Material and Methods

DNA Constructs

Phosphorylated and PAGE purified shRNA oligonucleotides were inserted into pLLox3.7 at HpaI and XhoI restriction sites. In short, Oligonucleotides are diluted to 3µg/µl and 1µl each is added to 48µl annealing buffer (100 mM NaCl, 20mM HEPES pH 7.4). Annealing is performed in PCR cycler with denaturing at 94°C for 4 min, then 80°C for 4 min followed by 2.5°C steps every 2 min to 20°C and cooling at 4 degrees. 1µl of annealed oligonucleotides is ligated with cut vector in 10 µl ligation reaction. Digestion with EcoRV, followed by agarose gel electrophoresis (98) verifies proper insertion of annealed oligonucleotides as the loop region contains an additional site. Figure 22 depicts sequences of all shRNA constructs tested with most efficient constructs indicated by red star. Targeting sequences for PSD-95 and PSD-93 completely overlap in mouse and rat. Rat cDNAs of PSD-95, PSD-93, SAP97, and SAP102 were inserted into pGWI vector. Silent mutations in cDNA pointmutant constructs were generated using Quick-Change II (Stratagene) following the manufacturer's instructions. Oligonucleotides used in mutagenesis reactions are shown in Figure 22. Exchanged nucleotides are indicated in red. All DNA constructs were verified by sequencing.

A

shRNA PSD-95

Accession: M96853
Gene ID: 206454
Definition: Rat postsynaptic density protein (PSD-95), homologue of discs-large tumor supressor protein mRNA.
Sequence Length: 3083
Specified Region: 58 -- 2232
GC% Range: 30% -- 60%
Organism: rat

PSD-95 #1 oligos •
for {P}G**TATACTGAGCGATGATCGTGA**TTGATATCCG**TCACGATCATCGCTCAGTATA**TTTTTC
rev {P}TCGAGAAAAA**TATACTGAGCGATGATCGTGA**CGGATATCAA**TCACGATCATCGCTCAGTATA**C

PSD-95 #2 oligos
for {P}G**TAGCTGCTATGACTGATCTCA**TTGATATCCG**TGAGATCAGTCATAGCAGCTA**TTTTTC
rev {P}TCGAGAAAAA**TAGCTGCTATGACTGATCTCA**CGGATATCAA**TGAGATCAGTCATAGCAGCTA**C

PSD-95 #3 oligos
for {P}G**TCTTGGCCTCGAATCGACTAT**TTGATATCCG**ATAGTCGATTCGAGGCCAAGA**TTTTTC
rev {P}TCGAGAAAAA**TCTTGGCCTCGAATCGACTAT**CGGATATCAA**ATAGTCGATTCGAGGCCAAGA**C

PSD-95 #4 oligos
for {P}A**TTCACCTGCAACTCATATCC**TTGATATCCG**GGATATGAGTTGCAGGTGAAT**TTTTTC
rev {P}TCGAGAAAAA**ATTCACCTGCAACTCATATCC**CGGATATCAA**GGATATGAGTTGCAGGTGAAT**

Mutagenesis primers for #1
95i#1muta-for
GGTCAGACG**GT**G**AC**T**AT**A**AT**T**GCTCAGTATA**AACCAGAAGAG
95i#1muta-rev
CTCTTCTGGTT**TATACTGAGC**A**AT**T**AT**A**GT**C**AC**CGTCTGACC

B

shRNA PSD-93

Accession: NM_022282
Gene ID: gi:11560112
Definition: Rattus norvegicus discs, large homolog 2 (Drosophila) (Dlgh2), mRNA.
Sequence Length: 3002
Specified Region: 277 -- 2835
GC% Range: 30% -- 60%
Organism: rat

PSD-93 #1 oligos
for {P}G**TCTAACGTGCCATTGTTACCA**TTGATATCCG**TGGTAACAATGGCACGTTAGA**TTTTTTC
rev {P}TCGAGAAAAAA**TCTAACGTGCCATTGTTACCA**CGGATATCAA**TGGTAACAATGGCACGTTAGA**C

