Vinculin-dependent Cadherin mechanosensing regulates efficient epithelial barrier formation

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Summary

Proper regulation of the formation and stabilization of epithelial cell–cell adhesion is crucial in embryonic morphogenesis and tissue repair processes. Defects in this process lead to organ malformation and defective epithelial barrier function. A combination of chemical and mechanical cues is used by cells to drive this process. We have investigated the role of the actomyosin cytoskeleton and its connection to cell–cell junction complexes in the formation of an epithelial barrier in MDCK cells. We find that the E-cadherin complex is sufficient to mediate a functional link between cell–cell contacts and the actomyosin cytoskeleton. This link involves the actin binding capacity of α-catenin and the recruitment of the mechanosensitive protein Vinculin to tensile, punctate cell–cell junctions that connect to radial F-actin bundles, which we name Focal Adherens Junctions (FAJ). When cell–cell adhesions mature, these FAJs disappear and linear junctions are formed that do not contain Vinculin. The rapid phase of barrier establishment (as measured by Trans Epithelial Electrical Resistance (TER)) correlates with the presence of FAJs. Moreover, the rate of barrier establishment is delayed when actomyosin contraction is blocked or when Vinculin recruitment to the Cadherin complex is prevented. Enhanced presence of Vinculin increases the rate of barrier formation. We conclude that E-cadherin-based FAJs connect forming cell–cell adhesions to the contractile actomyosin cytoskeleton. These specialized junctions are sites of Cadherin mechanosensing, which, through the recruitment of Vinculin, is a driving force in epithelial barrier formation.

Key words: E-cadherin, Vinculin, Actomyosin, Mechanotransduction, Junction formation, Cell–cell adhesion

Introduction

Multicellular epithelial tissues line all cavities in an organism and they serve as a selective permeability barrier. In polarized epithelial cells, junctions are organized in an adhesion belt in which they are connected to the actomyosin cytoskeleton. Tight Junctions (TJs) are located most apically, and serve as a selective barrier that limits water, solutes and immune cells from passing between cells. The tightness of the barrier is dependent on the expression of different combinations of transmembrane proteins in the TJs, such as Claudins, Occludin, and Junctional Adhesion Molecule (JAM) (Turksen and Troy, 2004). Adherens Junctions (AJs) and Nectin-based Junctions (NJs) are also part of the adhesion belt, and are located immediately below the TJs. AJs and NJs are also found outside the adhesion belt in both epithelial and non-epithelial cells, in a more discontinuous pattern (Niessen, 2007).

During tissues remodeling, the barrier function must remain intact and is therefore tightly regulated. The junctional complexes that connect to the actomyosin cytoskeleton (AJs, NJs, and TJs) are the targets of many signaling pathways that regulate tissue remodeling. One of the most used model systems for junction remodeling is the formation of epithelial cell–cell junctions in tissue culture. This entails a complex interplay between actomyosin and junctional complexes. As lamellipodia of two cells come into contact with each other, small adhesive puncta are formed, with thin actin bundles extending from the circumferential actin belt to these puncta. As the contact extends, more adhesive puncta arise, which merge and are still connected to radial actin bundles. These actin bundles are proposed to stabilize the adhesive puncta. Finally, the puncta mature into cell–cell junctions, and actin is remodeled back into the circumferential belt (Adams and Nelson, 1998; Meng and Takeichi, 2009). Although mainly studied in 2D cell culture models, it is likely that junction formation *in vivo* (for instance during dorsal closure, angiogenesis, immune responses, wound healing and tumorigenesis) is governed by the same basic principles (Cavey and Lecuit, 2009).

Engagement of cell–cell junction receptors activates several signaling pathways that regulate actin conformation. For instance, nectin–nectin engagement results in activation of c-Src, Rap1, Cdc42, and Rac small GTPases (Ogita et al., 2010; Takai et al., 2008). Engagement of Cadherin adhesion induces Myosin II activation, which in turn promotes the accumulation of Cadherins at sites of cell–cell adhesion (Shewan et al., 2005). Cadherin-induced activation of PI3-kinase and Rac1 leads to membrane and actin dynamics to further stimulate junction formation along the membrane (Noren et al., 2001). Furthermore, Cadherin adhesion leads to recruitment and activation of several actin regulators such as the Arp2/3 complex (Kovacs et al., 2002), cortactin (Helwani et al., 2004), N-WASP (Kovacs et al., 2011), formin (Kobielak et al., 2004) and Ena/VASP (Vasioukhin et al., 2000). Thus, much is known about the regulation of actin dynamics downstream of cell–cell junction formation. Conversely, however, the conformation of the actin cytoskeleton also influences cell–cell adhesion complexes. For example, perturbing actomyosin contractility strongly affects cell–cell adhesion formation and maturation (Angres et al., 1996; de Rooij et al., 2005; Gloushankova et al., 1998; Lambert et al., 2007; Miyake et al., 2006; Shewan et al., 2005), indicating that actomyosin based forces play a promoting or stabilizing role in this process. Exactly how physical forces from contractile actomyosin are transmitted to cell–cell junctions and by which mechanisms this influences their formation is not well understood.

Recently, we showed by magnetic twisting cytometry (MTC) that the E-cadherin complex is a mechanosensor that directly responds to forces exerted on it and that the actin-binding protein Vinculin is important in this process (le Duc et al., 2010). Concomitantly, it was shown that in apical Adherens Junctions force-dependent stretching of the E-cadherin-actin linker α-catenin results in recruitment of Vinculin to these junctions (Yonemura et al., 2010).

During junction formation it is not clear which of the different adhesion complexes forms a functional link with actomyosin. Early experiments showed that the E-cadherin complex is a master regulator of cell–cell adhesion, because the formation of all junctions can be inhibited by E-cadherin-blocking antibodies (Gumbiner et al., 1988). However, Nectins are also crucial for the formation of all other cell–cell junctions (Honda et al., 2003; Ikeda et al., 1999; Sakisaka et al., 2007). As TJ complexes form only after Nectin and Cadherin junctions have formed it is not likely that these complexes are crucial in the actin-dependent initial formation of cell–cell adhesion. Nevertheless, the TJ complex actin linker proteins Zonula Occludens-1 (ZO-1) and ZO-2 have been found in early junctions (Ooshio et al., 2010) preceding the formation of apical TJs (Fanning and Anderson, 2009).

For Cadherin–actin linkage, α-catenin is crucial, but additional proteins, including EPLIN and Vinculin could be needed as well (Abe and Takeichi, 2008; Watabe-Uchida et al., 1998). The latter two seem to be involved in specific phases of junction dynamics as their presence in junctions is not ubiquitous (le Duc et al., 2010; Miyake et al., 2006; Taguchi et al., 2011). For Nectin–actin linkage, Afadin is crucial (Takahashi et al., 1999) and for TJs the ZO proteins are vital (Fanning et al., 1998; Itoh et al., 1999). Complicating the understanding of early junction formation, Afadin, α-catenin and ZO proteins can interact among each other and a number of proteins, including α-actinin, LMO7 (Ooshio et al., 2004), ADIP (Asada et al., 2003), and ponsin (Ikeda et al., 1999), can make additional links by binding two of these proteins simultaneously. Because these actin-connected cell–cell junction complexes are not well separated in space during junction formation and because of the complicated interaction profile between their intracellular components it is not clear whether they can be functionally separated during junction formation.

In this study, we investigated the regulatory role of actomyosin during epithelial barrier formation in calcium switch assays and we investigated which cell–cell junction complexes mediate the functional link to actomyosin. We find that actomyosin-based force promotes epithelial barrier formation independent of the structural supportive role of F-actin. This force-dependent effect is mediated by α-catenin and Vinculin in the Cadherin complex specifically. Radial actin-contacted Focal Adherens Junctions (FAJ) are formed rapidly upon calcium, in which Vinculin responds to force on the E-cadherin complex to induce force-dependent reinforcement of cell–cell adhesion, resulting in accelerated barrier formation.

Results

E-cadherin and F-actin are inter-dependently remodeled upon calcium switch

To pinpoint the proteins most likely involved in connecting cell–cell junctions to F-actin in junction formation, we subjected MDCK cells to a calcium switch assay to induce the formation of Cadherin based adhesions. We fixed the cells at different time-points and performed immunofluoresence (IF) staining for key proteins from AJs, TJs and NJs including the main actin-binding proteins in these complexes. In MDCK cells in low calcium conditions (20 μM), no members of the Cadherin complex are present at cell–cell contact sites, and the actin cytoskeleton is present in bundles that run parallel to the cell–cell contacts (Fig. 1A). Surprisingly, in this experimental setup, Tight Junction- and Nectin Junction complexes were visible at many cell–cell contact sites in low calcium as shown by the presence of Occludin (TJs) and Afadin (NJs) (Fig. 1A). We considered the possibility that the low concentration of FBS used in this setup induced unusual responses. Nevertheless, increasing the serum content to 5%, which has been used in several previous calcium switch studies, did not affect the localization of either Afadin, Occludin, E-cadherin or actin (supplementary material Fig. S1). After the calcium switch (by adding 2 mM Calcium Chloride) Cadherin complex members appeared in small structures that orientated perpendicular to the plane of cell–cell contact. The actin cytoskeleton also remodeled significantly as the parallel bundles disappeared and radial bundles now extended to the cell–cell contact zone terminating in the Cadherin positive structures (Fig. 1B). This confirms earlier studies showing that the formation of Cadherin-dependent cell–cell junctions is a driving force in the remodeling of actin through the recruitment of actin regulating proteins like VASP, formins, cortactin, ARP2/3 and α-actinin (DeMali and Burridge, 2003; Helwani et al., 2004; Kobielak et al., 2004; Ooshio et al., 2004; Tang and Brieher, 2012; Vasioukhin et al., 2000). Conversely, the altered cytoskeleton appears to have profound effects on the morphology of cell–cell junctions in general, because also the pre-assembled NJ and TJ complexes re-orientated into perpendicular junctions upon calcium (Fig. 1B).

