α-Catenin mediates initial E-cadherin-dependent cell–cell recognition and subsequent bond strengthening

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α-Catenin is essential in cadherin-mediated epithelium development and maintenance of tissues and in cancer progression and metastasis. However, recent studies question the conventional wisdom that α-catenin directly bridges the cadherin adhesion complex to the actin cytoskeleton. Therefore, whether α-catenin plays a direct role in cadherin-dependent cell adhesion is unknown. Here, single-molecule force spectroscopy measurements in cells depleted of α-catenin or expressing the hereditary diffuse gastric cancer associated V832M E-cadherin germ-line missense mutation show that α-catenin plays a critical role in cadherin-mediated intercellular recognition and subsequent multibond formation within the first 300 ms of cell contact. At short contact times, α-catenin mediates a 30% stronger interaction between apposing E-cadherin molecules than when it cannot bind the E-cadherin–β-catenin complex. As contact time between cells increases, α-catenin is essential for the strengthening of the first intercellular cadherin bond and for the ensuing formation of additional bonds between the cells, all without the intervention of actin. These results suggest that a critical decision to form an adhesion complex between 2 cells occurs within an extremely short time span and at a single-molecule level and identify a previously unappreciated role for α-catenin in these processes.

cancer | cell adhesion | single-molecule force spectroscopy | actin

Intercellular adhesion depends critically on the cadherin family of transmembrane proteins, which play a central role in the normal development and maintenance of solid tissues and during cancer progression and metastasis (1, 2). The study of early invasive diffuse gastric cancers in carriers of E-cadherin germline mutations demonstrates that its deregulation may also be an initiating event in tumorigenesis (3, 4). When a cell–cell contact is formed, cadherins expressed on neighboring cells interact through their extracellular domain whereas their cytoplasmic domain interacts with the cytoskeleton through the catenin family of cadherin-binding proteins. The basic molecular entity responsible for cell adhesion in epithelial cells is a 1:1:1 complex comprising E-cadherin, β-catenin, and α-catenin (5). In the intracellular space, the cytoplasmic domain of E-cadherin binds β-catenin, which in turn binds α-catenin through its N terminus (Fig. 1*A*). On the extracellular side, adhesive interaction between E-cadherins expressed on apposing cells occurs through a little understood molecular mechanism. Surface force apparatus measurements suggest that this mechanism involves full-length E-cadherin *cis*-dimers, which bind to other *cis*-dimers on apposing cells, through a *trans*-configuration (6, 7). However, experiments using recombinant proteins suggest that E-cadherins dimerize through the EC1 domain of E-cadherin through a site that promotes both lateral and adhesive interactions (8, 9). It has also been reported that formation of *cis*- or *trans*-dimers is governed by the presence of Ca^{2+} (10). Given the widely debated nature of E-cadherin oligomerization, further studies are needed in addressing this issue.

Until recently, α-catenin was believed to bind both β-catenin and actin, serving as the direct linkage molecule between the adhesion complex and the actin cytoskeleton. α-Catenin knockout cells show a reduced ability to adhere to each other, which was thought to be caused by the disconnection of cadherins from the actin filament network because of their mutual linker, α-catenin. However, a critical study recently showed that, although α-catenin associates with β-catenin and actin individually, it does not bind to them simultaneously (5). Moreover, several actin-binding proteins (α-actinin, vinculin, etc.), which also bind α-catenin separately, do not appear in significant or stoichiometric amounts in the complex in immunoprecipitation pull-down assays (5). Because α-catenin does not seem to be the direct linkage between the E-cadherin–β-catenin complex and the actin cytoskeleton, whether α-catenin plays a direct role in cadherin-mediated cell–cell adhesion, especially at early times of cell contact, is unclear.

Results and Discussion

Our overall hypothesis is that α-catenin modulates cadherin-mediated intercellular adhesion at the earliest step possible in the formation of an intercellular junction, i.e., when homotypic molecular recognition between individual cadherin molecules on neighboring cells has just occurred. If so, then in the absence of α-catenin from the E-cadherin–β-catenin complex, early homotypic cadherin binding (if it occurs) is quickly weakened. To test this hypothesis, we generated 2 stable Chinese hamster ovary (CHO) cell lines, one expressing human wild-type (WT) E-cadherins and the other expressing mutated E-cadherins exhibiting the V832M missense point mutation, which has been implicated in hereditary diffuse gastric cancer (11). The V832M mutation affects the first residue of the β-catenin-binding site of the E-cadherin cytoplasmic domain (11). Functionally, it has been shown that CHO cells expressing V832M mutated E-cadherins have abrogated motility, have the ability to invade a collagen or matrigel matrix, and do not form aggregates in cell aggregation assays (12). Importantly for this work, the V832M

