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Calcium-dependent dynamics of cadherin interactions at cell–cell junctions

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Cadherins play a key role in the dynamics of cell-cell contact formation and remodeling of junctions and tissues. Cadherincadherin interactions are gated by extracellular Ca^{2+} , which serves to rigidify the cadherin extracellular domains and promote trans junctional interactions. Here we describe the direct visualization and quantification of spatiotemporal dynamics of N-cadherin interactions across intercellular junctions in living cells using a genetically encodable FRET reporter system. Direct measurements of transjunctional cadherin interactions revealed a sudden, but partial, loss of homophilic interactions ($\tau = 1.17 \pm 0.06 \text{ s}^{-1}$) upon chelation of extracellular Ca²⁺. A cadherin mutant with reduced adhesive activity (W2A) exhibited a faster, more substantial loss of homophilic interactions ($\tau = 0.86 \pm 0.02 \text{ s}^{-1}$), suggesting two types of native cadherin interactions-one that is rapidly modulated by changes in extracellular Ca²⁺ and another with relatively stable adhesive activity that is Ca²⁺ independent. The Ca²⁺-sensitive dynamics of cadherin interactions were transmitted to the cell interior where β -catenin translocated to *N*-cadherin at the junction in both cells. These data indicate that cadherins can rapidly convey dynamic information about the extracellular environment to both cells that comprise a junction.

cell adhesion | fluorescence resonance energy transfer | trans binding

The junctions between cells are populated with a variety of cell adhesion molecules that drive the recognition, assembly, and dynamics of cell–cell interactions. Parsing the distinct functions of different adhesion molecules has been challenging, in part due to a paucity of truly revealing in vivo assays. Among cell adhesion molecule families, the classic cadherins exhibit a unique dependence on extracellular Ca²⁺ to rigidify the extracellular domains and enable *trans* junctional homophilic interactions. Three Ca²⁺ ions bind with different affinities to each of the pockets between cadherin extracellular domains (1, 2). The affinity of the various Ca²⁺ binding sites is in the micromolar to millimolar range (3–8), suggesting the possibility that cadherins can respond dynamically to changes in junctional Ca²⁺ levels, and by virtue of physical interactions with cytoplasmic molecules, signal information about the junctional status to the cell interior.

3D reconstructions of desmosomes by cryoelectron tomography reveal cadherins interact across intercellular interfaces (9, 10), and crystallographic data of several cadherin domains (4, 11, 12) implicate the first two extracellular repeats (ECs 1 and 2) as critical for homophilic interactions. Previous studies have indicated the importance of a highly conserved tryptophan residue (Trp2), present in the first EC domain, for cadherin-dependent adhesion (2, 12). This tryptophan inserts into the hydrophobic pocket of the partner N-cadherin (Ncad) molecule to form a strand-swapped dimeric structure (11, 12). Mutation of Trp2 to an alanine residue (W2A) prevents strand swapping (13) and results in a loss of adhesive function (2, 14, 15). Although extensive work has elucidated the structure (4, 9-14), biochemistry (2, 16) and single-molecule characterization (15, 17) of cadherin adhesion, relatively little is known about these interactions and their dynamics in living cells.

In this work we develop a genetically encoded fluorescence reporter system that enables the visualization and quantification of spatiotemporal dynamics of N-cadherin interactions across intercellular junctions in live cells. We show that N-cadherin molecules exhibit a sudden, partial loss of interaction upon rapid removal of extracellular Ca^{2+} . In comparison, a variant with a point mutation (W2A) that substantially decreases adhesive activity exhibited a faster, more substantial loss with Ca²⁺ chelation, These data reveal a much greater structural change due to Ca²⁺ depletion in the absence of the Trp2-hydrophobic pocket interaction of EC1 and suggest a cadherin-cadherin interaction in the wild-type protein that is Ca²⁺ independent but Trp2 dependent. Furthermore, β-catenin, an intracellular binding partner of N-cadherin that mediates the association of cadherins with the actin cytoskeleton, translocates to the junction in both cells upon Ca^{2+} removal. We postulate that the rapid Ca^{2+} sensing property of cadherin molecules may coordinate adhesion and allow for both cells to access information about junctional status simultaneously and signal this information to the cell interior.

