transporter) (Synaptic Systems, 1:2,000), monoclonal mouse anti-collybistin (anti-Cb) (BD Biosciences, 1:500), polyclonal mouse anti-TC10 (ab168645, Abcam, 1:100), polyclonal rabbit anti-HA (Zymed Laboratories, Invitrogen, 1:2,000), monoclonal mouse anti-HA (Covance, 1:2,000), polyclonal rabbit anti-c-Myc (C3956, Sigma-Aldrich, 1:1,000), and monoclonal mouse anti-c-Myc (clone 9E10, Sigma-Aldrich, 1:1,000). As secondary antibodies, Alexa Fluor 488 or 555 goat anti-mouse or goat anti-rabbit IgG (Invitrogen, 1:2,000), Alexa Fluor 555 or 633 goat anti-guinea pig IgG (Invitrogen, 1:2,000) or goat anti-mouse IgG Cy5 conjugate (Millipore, 1:1,000) were used. For immunoblotting, polyclonal rabbit anti-TC10 (ab107573, Abcam, 1:250), polyclonal rabbit anti-TC10 (T8950, Sigma-Aldrich, 1:4,000), monoclonal mouse anti-gephyrin (3B11, Synaptic Systems, 1:3,000), monoclonal rat anti-HA conjugated with peroxidase (Roche, 1:10,000), polyclonal rabbit anti-c-Myc (C3956, Sigma-Aldrich, 1:2,000), or anti-GST-HRP conjugate (RPN1236V, GE Healthcare, 1:10,000) were used. As secondary antibodies, peroxidase conjugated AffiniPure goat anti-mouse or goat anti-rabbit IgGs (Jackson ImmunoResearch Laboratories, 1:10,000) were used.

cDNA Constructs. The pEGFP-C2-gephyrin plasmid (1) and the C-terminally HA-tagged and N-terminally Myc-tagged CbII constructs (2–5) have been described previously. The Myc-tagged SH3(+)- or ΔSH3CbII NE/AA and RR/AA mutants were generated by oligonucleotide-directed mutagenesis using the QuikChange mutagenesis kit (Stratagene) according to the manufacturer's instructions. GST tagged SH3(+)- and ΔSH3CbII were generated by cloning the CbII cDNAs (2) into the EcoRI/XhoI sites of the pGEX-4T-1 vector (GE Healthcare). The GST-ΔSH3CbII RR/AA mutant, as well as the GST-SH3(+)CbIIΔPH and GST-PH deletion mutants, were generated in pGEX-4T-1 by oligonucleotide-directed mutagenesis as described above, and the sequences of all mutagenized cDNAs were confirmed by automated DNA sequencing. The N-terminally HA- or Myc-tagged TC10 constructs [WT, constitutively active (CA), or dominant-negative (DN)] were generous gifts from Jeffrey E. Pessin (Albert Einstein College of Medicine, Bronx, New York). GST-TC10 was generated by cloning the *BamHI/EcoRI* fragment from pKMyc-TC10 into the pGEX-4T-1 vector. His-TC10 in pRSET-A was kindly provided by Perihan Nalbant (University of Duisburg-Essen, Essen, Germany). The HA-tagged Cdc42 construct was obtained from the Guthrie cDNA Resource Center (Guthrie Research Institute) and pRK5Myc-Cdc42 from Addgene Inc.

Purification of GST- and His-Tagged Proteins. GST-CbII constructs were expressed in the Rosetta2 *Escherichia coli* strain, which was grown in Terrific Broth at 37 °C to an OD_{600} of 0.6–0.8, transferred to 23 °C, and induced with 0.2 mM isopropyl-α-D-thiogalactopyranosid (IPTG) for 6 h. Bacteria were harvested by centrifugation (4,550 × *g*, 20 min) and resuspended in PBS [140 mM NaCl, 2.5 mM KCl, 1.8 mM KH_2PO_4, and 1 mM Na_2HPO_4 (pH 7.5), 30 mL per 500 mL culture] containing protease inhibitors (1 μg/mL aprotinin, 0.5 μg/mL leupeptin, 1.74 μg/mL PMSF, and 10 mM EDTA). After addition of lysozyme, DNase I, and 1 mM $MgCl_2$, cells were lysed by sonication (VS-70, Sonoplus, Bandelin) and incubated in the presence of 1% Triton-X 100 (vol/vol) for 30 min at 4 °C. Cellular debris was removed by centrifugation (10,000 × *g*, 20 min). Then, 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) was added to the supernatant to a final concentration of 0.5% (wt/vol). The lysate was cleared by ultracentrifugation (148,000 × *g*, 60 min), and the supernatant was loaded onto glutathione Sepharose 4B beads (GE Healthcare) and incubated for 2 h at 4 °C on a rotator. Beads were washed twice with PBS containing 0.5% (wt/vol) CHAPS and protease inhibitors (as above), and three times with 50 mM Tris·HCl (pH 8.0), 500 mM NaCl, 0.5% (wt/vol) CHAPS, and protease inhibitors. Beads were resuspended in elution buffer [50 mM Tris·HCl (pH 8.0), 150 mM NaCl, 10 mM EDTA, 0.5% (wt/vol) CHAPS, and 1 mM DTT].

For kinetic measurements, 5 U thrombin (GE Healthcare) were added per 200 μL of beads, followed by an incubation for 16 h at room temperature. Supernatants were collected and concentrated in Centriprep YM-3 centrifugal filter devices (Amicon, Millipore).

GST-TC10 was expressed and purified as described previously (6). His-TC10 was transformed into BL21 DE3 *E. coli*, grown to an OD_{600} of 0.6–0.8, cooled to 25 °C, and induced with 200 μM IPTG. Cells were harvested after 20 h as described above, resuspended in 10 mL lysis buffer [50 mM Tris·HCl (pH 8.0), 300 mM NaCl, 10 mM imidazole, and protease inhibitors] and lysed as described. After ultracentrifugation (148,000 × *g*, 1 h, 4 °C), 2 mL of a 50% Ni-NTA slurry (Qiagen) and GDP (final concentration 100 μM) were added to the supernatant prior to an incubation for 60 min at 4 °C. After three washes with 50 mM Tris·HCl (pH 8.0), 300 mM NaCl, 20 mM imidazole, 1% (vol/vol) Triton-X-100, and protease inhibitors, and two washes with 50 mM Tris·HCl (pH 8.0), 300 mM NaCl, 50 mM imidazole, 1% (vol/vol) Triton-X-100, and protease inhibitors, elution was performed in two steps, first with 50 mM Tris·HCl (pH 8.0), 300 mM NaCl, 250 mM imidazole, and protease inhibitors and then with 50 mM Tris·HCl (pH 8.0), 300 mM NaCl, 1 M imidazole, and protease inhibitors. Eluates were pooled and dialyzed against a buffer containing 25 mM Tris·HCl (pH 8.0), 200 mM NaCl, and protease inhibitors to remove imidazole in a Slide-A-Lyser Dialysis Cassette, 10,000 molecular weight cutoff (Thermo Scientific).

