Cadherin exits the junction by switching its adhesive bond

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The plasticity of cell–cell adhesive structures is crucial to all normal and pathological morphogenetic processes. The molecular principles of this plasticity remain unknown. Here we study the roles of two dimerization interfaces, the so-called strand-swap and X dimer interfaces of E-cadherin, in the dynamic remodeling of adherens junctions using photoactivation, calcium switch, and coimmunoprecipitation assays. We show that the targeted inactivation of the X dimer interface blocks the turnover of catenin-uncoupled cadherin mutants in the junctions of A-431 cells. In contrast, the junctions formed by strand-swap dimer interface mutants exhibit high instability. Collectively, our data demonstrate that the strand-swap interaction is a principal cadherin adhesive bond that keeps cells in firm contact. However, to leave the adherens junction, cadherin reconfigures its adhesive bond from the strand swap to the X dimer type. Such a structural transition, controlled by intercellular traction forces or by lateral cadherin alignment, may be the key event regulating adherens junction dynamics.

Introduction

The transmembrane receptor cadherin forms morphologically diverse adhesive structures collectively called adherens junctions. These junctions don't just hold cells together, their remodeling is critical for a proper tissue morphogenesis (Gumbiner, 2005; Nishimura and Takeichi, 2009). Despite their importance, the basic mechanisms underlying cadherin adhesion are not completely understood. The structure of the cadherin adhesive bonds and their assembly and disassembly are among the most controversial issues in the field of cell–cell adhesion.

Adherens junctions form rapidly once two cells come into contact (Adams et al., 1998; Vasioukhin et al., 2000; Kametani and Takeichi, 2007; Yamada and Nelson, 2007). This process culminates in the formation of adhesive clusters in which cadherin trans-homodimers are proposed to be stacked laterally by cis interactions (Boggon et al., 2002; Patel et al., 2003). In their steady-state condition, adherens junctions continuously gain and lose cadherin molecules (Troyanovsky et al., 2006; de Beco et al., 2009; Hong et al., 2010). This dynamic suggests the existence of a mechanism responsible for the gentle but rapid removal of cadherin from the junctions. Importantly, cadherin disengagement from cell–cell contacts is an active process that is blocked by ATP depletion or, in some cases, by inhibitors of endocytosis (Troyanovsky et al., 2006; de Beco et al., 2009; Hong et al., 2010). The mechanisms of such fast renewal of cadherin molecules in adherens junctions are completely unknown. These mechanisms must be essential to one of the crucial features of cadherin adhesion: its high plasticity.

In this work, we evaluated the possibility that cadherin is released from the junctions by remodeling its adhesive bonds. The energy-consuming conformational switch of the key structural bonds is a common mechanism used by many multiprotein structures, e.g., microfilaments and microtubules, to maintain their constant renewal. This idea is also in line with much data demonstrating the complexity of intercadherin interactions (Häussinger et al., 2004; Troyanovsky et al., 2007; Leckband, 2008; Sivasankar et al., 2009). Specifically, our cross-linking studies revealed stable and unstable cadherin dimers (Troyanovsky et al., 2007). Contiguous binding sites located at the EC1 domain apparently mediate the formation of these dimers. Structural analysis suggests that the stable dimer corresponds to a strand-swap dimer that is formed by the reciprocal insertion of the W2 residue (W156 by numbering from start codon) into the hydrophobic pocket of the paired molecule (Shapiro et al., 1995; Boggon et al., 2002; Häussinger et al., 2004). Point mutagenesis compellingly showed that this strand-swapped dimer forms a cadherin adhesion bond (Chitaev and Troyanovsky, 1998; Tamura et al., 1998; Troyanovsky, 2005: Zhang et al., 2009). The second type of dimers is the "X dimer," in which

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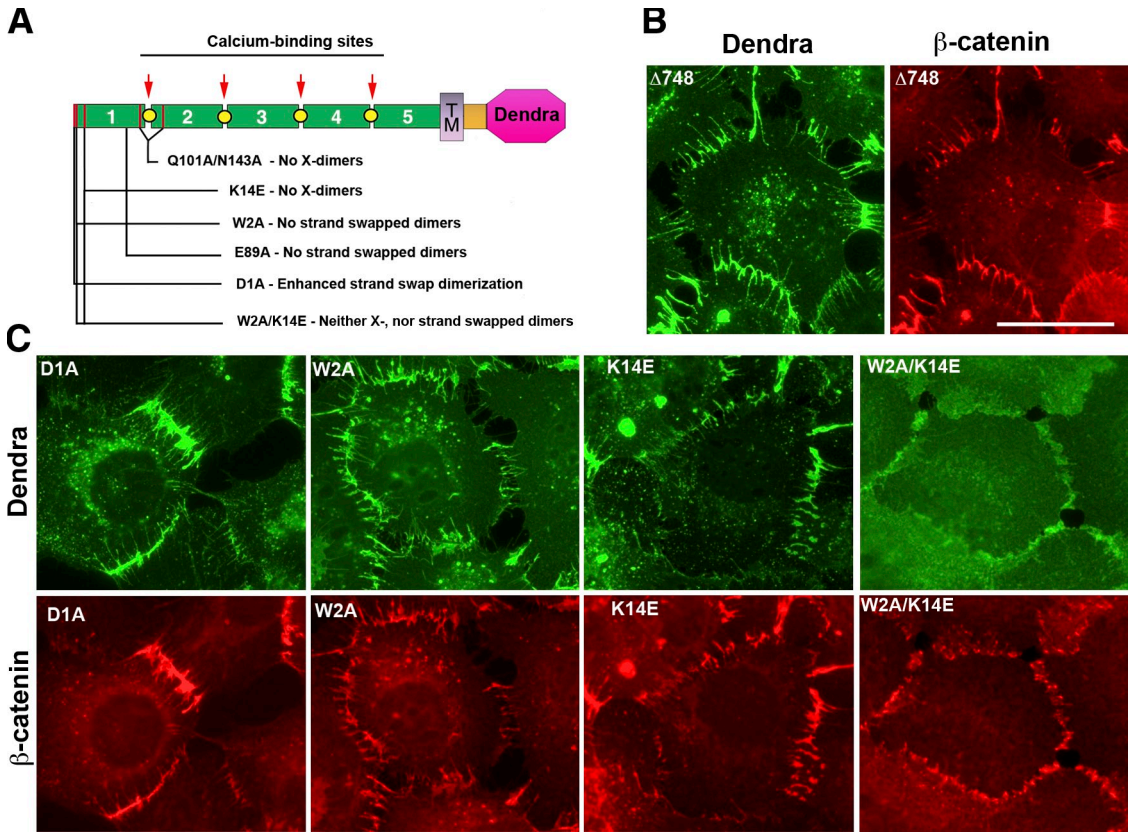

Figure 1. **General structures and subcellular distribution of catenin-uncoupled tailless cadherin mutants.** (A) Schematic representation of the tailless EcDendra-Δ748-KL mutant. The extracellular cadherin-like repeats (1–5); the transmembrane domain (TM); the short, 17-aa-long fragment that is located between the transmembrane and the p120-binding domains in the intact E-cadherin (yellow box); and the Dendra tag (Dendra) are shown. To stabilize the mutant on the cell surface, two endocytic signals (K738 and LL motif) that are present in the remaining intracellular fragment are point inactivated (not depicted). Point mutations used in our study and their effects on cadherin dimerization are indicated (see also Table S1). (B) Double immunofluorescence microscopy of A-431 cells expressing EcDendra-Δ748-KL. The cells were stained with rabbit anti-Dendra (Dendra) and mouse anti–β-catenin (β-catenin) antibodies. Note the precise colocalization of the mutant to the endogenous cadherin–catenin complex. (C) The same experiment as in B with A-431 cells expressing EcDendra-Δ748-KL mutants harboring different point mutations (shown in A). Note that mutations that change only X or only strand-swap dimerization do not prevent targeting of the mutant to the cell–cell contact and its colocalization with the endogenous cadherin. Only a double mutation (W2A/K14E) inactivating both interactions completely uncouples the mutant from endogenous cadherin. Bar, 40 µM.

paired molecules contact one another at a calcium-binding EC1–EC2 interface (Nagar et al., 1996; Pertz et al., 1999; Häussinger et al., 2004). By many properties, this dimer corresponds to the unstable dimer detected in our biochemical experiments (Troyanovsky et al., 2007). Targeted inactivation of the X dimer interface was recently shown to abolish cadherin function (Harrison et al., 2010). Although this data demonstrated that both X dimers and strand-swapped dimers are involved in cell–cell adhesion, their exact roles and mechanisms of assembly have not been identified.

