Supporting Information

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SI Materials and Methods

Transposon-Mediated in Vitro Transposition and Molecular Cloning. ***Transposon-mediated in vitro transposition.*** Mouse *N*-cadherin cDNA in a mammalian expression vector (pCXN2-Ncad) was used as the target for in vitro transposition (1). The procedure was carried out as described previously with some modifications (2). In brief, from an in vitro transposition screen of 509 colonies, we identified 23 clones with the correctly inserted GFP using colony PCR. This method allowed for screening of hundreds of colonies without DNA amplification and purification. Three 5′-end primers were designed to cover the entire *N*-cadherin coding region and one 3′-end primer within GFP. To reduce the number of reactions on a first pass, approximately five colonies were pooled in one reaction for rapid elimination of those colonies that failed to produce a positive result. During the transposition, a stop codon was inserted between the GFP and kanamycin-resistant gene, which created a truncated *N*-cadherin GFP fusion. A simple restriction enzyme (SmaI) digest followed by self-ligation and bacterial transformation restored the full length *N*-cadherin with intramolecular GFP insertion. All 23 candidates were restored to the full-length *N*-cadherin with GFP insertion. After the full restoration procedure, all clones were transiently transfected into a cultured mammalian cell line (HEK293 cells) for direct visualization of GFP fluorescence to determine if the fusion protein was in-frame and had good junctional localization. When the fluorescence was visualized in intact transfected cells, 10 clones exhibited localization at the cell–cell contacts, the expected location for a functional cadherin protein. These clones were then subjected to DNA sequencing to identify the exact site of insertion—six of 10 clones had GFP inserted in the extracellular domain of *N*-cadherin.

Two clones were chosen from the screen for FRET experiments, one in which GFP was inserted near EC2 (FP-NCad) and one in which GFP was inserted near the plasma membrane (FP_{prox}-NCad). In the FP-Ncad clone a few extra amino acids were introduced by the transposon, along with a flanking restriction enzyme site, AscI, on both sides of the GFP (**MLRYR*ILS***-LSLIHIWRA-GFP-GRARADVYKRQ***ILS***-QAPSTPSP**; in bold: original *N*-cadherin sequence; bold italic: repeated *N*-cadherin sequence; normal: extra amino acids introduced by the transposon; underlined: abbreviation for green fluorescent protein). The insertion was at amino acid position 311. In the FP_{prox}-Ncad clone a few extra amino acids were also introduced by the transposon, along with a flanking restriction enzyme site, AscI (**NGDCT*DVD***-LSLIHIWRA-GFP-GRARADVYKRQ***DVD***-**RIVGAG**; in bold: original *N*-cadherin sequence; bold italic: repeated *N*-cadherin sequence; normal: extra amino acids introduced by the transposon; underlined: abbreviation for GFP). The insertion was at aa 714. The GFP was replaced with Venus and Cerulean in the fusion proteins of both constructs.

Molecular cloning. The entire coding region of the *N*-cadherin fusion protein (EcoRI-XmnI) was moved to a different expression vector, pcDNA3.1Zeo(−) (Invitrogen), and a Kozak consensus sequence (CGCCACC) with a XbaI site was introduced in front of the start codon of the *N*-cadherin protein by PCR. A fragment of *N*-cadherin containing the W2A mutation was synthesized (GenScript) and used to replace the corresponding WT fragment in each of the FRET pair constructs (W2A-Ncad). All constructs were verified by DNA sequencing. The construction of the β-catenin–GFP plasmid was described previously (3).

Cell Culture and Transfections. ***Cell culture.*** L- and COS-7 cells (ATCC) were maintained in DMEM supplemented with 10% FBS and sodium pyruvate (Invitrogen) in a humidified 5% CO_2 incubator at 37 °C.

Transfections for FRET experiments. For FRET experiments, COS-7 cells were transiently transfected in suspension using either FuGENE6 (Roche Applied Science) or Lipofectamine 2000 (Invitrogen), as described by the manufacturer, in a six-well plate. Twenty-four hours posttransfection, cells were washed three times and then dissociated from the surface by incubating cells with 1 mL of D-PBS + 1.5 mM EDTA at 37 °C for 5 min. Equal numbers of Venus and Cerulean *N*-cadherin–expressing cells were plated on 35-mm glass-bottomed dishes (MatTek Corp.) that were pretreated with phenol red-free Matrigel. After recovery overnight, cells were switched into phenol red-free media supplemented with 2 or 5 mM Ca^{2+} for 24 h before imaging to reduce autofluorescence.

β-Catenin transfections. Transient transfections for β-catenin–GFP in COS-7 cells using Lipofectamine 2000 were performed as described by the manufacturer (Invitrogen). Cells were incubated for 24 h or less before imaging experiments.

