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# Early events in the assembly of E-cadherin adhesions

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ABSTRACT

E-cadherin is a calcium dependent cell adhesion molecule that is key to the organization of cells in the epithelial tissue. It is a multidomain, *trans*-membrane protein in which the extracellular domain forms the homotypic, adhesive interaction while the intracellular domain interacts with the actin cytoskeleton through the catenin family of adaptor proteins. A number of recent studies have provided novel insights into the mechanism of adhesion formation by this class of adhesion proteins. Here, we describe an updated view of the process of E-cadherin adhesion formation with an emphasis on the role of molecular mobility, clustering, and active cellular processes.

## 1. Introduction

Organization of cells into tissues is at the heart of multicellular organism development. In animals, cells in different tissues are organized primarily by the interaction of cell membrane-localized receptors with either receptors on other cells or ligands in the extracellular matrix. Cadherins are a large family ( > 100 members) of cell membrane-localized receptors with multiple extracellular cadherin repeats that are instrumental in the adhesion of cells in a variety of tissues. A subset of cadherins, known as classical cadherins, share a conserved cytoplasmic tail that binds catenins and links them with the actin cytoskeleton. Prominent among these are the epithelial (E)-, neuronal (N)-, and vascular endothelial (VE)-cadherins that forms adhesion between epithelial, neuronal and vascular endothelial cells, respectively. In this review we will be focusing primarily on E-cadherin.

A large number of studies have helped uncover the molecular structure of cadherins, which has been excellently reviewed elsewhere [1]. Briefly, the multidomain E-cadherin protein is expressed as a 120 kDa glycosylated protein with an extracellular domain containing five cadherin repeats (EC1-5), a trans-membrane domain, and a short unstructured intracellular domain (Fig. 1A) [1,2]. Each of the 5 EC repeats is ~110 amino acids long, and assume an immunoglobulin-like structure. Importantly, the structure of the extracellular domain is significantly altered by the binding of $Ca^{2+}$ ions at four sites in between the five EC repeats, from a floppy to a curved, rod-like rigid conformation [3–5]. The *trans*-membrane domain of E-cadherin contains a leucine-zipper motif that may promote its oligomerization [6], and by analogy to VE-cadherin may promote interaction with other transmembrane proteins [7]. The highly conserved intracellular domain [8] is ~150 amino acids long and binds adaptor proteins such as p120- and β-catenin, which in turn interact with a multitude of structural and signaling proteins, connecting E-cadherin with a variety of cellular machineries, most notably the actin-cytoskeleton [9–11]. Thus, cadherins not only allow cells in a tissue to adhere to each other but also organize signaling in the cells [12].

Here, we describe the mechanism of E-cadherin adhesion formation from a biophysical and mechanical perspective based on recent reports in the literature. We begin by asking how the extracellular domain of E-cadherin initiates adhesion between two apposing cells. We then ask how cellular protrusions such as filopodia and lamellipodia impinge on assembly of E-cadherin-mediated adhesion sites. Finally, we ask how mechanically sensitive adaptor proteins such as α-catenin could be impacted in these processes.

## 2. Molecular basis of E-cadherin adhesion

At the center of E-cadherin-mediated cell-cell adhesion is the homotypic *trans*-interaction formed by its extracellular domains present on apposing cell membranes (Fig. 1B). The *trans*-interaction involves a partial swapping of the N-terminal A* β strand present in the EC1 domains [13]. These swapped strands are stabilized by the docking of a Trp (W2) residue from one monomer into a pocket in the EC1 domain of the other monomer, and other salt bridge interactions formed by positively charged N-terminal residues [4,13–20].