Intracellular proteins called catenins bind to the intracellular cadherin region and control cadherin function (Provost and Rimm, 1999; Nelson, 2008). These proteins can potentially "sense" even minor junctional abnormalities and consequently annihilate the entire adhesive structures. To avoid such destructive catenin-based response to abnormalities in intercadherin interactions, in this paper we monitored the formation and dynamics of cell–cell junctions formed by the tailless E-cadherin mutant EcDendra-Δ748-KL. This mutant is unable to interact with any known intracellular proteins. Despite uncoupling from catenins, it is recruited into the junctions with approximately the same kinetics as the intact E-cadherin (Hong et al., 2010). Furthermore, the resulting junctions exhibit a continuous, albeit slow and ATP-independent, turnover of the mutant. These properties of the mutant allowed us to examine the roles of the strand-swap and X dimer cadherin interfaces in adherens junction homeostasis.

Results

Strand-swap interface is responsible for the stability of cadherin clusters

This study focused on adherens junction–like structures formed in A-431 cells expressing the tailless cadherin mutant EcDendra-Δ748-KL. These adhesive structures form through co-assembly of both the mutant and endogenous cadherin (Fig. 1 A; Hong et al., 2010). Because the tailless cadherin mutant cannot be directly influenced by intracellular factors, this mutant allows us to study the contribution of specific intercadherin-binding interfaces to the homeostasis of cadherin adhesive clusters. To analyze the role of the strand-swap interface, we expressed at similar levels (Fig. S1) three point mutants of this tailless mutant.

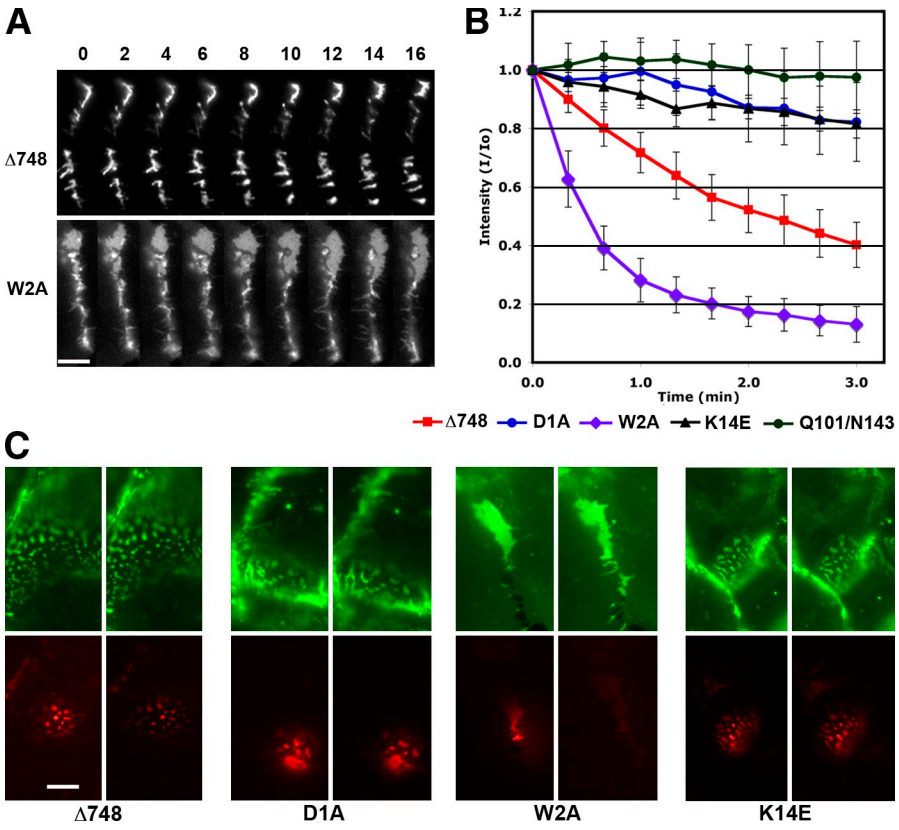

Figure 2. **Targeted modifications in dimerization interfaces differently change dynamics of cadherin clusters.** (A) Sequences of time-lapse images of cell–cell contacts between cells expressing the EcDendra-Δ748-KL mutant (Δ748, top) or its strand swap–incompetent version (W2A, bottom) acquired at 2-min intervals (see Videos 1 and 2). The junctions containing the parental mutant are stable along the entire sequence, whereas junctions containing the W2A mutant change their shapes and numbers. Bar, 10 µM. (B) Junctional Dendra activation assay of different EcDendra-Δ748-KL mutants. The graph shows changes in intensity of the red fluorescence in the individual junctions after Dendra2 activation (an average of four independent experiments). The error bars represent SD (n = 30). (C) Time-lapse analysis of the photoactivated adherens junctions in cells expressing different EcDendra-Δ748-KL mutants. The green channel shows normal Dendra2 fluorescence. The red channel reveals a photoconverted Dendra2 form. Frame 0 shows the cells right after photoactivation. Frame 3 is 3 min later. Note that the W2A mutant strongly accelerates the mutant's exit from the junctions. In contrast, D1A and K14E significantly delay this process. All images in C have the same magnification. Bar, 5 µM.

The first mutant, D1A-EcDendra-Δ748-KL, harbored point mutation D1A (Fig. 1 A and Table S1), which had been shown to facilitate production of strand-swapped dimers (Laur et al., 2002; Troyanovsky et al., 2007). Immunofluorescence and live imaging microscopy showed that the D1A mutation changed neither the morphology of the cadherin clusters nor their general behavior (Fig. 1 C). However, a junctional Dendra activation assay revealed that this mutation dramatically increased retention time of the mutant in adhesive clusters (Fig. 2, B and C). Such a change in internal cluster dynamics should result in an increase in the junctional pool of the mutant. Quantitative analysis did show that the D1A mutation raised the amount of the mutant in the junctional clusters (Fig. S1).

Two other mutations, W2A and E89A, had been shown to abolish cadherin strand swapping (Laur et al., 2002; Harrison et al., 2005, 2010). The first mutation inactivates W2 residue, which is a most crucial element in strand-swap dimerization. The second mutation prevents the formation of a salt bridge that stabilizes strand exchange. Because both mutations resulted in the same effects, only data with the W2A mutant is shown in this study. Unexpectedly, these mutations prevented neither the recruitment of the tailless mutant into the contacts nor its co-localization with endogenous cadherin (Fig. 1 C). However, the morphology of cadherin clusters in these cells changed remarkably: in general, clusters were larger in size but their fluorescence was significantly weaker than that in cells with the parental mutant. Live imaging also revealed a dramatic difference: instead of being stationary, these clusters became extremely mobile, continuously and rapidly changing their shape and distribution (Fig. 2 A and Videos 1 and 2). The Dendra activation assay revealed that the residence time of these two mutants in the clusters was much shorter than that of the parental mutant (Fig. 2). Collectively, these observations confirmed that strand swapping is an essential intercadherin interaction that maintains adhesion in adherens junctions. However, these data also suggested that in addition to strand swapping, other intercadherin interactions were able to co-cluster the mutant with endogenous cadherin.