In Vitro Binding Assays. In vitro binding assays were performed by incubating purified GST-tagged proteins coupled to glutathione-Sepharose beads for 2 h at 4 °C in 50 mM Tris·HCl (pH 7.5), 1 mM DTT, 0.5% (vol/vol) Triton X-100, 200 mM NaCl, and, for nucleotide-free conditions, 5 mM EDTA; for nucleotide-bound conditions, 5 mM $MgCl_2$ was included. After washing four times with at least 20 bead volumes, the beads were resuspended in 50 μL sample buffer, heated at 60 °C for 30 min, and analyzed by Western blotting. MemCode staining (MemCode Reversible Protein Stain Kit for Nitrocellulose Membrane, Thermo Scientific) was used to visualize GST-tagged proteins, and TC10 was detected by immunoblotting using the polyclonal rabbit TC10 T8950 antibody.

In Vivo TC10 and Cdc42 Activation Assays. Glutathione-Sepharose immobilized GST-PAK1-PBD, a GST fusion protein containing the p21-binding domain (PBD, residues 67–150) of human PAK-1, was purchased from Cytoskeleton (PAK02, Tebu-bio). HEK 293 cells were cultured and transfected as described previously (5). Twenty-four hours after transfection, cells were washed twice with ice-cold PBS and lysed for 20 min by adding 0.8 mL of lysis buffer [25 mM Hepes (pH 7.5), 150 mM NaCl, 1% (vol/vol) Igepal CA-630, 2% (vol/vol) glycerol, 25 mM NaF, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM sodium orthovanadate, 10 μg/mL leupeptin, and

10 μg/mL aprotinin]. After centrifugation at 14,000 × *g* for 10 min at 4 °C, the supernatants of the lysates were incubated at 4 °C for 1 h with 15 μg of immobilized GST-PAK1-PBD under constant agitation. The beads were washed four times with lysis buffer and the proteins eluted with sample buffer. Aliquots of the input fractions and eluates were separated on Bis-Tris 4–12% acrylamide gradient NuPAGE gels (Invitrogen). The amounts of total and bound recombinant Myc-TC10 or Myc-Cdc42 were detected by Western blotting using an antibody against the Myc epitope tag. Western blotting was performed in NuPAGE transfer buffer as described previously (7).

In Vitro Guanine Nucleotide Exchange Assays. The kinetic assays were performed as described previously for the measurement of guanine nucleotide exchange factor (GEF)-catalyzed nucleotide exchange reactions (6). In brief, 0.1 μM methylanthraniloyl-GDP (mant-GDP) loaded Cdc42 (amino acids 1–181, human sequence) or TC10 (amino acids 2–193, human sequence) and 2 μM Cb were preincubated in degassed GEF buffer [30 mM Tris·HCl (pH 7.5), 50 mM $MgCl_2$, 3 mM DTT, 10 mM K_2HPO_4/KH_2PO_4 (pH 7.4), and 0.05% (wt/vol) CHAPS] in a fluorescence cuvette at 25 °C for 5 min. The mant fluorescence signal was recorded in a Luminescence Spectrometer LS50B (Perkin-Elmer) using an excitation wavelength of 366 nm and an emission wavelength of 450 nm. After recording a stable baseline fluorescence signal, GDP was added to a final concentration of 20 μM and mixed in rapidly to start the reaction. An exponential decrease in fluorescence was observed in the following 20 h owing to a slow release of mant-GDP into the aqueous solution. The data were fitted exponentially using Grafit software (Erithacus Software Ltd.) to determine the dissociation rates of mantGDP-Cdc42 and mant-GDP-TC10 in the presence and absence of Cb.

Transfection and Immunostaining of COS-7 Cells. COS-7 cells were cultured in DMEM (Gibco, Life Technologies), 10% (vol/vol) FCS (Gibco, Life Technologies), 50 U/mL penicillin, and 50 U/mL streptomycin at 37 °C in 5% CO_2. For transfection, cells were plated in 24-well plates on 12-mm coverslips. Sterile coverslips were coated with poly-L-lysine [Sigma, 0.001% (wt/vol), diluted from 0.01% stock in Dulbecco's PBS, PAA Laboratories] for at least 2 h and washed three times with Dulbecco's PBS before plating. Medium was exchanged to DMEM devoid of supplements before transfection, at ∼80% confluency. For transfections, 200 ng GFP-gephyrin, 100 ng HA-TC10 (WT, CA, or DN) or HA-Cdc42 (WT), and/or 100 ng Myc-Cb DNA were used per well; pcDNA 3.1 was used to equalize the total amount of DNA per transfection to 400 ng. Cells were transfected using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. DMEM containing 10% (vol/vol) FCS and antibiotics was added 4 h after transfection. Cells were fixed 10 h after transfection in 4% (wt/vol) paraformaldehyde in PBS for 10 min. After three washes in PBS, cells were permeabilized using 40 μg/mL digitonin or 1% (vol/vol) Triton X-100 in PBS. Blocking was performed in 10% (vol/vol) goat serum in PBS for at least 60 min. For immunostaining, antibodies were diluted as indicated above in 10% (vol/vol) goat serum in PBS, and cells were incubated for 1.5 h with primary and 45 min with secondary antibodies. Cells were washed three times with PBS before mounting with Aqua Poly/Mount (Polysciences) and inspected under an AxioImager Z1 equipped with a Zeiss apochromat 63× objective (Zeiss). For GFP-gephyrin, exposure times were kept constant at 170 ms. Quantifications were performed using the ImageJ software package (http://rsb.info.nih.gov/ij).