Results and Discussion

To examine the spatiotemporal dynamics of cadherin interactions, we designed a genetically encodable intermolecular FRETbased reporter system that exploits the physical interaction of cadherins at junctions and uses Cerulean fluorescent protein (FP) as the FRET donor (18) and Venus FP as the FRET acceptor (19) (Fig. 1*A*). The dimensions of each cadherin EC are known to be ~45 Å long × 25 Å diameter. Based on this, we hypothesized that Cerulean and Venus (~42 Å in length × 24 Å diameter barrel) (20) inserted within the first two distal EC domains would be within the calculated Förster distance ($R_0 = 54$ Å for Cerulean and Venus) (21) and efficiently report cadherin interactions across junctions (Fig. 1*A*).

To insert a GFP into the compact extracellular domain of N-cadherin without affecting its function, we performed an in vitro transposition screen to isolate functional clones (22). We screened 509 colonies and found four clones that had GFP inserted in the distal extracellular region (from the N terminus of the first EC to the C terminus of the second EC). From this screen, we chose a clone with GFP inserted in one of the solvent-exposed helices in extracellular domain 2 (FP-Ncad; Fig. 1*B*) (2) that had the brightest expression at cell-cell contacts. We

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Fig. 1. Design and characterization of *N*-cadherin FRET reporter. (*A*) Schematic of *N*-cadherin–*N*-cadherin interactions at cell–cell junctions monitored by FRET. Light blue ovals represent the five EC domains; the inserted fluorescent protein, Venus (yellow) or Cerulean (cyan), is located in the EC2 domain. Dynamic interactions of cadherins monitored by FRET result in intensity changes of each fluorescent protein. (*B*) Domain structure of fluorescent protein insertion site in *N*-cadherin (gray, signal peptide; light blue, EC domains; dark blue, transmembrane domain; blue arrow, FP insertion site). Amino acid numbers are indicated below the bar. (*C*) *N*-cadherin fusion proteins. Cerulean (*Left*), Venus (*Center Left*), and Cerulean/Venus (*Center Right*) localize to the membrane and form cell–cell contacts similar to the localization of endogenous Ncad (*Right*) in COS-7 cells. (Scale bar: 10 µm.) (*D*) L-cells expressing Ncad or V-Ncad constructs show robust cell aggregation under high Ca²⁺ (1 mM Ca²⁺). (Scale bar: 50 µm.) (*E*) Ncad and V-Ncad stable cell lines show similar and increasing aggregation with increasing extracellular Ca²⁺ concentrations. No statistically significant differences (Student *t* test; *P* < 0.05) were seen at higher Ca²⁺ concentrations (300–1,000 µM). Error bars indicate \pm SEM for *n* = 3–4 for each condition. Untransfected L-cells showed no aggregation under any conditions.

replaced the GFP with Cerulean or Venus and then transfected two separate populations of COS-7 cells with either Cerulean (C-Ncad) or Venus cadherin (V-Ncad). Later, individual populations or mixed populations were plated to examine the junctional localization. Junctions comprising either a single cadherinfluorescent species (C-Ncad or V-Ncad) or mixed junctions (C-Ncad and V-Ncad) exhibited the appropriate membrane localization of cadherin molecules at cell–cell interfaces, similar to endogenous cadherin (Fig. 1*C*).

We next examined whether our fluorescently labeled cadherin fusion proteins retained Ca²⁺-dependent adhesion with similar Ca²⁺ binding properties to wild-type cadherin. L-cells that lack endogenous cadherins have been used extensively for cadherindependent adhesion function assays (23, 24). In L-cells stably transfected with either unlabeled wild-type Ncad or V-Ncad, comparable levels of total cadherin were expressed in each cell line with the majority of expression on the cell surface (Figs. S1 and S2). We assessed Ca^{2+} -dependent cell aggregation of these stable lines using a quantitative short-term aggregation assay. V-Ncad conferred Ca²⁺-dependent adhesion to L cells similar to wild-type Ncad within a physiologically relevant Ca2+ concentration range (Fig. 1 D and E and Fig. S3). In contrast, the untransfected L-cells showed no detectable aggregation under any conditions, as expected. Furthermore, fluorescently labeled cadherin fusion proteins interacted with their intracellular binding protein, β-catenin, as detected by immunoprecipitation and immunoblotting (Fig. S4). These results indicate that the fluorescent N-cadherin protein fusions exhibit features similar to endogenous *N*-cadherins, including membrane localization, Ca^{2+} -dependent adhesion, and interaction with β -catenin.