Immunoprecipitation. Adherent COS-7 cells were transiently transfected using Lipofectamine 2000 (Invitrogen) as described by the manufacturer in a six-well plate. Forty-eight hours after the addition of the DNA/liposome mixture, cells were washed three times with ice-cold PBS-MC (PBS, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$) and lysed with Nonidet P-40 lysis buffer [1% Nonidet P-40, 150 mM NaCl, 50 mM Tris·HCl (pH 8.0)] with freshly added protease inhibitor mixture (Roche). Cells were incubated on ice for 20 min and centrifuged for 15 min at 4 °C. The centrifuged extracts were mixed with lysis buffer-washed Protein G beads (Pierce) coupled to an anti-GFP antibody or rabbit IgG and incubated at 4 °C overnight with gentle rotation. Beads were washed three times with lysis buffer at room temperature for 10 min per wash and boiled in 5× SDS/PAGE sample buffer for 5 min. The eluate and 2% of the supernatant were resolved by a 5–15% SDS/PAGE and transferred onto polyvinylidene fluoride membrane (BioRad). Immunoblotting was performed with either β-catenin or *N*-cadherin antibodies.

Generation of *N*-Cadherin Stable Cell Lines. ***L-cell transfections.*** To generate stable lines, L-cells were transfected using 0.5 μg DNA with 1.5 μL FuGENE 6 transfection reagent and 0.5 μL CombiMag (Oz Biosciences) in a 24-well plate. DNA/FuGENE complexes were formed as described by the manufacturer before the addition of CombiMag beads. After a 20-min incubation at room temperature, complete media was added to the DNA/FuGENE/CombiMag mix. After the complete mix was applied to the cells, the 24-well plate was placed on a magnetic plate. The magnetic plate was removed after 20 min in the incubator.

L-cell stable line production. After 24 h, cells were trypsinized, diluted, and plated in a six-well plate in media supplemented with 800 μg/mL Geneticin (Invitrogen) for selection. After 7–10 d, individual colonies were observed in the transfected well. Fresh Geneticin-containing media was exchanged every other day for 1.5 wk. Colonies were then trypsinized and transferred to a new six-well plate for another 2–4 d or until the cell density reached 50–70%. Monoclonal selection was achieved by diluting cells to 2 cells/mL and plating at 100 μL per well in a 96-well plate.

Stable line screening. Wells containing colonies derived from a single cell were selected and expanded into a 24-well plate. Protein expression levels were determined by immunoblotting with an anti–*N*-cadherin antibody (BD Biosciences). Colonies expressing high total protein levels were further screened for protein localization by direct fluorescence of the genetically encoded tag or immunofluorescence for untagged constructs using a LSM510 Meta confocal laser scanning microscope with a 40× 1.3 N.A. oil immersion plan apochromat objective lens (Carl Zeiss MicroImaging, Inc.). Cell lines expressing high protein levels at the cell membrane were selected for experiments. Methods for immunoblotting and immunofluorescence have been described previously (4).

Quantitative Short-Term L-Cell Aggregation Assay. ***Short-term L-cell aggregation assay.*** L-cell aggregation assays were accomplished as previously described with some modifications (5). *N*-cadherin stable cell lines were passaged into T-75 flasks until 85–95% confluence was obtained. All flasks were washed three times with 10 mL of prewarmed Hepes buffer, Ca^{2+} and Mg^{2+} free (HCMF) + 5 mM $CaCl_2$ [HCMF, in mM: 137 NaCl, 5.4 KCl, 0.34 Na_2HPO_4, 0.1% glucose, 10 Hepes (pH 7.4)], and each flask was incubated in 5 mL of HCMF + 5 mM $CaCl_2$ containing 0.001% trypsin at 37 °C for 15 min. Trypsinization was terminated by the addition of a stop solution (0.02% soybean trypsin inhibitor, 10 ng/mL DNase I, 10 mM $MgCl_2$). Trypsinized cell suspensions were transferred into 15-mL conical tubes and centrifuged at 4 °C for 5 min. Pelleted cells were washed three times with 10 mL of ice-cold HCMF. Each cell suspension was then resuspended in growth media at 2×10^6 cells/mL. To prevent adhesion of the cells to the bottom surface, 24-well plates were precoated with 200 μL of 1% agarose. Aggregation assays were carried out in a humidified 5% CO_2 incubator at 37 °C at varying Ca^{2+} concentrations, with a total of 1×10^6 cells per well. No agitation was applied to minimize the shear force to avoid breaking apart weak interactions. After 1 h, aggregation was terminated by placing the cells on ice and adding an equal volume of 8% formaldehyde solution.

Quantitation of aggregation assay. Aggregates were transferred to an Accuvette (containing 22 mL Isoton II Diluent; Beckman Coulter) with a precut P1000 pipette tip. Particles volume was quantitated using a Multisizer3 Coulter Counter (Beckman Coulter) using a 280-mm aperture and volumetric control mode (2 mL total assayed volume). Data were analyzed using particle volume without coincidence correction. Populations of cells were expressed by normalized cumulative distributions of percent volume and median particle volume.