As the junctions matured, the AJ, TJ and NJ complexes lost their perpendicular orientation and changed into a linear junction, concomitant with a re-organization of the actin cytoskeleton to

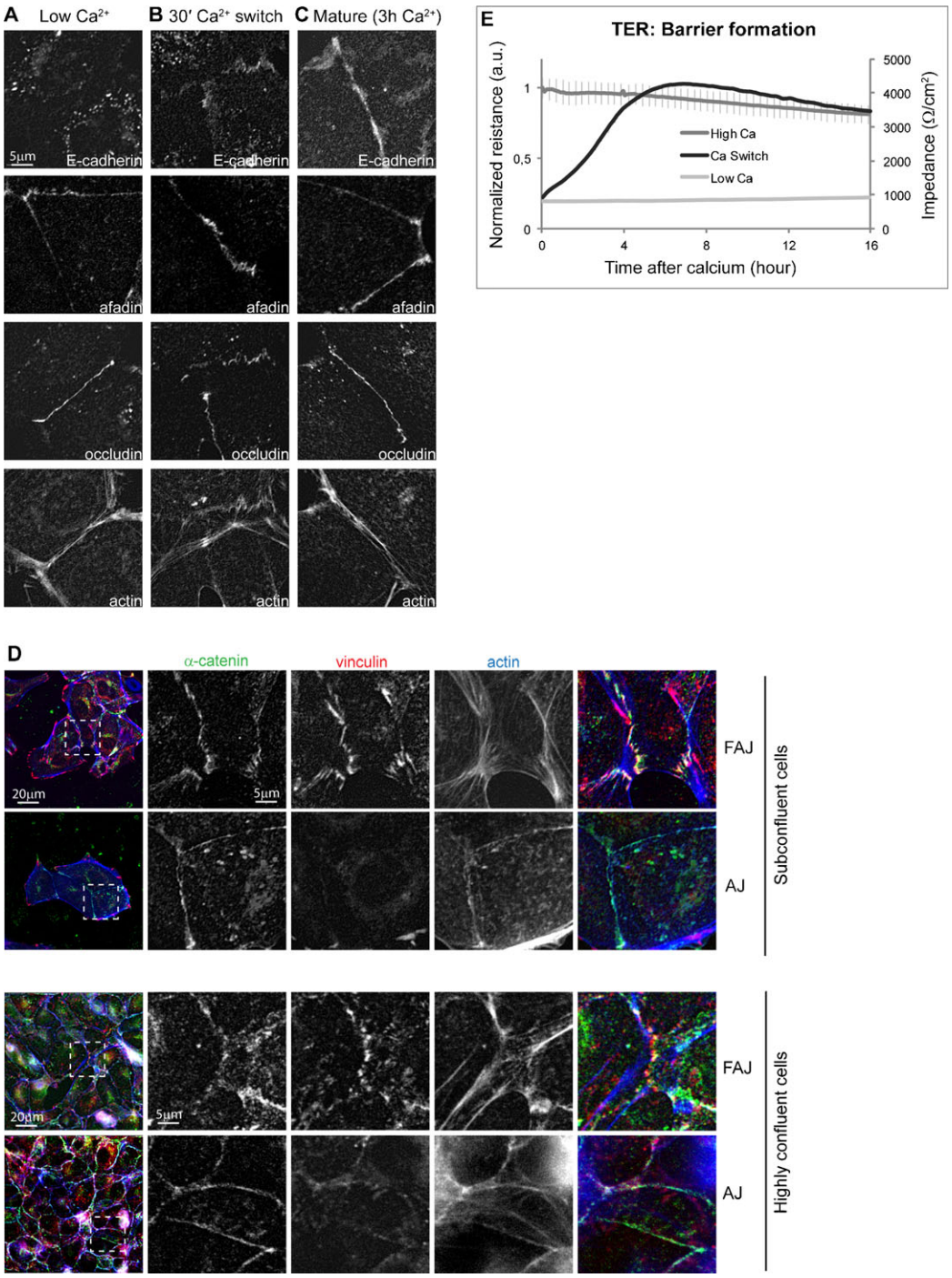

Fig. 1. E-cadherin and actin are inter-dependently remodeled upon calcium switch. (**A–C**) IF images of AJ (E-cadherin), NJ (Afadin), TJ (Occludin) markers and actin (from the same cells as the Occludin staining) during a calcium switch in MDCK cells. (**D**) IF images of FAJs and mature AJs after a calcium switch (30 min. for FAJs, 3 hours for AJs) stained for α-catenin (green) F-actin (blue) and Vinculin (red) in subconfluent and highly confluent conditions. (**E**) TER measurement of MDCK cells seeded in low calcium medium (light grey line), normal calcium medium (dark grey line) or during a calcium switch (black line). Like in all further TER experiments in this study, values normalized to the maximum value of the control sample are shown on the left Y-axis. Actual values in Ohm/cm^2 are shown on the right Y-axis for comparison. Error bars indicate standard error, $n>3$. Scale bar = 5 µm or 20 µm as indicated.

form bundles running parallel to them (Fig. 1C). The actin-driven regulation of junction morphology in the early phases after calcium strongly indicates that a physical link exists between the newly formed radial actin bundles and cell–cell junction complexes. Indeed, there is visible overlap between the radial actin bundles and adhesion proteins (Fig. 1B,D) and the actin binding and junctional protein Vinculin is present in the perpendicular nascent adhesions (Fig. 1D). These structures are very much like the Focal Adherens Junctions (FAJs) we characterized recently in endothelial cells (Huveneers et al., 2012). In that paper, we proposed the name FAJ to emphasize their distinction from stable AJs and their analogy to Focal

Adhesions; typical punctate morphology, connection to radial actin bundles and dependence on cytoskeletal tension. Structures like these have been described under various names in literature (Adams et al., 1998); adhesions zippers described during junction formation in sparse epithelial cells (Vasioukhin et al., 2000); spot-like Adherens Junctions in epithelial cells and fibroblasts (Yonemura et al., 1995); punctate Adherens Junctions in epithelial cells in wound healing (Taguchi et al., 2011). It is very likely that all of these structures are very similar in nature to the FAJs we describe here and in HUVECs.

The fact that TJ and NJ complexes did assemble at cell–cell contacts in low calcium led us to determine Trans Epithelial Electrical Resistance (TER), as a measure for epithelial barrier function of the MDCK monolayer. A confluent monolayer in low calcium showed very low barrier compared to a confluent monolayer grown in normal calcium conditions (Fig. 1E), indicating that although the TJ protein Occludin is present at sites of cell–cell contact (Fig. 1A), the monolayer has no barrier function. After the addition of calcium, a barrier function was gradually established (Fig. 1E). The phase of most rapid barrier increase (between 1 and 4 hours post calcium) coincided remarkably with the phase in which FAJs are observed. The number of FAJs after 4 hours of calcium is very low, whereas barrier function is then maximal. We confirmed that also in the highly confluent conditions that cells are seeded in for TER measurements, FAJs are formed in the first few hours after calcium switching that contain high levels of Vinculin, while Vinculin levels became very low when junctions matured into linear Adherens Junctions (Fig. 1D). Also junctional localization of Occludin and Afadin under low calcium conditions was clearly observed in these conditions (supplementary material Fig. S2). Finally, raising the serum concentration to 5%, did not strongly affect the speed of barrier formation (supplementary material Fig. S3).

In conclusion, upon the induction of E-cadherin adhesion by means of a calcium switch, the actin cytoskeleton is remodeled to form radial bundles, which in turn determine the organization of cell–cell junction complexes into perpendicular orientated FAJs. In this transient phase of active actin and junction remodeling the fastest increase in epithelial barrier function takes place.

Coupling of F-actin to cell–cell junctions requires α-catenin and is necessary for the establishment of the barrier

To understand the requirement of an intact actin cytoskeleton for both the formation and maintenance of the barrier, we treated MDCK cells with Cytochalasin D (CytoD), to induce the breakdown of actin filaments. This resulted in a failure to form a barrier after a calcium switch (Fig. 2A, dashed green line), or in a disruption of the barrier function of a pre-existing monolayer (Fig. 2A, dark green line). As expected from its crucial role in E-cadherin adhesion, MDCK cells with a constitutive α-catenin knockdown to levels below 10% (supplementary material Fig. S4) failed to form cell–cell junctions and consequently did not build any barrier function upon calcium (Fig. 2D, red line). We used this α-catenin-depleted MDCK cell line to further investigate the domains and interactions of α-catenin that are needed for efficient epithelial barrier formation. First of all, we tested an α-catenin mutant that lacks the C-terminal α-helix that is essential for F-actin binding (α-catenin-1-848) (Pokutta et al., 2002) (Fig. 2B). Contrary to rescues with α-catenin WT, this mutant, expressed at relatively high levels (Fig. 2C), could not