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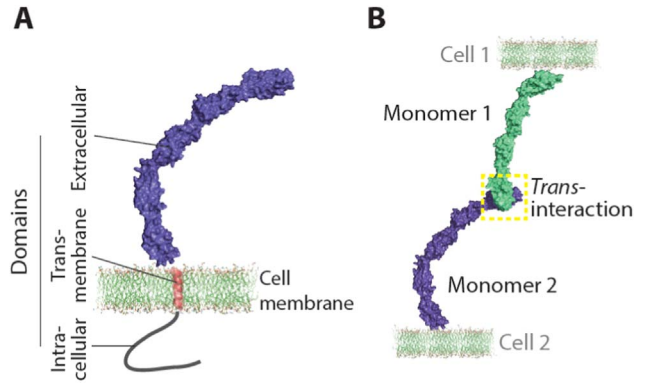


**Fig. 1. Schematic representation of E-cadherin.** (A) A cartoon showing the domain architecture of E-cadherin [28] on a membrane. (B) A cartoon showing a pair of E-cadherin molecules (extracellular domain) from apposing cells interacting with each. The extracellular domain cartoons were recreated using the Pymol (The PyMOL Molecular Graphics System, Version 1.8 Schrödinger, LLC).

Importantly, the *trans*-interaction is a relatively low affinity interaction with reported $K_d$ values ranging from 100 to 700 μM obtained from solution-based biochemical assays with the full-length versions of the extracellular domain [13,18,21,22], and involves a high activation energy barrier as determined from Nuclear Magnetic Resonance (NMR) assays [21,22]. Key to the E-cadherin homotypic *trans*-interaction is its high stability with a very slow rate of dimer-monomer interconversion [13].

The low affinity *trans*-interaction between E-cadherin extracellular domains is regulated in multiple ways at the molecular level. First, $Ca^{2+}$ ion binding to, and subsequent structural stabilization of the extracellular domain strongly affects homodimerization, a feature which has been successfully exploited in the traditional 'calcium switch assays' wherein the adhesion of cells is controlled by reversible exchange of $Ca^{2+}$ ions in the media [3,4,23]. Second, the final strand-swapped homodimer is formed from the conversion of an X-dimer intermediate that is formed during the initial encounter of the monomers [3,4,13,24]. The formation of the X-dimer intermediate has been implicated in the dynamics of both assembly and disassembly of cadherin adhesions based on the mutational studies of residues involved in this interaction [25]. Importantly, the X-dimer intermediate has been proposed to form a force-sensitive catch bond that could potentially be directly regulated by the mechanical tension in a cell-cell junction [26,27]. Third, the monomeric extracellular domain has been found to interact in *cis* via residues in the EC1 and EC2 domains that could potentially alter the adhesive function of cadherins by cooperatively regulating the *trans*-interaction [28,29]. Thus, it is clear that the process of cadherin adhesion formation could be regulated by different factors working at the molecular level.

## 3. Role of molecular mobility of E-cadherin in adhesion formation

While biochemical and biophysical studies have laid out the molecular basis of the *trans*-interaction between the extracellular domains of E-cadherin *in vitro*, the formation of cell-cell adhesions in cells is more complex. Basic molecular chemistry posits that E-cadherin molecules encounter each other by random physical translocation, and eventually form the *trans*-interacting dimers. The random translocation of E-cadherin molecules on the cell membrane will be dictated by laws of diffusion in two dimensions. However, single particle tracking experiments with E-cadherin in live cells have revealed three types of diffusion: unrestricted, free diffusion; restricted, corralled movements; and directed movements [30,31]. These different diffusion behaviors of E-cadherin could be attributed to its interaction with the actin and spectrin cytoskeleton underlying the cell membrane [32–35] or to the *cis*-interaction between non-ligated cadherin molecules [28]. Additionally, a general effect of the physical properties of the membrane on the mobility of E-cadherin, due to complex lipid compositions, cannot be ruled out. In fact, some members of the cadherin family have been found to be associated with 'lipid rafts', membrane structures that could drastically reduce their long-range molecular mobility [36–39]. Further, the mean diffusion coefficient of E-cadherin has been shown to be dependent on its oligomeric state [33,40] as well as the formation of adhesion [41].

