The formation of ordered nanoclusters controls cadherin anchoring to actin and cell–cell contact fluidity

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Oligomerization of cadherins could provide the stability to ensure tissue cohesion. Cadherins mediate cell–cell adhesion by forming trans-interactions. They form cis-interactions whose role could be essential to stabilize intercellular junctions by shifting cadherin clusters from a fluid to an ordered phase. However, no evidence has been provided so far for cadherin oligomerization in cellulo and for its impact on cell–cell contact stability. Visualizing single cadherins within cell membrane at a nanometric resolution, we show that E-cadherins arrange in ordered clusters, providing the first demonstration of the existence of oligomeric cadherins at cell–cell contacts. Studying the consequences of the disruption of the cis-interface, we show that it is not essential for adherens junction formation. Its disruption, however, increased the mobility of junctional E-cadherin. This destabilization strongly affected E-cadherin anchoring to actin and cell–cell rearrangement during collective cell migration, indicating that the formation of oligomeric clusters controls the anchoring of cadherin to actin and cell–cell contact fluidity.

Introduction

Around 35% of proteins in cells are in an oligomeric state (Goodsell and Olson, 2000). Oligomerization provides several functional advantages such as a mechanism to resist degradation and, more importantly, to make higher order long-living subcellular structures such as cytoskeletal filaments and functional nanomachines. Tissue cohesion is ensured by cell adhesion molecules that establish short living intercellular protein–protein bonds at the single molecule level (Perret et al., 2004). Oligomerization could provide the necessary strength to support intercellular adhesion and resistance to mechanical stress. Cadherins are major cell adhesion molecules in animal cells (Hulpiau et al., 2013). Cadherins diffusing at the plasma membrane initiate cell–cell interactions by establishing homophilic intercellular bonds (Mège et al., 2006). These trans-interactions analyzed by atomic force microscopy or biomembrane force probe have been shown to be short living (Baumgartner et al., 2000; Perret et al., 2004), implying that some higher order processes must take place for cadherin-mediated adhesion to reach sufficient stability to sustain physiologically relevant resistance to mechanical stress. Nascent cell–cell contacts initiated by cadherin trans-interactions evolve in adhesion plaques by the growth of cadherin clusters gathering additional trans-interacting cadherin molecules by a diffusion trapping mode (Adams et al., 1998; Lambert et al., 2007). Upon anchorage to the underlying actin cytoskeleton, which may bring additional cooperativity in cadherin recruitment as well as stability (Lambert et al., 2002; Hong et al., 2013), these adhesion plaques eventually evolve in adherens junctions (AJs; Mège et al., 2006). However, whether cadherin clusters found in AJs are organized in oligomeric structures as connexins in gap junctions (Ravil and Gilula, 1975) or desmosomal cadherins in desmosomes (Al-Amoudi et al., 2007), or have no particular organization as contradictorily reported for desmosomal cadherins (He et al., 2003), remains an open question.

Structural data have brought important information on the organization of cadherins (Overduin et al., 1995; Shapiro et al.,...
The current hypothesis is that adhesion starts with trans-interaction of EC1 domains of cadherins from apposed cells. More recently, a cis-interface for E-cadherin (Ecad) has been identified in crystal lattices. It involves the nonsymmetrical interaction of the EC1 domain of one cadherin with the EC2 domain of a neighboring cadherin (Harrison et al., 2011). Site-directed mutagenesis in EC1 (V81D) and EC2 (L175D) domains abolishes the formation of a cis-interface in the crystal without affecting the formation of the trans-interface. V81D, L175D-mutated Ecad ectodomain failed to form ordered junction-like structures in a liposome system, whereas wild-type (wt) Ecad did. Further theoretical and simulation work predicted that Ecad organizes in linear or more complex nanometric arrays as a result of trans- and cis-interactions (Wu et al., 2011, 2013). However, although Ecad cluster size and distribution have been reported with unprecedented resolution in tissues thanks to super-resolution microscopy (Truong Quang et al., 2013; Wu et al., 2015), cadherins have never been imaged at a nanometric resolution and thus no direct proof of ordered organization of cadherin in clusters has been provided so far in cells. Harrison et al. (2011) data suggest that the cis-interface stabilizes junctional Ecad. However, these data have been obtained by expressing wt and cis-Ecad forms deleted from the cytoplasmic domain. Because anchorage of cadherin cytoplasmic domain to actin via catenins is a major factor of AJ formation and strengthening (Lambert et al., 2002; Cavey et al., 2008; Hong et al., 2013), one may ask questions on the influence of cadherin oligomerization on cadherin cytoplasmic tail anchoring to F-actin.

The purpose of this work is to provide evidence for the predicted formation of arrays of oligomeric Ecad in cellulo and to study the functional implication of Ecad oligomerization on the formation and maturation of cell–cell contacts. We expressed full-length wt and cis-Ecad (V81D, L175D double mutant) in Ecad-deficient A431D cells and used an electron microscopy approach to visualize single Ecad molecules at the cell membrane. We also studied the consequences of the disruption of the cis-interface on cell–cell contact formation and stability. Our data provide evidence for an ordered organization of Ecad in clusters, depending on the cis-interface. We show that the cis-interface was, however, not required for AJ formation. The effects of the V81D, L175D mutations on Ecad turnover, association to catenin, and anchoring to actin were analyzed by cell imaging, FRAP analysis, and manipulation of Ecad-coated beads with magnetic tweezers. Ecad cis-oligomerization perturbations had moderate effects on Ecad complex stability but dramatically impaired Ecad anchoring to the actin cytoskeleton, the overall stability of cell–cell contacts, and collective cell behavior.

Results

Effect of the disruption of the cis-interface on Ecad expression and cell-cell adhesion

To visualize Ecad oligomers at the cell membrane, we used A431D cells, as they do not express Ecad (Lewis et al., 1997). Both wt Ecad-GFP and cis-Ecad-GFP were accumulated at cell–cell contacts where they recruited α- (Fig. 1) and β-catenin (Fig. S1, A and B) as previously reported (Troyanovsky et al., 2015). Western blot analysis indicates that wt Ecad-GFP and cis-Ecad-GFP were expressed at similar levels. Similar levels of α- and β-catenin were coimmunoprecipitated with the wt and cis-mutant Ecad, indicating that the impairment of Ecad cis dimerization did not affect the association to catenin. Cell surface fluorescence imaging indicates that cis-Ecad-GFP was as efficiently accumulated at the plasma membrane as wt Ecad-GFP and even slightly more (Fig. 1 C).