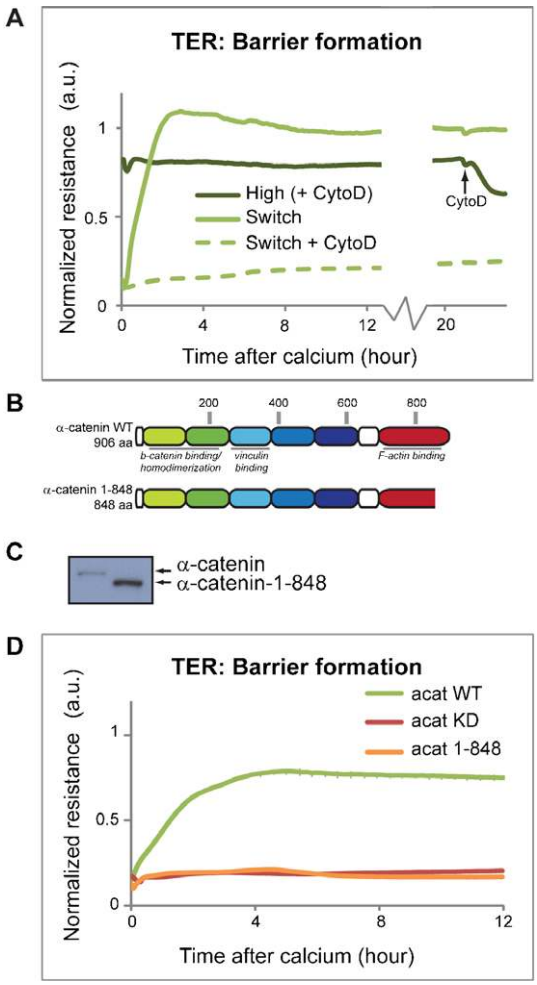

Fig. 2. Coupling of actin to cell–cell junctions requires α-catenin and is necessary for the establishment of the barrier. (**A**) Effect of Cytochalasin D on barrier formation in MDCK cells. CytoD was added during a calcium switch (dashed green line) or to a pre-existing monolayer (dark green line). Error bars indicate standard error, $n>3$. (**B**) Schematic representation of α-catenin-WT construct and α-catenin-1-848 mutant lacking the C-terminal F-actin binding site. (**C**) Expression levels of α-catenin-WT or α-catenin-1-848 in α-catenin depleted MDCK cells assessed by Western Blotting with α-catenin specific antibodies. (**D**) TER measurement in α-catenin depleted MDCK cells (red line), or the same cells rescued with α-catenin-WT (green line) or α-catenin-1-848 (orange line). Error bars indicate standard error, $n>3$.

rescue the formation of the barrier even under the high density plating conditions of the TER measurement (Fig. 2D, orange line). These results show that both intact F-actin and the actin binding region of α-catenin are needed to support epithelial barrier formation.

Coupling of F-actin to the Cadherin complex is sufficient for the induction of cell–cell adhesion

Since α-catenin can bind to the NJ complex through Afadin (Takai et al., 2008), is a key component of the E-cadherin complex in AJs, and can associate with the TJ complex via ZO-1, it was not *a priori* clear which of these complexes recruited α-catenin for a functional link to actin in barrier formation. To further investigate this, we performed IF on MDCKs fixed during a calcium switch. The images and linescans in Fig. 3A show that both α-catenin and Vinculin are in perpendicular structures that

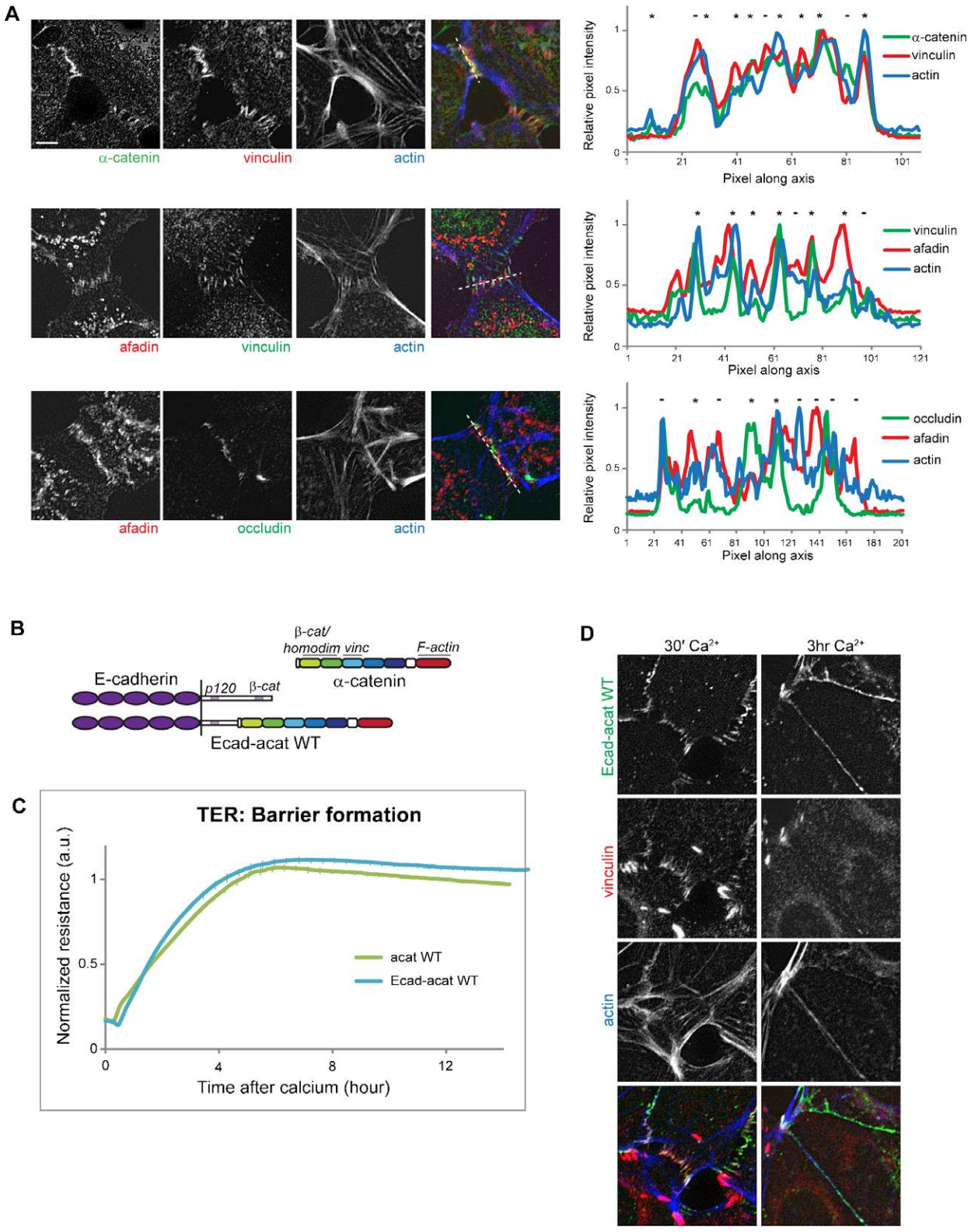

Fig. 3. Coupling of actin to the E-cadherin complex is sufficient for induction of cell–cell adhesion. (**A**) IF images of FAJs and radial actin bundles during a calcium switch. Top row: α-catenin (green) and Vinculin (red) show substantial overlap with radial actin bundles (blue). Middle row: Afadin (red) and Vinculin (green) also show overlap with radial actin bundles (blue). Bottom row: Afadin (red) shows more overlap with radial actin (blue) than Occludin (green). Right panels: linescan through the remodeling junctions showing overlap between α-catenin, Afadin, Vinculin, Occludin and radial actin bundles. (**B**) Schematic representation of the E-cadherin-α-catenin fusion construct used. E-cadherin is C-terminally truncated so that it lacks the β-catenin binding site, and fused to full length α-catenin. (**C**) TER measurement of α-catenin-depleted MDCK cells rescued with either α-catenin-WT (green) or Ecad-αcat (turquoise). (**D**) IF images of FAJs in α-catenin-depleted MDCK cells rescued with Ecad-αcat (green), containing vinculin (red) and connecting to radial actin bundles (blue). Scale bar = 5 µm.

often also overlap with radial actin bundles (Fig. 3A, top row). Similar to this, the main actin linker of NJs, Afadin, also showed a perpendicular staining that overlapped with Vinculin and was often localized at the terminus of radial actin bundles (Fig. 3A, middle row). Although TJ proteins like Occludin and the actin linker ZO-1 also remodeled during a calcium switch, their localization showed less overlap with Afadin or the radial actin bundles (Fig. 3A, bottom row). Because of these data, E-cadherin and Nectin complexes are the primary candidates for functional linkage to the actin cytoskeleton during junction formation.

To further decipher the importance of AJs or NJs in α-catenin-dependent F-actin coupling during junction formation, we expressed a fusion between E-cadherin and α-catenin (Fig. 3B, Ecad-αcat WT) in the α-catenin negative MDCK cells to bypass the possibility of α-catenin associating with the NJ complex. Such fusions have been used before and were shown fully functional in rescuing (D)E-cadherin negative cell lines and epithelial cell clones in organisms (*Drosophila*) (Gottardi et al., 2001; Pacquelet and Rørth, 2005). The fusion used here contained the extracellular and most of the intracellular domains of E-cadherin, except for the β-catenin binding domain. This domain was replaced by full length α-catenin. When α-catenin negative MDCK cells were rescued with this Ecad-αcat fusion, their ability to form junctions was restored to be very similar to that of WT MDCK cells: During early stages of junction formation, Vinculin-containing FAJs that were formed connected to radial actin bundles (Fig. 3D) and when the junctions matured, actin remodeled into the parallel conformation and Vinculin was mostly absent (Fig. 3D). The formation of the barrier function was also restored to be very similar to that in cells rescued with α-catenin-WT (Fig. 3C). Since there was no detectable endogenous α-catenin available in these cells to bind to NJ or TJ complexes, this demonstrates that recruitment of α-catenin to the E-cadherin complex is sufficient for a functional link between actin and cell–cell junctions during epithelial barrier formation. The transient participation of Vinculin in junction formation is also fully restored by the E-cadherin-α-catenin fusion. This suggests that indeed Vinculin recruitment and function at early cell–cell junctions depends on the Cadherin complex specifically.