X dimerization is responsible for the turnover of cadherin in the clusters

Another structurally characterized mode of cadherin dimerization is the X dimer. We tested its function using two EcDendra-Δ748-KL mutants with abnormalities in the X dimer interface. The first mutation, Q101A/N143A, changed two residues, the side chains of which had been shown to coordinate water molecules critical for X dimerization (Nagar et al., 1996). The second mutation, K14E, had been shown to specifically inactivate X dimerization by electrostatic repulsion (Harrison et al., 2010). Inspection of the cells expressing these mutants by fluorescence and live imaging microscopy showed that defects in the X dimerization had little effect on the cadherin clusters' appearance (Fig. 1 C). However, the Dendra activation assay revealed a crucial difference between clusters formed by the parental mutant and both of its X dimerization–incompetent counterparts: the junction turnover of the latter mutants was almost completely stalled (Fig. 2, B and C). In addition, these mutations significantly expanded the area of cadherin clusters, leaving their fluorescence intensity unchanged (Fig. S1). Thus, inactivation of the X dimer interface stabilized cadherin clusters.

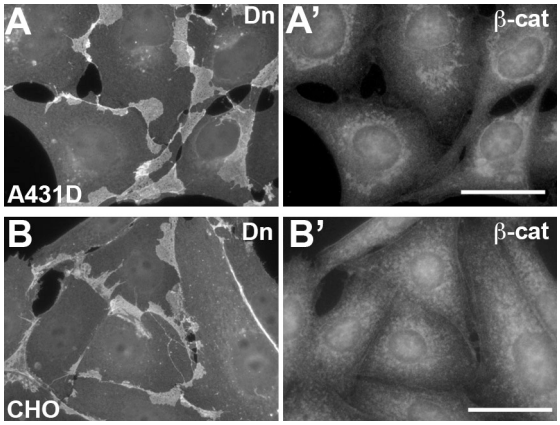

Figure 3. **The strand swap–incompetent mutants form clusters in cadherin-deficient cells.** A431D (A and A′) and CHO (B and B′) cells lacking endogenous cadherin were stably transfected with a plasmid encoding the W2A-EcDendra-Δ748-KL mutant and double stained for Dendra (Dn) and β-catenin (β-cat). The complete absence of β-catenin staining in the Dendra-positive junctions confirms their complete deficiency of endogenous cadherin. Bars, 40 µM.

X dimerization interface recruits cadherin mutants into highly dynamic clusters

In the next experiment, we tested whether the clusters observed in cells expressing the strand swap–incompetent mutants (Fig. 1 C) were based on X dimerization. Indeed, besides X dimerization, these mutants can potentially form clusters by other types of intercadherin trans or cis interactions suggested by different studies (Shapiro et al., 1995; Chappuis-Flament et al., 2001; Leckband, 2008). Furthermore, they could also be clustered through residual strand swapping with endogenous cadherin. To elucidate the role of endogenous cadherin in clustering of these mutants, we expressed W2A mutant in cadherin-deficient A431D and CHO cells. Fig. 3 shows that the endogenous cadherin is not required for the W2A tailless mutant to produce clusters.

Second, by combining two point mutations W2A and K14E, we tested whether the clustering of the W2A tailless mutant was based on the X dimer interface. This double mutant was unable to form clusters and was randomly distributed on the surface of A-431 cells. Parallel staining for β-catenin revealed, furthermore, that these cells, in contrast to cells expressing W2A mutant, exhibit normal adherens junctions (Fig. 1 C). These results showed that the clustering of the swapping-incompetent W2A mutant was based on X dimerization and that the inactivation of both the X and the strand-swap interfaces uncouples the mutant from endogenous cadherin.

X interface mutations stabilize cadherin clusters

Extraordinary long retention of the X dimer–incompetent mutants in cadherin clusters demonstrated by the junctional Dendra activation assay suggested the high stability of the strand-swapped dimers formed by these mutants. To confirm this assumption, we studied disassembly of cadherin cluster in calcium depletion experiments. These experiments were performed on the metabolically inactive cells to minimize the sharp morphological changes associated with calcium depletion that could nonspecifically

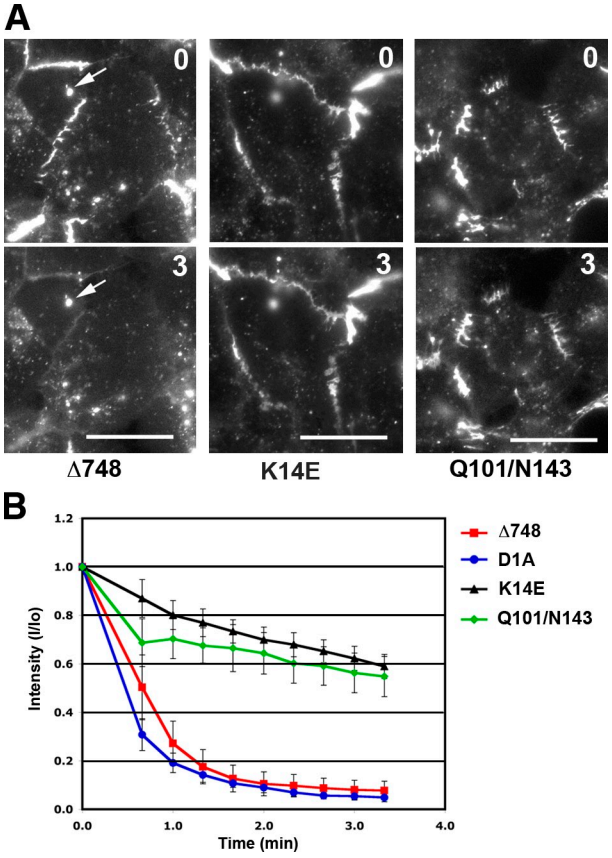

Figure 4. **X dimer mutations stabilize cadherin clusters in low calcium.** (A) Time-lapse images (the first and the last frames from videos acquired at 20-s intervals, total duration of 3 min) of cells expressing EcDendra-Δ748-KL (*Δ748*) and its X dimer mutants K14E (K14E) and Q101A/N143A (Q101/N143) during calcium depletion (numbers indicate min after placing cells in low-calcium/ATP-depletion media; 0, cells right before the media exchange). Note that in low calcium, the parental mutant rapidly exits the junction, whereas Dendra fluorescence in intracellular vesicles (one of which is indicated by an arrow) is not affected. Bars, 40 µM. (B) Kinetics of the release of EcDendra-Δ748-KL mutants (abbreviated as in Fig. 1) from the junctions in low calcium. Note that inactivation of the X dimer interface significantly delays cadherin release (n = 10). The error bars indicate SD.

destroy adherens junctions. Fig. 4 shows that ATP depletion, although preventing vast morphological changes after placing cells into low-calcium media, failed to prevent the fast disappearance of the parental tailless cadherin from the junctions. In contrast, both its X dimer mutants were retained for much longer in the junctions after calcium depletion. In complimentary experiments, we studied the kinetics of cadherin clusters disassembly at standard calcium concentration using an E-cadherin function-blocking antibody. Again, X dimer mutations dramatically increased the stability of cadherin clusters in this assay (Fig. S2). Interestingly, the D1A mutation failed to extend the lifetime of cadherin clusters in low calcium (Fig. 4). Therefore, the D1A mutation that facilitates strand swapping and mutations that inactivate X dimer interface inhibit cadherin turnover by different mechanisms.