To test whether the impairment of cis dimerization alters cadherin-mediated cell adhesion, we measured the ability of Ecad-Fc–coated beads to bind to transfected cells (Fig. S2, C...
No significant difference was seen in the number of Ecad-Fc coated beads bound on cis and wt Ecad expressing cells. These observations indicate that the disruption of the cis-interface did not affect the ability of Ecad to mediate cell adhesion, extending previous observations showing that the disruption of the cis-interface did not impair cell aggregation in the context of cells expressing tailless Ecad (Harrison et al., 2011).

In cellulo visualization of Ecad cis-oligomers

In an attempt to visualize cadherin oligomeric organization in cellulo, we used an electron microscopy approach, allowing to stoichiometrically label GFP-tagged proteins on membrane patches (Fig. 2 A). Cells were grown on Ecad-Fc–coated electron microscope grids, and then ripped off from the surface, exposing the cytoplasmic face of the plasma membranes. Membrane rip-offs were then incubated with gold nanoparticles (NP) functionalized at a 1:1 stoichiometry with anti-GFP-NP. NPs were then observed by transmission electron microscopy (TEM), allowing the visualization of single GFP-tagged cadherins. Few areas with very high density of anti-GFP nanobodies were observed for wt Ecad (up to 2698 NP/µm²) and were never observed for cis-Ecad (up to 279 NP/µm²; Fig. S2, A and E). Because wt and cis-Ecad were expressed at similar levels and only a tiny area can be visualized by TEM, this difference could be the consequence of differences in distribution of wt and cis-Ecad or of a greater propensity of cis-Ecad membrane to be torn off when scratching the cell roof from the Ecad-coated grid.

To further compare the NP distribution between wt and cis-Ecad expressing cells only fields having a similar density of labeling (4–180 NP/µm²) were considered for the analysis (Fig. 2 and Fig. S2, B and E). A fraction of NP-bound wt Ecad was characterized by K-function analysis. Curves with solid symbols correspond to the mean L(r) − r values calculated for all images. Curves with open symbols correspond to the mean L(r) − r values calculated for individual images presenting significant clustering. Values of L(r) − r above the 99% confidence interval (CI; black line) indicate significant clustering. Proportion of images having clustering of NP for wt and cis-Ecad is significantly different according to χ² test (P < 0.01).
7.6% of the NPs at <15 nm, a peak in the distance distribution between 30 to 55 nm, and very few NPs with a distance <10 nm (Fig. 2 C and Fig. S2 F). To determine whether the oligomeric forms observed reflect significant clustering, Ripley’s K-function was used (Prior et al., 2003). 34% of the images acquired for wt Ecad cells present a significant clustering within a 15- to 120-nm range, with a maximum deviation out of the 99% confidence interval occurring at a radius of 30 nm (Fig. 2 D). In contrast, only 4.9% of the images acquired for cis-Ecad cells present a significant clustering within a 15- to 51-nm range, with a maximum deviation out of the 99% confidence interval occurring at a radius of 30 nm. cis-Ecad molecules were more randomly distributed, indicating that the cis-interface largely participates in the clustering of Ecad molecules. Altogether, these data provide supporting evidence that Ecad engaged in trans-interactions organize in ordered oligomers that require a proper cis-interface. In addition, the observed organization of particles within clusters, in particular in higher density images (Fig. S2 D), was highly reminiscent of the one predicted by theoretical models (Wu et al., 2011, 2013). This is, to our knowledge, the first time nanometric distribution of Ecad molecules can be visualized in a cellular context.

**AJs form independently of Ecad cis-oligomerization**

We then asked whether the disruption of this interface would affect the formation of AJs, which are believed to result from clustering of Ecad in restricted domains of cell–cell contacts (Mege et al., 1988; Harrison et al., 2011). We analyzed the propensity of transfected cells to organize Ecad in discrete cadherin adhesions when spread on Ecad-Fc, as a proxy of the ability of cells to form AJs (Gavard et al., 2004b). Both cis-Ecad and wt Ecad expressing cells spread on Ecad-Fc, and wt Ecad and cis-Ecad molecules were similarly recruited in radial cadherin adhesions, colocalizing with actin fibers (Fig. 3 A), suggesting that the formation of AJs may not involve the cis-interface.

To directly investigate the ability of cis-mutant proteins to induce the formation of AJs, we performed an ultrastructural examination of transfected cell monolayers by TEM. We searched for the presence of AJs, defined as zones of straight membrane apposition associated with the presence of dense material (Perez-Moreno et al., 2003). As reported by Lewis et al. (1997), A431D cells formed neither desmosomes nor AJs (unpublished data), allowing unambiguous interpretation of the observations on wt Ecad and cis-Ecad expressing A431D cells. The expression of wt Ecad drastically changed the cell–cell contact ultrastructure with the appearance of AJs with a mean intermembrane distance of 18.4 ± 0.7 nm (Fig. 3 B). Typically, cis-Ecad cells presented indistinguishable intercellular junctions with similar mean intermembrane spacing (18.0 ± 0.5 nm). Moreover, the mean lengths of these structures were similar. Altogether, TEM analysis demonstrates that the cis-interface is not required for the formation of AJs.