Myosin II activity promotes epithelial barrier formation

Previously we have shown that FAJs are linked to radial actin bundles and are under actomyosin-dependent tension during junction remodeling in endothelial cells (Huveneers et al., 2012). To test whether contractile cytoskeletal force is important in the process of junction formation, we performed the calcium switch in the presence of the myosin inhibitor blebbistatin. As shown in Fig. 4 (dashed line), the establishment of the barrier is strongly delayed, but the final resistance reached is equal between control and blebbistatin treated samples. This shows that the rate of epithelial barrier formation is enhanced by actomyosin contractility and suggests that besides a structural role in the maintenance of cell–cell adhesions, the actomyosin cytoskeleton also has an instructive function that regulates the efficiency of cell–cell junction formation. It is likely that this response to force is generated at the FAJs where contractile radial actin bundles terminate and where the presence of Vinculin indeed indicates that tensile forces apply (le Duc et al., 2010; Miyake et al., 2006; Yonemura et al., 2010).

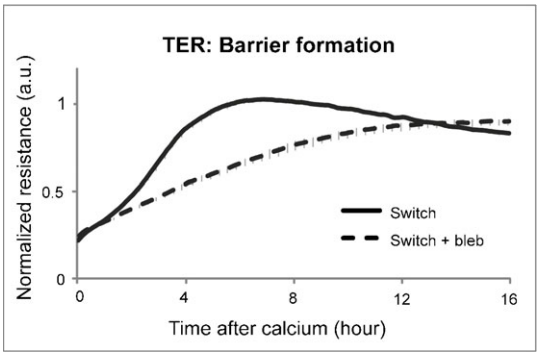

Fig. 4. Actomyosin contractility promotes barrier formation. TER measurement of MDCK cells in the presence or absence of 50 μM blebbistatin. Error bars indicate standard error, $n>3$.

Loss of Vinculin from FAJs does not disturb their physical connection to actomyosin

Vinculin is present at FAJs that are under tension, but Vinculin is also involved in overall F-actin organization and myosin-based contraction (Mierke et al., 2008), both of which likely depend on its role in integrin-based Focal Adhesions (FAs) (Puklin-Faucher and Sheetz, 2009). To specifically perturb Vinculin's role at FAJs, we used the α-catenin-ΔVBS mutant recently developed in our lab. In this construct, the Vinculin binding site (VBS) in α-catenin is replaced with the homologous domain from Vinculin (domain 3A), the closest homolog of α-catenin (Fig. 5A). This leaves α-catenin's structural conformation intact, but prevents Vinculin recruitment to tensile FAJs (Huveneers et al., 2012). To specifically enhance Vinculin's function at cell–cell junctions, we used a truncated version of α-catenin (Fig. 5A, α-catenin-1-402) that constitutively binds and recruits Vinculin (Yonemura et al., 2010).

First, to biochemically characterize the interactions between Vinculin and the α-catenin mutants in more detail, they were co-expressed in and immunoprecipitated (IP) from Cos7 cells. As expected from previous work (Johnson and Craig, 1995) full length Vinculin did not co-IP with any of the α-catenin-GFP constructs (Fig. 5B, lanes 12–15), whereas a truncated mutant that lacks the tail domain (Vinculin 1-881) showed strong α-catenin binding (Fig. 5B, lanes 17, 19). Importantly, all α-catenin-ΔVBS constructs showed a strong reduction in Vinculin 1-881 binding (Fig. 5B, lanes 18, 20). Thus indeed replacement of the Vinculin binding domain in α-catenin strongly perturbs its interaction with Vinculin. Interestingly, whereas α-catenin in cell–cell junctions clearly requires an activation step involving contractile actomyosin before it can recruit Vinculin (Yonemura et al., 2010), in these cell lysates, over-expressed full length α-catenin pulls down Vinculin with similar efficiency as the α-catenin-1-402 mutant that constitutively recruits Vinculin to cell–cell junctions. This suggests that at cell–cell junctions a specifically folded α-catenin is retained or that additional proteins interacting with α-catenin at the junction prevent Vinculin binding. Moreover, in these lysates it is clear that Vinculin also requires an activation step for its interaction with α-catenin. To investigate if β-catenin could be involved in such inactivation of α-catenin or activation of Vinculin, we co-expressed also β-catenin-mCherry in these cells and performed the same IP for α-catenin-GFP. A ternary complex containing α-catenin, β-catenin and Vinculin 1-881 was readily precipitated

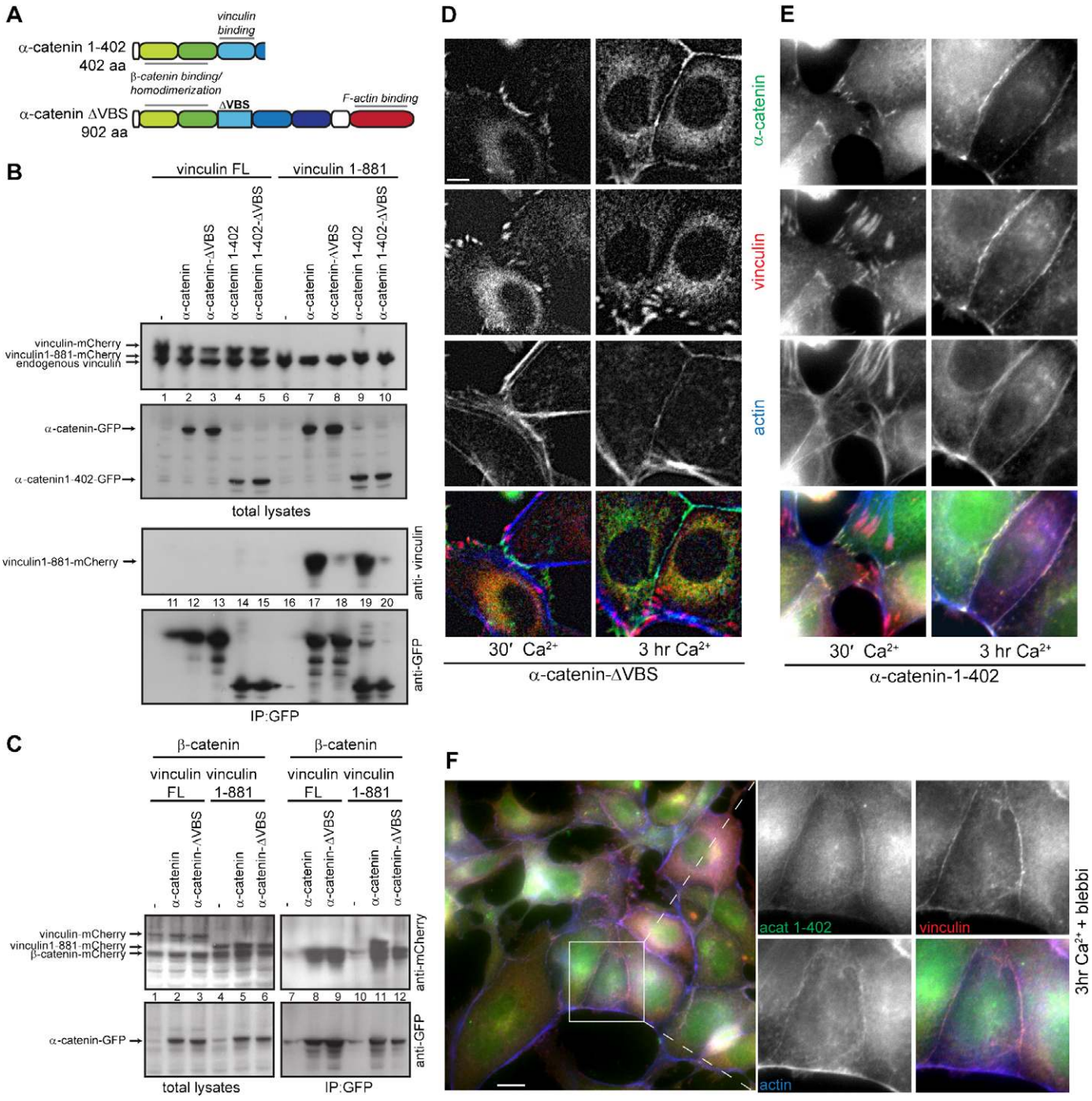

Fig. 5. α-catenin mutants that abolish or enhance Vinculin recruitment to cell–cell junctions. (**A**) Schematic representation of the α-catenin-1-402 truncation mutant and α-catenin-ΔVBS mutant in which the vinculin binding site (VBS) from α-catenin is replaced by the homologous domain from vinculin. (**B**,**C**) Western Blot results of IP of the indicated GFP-α-catenin constructs from MDCK cells and coprecipitation of mCherry-vinculin or mCherry-vinculin 1-881 in the absence (B) or presence of co-expressed mCherry-β-catenin (C). (**D**,**E**) IF images of FAJs and mature junctions that are formed during a calcium switch in α-catenin negative MDCK cells rescued with α-catenin-ΔVBS or α-catenin-1-402 (green), showing absence (D) or constitutive presence of Vinculin (red, E). (**F**) IF images show vinculin (red) recruitment to α-catenin-1-402 (green) is tension-independent, it still occurs in the presence of 50 µM blebbistatin. Scale bars: (D) 5 µm; (F) 10 µm.

(Fig. 5C, lane 11), while a complex between full length Vinculin and α-catenin did still not form in the presence of β-catenin (Fig. 5C, lane 8). This indicates that β-catenin is not directly regulating the interaction between α-catenin and Vinculin.