To assess the molecular interaction of cadherins under basal conditions in living cells, we performed acceptor bleach FRET experiments on junctions of adjacent transfected COS-7 cells expressing either V-Ncad or C-Ncad fusion proteins (Fig. 24). Following acceptor bleaching of V-Ncad at well-defined twocolor junctions, donor dequenching of C-Ncad was observed (Fig. 2A), indicating that the fluorescent molecules from the two different cell membranes were well within the distance required for FRET. The average FRET efficiency ($34.1 \pm 2.32\%$; mean \pm SEM; n = 10; Fig. 2E) for the wild-type Ncad interactions in living cells most likely represents the molecules in a strandswapped trans dimer configuration (13). Because FRET is distance dependent, we examined the spatial limits for transjunctional FRET using a different clone with an insertion site between EC5 and the transmembrane domain found in our transposition screen (Fig. 2F, red arrow, referred to as FP_{prox}-Ncad). Although FP_{prox} -Ncad localized to cell-cell contacts (Fig. 2B), the average FRET efficiency was markedly less than FP-Ncad ($6.6 \pm 1.01\%$; mean \pm SEM; n = 10; Fig. 2E), as expected from the relatively remote positions of the fluorophores (Fig. 2F). As a control, measurements in well-defined single-color junctions of cells expressing V-Ncad or C-Ncad alone indicated no evidence of photoconversion of Venus into a Cerulean-like species as previously reported (25). In additional analyses we were able to quantify and qualitatively map the Ncad interactions assessed by FRET within live cellular contacts, revealing an apparent spatial heterogeneity of Ncad binding within junctions with small



Fig. 2. Detecting *N*-cadherin interactions under basal conditions across cell–cell junctions. A representative example of a cell–cell junction of two adjacent transfected COS-7 cells expressing (*A*) V-Ncad or C-Ncad, (*B*) V_{prox}-Ncad or C_{prox}-Ncad, and (*C*) V-W2A or C-W2A fusion proteins under basal high Ca²⁺ conditions (1.8 mM Ca²⁺). The acceptor (Venus fusion protein) was bleached within the ROI (red box); images were acquired before and after bleaching (see *D* for color look-up table). (Scale bars: images, 10 µm; corresponding ROIs, 1 µm.) (*D*) Quantitative spatial FRET maps for each of the examples shown in *A*–*C*, respectively, show the heterogeneity of cadherin interactions at cell–cell contact. (Scale bar: same as for corresponding ROIs in *C*.) (*E*) Average FRET efficiencies for WT, W2A, and WT_{prox}*N*-cadherin junctions. Error bars indicate \pm SEM for *n* = 10 cells each. (*F*) *N*-cadherin domain structure indicating the W2A mutation (green; FP-W2A), and distal (blue; FP-Ncad) and proximal (red; FP_{prox}-Ncad) insertion sites for FRET reporter constructs.

(<1–2 μ m) regions showing much stronger FRET than adjacent areas (Fig. 2D).

An important amino acid responsible for cadherin-cadherin adhesion is the highly conserved Trp2, present in the first EC domain, which inserts into the hydrophobic pocket of the partner N-cadherin molecule (2, 3, 12). Mutation of Trp2 to an alanine residue (W2A) prevents strand-swapping and results in substantially reduced cadherin homophilic interactions (2, 3). Similar to N-cadherin (FP-Ncad) expression in L cells, FP-W2A exhibited membrane localization but with less of the total cellular surface area comprising contacts with neighboring cells (Figs. S1 and S2). The V-W2A cell line exhibited modest aggregation but only at higher Ca^{2+} concentrations (Student *t* test; P < 0.05; Figs. S3 and S5), reflecting a markedly weaker binding and confirming similar previous findings (13, 15). Expression of FP-W2A in COS-7 cells, which also possess endogenous cadherins, allowed us to assess the nature of the relationship of FP-W2A cadherins at intact junctions. Although reduced adhesion was observed in L-cells expressing FP-W2A, FP-W2A cadherin mutants exhibited a higher average FRET efficiency (47.3 \pm 2.30%; mean \pm SEM, n = 10; Fig. 2E) compared with FP-Ncad (Student t test; P < 0.001), indicating that the W2A molecules are closer together than wild-type NCad. These data are consistent with recent structural data comparing wild-type and W2A dimeric configurations, in which the wild-type trans dimer in a

strand-swapped configuration was found to have a markedly wider angle between paired EC1-2 domains compared with the W2A mutant (13). Due to the placement of our label within the EC2 domain, our FRET measurements directly demonstrate these structural differences in living cell–cell junctions (Fig. 2*F*).