**Ecad cis-oligomerization stabilizes junctional cadherins**

We hypothesize that the cis-interface may, however, affect the dynamics of junctional molecules. To test whether mutations affecting the cis-interface had an effect on full-length Ecad dynamics, we performed FRAP experiments on wt and cis-Ecad expressing cells (Fig. 4, A and B). The disruption of the cis-interface induced a moderate but significant increase of the mobile fraction of Ecad. The \( t_{1/2} \) value was not affected (Fig. 4 C), suggesting that the mobility of fast diffusing molecules was not altered. To have access to the dynamics of the cytoplasmic partners of Ecad, dual wavelength FRAP was performed on cells coexpressing Life-ACT-Ruby were fixed after 2 h of spreading on Ecad-Fc-coated surfaces and imaged for Ecad-GFP and F-actin. Bar, 20 µm. The disruption of the cis-interface did not impact the ability of transfected cells to spread on Ecad-Fc and to recruit Ecad in cadherin adhesions (arrowheads). (B) Transmission electron micrographs showing the ultrastructure of intercellular contacts of wt Ecad-GFP (wt Ecad) and cis-Ecad-GFP (cis Ecad) cells expressing Life-ACT-Ruby. Bar, 100 nm. Insets show the junctional areas. (C) Table showing the mean length (± SEM) and the mean width (intermembrane distance; ± SEM) of AJs formed by wt Ecad (\( n = 36 \)) and cis-Ecad-GFP expressing cells (\( n = 24 \)). The disruption of the cis-interface does not prevent the formation of AJ.
to a proportional increase of the α-catenin mobile fraction, suggesting that the increase in cadherin stability brought by ectodomain oligomerization has a direct intracellular impact through modifications of α-catenin dynamics. This may in turn affect cadherin anchoring to actin filaments. Such moderate changes in cadherin dynamics are expected to have limited impact on the steady-state recruitment of cadherin and catenin molecules and formation of AJs in agreement with photon end electron microscopy observations.

**Ecad cis-oligomerization stiffens the mechanical coupling of cadherin adhesions to actin**

The functional anchorage of cadherin–catenin complexes to F-actin has emerged as a major signaling pathway downstream of cadherins (Giannone et al., 2009; Takeichi, 2014), acting on the reinforcement of cell–cell contacts (le Duc et al., 2010; Yonemura et al., 2010; Thomas et al., 2013). To compare the functional anchorage of wt Ecad and cis-Ecad to actin we first studied actin dynamics in the lamellipodia of cells spread on Ecad-Fc–coated surfaces. Indeed, according to Mitchison and Kirschner (1988), a decreased actin treadmilling speed correlates with an increased friction between the cytoskeleton flowing underneath the plasma membrane and the membrane-anchored adhesion sites. Actin retrograde flow was visualized by live-cell imaging, thanks to the coexpression of LifeAct-Ruby (Fig. 5 A and Videos 1 and 2). The speed of the rearward flow of actin was increased by 30% in cis-Ecad compared with wt Ecad expressing cells, suggesting that cadherin oligomerization is involved in the coupling of the actin cytoskeleton to the plasma membrane. Thus, the increase in actin retrograde flow observed when the Ecad cis-interface was disrupted reflects a reduced association of Ecad clusters to the actin network. This increase in actin dynamics was correlated with an increase in cell protrusion (Fig. 5 B). Both the maximum amplitude and the frequency of lamellipodia edge back and forth movements were increased when the cis-interface was mutated (Fig. 5 C). These increases in actin rearward flow and protrusion activity in the lamellipodia of cells spread on Ecad-Fc are also suggestive of a decreased anchoring of cis-Ecad clusters to actin.

To directly test the mechanical coupling of wt and cis-Ecad to the underlying cytoskeleton, we probed the response to force of Ecad-coated magnetic beads bound to wt and cis-Ecad cells, using magnetic tweezers (Kollmannsberger and Fabry, 2007). We generated a magnetic field in the vicinity of beads applying forces ~20 pN (Fig. 6 A). The semi-quantitative analysis of bead behavior as the magnetic power was turned on indicates that beads adhered less firmly to cis-Ecad expressing cells than to wt Ecad expressing cells (Fig. 6 B). Single beads bound to wt Ecad cells and cis-Ecad cells were then subjected to successive steps of force application (Fig. 6 C and Videos 3 and 4). No clear trend in the changes of the displacement amplitude in function of the number of cycles performed was noticed, suggesting that bead–cell mechanical coupling was not subject to force-dependent reinforcement as previously reported (Lambert et al., 2002). However, the amplitude of bead displacement was consistently higher for Ecad-Fc beads attached to cis-Ecad cells than for those bound to wt Ecad cells (Fig. 6 D). Altogether, these data indicate that Ecad cis-oligomerization increases the stiffness of Ecad complex coupling to internal structures.

We also took advantage of this experimental setup to extract information on the mobility of wt Ecad and cis-Ecad molecules in the cell membrane, which has been demonstrated to reflect anchoring of the molecules to the actin cytoskeleton (Lambert et al., 2002). Fig. 6 E shows successive x-y trajectories undergone by single beads bound to wt Ecad and cis-Ecad expressing cells in the absence of force. Beads bound on cis-Ecad expressing cells moved over larger areas than
Impairment of Ecad cis dimerization alters the actin dynamics of cells spread on Ecad-Fc. Cells coexpressing wt Ecad-GFP or cis-Ecad-GFP and LifeAct-Ruby were seeded on Ecad-Fc substrates for 2 h and then subjected to spinning disk live-cell imaging for 3 min at a frequency of one image per 500 ms. (A) Still Images of LifeAct-Ruby distribution. Bar, 25 µm. The actin retrograde flow was quantified by kymograph analysis [yellow lines 1–3, 1 pixel width, perpendicular to the cell membrane in Ecad dense region]. Superimposed on the kymographs are the means of actin retrograde flow speed for wt Ecad (n = 156 kymographs from 26 cells) and cis-Ecad (n = 192 kymographs from 32 cells) cells. The actin retrograde flow was significantly faster for cis-Ecad expressing cells than for cells expressing wt Ecad (P ≤ 0.0002, Student’s t test). (B) Similar kymographs of the LifeActRuby signal extending on a longer time window revealed the cyclic protrusion of the edge of wt Ecad and cis-Ecad expressing cells. (C) Quantification of the maximum amplitude and frequency of membrane protrusions [mean values ± SEM; n = 100 kymographs from 26 cells for wt Ecad cells and n = 130 kymographs from 32 cells for cis-Ecad expressing cells]. ****, P ≤ 0.0001; ***, P ≤ 0.005, Student’s t test.

