

# Afadin controls cadherin cluster stability using clathrin-independent mechanism

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Afadin is an actin-binding protein that interacts with the intracellular region of the transmembrane proteins, nectins. In collaboration with other transmembrane proteins, cadherins, nectins form adherens junctions, a major type of cell-cell adhesive structures in the multicellular organisms. To elucidate the afadin function, we studied adherens junction defects induced by afadin depletion in epithelial A431 cells. We have found that the cells lacking afadin exhibit no abnormalities in morphology or in general dynamics of adherens junctions in the confluent cell cultures. The only observed difference is a slight increase in the rate of cadherin turnover in these junctions. However, afadin depletion strongly affects the assembly of new adherens junctions immediately after two cells touch one another: initiation of new junctions is significantly delayed, the growth of the nascent junctions stagnates, and their lifetime shortens. As a result, the afadin-depleted cells need much more time to establish the mature junctional structures. This defect is not caused by the clathrin-dependent endocytosis of cadherin clusters that was monitored using live-cell imaging of A431 cells co-expressing GFP-tagged E-cadherin and mCherry-tagged clathrin light chain. Taken together our data show that afadin reinforces adherens junctions and that this process is crucial for the fast formation of adherens junctions at the sites of new cell-cell contacts.

## Introduction

Nectins constitute the family of single-pass transmembrane adhesive receptors that, together with cadherins, form adherens junctions – the major type of intercellular junctions of most vertebrate cells. Within the same junctions, cadherin and nectin reside in separate clusters.<sup>1</sup> The adhesive strength of the adherens junctions and their association with the cytoskeleton is believed to be determined by the cadherin clusters. Despite the genetic data showing that nectin adhesion plays an important role in development,<sup>2,3</sup> the exact function of nectin clusters in adherens junctions is not completely understood. It has been suggested that nectin controls initiation of cadherin clustering by preventing cadherin endocytosis.<sup>4</sup> No data, however, were reported that might help to understand the role of nectin-afadin complex in the mature adherens junctions in which these two proteins are the abundant components. One of the many missing pieces is the time-lapse microscopy of adherens junctions in cells with abnormalities in nectin-afadin adhesion. Our work, therefore, is focused on adherens junction dynamics in cells lacking afadin.

Nectin's extracellular region is composed of three Ig-like domains.<sup>5,6</sup> Recent structural studies of nectin extracellular regions confirmed previous data suggesting that a single interface within the N-terminal V-type Ig domain mediates nectin adhesive dimerization.<sup>7,8</sup> Nectins do not exhibit any sequence

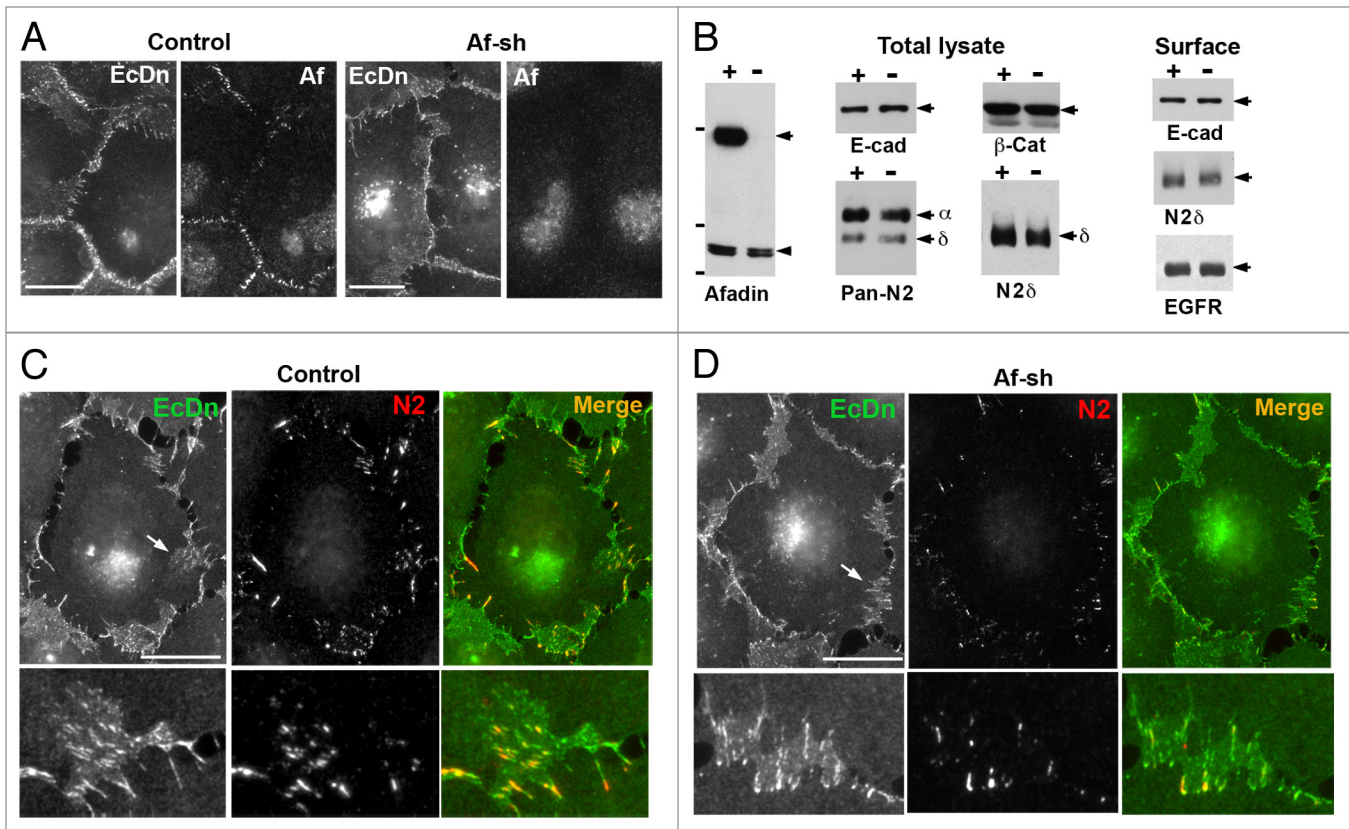
similarity within their intracellular regions, though some of them share a C-terminal motif that binds to PDZ domain of large scaffolding proteins, afadin<sup>9</sup> or PICK-1.<sup>10</sup> Directly or indirectly, afadin, which itself possesses an actin-binding domain, bridges nectins to a large array of cytosolic proteins such as  $\alpha$ -catenin, ponsin, ADIP, LMO7, vinculin, ZO1, IQGAP, and several others,<sup>11-16</sup> which are thought to integrate nectin with adherens junctions, tight junctions and the actin cytoskeleton. Binding to afadin, however, is not required for nectin's cell-to-cell adhesive activity.<sup>17,18</sup> Some experiments suggest that afadin might be important for nectin clustering,<sup>9,17</sup> albeit other data<sup>18,19</sup> did not support this conclusion. Understanding the contribution of afadin to nectin adhesion is important in order to shed light on the adherens junction assembly and, in particular, the role of nectin in formation of cadherin adhesive clusters.<sup>20</sup>