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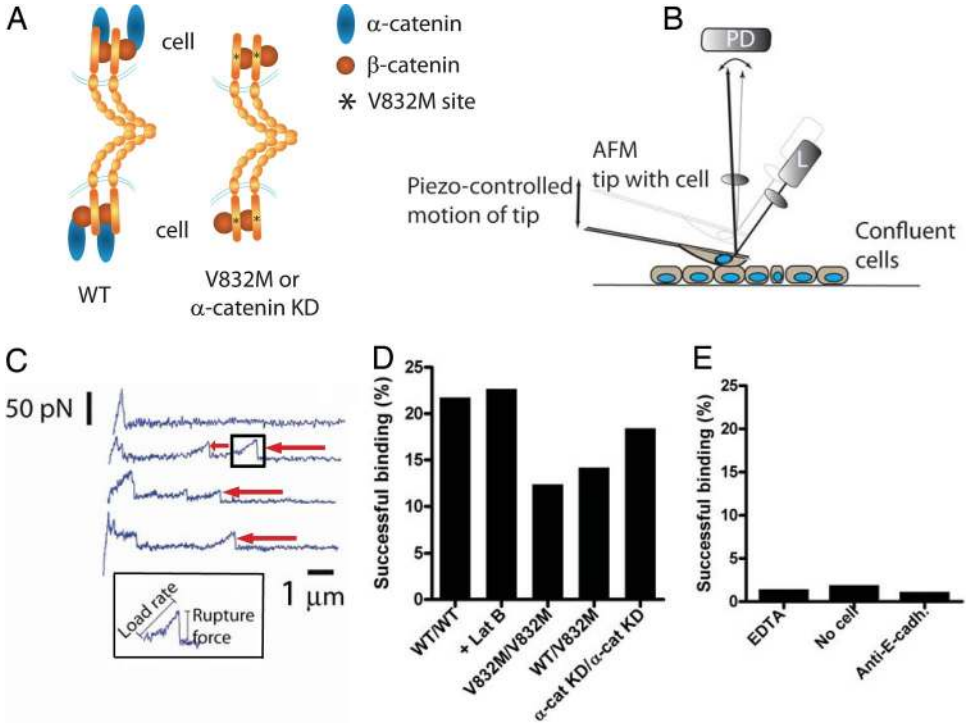

Fig. 1. Live-cell single-molecule force spectroscopy is used to probe the putative modulation of cadherin–cadherin bond tensile strength by α-catenin binding to the E-cadherin–β-catenin complex. (*A*) Schematic of the E-cadherin–catenin complex at the cell–cell adhesion interface after between 2 neighboring cells. The disease-causing V832M mutation prevents binding of α-catenin to the E-cadherin–β-catenin complex. (*B*) Typical succession of force–displacement curves obtained by single-molecule force spectroscopy, whereby E-cadherin-expressing CHO cells, placed in contact with each other for <1 ms with both controlled speed and force, are pulled in opposite directions at a controlled retraction velocity. One cell is placed on a flexible cantilever of known stiffness and whose displacements are laser-tracked with nanometer spatial and millisecond temporal resolutions, and the other cell is placed on a bottom culture dish. Force–displacement curves reveal cadherin-bond ruptures as abrupt falls indicated by arrows. (*Inset*) The loading rate (pico-Newtons per second) applied on each bond is computed as the product of the slope of the force–displacement curve before bond rupture (pico-Newtons per micrometer) with the computer-controlled retraction velocity (micrometers per second). (*C*) Experimental setup used in this work to measure intercellular interaction at single-molecule resolution. (*D*) Probability of bond formation between 2 CHO cells expressing both WT E-cadherin, WT E-cadherin and treated with latrunculin B, both V832M-E-cadherin, 1 V832M-E-cadherin and the other 1 WT E-cadherin or WT-cadherin with α-catenin knocked down. (*E*) Probability of bond formation between 2 E-cadherin-expressing CHO cells in the presence of EDTA, an anti-cadherin antibody, and with no cell placed on the cantilever.

mutation was shown to reduce the binding affinity of α-catenin to the E-cadherin–β-catenin complex (Figs. 1*A* and 2*D*), presumably through an allosteric effect, and correlates with global loss of cell–cell adhesion (12). The interaction between E-cadherin and β-catenin is not affected by the V832M mutation, at least in our cell model. Using a point mutant instead of domain deletion to eliminate α-catenin binding to the E-cadherin–β-catenin complex serves 2 purposes. It reduces the chances of uncontrolled effect on E-cadherin structure and tests the functional relevance of a pathological mutation.

To test whether α-catenin binding to the adhesion complex affected the strength of a single bond made of cadherin pairs on the surfaces of 2 apposing cells, single-molecule force spectroscopy measurements (13, 14) were conducted on live cells expressing either WT or V832M E-cadherins. In this assay, a cell attached to a cantilever, whose position is detected with a laser and a quadrant detector (Fig. 1*B*), is brought into contact with a cell on a bottom dish for a controlled time of contact (minimum 1 ms). The cantilever is then retracted at a controlled velocity, and force-deflection curves are recorded with high-temporal resolution (Fig. 1*C*). An abrupt deflection in these curves corresponds to the rupture of an individual cadherin–cadherin bond that formed during the controlled contact between the 2 adjoining cells (Fig. 1*C*, arrows). The magnitude of this deflection is the adhesion force or tensile strength of that single bond (Fig. 1*C Inset*).

Without the expression of specific surface adhesion molecules, WT CHO cells interact weakly with each other. Nevertheless, to test further the specificity of our single-molecule force spectroscopy measurements, we conducted 3 negative controls [see details in *Experimental Procedures* and in supporting information (SI) *Text* and Figs. S1 and S2]. We found a greatly diminished probability of binding between 2 cells (i.e., the fraction of force-deflection curves with abrupt deflections) when EDTA was added to the cell medium or a function-blocking antibody against E-cadherin was present in the cell medium or when no cell was present on the cantilever (Fig. 1, *D* and *E*).