Disruption of the cis-interface strongly impacts collective cell migration

Although wt Ecad and cis-Ecad cells did not display differences in the ability to form cell–cell contact at confluence, we postulated that the formation of structured clusters by its action on the strengthening of the Ecad–actin mechanical link may have an impact on the stability of cadherin adhesions, which may be revealed only when cell–cell adhesions are challenged, for example, during collective cell movement. Indeed, previous studies showed that changes in intercellular adhesion had a strong impact on collective cell migration behaviors (Petitjean et al., 2010; Tambe et al., 2011; Vedula et al., 2014). To unravel the impact of Ecad cis-interface disruption, we performed a cell layer expansion assay after release of confinement (Fig. S3 A). When confluence was reached the block was removed, freeing space for cell layer expansion. The migration front was followed over 24 h and analyzed for its progression and roughness (Fig. 7 A). Both wt and cis-Ecad cell monolayers expended linearly. However, this displacement was 1.6 times faster in the case of cis-Ecad cells. The roughness of the migration front increased during the first 12 h as front cells moved toward the freed area and then stabilized at a plateau. However, the roughness at the front of cis-Ecad expressing cell layers both increased more quickly and reached a higher value at plateau (Fig. 7 A). This was because of individual cis-Ecad expressing cells that tend to escape the monolayer. In addition, some cis-Ecad cells transiently detached from the monolayer, in such a way that has never been observed with wt Ecad expressing cells (Videos 5 and 6).

This analysis suggests that cis-Ecad expressing cells migrate faster as a result of reduced cell–cell cohesion releasing the constraints imposed by neighboring cells. However, faster progression of the migration front might also result from increased cell autonomous migratory behavior. We thus analyzed the migration of isolated wt Ecad and cis-Ecad cells (Fig. S4). Both cell types displayed comparable individual cell behavior, indicating that the faster migration of cis-Ecad cells was a result of collective cell behavior. Alternatively, during the extension of the monolayer, numerous divisions were taking place, suggesting that changes in the division rate might directly have an impact on monolayer front progression. To put aside this possibility, we verified that occurrence of mitosis in the extending monolayer was not different for wt Ecad and cis-Ecad cells (Fig. S5 A). In addition, 5-ethynyl-2-deoxyuridine incorporation showed that there was no difference in the proportion of cells in S phase in each population (Fig. S5 B). Thus the differences in the migratory behavior do not result from altered cell autonomous properties but is specific of collective behavior.

To further support this hypothesis we analyzed the trajectories of cells located either at the migration front or in the back (at least at the fourth row) For both cell types, individual cells at the front had very directional trajectory projecting for the majority in a 40–50° angle cone perpendicular to the migration front. In contrast, cells at the back had more randomly oriented trajectories (Fig. 7 B). However, cis-Ecad cells covered much larger distances both at the front and rear. cis-Ecad cells migrated almost two times faster than wt Ecad cells both at the front and rear (Fig. S3 B). To further describe the migration behavior of these cells, we extracted MSD values from individual cell trajectories (Fig. 7 C). The evolution of MSD as a function of time further showed that cells with the largest displacement...
were front cis-Ecad cells followed by rear cis-Ecad, and then front wt Ecad and rear wt Ecad cells. MSD curves were fitted using the equation $\text{MSD} = 4D \tau + v^2 \tau^2$, where $4D \tau$ represents the Brownian motion component and $v^2 \tau^2$ represents the directed motion component (Fig. 7 D). Diffusion coefficient ($D$) value for cis-Ecad cells both at the front and rear were significantly higher than the ones of wt Ecad cells, revealing the increased exploratory behavior of the cis-mutant cells versus their wt counterparts. This can be interpreted as an increased fluidity of the cis-Ecad monolayers caused by a higher instability of cell–cell contacts and an increased ability of cells to exchange partners. The comparison of the second term of the equation ($v$: velocity of the directed movement) shows that front cells of both phenotypes display a more directed motion than the cells at the rear, which is expected because these cells face an empty space. However, the directed motion velocity of cis-Ecad cells is twice higher than the one of wt cells. Altogether these results suggest that the migration of wt Ecad cells is restrained by stable cell–cell contacts formed between neighboring cells within the monolayer. These results thus demonstrate that the stabilization of cell–cell contacts brought by the formation of the cis-oligomers is essential for coordinated cell behavior during collective cell migration.

**Disruption of the cis-interface strongly reduces cell-cell coordination**

To confirm that cell–cell coordination was affected by cis-interface impairment, we further analyzed the dynamic properties of expanding wt Ecad and cis-Ecad cell monolayers by particle image velocimetry (PIV; Fig. 8). PIV has been used as a powerful tool for quantitative analysis of tissue fluidity resulting from cell–cell rearrangements during collective cell migration (Vedula et al., 2012; Doxzen et al., 2013). PIV analysis confirmed that the mean instantaneous migration speed of cis-Ecad expressing cells was twice the one of wt Ecad cells (Fig. 8, A and B). From the velocity fields we calculated an order parameter as well as a correlation length. The order parameter reflects the degree of orientation of the velocity field in respect to a given direction, which is here defined as perpendicular to the initial migrating front (Fig. 8 C). Order parameter was maximum at the front but significantly lower for cis-Ecad than for wt Ecad cells. Finally, the correlation length, reflecting the mean distance at which velocity vectors are maintained in the same orientation, was also decreased.
when the cis-interface was impaired (Fig. 8 D). This parameter reflects the length over which cells are mechanically coupled, which is in the order of two to three cells for wt Ecad cells and only one cell for cis-Ecad cells. Altogether these observations show that cis-Ecad cell monolayers behave as a more fluid material, implying more rearrangement between individual cells. In agreement with single cell tracking and front displacement analysis, these observations demonstrate that when the cis-interface was impaired the apparent fluidity of the cell monolayer was increased as a direct consequence of decreased cell–cell cohesion, allowing more cell–cell rearrangements.

**Discussion**

It is proposed that oligomerization of cell adhesion molecules and their association to the underlying cytoskeleton provide to intercellular junctions the necessary strength to ensure tissue cohesion. To address this hypothesis, we determined the nanometric organization of Ecad in the plasma membrane and studied the consequences of the disruption of the cis-interface on single molecule organization up to the multicellular scale. We provide the first description of the nanometric distribution of Ecad in cellulo. Surprisingly, Ecad cis-interface is not required for AJ formation. However, its mutation strongly impaired the mechanical coupling of adhesion complexes to actin filaments affecting cell–cell contact strength. This reduced linkage to actin strongly affects cell movement coordination, leading to increased cell migration. Altogether, we show that Ecad oligomerization occurs in a cellular context and we provide direct evidence that it participates in mechanical anchoring of cadherin clusters to the cytoskeleton.

