Figure S1

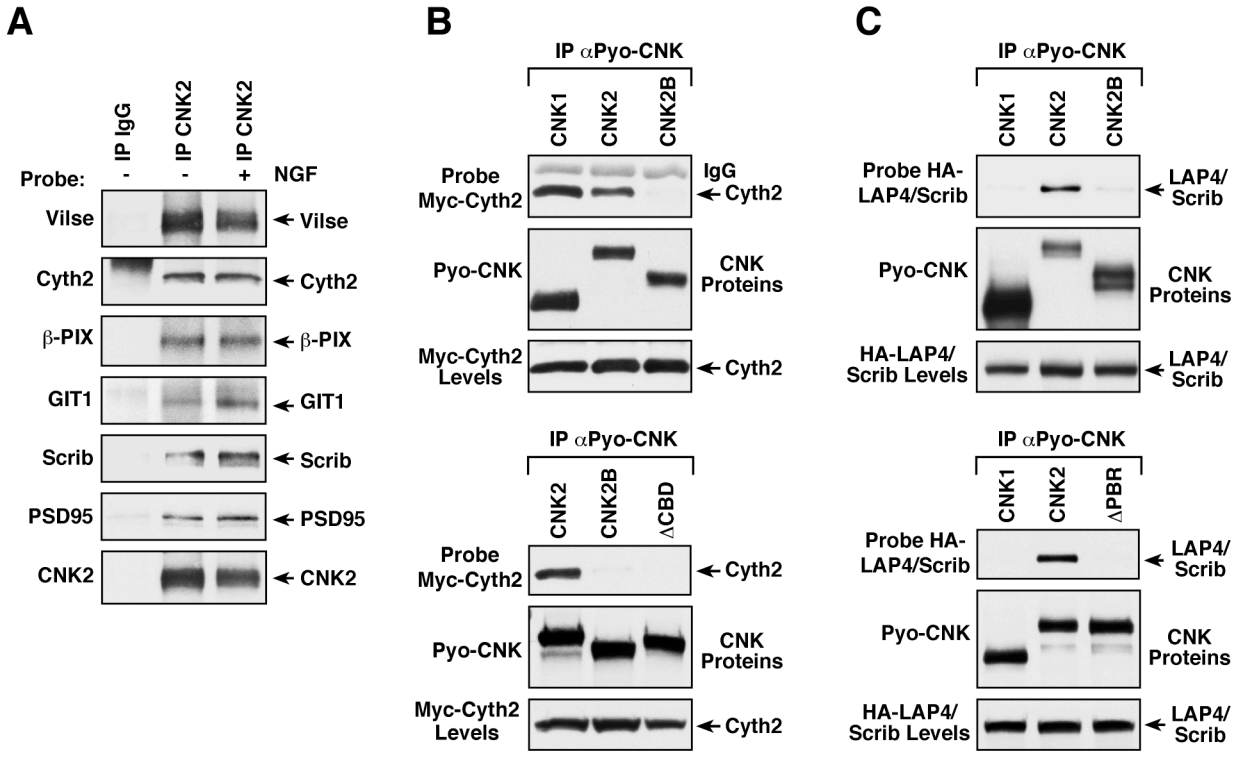

Figure S1. Analysis of CNK2 Protein Interactions (Related to Figure 1)

(A) Endogenous CNK2 proteins were immunoprecipitated from NG108 cells that were untreated or treated with NGF for 18 hrs. Control IgG immunoprecipitates were also prepared. The control and CNK2 complexes were then probed for the binding of the indicated proteins by immunoblot analysis. (B) Pyo-CNK proteins were immunoprecipitated from lysates of 293 cells co-expressing the indicated Pyo-CNK and Myc-Cytohesin-2 (Cyth-2) proteins. The immune complexes were then probed for the presence of Myc-Cyth-2 by immunoblot analysis. CNK1, CNK2, CNK2B (short isoform ending at amino acid 899), and a CNK2 mutant lacking the coil-coiled cytohesin binding domain (residues 907-990; ΔCBD) were used in this analysis. (C) Pyo-CNK proteins were immunoprecipitated from lysates of 293 cells co-expressing the indicated Pyo-CNK and HA-LAP4/Scrib (Scrib) proteins. CNK1, CNK2, CNK2B, and a CNK2 mutant lacking the PDZ domain binding residues ETHV (ΔPBR) were used in this analysis.