Structural, biochemical, and live imaging studies showed that classical cadherin produces an adhesive bond through strand-swap *trans*-dimerization of its N-terminal EC1 domain. The resulting *trans*-dimers are stabilized by their *cis*-interactions. These two cooperative binding reactions result in spontaneous formation of cadherin adhesive clusters.<sup>21,22</sup> The mechanisms controlling the size and stability of these clusters, as well as the nucleation of their assembly, remain to be studied. These mechanisms require additional proteins, one of which could be nectin, that might control lateral cadherin organization,

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**Figure 1.** Nectin junctions in control and afadin-depleted cells. **(A)** Control (*Control*) and afadin-depleted (*Af-sh*) A431D cells stably expressing EcadNn were double-stained with anti-cadherin (*EcDn*) and anti-afadin (*Af*) antibodies. Afadin co-localizes with cadherin in adherens junctions of the control cells but is completely absent in the afadin-depleted cells. Bars, 20  $\mu\text{m}$ . **(B)** Comparison of total and surface levels of some cadherin adhesion (E-cadherin, *E-cad*, and  $\beta$ -catenin,  $\beta$ -*Cat*) and nectin adhesion (nectin-2, *Pan-N2*, nectin-2 isoforms, *N2 $\alpha$*  and *N2 $\delta$* , and afadin, *afadin*) components in control (+) and afadin-depleted (-) A431 cells. As loading control, the samples containing surface proteins were stained for EGF receptor (*EGFR*). Specific bands are indicated by the arrows, a non-specific one by the arrowhead. **(C and D)** Control A431D cells expressing EcadNn **(C)** and their afadin-depleted variant **(D)** were stained for cadherin (*EcDn*, green), and nectin-2 (*N2*, red). Higher magnifications of the selected regions (indicated by arrows) are shown in the bottom row. Note that the lack of afadin significantly decreases the amounts of nectin-2 in the adherens junctions. The remaining nectin is still co-localized with E-cadherin. Bars, 20  $\mu\text{m}$ .

modes of cadherin *trans*-dimerization,<sup>20,23,24</sup> and/or cadherin endocytosis.<sup>25-27</sup> Loss-of-function experiments in cultured cells as well as experiments with inhibitors of nectin adhesion and with nectin-coated beads suggested that the formation of a nectin-based intercellular adhesive sites initiates cadherin recruitment.<sup>4</sup> In the case of *Xenopus*' N-cadherin, this process could be caused by a direct interaction between extracellular cadherin and nectin regions.<sup>28</sup> In the case of mammalian E-cadherin, afadin in the proximity to nectin-based junctions was suggested to suppress clathrin-dependent cadherin endocytosis thereby promoting intercadherin *trans*-interactions.<sup>29-31</sup> By contrast, genetic loss-of-function experiments supported a crucial role of nectin-afadin adhesion in development, but did not reveal an involvement of this system in adherens junction structures per se.<sup>32-34</sup>

We sought to clarify the role of nectin-afadin adhesion in cadherin junction assembly using A431 epithelial cells. These cells produce two types of adherens junctions, stable apical and dynamic lateral. By many features, the apical junctions are similar to the zonula adherens in polarized epithelial cells.<sup>1</sup> Spot-like lateral adherens junctions continuously and rapidly form at the

basolateral surface and then move apically where they eventually merge with the apical junctions.<sup>21,35</sup> If nectin does contribute to the assembly of adherens junctions, one may expect that a loss of nectin adhesion would induce some distortions, at least, to highly dynamic spot-like adherens junctions in these cells. Therefore, the main objective of this work was to study adherens junctions in A431 cells after inactivation of the nectin-afadin adhesion system. Our data support the role of afadin as a positive regulator of cadherin clustering. But no evidence was obtained that this regulation is based on the clathrin-dependent endocytosis of cadherin.