PSD-93 #2 oligos
for {P}G**TTTCTGCTACAAATCTCACAG**TTGATATCCG**CTGTGAGATTTGTAGCAGAAA**TTTTTTC
rev {P}TCGAGAAAAAA**TTTCTGCTACAAATCTCACAG**CGGATATCAA**CTGTGAGATTTGTAGCAGAAA**C

PSD-93 #3 oligos
for {P}G**TAAGAGTGAGTGATATCCGGT**TTGATATCCG**ACCGGATATCACTCACTCTTA**TTTTTTC
rev {P}TCGAGAAAAAA**TAAGAGTGAGTGATATCCGGT**CGGATATCAA**ACCGGATATCACTCACTCTTA**C

PSD-93 #4 oligos •
for {P}G**TAAGTCCTTGGCTAGGCAGTC**TTGATATCCG**GACTGCCTAGCCAAGGACTTA**TTTTTTC
rev {P}TCGAGAAAAAA**TAAGTCCTTGGCTAGGCAGTC**CGGATATCAA**GACTGCCTAGCCAAGGACTTA**C

Mutagenesis primers for #4
93i#4muta-for
GGACAGTG**GACT**A**CC**G**AG**T**CA**G**GGACTTA**GTTTTAAATATG
93i#4muta-rev
CATATTTAAAAC**TAAGTCC**C**TG**A**CT**C**GG**T**AGTC**CACTGTCC

C
shRNA SAP-97

Accession: NM_012788
Gene ID: 6978762
Definition: Rattus norvegicus discs, large homolog 1 (Drosophila) (Dlgh1), mRNA.
Sequence Length: 3256
Specified Region: 452 -- 3187
GC% Range: 30% -- 60%
Organism: rat

SAP-97 #1 oligos
for {P}G**TTAAAGCCTGAAAGAGGTTGC**TTGATATCCG**GCAACCTCTTTCAGGCTTTAA**TTTTTTC
rev {P}TCGAGAAAAAA**TTAAAGCCTGAAAGAGGTTGC**CGGATATCAA**GCAACCTCTTTCAGGCTTTAA**C

SAP-97 #2 oligos
for {P}G**TTTGTCAGGAAATTCTGAGAT**TTGATATCCG**ATCTCAGAATTTCCTGACAAA**TTTTTTC
rev {P}TCGAGAAAAAA**TTTGTCAGGAAATTCTGAGAT**CGGATATCAA**ATCTCAGAATTTCCTGACAAA**C

SAP-97 #3 oligos
for {P}G**TTTAATGCGGCAGCTGCTTGT**TTGATATCCG**ACAAGCAGCTGCCGCATTAAA**TTTTTTC
rev {P}TCGAGAAAAAA**TTTAATGCGGCAGCTGCTTGT**CGGATATCAA**ACAAGCAGCTGCCGCATTAAA**C

SAP-97 #4 oligos •
for {P}G**TAGGTCCTAATATGATGACTG**TTGATATCCG**CAGTCATCATATTAGGACCTA**TTTTTTC
rev {P}TCGAGAAAAAA**TAGGTCCTAATATGATGACTG**CGGATATCAA**CAGTCATCATATTAGGACCTA**C

Mutagenesis primers for #4
97i#4muta-for_new
TATACCCGAC**CAGTTATAATCTTGGGACCTA**TGAAAGACAG
97i#4muta-rev_new
CTGTCTTTCA**TAGGTCCCAAGATTATAACTG**GTCGGGTATA

D
shRNA SAP-102

Accession: U50147
Gene ID: 1236952
Definition: Rattus norvegicus synapse-associated protein 102 mRNA, complete cds.
Sequence Length: 3736
Specified Region: 343 -- 2892
GC% Range: 30% -- 60%
Organism: rat