The distributions of tensile (adhesion) strengths of individual WT E-cadherin and mutant E-cadherin bonds formed between 2 adjoining cells were qualitatively and quantitatively different. For WT E-cadherin intercellular bonds, the distribution of tensile strengths was relatively symmetric (Fig. 2*A*). This distribution showed no second or third peak at quantitated values of tensile strength, which would have indicated the rupture of multiple bonds simultaneously during de-adhesion of the top cell from bottom cell (15). For V832M E-cadherins, the distribution of bond tensile strengths was wide and asymmetric (Fig. 2*B*). Fits of these tensile strength distributions revealed that the depth of the potential of the energy well describing the interaction between WT E-cadherin pairs was significantly deeper than for the V832M E-cadherin pairs (9 k_BT vs. 7 k_BT where $k_BT \approx 4.1$ pN.nm), which indicates that the WT bond was approximately $\exp(9/7) \approx 4$ times more stable than the mutant bond. Also, the

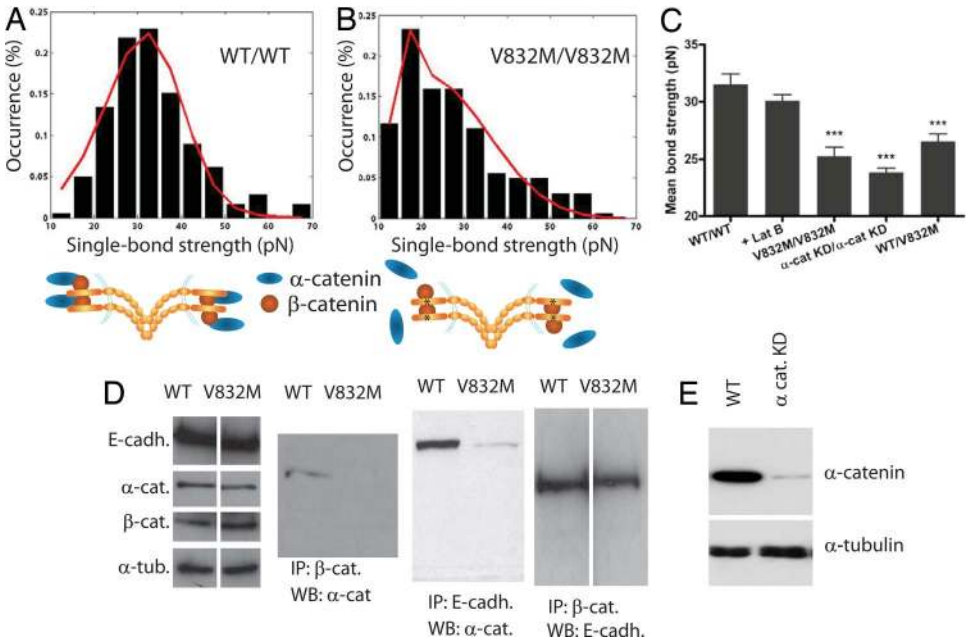

Fig. 2. Difference in tensile strength of intercellular E-cadherin bonds is mediated by α-catenin. (*A* and *B*) Distributions of tensile strengths of (*A*) single WT–WT E-cadherin bonds formed between 2 neighboring cells for which α-catenin can bind the cadherin–β-catenin complex and (*B*) single V832M–V832M E-cadherin between 2 mutant cells, for which α-catenin cannot bind the complex. Both distributions were obtained with a constant cantilever retraction velocity of 5 μm/s. Time of cell–cell contact, < 1 ms. The lines depict fits obtained using Hummer and Szabo model (27) to estimate interaction energies. (*C*) Mean tensile strengths of individual WT–WT E-cadherin, V832M–V832M E-cadherin, WT-WT E-cadherin with α-catenin knocked down, and WT–V832M E-cadherin bonds in untreated cells, and WT–WT E-cadherin bonds formed between 2 cells treated with the actin-depolymerizing drug latrunculin B. Retraction velocity of the cantilever, 5 μm/s. ***, $P < 0.001$ compared with WT E-cadherin cells. (*D*) Western blotting and immunoprecipitation (IP) studies indicate a reduced stability of the interaction between α-catenin and β-catenin in V832M CHO-expressing cells. (*E*) Western blotting indicates the presence and absence of α-catenin in WT CHO cells and α-catenin knocked-down CHO cells, respectively.

molecular length over which WT E-cadherin pairs interacted was much longer, 1.1 nm vs. 0.3 nm, which corresponds to a fraction of the length of single-repeat domain of E-cadherin that is ≈2 nm in length. Moreover, the mean tensile strength of a single WT E-cadherin bond (equal to the mean force required to rupture the bond) between 2 cells was significantly higher than the tensile strength of both the homotypic bond formed between V832M E-cadherins on adjoining cells and higher than the heterotypic bond between V832M E-cadherins and WT E-cadherins (Fig. 2*C*).