Next, to assess their capacity to mediate junction formation, we expressed these α-catenin mutants in α-catenin negative MDCK cells. As shown in Fig. 5D, in a calcium switch assay, FAJs are still formed in α-catenin-ΔVBS cells: α-catenin-ΔVBS is present in typical perpendicular structures, and F-actin staining shows radial bundles that terminate at these α-catenin structures. Nevertheless, Vinculin is completely absent from these junctions. In these α-catenin-ΔVBS-rescued cells, junctions do mature and

linear junctions look very similar to those in α-catenin-WT-rescued cells (compare Fig. 5D, right column to Fig. 1D, second row). From these experiments it is clear that α-catenin-ΔVBS can mediate the physical link between cell–cell junctions and F-actin, can restore the formation of FAJs and can support their maturation into linear AJs. Apparently, Vinculin is dispensable for each of these functions.

Also in α-catenin-1-402 expressing cells, junction formation appears to be fully rescued: FAJs appear with connecting radial actin bundles and actin remodels back to parallel bundles as the junctions mature (Fig. 5E). Importantly, Vinculin is constitutively recruited to α-catenin-1-402, not only in FAJs, because it remains present even as the junctions mature (Fig. 5E). To definitively show that Vinculin recruitment by α-catenin-1-402 is independent of actomyosin-induced tension these cells were subjected to a calcium switch in the presence of the myosin inhibitor blebbistatin. Although junction formation was clearly affected by blebbistatin, intact cell–cell junctions contained both α-catenin-1-402 and Vinculin at all times (Fig. 5F). We attribute the capacity to sustain junction formation of this α-catenin mutant that lacks F-actin binding capacity to the constitutive presence of the F-actin binding domain of Vinculin.

In conclusion, these mutants can be used to abolish or enhance the recruitment of Vinculin to cell–cell junctions, without obvious perturbation of the organization of F-actin at cell–cell contacts or of its functional connection to the E-cadherin complex.

Failure to recruit Vinculin blocks Cadherin mechanosensing

To investigate whether the α-catenin-dependent recruitment of Vinculin to the E-cadherin complex is important for Cadherin mechanosensing, the capacity to sense force and generate a proportional biochemical response (Vogel and Sheetz, 2006), as suggested by our earlier work (le Duc et al., 2010) and that of Yonemura (Yonemura et al., 2010), we employed magnetic twisting cytometry (MTC). Fc-E-cadherin-coated ferro-magnetic beads were placed on α-catenin negative DLD1-R2/7 cells rescued with α-catenin-WT or α-catenin-ΔVBS and allowed to adhere for 15 min. During this time of adhesion, maximal bead–cell interaction was established and the cellular cortical stiffness at the bead cell interface was measured by a short twisting of the bead through an oscillating magnetic field and image based analysis of bead displacement as described previously (le Duc et al., 2010; Wang and Ingber, 1995). Although bead–cell interactions are formed, the inability of α-catenin to recruit Vinculin strongly reduces the stiffness at the bead cell interface (Fig. 6A). Next, a continuous oscillating magnetic field was applied to the beads to induce prolonged tension on the bead–cell junctions and the bead displacement was measured over time. As shown in Fig. 6B, cadherin-mediated force-actuated stiffening was observed in α-catenin-WT-rescued cells, very similar to the stiffening response in F9 cells we reported previously (le Duc et al., 2010). Strikingly, in the α-catenin-ΔVBS cells the force-actuated stiffening was completely absent (Fig. 6B). This shows that the Vinculin-binding domain of α-catenin is crucial for E-cadherin mechanosensing. This not only reinforces previous findings in cells from which Vinculin was totally absent, but also proves definitively that the Vinculin dependence of E-cadherin-based mechanosensing is localized to interactions at the bead–cell interface. In conclusion, the α-catenin-ΔVBS mutant thus specifically perturbs E-cadherin's mechanosensitive function,

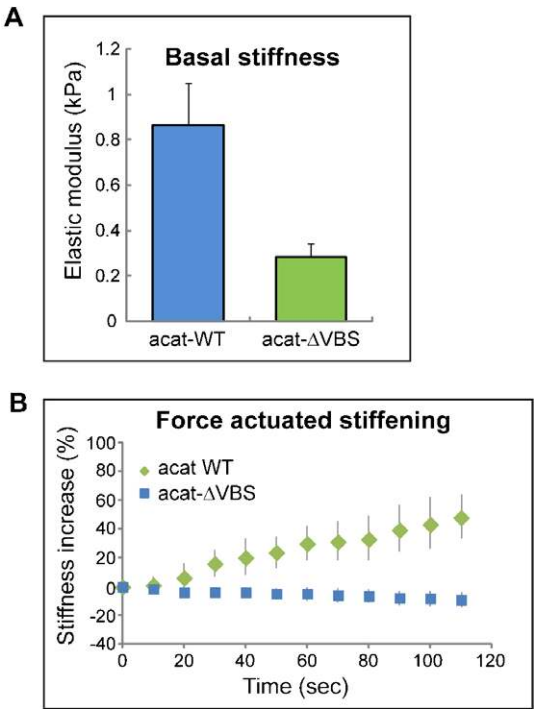

Fig. 6. Failure to recruit Vinculin abolishes E-cadherin mechanosensing. (**A**) Basal stiffening of the interface between Fc-E-cadherin-coated magnetic beads and DLD1-R2/7 cells rescued by expression of α-catenin-WT or α-catenin-ΔVBS, as calculated from bead displacement in 10 sec. MTC measurements after 15 min. of bead–cell contact. Error bars represent standard deviations. $n>369$ beads. (**B**) Force-actuated stiffening as measured by prolonged continuous MTC measurements. Error bars represent standard deviations. $n>300$ beads.

while leaving its capacity to link F-actin to the Cadherin complex in junction assembly and concomitant force transmission intact.

Vinculin recruitment promotes efficient barrier formation

Our IF stainings did not reveal overt structural defects in junction formation by abolishing or increasing Vinculin recruitment to cell–cell junctions, yet E-cadherin mechanosensing is completely inhibited in the α-catenin-ΔVBS cells. To investigate the effects on epithelial barrier formation quantitatively and in live cells, we performed TER experiments with the cells expressing α-catenin-WT, α-catenin-ΔVBS, or α-catenin-1-402. As shown in Fig. 7, the establishment of the barrier function of the monolayer was strongly delayed in the α-catenin-ΔVBS expressing cells compared to cells rescued with α-catenin-WT. The same delay was observed when comparing cells expressing a fusion between E-cadherin and α-catenin-WT or α-catenin-ΔVBS (Fig. 7B). This further supports the view that the E-cadherin complex mediates mechanosensitive F-actin coupling at cell–cell junctions. Conversely, in α-catenin-1-402 cells, with enhanced recruitment of Vinculin, epithelial barrier function is established much more rapidly than in α-catenin-WT-rescued cells. The delayed barrier formation in α-catenin-ΔVBS cells is also reflected in the increased number of immature cell–cell junctions present at 3 hours after a calcium switch (Fig. 7C). The magnitude of the delay is very similar to that induced by blebbistatin (Fig. 4). These results strongly suggests that Vinculin indeed mediates a response of the E-cadherin complex to actomyosin-based force that results in more efficient epithelial barrier formation. The fact that force-independent Vinculin

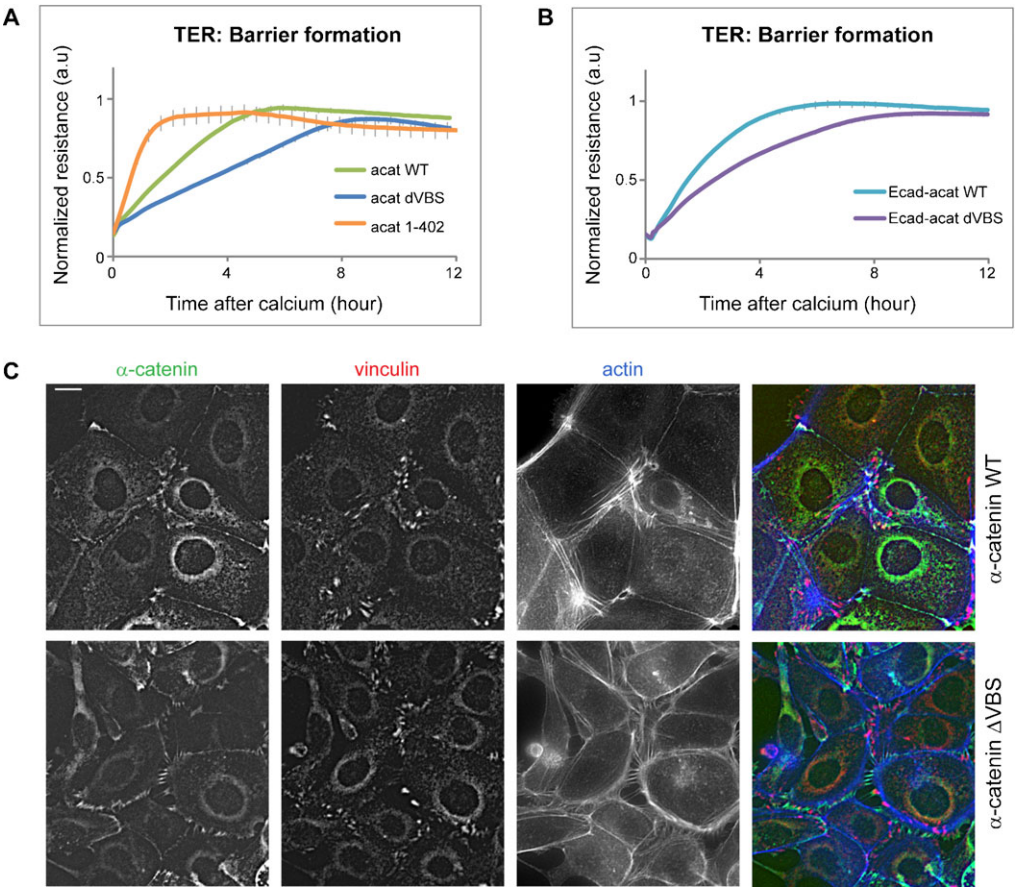

Fig. 7. Vinculin recruitment enhances the efficiency of barrier formation. (**A**) TER measurements after a calcium switch in α-catenin-depleted MDCK cells rescued with the indicated constructs. Error bars indicate standard error, $n>3$. (**B**) TER measurements after a calcium switch in α-catenin-depleted MDCK cells rescued with Ecad-αcat fusions that contain α-catenin-WT or α-catenin-ΔVBS show that mechanosensing at the Cadherin complex is sufficient for force-enhanced barrier formation. Error bars indicate standard error, $n>3$. (**C**) IF images of α-catenin-depleted MDCK cells, rescued with α-catenin-WT or α-catenin-ΔVBS, fixed at 3 hours after calcium showing the delayed maturation into AJs when vinculin cannot be recruited. Scale bar = 10 μm.