The idea that the freely diffusing cadherin molecules encounter each other and form *trans*-interaction has been applied to *in silico* simulations, which led to the proposition of a 'diffusion trap' model, in which freely diffusing monomers from apposing membranes bind to each other and are eventually trapped at the initial point of contact between the two membranes [29]. Indeed, a reduction in the mobility of cadherin has often been utilized as a marker for stable adhesion formation [32,42]. However, this simplistic idea has been questioned by recent observations made in hybrid assays involving live cells and cadherin-functionalized supported lipid bilayers [43]. It is important to point out that the role of molecular mobility on the assembly of cadherins adhesions is difficult to assess purely from cells in culture. This is primarily due to the fact that any perturbation of molecular mobility on live cell membranes such as by changing the membrane composition or deleting the intracellular domain of E-cadherin will affect a multitude of processes in the cell. In contrast, a synthetic supported lipid bilayer allows control over the molecular mobility of proteins anchored to the bilayer by simply altering the constituent lipid molecules. The supported lipid bilayer-based assays revealed that cells rarely formed adhesion with bilayers displaying highly mobile E-cadherin molecules (diffusion coefficient, $D$ of $1.6 \pm 0.2$ μm$^2$/s). In contrast, cells readily formed adhesion with viscous bilayers that display low mobility E-cadherin molecules. These results indicate that it is the immobile fraction of E-cadherin molecules that are capable of forming *trans*-interacting homodimers, and serve as the seed for nucleating extended cadherin adhesions [43].

## 4. Nanoscale E-cadherin clusters as building blocks for cell adhesion formation

A very basic observation made with cadherin adhesions is the enrichment of *trans*-interacting homodimers at the cell-cell interface [41]. This is not surprising as the local concentration of cadherin molecules at the adhesion interface is a sum of the interacting (ligated) dimers as well as non-interacting (non-ligated) monomers. The *cis*-interaction between the extracellular domains of cadherin [28] could further enhance the local enrichment of cadherins, as suggested by *in silico* molecular dynamics studies [29]. However, unlike the uniform distribution of *trans*-interacting cadherin molecules seen between two adhering vesicles *in vitro* [28], they are not uniformly distributed at the adhesive interface of living cells. Instead, they are organized into micron-scale clusters [41,43–49]. These micron-scale clusters have been further resolved in recent super-resolution (3 dimensional-Stochastic Optical Reconstitution Microscopy; 3D-STORM) studies into nanometer-scale clusters that reach molecular densities close to that seen in crystals [50,51]. Interestingly, molecular densities in these nanometer-scale clusters of E-cadherin are regulated by the *cis*-interaction between the extracellular domains, while the size of these clusters are limited by the underlying actin cytoskeleton through interaction with the intracellular domain [50]. The nanometer-scale clustering behavior of *trans*-interacting cadherins observed in the super-resolution studies is further corroborated by the Fluorescence Correlation Spectroscopy (FCS) measurements in the hybrid live cell-supported lipid bilayer experiments [43]. These experiments revealed a marked reduction in the diffusion coefficient as well as formation of large oligomers of E-cadherin at the adhesive interface [43].

One of the key observations, however, made with the super-

resolution imaging of epithelial cells in monolayer culture is the presence of the smaller clusters of E-cadherin molecules on the non-adhering membranes [50]. These clusters were observed irrespective of the *trans*- or the *cis*-interaction between the extracellular domains. The fact that a construct consisting of just the intracellular domain of E-cadherin was also found to be clustered on the cell membrane points to the role of the actin cytoskeleton in the formation of these clusters. In agreement with these results, adhesion-independent cadherin clusters have also been found on the surface of early *Caenorhabditis elegans* embryos, where they play a role in regulating stability and contractility of the actomyosin cortex [52]. This implies that clustered E-cadherin molecules form the building blocks of cadherin-mediated cell-cell junctions, instead of individual molecules.

## 5. Role of cellular protrusions in E-cadherin adhesion formation

Given that the immobile cadherin molecules are the ones that are functional with respect to adhesion formation, it becomes imperative to elucidate the basis of their physical collision leading to the formation of the *trans*-interacting homodimers. It has been established that cadherin junction formation is an active process requiring cellular energy expenditure. A reduction in the cellular ATP levels by treating cells with oxidative phosphorylation inhibitor antimycin A and glycolysis inhibitor 2′-deoxy glucose abrogates cell-cell adhesion formation [43]. Further, inhibition of myosin II by treating cells with blebbistatin results in the loss of cell-cell adhesion [53–55].