α-Catenin is not essential for intercellular "molecular recognition," because V832M/V832M and WT/V832M pairings between 2 cells were both permissible. However, these results indicate that, already for short contact times (<1 ms), the binding strength between E-cadherin pairs and associated resistance to dissociation depend critically on the binding state of α-catenin to the E-cadherin–β-catenin complex. Intermolecular adhesion strength is also greatly diminished when α-catenin is absent from the complex in 1 of the 2 cells. This is consistent with the notion that cells carrying the disease-causing germ-line missense mutation V832M acquire the ability to invade only upon loss of the WT allele (12, 16).

The key role of the cytoplasmic protein α-catenin in the earliest binding interactions between E-cadherin was confirmed through gene silencing (Fig. 2). Depletion of α-catenin from E-cadherin-expressing cells (Fig. 2*E*) resulted in the reduction (but not the abrogation) of intercellular interactions. The same result was obtained with different siRNAs directed at different regions of α-catenin (data not shown). Indeed, the tensile strength of individual E-cadherin–E-cadherin bonds between 2 adjoining α-catenin-depleted cells was reduced to the same extent as caused by the V832M mutation (Fig. 2*C*).

We hypothesized that the maturation of an E-cadherin bond over time would enhance its strength against tensile forces. This hypothesis was tested by increasing the time of contact between 2 neighboring cells and then, upon de-adhesion, measured how the magnitude of the bond tensile strength would change. These measurements showed that shape of adhesion force distributions did not change qualitatively and remained single-peaked (Fig. S2), but the mean tensile strength of a single WT E-cadherin bond between cells increased significantly with the time of cell–cell contact (Fig. 3*A*). Moreover, the mean depth of the interaction energy well of the WT E-cadherin bond increased from 9 to 13 k_BT, indicating a rapid stabilization of the bond. In contrast, the mean tensile strength of a single V832M E-cadherin bond and its mean interaction energy significantly decreased from 7.5 to 5 k_BT within only 300 ms. At this point in time, the WT cadherin bond was 13 times more stable than the mutant cadherin bond. These results indicate that individual WT cadherin bonds, for which α-catenin is bound to the E-cadherin–β-catenin complex, strengthen and stabilize over time. In contrast, single mutant cadherin bonds weaken, become shorter-lived and more prone to rupture over time when the α-catenin–β-catenin interaction is not permissible: an already weak V832M E-cadherin bond quickly becomes even weaker.

The key role of α-catenin in mediating the rapid strengthening of an E-cadherin–E-cadherin bond after their initial interaction was confirmed by depleting α-catenin from E-cadherin-expressing cells. For α-catenin-depleted cells, E-cadherin–E-cadherin bond strength rapidly decreased at the same pace and to the same extent as for V832M E-cadherin cells (Fig. 3*A*). Moreover, treatment of WT E-cadherin cells with F-actin depolymerization drug latrunculin B led to no change in single-bond adhesion strength (Fig. 3*A*). This result suggests that F-actin plays no significant role in early E-cadherin-mediated cell–cell interactions.

When 2 cells were pulled apart, the majority of force-deflection curves (>78%; Fig. 1*C*) displayed no bond rupture

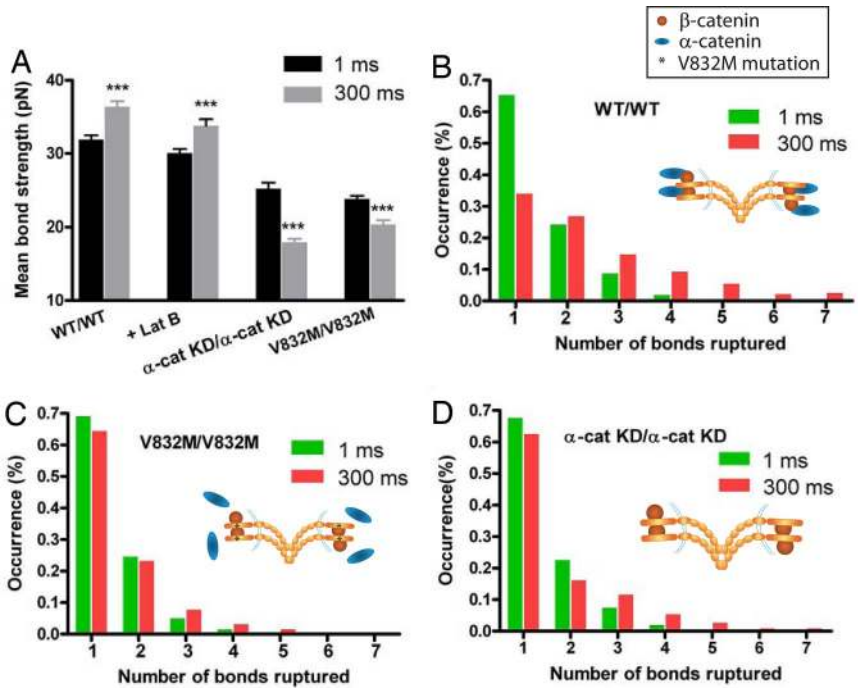

Fig. 3. α-Catenin mediates the strengthening of individual E-cadherin bonds over time and the formation of additional cadherin bonds between cells. (*A*) Mean tensile strength of a single cadherin–cadherin bond as a function of the time of interaction between 2 WT E-cadherin cells, 2 V832M E-cadherin cells, 2 WT E-cadherin–α-catenin-depleted cells, or 2 WT E-cadherin cells treated with latrunculin B. ***, $P < 0.001$ between values obtained for 1-ms and 300-ms contact times. (*B–D*) Probability for the formation of only 1, or 2, or 3, etc. cadherin bonds when 2 cells expressing either WT E-cadherin (*B*), V832M E-cadherin (c), E-cadherin in α-catenin-depleted cells (*D*) are allowed to interact for an increasing time of contact after a first bond has been formed.