Transfection and Immunostaining of Cultured Rat Hippocampal Neurons. Cultures of hippocampal neurons were prepared from embryonic day 18 (E18) rats. Hippocampi were treated with trypsin in HBSS (Gibco, Life Technologies) for 15 min at 37 °C and mechanically triturated. Cells were plated on poly-L-lysine–coated glass coverslips at a density of 120,000 cells/mL in Neurobasal medium supplemented with B27 (Gibco, Life Technologies), Glutamax (Gibco, Life Technologies), and penicillin–streptomycin (Roche). Neurons were transfected at days in vitro (DIV) 4 using the CalPhos mammalian transfection kit (Clontech) and either 2 μg Myc-SH3(+)- or ΔSH3CbII and 1 μg pcDNA3 empty vector (Invitrogen) or 2 μg Myc-SH3(+)CbII and 1 μg HA-TC10 (CA or DN), respectively, per well of a six-well plate. For electrophysiological recordings and related immunostainings, 1 μg pEGFP-N1 (Clontech) was additionally included in the transfection mixture. Immunocytochemistry with the antibodies listed above was performed as described previously (5), except for endogenous TC10 immunostaining. Here, neurons were fixed for 5 min at −20 °C with methanol and processed directly for immunocytochemistry using a polyclonal mouse TC10 antibody, which was preincubated in PBS for 2 h at room temperature on a rotator, in the absence or presence (antibody preabsorption) of GST-TC10 immobilized on glutathione-Sepharose beads. Pictures of transfected hippocampal neurons were taken with an AxioImager Z1 equipped with a Zeiss apochromat 63× objective and an Apotome module (Zeiss), or an inverse Leica DMIRE2 microscope equipped with a 63× oil-immersion objective and connected to a Leica TCS SP2 AOBS confocal laser scanning setup (Leica Microsystems). Acquired images were processed identically using the ImageJ software package (http://rsb.info.nih.gov/ij). Single channels were recorded using the same standardized threshold levels. Subsequently a binary image was generated, and particles were counted automatically by the ImageJ software. All values represent means ± SEM. Statistical significance was evaluated with the one-way ANOVA variance test followed by Tukey's multiple comparison test, always applying a 95% confidence interval.

Electrophysiological Recording of GABAergic Miniature Inhibitory Postsynaptic Currents from Dissociated Rat Hippocampal Neurons. Between DIV 12 and DIV 15, neurons were whole-cell voltage-clamped at −70 mV using a MultiClamp 700B amplifier (Axon Instruments, Molecular Devices) under the control of the Clampex program 10.1 (Molecular Devices). In all experiments the following solutions were used: extracellular solution (140 mM NaCl, 2.4 mM KCl, 10 mM Hepes, 10 mM glucose, 4 mM $CaCl_2$ and 4 mM $MgCl_2$, 320 mOsmol/L, pH 7.4); patch-pipette solution (136 mM KCl, 17.8 mM Hepes, 1 mM EGTA, 0.6 mM $MgCl_2$, 4 mM NaATP, 0.3 mM Na_2GTP, 15 mM creatine phosphate, and 5 U/mL phosphocreatine kinase, 315–320 mOsmol/L, pH 7.4). Miniature inhibitory postsynaptic currents (mIPSCs) were recorded in the presence of 300 nM tetrodotoxin (TTX) and 10 μM 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo (*F*)quinoxaline (NBQX). High [K^+] evoked inhibitory postsynaptic currents (eIPSCs) were elicited in a solution containing 300 nM TTX, 10 μM NBQX, 90 mM NaCl, 50 mM KCl, 10 mM Hepes, 4 mM $CaCl_2$, 4 mM $MgCl_2$, and 10 mM glucose (320 mOsmol/L, pH 7.3). GABA-induced currents were recorded with 10 μM GABA, 300 nM TTX, and 10 μM NBQX in the extracellular solution. All extracellular solutions were applied with a custom-built fast flow system consisting of an array of flow pipettes controlled by a stepper motor that allows for complete and rapid solution exchange with time constants of ∼30 ms. Except for TTX (Tocris Bioscience), chemicals were purchased from Sigma-Aldrich. Data were analyzed using Axograph X version 1.3.1.

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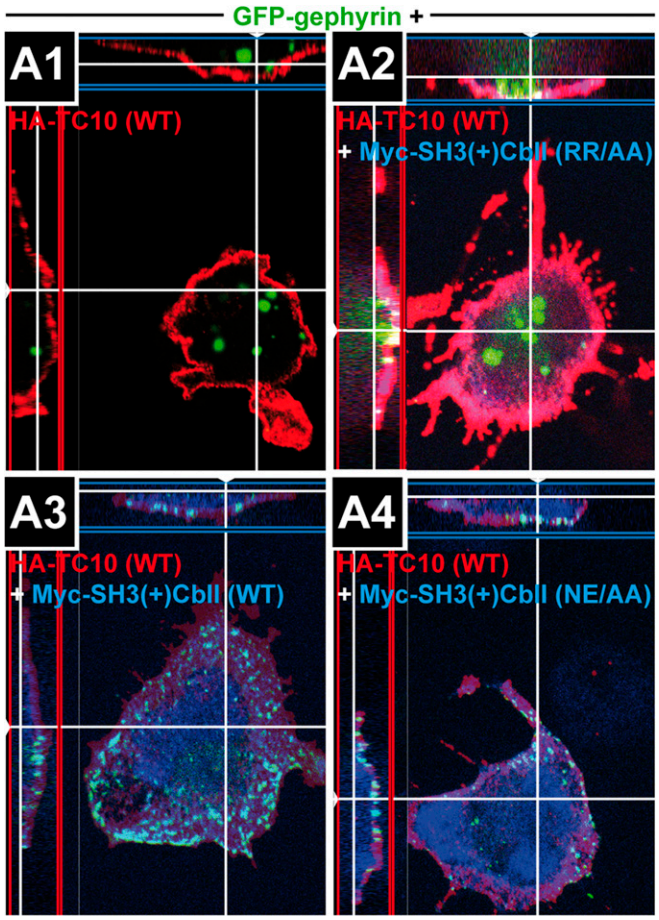

Fig. S1. TC10 triggers Cb-mediated redistribution of GFP-gephyrin into submembraneous microclusters. (*A1–A4*) 2D compressed Z-stack overlaid images of COS-7 cells expressing GFP-gephyrin and HA-TC10 in the absence of Cb (*A1*) or together with Myc-SH3(+)CbII WT (*A3*), its corresponding PI(3)P-binding deficient RR/AA mutant (*A2*), or the GEF-deficient mutant NE/AA (*A4*). Images were processed with the CutView algorithm provided with AxioImager software (Zeiss) using a maximum intensity projection algorithm. The CutView panels depict two perpendicular transverse sections per 2D compressed image as indicated by white lines.