SAP-102 # 1 oligos
for {P}G**TTATAGCGTTGCCGGACACGT**TTGATATCCG**ACGTGTCCGGCAACGCTATAA**TTTTTC
rev {P}TCGAGAAAAA**TTATAGCGTTGCCGGACACGT**CGGATATCAA**ACGTGTCCGGCAACGCTATAA**C

SAP-102 # 2 oligos
for {P}G**ATTCTTGCTCCAGTTTCATGG**TTGATATCCG**CCATGAAACTGGAGCAAGAA**TTTTTTC
rev {P}TCGAGAAAAAATTCTTGCTCCAGTTTCATGGCGGATATCAACCATGAAACTGGAGCAAGAATC

SAP-102 # 3 oligos •
for {P}G**TAAGTGCTTCGATGGACTTGG**TTGATATCCG**CCAAGTCCATCGAAGCACTTA**TTTTTC
rev {P}TCGAGAAAAA**TAAGTGCTTCGATGGACTTGG**CGGATATCAA**CCAAGTCCATCGAAGCACTTA**C

SAP-102 # 4 oligos
for {P}G**ATCTTGGATTCAAAGCGACTG**TTGATATCCG**CAGTCGCTTTGAATCCAAGA**TTTTTTC
rev {P}TCGAGAAAAA**ATCTTGGATTCAAAGCGACTG**CGGATATCAA**CAGTCGCTTTGAATCCAAGAT**C

Mutagenesis primers for #3
102i#3muta-for_new
TTCATCAAGC**CCAAATCTATAGAGGCACTTA**TGGAAATGAAC
102i#3muta-rev_new
GTTCATTTCCA**TAAGTGCCTCTATAGATTTGG**GCTTGATGAA

Mouse variant SAP-102 #3
for {P}G**TAAGTGCTTCAATGGACTTGG**TTGATATCCG**CCAAGTCCATTGAAGCACTTA**TTTTTC
rev {P}TCGAGAAAAA**TAAGTGCTTCAATGGACTTGG**CGGATATCAA**CCAAGTCCATTGAAGCACTTA**C

Figure 22 shRNA targeting sequences and mutagenesis oligonucleotides. Database information identify PSD-MAGUK sequences that targeting sequences were based on. Four oligonucleotide pairs for each PSD-MAGUK are constructed around targeting sequences (bold) with the most efficient identified by a red star. Mutagenesis primers used to introduce silent pointmutations into PSD-MAGUK cDNAs are depicted with changed nucleotides indicated in red. A) shRNA PSD-95, B) PSD-93, C) SAP97 and D) SAP102.

Antibodies

Mouse PSD-95 and mouse pan-PSD-95/PSD-93 antibodies were from ABR. Rabbit anti-GluR2/3 and anti-GluR1 and mouse anti-GluR2 were obtained from Chemicon. Mouse NR-1 antibody was from Pharmigen. Mouse Tubulin and goat actin antibodies were from Santa Cruz. Homer antibody was a generous gift from A. Kato. PSD-93 antibody was from Zymed. Guinea pig SAP102 was described in (99). Rabbit SAP97 antibody was described in (49).

Knock-out mouse strains and genotyping

Generation of gene targeted deletion mouse strains are described in (4, 89). Tail samples from two to three week old knock-out mice were digested in 200µl tail lysis buffer (50 mM KCl, 10 mM TrisHCl pH 8.3, 2.5 mM MgCl2, 0.1 mg/ml gelantin, 0.45% NP40 (v/v), 0.45% Tween20 (v/v) add 100µg/ml proteinase K). Digest 55°C over night. Proteinase K was inactivated by heating samples to 95°C for 90 minutes. Lysates are diluted to 1ml total volume with water and mixed vigorously. Debris was pelleted for one minute at 20.000 g. Genotyping polymerase chain reaction (PCR) (100) was carried out on 1µl tail lysate with Phusion DNA polymerase (NEB) with HF buffer according to manufacturers specifications in 25 µl reactions. Oligonucleotides used for PSD-