## Results

### Afadin-depleted adherens junctions retain their morphology and distribution

In order to determine the role of the nectin-afadin adhesion complex in adherens junctions, we first analyzed the morphological changes of the cell-cell contacts in cells, which afadin expression

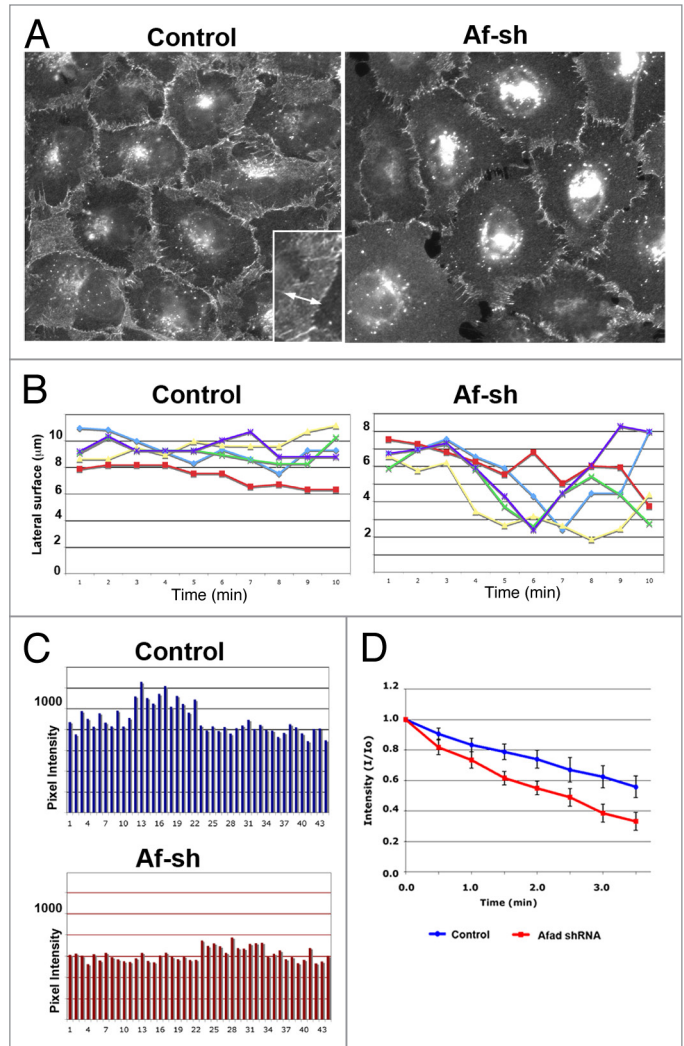
was suppressed by afadin-specific shRNAs. Two cell lines, both of which express nectin-2 and different sets of classical cadherins, were used in our experiments. Human A431 cells derived from a vulvar squamous cell carcinoma express E- and P-cadherins.<sup>36,37</sup> In culture these epithelial cells produce numerous laterally located dynamic adherens junctions.<sup>23,35</sup> To monitor these dynamics, we used A431 cells stably expressing E-cadherin tagged with EGFP (EcadGFP) and, in some cases, co-expressing EcadGFP and mCherry-tagged mouse nectin-2 (Ntn2-CH; ref. <sup>1</sup>). To simplify interpretation of our imaging experiments, we also used A431D cells stably expressing E-cadherin tagged with photoswitchable protein Dendra2 (EcadDn). The advantage of these cells was that the recombinant EcadDn was the only classical cadherin these cells expressed since the parental A431D cells are cadherin-deficient. Both these cell lines yield identical results suggesting that the afadin-nectin function is not cadherin-specific.

The afadin-specific shRNAs expression in any of these cells resulted in a complete loss of afadin-specific staining of cell-cell contacts (Fig. 1A, ref. <sup>1</sup>). It also significantly reduced their anti-nectin-2 staining. However, the remaining nectin was still co-localized with cadherin-positive junctions (Fig. 1C and D). Importantly, no clear abnormalities in the morphology or in the localization of adherens junctions stained by the anti-cadherin antibody were detected in the afadin-depleted cells (Fig. 1). There were no differences detected in the motility of adherens junctions using EcadGFP-expressing A431 cells (not shown). Therefore, our initial examination of the afadin-depleted cells showed that the nectin-afadin complex is not an essential component of adherens junctions: the latter retained their integrity and distribution. The retention of nectin-2 in adherens junctions lacking afadin suggests that some afadin-independent mechanisms could target nectin into adherens junctions.

To complete general characterization of the afadin-depleted cells, we studied whether they changed the total or the surface expression levels of their E-cadherin or nectin-2. To this end, the cell lysates and the precipitates obtained from the surface-biotinylated A431 cells using streptavidin-agarose were analyzed by western blotting. Again, no differences in total or surface levels of these two proteins were detected (Fig. 1B). The total expression levels of the major cadherin-associated proteins, such as  $\beta$ -catenin,  $\alpha$ -catenin, or p120 were also unchanged. Western blotting also confirmed nearly complete depletion of afadin in the afadin shRNA-expressing cells.