X dimer mutants have slow kinetics of junction assembly in a calcium switch assay

The most obvious interpretation of the data described in the previous section is that strand-swap dimers dissociate through

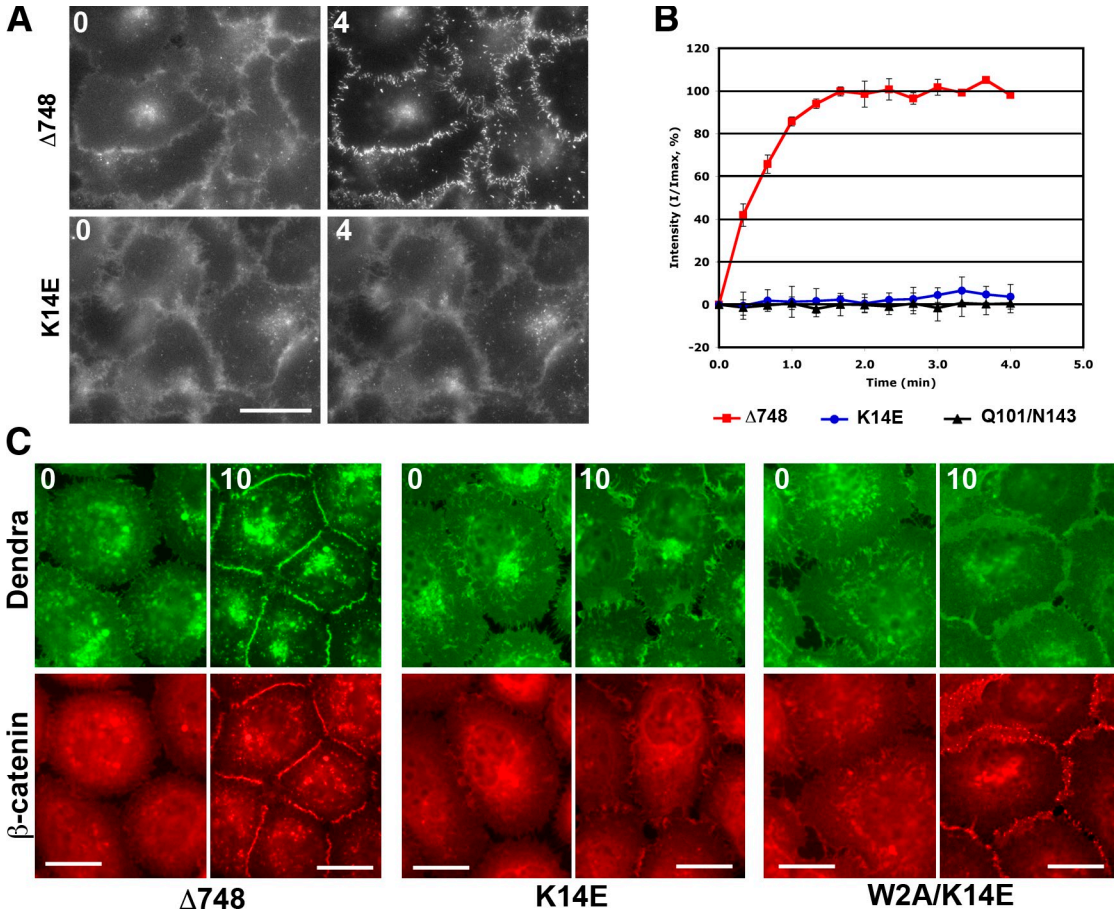

Figure 5. **X dimer mutations delay cadherin clustering in the calcium switch assay.** (A) Time-lapse images (the first and the last frames from Videos 3 and 4) acquired at 20-s intervals (total duration 4 min) of cells expressing EcDendra-Δ748-KL (Δ748) and its X dimer K14E mutant (K14E) during the calcium switch assay in ATP depletion media (numbers indicate min after a switch; 0, immediately before the switch). Note that calcium induced the formation of numerous junctional clusters in cells expressing the parental, but not the X dimer, mutant. (B) Clustering kinetics of EcDendra-Δ748-KL (Δ748) and its X dimer mutants K14E (K14E) and Q101A/N143A (Q101/N143) after the addition of calcium (n = 10). The error bars indicate SD. (C) A-431 cells expressing the parental EcDendra-Δ748-KL (Δ748) mutant, X dimer mutant K14E (K14E), or X/strand-swap dimer–incompetent W2A/K14E mutant (*W2A/K14E*) were incubated in low calcium overnight. Then, to block morphological changes, they were depleted of ATP by a 10 min-long incubation in low-calcium/ATP-depletion media (0) and subsequently treated with high-calcium/ATP-depletion media for 10 min (10). Subcellular localization of the mutant and endogenous cadherin was determined by double staining as in Fig. 1. Note that the X dimer–incompetent K14E mutant inhibits the formation of adherens junctions. The inactivation of the strand-swap interface of this mutant by W2A mutation blocks this effect. Bars, 40 μM.

X dimer intermediates: the inability to form X dimers entraps cadherin in cell–cell junctions by locking it in the strand-swap configuration. This hypothesis is fully consistent with recent biophysical measurements (Harrison et al., 2010). It thoroughly explains the long retention of X dimer mutants in junctions in a steady-state as well as upon calcium depletion or in the presence of the functional blocking antibody. In the following experiments, we addressed a parallel question: is X dimerization also an intermediate step in the formation of strand-swap dimers?

To answer this question, we compared the clustering kinetics of the parental and X dimer mutants in the calcium switch assay. To minimize the contribution of various intracellular activities like actin polymerization on cadherin clustering, the cells were treated with ATP depletion agents 10 min before calcium switch. Consistent with previous data (Hong et al., 2010), the clustering of the parental mutant was completed in ∼1 min (Fig. 5). In contrast, both X dimer mutants were unable to form clusters during 4-min-long observation periods (Fig. 5, A and B). Additional experiments determined that the X dimer interface mutants needed >1 h to eventually produce clusters (Video 5). Strikingly, anti–β-catenin immunostaining revealed that endogenous cadherin, which is expressed at the same level as in wild-type A-431 cells (Fig. S1), also remained unclustered in these cells (Fig. 5 C).

Slow clustering of X dimer mutants appears to support the role of X dimers as intermediates in cadherin strand swapping. However, the negative influence of X dimer mutants on endogenous cadherin clustering suggested an alternative possibility: the strand-swap cis dimers, which formed in cells at low calcium (Klingelhöfer et al., 2002) and are abnormally stable because of X dimer interface inactivation, depleted the pool of monomeric cadherin, thereby blocking adhesion after a calcium increase. To test this possibility, we studied whether inactivating the strand-swap interface in the X dimer mutant abolished its inhibitory effect on endogenous cadherin clustering. The results showed that the W2A mutation completely suppressed the negative influence of the K14E mutant on the assembly of endogenous cadherin in the calcium switch assay (Fig. 5 C).