We used NP labeling to reveal an ordered nanometric organization of Ecad molecules at the cell membrane. The minimal distance between two Ecad was 8 nm. However, the mean distance (10.1 nm) between two adjacent Ecad is significantly higher. This may be explained by steric hindrance between functionalized NP, which are ~9 nm in diameter plus the flexible GFP-bearing arm. This flexible long arm may, however, favor efficient labeling of adjacent molecules. The fact that the
cis-interface disruption significantly decreased the fraction of NP spaced <10 nm, as well as their clustering, strongly suggests that we indeed visualized predicted Ecad arrays stabilized by trans- and cis-interactions in the crystal (Harrison et al., 2011). We frequently observed a few (three to six) particles arranged in straight or broken lines as well as clusters of particles organized in two-dimensional arrays corresponding to the predicted organization of arrays predicted by modeling (Wu et al., 2011). The size of the nanoclusters is below the estimate of cluster size made by Truong Quang et al. (2013) using super-resolution imaging in Drosophila melanogaster embryos, although the comparison is difficult. Indeed, on the one hand, each Ecad molecule within a cluster might not be decorated with a NP and, on the other hand, super-resolutive imaging did not reach nanometric resolution, allowing to address whether Ecad molecules are packed in oligomeric nanoclusters. However, our data fit very well with the estimate of the number of molecules per cluster as well as of the surface of these clusters obtained by Wu et al. (2015) in mammalian cells.

The absence of the cis-interface did not prevent the formation of AJIs in epithelial cells. This result recalls modeling data predicting the assembly of membrane-bound ligand–receptor complexes in microdomains (Weikl et al., 2002; Krobath et al., 2011; Bihr et al., 2012; Schmidt et al., 2012). In these models, an initial interaction brings locally the two membranes in close contact, increasing the probability of association of other freely diffusing ligands. This diffusion trap mechanism, similar to the one we proposed earlier for cadherin adhesions (Mège et al., 2006), leads to the buildup of densities of ligand–receptor complexes. Outside of these densities, the spacing of the two membranes superior to the length of the ligand–receptor complex prevents their growth from leading to the formation of discrete regularly spaced clusters. This remoteness has been attributed either to membrane thermal fluctuations or accumulations of membrane-bound glycoproteins. In these models, the increase in cooperatively brought by low energy cis-interactions (Wu et al., 2015) may be negligible. The absence of effect of cis-interface disruption on cadherin adhesion formation further supports this hypothesis. Indeed, cadherin adhesions formed on Ecad-Fc surfaces are found in areas of close contact between the plasma membrane and the substratum, whereas inter-cadherin adhesion areas are characterized by larger membrane-substratum spacing (Lambert et al., 2007). Although we cannot exclude that mutation of the cis-interface slightly destabilizes trans-interactions, the fact that cadherin adhesion and AJ formation are not affected does not advocate for this hypothesis. This is further supported by equilibrium analytical ultracentrifugation data showing that the $K_p$ for Ecad ectodomain dimerization is not affected by this mutation (Harrison et al., 2011).

Oligomerization through cis-interactions slightly stabilizes cadherins at cell–cell contacts, in confirmation of previous FRAP experiments performed with tailless cadherins (Harrison et al., 2011). This increase in stability of Ecad was associated with an increase in stability of α-catenin at contact sites, indicating that Ecad ectodomain stability drives the dynamics of its associated partners. The formation of ordered clusters by stabilizing α-catenin may thus favor the association of the clusters to actin filaments. The disruption of the cis-interface significantly increases the retrograde flow of the actin networks in the lamellipodia of cells plated on Ecad-Fc. As reported previously (Plestant et al., 2014), this may result from a weaker coupling of the actin retrograde flow to the adhesion complexes. In addition, the amplitude and the frequency of membrane protrusion are increased in cis-Ecad expressing cells, which could also result from a weaker association to actin. To directly evaluate the anchoring of Ecad clusters to actin we analyzed the mobility of Ecad-Fc–coated beads bound to the surface of transfected cells because the restriction of cadherin mobility in the membrane has been associated with its anchoring to actin (Sako et al., 1998; Lambert et al., 2002). The binding of Ecad-Fc beads was not affected by the oligomeric status of Ecad. In contrast, the bead mobility was significantly higher when the cis-interface was mutated. The displacement of the beads under force was greater for cis-Ecad than for wt Ecad, indicating a weaker
mechanical coupling of cellular Ecad to the underlying actin networks. Altogether these data indicate a contribution of oligomerization in the stiffening of the link between Ecad and actin. How can the stabilization of oligomeric clusters have such a strong influence on their anchoring to actin? The slight reduction in α-catenin accumulation may locally alter F-actin dynamics by regulating binding of nucleation and disassembly factors (Hansen et al., 2013). However, an obvious hypothesis is that the organization of Ecad molecules in clusters decreases the probability of rupturing their link to actin by a cooperative effect. Cadherin–catenin complexes within a nonorganized, fluid cluster would behave independently, preventing their cooperative binding to actin (Fig. 9).

The mechanical stabilization controlled by cadherin oligomerization had strong functional incidence on collective cell behavior. The disruption of the cis-interface increased by >70% of the speed of monolayer expansion. It was associated with a decrease of the order parameter. In other words, the monolayer composed of cis-Ecad cells behaves as a more fluid system. These changes in the fluidity of the monolayer can be directly attributed to a destabilization of the cell–cell contacts facilitating cell–cell contact reshaping and cell partner exchange. Thus, disruption of the cis-interface, albeit having a mild effect on junction formation, drastically alters cell cohesion. In conclusion, we show that cis-interactions stiffen Ecad molecule anchoring to the actin cytoskeleton, allowing cells to acquire more stable contacts and behave more collectively. Thus our results provide direct evidence that cadherin oligomerization indeed supplies the necessary strength to maintain tissue cohesion. Whether this pathway is modulated in vivo during collective cell migration, cell intercalation, wound healing, or tissue cohesion remains to be investigated.