recruitment by α-catenin-1-402 leads to enhanced barrier formation suggests that Vinculin recruitment invokes downstream biochemical processes that enhance barrier formation independent of force.

Discussion

In this study we have shown that actomyosin contractility regulates efficient barrier formation through Cadherin dependent Vinculin recruitment. Upon a calcium switch, Tight Junction-, Nectin- and E-cadherin complexes as well as the actomyosin cytoskeleton itself all participate in irregular or punctate cell–cell junctions. Nevertheless, functional, α-catenin-dependent coupling of actomyosin to the E-cadherin complex specifically is sufficient for normal barrier formation. Actomyosin connects to the E-cadherin complex in FAJs that are characterized by the presence of Vinculin. Vinculin is not essential for FAJ formation, their actin linkage or final barrier formation, but mediates the force-dependent reinforcement that determines the rate of epithelial sealing. Constitutive junctional Vinculin recruitment, uncoupled from cytoskeletal pulling forces, enhances epithelial barrier formation independent of cytoskeletal force. Thus pulling forces from contractile actomyosin on the Cadherin complex directly, are a driving factor in epithelial barrier formation, through Vinculin dependent Cadherin mechanotransduction.

The connection between E-cadherin and actin

The simple model in which E-cadherin is connected to F-actin via β- and α-catenin was challenged by the Nelson and Weis labs when they showed that α-catenin cannot bind to F-actin and β-catenin simultaneously (Drees et al., 2005). Nevertheless, α-catenin is required for tight epithelial cell–cell adhesion and this depends on the domain that is essential for its F-actin binding activity (Fig. 2) (Pokutta et al., 2002; Yonemura et al., 2010). Using an E-cadherin-α-catenin fusion, we showed that the interaction of α-catenin with the Cadherin complex is sufficient for a functional link between early cell–cell junctions and contractile actomyosin that sustains apparently normal barrier formation (Fig. 3). Although intermediates between α-catenin and F-actin cannot be excluded, the most obvious candidates EPLIN (Taguchi et al., 2011) and Vinculin (Fig. 5) can be absent from FAJs without perturbing their connection to F-actin, so they are clearly not obligatory. The most straightforward hypothesis to explain all current observations is that a conformational activation of α-catenin occurs in mammalian cells that allows simultaneous binding to the Cadherin complex and actomyosin, which cannot be recapitulated in lysates with mammalian expressed α-catenin proteins.

Vinculin recruitment to cell–cell adhesions

Vinculin has been shown to be present in punctate basolateral cell–cell junctions, which we now call FAJs, in keratinocytes (Vasioukhin et al., 2000), MCF10 cells (Peng et al., 2010) and MDCK cells (le Duc et al., 2010; Miyake et al., 2006; Taguchi et al., 2011). In other studies in tissues (Geiger et al., 1980), MCF7 (Maddugoda et al., 2007), DLD-1 (Taguchi et al., 2011; Yonemura et al., 2010) and MDCK cells (Miyake et al., 2006; Yonemura et al., 2010), Vinculin has been found in the apical zonula adherens (ZA). Several pieces of evidence show that

Vinculin is needed for the structural integrity of this apical ZA (Maddugoda et al., 2007; Watabe-Uchida et al., 1998). In both early junctions and mature apical junctions, the presence of Vinculin depends on myosin activity (le Duc et al., 2010; Miyake et al., 2006; Yonemura et al., 2010). In this study we have only investigated the role of Vinculin in early cell–cell junctions, not in ZA formation. Within the 24 hours after calcium addition that our experiments maximally lasted, there is no clear apical ZA formed in MDCK cells and after the initial phase, Vinculin is mostly absent from cell–cell junctions (Fig. 1). This may reflect reduced tension on these junctions compared to early junctions and ZA junctions, but this has not been experimentally addressed so far. Notably, in DLD1-R2/7 cells rescued with α-catenin, Vinculin is recruited in a myosin dependent manner to the apical-most ZA regions of these cells only, even though E-cadherin and α-catenin are present along the basolateral membrane as well. Force-independent binding of Vinculin to α-catenin-1-402 occurs along the entire lateral membrane (Yonemura et al., 2010). Clearly, the magnitude and direction of the mechanical stresses at different cell–cell junction areas remain to be established to fully understand how junctions are regulated by force.

The role of Vinculin in early junctions remained unclear so far, and there is also conflicting evidence as to which protein recruits it to the Cadherin complexes. Both β-catenin and α-catenin have binding sites for Vinculin and it was shown in MDA_MB-468 cells, lacking α-catenin, that Vinculin could still associate with the Cadherin complex, and this is mediated through β-catenin. This study suggested that Vinculin may be able to replace α-catenin in cell–cell adhesion (Hazan et al., 1997). Vinculin recruitment via β-catenin was also shown to regulate cell surface E-cadherin expression in MCF10a cells (Peng et al., 2010). On the other hand Weiss and colleagues demonstrated that Vinculin can be recruited to the junctional complex through α-catenin (Weiss et al., 1998). Vinculin recruitment via either α- or β-catenin may have different functions in different cell types or at certain stages of junction formation. In MDCKs, we find no evidence for the hypothesis that Vinculin can be recruited to junctions by β-catenin to perform α-catenin-independent functions: KD of α-catenin in MDCK was not rescued by recruitment of Vinculin to junctions. In our α-catenin-ΔVBS mutant, we do not see any Vinculin present in any of the junction types. Furthermore, the E-cadherin-α-catenin fusion protein shows no obvious defects despite the fact that it lacks the β-catenin binding site. Apparently, how Vinculin is recruited to cell–cell junctions is dependent on the cell type and state of the junctions, and more details need to be known for all observations to be reconciled. Speculatively, one could envision a cell-type specific situation in which junction maturation is prevented and all Cadherin-based adhesions remain in the tensile FAJ stage. Loss of Vinculin could render these junctions less stable and result in their overall reduction and as a consequence a reduction of E-cadherin retained at the plasma membrane. We conclude from our present work that the presence and function of Vinculin during cell–cell junction formation in MDCK cells mainly depends on myosin activity and α-catenin connected to the E-cadherin complex.

Regulation of the Vinculin–α-catenin interaction

Whereas F-actin binding by α-catenin may require an elusive activation step (see above), force-dependent recruitment of Vinculin by α-catenin (Yonemura et al., 2010) may first require an inactivation step: our IP results (Fig. 5) show that full length α-catenin binds the Vinculin head domain in the cytoplasm of highly expressing Cos-7 cells quite efficiently (similar to truncated α-catenin-1-402). This is in apparent contrast to the very inefficient co-IPs between Cadherin complex members and Vinculin in MDCK cells (le Duc et al., 2010). It is highly unlikely that forces act on α-catenin in the cytoplasm of Cos-7 cells. Moreover, also when purified from cell lysates, α-catenin does not have the same globular conformation as Vinculin (Drees et al., 2005), indicating that α-catenin is in a more open conformation. This suggests that specifically at cell–cell junctions a closed conformation of α-catenin is stabilized or that α-catenin is bound to another protein that blocks the VBS domain to exclude Vinculin binding. Even though β-catenin does influence α-catenin conformation in solution (Drees et al., 2005) and the interaction between the two is clearly observed in Cos-7 cells (Fig. 5), there is no apparent effect on Vinculin binding to α-catenin in these Cos-7 lysates (Fig. 5). Thus, an elusive inactivation step of α-catenin might occur at cell–cell junctions to prevent constitutive Vinculin recruitment and enable force-sensing.

One alternative explanation would be that not α-catenin, but Vinculin is activated by myosin-dependent force to induce their interaction. Indeed full length Vinculin in solution or in Cos-7 cells (Fig. 5) does not bind α-catenin, whereas the head domain of Vinculin binds to α-catenin very efficiently (Watabe-Uchida et al., 1998; Weiss et al., 1998; Yamada et al., 2005). By inserting a tension sensing FRET element in Vinculin, it was shown that in integrin-based Focal Adhesions Vinculin itself is under actomyosin-based tension (Grashoff et al., 2010). It is difficult to envision, however, how force at cell–cell junctions can activate Vinculin prior to its visible presence. Moreover, recent work by DeMali shows that the presence of unforced F-actin already stimulates the interaction between full length Vinculin and α-catenin (Peng et al., 2012). Thus it is not clear how Vinculin gets activated for α-catenin binding in cell–cell junctions and this means that there is an additional elusive signaling step that participates in Cadherin mechanosensing. Taken together, it is likely that the Yonemura model in which α-catenin is the force-activated molecule at the Cadherin complex applies to FAJs in junction formation, but it is clear that several key details are missing to fully understand the mechanism of Cadherin mechanosensing.