Imaging of aggregation assay. The entire contents of one well was plated into a new 24-well plate without agar. Images of cell aggregates were acquired using a LSM510 Meta confocal laser scanning microscope with a 10× 0.3 N.A. Plan-Neofluar objective lens (Carl Zeiss MicroImaging, Inc.) and 488-nm argon ion laser excitation for differential interference contrast (DIC) imaging.

FRET Imaging and Analysis. ***FRET spectral imaging.*** Spectral imaging (12-bit, 512 × 512) was accomplished using a LSM510 Meta confocal laser scanning microscope with a 40× 1.3 N.A. oil immersion plan apochromat objective lens (Carl Zeiss MicroImaging, Inc.). A 458-nm argon ion laser was used for excitation. Emitted light was collected over a spectrum of wavelengths between 463 and 580 nm, with band widths of 10.7 nm (6). Because of the significant overlap in the emission spectra of Cerulean and Venus, the fluorescence contribution of each fluorophore at each pixel was separated using a linear unmixing algorithm based on the spectral signatures of Cerulean and Venus created from reference lambda stack images of single-color cell junctions on the same day (6).

Acceptor bleach FRET. Acceptor bleach FRET was performed by continuous bleaching with the 514-nm laser (100% power, 2,000 iterations) within a chosen ROI. In general, Venus was photobleached greater than 70–80% in the ROI. Acceptor bleach FRET experiments were performed in Hepes buffered saline [HBS; in mM: 119 NaCl, 5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 30 glucose, 10 Hepes (pH 7.4)].

Acceptor bleach FRET analysis. Out-of-focus images were discarded before analysis. Before analysis, all images were background subtracted and smoothed (ImageJ). Within the bleached ROI, adherens junctions were straightened and cropped to include only the regions with a colocalized FRET reporter signal. Custom-written macros and the FRETcalc v3.0 plug-in for ImageJ were used to assemble and quantify FRET efficiencies. When possible within the same image, single-color junctions (Venus/Venus and Cerulean/Cerulean) and nonbleached areas on the same junction as the ROI were used as a negative control and showed zero or negative FRET changes. Settings for the FRETcalc v3.0 plug-in (bleach threshold = 0–100%; donor and acceptor thresholds = 1 pixel and FRET threshold = −100–100%). Output included analysis of all pixels and spatial FRET maps, which were pseudocolored on an 8-bit scale, where 0 corresponds to 0% FRET, 128–50% FRET, and 255–100% FRET.

The FRET efficiency, E, is inversely proportional to the sixth power of the donor-acceptor distance, R:

$$E = R_0^6/(R_0^6 + R^6), \quad [1]$$

where R_0 is the Forster distance (54 Å for the Cerulean/Venus pair) (7).

For acceptor bleach FRET experiments, FRET efficiencies were calculated using Eq. **2**:

$$E = (F_{CerPost} - F_{CerPre})/F_{CerPost} \quad [2]$$

Time-lapse ratiometric FRET and analysis. To resolve faster kinetics for time-lapse ratiometric FRET measurements, continuous fast scans (100–250 ms) of smaller ROIs were used to monitor FRET changes. The mean fluorescence intensity was measured for each fluorophore, and the FRET efficiency was expressed as the emission ratio of Venus:Cerulean. Ratiometric spectral FRET experiments were performed in HibernateA low-fluorescence media with freshly added SCAVEGR (BrainBits LLC) to minimize photobleaching. The Ca^{2+} chelator EGTA (final concentration 20 mM; Sigma) or 1,2-bis(*o*-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid (BAPTA; final concentration 20 mM; Invitrogen) was manually added as a small volume of concentrated stock solution after 15 s of baseline imaging. For all experiments, the entire aliquot of EGTA or BAPTA was added within 1–2 s close to the cells of interest. Cerulean fluorescence exhibited a rapid photobleaching component upon acquisition of the first few images. Therefore, for normalization purposes, the baseline was averaged 10 s before the addition of EGTA.

β-Catenin Live-Cell Imaging Experiments. ***Live-cell imaging.*** For β-catenin–GFP experiments, images were acquired using a LSM510 Meta confocal laser scanning microscope with a 40× 1.3 N.A. oil immersion plan apochromat objective lens (Carl Zeiss MicroImaging, Inc.) and 488-nm argon ion laser excitation. The same EGTA treatment was applied to two neighboring cells with similar levels of modest expression after 30 s of baseline (1 s per frame) with some signal fluctuation observed due to *z*-axis drift. Only images with minimal drift in focus were analyzed. For

normalization, the baseline was averaged 10 s before the addition of EGTA.