Materials and methods

Cell culture and transient cell transfections
A431D Ecad-deficient epidermoid carcinoma cell line (Lewis et al., 1997; Hong et al., 2010) was grown in DMEM supplemented with 10% FBS, 2 mM l-glutamine, 100 IU of penicillin, and 100 µg/ml streptomycin at 37°C in 5% CO2. The plasmids encoding human wt Ecad, cis-Ecad, CAAX-GFP, α-E-catenin-mCherry, and LifeAct-Ruby thanks to the Amaxa Cell Line Nucleofector Kit T (program X-001; Lonza), resulting in >80% transfection efficiency. Assays were performed 24–48 h after transfection. Cells were always used at P < 20.

Protein extraction and coimmunoprecipitation
Proteins were extracted from 5–10 × 10⁶ transfected cells. Cells were rinsed in ice-cold PBS, detached with a cell scraper in cold PBS, and centrifuged at 1,000 rpm for 10 min at 4°C. Whole cell extracts were obtained by lysing cells into cold RIPA buffer (10 mM sodium phosphate buffer, pH 7.8, 60 mM NaCl, 1% Triton X-100, 10% glycerol, 25 mM β-glycerophosphate, 50 mM sodium fluoride, 2 mM sodium pyrophosphate, 1 mM orthovanadate, and protease inhibitor cocktail [Complete, Roche]). Lysates were agitated for 15 min at 4°C and cleared by centrifugation at 13,000 rpm for 10 min at 4°C, and protein concentration was estimated by micro-BCA assay (Thermo Fisher Scientific). GFP-tagged proteins were then coimmunoprecipitated using GFP-Trap according to the instructions of the manufacturer (ChromoTek). Protein samples (input and bound) were then subjected to SDS-PAGE and transferred on nitrocellulose membranes at 4°C. Membranes were then blocked with 5% nonfat milk and incubated with the corresponding primary antibody and then with IRDye-coupled secondary antibody (Rockland) against rabbit or mouse immunoglobulins, which were detected with Odyssey Imaging System (LI-COR Biosciences).

Immunocytochemistry
Cells were fixed for 12 min at room temperature using 4% paraformaldehyde in PBS, and then rinsed with PBS and permeabilized for 45 min in PBS supplemented with 1.5% BSA and 0.1% Triton X-100. Cells were then incubated for 1 h with mouse anti–α-catenin (BD) at 1:400 or rabbit anti–β-catenin (Sigma-Aldrich) at 1:400 dilution in PBS-BSA, rinsed, and incubated 1 h with anti–mouse or anti–rabbit Cy3-conjugated antibodies (Jackson ImmunoResearch Laboratories, Inc.) at 1:500 dilution. Preparations were mounted in Mowiol, 90% glycerol, and PBS. Images were taken with a microscope (DM6000; Leica) equipped with a 63× oil objective and Micromax charge coupled device (CCD) camera (Roper Scientific).

Cell membrane fluorescence analysis
Transfected cells were trypsinized, washed, fixed in 3.5% formaldehyde for 15 min, washed again, and imaged under flow using ImageStream X (Amnis) set with the 488-nm laser and 480–560 filter. Data were analyzed using the IDEAS software (Amnis). Regions corresponding to the cell membrane were extracted from bright field images. In brief, two masks were created by eroding and expanding the object by 4 pixels, respectively. The subtraction of the two masks corresponding to the cell membrane region was then applied on the fluorescence image to extract the cell membrane fluorescent intensity.
Data acquisition was performed for 300–1,200 cells for each condition and repeated three times.

**Ecad-Fc and fibronectin coating procedure**

Silanized glass coverslips or electron microscopy Formvar/carbon-coated gold grids (Oxford Instruments SAS) were coated with a human Ecad-human Fc chimera (R&D Systems) or fibronectin (EMD Millipore) as described previously (Gavard et al., 2004a). In brief, 5 µg of anti-human IgG antibodies (Jackson ImmunoResearch Laboratories, Inc.) in 130 µl Ca²⁺ Mg²⁺ PBS were left to adsorb overnight at 4°C. The surfaces were washed three times with PBS, and then 10 µg of Ecad-Fc chimera proteins in PBS were allowed to bind for 2–3 h at room temperature. After three washes, coverslips were blocked for 45 min with PBS and 2.5% BSA.

**Preparation of mix-capped gold NPs conjugated to GFP-NPs**

Gold NPs used in this study were obtained from British Biocell International Ltd (5-nm diameter). PEGylated alkanethiol, HS-EC₃₃-EG₃ (HS-PEG), was purchased from ProChimia Surfaces and Gold NPs used in this study were obtained from British Biocell Preparation of mix-capped gold NPs conjugated to GFP-NPs Silanized glass coverslips or electron microscopy Formvar/car-

**Conjugated NP size**

The actual measured diameter of the gold NPs was 6.9 ± 1.8 nm (n = 2,667 NPs from three independent images). They were surrounded by self-assembling monolayers of ~2.5 nm (Harder et al., 1998; Duchesne et al., 2008), which give an inferred diameter of the functionalized NP of ~9 nm. The length of the flexible Ni-trisNTA alkyl-PEG-thiol ligand (4.3 nm; Tinazli et al., 2005), of the nanobody itself, and of the GFP of both is in the range of 3 to 4 nm.

**TEM experiments**

For visualization of individual cadherin molecules, wt Ecad-GFP or cis-Ecad-GFP expressing cells were mechanically detached from the culture flask in the presence of PBS, 3.5 mM EDTA, and 2% BSA on ice and allowed to adhere for 2 h on Ecad-Fc– or fibronectin-coated electron microscopy grids, at 37°C in the absence of serum. After three washes with DME to remove the nonadherent cells, plasma membrane sheets on the electron microscopy grids were prepared (“rip-off” procedure) as described previously (Prior et al., 2003; Hancock and Prior, 2005) with some modifications. In brief, cells on grids were pressed onto a clean glass coverslip. The coverslip was turned over and 200 µl PBS was added quickly around the grids to separate them from the coverslip and to generate plasma membrane sheets on the grids (inner leaflet face up). Samples were then fixed with a solution of 0.1% glutaraldehyde (vol/vol) and 4% formaldehyde (wt/vol) for 10 min. The fixative was then quenched with three washes in 100 mM glycine. After three bashes in PBS, nonspecific sites were blocked for 10 min with PBS supplemented with 0.25% BSA. Grids were then incubated for 30 min with 3 nM anti-GFP-NP (or control uncoupled Ni-trisNTA-NP) in PBS and 0.25% BSA. After extensive washes with PBS and then deionized water, grids were treated with a mixture of 0.3% uranyl acetate (wt/vol) and 1.8% methylcellulose (wt/vol) for 10 min on ice. Grids were then individually picked up with homemade 5-mm-thick wire loops and left to dry overnight before storage. Preparations were digitally imaged using an 80-kV transmission electron microscope (CM100; Philips) equipped with an Orius CCD Camera (Gatan) or a 200-kV (Tecnai G2 T20 Spherea; FEI) transmission electron microscope equipped with a LaB6 electron source and a US4000 CCD camera (Gatan). For the analysis, 3-µm² images acquired with the CM100 were cropped down to 0.55-