#### Cadherin junctions and intercellular contacts are less stable in afadin-depleted cells

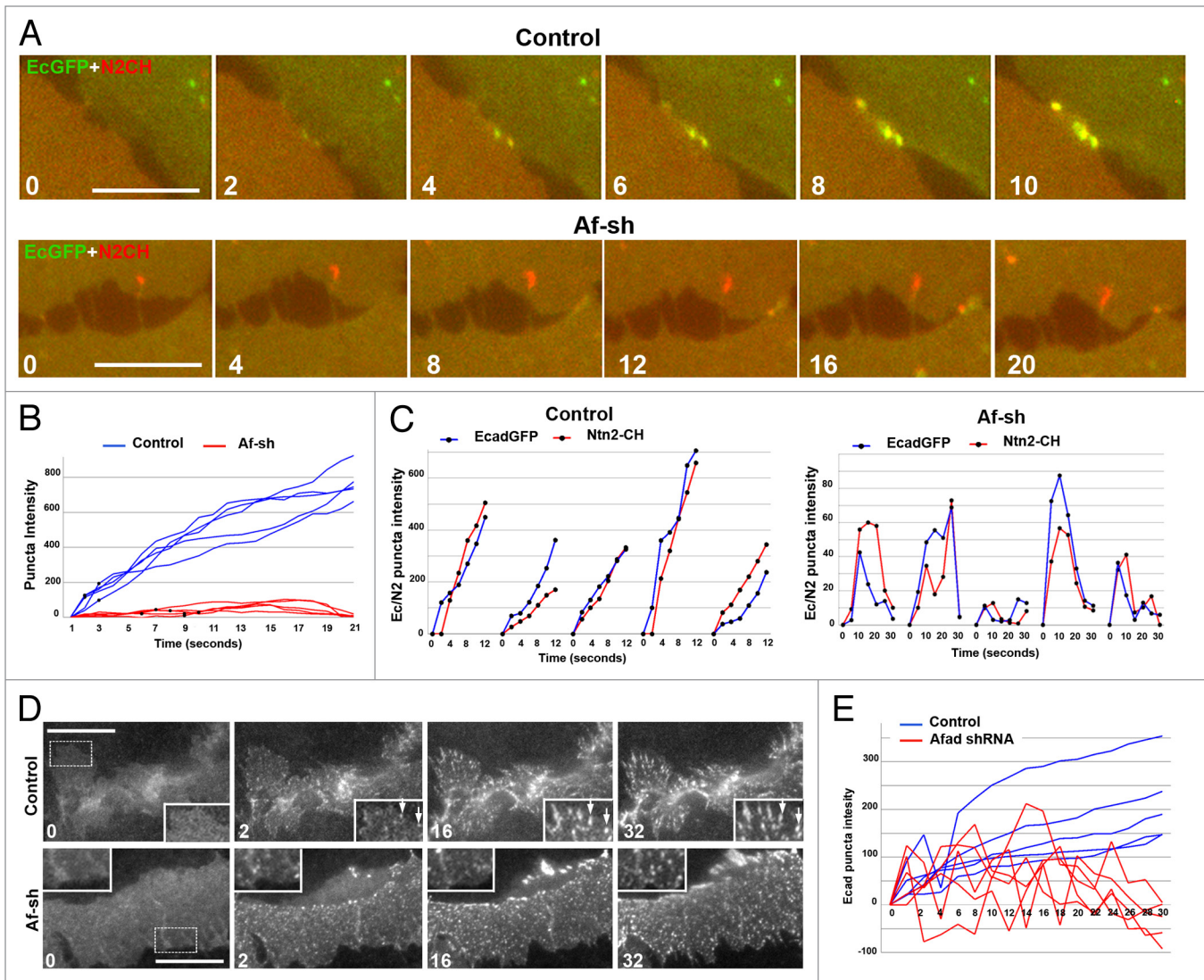
A more detailed inspection of the afadin-depleted A431 or A431D-EcadDn cells revealed, however, an interesting feature: their intercellular contacts in the overnight cultures or on the periphery of epithelial colonies were often unsealed and exhibited many openings (Fig. 2A). To get a better understanding of this phenomenon, we performed a series of 10 min-long time-lapse sequences of the EcadDn-expressing A431D cells and their afadin-depleted counterparts. We then plotted the apical-to-basal distances of the individual cell-cell contacts over the time span (Fig. 2B). These imaging experiments revealed more frequent fluctuations in the length of the lateral intercellular interfaces in



**Figure 2.** Properties of adherens junctions in control and afadin-depleted cells. (A) Overnight cultures of control (*Control*) and afadin-depleted (*Af-sh*) A431D-EcadDn cells stained for E-cadherin. Note that afadin-depleted cells exhibit multiple openings in their intercellular contacts. (B) The distances between apical and basal margins of the randomly selected cell-cell contacts (see as the example the arrow line in A, insets) in these two cultures were monitored over 10 min and plotted over the time. Note that the afadin-depleted cells show significant fluctuations in this parameter. (C) Fluorescence intensities of the individual, randomly selected lateral adherens junctions. (D) Dendra-photoconversion assay. The graph shows changes in intensity of the red fluorescence in EcadDn adherens junctions after Dendra2 photoconversion. The initial red fluorescence of the junctions is considered 1.0. The graph represents the average of four independent experiments ( $n = 30$ ), the error bars is SD ( $n = 20$ ). Note that the afadin depletion increases the rate of cadherin turnover in the junctions.

the afadin-depleted cells than in the control cells. Similar results were obtained with A431 cells expressing EcadGFP. Therefore, relative to the control cells, the cell-cell contacts of the afadin-depleted cells are significantly less stable.