Reagents. ***Antibodies.*** Anti-GFP, β-catenin, *N*-cadherin, and control rabbit IgG antibodies were purchased from Invitrogen, Zymed, BD Transduction Lab, and Jackson ImmunoResearch, respectively.
Chemicals and other reagents. All chemicals were purchased from Mallinckrodt Chemicals with the exception of Hepes and BAPTA (Invitrogen); trypsin (Worthington); soybean trypsin inhibitor and DNase I (Sigma); formaldehyde (Polysciences Inc.); and Isoton II (Beckman Coulter).

Image Analysis and Processing. ***Analysis.*** All postacquisition processing and analyses were carried out with the combination of Zeiss AIM or ZEN software and ImageJ. Statistics were carried out using Excel (Microsoft) or Origin (OriginLab).

1. Shan WS, et al. (2000) Functional *cis*-heterodimers of *N*- and *R*-cadherins. *J Cell Biol* 148: 579–590.
2. Sheridan DL, et al. (2002) A new way to rapidly create functional, fluorescent fusion proteins: Random insertion of GFP with an in vitro transposition reaction. *BMC Neurosci* 3:7.
3. Murase S, Mosser E, Schuman EM (2002) Depolarization drives beta-Catenin into neuronal spines promoting changes in synaptic structure and function. *Neuron* 35:91–105.
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5. Takeichi M, Nakagawa S (2001) Cadherin-dependent cell–cell adhesion. *Curr Protoc Cell Biol*, 10.1002/0471143030.cb0903s00.
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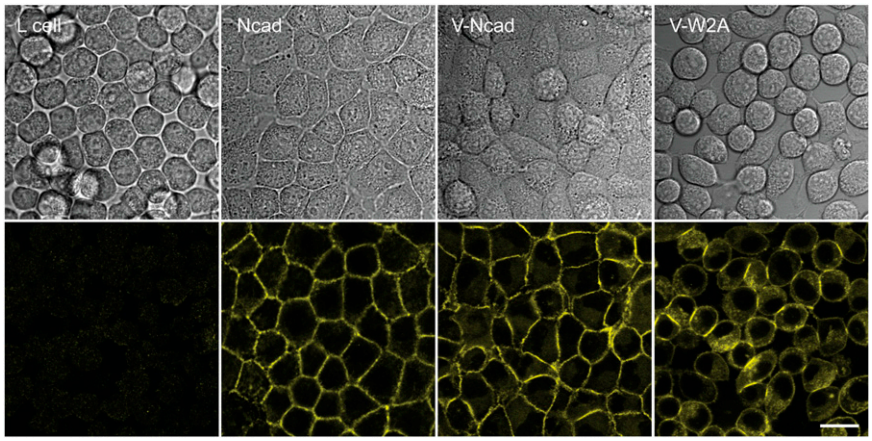

Fig. S1. Morphology and expression of *N*-cadherin stable cell lines. Examples of DIC (*Upper*) and fluorescence (*Lower*) images of untransfected (L-cell; *Far Left*), unlabeled wild-type *N*-cadherin (Ncad; *Center Left*), Venus-tagged *N*-cadherin (V-Ncad; *Center Right*), and Venus-tagged W2A-*N*-cadherin (V-W2A; *Far Right*) cell lines. Clear morphological differences are evident in the quality of the junctions between cells in the tight monolayers of Ncad and V-Ncad populations in comparison with L-cell and V-W2A cell lines. Immunocytochemistry was used to localize *N*-cadherin expression in L- and Ncad cell lines. These images do not correspond with the live DIC images shown above them. (Scale bar: 10 μm.)