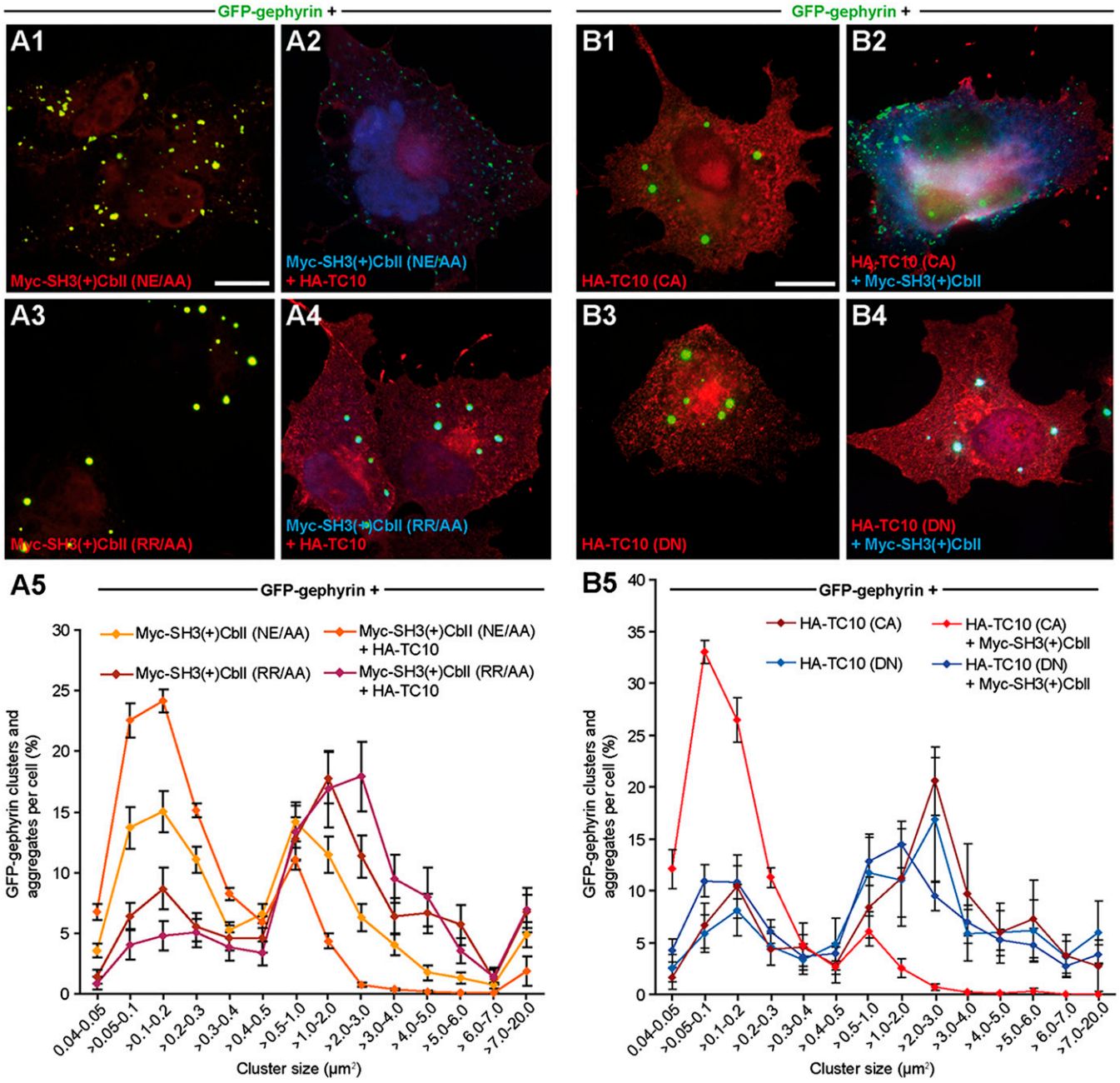

Fig. S2. (*A*) Lipid binding of Cb is required for TC10-mediated gephyrin redistribution. (*A1–A4*) Epifluorescent images of GFP-gephyrin in COS-7 cells coexpressing Myc-SH3(+)CbII NE/AA (*A1*), Myc-SH3(+)CbII NE/AA and HA-TC10 (*A2*), Myc-SH3(+)CbII RR/AA (*A3*), or Myc-SH3(+)CbII RR/AA and HA-TC10 (*A4*). The GEF-deficient mutant, NE/AA, triggers the redistribution of GFP-gephyrin into microclusters together with HA-TC10 (*A2*), whereas the PI(3)P-binding deficient RR/AA-mutant prevents HA-TC10 from triggering gephyrin microcluster formation (*A4*). (Scale bar, 10 μm.) (*A5*) Gephyrin puncta in cells transfected as indicated, binned according to their size. Results are mean values ± SEM (n = 29–31 cells per transfection condition). (*B*) TC10 CA, but not TC10 DN, stimulates SH3(+)CbII-mediated gephyrin microcluster formation. (*B1–B4*) Epifluorescent images of GFP-gephyrin in COS-7 cells coexpressing HA-TC10 CA (*B1*), Myc-SH3(+)CbII and HA-TC10 CA (*B2*), HA-TC10 DN (*B3*), or Myc-SH3(+)CbII and HA-TC10 DN (*B4*). HA-TC10 CA stimulates the SH3(+)CbII-mediated redistribution of GFP-gephyrin into microclusters (*B2*), whereas HA-TC10 DN does not promote SH3(+)CbII-induced gephyrin microcluster formation (*B4*). (Scale bar, 10 μm.) (*B5*) Gephyrin puncta in cells transfected as indicated, binned according to their size. Results are mean values ± SEM (n = 9–30 cells per transfection condition).