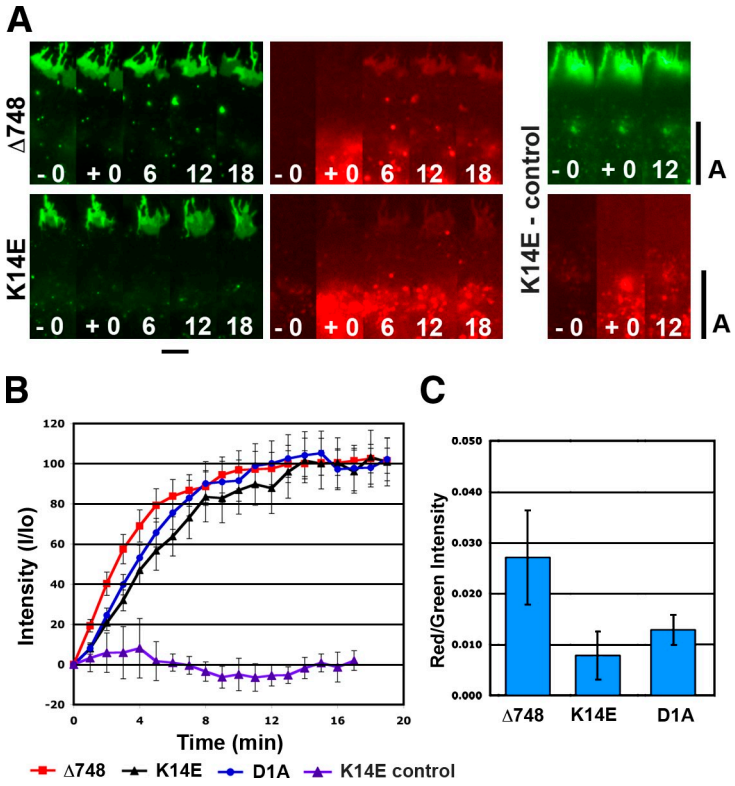

Figure 6. **X dimerization interface is not essential for mutant recruitment into the junctions.** (A, left) Central extrajunctional regions of the cells expressing the control mutant EcDendra-Δ748-KL (Δ748) or its X dimer–incompetent variant (K14E) were photoactivated (the activated area is marked on the right; A), and recruitment of the activated proteins into the junction was then followed by live cell imaging. At each time point (indicated in min), cells were imaged in green and red channels. Note that junctions in the case of the K14E mutant exhibit very weak red fluorescence. (A, right) The same experiment but performed with K14E mutant–expressing cells immediately after placing cells in low-calcium media. Note that activated cadherin is not recruited into the junction. Bar, 5 µM. (B) The graph (average of four independent extrajunctional Dendra activation experiments; n = 30) shows red fluorescence in individual junctions over time. The error bars represent SD. (C) The quantification of red/green individual junction fluorescence 10 min after activation of the cellular center. The mean values of eight junctions from three independent experiments are shown (n = 20). The error bars indicate SD.

Therefore, the very slow formation of the junctions in cells expressing X dimer mutants in the calcium switch assay can be, at least in part, caused by the accumulation of the stable cadherin cis dimers at low calcium conditions.

X dimer interface is not required for cadherin to enter the junction

To elucidate the role of X dimerization in junction assembly, we studied the entry of the mutants into the junctions by the extrajunctional Dendra activation assay. In this assay, mutant molecules located in the central, extrajunctional cellular area were activated, and their recruitment into the junctions was monitored over time. This assay showed that all tested mutants including the parental mutant and its D1A and K14E counterparts entered the junctions with essentially the same kinetics: their amounts in the junctions reached a plateau ∼10 min after activation (Fig. 6, A and B). Significant differences between the mutants, however, were noted in the amounts of activated cadherin recruited into the junctions: 10 min after activation, the junctional fluorescence intensity of the activated K14E mutant was approximately threefold weaker than that of the parental mutant (Fig. 6 C). This observation, as well as increased sizes of cadherin clusters in cells expressing X dimer mutants (Fig. S1), suggested that the free extrajunctional pool of cadherin is severely depleted in these cells. However, these experiments also suggest that the X dimer interface is not essential for cadherin to enter the junction.

Strand-swap and X dimerizations have different calcium requirements

The obtained results with the extrajunctional Dendra activation assay are at odds with a biophysical study that suggests X dimerization is an intermediate in both the assembly and disassembly of strand-swap cadherin dimers (Harrison et al., 2010). Therefore, we designed an alternative experiment that could confirm this unexpected observation. In this experiment, we compared the calcium requirements for the clustering of the parental mutant and for its swapping-incompetent counterparts (W2A and E89A mutants), which form clusters exclusively through the X dimer interface. These two mutations, both of which target W2 residue by different mechanisms, are unlikely to decrease cadherin affinity to calcium. It was directly shown for analogous W2-targeting mutations (Courjean et al., 2008). Therefore, if X dimerization is an intermediate in strand swapping, one may expect that calcium concentration that affects X dimerization would also affect strand swapping. Our experiments showed the opposite. W2A or E89A cadherin mutants failed to form clusters at calcium concentrations 0.5 mM or below. At 1 mM or above, the kinetics of these mutant clustering were nearly the same (Fig. 7; the data are the same for E89A mutant). Thus, X dimerization is activated at calcium concentrations between 0.5 and 1 mM. The parental mutant, in contrast, exhibited no change in clustering at calcium concentrations between 0.2 to 1 mM. It clustered, albeit slowly, even at 0.1 mM calcium (Fig. 7 B). This remarkably more strict calcium requirement for X dimerization than for the entire strand swap process suggested that X dimerization is unlikely to be its intermediate.

X dimerization interface is not required for adhesive dimer formation

An alternative way to determine the role of X dimerization in strand swapping was a direct biochemical measurement of strand-swapped dimer assembly in cells expressing the parental

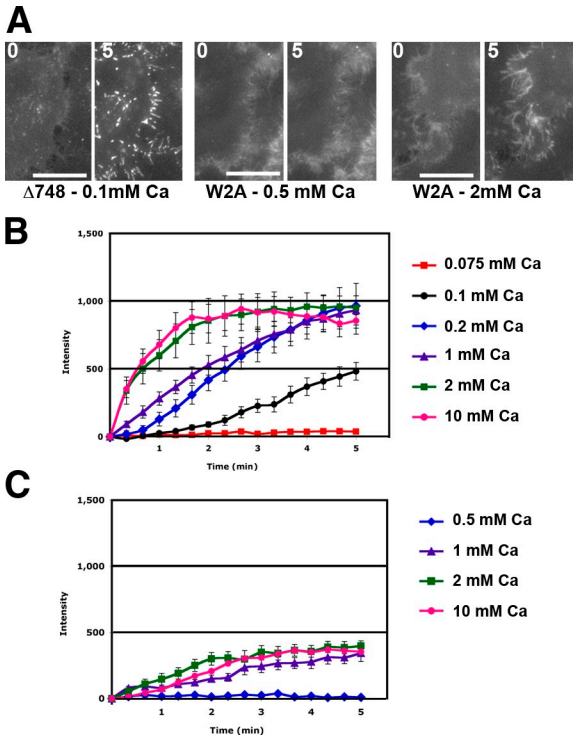

Figure 7. **Strand-swap and X dimerizations have different calcium requirements.** (A) Live-imaging of the calcium switch experiment with cells expressing the parental mutant and its strand-swap–incompetent W2A variant. A calcium switch was performed as in Fig 4 A, but only with different calcium concentration in high-calcium media (indicated). Note that the parental mutant forms clusters at 0.1 mM calcium. The W2A mutant forms clusters at much higher calcium concentrations. Bars, 10 μM. (B and C) Clustering kinetics of the parental mutant (B) and its W2A variant (C) at different calcium concentrations. The error bars indicate SD.