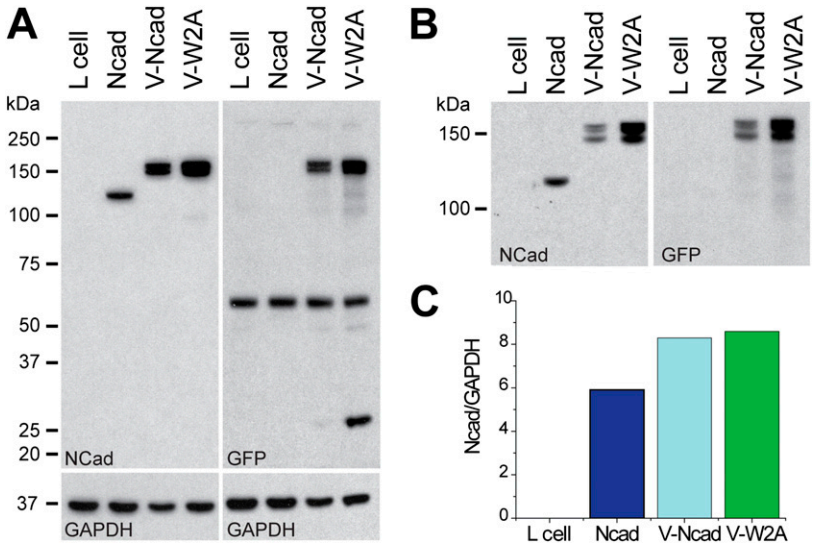

Fig. S2. Relative quantification of *N*-cadherin in stable cell lines. (*A*) Whole-cell lysates from untransfected (L-cell), unlabeled wild-type *N*-cadherin (Ncad), Venus-tagged *N*-cadherin (V-Ncad), and Venus-tagged W2A-*N*-cadherin (V-W2A) cell lines were resolved by SDS/PAGE and immunoblotted using antibodies against *N*-cadherin and GFP, which also recognizes Venus. The V-W2A cell line showed a band of Venus protein uncoupled from *N*-cadherin. When probing for GFP, the band at ~60 kDa is nonspecific and appears in all cell lines. (*B*) A double band of tagged *N*-cadherin (~150 kDa) was resolved for both V-Ncad and V-W2A cell lines using both antibodies, in which the upper band corresponds to the immature form of the *N*-cadherin fusion protein (including the leader peptide and propeptide sequences), and the lower band corresponds to the mature full-length *N*-cadherin plus Venus (27 kDa). (*C*) For determining relative protein levels, GAPDH was used as a loading control (*A Lower*), and relative quantification of the mature full-length *N*-cadherin (*B*) was accomplished using densitometry. Similar quantification was found for V-Ncad and V-W2A probing for GFP.

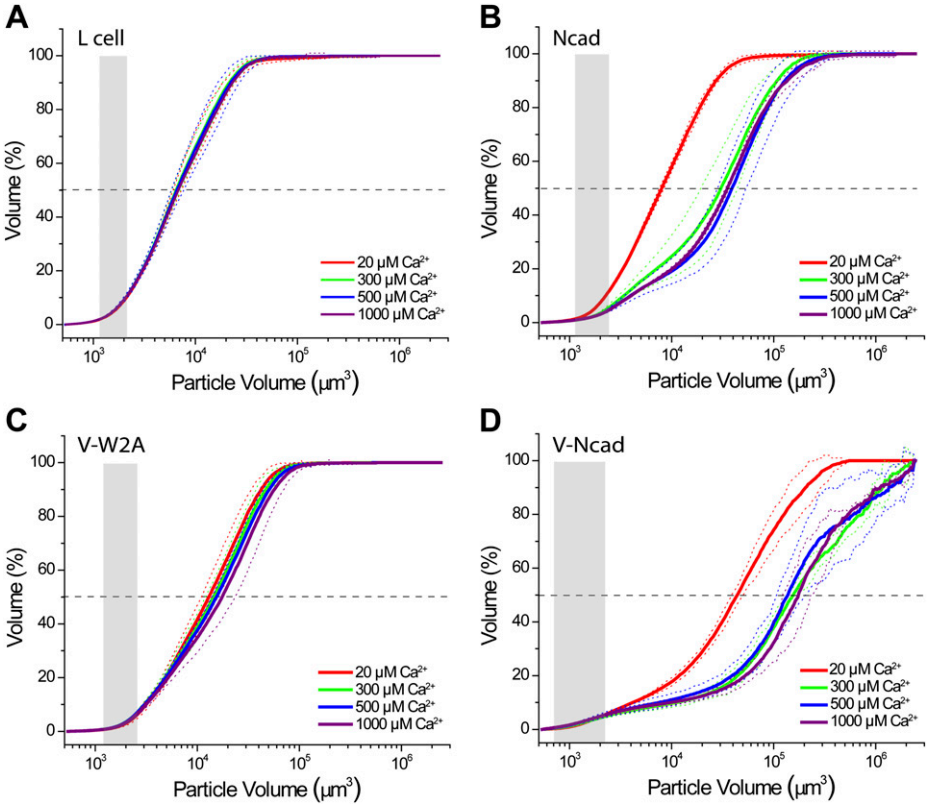

Fig. S3. Ca^{2+}-dependent aggregation of *N*-cadherin stable lines. Average normalized cumulative distributions (solid lines) of percent volume with two SD (dotted lines) of (*A*) L-, (*B*) Ncad, (*C*) V-W2A, and (*D*) V-Ncad cell lines measured at varying Ca^{2+} concentrations after 60 min. Dashed horizontal lines (black) indicate the median values, and the gray shaded region indicates mean volume for a single cell ± SEM. Both Ncad and V-Ncad populations showed increasing aggregation with higher Ca^{2+} concentration. In contrast, the untransfected L-cell line showed no aggregation and the V-W2A stable line showed some aggregation at higher Ca^{2+} concentrations.