93 genotyping: PSD-93 intron (GTGCGGAATGTTGTGCAGTGCAGG), PSD-93 exon (CACAACAGTCTCCAATATGGGTCGCC), neo (CCTTCTATCGCCTTCTTGACGAGTTC). Oligonucleotides used for PSD-95 genotyping: PSD-95 forward (CTCTACCTACCCTGTGATCCAGAGCTGG), PSD-95 reverse (CTCTGGGAGAGAACGTCTCCTCCG). The following PCR program was used for both genotypes: 98°C 2min, repeat 35 times 98°C for 15 sec, 72°C for 40 sec, followed by 72°C for 4 min then holding at 4°C. PCR reactions were analyzed on 2% agarose gels containing ethidium bromide, visualized by exposure to ultraviolet light (98).

Overexpression in heterologous cells

HEK293T or COS-7 cells were transfected with Lipofectamine 2000 (Invitrogen) with cDNA expression vectors as well as corresponding shRNA vectors in a molar ratio of 1:3 according to manufactures instructions (***). Forty-eight hours after transfection cells were lysed with SDS-PAGE sample buffer. Lysates were sonicated and denatured at 67°C for 10 min and analyzed by SDS-PAGE (101) and Western blots analysis (102). Blots were probed with antibodies PSD-95 (1:1000), PSD-93 (1:2000), SAP97 (1:2000) and SAP102 (1:2000). Actin served as a loading control for each blot. Immuno-reactivity was visualized with the ECL Pico Western system (Pierce).

Neuronal Culture and Transfection

Dissociated hippocampal neuron cultures were prepared as previously described (50). In short, embryonic day 18 (E18) rat pups were sacrificed, and hippocampi were isolated and digested using papain protease. Neurons were dissociated and resuspended in Neurobasal medium containing B27, GlutaMax, and antibiotics (all Invitrogen). Neurons were plated at 50K or 100K per cm2 on Poly-D-lysine-treated support. Hippocampal neurons were transfected at day *in vitro* (DIV) 14–16 with 0.5–1 mg pLLox3.7 vectors using 0.5–1 ml Lipofectamine 2000 (Invitrogen) and incubated for 5 more days before analysis. Immunocytochemistry and Surface GluR2 Staining Transfected neurons were used for immunocytochemistry at DIV 14–16+5. Neurons were fixed with 4% paraformaldehyde/4% sucrose in PBS for 12 min on ice followed by 100% methanol for 10 min at –20°C, washed twice with PBS for 5 min on ice. Cells were permeabilized with 0.1% Triton-X100 in PBS for 10 min and washed with PBS for 5 min. After blocking with normal goat serum (3%) in PBS for 1 hr, the cells were incubated with mouse anti-PSD-95 (1:1000) and rabbit anti-PSD-93 (1:1000) antibodies followed by Alexa 546 or 647 conjugated secondary antibodies (Molecular Probes). Images were captured with a laser scanning fluorescent microscope LSM510 (Carl Zeiss) equipped with a 63x oil emersion objective. Co-localization was analyzed using LSM510 analysis software. At least 500 PSD-93 positive clusters were analyzed for PSD-95 co-localization in at least seven independent cells per condition. Statistical significance was determined by Student's t test.