Electron microscopic observations of purified E-cadherin EC domains indicate a progressive rigidification of the modular rodlike structure as the Ca^{2+} concentration is increased from <50 μ M to >1 mM (4). In vivo, the average basal extracellular Ca²⁺ concentration is 1.5-2 mM (26), well within the range where cadherins engage in trans interactions. To test directly whether a change in extracellular Ca²⁺ can be detected by cadherins, we continuously monitored FRET at two-color N-cadherin (FP-NCad) junctions in live cells before and after the chelation of extracellular Ca^{2+} (via the addition of EGTA; Fig. 3 A and B, Upper). The rapid addition of EGTA allowed us to evaluate dynamic changes in the molecular geometry of the labeled cadherins and couple the consequences to signaling. Addition of EGTA triggered a rapid and substantial loss of FRET efficiency (Fig. 3D and Fig. S6), resulting from simultaneous and reciprocal changes in C-Ncad and V-Ncad fluorescence intensities. Emission spectra before and after Ca²⁺ chelator addition indicated a decrease in the Venus emission signal near 530 nm and a corresponding increase in the Cerulean emission signal near 497 nm (Fig. 3C). In control measurements, single-color junctions showed



no significant changes in fluorescence intensity upon extracellular Ca^{2+} chelation (Fig. S7). The reduction in cadherin–cadherin interaction at these junctions exhibited kinetics that was well fit by a single exponential ($\tau = 1.17 \pm 0.06 \text{ s}^{-1}$, mean \pm SEM; n = 17; Fig. 3D). In the continued presence of EGTA, the FRET loss at FP-Ncad junctions reached steady-state and remained stable for the duration of the experiment (Fig. 3 and Fig. S6). Another Ca²⁺ chelator, BAPTA, also elicited similar results.

Given the residual cadherin–cadherin interaction that appeared Ca²⁺ insensitive in the above experiment, we considered the possibility that the molecularly distinct adhesive interface conferred by the Trp2 residue might provide structural stability that maintains some cadherin proximity in the absence of Ca²⁺. To address this, we examined the behavior of the FP-W2A cadherin mutants following a rapid reduction in Ca²⁺. Upon chelation of Ca²⁺, FP-W2A junctions exhibited significantly faster kinetics ($\tau = 0.86 \pm 0.02 \text{ s}^{-1}$; mean \pm SEM; n = 17; Fig. 3F and Fig. S8) and a substantially greater decrease in FRET compared with FP-Ncad junctions (FRET change for FP-Ncad: 14.43 \pm 1.33%, FP-W2A: 57.36 \pm 2.13%; n = 17 each; P < 0.0001; Fig. 3E). The estimated change in intermolecular distance between FP-W2A before and after the addition of EGTA was much larger than for the FP-NCad.

Fig. 3. N-cadherin interactions across junctions exhibit a rapid sensitivity to extracellular Ca2+ changes. (A) Images of COS-7 cells expressing either FP-Ncad or FP-W2A fluorescent fusion proteins of the FRET reporter. ROIs (red boxes) were chosen encompassing well-defined junctions for faster acquisition. ROIs were colorized according to the emission ratio of V-Ncad: C-Ncad fluorescence intensity (V/C ratio). (Scale bar: 10 µm.) (B) Quantitation of Ca2+-dependent cadherin FRET changes over time. ROIs (red boxes in A) at two-color junctions were imaged before and after the addition of the Ca²⁺ chelator, EGTA (20 mM final), at 15 s (gray arrow). The fluorescence intensity of each channel was normalized to the average baseline (vellow for V-Ncad and cyan for C-Ncad) and plotted together with the V/C ratio as a measure of FRET (red). (C) Representative emission spectra of a two-color FP-Ncad junction before and after EGTA treatment. (D and F) Kinetics of the FRET decrease upon Ca²⁺ removal. EGTA was added at 15 s (see gray arrow; FP-Ncad (D) and FP-W2A (F); n = 17 each; mean \pm SEM). Data were fit with a single exponential decay curve (red). (E) Summary of the magnitude of FRET changes upon Ca2+ depletion in FP-Ncad and FP-W2A junctions (n = 17 each; P < 0.0001).