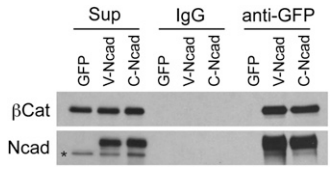

Fig. S4. β- Catenin interacts with the *N*-cadherin fusion proteins. COS-7 cells were transiently transfected with GFP, V-Ncad, or C-Ncad, and whole-cell lysates were harvested. Each fusion protein (GFP, V-Ncad, or C-Ncad) was immunoprecipitated using an anti-GFP antibody. The supernatant before the immunoprecipitation (Sup), the pull-down of the IgG beads alone (IgG), and the IgG beads with anti-GFP antibody (anti-GFP) were resolved by SDS/PAGE (5–15%) followed by immunoblotting using anti–β-catenin and anti–*N*-cadherin antibodies. After immunoprecipitation with an anti-GFP antibody, bound β-catenin was detected for V-Ncad and C-Ncad by immunoblotting with an anti–β-catenin antibody. No bands were seen with the IgG beads alone. A lower band was seen for *N*-cadherin because COS-7 cells possess endogenous *N*-cadherin (*).

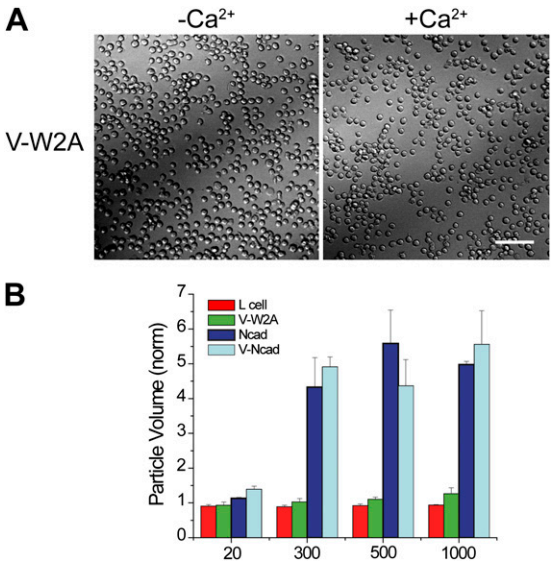

Fig. S5. Venus/W2A/*N*-cadherin stable cell line in a Ca^{2+}-dependent aggregation assay. (*A*) After 60 min, V-W2A shows slight aggregation in the presence of Ca^{2+} (2 mM). (*B*) The Ca^{2+} titration curve for V-W2A has been added to the bar graph for the other cell lines (Fig. 1*E*) for comparison. V-W2A shows modest aggregation at higher Ca^{2+} concentrations compared with untransfected L-cells.