event for short cell–cell contact times. For an increasing time of interaction between cells that express WT E-cadherin, force–distance curves rapidly began to display multiple bond ruptures. The proportion of curves showing at least 1, 2, 3, etc. bond ruptures represents the probability of forming these multiple bonds during initial cell contact. In just 300 ms, when at least 1 bond was formed between the cells, the probability to form only that single bond was halved, and the probability to form 3 and 4 bonds was increased 3-fold and 6-fold, respectively, compared with the case of 1-ms dwell time. In contrast, within the same time of cell contact, the probability of forming additional bonds between mutant cadherin-expressing cells once a first bond was formed remained largely unchanged as time elapsed (Fig. 3*C*). The role of α-catenin in mediating the formation of multiple bonds between adjoining E-cadherin-expressing cells was confirmed by depleting α-catenin from these cells (Fig. 3*D*). In this analysis, treatment of WT E-cadherin cells with latrunculin B did not affect the probability of intercellular bond formation (data not shown). Together, these results indicate that the probability of forming additional intercellular bonds once the first bond is formed increases dramatically with time when α-catenin is in the adhesion complex, independently of actin, whereas it remains low and constant when α-catenin is absent from the adhesion complex.

A plausible mechanism for the rapid α-catenin-mediated formation of multiple intercellular bonds stems directly from our results (Fig. 4). Once a first E-cadherin bond is formed between 2 neighboring cells, this initial intercellular bond increases the probability of formation of additional bonds because of both its own rapid strengthening and stabilization (Fig. 4), which are accompanied by the rapprochement of the plasma membranes of the adjoining cells. This accelerated formation of additional bonds between neighboring cells is akin to a "Velcro" effect, which drives cell–cell junction maturation. By contrast, V832M E-cadherins bonds or WT E-cadherins bonds between neighboring cells lacking α-catenin are able to form, but weaken extremely rapidly, with a stability against tensile forces decreasing, in <300 ms, 13-fold compared with the WT E-cadherin bond. This instability reduces the chance of other mutant cadherin pairs joining that first bond to help form a nascent cell–cell junction. Here, V832M E-cadherins cannot synergistically build a firm intercellular junction, resulting in a rapidly weakening of cell–cell adhesion (Fig. 4). Indeed, previous work has shown that global cell–cell adhesion of CHO cells expressing V832M E-cadherins is much weaker than for control cells (12).

Another possible molecular mechanism that may explain the enhanced recruitment of E-cadherins when α-catenin is bound to the E-cadherin–β-catenin complex arises from the fact that α-catenin is known to dimerize. Although no report of α-catenin dimerization across E-cadherin–β-catenin complexes has appeared, there is a distinct possibility that α-catenin could act as

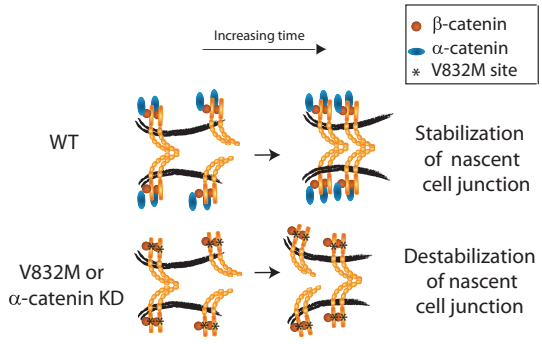

Fig. 4. Proposed mechanism by which α-catenin mediates the strengthening of individual E-cadherin bonds over time and the formation of additional cadherin bonds between 2 cells after the formation of a first intercellular bond.

a "bridge" spanning across 2 E-cadherin–β-catenin complexes and, thereby, enhance the recruitment of E-cadherins to cell–cell contact site. However, binding screening results show that the dimerization site of α-catenin overlaps with the β-catenin-binding site (17). By using chimeras, it was found that β-catenin binding disrupts the dimerization of α-catenin molecules, potentially ruling out the possibility that α-catenin dimerization mediates in E-cadherin recruitment. Hence, it would thus be interesting to visualize in a future study the recruitment of E-cadherins, under varied levels of α-catenin expression and with alterations in the dimerization sites.

Our assay probes cadherin–cadherin interactions between 2 individual living cells. Analyses using live cells (18, 19) rather than recombinant proteins (7, 20–22) ensures that the physiological orientation of cell receptors on the cell surface and the posttranslational modifications of these receptors (e.g., glycosylation) are preserved. Moreover, using living cells rather than recombinant proteins ensures that the cytoplasmic domain of transmembrane receptors can interact with cytoplasmic proteins, thereby ensuring that cell signaling pathways that may well influence adhesion remain functional, which is of critical importance to this work.