**Oligomer analysis**

Centrifugation was performed for 90 min at 17,000 g at 4°C, and the supernatant, corresponding to free soluble anti-GFP-NP (uncoupled), was removed. The pellet was resuspended in 200 µl PBST and centrifuged again; a total of five cycles of centrifugation were performed. At the end, the pellet, which corresponds to the purified anti-GFP-NP conjugate (stoichiometry 1:1), was resuspended in PBS at a final concentration of 10 nM. The final concentration of conjugated NPs was calculated using ε₂₅₀nm of gold NPs given by the manufacturer.

Data acquisition was performed for 300–1,200 cells for each condition and repeated three times.
To interpret the statistical significance indicating clustering, a 99% confidence interval for $L(r) - r$ is generated using Monte Carlo simulations. Plots were generated for each individual image and $L(r) - r$ values were then standardized on the confidence interval for each image to allow comparison and averaging. Averaging was done for all images and specifically for images presenting a significant clustering between 0- to 300-nm radius. Values above 1 for the standardized $L(r) - r$ function indicate significant clustering (99% confidence interval) at the radius $r$ and no deviation ($0 < L(r) - r < 1$) indicates a random pattern.

Graphs and statistical analyses were performed using OriginPro 8.6 software. Kolmogorov-Smirnov nonparametric test was used to compare distribution and $\chi^2$ test was performed to compare proportions.

For analysis of intercellular junctions, transfected cells were deposited on a 12-well format cell culture insert at high density ( pore size of 0.4 µm; BD). When the monolayer was confluent, cells were fixed with 3% glutaraldehyde in phosphate buffer for 1 h at room temperature. Samples were kept at 4°C in 0.1 M phosphate buffer, pH 7.4, until further treatment and embedding in epon resin was performed. Thin section chromatography was performed and samples were stained with uranyl acetate and observed using a transmission electron microscope (1011; JEOL) equipped with an Orius CCD camera.

**FRAP**

FRAP was measured at 37°C on cells coelectroporated with wt Ecad-GFP or cis-Ecad-GFP and de-catenin-mCherry using a confocal microscope (TCS SP5; Leica) equipped with a 40x water immersion objective. After five prebleach scans (0.347 s), a rectangular region of interest (3.5 x 2.9 µm) was bleached and fluorescence recovery was acquired every 0.347 s (20 scans), then every 2 s (20 scans), and finally every 10 s (20 scans). The normalized recovery of fluorescence was expressed as a ratio of prebleach fluorescence rate after correction for photobleaching, as reported previously (Lambert et al., 2007). Fluorescence recovery in function of time were best fitted with a one-term exponential equation, allowing to extract a plateau value representing the fraction of diffusion-limited molecules (mobile fraction) and a recovery $t_{1/2}$ proportional to the apparent diffusion coefficient of diffusion-limited molecules (Thoumine et al., 2006). The mobile fraction and the $t_{1/2}$ were determined by fitting the normalized recovery curves using one-phase decay nonlinear regression function of the Prism 5.01 software (GraphPad Software).

**Collective cell migration assay**

A431D cells expressing wt Ecad or cis-Ecad were high density plated in 3.5-cm Petri dishes where a polydimethylsiloxane (PDMS) block was previously deposited to impose cells to grow on a restricted area of the dish. When cells reached confluence, the PDMS block was removed. Images were then acquired every 5 min during 24 h under a controlled temperature and CO2 environment (5% CO2 at 37°C; 10x objective; BioStation; Nikon). The surface occupied by the monolayer determined thanks to ImageJ was plotted as a function of time. Manual tracking of individual cells at the front or rear (at least four rows of cells away from the front) was performed with the MTrack plugin during the first 8 h. Individual trajectories were positioned on an orthonormal axis with the coordinates of the cell at $t_0 = (0, 0)$. The MSD was then extracted for each condition and plotted versus time. The direction persistence was calculated as the ratio of the cumulative distance over the Euclidian distance between the position of the cell at time 0 and its position at time $t$.

**Actin dynamics and lamellipodial activity measurement**

Cells coexpressing LifeAct-Ruby and wt Ecad-GFP or cis-Ecad-GFP were mechanically detached as described previously (Plestant et al., 2014) and plated in live cell imaging buffer (10 mM Hepes, pH 7.4, 128 mM NaCl, 6 mM KCl, 1 mM MgCl2, 2 mM CaCl2, 5.5 mM glucose, and 0.2% BSA) at low cell density ($< 5 \times 10^5$ cells/cm²) on Ecad-Fc–coated glass-bottom dishes for 2 h. Cells were then imaged every 500 ms for 3 min at 63x with a time-lapse confocal video microscope equipped with a Nipkow disk (spinning disk). Kymographs were made by generating time-lapse montages of a single line perpendicular to the cell edge for each frame of the video (ImageJ) along three lines normal to the free edge of each analyzed cell. Three flow rates were calculated for each kymograph (15 cells analyzed for each condition).

**Preparation of the Ecad-coated beads**

2.8 µm of magnetic protein A–coated beads (Dynabeads; Invitrogen) were coated with Ecad-hFc. In brief, 10 µl of the blury solution was washed three times and resuspended in 200 µl of 0.1 M borate buffer, pH 8.0, before 2 x 30-s sonication. Then, 50 µl of goat anti-human IgG Fc fragment (2.4 mg/ml; Jackson ImmunoResearch Laboratories, Inc.) was added and left to incubate overnight on a wheel at room temperature. Beads are then washed three times and resuspended in 200 µl PBS (Life technologies) before 2 x 30-s sonication. Then 5 µl of recombinant Ecad-Fc were added and left to incubate for 3 h on a rotating wheel at room temperature. Finally, beads were washed and resuspended in 1 ml PBS supplemented with 1% BSA. 50 µl of this Ecad-coated bead solution was added to cells grown on a 22 x 22-mm glass coverslip placed on a 3.5-cm Petri dish for 1 h. After extensive washes to remove unbound beads, the medium was changed for phenol red–free DMEM supplemented with 20 mM Hepes, pH 7.4.