Taken together, our data reveal a much greater structural change due to Ca^{2+} depletion in the absence of the Trp2hydrophobic pocket interaction of EC1 and indicate a cadherincadherin interaction in the wild-type molecule that is Ca^{2+} independent. These findings may represent (*i*) a mixed population of dynamic molecules, some which unbind, similar to the W2A dimer configuration, and others that exhibit stable, EGTAresistant interactions (represented by the remaining residual FRET efficiency); (*ii*) a population that converts to a different stable state (following EGTA treatment) where the fluorophores move to a less favorable FRET orientation; or (*iii*) a combination of all of the above. The current data does not allow us to determine which of these interpretations is correct, but future research will provide more insight into these different states of wild-type *N*-cadherin interactions upon unbinding.

The homophilic nature of cadherin associations allows, in principle, for the transmission of information to the interior of both cells that comprise a junction. To monitor whether a change in signaling can be invoked by extracellular Ca²⁺ dynamics and the loss of cadherin interactions, we examined the dynamics of β -catenin, an intracellular binding partner of *N*-cadherin that regulates the interaction of cadherins with the actin cytoskeleton (27). COS-7 cells were transfected with a β -catenin–GFP construct, and two neighboring cells were imaged before and after

chelation of extracellular Ca²⁺. Upon addition of EGTA, a bidirectional translocation of β-catenin-GFP to cell-cell contacts was observed (immediate onset upon treatment; $\tau = 4.38 \pm$ 0.25 s; mean \pm SEM; n = 7; Fig. 4 A and D). This increase in β -catenin–GFP fluorescence at the junction was associated with a concomitant decrease in cytosolic fluorescence in both cells during Ca^{2+} removal (Fig. 4C). When the same experiment was performed in L-cells that lack endogenous cadherins, β-catenin failed to translocate to the junction (Fig. 4B), indicating that the junctional translocation is cadherin dependent. The fast membrane translocation of β -catenin upon Ca²⁺ chelation indicates that information about the extracellular milieu can be rapidly transmitted to the cytoplasm. The recruitment of β -catenin to the junctional membrane likely represents a compensatory mechanism to stabilize cadherin interactions affected by Ca²⁺ chelation. It has been shown previously that catenins can directly influence the adhesive state of cadherins (28).

Our results show that cadherin molecules localized at junctions in living cells adopt structurally distinct conformations and can sense changes in extracellular Ca²⁺ on a rapid timescale. The FRET measured via fluorophores associated with EC2 provides evidence in living cells for recently described cadherin-cadherin structural interactions comparing wild-type and W2A dimeric configurations (13, 15). The W2A mutant described as the "initial encounter complex" (13) or "X-dimer" (17) has been proposed to be an important intermediate step in the assembly and disassembly of strand-swapped dimers. A recently published paper suggests that the X-dimer may not be an essential step in cadherin assembly at cell-cell contacts but instead is likely a critical transition stage for the release of cadherins from junctions (29). In our experiments we found that the W2A mutant was able to form weak adhesive complexes and possessed rapid dissociation kinetics, consistent with it being an intermediate step in disassembly of strand-swapped dimers.

Furthermore, we show that underlying a dynamic Ca^{2+} -dependent change is a residual, EGTA-insensitive component to

the wild-type cadherin–cadherin interactions found at cell junctions. The dissociation rate of strand-swapped dimers at cell–cell contacts in vivo may be slower than dissociation kinetics measured in vitro because in vivo dimers are likely reinforced by *cis* interactions (30, 31). A similar Ca^{2+} -independent stability has been described using cross-linking techniques for lateral dimers (32, 33). Previous work examining cadherin dynamics (34) and the Ca^{2+} dependence of cadherin interactions (35) demonstrated changes with a much longer latency (minutes or hours, rather than seconds) than we have observed here. Our data indicate that extracellular Ca^{2+} fluctuations result in a rapid regulation of cadherin interactions, and that these dynamics can invoke changes in intracellular signaling.

The presence of Ca²⁺-permeable channels and pumps, as well as a large extra/intracellular Ca²⁺ concentration gradient, suggests that the extracellular ionic environment is dynamic. Indeed, extracellular Ca²⁺ fluctuations have been documented in bone (36), cardiac muscle (37), and neuronal synapses (38, 39). Of particular interest is the neuronal synapse where fluctuations of extracellular Ca2+ have been described and characterized. Simulations of active synaptic clefts (26, 40) predict that during synaptic activity, Ca^{2+} concentrations in the cleft drop into the range where local synaptic and/or perisynaptic cadherins would lose transinteractions (~0.8-1 mM). In addition, experimental measurements in the synaptic cleft during high-frequency stimulation have shown that Ca^{2+} concentration may drop to as low as 0.3-0.8 mM (41, 42), and Ca²⁺-dependent inhibition of cadherin function has been observed during synaptic plasticity (43). Biophysical measurements of purified cadherin extracellular domains, using laser tweezers, showed that an extracellular drop in Ca^{2+} from 1.5 to 0.8 mM is predicted to decrease binding of N-cadherin by $\sim 40\%$, whereas a further drop to 0.3 mM would result in a reduction of binding by 85% (44). Thus, the Ca² dependence of cadherin interactions seems to be appropriately tuned to monitor physiological fluctuations of Ca2 concentrations. The rapid Ca²⁺ sensing property of cadherin molecules