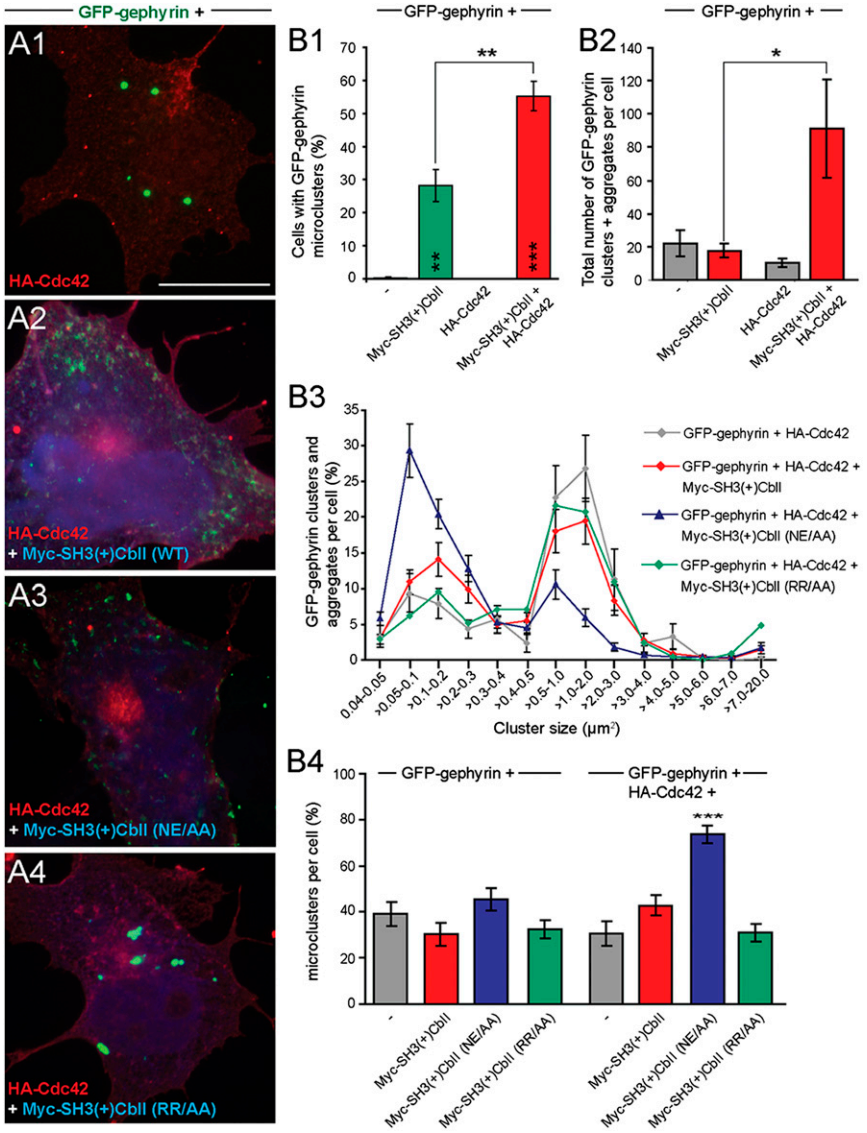

Fig. S3. Cdc42 triggers Cb-dependent gephyrin microcluster formation in COS-7 cells. (*A1–A4*) Epifluorescent images of transfected COS-7 cells, as indicated. GFP-gephyrin accumulates in large cytoplasmic aggregates when coexpressed together with HA-Cdc42 (*A1*). HA-Cdc42 together with Myc-SH3(+)CbII WT (*A2*) or its corresponding GEF-deficient NE/AA mutant (*A3*) jointly trigger GFP-gephyrin microcluster formation. In contrast, the PI(3)P-binding–deficient RR/AA mutant prevents HA-Cdc42 from triggering gephyrin microcluster formation (*A4*). (Scale bar, 20 μm.) (*B1*) Quantification of the percentage of GFP-gephyrin (co)transfected cells classified as having GFP-gephyrin microclusters (>50 puncta per cell). Results represent mean values ± SEM (n = 3 independent transfections, n = 284–500 cells per transfection condition); significance levels compared with cells transfected only with GFP-gephyrin (first bar) are indicated at the bottom of the bars (*P < 0.05; **P < 0.01; ***P < 0.001). (*B2*) Quantification of the total number of GFP-gephyrin clusters and aggregates from epifluorescent images of transfected COS-7 cells. Results are mean values (± SEM) obtained from 23–37 transfected cells per transfection condition (*P < 0.05). (*B3*) Gephyrin puncta counted in *B2* were binned according to their size. Results are mean values ± SEM (n = 23–37 cells per condition). (B4) Quantification of the percentage of microclusters (0.04–0.4 μm²) per cell from epifluorescent images of transfected COS-7 cells, as indicated. Results are mean values (± SEM, n = 30 cells per condition); statistical significance levels are compared between conditions without coexpression of HA-Cdc42 (first four columns) and the corresponding co-transfections with HA-Cdc42 (***P < 0.001).