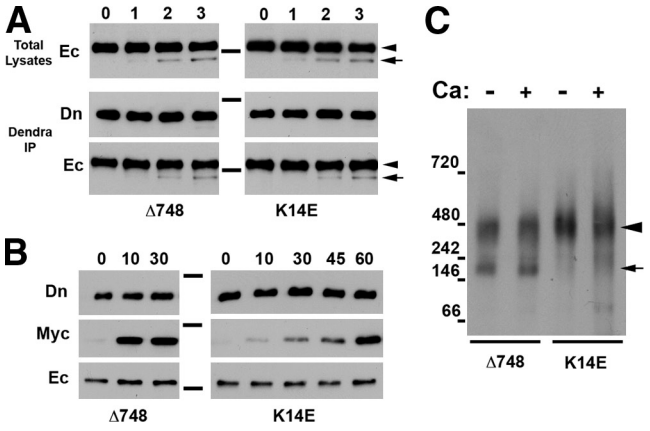

Figure 8. **Role of X interface in the association and dissociation of strand-swap dimers.** (A) Cells expressing the parental mutant EcDendra-Δ748-KL (Δ748) and its X dimer mutant K14E (K14E) were chased in media containing 1 μM tunicamycin for 1, 2, or 3 h (indicated above the lanes). In the presence of this inhibitor, cadherin is not glycosylated (arrows) and can be distinguished from the intact form (arrowheads) that had been present in the cells before the addition of tunicamycin. Top blots show total cell lysates (Total Lysates) of these cultures stained with an antibody specific for endogenous cadherin (Ec). Note the gradual accumulation of the unglycosylated form. The same lysates were immunoprecipitated using anti-Dendra2 antibody, and Dendra-tagged cadherin mutants (Dn) and co-immunoprecipitated endogenous cadherin (Ec) were revealed by Western blotting. Note that the X dimer mutation does not delay the formation of strand-swap dimers between the Dendra-tagged mutants and endogenous cadherin. (B) A-431 cells expressing myc-tagged mutant EcMyc-Δ748-KL were co-cultured overnight in low-calcium media with A-431 cells expressing either the same version of the Dendra2-tagged mutant (Δ748) or its K14E mutant (K14E). After different incubation periods in high calcium (indicated in min), cells were lysed, anti-Dendra precipitated, and analyzed for adhesive dimers using anti-myc or, for total (lateral and adhesive dimers), using the endogenous E-cadherin–specific antibody. Note that at the starting point (0), neither cell co-culture has adhesive dimers. High calcium triggers the rapid assembly of adhesive dimers between myc- and Dendra-tagged parental mutants. The X dimer K14E mutant forms adhesive dimers very slowly; cis dimer is a predominant form even after 30 min in high calcium. Note also that at any time, the total amounts of dimers detected by anti-E-cadherin remain at the same level in both co-cultures. Bars in A and B indicate denote the relative positions of 116-kD molecular marker (β-galactosidase). (C) Native gel electrophoresis of total cell lysates (anti-Dendra staining) detects two major (low, arrow; high, arrowhead) molecular weight complexes. Cells were cultured overnight in low calcium (−) and placed to high calcium for 30 min (+). Note, the cells expressing K14E mutant completely lack the low-molecular-weight complex. Bars at the left margin denote the relative positions of molecular markers (molecular weights are indicated).

and X dimer mutants. To this end, we examined the incorporation of the monomeric, newly synthesized cadherin mutants into the dimers. To mark the newly synthesized cadherin, we blocked its glycosylation by adding tunicamycin. This inhibitor was shown to reduce the molecular weight of cadherin but to leave intact its adhesive functions (Shirayoshi et al., 1986). After different chase periods with the inhibitor, the mutants were immunoprecipitated by an anti-Dendra antibody. The amount of the endogenous cadherin that was synthesized during these periods and dimerized with the mutants was then determined by Western blotting. Fig. 8 A shows that the newly synthesized endogenous cadherin incorporated into the complex with both mutants with exactly the same kinetics: a short form of cadherin started to appear in both precipitates after 2 h of chase. These data are fully consistent with our extrajunctional Dendra activation assay and strongly support our assumption that the >1 h of delay of junction assembly induced by X dimer mutants in calcium switch experiments is based on abnormally stable cis dimers.

In our final approach to address the role of X dimerization in strand swapping, we determined how fast strand-swapped cis dimers that are abundant in cells at low calcium are replaced with strand-swapped trans dimers during calcium switching. For intact cadherin, this process is so fast that it cannot be separated in time (Klingelhöfer et al., 2002; Troyanovsky et al., 2003). In addition, in wild-type cells, the pool of monomeric cadherin is likely to be sufficient to produce adhesion even without immediate dissociation of cis dimers. If, as we proposed, cis dimerization with the X dimer mutant inactivates endogenous cadherin, one may expect such cis dimers to persist for a long time after the addition of calcium. Our experiments tested this possibility.

We co-cultured cells expressing EcDendra-Δ748-KL with cells expressing its myc-tagged counterpart. As expected, when the co-culture was maintained at low calcium, the anti-Dendra antibody coimmunoprecipitated only endogenous cadherin (Fig. 8 B). This indicated that the cells in low calcium contained only cis dimers that caused Dendra-tagged and endogenous cadherin coimmunoprecipitation. Just 10 min after the addition of calcium, the amount of myc-tagged cadherin in the anti-Dendra immunoprecipitates reached a maximum, whereas

the amount of endogenous E-cadherin (reflecting the sum of cis and trans dimers) remained the same. These data show that the strand-swap dimers in control cells were very unstable; during the 10 min after calcium switch, cis dimers were replaced by trans dimers. This switch from cis to trans dimers was notably delayed in a co-culture of K14E-EcDendra-Δ748-KL– and EcMyc-Δ748-KL–expressing cells (Fig. 8 B): strand-swapped trans dimers in these cells only appeared ∼1 h after the addition of high-calcium media, which is consistent with our live-imaging experiments (Fig. 5).

We also analyzed mutant dimerization using native electrophoresis, Blue native PAGE (BN PAGE; Fig. 8 C). Extracted from the cells maintained at low calcium, the parental EcDendra-Δ748-KL mutant exhibited two (low and high) molecular weight bands, which likely represented monomer and dimer forms. The same ∼1:1 ratio between these two forms remained 30 min after a rise in the calcium concentration. In contrast, the K14E mutant migrated only as a high molecular weight band at both low and high calcium. Collectively, our biochemical experiments corroborated our hypothesis that the X dimer mutation significantly decreased the cadherin monomeric pool and that stable cis dimerization of X dimer mutants delayed junction assembly in the calcium switch assay.

X dimer mutation inactivates clustering of the full-size cadherin

To validate the role of the X interface in adherens junctions, we expressed the full-size Dendra-tagged E-cadherin (EcDendra-KL) and its K14E mutant (K14E-EcDendra-KL) in cadherin-deficient A431D cells. To minimize cadherin endocytosis, which can potentially destroy junctions stabilized by the K14E mutation, both recombinant proteins harbored point inactivation of their endocytic signals, K738, and a dileucine motif (Hong et al., 2010). In striking contrast to the experiments with catenin-uncoupled cadherin mutants, the K14E mutant of EcDendra-KL was randomly localized in cell–cell contact areas and did not form the definitive junctional structures common for control Ec-Dendra-KL–expressing cells (Fig. S3). These data suggests that the intracellular catenin-binding region could be incompatible with K14E mutant clustering. Alternatively, it is possible that endogenous cadherin promoted clustering of the K14E mutant in our previous experiments with A-431 cells. To discard one of these possibilities, we expressed the tailless K14E-EcDendra-Δ748 mutant in A431D cells. The result of this experiment clearly shows that endogenous cadherin is not needed for clustering of the K14E tailless mutant (Fig. S3 B). Finally, using a coimmunoprecipitation approach, we studied whether the K14E mutation prevented clustering of the full-size cadherin by blocking its strand-swap dimerization. To this end, we co-cultured A431D cells expressing EcDendra-KL and its K14E mutant with wild-type A-431 cells. Immunoblot analyses of anti-Dendra immunoprecipitates obtained from these co-cultures showed that the both Dendra-tagged proteins formed similar amounts of trans dimers with endogenous E-cadherin of A-431 cells (Fig. S3 D). As predicted, trans dimers incorporating the K14E mutant were highly resistant to low calcium. Collectively, these data suggest that the catenin-binding region controls some step during adherens junction assembly that includes cadherin X dimerization and that is downstream from strand-swap dimerization.

Discussion

Recent live-imaging experiments revealed a very important feature of adherens junctions: instead of being static, they exhibit constant cadherin turnover (de Beco et al., 2009; Hong et al., 2010). However, this turnover is not based on the weakness of intercadherin interactions: the release of cadherin from the junctions is driven by active mechanisms (Troyanovsky et al., 2006; de Beco et al., 2009; Hong et al., 2010). To support this dynamic process, a cadherin adhesive bond must combine sufficient strength to drive fast recruitment of cadherin into the junctions with sufficient weakness to be released from the junctions. This dual property of the adhesive bond may be based on its ability to switch between tight and relaxed states. Although numerous structural studies have suggested that intercadherin interactions are diverse (Patel et al., 2003; Troyanovsky, 2005; Leckband, 2008), their individual contributions to adhesion have remained unknown. Here, we studied this issue using the targeted inactivation of the specific binding interfaces of the catenin-uncoupled cadherin mutant. The use of this mutant allowed us to minimize the influence of intracellular factors on extracellular cadherin-binding events.