Experiments with cells on culture dishes revealed that the initial encounter of adhesive cells results in the formation of multiple finger-like, filopodial projections containing cadherin clusters and filamentous actin [44]. Similar structures were also observed in nematode, fly and mouse embryos [56–58]. A clear picture on the role of cellular filopodia in cadherin clustering came from reconstitution experiments with cadherin functionalized supported lipid bilayers and live cells [43]. Cells forming adhesion on supported lipid bilayers displayed extension of filopodia on the bilayer, and clustering of cadherin molecules coincided with the retraction of filopodia as seen from epi-fluorescence and Reflection Interference Contrast Microscopy (RICM) imaging. Indeed, abrogation of filopodia formation by the pharmacological inhibition of the Rho family GTPase, Cdc42, which is required for filopodia formation [59], resulted in the loss of cadherin clustering and adhesion formation. These experiments also confirmed the requirement for cellular actomyosin tension and energy in the form of ATP. A different set of experiments with solid, N-cadherin-functionalized substrates pointed out a role for cellular lamellipodia in the formation of focal adhesion-like cadherin clusters [60,61]. Similar to the experiments with supported lipid bilayers, cadherin clustering on the solid substrates strongly associated with the actin polymerization in the cell periphery. These observations clearly established that cadherin adhesion formation is an active process, and actin polymerization is a key actuator of cadherin clustering. Thus, unlike many other receptor-ligand interactions in the cell that are purely driven by biochemical affinities, E-cadherin-mediated cell-cell adhesion formation is an active process requiring the interaction of its intracellular domain with the cellular actin cytoskeleton.

## 6. Mechanical signal transduction in early E-cadherin adhesion

The short intracellular domain of E-cadherin forms a high affinity interaction with β-catenin that extends across almost two-thirds of the length of the intracellular domain [62]. Such an extended interaction is allowed due to the lack of any specific structure of the intracellular domain. Additionally, the modular nature of the interaction interface could allow post-translational regulation of the intracellular domain-β-catenin complex, and it enables the withstanding of mechanical tension developed at cell-cell junctions [63,64]. β-catenin, in turn, interacts with α-catenin, which undergoes a force-induced 'closed' to 'open' conformational transition, based on results obtained with a conformation sensitive antibody staining and truncation mutation experiments [65]. This has been confirmed with single molecule in vitro experiments, either with the isolated α-catenin protein [66] or with a trimeric complex of E-cadherin, β-catenin and α-catenin [67]. This structural transition is critical for it to be able to bind to both F-actin as well as vinculin, a protein that is well known for its role in mechanical signal transduction at integrin-based focal adhesions [68]. This conformational change and the associated interaction of F-actin and vinculin has been proposed to allow mechanical tension sensing at cadherin adhesions [65,66].

While this appears to be a convincing hypothesis, it generates the question as to what causes α-catenin to assume the 'open', activated conformation to bind F-actin at the first place? *In vitro* reconstitution experiments with purified proteins have shed some light on this [67]. The interaction of α-catenin with F-actin is enhanced upon application of force on the actin filament. Importantly, this binding was found to be highly cooperative i.e. once one α-catenin binds to the F-actin, the probability of binding of other α-catenin is much higher. Thus, it is likely that a small fraction of α-catenin molecules is present in the 'open' conformation at any given instance, and these molecules are poised to bind F-actin. Binding of these molecules then increases the probability of binding of other molecules due to physical proximity.

Hybrid live cell-supported lipid bilayer experiments revealed that α-catenin is activated upon filopodia retraction-mediated nucleation and clustering of E-cadherin into micron-scale clusters, as assessed by the binding of the vinculin head domain (a vinculin construct lacking the C-terminal, inhibitory domain) and the conformation specific antibody, α18 [49]. The role of this micron-scale clustering on α-catenin activation was investigated by utilizing the 'spatial mutation' technology wherein physical barriers created on the glass substrate restrict the mobility of proteins on the bilayer [69]. Abrogation of the micron-scale clustering of E-cadherin resulted in an inhibition of the conformational activation of α-catenin [49]. Importantly, α-catenin was found to be stably activated upon micron-scale clustering of E-cadherin, and did not return to the 'closed', inactive state upon reduction in the actomyosin tension in cells as expected from the reversible conformational transition observed in vitro [66,67]. The later is indicative of secondary events such as a phosphorylation of residues in the linker region between the central and C-terminal domain [70]. Alternatively, α-catenin could be stabilized in the active state due to the stable binding of a protein that interacts with the central, regulatory domain such as the actin crosslinking protein, α-actinin [71].