Surface GluR2 staining was performed as described in (88). In short, surface GluR2 receptors were ‘‘live’’-labeled with an antibody to an extra cellular epitope of GluR2 (1:10 dilution) (Chemicon) by incubating neurons in conditioned medium for 15 min at 37 C. Neurons were fixed with 4% paraformaldehyde/4% sucrose/PBS for 8 min and blocked with PBS containing 3% normal goat serum. Surface GluR2 was visualized with Alexa 546 conjugated secondary antibody. Stacks of fluorescent images were acquired with a LSM Pascal confocal laser microscopy system (Carl Zeiss) with a 633 oil emersion objective. Quantification was carried out on proximal dendrites in at least nine independent, transfected neurons. For quantification of spine localization of surface GluR2 clusters, only mushroom-shaped protrusions from the dendrite as visualized by GFP expression were analyzed using LSM Pascal co-localization analysis software. A spine was considered GluR2 positive if it co-localized with at least one surface GluR2 cluster. The total number of spines along the analyzed dendrite was recorded. The length of a given dendrite was measured using LSM Pascal analysis software tools (Carl Zeiss). The number of spines was then normalized to 10 µm. Filopodia-like protrusions were excluded from the analysis. At least 250 spines were analyzed under each condition. Total surface GluR2 staining was assessed with the following criteria. Surface GluR2 clusters co-localized with the GFP marker anywhere on the dendrite were analyzed. The number of clusters along the dendrite was counted and the length of the analyzed dendrite was measured using LSM Pascal analysis software tools. The number of clusters was normalized to 10 µm. At least 275 surface GluR2 clusters were

analyzed in each condition in at least nine independent neurons. Statistical significance was determined by Student's t test.

Lentivirus Production and Neuronal Infection

For Lentiviral particle production, $2x10^6$ HEK293T cells were cotransfected with 9µg pLLox3.7 and helper vectors 9µg pDelta8.9 and 6µg pVSVg using 24µl Lipofectamine 2000 (Invitrogen) or 70 µl FuGeneHD (Roche). Twelve hours after transfection, medium was changed to 20 ml production medium containing Neurobasal substituted with Transferrin/Insulin/Selenium-Mix (all Invitrogen). Forty-eight hours after transfection, supernatant was collected and concentrated by ultrafiltration in Centricon Plus 100 (Millipore). Particles were aliquoted and stored at -80°C. Particle titer was determined by infection of $2x10^5$ HEK293T cells in serial dilutions. Forty-eight hours after infection, HEK293T cells were analyzed with a FACScalibur flow cytometer to determine titer. Titer is determined as followed: $T=[F \times C_o/V] \times D$, where T is titer in [ml], F frequency of positive cells, C_o number of cells at time of infection, V virus volume added [ml] and D dilution factor. Dissociated hippocampal neurons (see above) were infected at DIV 9–10 with MOI 2 and incubated for 6 more days in the presence of 5-Fluorodeoxyuridine and Uridine (both Sigma). This achieved about 70% of infection efficiency. Neurons were then lysed with SDS-PAGE sample buffer. Lysates were analyzed with SDS-PAGE and western blot analysis. Blots were probed with PSD-95, PSD-93, SAP97, and SAP102 as described above as well as anti NR-1 (1:2000) and anti Tubulin (1:1000) as loading controls.

Brain Lysates and Solubilization

PSD-95/PSD-93 double knockout mice and littermate control animals were sacrificed and the hippocampus was isolated. Hippocampi were homogenized in 10 volumes H-Buffer (320 mM sucrose, 2mM EDTA, 20mM TrisHCl [pH 8.0], 1mMPMSF). Homogenate was centrifuged for 10 min at 1000xg. Supernatants S1 were used for further experiments. For total protein extracts SDS was added to a final concentration of 1% and agitated for 1 hr at 4°C. Extracts were sonicated and the protein concentration was determined by Bradford assay. Twenty-five micrograms of total protein was loaded per lane and analyzed by SDS-PAGE and western blotting. P2 crude synaptosome fractions, prepared from the supernatant S1, were resuspended in an equal volume of TET buffer (1% TritonX-100, 2 mM EDTA, 20 mM TrisHCl [ph 7.4], 1 mM PMSF) and agitated for 1 hr at 4°C. Extracts were centrifuged at 100,000 x g for 1 hr. The supernatant is the TritonX-100 soluble fraction. The pellet was resuspended in 0.1 vol of 1% SDS, 2 mM EDTA, and 20 mM TrisHCl (pH 7.4). Then 0.9 volume of TET buffer was added and the extracts agitated for 1 hr at 4°C (103). Protein concentration was determined by Bradford assay (BioRad). Five micrograms of protein were loaded per lane and analyzed by SDS-PAGE followed by western blotting. Densitometric analysis was carried out using ImageJ software. Significance was determined by Student's t test.