Figure S2

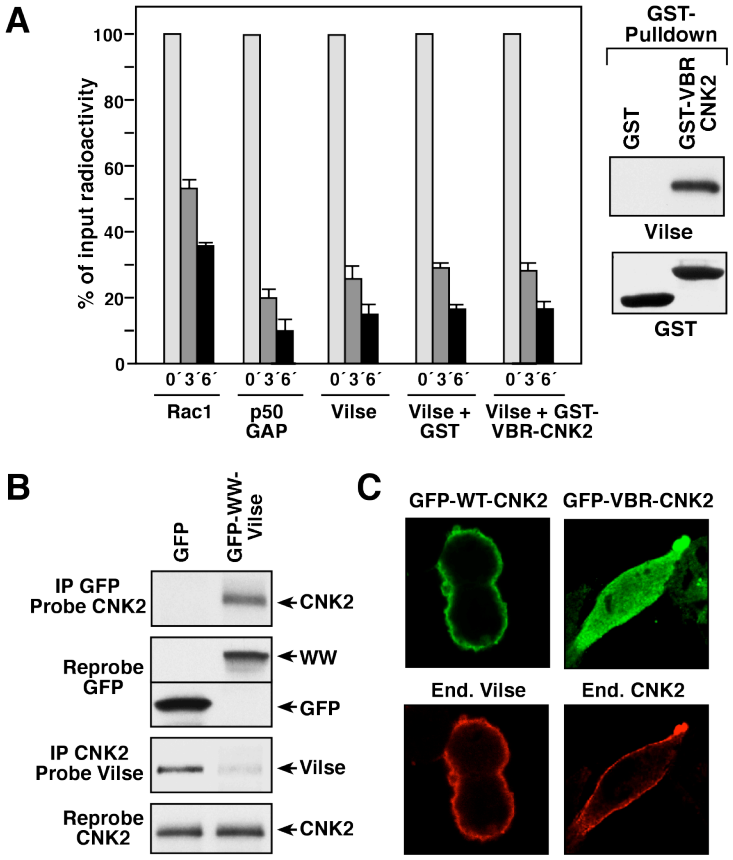

Figure S2. Effects of the CNK2/Vilse Interaction (Related to Figure 2)

(A) *In vitro* Rac GTPase (GAP) activity assay of Rac1, p50GAP, Vilse, and Vilse bound to VBR-CNK2. 100% corresponds to the input of ^{32}P-GTP-bound protein and error bars indicate SD. These results indicate that direct binding of the Vilse WW domain to the P1m motif of CNK2 does not alter the GAP activity of Vilse. (B) NG108 cells expressing GFP or GFP-WW-Vilse were examined for endogenous complex formation between Vilse and CNK2. Expression of GFP-WW-Vilse disrupts the endogenous CNK2/Vilse interaction. (C) Intracellular localization of the indicated proteins was determined by confocal microscopy. Endogenous Vilse colocalizes with GFP-WT-CNK2 at the plasma membrane. Expression of GFP-VBR-CNK2 has no effect on the plasma membrane localization of endogenous CNK2.

Figure S3

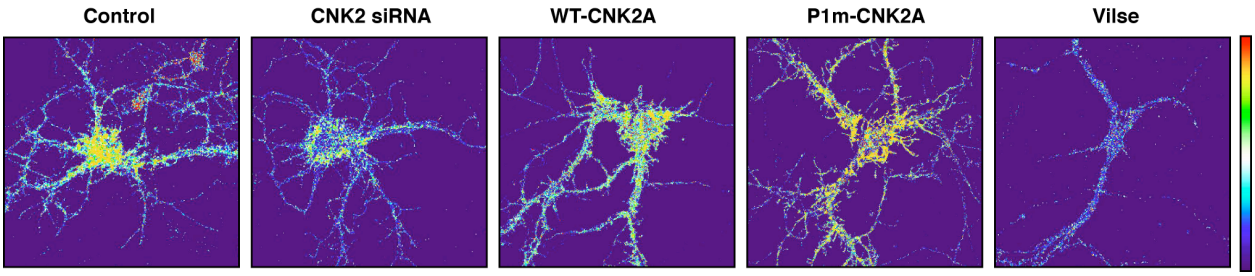

Figure S3. FRET Analysis of Primay Rat Hippocampal Neurons (Related to Figure 4)

Primary rat hippocampal neurons were transfected with the RaichuEV-Rac1 FRET reporter and CNK2-siRNA or Pyo-WT-CNK2, Pyo-P1m-CNK2, Flag-Vilse constructs at 8 DIV and 48-60 hrs later. Ratio images of FRET efficiency from a representative neuron is shown.