Function of Vinculin at cell–cell junctions

Vinculin has been found at forming cell–cell junctions (Vasioukhin et al., 2000; this study), remodeling junctions (Huveneers et al., 2012; le Duc et al., 2010) and apical adhesions (Watabe-Uchida et al., 1998; Yonemura et al., 2010). The main common theme in all of these instances appears to be the increased presence of contractile actomyosin, but the precise function of Vinculin's presence at such diverse adhesion sites remains somewhat unclear. Vinculin is involved in compaction of epithelial apical junctions (Maddugoda et al., 2007; Watabe-Uchida et al., 1998) and protects FAJs from opening in thrombin-treated HUVEC cells (Huveneers et al., 2012). We now show that Vinculin enhances the efficiency of epithelial junction sealing upon early contact formation. Combining these observations, a general function for Vinculin in force-dependent junction reinforcement becomes apparent. This is in agreement with the force-dependent reinforcement of the junctions between cells and

E-cadherin-coated beads measured in MTC (le Duc et al., 2010) that is lost in α-catenin-ΔVBS expressing cells (Fig. 6). The next question is how exactly Vinculin can bring about this reinforcement. Vinculin can bind to α-catenin and F-actin and could potentially simply reinforce adhesion by supplying additional bonds between E-cadherin and F-actin. In analogy to integrin dependent force sensing, this would increase the adhesive clutch between E-cadherin and retrograde flowing contractile F-actin, thus slowing this retrograde movement at FAJs and enhancing local membrane protrusion, which is also driven by actin polymerization (Gardel and Schwarz, 2010; Hu et al., 2007; Moore et al., 2010). This in turn would enhance the formation of new Cadherin–Cadherin interactions and result in more efficient epithelial barrier formation. On the other hand, Vinculin can also affect actin polymerization itself (Le Clainche et al., 2010; Wen et al., 2009) or by bringing in additional factors such as VASP (Brindle et al., 1996) and ARP2/3 (DeMali and Burridge, 2003). Especially the enhanced barrier formation in the α-catenin-1-402 expressing cells, that have a reduced number of F-actin binding sites in the Cadherin complex due to the truncation of α-catenin, suggests that increased actin polymerization downstream of Vinculin recruitment could indeed a driving factor for barrier formation. To further investigate this, one would need to develop a system in which Vinculin mutants can be used to specifically rescue the absence of Vinculin at cell–cell junctions. Replacing overall Vinculin with mutants is likely to affect FAs and cytoskeletal integrity and will have indirect effects on the mechanics of cell–cell junctions. Clearly integration of the different observations so far into a complete model for Vinculin function awaits further experiments.

Concluding remarks

In conclusion, we have shown that actomyosin contractility is a driving factor in epithelial barrier formation. The crucial involvement of Vinculin at radial actin contacted FAJs shows that force-generated signaling at the Cadherin complex mediates this function of actomyosin. This has implications for embryogenesis, tissue morphogenesis, wound healing and endothelial remodeling processes where mechanical forces and cell–cell junction formation converge to build and maintain properly functioning tissues (Cavey and Lecuit, 2009).

Materials and Methods

Cell lines and culture

Madin Darby Canine Kidney strain II (MDCK-II) cells were cultured in high glucose DMEM containing GlutaMAX (Gibco) and supplemented with 10% Fetal Bovine Serum (FBS, Sigma) and Penicillin/Streptomycin (Gibco) in standard 10 cm culture dishes. MDCK cells negative for α-E-catenin were a kind gift from James Nelson and used previously by Loerke et al. (Loerke et al., 2012). They were selected by neomycin upon integration of a lentiviral vector containing a specific shRNA sequence against α-E-catenin. These cells were cultured under constant neomycin selection (250 μg/ml). α-E-catenin negative MDCK cells stably rescued with α-catenin mutants or E-cadherin-α-catenin fusions were generated by lentiviral transduction, followed by continuous puromycin (1.3 μg/ml) and neomycin (250 μg/ml) selection. These rescue cell lines were kept in culture for a maximum of 6 passages after selection, to prevent endogenous α-catenin from being re-expressed. DLD-1 R2/7 cells were cultured in high glucose DMEM containing GlutaMAX (Gibco) and supplemented with 10% Fetal Bovine Serum (FBS, Sigma) and Penicillin/Streptomycin (Gibco) in standard 10 cm culture dishes.

Immunocytochemistry and microscopy

For immunofluorescence, cells were plated overnight on glass coverslips, coated with 30 mg/ml collagen type I (Calf collagen I, Sigma) in DMEM lacking calcium (Gibco), supplemented with L-glutamine, Sodium Pyruvate (Gibco), 0.5% FBS (Sigma) or 5% FBS (10 kDa dialyzed, Gibco) and 20 μM $CaCl_2$. Calcium switch was performed by adding $CaCl_2$ to a final concentration of 2 mM. At different time-points, cells were washed with PBS and fixed in 2% paraformaldehyde in PBS for 20 min. at RT, permeabilized with 0.4% Triton X-100 for 5 min. and blocked in 2% BSA for 1 hour. Phalloidin, primary and secondary antibody stainings were performed in 2% BSA for 1 hour. Coverslips were mounted in Mowiol4-88/DABCO solution (Sigma). Fixed cells were imaged using a widefield microscope (Nikon Eclipse Ti) with a 60× 1.49 NA Apo TIRF objective and an EMCCD camera (Luka, Andor). Images were enhanced for display with a background subtraction by rolling ball (r=20), unsharp mask (r=3, weight=0.6), gaussian blur (r=1) and brightness/contrast adjustments in ImageJ (National Institutes of Health). Linescans were made on background-subtracted images, using Metamorph 7.5 software.

DNA constructs and viral transduction

GFP-α-catenin-ΔVBS was generated as described before (Huveneers et al., 2012). α-catenin-1-402 was made by digesting α-catenin with ScaI and XbaI, filling in the recessed 3′ ends of the XbaI sites, followed by blunt-ended ligation. This yields a new stop codon after amino acid 402 of α-catenin. Constructs were cloned into the self-inactivating lentiviral pLV-CMV-ires-puro vector using NdeI and HpaI, or SnaBI and XbaI sites. A-catenin 1-848 was cloned directly into the lentiviral vector by ligating the PCR product into the NdeI/HpaI restriction sites. A stop codon and the HpaI site were inserted after amino acid 848 of α-catenin using the following primers: 5′-GTGTATCATATGCCAAGTACGCCC-3′ (Fw) and 5′-CAGAGTTAACTTACCCCTGTGACTT CTGGTATTTGG-3′ (Rv). mCherry-vinculin was made by replacing EGFP for mCherry in the previously described pEGFP-C3-vinculin vector (le Duc et al., 2010). mCherry-vinculin 1-881 and mCherry-vinculin 1-258 were made by digesting the PCR product of truncated mCh-vinculin with AgeI and KpnI and ligating it into the pEGFP-C1 vector. PCR primers used: 5′-CGCTACCGGTATGGTGAGCAA-3′ (Fw) and either 5′-CAGAGGTACCTTATTTTTCTTCAGG GGGTGGTGGTC-3′ (Rv), introducing a stop codon after amino acid 881 of vinculin, followed by a KpnI restriction site, or 5′- CAGAGGTACCTTACCAGGCATCTT CATCCCAGG-3′ (Rv), introducing a stop codon after amino acid 258 of vinculin, followed by a KpnI restriction site. β-catenin-mCherry was made by replacing EGFP with mCherry in the previously published pEGFP-β-catenin vector (Yamada et al., 2005). E-cadherin-α-catenin fusions were generated by fusing either full length α-catenin WT or ΔVBS in frame after amino acid 811 of mouse E-cadherin (Ecad-DEIGN-RS-MTAVH-acat), thereby replacing part of the intracellular domain, including the β-catenin binding domain of E-cadherin. PCR product of E-cadherin was obtained using the following primers: 5′-CAGAGCTAGCATGGGAGCCCG-GTGCC-3′ (Fw) and 5′-CAGAAGATCTGTTTCCAATTTCATCAGGATTGGC-AGG-3′ (Rv), introducing a BglII site behind E-cadherin. PCR product of α-catenin was obtained using the following primers: 5′-CAGAAGATCTATGAC-TGCCGTCCACGCAG-3′ (Fw) and 5′-CAGACCGCGGGATGCTGTCCAT-GGCTTTGAACTC-3′ (Rv). All clones were verified by sequencing. Lentiviral particles were isolated from HEK293T cells transiently transfected with third-generation packaging constructs and the lentiviral expression vectors. MDCK cells were transduced with supernatant containing lentiviral particles in the presence of 8 μg/ml polybrene overnight. To create stable cell lines, cells were selected with puromycin (1.3 μg/ml) for at least two weeks before being used in experiments.

Trans Epithelial Electrical Resistance (TER)

To measure epithelial barrier formation, collagen-I coated E-plate 16 electrodes (Roche) were incubated with medium for 30 min., and a background measurement was taken. Then, 0.5×10^5 α-catenin negative MDCK cells rescued with indicated constructs were plated the electrodes, and measurement on the xCELLigence Real Time Cell Analyzer (RTCA) DP instrument (Roche) was started immediately. After 24 hours, junction formation was induced by adding $CaCl_2$ to a concentration of 2 mM, while the measurement continued for another 24 hours. For each well, the measured impedance value was divided by the individual background values, yielding the dimensionless parameter Cell Index (CI). Alternatively, 1.5×10^5 MDCK WT cells were plated onto L-cysteine reduced, collagen-I coated 8W10E electrodes (Applied Biophysics) and grown for 24 hours in low calcium medium before starting measurement. Electrical impedance during a calcium switch was measured in real time at 37℃ and 5% CO_2 using a 1600R Electrical Cell Impedance Sensing (ECIS) system (Applied Biophysics) at 4000 Hz. Importantly, results obtained by these two were almost identical and in displayed experiments values were normalized to the maximum value of the control sample measured in the same experiment.