Fig. 4. Extracellular Ca²⁺ chelation results in a translocation of β -catenin to the membrane. β -catenin-GFP is expressed in two neighboring cells in either COS-7 cells with endogenous N-cadherin (A) or in L-cells in the absence of N-cadherin (B). Cells were imaged every 1 s before and after the addition of the Ca²⁺ chelator EGTA at 30 s (A and B Upper). An ROI (red box) of the junctions (A and B Right) shows pre- and post-EGTA treatment and is pseudocolored according to fluorescence intensity (see C for color look-up table). (Scale bars: images, 5 µm; corresponding ROIs, 2 μm.) β-catenin–GFP fluorescence intensity changes in the ROIs were normalized to baseline and plotted over time (A and B Lower). (C) Kymograph showing β-catenin–GFP fluctuations at the junction and neighboring cytosol over time. The one-pixel line scan (marked by the two red arrowheads in A) was colorized, and the summary graph indicated a corresponding drop in the cytosol near the junction. (D) Kinetics of the β-catenin-GFP fluorescence change upon Ca2+ removal. EGTA was added at 30 s (n = 7; mean \pm SEM). Data were fit with a Boltzmann function (red).

that we document here may coordinate adhesion and signaling across cell–cell junctions, allowing both cells that comprise a junction access to the same information at the same time.

Materials and Methods

FRET Constructs. To generate a functional *N*-cadherin fusion protein with GFP, we used the bacterial transposon-mediated random insertion technique (22) (*SI Materials and Methods*). From our screen, we chose one particular clone with GFP inserted in the extracellular domain (EC2) of cadherin because of its close proximity to the adhesion site at the tip as well as a brighter expression on the membrane and at cell–cell junctions in HEK293 cells. For FRET measurements, the GFP was replaced by Venus and Cerulean. Methods for immunoprecipitation and immunofluorescence have been described previously (45) (*SI Materials and Methods*).

FRET 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 (21). Acceptor bleach FRET was performed by

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continuous bleaching with the 514-nm laser (100% power, 2,000 iterations) within a chosen ROI. To resolve faster kinetics for time-lapse ratiometric FRET measurements, continuous fast scans (100 ms) of smaller fields of view were used to monitor FRET changes. 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 (21). The mean fluorescence intensity was measured for each fluorophore, and the FRET efficiency was expressed as the emission ratio of Venus:Cerulean. Full methods are provided in *SI Materials and Methods*.

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Supporting Information

Kim et al. 10.1073/pnas.1019003108

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 (MLRYRILS-LSLIHIWRA-GFP-GRARADVYKRQILS-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 (NGDCTDVD-LSLIHIWRA-GFP-GRARADVYKRQDVD-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% CO₂ 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 Ca²⁺ 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 MgCl₂, 0.1 mM CaCl₂) 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, Ca²⁺ and Mg²⁺ free (HCMF) + 5 mM CaCl₂ [HCMF, in mM: 137 NaCl, 5.4 KCl, 0.34 Na₂HPO₄, 0.1% glucose, 10 Hepes (pH 7.4)], and each flask was incubated in 5 mL of HCMF + 5 mM CaCl₂ 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 MgCl₂). 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% CO2 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 co-incidence 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 CaCl₂, 2 MgCl₂, 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. Customwritten 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 = 1pixel 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:

$$E = R_0^6 / (R_0^6 + R^6),$$
 [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:

$$E = (F_{CerPost} - F_{CerPre})/F_{CerPost}$$
^[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 Ca2+ 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

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(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).

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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 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 \pm 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 twocolor 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 μ m.)



Fig. 57. 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 μ m.)

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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 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-W2A/C-W2A; red) reflected loss of interactions due to the removal of Ca^{2+} . (Scale bar: 10 µm.)

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