These differences in cell-cell contact stability prompted us to study the dynamics of cadherin in the individual adherens junctions. First, we compared Dendra2-specific junctional



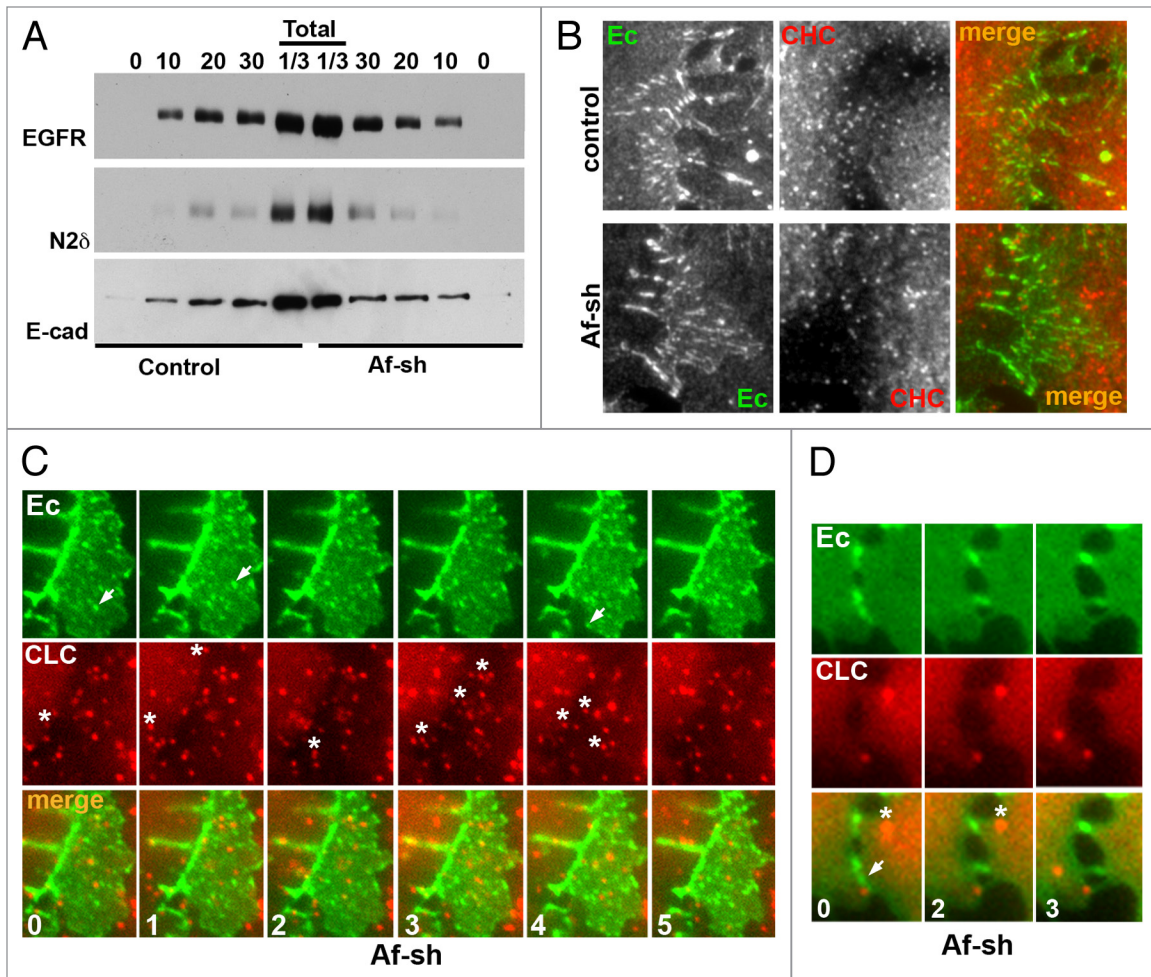
**Figure 3.** Dynamics of adherens junction assembly. **(A)** A431 cells co-expressing EcadGFP (*EcGFP*, green) and N2mCh (*N2CH*, red) were imaged in one sec intervals and with the one sec-long acquisition. Numbers indicate time (in sec). Time 0 is the moment when the cells touch one another. Note that cadherin and nectin-2 in the control cells produced cluster as fast as 2 s after 0 time. It takes about 10 s for cadherin to produce the punctum in the afadin-depleted cells. The fluorescence of the afadin-depleted punctum is much weaker. Bars 5  $\mu$ m. **(B)** Changes in fluorescent intensities of the individual EcadGFP puncta (the moments of their formation are marked by the dots) in the control and afadin-depleted cells determined from several movies like those presented in A. Before formation of the visible puncta, the graph shows the fluorescent intensities at the corresponding spots. **(C)** The changes in fluorescent intensities of EcadGFP (blue) and N2mCH (red) in punctum after their de novo formation in the experiments depicted in A. The changes are shown for 5 different punctum in each of the cultures taken from different movies. **(D)** Formation of adherens junctions in the control and afadin-depleted EcadGFP-expressing A431 cells in a calcium switch assay (numbers indicate seconds after addition of calcium). The boxed regions are magnified in the insets. Note that in contrast to the experiments presented in A, adherens junctions are rapidly produced in both types of cells. However, the fluorescence of the individual puncta (some of them are marked by arrows in the insets) steadily grow in the control cells but show significant fluctuations in the afadin-depleted cells. Bars 5  $\mu$ m. **(E)** Changes in fluorescent intensities of the individual EcadGFP puncta after their formation in the control and afadin-depleted A431 cells determined from the calcium switch experiments.