In addition to α-catenin, some studies have implicated vinculin, which is homologous to α-catenin, in the mechanical signal transduction at cadherin adhesions [65,72–74]. Similar to α-catenin, vinculin also shows a 'closed' to 'open' conformational activation leading to accessibility of its F-actin as well as α-catenin binding sites [75]. Super-resolution microscopy revealed that following activation, vinculin can extend ~30 nm to link the cadherin-catenin complex with F-actin [73]. Importantly, vinculin conformational activation requires both tension and tyrosine phosphorylation, regulated by Abl kinase and PTP1B phosphatase [73]. While some studies have suggested that vinculin can directly interact with β-catenin [76], a more generally accepted view is that it is recruited to cadherin adhesions via α-catenin [73]. It is clear from biochemical studies that for vinculin to bind α-catenin at the sites of adhesion, both α-catenin as well as vinculin need to be present in the 'open', active conformation. Indeed, in cells vinculin was shown to associate only with the subset of adhesions that are under tension [65], and vinculin associated only with the peripheral cadherin clusters in adhesions reconstituted on supported lipid bilayers [49]. The fact that α-catenin-bound vinculin could be further stretched under conditions of increased tension between adhering cells and subsequently, F-actin

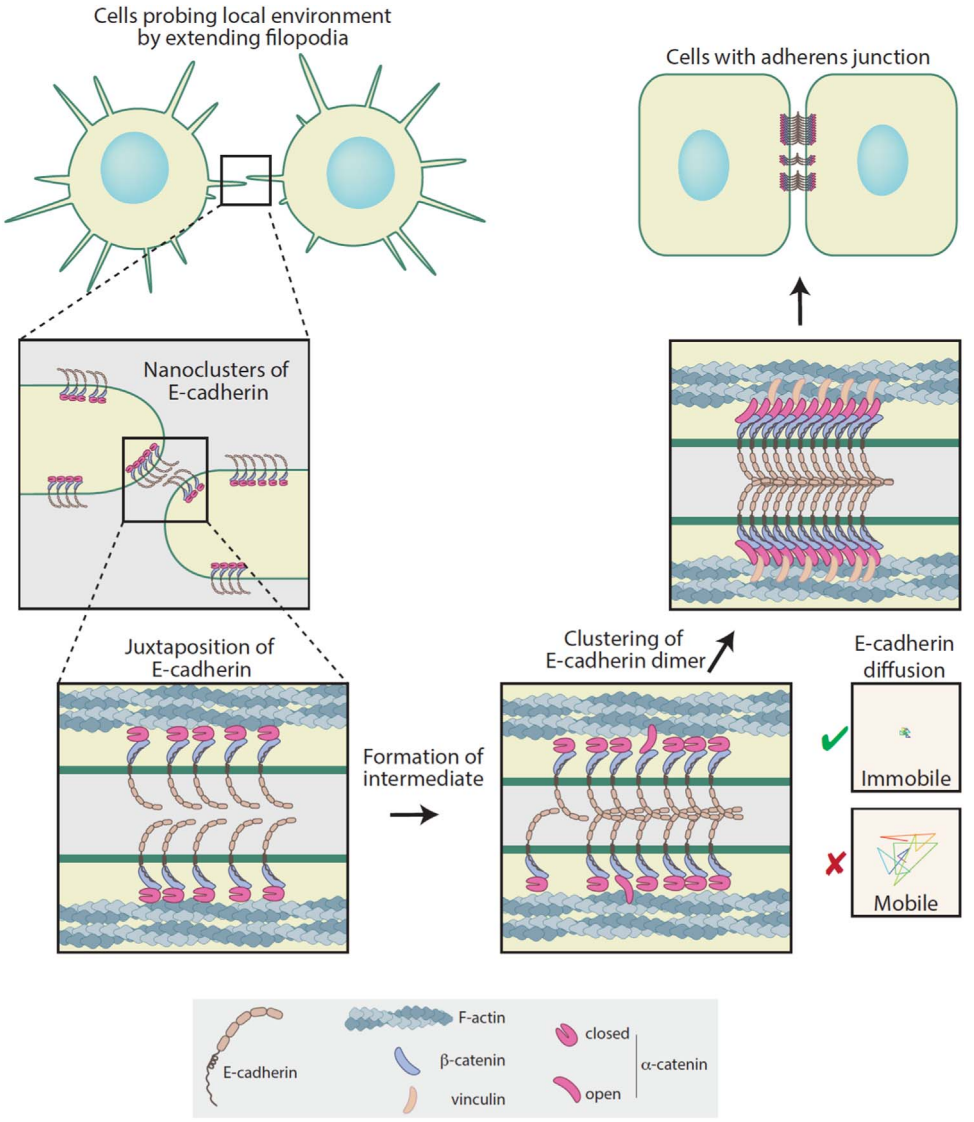


**Fig. 2. Schematic illustration of the process of E-cadherin adhesion assembly.** Cells explore their environment by extending protrusions such as filopodia. A zoomed in view of two filopodia showing the nanoscale clusters of E-cadherin. The assembly of E-cadherin adhesion starts with the close juxtaposition of membranes of the cells. For instance, filopodia bearing E-cadherin molecules may randomly come in close proximity. This probably leads to the formation of the intermediate, X-dimer structure, which then results in the formation of the final, strand-swapped dimer. Note that the probability of completion of these steps will be enhanced due to the presence of the nanoscale clusters of E-cadherin. Formation of the strand-swapped dimer is coincident with the micron-scale clustering of a large number of E-cadherin molecules, a process during which α-catenin is activated from the 'closed' to the 'open' state. These are then organized into apical and lateral clusters at the interface between the two apposing cells.