Table S1. Mass Spectrometry Analysis of CNK2 Complexes

Supplemental Experimental Procedures

Antibodies, Reagents and DNA Constructs

Glu-Glu and Hemagglutinin (HA) antibodies were from Covance; Cytohesin-2 and Flag antibodies were from Sigma-Aldrich; Myc (9E10) and Scribble antibodies were from Santa Cruz Biotechnology; rabbit GFP and Vilse (KIAA1688) antibodies were from Abcam; mouse GFP antibody was from Roche; β-PIX antibody was from Cell Signaling; GIT1 antibody was from BD Bioscience; Rac1 antibody was from Cytoskeleton; PSD-95 antibody was from Thermo Scientific. CNK2 antibody was a generous gift from Dr. Michael White (University of Texas Southwestern Medical Center). A human Vilse cDNA clone was obtained from Kazusa DNA Research Institute, Japan and the full-length human CNK2 cDNA construct (encoding amino acids 1-1034) was a kind gift from Dr. K-L. Guan (University of California at San Diego). pcDNA3 Flag-N´-WW-Vilse and Flag-C´-Vilse were generated by PCR amplification as were GFP-WW-Vilse, GFP-VBR-CNK2 (encoding CNK2 residues 321-390, that encompass the Vilse binding region), and GST-VBR-CNK2. siRNA-resistant WT- and P1m-CNK2 were generated by site-directed mutagenesis as were P1m-, P2m-, YIPm-CNK2. The RaichuEV-Rac1 (Raichu-2248X) FRET probe was provided by Dr. Michiyuki Matsuda (Kyoto University, Kyoto, Japan). For depletion of endogenous CNK2, siRNA recognizing both rat and mouse CNK2 sequences (siGENOME siRNA D-095509-01-0010; Sequence: UGAUUCAGCUGGCAAAUAU, nucleotides 644-662; Dharmacon) was used.

Cell Culture and Transfection

NG108 cells were cultured in DMEM containing 10% FBS, 1% HT supplement, and 1% L-Glutamine and transfected using Expressfect (Denville Scientific Inc.), Lipofectamine 2000, or Lipofectamine LTX (Invitrogen). Rat E18 embryonic hippocampal neurons were purchased from Genlantis. Dissociated neurons were seeded onto poly-D-lysine and laminin-coated

coverslips (~295 cells/mm^2) and cultured in Neurobasal medium containing B27 supplement and GlutaMax. Neurons were transfected with Lipofectamine 2000 or Lipofectamine LTX.

Co-immunoprecipitation Assays

For co-immunoprecipitation assays, cells were lysed in 1% NP-40 buffer (20 mM Tris pH 8.0, 137 mM NaCl, 10% glycerol, 1% NP-40 alternative, 0.15 U/mL aprotinin, 1 mM PMSF, 0.5 mM sodium vanadate, 20 μM leupeptin), and lysates were clarified by centrifugation. Equivalent amounts of protein lysate were incubated with the appropriate antibody and Protein G Sepharose beads for 3 hr at 4°C. Complexes were washed extensively with 1% NP-40 buffer and examined by immunoblot analysis.

RacGTP Activation Assay

To detect the levels of GTP-bound Rac present in cell lysates, a GST fusion protein containing the PAK1-binding domain (GST-PAK-PBD; Cytoskeleton) was used in pull-down assays. Briefly, cells were lysed in 1% NP-40 lysis buffer, and clarified lysates were incubated with glutathione beads containing GST-PAK-PBD for 1 hr at 4ºC with rocking. The beads were washed extensively with 1% NP-40 buffer, and binding of endogenous GTP-bound Rac to GST-PAK-PBD was determined by immunoblot analysis.