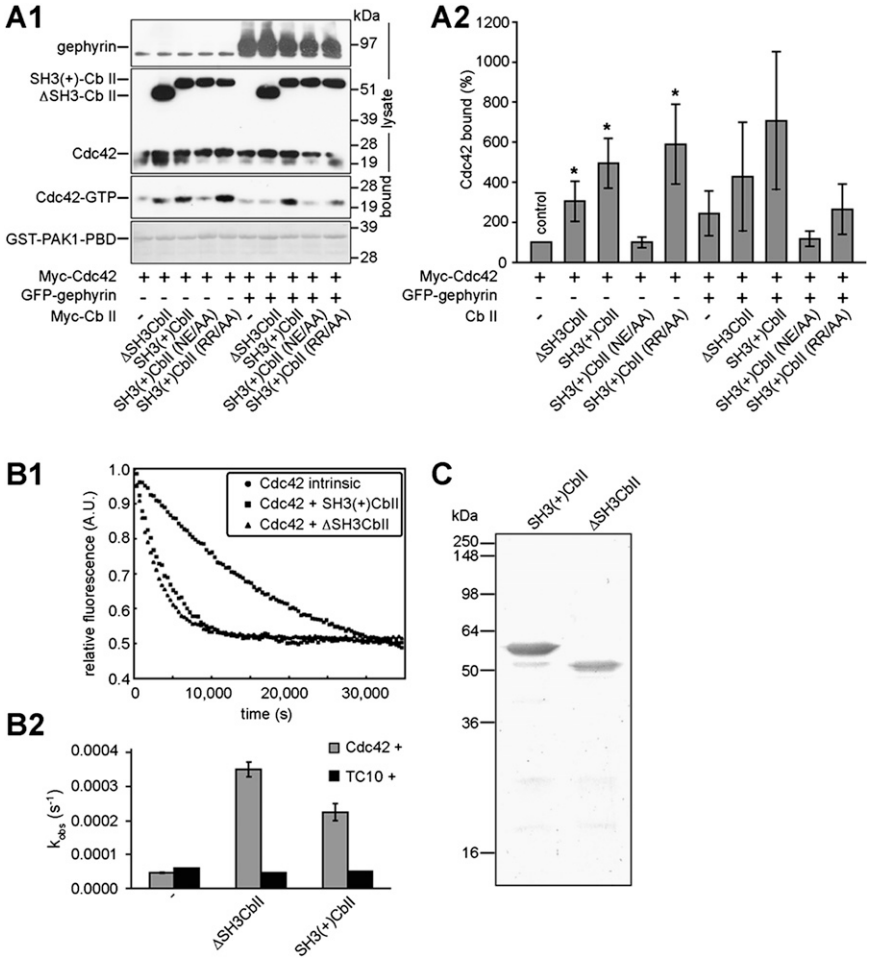

Fig. S4. Both ΔSH3CbII and SH3(+)CbII activate Cdc42 in nonneuronal cells and in vitro. (*A1* and *A2*) HEK 293 cells were transfected with Myc-Cdc42 either alone (-) or together with different Myc-tagged Cb constructs, as indicated, in the absence or presence (*A1, Top*, last 5 lanes) of GFP-gephyrin. Twenty-four hours after transfection, cells were lysed, and lysates were used for pull-downs with immobilized GST-PAK1-PBD. Bound GTP-Cdc42 was detected by Western blotting with an anti-Myc antibody. MemCode staining (*Bottom*) was used to confirm that equal amounts of GST-PAK1-PBD had been added to each lysate. (B) Relative band intensities of bound recombinant Cdc42. Results represent means (± SEM) from three independent experiments; statistical significance was compared with cells expressing only Myc-Cdc42 (first bar; $*p < 0.05$). (*B1* and *B2*) The ability of bacterially expressed and purified untagged ΔSH3CbII or SH3(+)CbII to activate bacterially expressed and purified C-terminally truncated Cdc42 or TC10 was tested in vitro. (*B1*) Exemplary traces of mant-GDP dissociation from 0.1 μM Cdc42, monitored spectroscopically after the addition of 20 μM unlabeled GDP either in the absence of Cb (circles) or in the presence of 2 μM SH3(+)CbII (rectangles) or 2 μM ΔSH3CbII (triangles). A.U., arbitrary units. (*B2*) The rate constants (k_{obs}) of the intrinsic, ΔSH3CbII-catalyzed or SH3(+)CbII-catalyzed exchange reactions determined in the presence of 20 μM GDP are shown for both 0.1 μM mant-GDP–bound Cdc42 and 0.1 μM mant-GDP–bound TC10. The k_{obs} values were obtained by single exponential fitting of the data. Note that SH3(+)CbII and ΔSH3CbII catalyzed rate accelerations of 4.8- and 7.5-fold, respectively. For Cdc42, mean values (± SEM) from three to four independent experiments are shown. (*C*) Analysis of samples taken at the end of the in vitro exchange assay by SDS/PAGE and Coomassie staining, indicating that the comparable GEF activities of both isoforms toward Cdc42 were not due to proteolytic cleavage of the SH3 domain of SH3(+)CbII during prolonged incubation at 25 °C. The SH3 domain thus does not inhibit the GEF activity of Cb toward Cdc42. The positions of the molecular mass markers (in kDa) are indicated by horizontal lines.