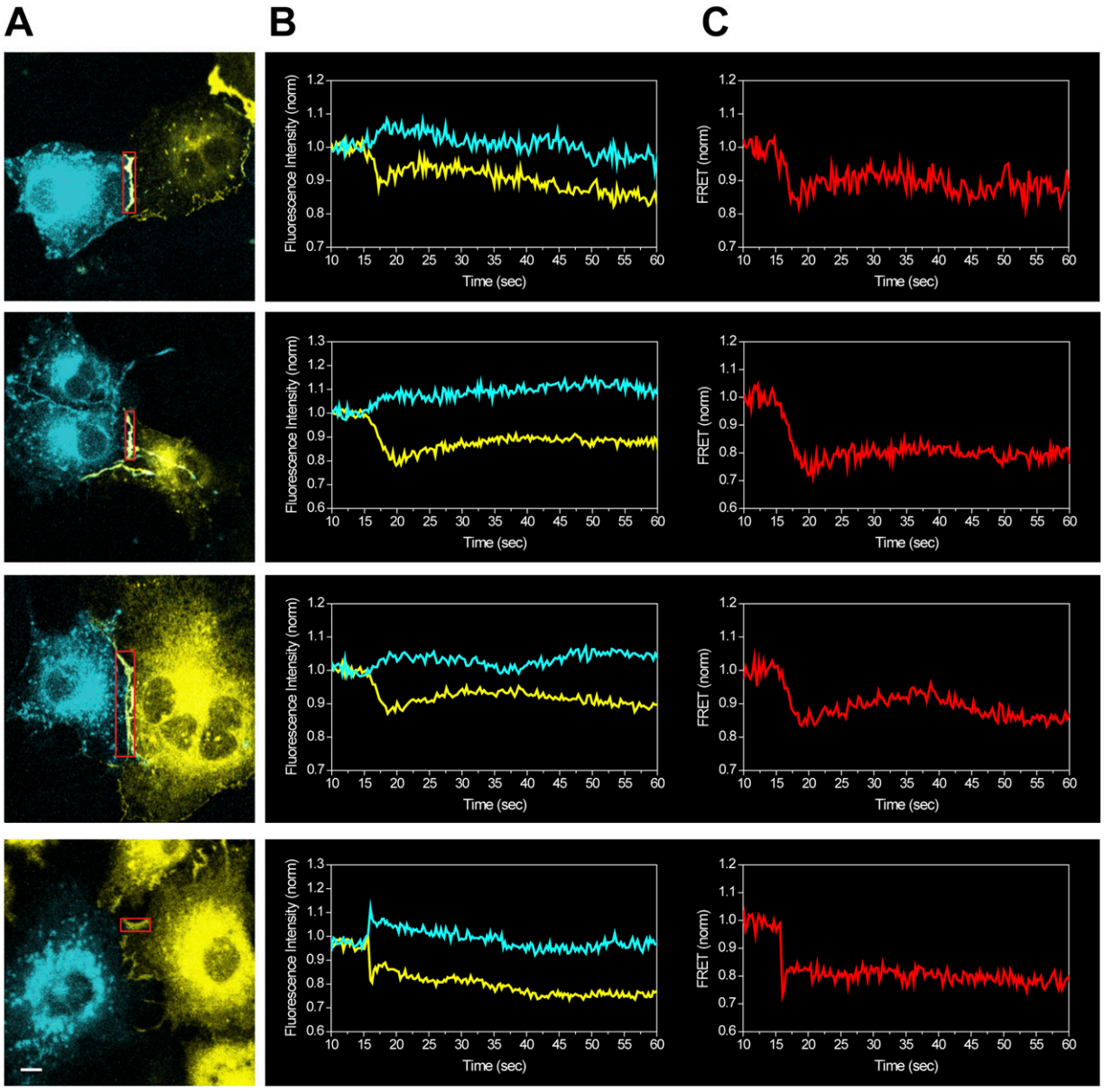

Fig. S6. Examples of changes in FP-Ncad interactions upon Ca^{2+} chelation. (*A*) Images of COS-7 cells expressing FP-Ncad FRET constructs. ROIs (red box) at two-color junctions were selected and imaged every 100–250 ms. (*B*) Quantitation of C-Ncad (cyan) and V-Ncad (yellow) fluorescence intensity changes with Ca^{2+} chelation. Baseline was defined as the average between 10 and 15 s, and all values were normalized to the baseline. Specific changes are seen as the two fluorescence intensities of the reporter anticorrelated upon the addition of EGTA (20 mM final; same condition for all rows) at 15 s. (*C*) Ca^{2+}-dependent FRET changes over time. Emission ratiometric spectral FRET (V-Ncad/C-Ncad; red) reflected loss of interactions due to the removal of Ca^{2+}. (Scale bar: 10 μm.)

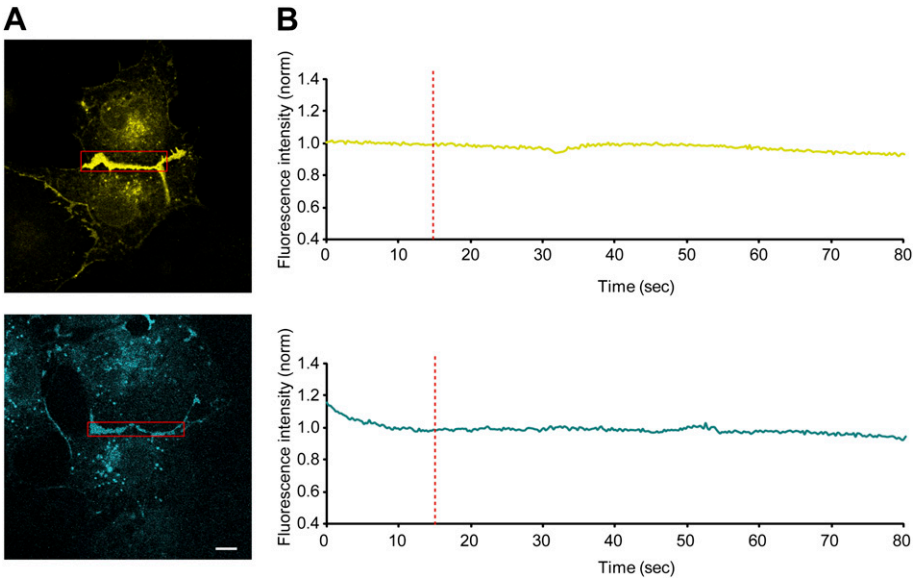

Fig. S7. Control measurements for FRET experiments. (*A*) For single-color junctions, COS-7 cells were transfected with either V-Ncad (*Upper*) or C-Ncad (*Lower*). ROIs (red box) at single-color junctions were selected and imaged approximately every 100 ms. (*B*) Normalized traces of C-Ncad and V-Ncad average fluorescence intensity showed no change in either case upon Ca^{2+} chelation (20 mM EGTA added at 15 s as marked by the red dotted line). (Scale bar: 10 μm.)