Antibodies and reagents

Vinculin was stained using mouse monoclonal vinculin antibody hVIN1 (Sigma–Aldrich). Mouse monoclonal E-cadherin and afadin antibodies were obtained from BD Biosciences. Rabbit polyclonal α-catenin antibody was obtained from Sigma. Rabbit polyclonal occludin antibody was obtained from Invitrogen. Rabbit polyclonal GFP antibody was from Covance and rabbit polyclonal mCherry

antiserum was home-made and a gift from Jacques Neefjes. Secondary antibodies coupled to Alexa fluor 488 and 594 were obtained from Molecular Probes. Phalloidin coupled to Promofluor 415 was obtained from Promokine. To check the expression of α-catenin 1-848 on blot, we used mouse monoclonal α-catenin antibody (BD Biosciences). Blebbistatin (used at 50 μM) was from Calbiochem, Cytochalasin D (used at 0.2 μg/ml) was from Sigma–Aldrich.

Immunoprecipitation

Cos-7 cells were transfected with the indicated constructs by standard transfection using polyethyleneimine (PEI). 48 hours post transfection, cells were lysed at 4℃ for 10 min. in lysis buffer (1% Nonidet P-40, 25 mM Tris pH 7.4, 100 mM NaCl, 10 mM $MgCl_2$, 10% glycerol, protease and phosphatase inhibitors). Lysates were cleared by centrifugation at 14,000 rpm for 1 min. GFP-tagged α-catenin was precipitated from the lysates using GFP-Trap beads (Chromotek) for 1 hour at 4℃. Precipitations were washed 3 times in lysis buffer and dissolved in Laemmli sample buffer for standard Western blot analysis.

Magnetic Twisting Cytometry

MTC measurements were performed as described (le Duc et al., 2010; Wang et al., 1993). Briefly, DLD1-R2/7 cells were cultured on collagen substrates and incubated with Spherotech, 4.5 μm ferromagnetic beads, covalently modified with Fc-tagged human E-cadherin on a heated microscope stage at 37℃. MTC imaging was done on a Leica inverted microscope using a 20×, NA 0.6 objective and a cooled CCD camera (Orca2, Hamamatsu). In all measurements, an initial, brief high field was applied to magnetize the beads. After defined time periods, the oscillating magnetic field perpendicular to the bead magnetic moment was turned on for a defined period in order to induce a modulating shear stress on the beads. The bead magnetic moment constant was calibrated in a viscosity standard by rotating the beads in the fluid and measuring the bead angular strain and determined to be 0.12 Pa per Gauss magnetic field. The bead displacements were directly measured and converted to the complex modulus/stiffness by taking the ratio of the applied stress (the bead magnetic moment constant times the applied magnetic field) to the bead displacement. Fourier transforms of the bead displacements and the specific torque were used to determine the complex modulus of the bead–cell junction. Each measurement (experimental condition) represents measurements with N>300 cells at ~1 bead/cell. The data follow a log-normal distribution, from which we obtain the mean and standard deviation.

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Competing Interests

The authors have no competing interests to declare.

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Supplementary Material

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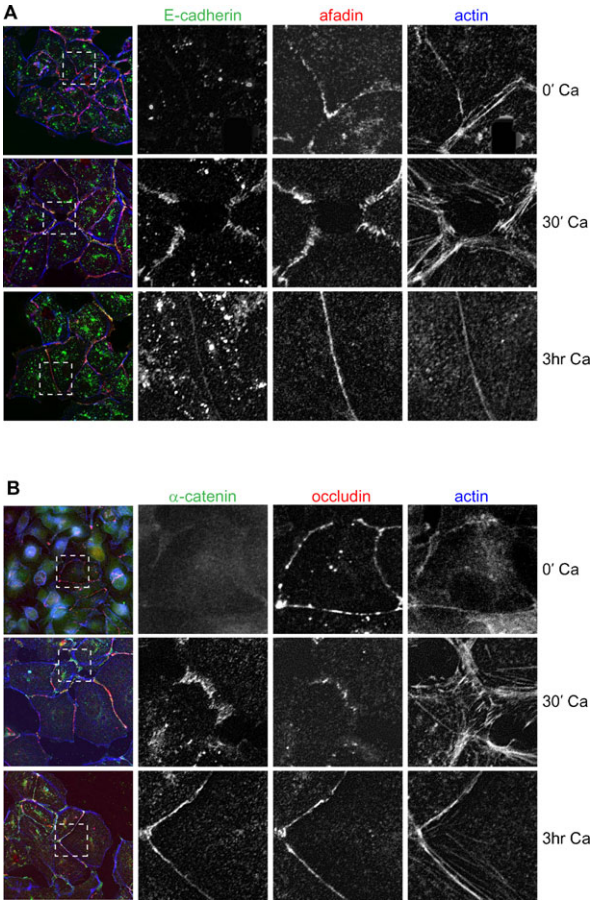

Fig. S1. Serum conditions do not affect Afadin and Occludin localization during a calcium switch. (**A**,**B**) IF images of AJ (E-cadherin, α-catenin), NJ (Afadin) and TJ (Occludin) markers and actin during a calcium switch in subconfluent MDCK cells in 5% FBS low calcium medium.

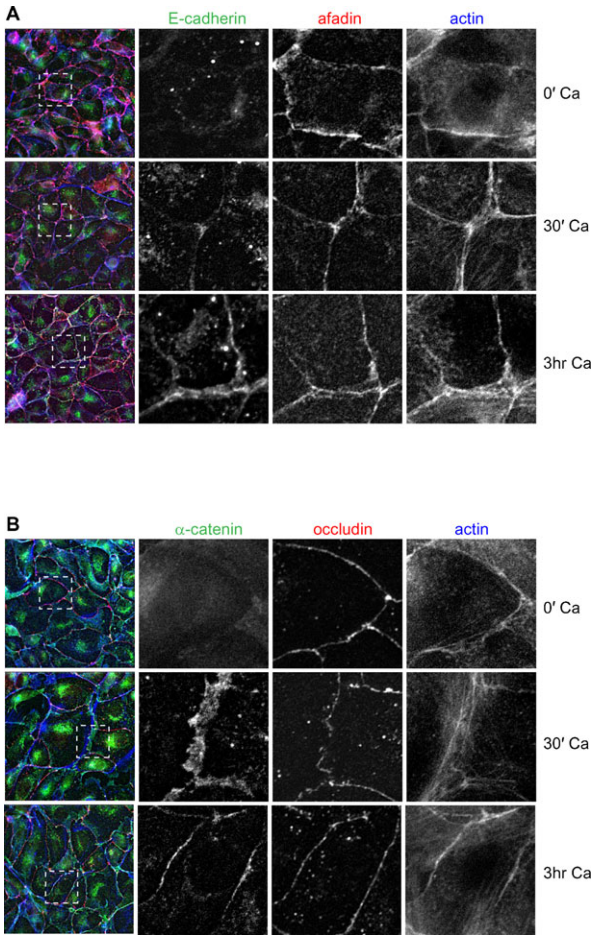

Fig. S2. Cell density does not affect Afadin and Occludin localization during a calcium switch. (**A**,**B**) IF images of AJ (E-cadherin, α-catenin), NJ (Afadin) and TJ (Occludin) markers and actin during a calcium switch in MDCK cells seeded at high confluency in 0.5% FBS low calcium medium.

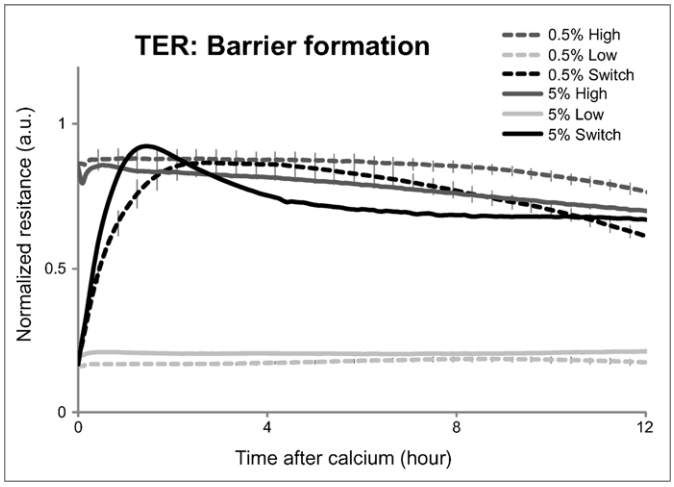

Fig. S3. Serum conditions do not affect barrier formation. TER measurement of MDCK cells seeded in low calcium medium (light grey line), normal calcium medium (dark grey line) or during a calcium switch (black line), containing either 0.5% FBS (dashed lines) or 5% FBS (solid lines). Error bars indicate standard error, $n>3$.

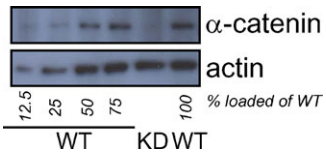

Fig. S4. α-catenin knockdown levels in MDCK cells. Western Blot result of MDCK α-catenin knockdown cells compared to α-catenin levels in MDCK WT cells.