RacGTPase Assay

Flag-Vilse protein was expressed in 293 cells and affinity purified using M2-coupled separose beads (Sigma) from cells lysed under stringent conditions using RIPA lysis buffer (20 mM Tris pH 8.0, 137 mM NaCl, 10% glycerol, 1% NP-40 alternative, 0.5% sodium deoxycholate and 0.1% SDS, 0.15 U/mL aprotinin, 1 mM PMSF, 0.5 mM sodium vanadate, 20 μM leupeptin). GST and GST-VBR-CNK2 proteins were expressed in E. coli and purifed using glutathione-Sepharose beads. It should be noted that full-length CNK2 could not be stably overexpressed in these systems. Protein concentrations were determined, and GST or GST-VBD-CNK2 was

incubated with purified Flag-Vilse at an equimolar concentration prior to RacGTPase (GAP) activity determinations. The GAP assays were conducted as described by Lundtrom et al. [1]. Briefly, 0.1 μg of recombinant Rac1 (Cytoskeleton) was preloaded with 10 μCi[γ-^{32}P]GTP in 20 μl of 20 mM Tris-HCL at pH7.5, 25 mM NaCl, 5 mM EDTA, and 0.1 mM DTT. The mixture was incubated for 10 min at 30ºC, after which the reaction was terminated by adding 5 μl 0.1M $MgCl_2$, and the resulting [γ-^{32}P]GTP-loaded Rac1 was stored on ice. For the GAP assays, equimolar amounts of Rac1 and p50RacGAP (Cytoskeleton) or purified Flag-Vilse were used. Three μl of the [γ-^{32}P]GTP-loaded Rac1 was added to a 30 μl mixture of 20 mM Tris-HCl at pH7.5, 1 mM nonradioactive GTP, 0.87 mg/mL bovine serum albumin, 0.1 mM DTT, with either p50RacGAP, Flag-Vilse alone, Flag-Vilse and GST, or Flag-Vilse and Flag-Vilse and GST-VBR-CNK2. The mixture was incubated at 30ºC, and 5 μl aliquots were removed at 0, 3, and 6 min; the reaction was stopped by the addition of 1 ml ice cold buffer A (50 mM Tris-HCl at pH 7.5, 50 mM NaCl, 5 mM $MgCl_2$). The samples were collected on nitrocellulose filters and washed with 10 ml ice cold buffer A. The portion of [γ-^{32}P]GTP remaining bound to Rac was determined by scintillation counting.

Immunofluorescent Staining

Primary rat hippocampal neurons were fixed in PBS containing 4% PFA and 4% sucrose for 10 min. After washing in PBS, neurons were blocked and stained in gelatin dilution buffer (0.1% gelatin, 0.3% Triton X-100, 16 mM sodium phosphate pH 7.4, 450 mM NaCl) containing primary antibodies for 2 hrs at 25ºC or for 18 hrs at 4ºC. NG108 cells were fixed in freshly prepared 4% paraformaldehyde/PBS for 10 min at 25°C and then permeabilized for 5 min with 0.1% Triton X-100 in PBS. Following blocking for 1 hr in 3% bovine serum albumin (BSA), cells were incubated for 1 hr at 25°C in the appropriate antibody diluted in blocking buffer. Neurons and NG108 cells were then incubated with secondary antibodies conjugated with Alexa fluor-568 or Alexa fluor-488 (Invitrogen). Images were captured using a Zeiss LSM 710

Confocal Laser Scanning Microscope at 63X magnification or a Leica Microsystems fluorescence microscope at 40X magnification.

FRET Imaging Analysis

Neurons were co-transfected with RaichuEV-Rac1 and CNK2 siRNA or various plasmids at 8 DIV and analyzed 48-60 hours after transfection using a Zeiss LSM 710 confocal microscope. Images of the FRET probe were obtained by illuminating cells with a 458 nm laser and collecting signals at a range of 468-530 nm for CFP and 538-596 nm for YPet. Neurons from three different cultures were examined, and the intensity of YPet and CFP from five different areas of dendritic shafts per neuron were measured and averaged. The ratio of YPet/CFP was calculated using ImageJ from background subtracted individual YPet and CFP images as described in Zhang et al [2]. To confirm the presence of FRET, images of CFP and YPet were obtained before and after illuminating cells with 514 nm, and intensities were compared before and after YPet photobleaching.

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2. Zhang, H., Webb, D.J., Asmussen, H., Niu, S., and Horwitz, A.F. (2005). A GIT1/PIX/Rac/PAK signaling module regulates spine morphogenesis and synapse formation through MLC. J Neurosci *25*, 3379-3388.