We found that two distinct types of cadherin dimerization, known as strand-swap and X dimerization (Harrison et al., 2010), can independently recruit cadherin into the cell–cell contact. Apparently, there is no third binding interface that is able to target cadherin into the junctions because simultaneous inactivation of the strand-swap and X dimer interfaces completely abolished the junctional localization of the mutant. Furthermore, the normal phenotype of the adherens junctions in cells expressing this strand-swap/X dimerization–incompetent mutant indicates that this mutant does not form any functional cis dimers with endogenous cadherin: cis dimers between endogenous cadherin and the mutant would either target the mutant into the junctions or inactivate endogenous cadherin.

Point mutation D1A, which promotes strand swapping (Laur et al., 2002; Troyanovsky et al., 2007), stabilizes cadherin in the junctions but does not change their morphology. Mutations that specifically prevent X dimerization change internal junctional dynamics but also have little effect on the general appearance of the junctions. These observations are consistent with the model in which adhesion in adherens junctions is based on strand-swap interactions. In contrast, cadherin clusters formed exclusively through the X dimer interface by the strand swap–incompetent mutants are remarkably plastic, exhibiting very fast turnover and disappearing almost instantly in low calcium. Therefore, some sort of cooperation between the strand-swap and X dimer interfaces may confer both strength and plasticity on a cadherin adhesive cluster. Our data allows us to discuss some details of this cooperation.

In vitro binding experiments showed that the cadherin X dimer functions as an "encounter complex" (Harrison et al., 2010) that initiates cadherin strand swapping. Weak interactions

detected between W2A cadherin mutant molecules were also attributed to the formation of such complex (Sivasankar et al., 2009). At first glance, our observation that the X dimer mutations delayed junction assembly in a calcium switch assay seems to support the role of X dimers as important intermediates in the cadherin strand-swapping reaction. However, the detailed analysis shows that this delay is caused by abnormally long retention of the X mutants in the low-calcium–induced cis dimers. Furthermore, our other results also suggest that X dimerization is not an essential step in junction assembly. First, the extrajunctional Dendra activation assay and biochemical experiments with tunicamycin-treated cells show that X dimer mutations did not affect cadherin recruitment into the junctions at steady state. Second, we found that strand-swap dimerization recruits cadherin into the junctions at a calcium concentration as low as 0.1 mM. It is unlikely that X dimerization is a part of this process because this type of dimerization recruits cadherin into the junctions at much higher calcium concentrations (1 mM). Such a high (>0.5 mM) calcium concentration requirement for cadherin X dimerization has been shown by experiments with the ECADCOMP experimental system. A very weak calcium-binding affinity of the EC1–EC2 calcium-binding sites was proposed to be responsible for this fact (Koch et al., 1997; Pertz et al., 1999). Collectively, our experiments indicate that a deficiency in X dimerization does not change (or only slightly changes) cadherin strand swap dimerization in cell junctions.

The obvious discrepancies between our experiments showing that cadherin strand swapping circumvents the X dimer step and the published protein-binding experiments showing that the X dimer is a kinetically important intermediate in the same reaction may be caused by several factors that specifically enhance the production of strand dimers in cell–cell junctions. The first factor that can intensify strand swapping in cells is cadherin presentation: in cell contacts, two encountering EC1 domains can be favorably oriented. Second, the diffusion of cadherin molecules on the cell surface is 2D and much slower than that in solution. Therefore, the initial cadherin–cadherin encounter in cells can be long enough to allow strand exchange being completed. These two factors (favorable cadherin presentation and the long duration of each cadherin-cadherin collision) may produce conditions that strongly facilitate cadherin strand exchange in cell–cell contacts. In fact, exactly this phenomenon (enhanced production of strand dimers in cells vs. in solution) was documented in our previous work (Troyanovsky et al., 2007). Direct strand-swap cadherin dimerization is not an unfeasible process; it has been shown by nuclear magnetic resonance–based studies for the cadherin 8 EC1 and E-cadherin EC1/EC2 fragments (Häussinger et al., 2004; Miloushev et al., 2008).

Not just association but also dissociation of strand dimers in solution and in cell–cell contacts can be very different: the dissociation rate of junctional strand dimers is likely to be much smaller than that in solution because these dimers in junctions are reinforced through cis interactions (Wu et al., 2010; Harrison et al., 2011). Therefore, strand-swap trans dimers in cadherin clusters may require a specific mechanism for their dissociation. Indeed, although our results do not show the X dimer playing a significant role in cadherin cluster assembly, they compellingly show that this dimer is essential for the exit of cadherin from the junctions. This is evident from the very slow junctional turnover of X dimer mutants and their slow release from the junctions in the calcium switch assay or in the presence of the cadherin-blocking antibody. The failure to form X dimers locks cadherin in cadherin clusters in the strand swap dimerization state that is fully consistent with a slowly exchanging equilibrium between monomer and strand dimer states for X dimer mutants in solution (Harrison et al., 2010). Collectively, properties of X dimer mutants suggest that cadherin adhesion is a directional process: cadherin clusters assemble and disassemble through two different pathways. Cadherin enters the junction by direct strand swapping, but it is released from the junction through a strand swap–to–X dimer transition.

The mechanism of such a reconfiguration of strand swap into X dimers appears to be key to understanding cadherin dynamics in adherens junctions. Because X dimer is much less stable than strand-swap dimer, such a transition can be the energy-consuming step of adherens junction disassembly that we have identified in our previous biochemical and live-imaging experiments (Troyanovsky et al., 2006; Hong et al., 2010). Two possibilities can be envisioned. The first is that a configuration of cadherin dimers is regulated by intercellular tension. Indeed, the difference in angles between the paired EC1 domains (Harrison et al., 2010) makes the total length of the X dimer (which must correspond to the intercellular distance in the cell–cell contact) shorter than that of its strand-swap counterpart (Fig. 9). Therefore, compression of the junction consisting of the strand-swap dimers by local actin polymerization would facilitate their conversion to X dimers. However, stretching the junction by actomyosin contraction would result in its strengthening because an increase in intercellular distance must obstruct the strand-swap–to–X dimer transition. Importantly, cadherin dimers in solution, where dimer configuration is undefined, can be much less stable than those in the contacts (see Fig. 9 for detail). The increased strength of cadherin adhesion under mechanical tension is supported by numerous observations (Gloushankova et al., 1998; Ivanov et al., 2005; Shewan et al., 2005; Bershadsky et al., 2006; Miyake et al., 2006; Ladoux et al., 2010; Yonemura et al., 2010).

The second possibility is that the strand swap–to–X dimer transition is controlled by a specific type of cis interactions between trans dimers. Indeed, in cadherin crystals, cis interactions pack cadherin trans dimers into 2D lattice (Boggon et al., 2002). Such cis alignment apparently reinforces strand-swap trans dimers in real junctions (Wu et al., 2010). Specific catenin- or cytoskeleton-induced changes in such lattice of trans dimers can trigger their reconfiguration into the X type, thereby facilitating junction disassembly. Indeed, our recent work showed that binding to catenins is required for fast cadherin exchange at steady state (Hong et al., 2010). Our experiments with full-size cadherin X dimer mutants further demonstrate a link between catenins and states of cadherin dimerization. They suggest that catenins bound to cadherin appear to sense defects in X dimerization and down-regulate adherens junction assembly. Although additional experiments are needed, these data shows that continuous assembly and disassembly of cadherin trans dimers is required for the integrity of adherens junctions.