Electrophysiology in Acute Slices

Transverse hippocampal slices (300–400 mm thick) were prepared from PSD-95 knock-out, PSD-93 knock-out, PSD-95/PSD-93 double knock-out, heterozygous, and wild-type littermates (7–40 days old) as previously described (104). Acute recordings were done at room temperature (24°C–28°C). Perfusion medium contained (in mM) 119 NaCl, 2.5 KCl, 1.3 $MgSO_4$ (4 $MgSO_4$, to measure AMPA/NMDA ratios and mEPSCs), 1 NaH_2PO_4, 26.2 $NaHCO_3$, 11 glucose, 2.5 $CaCl_2$ (4 $CaCl_2$, for AMPA/NMDA ratios and mEPSCs), and 0.1–0.15 picrotoxin, saturated with 95% O_2/5% CO_2. Field EPSP recordings were made in CA1 stratum radiatum following stimulation of Schaffer collaterals with monopolar glass electrodes filled with 1 M NaCl. Synaptic responses were recorded with glass electrodes (3–5 MU) filled with 1 M NaCl using a Multi-Clamp 700A amplifier (Axon Instruments). PPF was obtained by delivering two stimuli at an interval of 40 ms. Somatic whole-cell voltage-clamp recordings were made from visually identified CA1 pyramidal neurons using 3–5 MU glass electrodes filled with internal recording solution containing (in mM) 125 $CsMeSo_3$, 2.5 CsCl, 7.7 TEA, 5 QX-314, 4 Mg-ATP, 0.3 Na-GTP, 20 HEPES, 8 NaCl, and 0.2 EGTA (or 10 BAPTA) (pH 7.2) at 280–290 mOsm. Series and input resistance were monitored, and cells in which either parameter varied by 25% during a recording session were discarded. AMPA and NMDA EPSCs were obtained by evoking dual component responses while voltage-clamping neurons at +40 mV. 100 µM APV (or 10 µM CPP) was then added to obtain the pure AMPAR EPSC, and the NMDAR component was derived by subtracting the AMPAR EPSC from the

compound EPSC. Interstimulus interval was 10 s. For experiments using BAPTA, responses were evoked 2 min after establishing whole-cell configuration. For recordings not using BAPTA, only cells in which the AMPAR EPSC after NMDAR blockade did not differ from baseline responses recorded at 270 mV before voltage-clamping at +40 mV were included in the final analysis. mEPSCs were recorded at 270 mV in the presence of 0.5 mM tetrodotoxin, 0.1–0.15 mM picrotoxin, and 50 mM sucrose to increase the frequency of events. mEPSCs were analyzed off line with customized software using a threshold of 4 pA. All data are expressed as mean +/- SEM. Statistical significance was determined using two-tail unpaired t tests for between-group comparisons. For mEPSCs, the statistical significance between distributions was determined using Kolmogorov-Smirnov test.

Electrophysiology in Slice Culture

Standard procedures were used to prepare rat and mouse organotypic slice cultures from animals ranging from P2–9 (72, 90). All RNAi slice culture experiments were carried out in mature slices (from P9) except experiments regarding SAP102 early developmental role, which were carried out using immature slices (from P2). All slice cultures were injected near the pyramidal cell layer with shRNA-containing Lentiviral particles at a titer raging from 10^6–10^8 cfu/ml. Synaptic responses were evoked from pairs of infected and uninfected neurons simultaneously by electrically stimulating a common pathway with monopolar glass electrodes filled with external perfusion medium (see below).

