APPENDIX

MATERIALS AND METHODS

Constructs, viruses and antibodies

Mouse N-cadherin cDNA in a mammalian expression vector (pCXN2-Ncad) was provided by Dr. Deanna Benson. To enhanced the surface expression level of N-cadherin, the construct was modified by adding a Kozak sequence in front of the start codon of N-cadherin protein and moving the entire cDNA into a different expression vector, pcDNA3.1Zeo(-)(Invitrogen). All PCR-modified constructs have been confirmed by DNA sequencing. Anti-GFP and β-catenin antibodies are purchased from Molecular Probes and Zymed respectively. Transfection and Sindbis virus infection were performed as described previously (Aakalu, 2001).

Cultured hippocampal neurons

Dissociated hippocampal neurons were prepared and maintained as previously described (Aakalu, 2001). Briefly, hippocampi from postnatal day 2 Sprague-Dawley rat pups were enzymatically and mechanically dissociated and plated into poly-lysine coated glass-bottom petri dishes (Mattek). Neurons were maintained for 14-21 days at 37º C in growth medium (Neurobasal A supplemented with B27 and Glutamax-1, Invitrogen).

Cell culture and transfection

L, HEK293, and COS-7 cells were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and penicillin/streptomycin (Invitrogen). For transient transfection, cells were plated either in a Mattek dish for microscopy or in a 35 mm culture dish for immunoprecipitation, grown to 80% to 90% confluence, then transfected with the appropriate amount of DNA mixed with lipofectAMINE (Invitrogen). Transient expression was allowed for 40 to 48 hours. To generate stable lines, L cells were plated into 10 cm culture dishes and transfected with 10 μg of DNA (TS25 with either the YFP or CFP insertions) with LipofectAMINE (Invitrogen). After 48 hours of transient expression, 500 μg/mL of Geneticin (Invitrogen) was added into the medium. Fresh Geneticin-containing media was supplemented every day for 10 days until visible colonies were observed under the microscope. Twenty-four colonies of each construct were amplified sequentially through a series of clonal expansions from 96-well plate to 24-well plate. In preliminary experiments, ten clones survived and were analyzed by immunoblotting using an anti-GFP antibody.

Immunoprecipitation

COS-7 cells were transfected with plasmid DNA purified from either Maxiprep or QIAprep spin miniprep kits (Qiagen) with LipofectAMINE reagents overnight at 37^0C. Forty-eight hours after the addition of the DNA/liposome mixture, cells were washed three times with ice-cold PBS-MC (phosphate buffered saline, 1 mM $MgCl_2$, 0.1 mM

$CaCl_2$) and lysed with NP40 lysis buffer (1% NP40, 150 mM NaCl, 50 mM Tris HCl pH 8.0) with freshly added protease inhibitor cocktail (Roche). Cells were incubated on ice for 20 minutes and spin at 13,000 rpm for 15 minutes at $4^{\circ}C$ in a microfuge. The centrifuged extracts were then mixed with lysis buffer-washed Protein G beads (Pierce) and incubated at $4^{0}C$ overnight with gentle rotation. Beads were washed three times with the lysis buffer at room temperature for 10 minutes each and boiled in 5X SDS-PAGE sample buffer for 5 minutes. The entire eluate with 2% of the supernatant were loaded into 5%-15% SDS-polyacrylamide gel and transferred onto PVDF membrane (BioRad). Immunoblotting was performed with various antibodies against tags or specific proteins.

L cell aggregation assay

Stable lines were plated into 35 mm dishes until 80% to 90% confluent. All dishes were washed three times with prewarmed PBS+1mM $CaCl_2$, then incubated with 500 mL of 0.01% trypsin in PBS + 1 mM $CaCl_2$ at $37^{\circ}C$ for 30 minutes. The trypsinized cell suspensions were transferred into microfuge tubes and centrifuged at 1,000 rpm at room temperature for 5 minutes. The pelleted cells were washed twice with equal volumes of PBS + 1% BSA. The cell suspension was then divided into two tubes containing PBS + 1% BSA with or without 5 mM $CaCl_2$, and incubated at $37^{\circ}C$ for 1 hour with head-to-tail rotation. The contents of the tubes were plated into 35 mm dishes and cell aggregates are observed under the microscope.

Microscopy and image analysis

All images were acquired with a Zeiss 510 Meta confocal laser scanning microscope. YFP is excited with the 514 nm line of an argon ion laser using a Plan-Neofluor IR 40X 1.3 NA oil immersion objective (Zeiss), and emitted light was collected over a spectrum of wavelengths between 462 and 633 nm with bandwidths of 10.7 nm. CFP was excited with the 458 nm line of a argon ion laser, and emitted light was collected over the same spectrum of wavelengths used for YFP detection. Because there is significant overlap in the emission spectra of YFP and CFP, the fluorescence contribution of each fluorophore at each pixel was separated using a linear unmixing algorithm based on the spectral signatures of YFP and CFP created from reference lambda stack images of cells expressing either soluble YFP or soluble CFP, respectively. Between-dish comparisons on a given day, all images were acquired at the same settings, without knowledge of the experimental condition during image acquisition. All postacquisition processing and analysis was carried out with ImageJ (NIH) and Matlab (The MathWorks, Inc.).