fluorescence intensities in the A431D-EcadDn cells and their afadin-depleted counterparts. The intensities were determined in cells localized at the peripheries of the epithelial colonies. **Figure 2C** shows that the intensities were reduced by about 20% in the afadin-depleted cells. Considering that the surface levels of cadherin in both cell types were the same, this difference suggested that afadin depletion might increase the dynamics of cadherin molecules in the junctions. To directly test this

idea, we used a Dendra-photoconversion assay that determines the turnover rates of cadherin molecules in the junctions. Such examination confirmed that afadin depletion increased the exchange of cadherin molecules in the individual junctions (**Fig. 2D**,  $t_{1/2}$  ~2 min vs. ~4 min in control cells).

#### The lack of afadin destabilizes the initial cadherin puncta

The observation that the differences between the wild type and afadin-depleted cells were most dramatic in the sparse

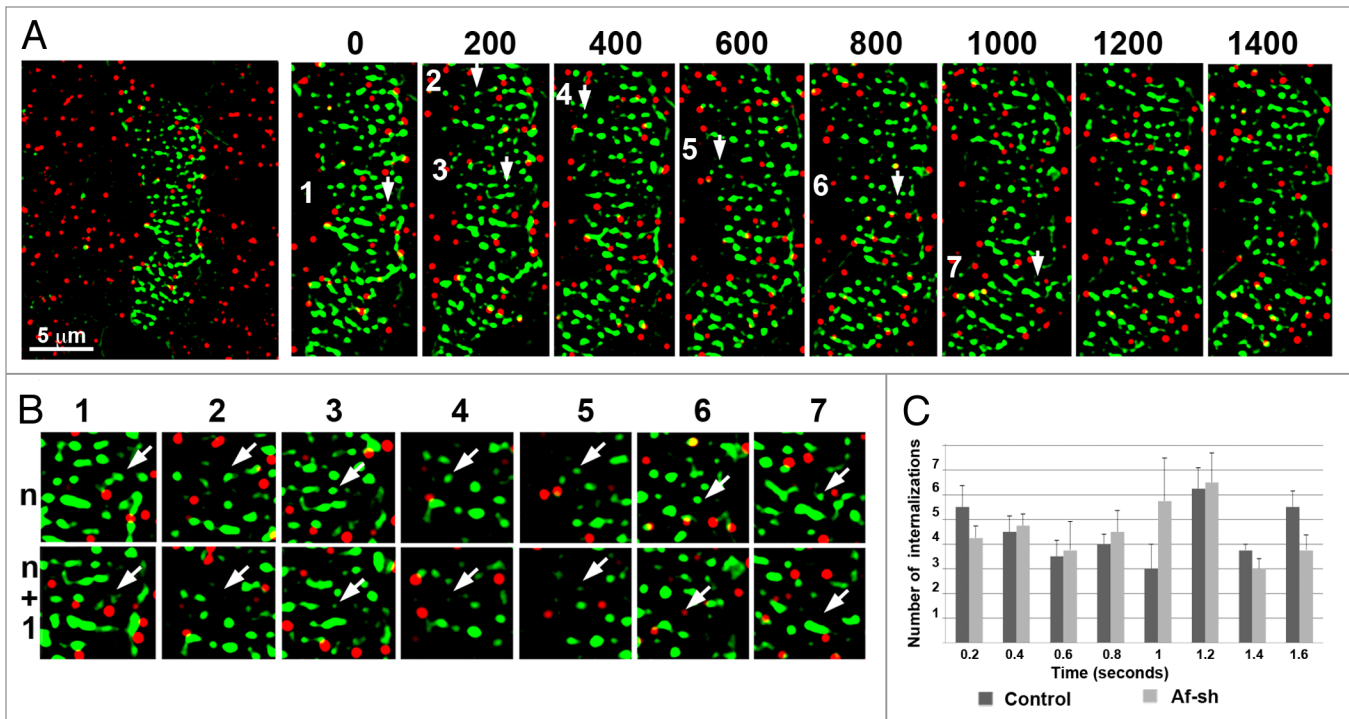


**Figure 4.** Increased cadherin turnover in the afadin-depleted cells is not caused by clathrin-dependent endocytosis. **(A)** Internalization of EGF receptor (*EGFR*), endogenous nectin-2 (*N2δ*), and endogenous E-cadherin (*E-cad*) in the control (*Control*) and afadin-depleted (*Af-sh*) overnight culture of A431 cells as measured by a biotin internalization assay. Numbers above the lanes show chase periods (in minutes). Lanes marked "Total" were loaded with the 30% of the total anti-biotin precipitates. **(B)** Control and afadin-depleted A431 cells were double-stained using anti-E-cadherin (*Ec*, green) and anti-Clathrin heavy chain (*CHC*, red) antibodies. **(C)** Time-lapse images of a cell-cell contact between two afadin-depleted A431D cells co-expressing *EcadGFP* (*Ec*, green) and mCherry-tagged Clathrin Light Chain (*CLC*) acquired at 1 s intervals (numbers show time in seconds). The contact exhibits numerous apically moving adherens junctions. Arrows indicate junctions that significantly decrease their fluorescence on the following frame. Asterisks indicate clathrin structures that disappear on the following frame apparently as a result of endocytosis. Note that the arrow-marked junctions reduce their fluorescence independently to the endocytic events. **(D)** Three consequent frames (numbers indicate seconds) from a movie of the afadin-depleted A431 cells that just establish the contact. The arrow indicates a punctum that disappears between second and third frames. Note that this punctum is devoid of clathrin. The endocytosed clathrin-coated pit is marked by the asterisk.

culture suggested that afadin is required during early steps of cell-cell contact formation. Therefore, we focused our subsequent experiments on investigating the nascent adherens junctions. To this end we performed time-lapse analyses of A431D cells co-expressing *EcadGFP* and *Ntn2-CH*. To detect earlier events of junction formation, we imaged (in 1 s intervals) the cells, starting from the moment they were closely located but not yet in a direct contact. The sequences that were obtained captured ten cases of the cell-cell contact formation in the control and afadin-depleted cell cultures. According to the published data,<sup>38</sup> in the control cells cadherin produced bright puncta immediately after two cells touch one another. The longest detected interval between the initial cell-cell contact and the following cadherin punctum formation was 3 s (Fig. 3A). After their appearance,

cadherin puncta rapidly intensified in their fluorescence (Fig. 3A and B). *Ntn2-CH* clusters always appeared at the same sites, in some cases a few seconds after the appearance of the cadherin puncta. Fluctuations of *Ntn2-CH* fluorescent intensities of puncta typically copied that of *EcadGFP* (Fig. 3C).