could be recruited to these tensed adhesions [74] indicates that the simple one step 'open' to 'close' transformation may not be sufficient. Instead, a multi-step conformational transition could be at play under conditions of increased cellular tension, as seen in single molecule force spectroscopy experiments [66]. Similar multi-step structural transitions have been envisaged for α-catenin at cadherin clusters [49,77]. Taken together, it appears that during the process of cadherin cluster formation, conformational activation of α-catenin allows binding of vinculin to the clusters. This association of vinculin with α-catenin may enhance the effect of the actin cytoskeleton on cadherin adhesion formation, as it does in the case of mature junctions upon increase in the junctional tension.

## 7. Conclusions

Given its essential role in the development and maintenance of tissue structures in multicellular organisms, cadherin adhesions have been the focus of a multitude of studies for a long time now. While these studies have laid out the fundamentals of cadherin adhesions, development of novel experiments and assays including the supported lipid bilayer system and super-resolution microscopy techniques have provided deeper insights into the mechanism of cadherin adhesion formation. Based on these advances, a picture of the mechanism of E-cadherin adhesion formation emerges (Fig. 2). Cells explore their microenvironment with actin polymerization-based processes such as filopodia or lamellipodia. These cellular processes bear nanometer scale clusters of E-cadherin molecules, which are brought in collision with each other by the physical movement of the cellular processes. Once in contact, these cadherin molecules form the intermediate X-dimer structure. Retraction of the cellular processes then provides the necessary force for the formation of long-lived catch-bonds between the interacting cadherin molecules. The high affinity, long-lived X-dimer is then converted to the final strand-swapped dimer during the retraction process. Concurrently, the cluster size as well as density of cadherin molecules grows during this initial phase of adhesion formation. This growth of the clusters leading to the formation of the micron scale clusters is associated with the conformational activation of α-catenin, which allows recruitment of vinculin to these clusters. In the

future, we envisage that a number of these steps in cadherin adhesion formation such as the X-dimer formation or α-catenin activation will be visualized in real-time in live cell assays.

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