Recordings were made from pairs of pyramidal neurons using 3–5 MU glass electrodes filled with an internal solution containing (in mM) 115 CsMeSo3, 20 CsCl, 10 HEPES, 2.5 MgCl2, 4 Mg-ATP, 0.4 Na-GTP, 0.6 EGTA, and 5 QX-314 (pH 7.2) at 290–300 mOsm. For AMPAR/NMDAR ratios of SAP102 shRNA infected cells from PSD-95/PSD-93 double knock-outs the following intracellular recording solution was used (in mM): 107.5 Cs-gluconate, 20 HEPES, 0.2 EGTA, 8 Na-gluconate, 8 TEA-Cl, 4 Mg-ATP, 0.3 Na3-GTP, and 5 QX-314 (pH 7.2) at 290 mOsm. External perfusion medium consisted of (in mM) 119 NaCl, 2.5 KCl, 4 MgSO4, 1 NaH_2PO_4, 26.2 $NaHCO_3$, 11 glucose, and 4 $CaCl_2$, saturated with 95% O_2/5% CO_2 and including 0.1–0.15 picrotoxin and 5–10 2-chloroadenosine to block inhibition and suppress epileptic activity. Series and input resistance were monitored and cells were discarded if they differed by more than 20%. AMPAR EPSCs were evoked while voltage clamping cells at 270 mV, and the amplitude was determined by measuring the peak of this response. NMDA EPSCs were obtained while voltage clamping cells at +40 mV, and the magnitude was determined by measuring the amplitude of the EPSCs 60-100 ms after the shock artifact. Statistical difference was determined using a two-tailed paired t test. mEPSCs were recorded at 270 mV in the presence of 0.5 mM tetrodotoxin, 0.1–0.15 mM picrotoxin, and 50mM sucrose to increase the frequency of events (100 mM sucrose was added for experiments of SAP102 knock-down in PSD-95/PSD-93 double knock-outs). mEPSCs were analyzed off line with customized software using a threshold of 4 pA. For mEPSCs, statistical significance between distributions was determined using a Kolmogorov-Smirnov

test. Minimal stimulation experiments were carried out as previously described (90).

Immunoprecipitations

Using a potter homogenizer, ~P30 mice hippocampi from heterozygote or knockout mice were homogenized in 3 volumes of H-buffer (320 mM sucrose, 20 mM Tris (pH 7.4) and 2 mM EDTA) containing 10 µg/ml leupeptin and 200 µg/ml PMSF. Homogenates were centrifuged at 20,000*g* for 1 h and pellets were resuspended in TET buffer (20 mM Tris (pH 8.0), 1 mM EDTA and 1.3% Triton X-100) containing 10 µg/ml leu-peptin and 50 µg/ml PMSF. After re-homogenization, samples were incubated for 1 h at 4^0C. Lysates were pelleted at 100,000*g* for 1 h. The supernatant was collected and precleared with protein A Sepharose (Amersham) for 1 h at 4^0C. Precleared lysates were immunoprecipitated with 5 µg of indicated antibody or control rabbit IgG for 2 h to over night at 4^0C. To collect immunoprecipitated protein complexes, 80 µl of a 50% protein-A Sepharose slurry was added to the lysates and incubated for 1 h at 4^0C. Immunoprecipitates were washed extensively and loaded onto SDS-PAGE to separate the proteins. Gels were either silver stained (BioRad) or transferred to nitrocellulose for western blot analysis.

Western blot analysis

After SDS-PAGE (101) gels are transferred to nitrocellulose membrane in transfer buffer (500mM Glycine, 50mM TrisHCl, 0.01% SDS, 20% methanol) buffer at 500 mA for 1h (102). Membranes are washed in TBS-T (10mM TrisHCl, 100 mM NaCl, 0.1% Tween at pH 7.4) and then blocked with 3% non-fat milk extract in TBS-T for 30min to 1h. Membranes are exposed to primary antibody in 1% BSA in TBS-T for 1h at room temperature to over night at 4°C. Membranes are washed with TBS-T three times for ten minutes. Then are incubated with appropriate secondary antibodies (Amersham and Jackson Labs) coupled to horse radish peroxidase (HRP) for 1h at room temperature to over night at 4°C. After washing the membranes three times for ten minutes, signals were visualized using Western Pico Super ECL reagent (Pierce).