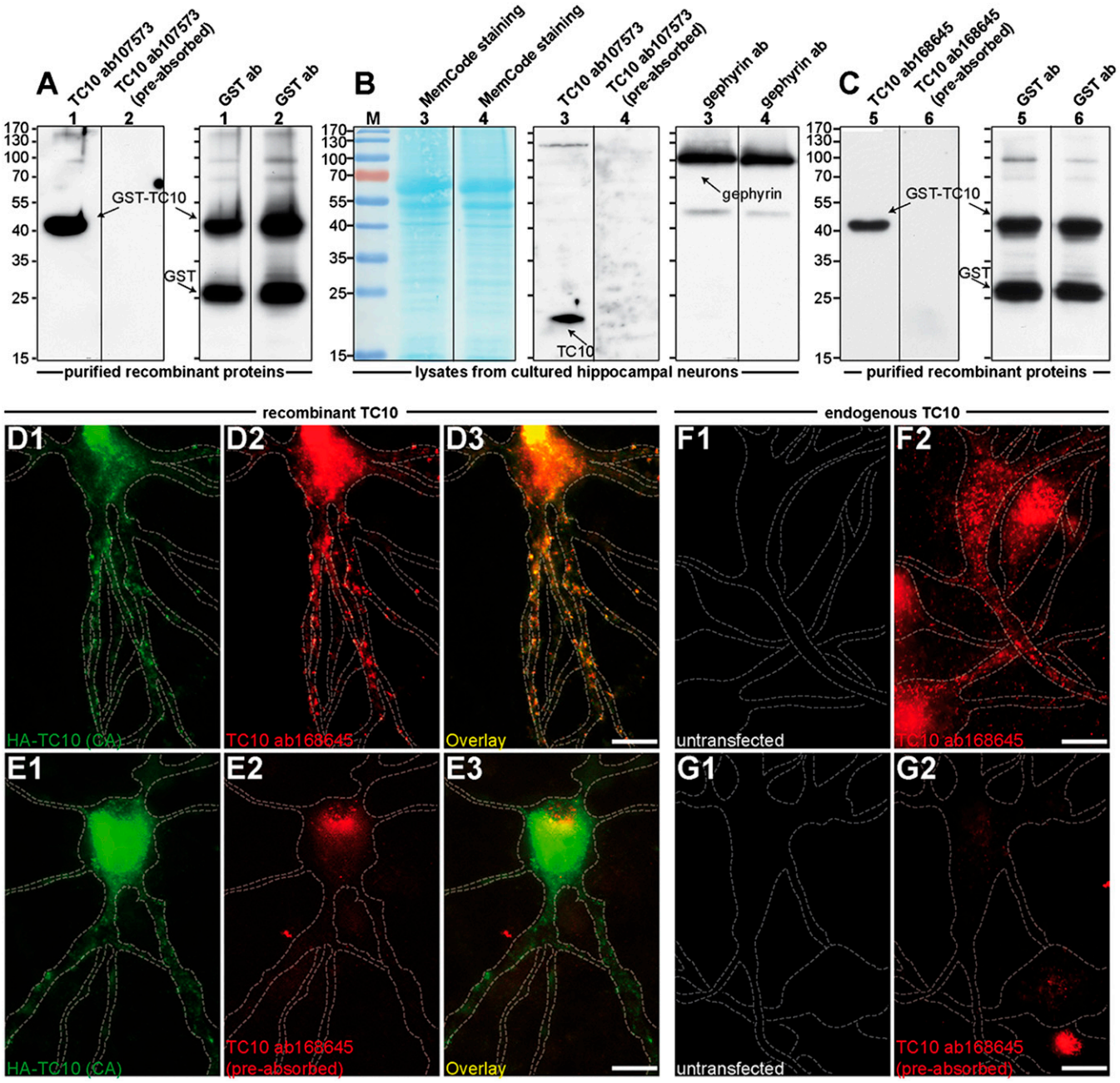

Fig. S5. Expression of TC10 in rat hippocampal neurons. (*A*) The specificity of a polyclonal rabbit TC10 antibody (ab107573, Abcam) was examined by Western blotting on membranes to which equal amounts of purified recombinant GST and GST-TC10 proteins had been transferred. GST-TC10 but not GST was detected only when using supernatants of a PBS solution in which the antibody had been preincubated for 2 h at 4 °C in the absence of purified GST-TC10 immobilized on glutathione-Sepharose beads (1, *Left*), followed by centrifugation (2,000 × *g*, 1 min), whereas preincubation with immobilized GST-TC10 eliminated antibody detection (2, *Left*). In contrast, a GST-specific antibody detected both GST-TC10 and GST on the same membranes (1 and 2, *Right*), indicating that equal amounts of recombinant proteins had been loaded. (*B*) Western blotting of protein lysates prepared from DIV 14 cultured rat hippocampal neurons using ab107573 (3 and 4, *Center*). Note that a TC10-specific band of 22 kDa could be only detected when GST-TC10 was not included in the preincubation solution (3, *Center*) but not upon preabsorption of the antibody with immobilized GST-TC10 (4, *Center*). MemCode stainings of the membranes before blocking and incubation with antibodies confirmed that equal amounts of protein had been loaded (3 and 4, *Left*). In addition, postincubation of the membranes with a gephyrin-specific antibody (3B11, Synaptic Systems) revealed bands of equal intensity on both membranes (3 and 4, *Right*). (*C*) The specificity of a polyclonal mouse TC10 antibody (ab168645, Abcam) was tested using the same procedure as described in *A*. (*D*–*G*) Cultured rat hippocampal neurons were either transfected at DIV 4 with cDNA encoding HA-TC10 CA (*D1* and *E1*) or left untransfected (*F1* and *G1*). At DIV 14, the neurons were fixed and immunostained for HA-tag and TC10 using the polyclonal mouse antibody (ab168645) preincubated in the absence (*D2* and *F2*) or presence of immobilized GST-TC10 (*E2* and *G2*), as described in *A*. After preincubation of ab168645 in the absence of immobilized GST-TC10, extensive punctate immunoreactivity was seen in neurites of both the HA-TC10 CA transfected (*D2*) and the untransfected (*F2*) neurons. In contrast, only weak nuclear immunofluorescence was seen after preabsorption of ab168645 with immobilized GST-TC10 in both classes of neurons (*E2* and *G2*). Moreover, in HA-TC10 CA transfected neurons ab168645 immunoreactivity largely colocalized with HA immunoreactivity (*D1*–*D3*). This result is consistent with ab168645 detecting endogenous TC10. (Scale bars, 10 μm.)