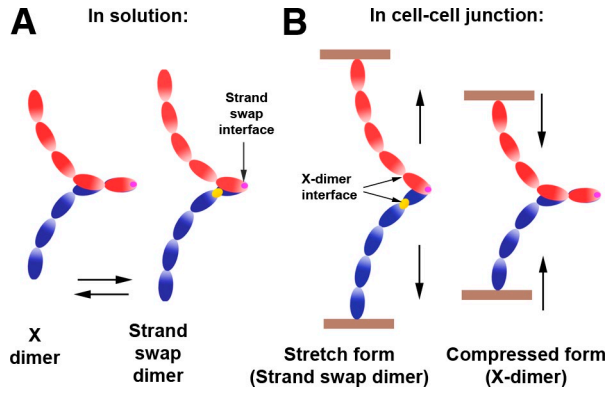

Figure 9. **Hypothetical mechanoregulation of the cadherin dimerization state.** (A) Cadherin dimer in solution cannot be regulated by traction forces and freely shuttles between X and strand-swap modes. Once in X mode, the dimer becomes unstable and may dissociate. Such uncontrolled strand swap–to–X dimer transitions must significantly decrease cadherin dimerization affinity in most in vitro binding assays. (B) The same cadherin dimer, engaged in cell–cell adhesion, cannot freely shuttle between two binding modes. If the junction is stretched by contracting forces, cadherin in the dimer interacts exclusively through strand swapping because their X dimer interfaces (positions of both interfaces are indicated) are far away from one another. In contrast, closing the intercellular gap (for instance, by actin polymerization) compresses the dimer. In such a compressed form, X dimer interfaces are perfectly aligned, facilitating the strand swap–to–X dimer transition, thereby strongly increasing the probability of dimer dissociation.

In conclusion, our work provides new evidence that the strand-swap interaction drives cadherin clustering and entraps cadherin in cell–cell junctions. To counterbalance these two events, cadherin exits the junctions in the course of an active, apparently complex process. Our experiments have determined a critical step in this process: the destabilization of strand-swap cadherin adhesive bonds by their transition to the X configuration. The identification of this step is important for further understanding the mechanisms regulating adherens junction renewal and plasticity. Our findings demonstrate that cadherin adhesion dynamics are based on the same molecular principle (change in the mode of intersubunit interaction) as the plasticity of many other structures, including microfilaments and microtubules.

Materials and methods

Cell culture, antibodies, plasmids, and DNA transfections

Transfection, growth, and immunofluorescence microscopy of human A-431, CHO, and A431D (provided by J.K. Wahl, University of Nebraska, Lincoln, NE) cells were performed as described previously (Hong et al., 2010). All cell lines were cultured in DME with 10% FCS. The plasmids encoding EcDendra-Δ748-KL and EcDendra-KL have been described previously (Hong et al., 2010). After transfection and selection, the cell colonies were screened for transgene expression by FACS, and only homogeneously positive clones were used. All clones exhibited the same expression level of Dendra-tagged proteins (Fig. S1). The point mutations inactivating dimer interfaces (Fig. 1 and Table S1) were incorporated into the pRc-EcDendra-Δ748-KL using site-directed mutagenesis. For coimmunoprecipitation experiments, the Dendra tag in this plasmid was replaced with 6xmyc. For convenience, the amino acid residues mutated in the tailless mutant were numbered starting from the amino-terminal residue of the fully processed E-cadherin. Such numbering had been used in many structural studies. Table S1 numbers the same mutations using an alternative way that starts from initiation Met codon of human E-cadherin.

The following antibodies were used: anti–E-cadherin (clone C20820 recognizing only the endogenous cadherin and mouse anti–β-catenin; BD), function-blocking anti–E-cadherin (clone SHE78-7; Invitrogen), rabbit anti-Dendra2 (Evrogen), and anti-myc (clone 9E10; Sigma-Aldrich). Depletion of ATP was achieved by ATP depletion media containing 2 mM 2-deoxy-D-glucose and 1 µM antimycin A (Troyanovsky et al., 2006). If not specifically indicated, a low-calcium media contained 20 µM calcium. Tunicamycin was obtained from Sigma-Aldrich and used at final concentration 1 µM.

Coimmunoprecipitation assay and Western blotting

For coimmunoprecipitation, cells were first extracted with 1 ml of 1% Triton X-100–containing immunoprecipitation lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, and 0.5 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride [AEBSF]), then subjected to subsequent incubations with anti-Dendra antibody and protein A–Sepharose (Troyanovsky et al., 2006). The resulting immunoprecipitates were analyzed by immunoblotting. BN PAGE was performed according to the manufacturer's instructions (Invitrogen). Cells were extracted with lysis buffer containing 1% digitonin as indicated previously (Kiss et al., 2008).

Live-cell imaging and data processing

These experiments were performed essentially as described previously (Hong et al., 2010). In brief, cell suspension (~10^5 cells) was plated into a homemade chamber built on cover glass. The next day, the culture media was replaced with imaging media (L-15 plus 10% FBS) and the chamber was imaged with a microscope (Eclipse Ti-E; Nikon) at 37°C controlled with NIS-Elements software (Nikon). The microscope was equipped with an incubator chamber, a camera (CoolSnap HQ2; Photometrics), Plan-Apochromat 60×/1.40 NA and Plan-Apochromat VC 100×/1.40 objective lenses, and halogen and mercury light sources. Time-lapse images were taken in both FITC and TRITC filter sets using halogen light that minimized phototoxicity and photobleaching. To analyze cadherin junctional turnover, we used a junctional Dendra photoactivation assay (Hong et al., 2010). A circular region of interest (ϕ = 5 µm) was photoactivated by a 3-s-long exposure to the 402-nm light using the mercury arc light and a pinhole insert. The entry kinetics of cadherins into junctions were studied using the extrajunctional Dendra photoactivation assay (Hong et al., 2010); the extrajunctional pool of cadherins was photoactivated by illuminating the center region of a cell (ϕ = 20 µm).

All images were saved as Tiff files and processed using ImageJ software (National Institutes of Health). A circular region of interest (ϕ = 0.65 µm) was positioned on a single adherens junction, and the mean value of fluorescent intensity in each frame was calculated in both photoactivation and calcium switch experiments. In the junction Dendra photoactivation assay, the red fluorescent intensity was normalized in such a way that 0 and 1 corresponded to the background and the initial (immediately after activation) values. The background value was obtained from the image taken right before the photoactivation. The time course of intensity change was produced from 10 sets of independent experiments. In the extrajunctional Dendra assay, red fluorescent of the junctions nearby to the activation region was normalized in a way that 0 and 1 corresponded to their initial and plateau intensity values. The total incorporation of activated cadherin into these junctions (Fig. 6 C) was estimated by the ratio of the red to green intensities in plateau. The intensity changes during the calcium switch were normalized to 0 and 100% for the minimum and maximum values.

Online supplemental material

Fig. S1 shows the morphology and the size of the cadherin clusters in cells expressing EcDendra-Δ748-KL and its mutants. The figure also shows that these cells expressed the same levels on transgenes. Fig. S2 shows the disassembly kinetics of cadherin clusters by an E-cadherin function-blocking antibody. Fig. S3 shows the subcellular distribution and dimer production of the Dendra-tagged full-size cadherin and its K14E point mutant in cadherin-deficient A-431D cells. Table S1 provides a detailed characterization of the cadherin extracellular domain point mutations used in the work. Videos 1 and 2 demonstrate the very different dynamics of cadherin clusters in cells expressing EcDendra-Δ748-KL and its strand-swap–incompetent W2A mutant. Videos 3 and 4 demonstrate a significant difference in the assembly kinetics of cadherin clusters in cells expressing EcDendra-Δ748-KL and its X dimer–incompetent K14E mutant. Video 5 shows that the latter cells require ~1 h to produce cadherin clusters. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201006113/DC1.

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