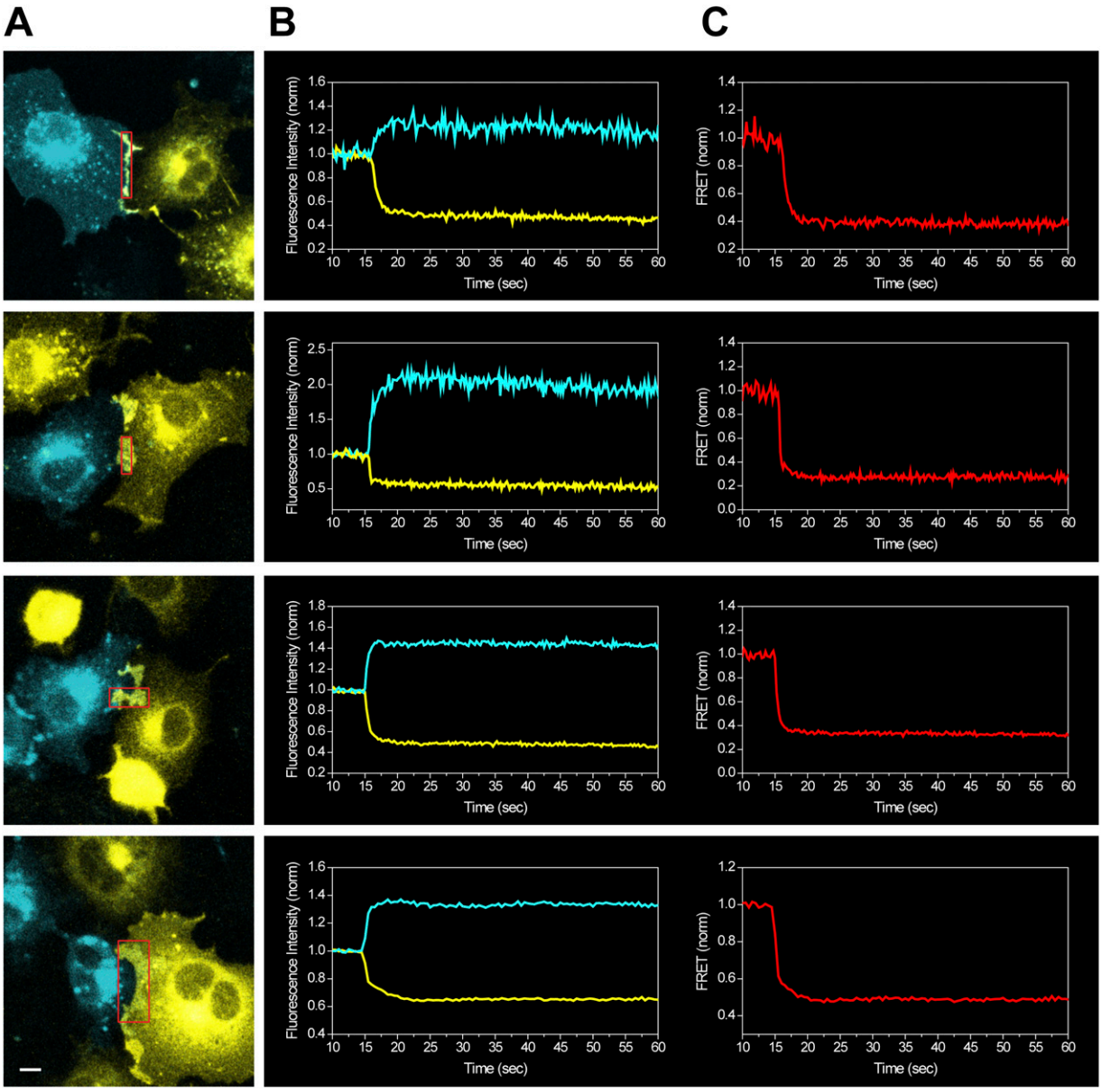

Fig. S8. Examples of changes in FP-W2A interactions upon Ca^{2+} chelation. (*A*) Images of COS-7 cells expressing the W2A variant of the *N*-cadherin FRET reporter. ROIs (red box) of two-color junctions were selected and imaged every 100–250 ms. (*B*) Quantitation of C-W2A (cyan) and V-W2A (yellow) fluorescence intensity changes with Ca^{2+} chelation. Baseline was defined as the average between 10 and 15 s, and all values were normalized to the baseline. Specific changes were seen as the two fluoresence intensities of the reporter anticorrelated upon the addition of EGTA (20 mM final; same condition for all rows) at 15 s. (*C*) Ca^{2+}-dependent FRET changes over time. Emission ratiometric spectral FRET (V-W2A/C-W2A; red) reflected loss of interactions due to the removal of Ca^{2+}. (Scale bar: 10 μm.)