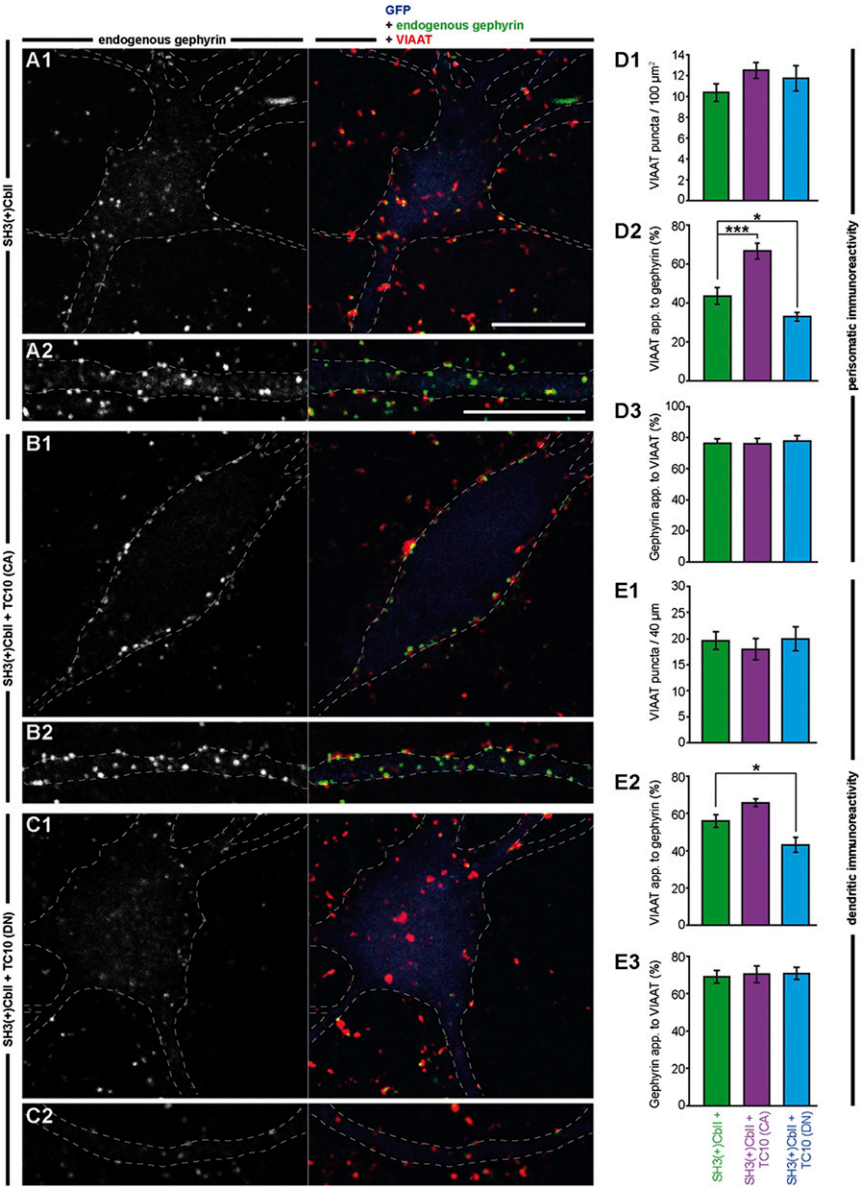

Fig. S6. TC10 activity regulates SH3(+)CbII-mediated gephyrin clustering without affecting presynaptic VIAAT immunoreactivity. (*A*–*C*) Cultured rat hippocampal neurons were transfected at DIV 4 with plasmids encoding GFP and Myc-SH3(+)CbII (*A1*–*A2*) or GFP, Myc-SH3(+)CbII, and either HA-TC10 CA (*B1* and *B2*) or DN (*C1* and *C2*). At DIV 14, neurons were fixed and stained with anti-gephyrin, anti-VIAAT, anti-Myc, or anti-HA antibodies. For clarity, only endogenous gephyrin (green) and VIAAT (red) immunoreactivities are shown in the confocal images from representative somatic (*A1*–*C1*) or dendritic (*A2*–*C2*) areas of transfected neurons. GFP (pseudocolored blue) was used to identify transfected neurons for electrophysiological recordings. (Scale bar, 10 μm in *A1*–*C2*.) Dotted lines in *A1*–*C2* indicate the somatic and dendritic borders of the transfected neurons. (*D* and *E*) Quantifications of perisomatic (*D1*–*D3*) or dendritic (*E1*–*E3*) VIAAT immunoreactivities and percentages of gephyrin clusters apposed to VIAAT or VIAAT puncta apposed to gephyrin in perisomatic (*D2* and *D3*; n = 423–562 analyzed clusters) or dendritic (*E2* and *E3*; n = 260–315 analyzed clusters) areas of neurons transfected as described above. Consistent with the differences observed in gephyrin cluster densities upon TC10 CA or DN coexpression (Fig. 4 *B1* and *C1*), the data shown in *D2* and *E2* indicate that at DIV14 (i.e., during synaptogenesis) TC10 activity increases the number of gephyrin scaffolds apposed to presynaptic sites. Bars in *D1*–*E3* correspond to values obtained from the perisomatic surface area and one randomly selected second-order dendrite (60–100 μm distal to the soma) per neuron, respectively (n = 13–15 cells). Data represent means (± SEM) from two independent experiments each (*$P < 0.05$, ***$P < 0.001$).

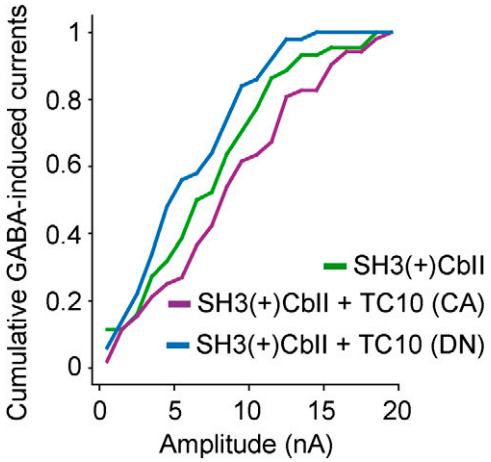

Fig. S7. Opposing effects of TC10 CA and TC10 DN on currents elicited by exogenously applied GABA. GABA-induced currents of neurons coexpressing GFP and Myc-SH3(+)CbII without (green) or together with HA-TC10 CA (purple) or HA-TC10 DN (blue), respectively. TC10 CA coexpression resulted in increased current responses to exogenously applied GABA, whereas TC10 DN coexpression reduced GABA-induced currents. The Kolmogorov-Smirnov test indicated a significant difference between TC10 CA and TC10 DN coexpressing neurons ($P = 0.011$). However, the current amplitudes of the two groups only showed a tendential but not statistically significant difference to those elicited in GFP, SH3(+)CbII only expressing neurons. We attribute this to the presence of extrasynaptic $GABA_A$ receptors, which are also activated by exogenous application of GABA. Data represent means of $n = 53–59$ neurons from 2 independent experiments.

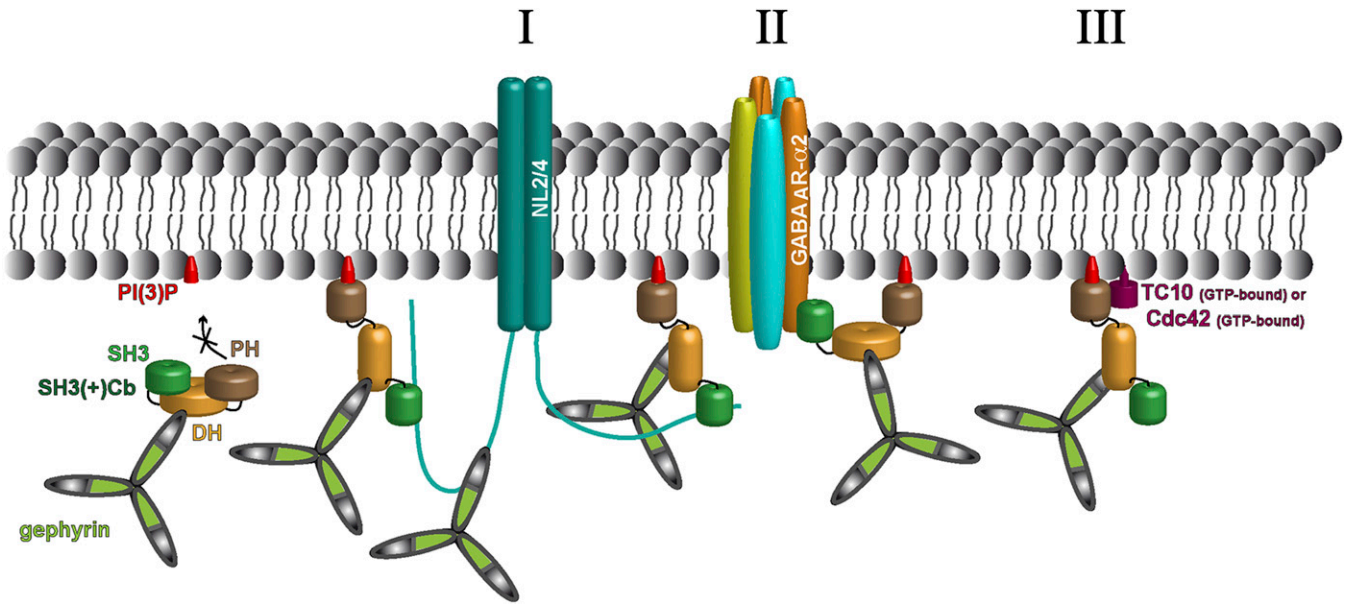

Fig. S8. Schematic representation of Cb activation mechanisms. Gephyrin trimers associated with Cb are transported to subsynaptic sites. During transport, Cb adopts a closed conformation, in which the SH3 and pleckstrin homology domains fold back onto the Dbl homology domain, similar to what is known for the autoinhibited form of Asef, the closest homolog of Cb (1). In this closed conformation, PI(3)P binding by the PH domain is masked due to steric hindrance (Left). At postsynaptic sites, NL2 or/and NL4 (mechanism I) as well as the α2-subunit of $GABA_AR_s$ (mechanism II) interact specifically with the SH3 domain of Cb (2–4). In addition, membrane-bound active TC10 or Cdc42 (mechanism III) are able to recruit Cb by binding to the PH domain (present study). All these intermolecular interactions interfere with intramolecular ones between the different domains of Cb, leading to a transition toward an open state, which allows the PH domain to bind PI(3)P. Together with Cb, gephyrin is corecruited to PI(3)P-rich membrane domains and subsequently assembles into the postsynaptic gephyrin scaffold.

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