For bead binding assays, Ecad-hFc– or hFc-coated beads were deposited on nontransfected and wt Ecad– or cis-Ecad–transfected cells, left to adhere for 1 h, and gently washed before fixation. Images were taken with a DM6000 microscope equipped with a 10x objective and a micromax CCD camera. The number of bound beads per squared millimeter was then manually scored.

**Magnetic tweezers assay**

The forces were locally applied on bead-bearing cells with magnetic tweezers made of an electromagnet and the superparamagnetic microbeads mentioned in Preparation of the Ecad-coated beads. The electromagnet was 816 turns of 0.5-mm copper wire coil surrounding a soft iron core 5 mm in diameter with a 30° cone-shaped tip. It was mounted on a micromanipulator (InjectMan NI2; Eppendorf) at a 45° vertical angle, and the tip initially aligned at 700 µm from the center of the observation zone. The current was provided by a home-made voltage-controlled current and a function generator (TG1010; TT Instruments). This function generator was directly controlled from the computer through a control card (USB1208HS; Measurement Computing). This function generator was mounted on a scope (DMIRB; Leica) equipped with a CCD camera (CoolSnap HQ2; Roper Scientific) through a 100x oil objective. Both the camera and the current in the coil were controlled by the µManager software (version 1.4.8). 2.8 µm of Ecad-Fc–coated magnetic beads were preincubated for 1 h on wt or cis-Ecad cells, and then the unbound beads were washed away. Applications of current ranging from 0.5 to 1.2 A drew the beads toward the tip. The force exerted by the electromagnet was calibrated by measuring the velocity of a bead moving through a viscous fluid (PDMS). Six steps of forces were applied, each step consisted of the following: at $t = 0$, current in the coil was set to 1.2 A and camera received signal to start acquiring images in the burst mode (frequency of ~13 frames/s) for 170 frames; the current was turned down and the camera was set to an acquiring rate of 2 frames/s for 114 s; the camera was then set to a 1-frame/s acquisition rate for an additional 125 s. Tracking of the bead position was done with Icy (Icy v1.4.3.5; Quantitative Image Analysis Unit, Institut Pasteur) using the Active Contour plugin.
PIV

PIV is an image correlation–based method usually used to obtain instantaneous velocity field measurements from the local displacements and heavily used in hydrodynamics. Images are divided into multiple interrogation subwindows. Each interrogation subwindow should contain sufficient numbers of tracers to enable comparison between the current time frame and the subsequent time frame. PIV analysis of monolayer movement (Petijean et al., 2010) was performed as described previously (Vedula et al., 2012) using MatPIV v. 1.6.1 package and implemented in Matlab (MathWorks). The analysis was done with 32 × 32-pixel (19 × 19-μm) interrogation windows with an overlap of 50% using the same size of initial migrating front (300 μm). Order parameter and correlation length were calculated using the formula previously described (Doxzen et al., 2013; Vedula et al., 2014).

Statistical analysis and curve fitting and image processing

Statistical analysis and curves fitting were performed with Prism 5.0 software. Differences were considered significant for p-values ≤ 0.05. Image processing was done in ImageJ (or Matlab when indicated), and then with Photoshop and Illustrator (Adobe).

Online supplemental material

Fig. S1 shows the recruitment of β-catenin at cell–cell contacts and binding of Ecad-Fc beads independent of Ecad cis-oligomerization. Fig. S2 shows the nanometric organization of wt and cis-Ecad-GFP at the cell membrane of A431D-transfected cells. Fig. S3 shows the nanometric organization of wt and cis-Ecad-GFP–transfected cells. Videos 1 and 2 show actin retrograde flow in wt Ecad-GFP expressing cell spread on Ecad-Fc (Video 1) and in cis-Ecad-GFP expressing cell spread on Ecad-Fc (Video 2). Videos 3 and 4 show Ecad-Fc magnetic bead displacement under force in wt Ecad-GFP (Video 3) and in cis-Ecad-GFP expressing cells (Video 4). Videos 5 and 6 show collective cell migration of wt Ecad-GFP (Video 5) and of cis-Ecad-GFP expressing cells (Video 6). Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201410111/DC1.

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The formation of ordered nanoclusters controls cadherin anchoring to actin and cell–cell contact fluidity

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In Figure 5 A, the authors noticed the inadvertent duplication of one kymograph in the wt Ecad and cis Ecad panels. Below is the amended version of the figure containing the correct cis Ecad kymograph.

The HTML and PDF versions of this article have been corrected. The error remains only in the print version.

Figure 5. Impairment of Ecad cis dimerization alters the actin dynamics of cells spread on Ecad-Fc. Cells coexpressing wt Ecad-GFP or cis-Ecad-GFP and LifeAct-Ruby were seeded on Ecad-Fc substrates for 2 h and then subjected to spinning disk live-cell imaging for 3 min at a frequency of one image per 500 ms. (A) Still Images of LifeActRuby distribution. Bar, 25 µm. The actin retrograde flow was quantified by kymograph analysis (yellow lines 1–3, 1 pixel width, perpendicular to the cell membrane in Ecad dense region). Superimposed on the kymographs are the means of actin retrograde flow speed for wt Ecad (n = 156 kymographs from 26 cells) and cis-Ecad (n = 192 kymographs from 32 cells) cells. The actin retrograde flow was significantly faster for cis-Ecad expressing cells than for cells expressing wt Ecad (P ≤ 0.0002, Student's t test). (B) Similar kymographs of the LifeActRuby signal extending on a longer time window revealed the cyclic protrusion of the edge of wt Ecad and cis-Ecad expressing cells. (C) Quantification of the maximum amplitude and frequency of membrane protrusions (mean values ± SEM; n = 100 kymographs from 26 cells for wt Ecad cells and n = 130 kymographs from 32 cells for cis-Ecad expressing cells). ****, P ≤ 0.0001; ***, P ≤ 0.005, Student's t test.