Afadin-depleted cells showed very different dynamics. A significant difference was in the time intervals between the initial cell-cell contact and the appearance of the first cadherin punctum: the afadin-depleted cells needed more than 5 s to produce the first punctum (Fig. 3B). Very stagnant growth of fluorescence intensities of the produced puncta was the second important feature of the afadin-depleted cells (Fig. 3B). Finally, the majority of cadherin puncta in afadin-depleted cells were unstable and disintegrated in 10–20 s after formation (Fig. 3C).



**Figure 5.** Clathrin-dependent endocytosis in cell-cell contacts. **(A)** A 1.4 s-long sequence obtained using N-SIM showing dynamics of clathrin-coated pits labeled by Clc-mCherry (red) and cadherin clusters labeled by EcadGFP (green) in the afadin-depleted A431D cells co-expressing EcadGFP and Clc-mCherry. The left image shows a low magnification of the analyzed area (time 0). Time points (in milliseconds) are indicated. The numbered arrows indicate the cadherin clusters that disappear on the following frame. **(B)** Zoomed areas containing the clusters that are numbered in A. Upper row (n) shows the last appearance of the labeled clusters; the bottom row (n+1) shows the same area 200 ms later. Note that in neither case the EcadGFP cluster disappearance corresponds to the endocytosis of the Clc-mCherry-labeled coated pits. **(C)** The bars show numbers of endocytic events noted in the 15  $\mu\text{m}^2$  areas of the cell-cell contact pooled from several movies like that shown in A.

Taken together, these imaging experiments confirmed that afadin is required for cadherin dynamics during formation of intercellular contacts.

In complimentary experiments, we analyzed cell-cell contact formation using a calcium switch assay. The EcadGFP-expressing A431D cells were cultured overnight in low calcium and started to survey just before the calcium was added into the media. **Figures 3D and E** show that calcium addition triggered immediate formation of numerous cadherin-containing puncta in both control and afadin-depleted cells. Notably, the fluorescent intensities of the resulting puncta steadily increased in the control cells, but exhibited significant fluctuations in cells lacking afadin (**Fig. 3E**).

#### Clathrin-mediated endocytosis does not consume cadherin clusters

The experiments reported above confirmed previous results obtained in static cultures that nectin-afadin system facilitates adherens junction formation. Furthermore, our results convincingly demonstrated that the nascent cadherin clusters in cells lacking afadin are significantly less stable. Such adherens junction instability was suggested to be based on the activation of the clathrin-mediated cadherin endocytosis that consumes cadherin at the sites of cell-cell contacts.<sup>29,30</sup> This possibility is consistent with our observation that afadin depletion significantly increases the rate of cadherin turnover in

the clusters, the process that might be driven by the clathrin-dependent cadherin internalization directly from the adherens junctions.<sup>25-27</sup> To test this possibility, we measured cadherin and nectin endocytosis in control and afadin-depleted cells using a cell-surface biotinylation assay. This assay failed to show any significant differences (**Fig. 4A**). However, given that only a very low fraction of the surface cadherin resides in the adherens junctions,<sup>25</sup> this biochemical assay might not determine targeted internalization of cadherin from the clusters.