In conclusion, this work identifies 2 key functions for α-catenin and provides mechanistic insight into the earliest stages of molecular recognition between cells and the formation of intercellular adhesion (Fig. 4). (*i*) When bound to the E-cadherin–β-catenin complex, α-catenin enhances the initial, homotypic interaction of individual WT E-cadherin molecules between the cells. (*ii*) By mediating the strengthening of the first intercellular E-cadherin bond within a short time span, α-catenin helps transform an initial cell–cell contact consisting of just 1 bond into a nascent junction by mediating the formation of multiple bonds between cells without apparent input from the actin cytoskeleton. Hence, the cytoplasmic protein α-catenin is essential for both cadherin-mediated intercellular recognition and the adhesive properties of individual cadherins in vivo.

Experimental Procedures

Cell Culture. Unless stated otherwise, all reagents were purchased from Sigma. Human WT, α-catenin-depleted, and V832M E-cadherin-expressing CHO-K1 cells were cultured in α-MEM (Lonza) sterile cell culture medium, supplemented with 2 mM L-glutamine (Lonza), 5% FBS, 1% penicillin/streptomycin, and 500 μg/mL G418. Plated cells were cultured in T-75 flasks (Falcon) at 37 °C and a humidified, 5% CO_2 environment. For passing, a 70% confluent cell layer was washed in HBSS (Invitrogen) and incubated with 1 mL of trypsin–EDTA (Invitrogen) for 5 min, suspended in medium, and plated at 1:10 ratio. For molecular force probe (MFP) measurements, cells were passed into a 35-mm Corning dish and incubated overnight. For all experimental procedures requiring the cells to be out of the environment-controlled incubator, the medium was supplemented with 2% Hepes (Invitrogen) for pH control. E-cadherin-expressing CHO cells stably depleted of α-catenin were generated through small interference RNA (siRNA) interference and selection (Fig. 2*E*). For some of the experiments, WT E-cadherin CHO cells were treated with 40 nM latrunculin B in serum-free medium for 2 h in the absence of serum before experiments. Through fluorescence microscopy, we found that this concentration induced significant disassembly of the actin filament network (data not shown). Immediately before loading the treated cells on the atomic force microscope (AFM), the medium was changed to latrunculin B containing prewarmed medium with 5% FBS.

siRNA Transfection. A set of 4 different siRNAs targeting α-catenin mRNA was purchased from Dharmacon and prepared according to the manufacturer's instructions. In parallel, nonsilencing siRNA duplexes were used as negative control. Before transfection, 60% confluent monolayers of CHO-WT cells plated onto 6-well plates were washed with PBS and incubated in serum and antibiotic-free medium. Cells were transiently transfected with 0–150 nM siRNA, using Lipofectamine 2000 transfection reagent (Invitrogen). At the end of each transfection, putative cytotoxic effects were evaluated, analyzing cell viability by the trypan blue dye exclusion test. Efficiency of depletion was maximum at 48 h, and cat-11 and cat-12 were chosen from the set of siRNAs and used at 150 nM (see Figs. S3 and S4).

Immunoprecipitation. Immunoprecipitation studies were performed by using the Immunoprecipitation Starter Pack (Amersham Bioscience). Confluent cells were lysed in cold PBS containing 1% Triton X-100, 1% Nonidet P-40, protease inhibitor mixture (1 tablet per 50 mL of buffer; Roche), and phosphatase inhibitor mixture (1:100 dilution; Sigma). Total protein was quantified by following the Bradford dye-binding procedure. An aliquot of 500 μg of protein was immunoprecipitated with protein G, according to the manufacturer's instructions; 1–10 μg of monoclonal antibodies against E-cadherin, α-catenin, or β-catenin (Transduction Laboratories) was used. Immunoprecipitated proteins were separated on a 7.5% SDS-polyacrylamide gel by electrophoresis, followed by transfer onto a nitrocellulose membrane (Hybond C-extra; Amersham Bioscience). Immunoblotting was performed with antibodies against E-cadherin (1:3,500; R&D Systems), α-catenin or β-catenin (1:1,000; Transduction Laboratories), with an ECL Western blotting detection kit (Amersham Bioscience).

Preparing AFM Cantilevers. Gold-plated SiN AFM cantilevers (model MLCT-AUHW; Veeco Instruments) were used for force spectroscopy measurements. The AFM cantilevers were washed with 70% EtOH, 10% HCl solution, ultrapure water, and 95% EtOH for 1 min each, taking care that the cantilevers at the end of the tips did not break because of interfacial tension. The cantilevers were subsequently washed in acetone for 5 min and allowed to dry on a clean, Pyrex glass dish. The cantilevers were then incubated in a PBS drop until needed for the AFM pulls. For experiments with dwell, the tip was incubated in a 0.5 mg/mL of streptavidin for 3 h. No streptavidin treatment was necessary for no-dwell experiments.

Biotinylation of E-Cadherins on Live Cells. Streptavidin–biotin linkages were used (only for the dwell experiments) to tether the cells to the cantilevers strongly because a prolonged cell–cell contact leads to greater adhesion forces. Biotinylation of cells with sulfo-NHS-LC-biotin (Pierce) was carried out according to the manufacturer's instructions. In short, cells were washed three times in ice-cold PBS and treated with 0.5 mg/mL biotin at 6×10^6 cells per mL. The biotin–cell suspension was stored at 4 °C for 30 min, and excess biotin was quenched with glycine and washed down to a final suspension of 5×10^8 cells per mL. For experiments involving no dwell, this step was eliminated, and the cells were centrifuged to a final suspension of 5×10^8 cells per mL for loading onto the AFM cantilever.

Loading Live Cells on AFM Cantilever Tips. An Eppendorf Transjector 5246 equipped with a modified borosilicate microneedle (Sutter Instruments) was used to deposit 4–5 cells on the tip of the AFM cantilever. The whole procedure was carried out in a temperature-controlled chamber mounted on a Nikon TE-2000 microscope, using a 10× Plan Fluor objective (N.A. 0.3). Hepes was used for pH control of the cell culture medium. The cantilever with the cells attached to its tip as incubated at 5% CO_2 and 37 °C overnight.

Single-Molecule Force Spectroscopy. Single-molecule, live-cell measurements were made by using a pico-Newton sensitive MFP (Asylum Research). The functioning of an MFP has been detailed elsewhere (23, 24). Briefly, it works on the principle of reflection of a directed laser from the surface of a flexible-cantilever that undergoes a sudden deflection as a result of sudden release of tethering force, in this case, the rupture of a single bond. The deflection of the cantilever is measured as a voltage-signal read-out through a photodetector while the vertical displacement of the cantilever is controlled through an accurately calibrated piezodrive. The raw output of the MFP comprises 2 parameters, the photodetector sensor output (PSPD, in volts) and the linear variable differential transformer output. Two calibration parameters are used to convert the PSPD output to force, the force constant (k, pN/nm) of the cantilever and the inverse optical lever sensitivity (InvOLS, nm/V). Whereas the force constant is measured through the thermal resonance technique (25), the InvOLS is measured by lowering the cantilever onto a hard substrate and measuring the slope of the displacement vs. PSPD output. Immediately before the MFP measurements, the 35-mm Corning dish with plated cells was washed thoroughly with HBSS (Invitrogen) and was filled with 5 mL of serum-free medium supplemented with 2% Hepes, preheated to 37 °C.

Statistical theory of cell adhesion predicts that when the probability of binding between 2 cells is reduced to 25%, then the probability of forming a single bond vs. 2 and 3 bonds is 84%, 12%, and <3%, respectively, and follows Poisson statistics (26). The probability of formation of a single functional cadherin bond between 2 cells may depend on many parameters, including their time of contact, the impinging force upon approach of the cells, the surface area of contact between the cells, and the density and diffusivity of cadherin pairs of the cell surface. Time of cell–cell contact (<1 ms) and force of approach (<150 pN) were manipulated to reach a targeted 30% success rate

of binding. We verified that, in this case, the probability of formation of multiple bonds followed Poisson statistics. Analysis of force–distance curves in terms of tensile strength (the force required to rupture that bond) and loading rate applied to that bond (Fig. 1*B Inset*) was confined to those showing 1 bond–rupture event. These precautions ensured that all reported data are only for single bonds between adjoining cells.

Single-Molecule Data Acquisition and Analysis. Cells were placed on the MFP base, and the cantilevers were attached to the tip holder and loaded onto the MFP. Because calibration of the cantilever involves large impingement forces leading to rupture of cell and/or detachment of the cell from the cantilever, calibration was conducted at the end of the experiments, according to the manufacturer's protocol. The force constant and the InvOLS were estimated to be a sufficiently high value, to overestimate the forces read-out of the PSPD. The tip was lowered over a confluent patch of cells, and retraction traces were obtained at preset retraction speeds and dwell times. The traces were obtained up to a maximum of 30 times for the same cell and experiments conducted on different days to ensure a widely sampled set of force curves. Analysis of the force curves was done by using the IgorPro 4.09 software (Wavemetrics, Inc.). The distribution of bond tensile strengths was obtained by counting the number of curves falling in a bin spanning 5 pN; all ruptures smaller than 10 pN were ignored (being comparable to the cantilever noise). Increasing retraction velocity subjected bonds formed between top and bottom cells to increasing loading rates (Fig. 3*B*), which were computed for each force–displacement curve as the product of the set retraction velocity and the measured slope of the curve just before bond rupture (Fig. 1*B*). For the loading rate, the bin span was taken to be 50 pN/s $<$100 pN/s, 100 pN/s for loading rates between 100 pN/s and 500 pN/s. All loading rates between 500 pN/s and 1000 pN/s were binned together.

Specificity of Measurements. First, we verified that when no cell was placed on the cantilever, few force–displacement curves ($<$3%) (Fig. 1, *C* and *D*) showed bond–rupture events, as indicated by few abrupt drops in force of at least 10 pN in magnitude along the curves. Second, the probability of (successful) binding between 2 WT E-cadherin-expressing CHO cells in the presence of chelating agent EDTA, as measured by the proportion of force–distance curves showing bond–rupture events $>$10 pN, was low and equal to 3% (Fig. 1*D*). Finally, the probability of binding between 2 WT E-cadherin-expressing CHO cells in the presence of a function-blocking antibody against cadherin was also low, $<$3% (Fig. 1*D*). Together, these controls indicate that single-molecule force spectroscopy measurements shown here are specific and involve interactions between cadherins, not other adhesion molecules expressed on the surface of CHO cells. For function-blocking controls, plated cells were treated with HECD1 anti-human E-cadherin monoclonal antibody (Zymed Laboratories) at a concentration of 200 μg/mL for 30 min at 37 °C and 5% CO_2. After incubation, 4 mL of serum-free medium was added, and the cells were immediately used for measurements.

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1. Gumbiner BM (2005) Regulation of cadherin-mediated adhesion in morphogenesis. *Nat Rev Mol Cell Biol* 6:622–634.
2. Mareel M, Leroy A (2003) Clinical, cellular, and molecular aspects of cancer invasion. *Physiol Rev* 83:337–376.
3. Guilford P, *et al*. (1998) E-cadherin germ-line mutations in familial gastric cancer. *Nature* 392:402–405.
4. Huntsman DG, *et al*. (2001) Early gastric cancer in young, asymptomatic carriers of germ-line E-cadherin mutations. *N Engl J Med* 344:1904–1909.
5. Yamada S, Pokutta S, Drees F, Weis WI, Nelson WJ (2005) Deconstructing the cadherin—catenin–actin complex. *Cell* 123:889–901.
6. Leckband D, Sivasankar S (2000) Mechanism of homophilic cadherin adhesion. *Curr Opin Cell Biol* 12:587–592.
7. Bayas MV, Leung A, Evans E, Leckband D (2006) Lifetime measurements reveal kinetic differences between homophilic cadherin bonds. *Biophys J* 90:1385–1395.
8. Klingelhofer J, Troyanovsky RB, Laur OY, Troyanovsky S (2003) Exchange of catenins in cadherin–catenin complex. *Oncogene* 22:1181–1188.
9. Klingelhofer J, Laur OY, Troyanovsky RB, Troyanovsky SM (2002) Dynamic interplay between adhesive and lateral E-cadherin dimers. *Mol Cell Biol* 22:7449–7458.
10. Ahrens T, *et al*. (2002) Analysis of heterophilic and homophilic interactions of cadherins using the c-Jun/c-Fos dimerization domains. *J Biol Chem* 277:19455–19460.
11. Suriano G, *et al*. (2003) E-cadherin germ-line missense mutations and cell phenotype: Evidence for the independence of cell invasion on the motile capabilities of the cells. *Hum Mol Genet* 12:3007–3016.
12. Suriano G, *et al*. (2003) The intracellular E-cadherin germ-line mutation V832M lacks the ability to mediate cell–cell adhesion and to suppress invasion. *Oncogene* 22:5716–5719.
13. Panorchan P, *et al*. (2006) Single-molecule analysis of cadherin-mediated cell–cell adhesion. *J Cell Sci* 119:66–74.
14. Panorchan P, George JP, Wirtz D (2006) Probing intercellular interactions between vascular endothelial cadherin pairs at single-molecule resolution and in living cells. *J Mol Biol* 358:665–674.
15. Benoit M, Gabriel D, Gerisch G, Gaub HE (2000) Discrete interactions in cell adhesion measured by single-molecule force spectroscopy. *Nat Cell Biol* 2:313–317.
16. Yabuta T, *et al*. (2002) E-cadherin gene variants in gastric cancer families whose probands are diagnosed with diffuse gastric cancer. *Int J Cancer* 101:434–441.
17. Pokutta S, Weis WI (2000) Structure of the dimerization and β-catenin-binding region of α-catenin. *Mol Cell* 5:533–543.
18. Dobrowsky TM, Panorchan P, Konstantopoulos K, Wirtz D (2008) Live-cell single-molecule force spectroscopy. *Methods Cell Biol*, in press.
19. Dobrowsky TM, Zhou Y, Sun SX, Siliciano RF, Wirtz D (2008) Monitoring early fusion dynamics of human immunodeficiency virus type 1 at single-molecule resolution. *J Virol* 82:7022–7033.
20. Baumgartner W, *et al*. (2000) Cadherin interaction probed by atomic force microscopy. *Proc Natl Acad Sci USA* 97:4005–4010.
21. Perret E, Leung A, Feracci H, Evans E (2004) *Trans*-bonded pairs of E-cadherin exhibit a remarkable hierarchy of mechanical strengths. *Proc Natl Acad Sci USA* 101:16472–16477.
22. Baumgartner W, Schutz GJ, Wiegand J, Golenhofen N, Drenckhahn D (2003) Cadherin function probed by laser tweezer and single-molecule fluorescence in vascular endothelial cells. *J Cell Sci* 116:1001–1011.
23. Hanley W, *et al*. (2003) Single molecule characterization of P-selectin/ligand binding. *J Biol Chem* 278:10556–10561.
24. Hanley WD, Wirtz D, Konstantopoulos K (2004) Distinct kinetic and mechanical properties govern selectin–leukocyte interactions. *J Cell Sci* 117:2503–2511.
25. Hutter JL, Beccoeffer J (1993) Calibration of atomic-force microscope tips. *Rev Sci Instr* 64:1868–1873.
26. Chesla SE, Selvaraj P, Zhu C (1998) Measuring two-dimensional receptor–ligand binding kinetics by micropipette. *Biophys J* 75:1553–1572.
27. Hummer G, Szabo A (2003) Kinetics from non-equilibrium single-molecule pulling experiments. *Biophys J* 85:5–15.