The difficulties in revealing specific junctional endocytosis by biochemical assays prompted us to use two alternative approaches: pharmacological inhibition and live-cell imaging. First, we attempted to block clathrin endocytosis in the cells using available inhibitors (see the list in the Materials and Methods) of the GTPase or the lipid-binding domains of Dynamin-2, the critical protein in the internalization of the clathrin-coated pits. By using transferrin internalization assay, we found that available inhibitors either did not block clathrin-dependent endocytosis or, at high concentrations, were toxic for A431 cells.

Another way to determine the role of clathrin endocytosis in cadherin junction instability was to directly image internalization of clathrin-coated pits. Double staining for clathrin and cadherin did not show any significant increase in number of clathrin-containing structures in A431 or A431D-EcadDn cells lacking afadin in comparison with their control counterparts (**Fig. 4B**).

Clathrin endocytosis, however, is a very dynamic process that might not be detected in the static images. In order to mitigate this, we performed live imaging of the afadin-depleted A431D cells co-expressing the EcadGFP and clathrin light chain tagged at the N-terminus by mCherry (Clc-mCherry). Expression of this recombinant Clc-mCherry protein had been successfully used in the imaging of clathrin-coated pit internalization and in understanding the molecular dynamics of these structures.<sup>39</sup> Indeed, in agreement with Taylor et al.,<sup>39</sup> the Clc-mCherry assembled coated pits, many of which were localized to the cell-cell contact areas. The major feature of these structures was their short lifetime – in many cases just a few seconds (see asterisks in Fig. 4C). However, we were not able to detect any relationship between the disappearances of these clathrin-containing dynamic structures (that apparently corresponds to the single endocytic event) with the disappearance of the cadherin clusters (Fig. 4C). Even when two separate A431D-shAF cells formed initial contacts, no endocytic events accompanied the disappearance of the cadherin clusters, which, as reported above, were particularly unstable at these sites (Fig. 4D).

Another possibility is that clathrin-mediated endocytosis targets nonclustered cadherin, the process that could destabilize the clusters by reducing the local cadherin concentration. To test this possibility, we assessed the total number of the clathrin-coated pits endocytosed in the cell-cell contacts of the control and afadin-depleted cells. In order to improve the detection of endocytic events, we imaged Clc-mCherry- and EcadGFP-co-expressing cells using the super-resolution structured illumination microscopy (Fig. 5). This technique allowed the rapid dynamics of the clathrin-coated structures to be observed. Again, no clear association between the dynamics of cadherin clusters and clathrin endocytosis was detected. To approximate the rate of endocytosis in cell-cell contact areas in our cells, we calculated the numbers of endocytic events detected in 15  $\mu\text{m}^2$  areas of the cell-cell contacts in the recorded movies. This quantification (Fig. 5C) showed that the number of endocytic events in the cell-cell contact areas of both cell types was quite similar (approximately 1.5 internalizations per sec per 1  $\mu\text{m}^2$ ).

## Discussion

It has been proposed that the nectin-afadin-based adhesion plays a key role in the organization of adherens junctions. Specifically, it has been shown that the inhibition of nectin adhesion or the afadin knockdown delay the formation of adherens junctions in calcium-switch experiments.<sup>31,40,41</sup> Contrary to this data obtained in cell culture, the conditional afadin knockout resulted in defects in paracellular permeability of the intestinal epithelia and in the mislocalization of nectin,<sup>34</sup> but these defects were not associated with any significant abnormalities in ultrastructure or localization of adherens junctions. Therefore, the nectin-afadin adhesion apparently regulates some specific processes during formation or maturation of the adherens junctions, but is not required for their maintenance. In order to identify such processes, we reinvestigated the role of afadin in adherens junctions using

the combination of biochemical and live imaging experiments. In our experiments, we used A431 epithelial cells, which have no functional tight junctions. This feature of A431 cells simplifies the interpretation of the data because it excludes tight junctions as an intermediate link in cadherin-nectin interrelations. The latter possibility is indicated by the data showing that afadin directly interacts with tight junction protein JAM-A<sup>42</sup> and the nectin-afadin function is linked to the tight junction assembly.<sup>15</sup>

To inactivate the nectin-afadin adhesion, we used an approach that has been successfully applied previously<sup>1,31,34,40</sup>—knockdown of afadin expression using afadin-specific shRNAs. Immunomorphological examination of the confluent cultures of the afadin-depleted A431 cells failed to show any prominent defects either in distribution or in the number of adherens junctions. Similarly, afadin depletion did not change the total or the surface levels of cadherin. Only more detailed inspection of these cultures showed a modest decrease in the amount of cadherin in the adherens junctions and its more rapid turnover. These data are consistent with previous observations showing no changes in major characteristics of adherens junctions in the cell cultures of various epithelial cells<sup>31,40</sup> or epithelial tissues *in vivo*<sup>32,34</sup> after afadin inactivation. Therefore, as previously suggested, the basic machinery responsible for cadherin-cadherin adhesive interactions, cadherin clustering, and association of cadherin molecules with the cytoskeleton is afadin-independent.

The most dramatic abnormalities in cadherin adhesion were evident in the afadin-depleted cells located at the periphery of the epithelial colonies or in the sparse cultures: these cells exhibit significantly more loose cell-cell contacts. The same phenotype was induced by two different afadin-specific shRNAs excluding that it is caused by the shRNA's off-target effect. This observation is also completely in line with the previous data showing that the lack of afadin affects adhesion between the cells located at the margins of epithelial colonies or after the calcium switch.<sup>31,40</sup>

Our live imaging examination of adherens junctions in the afadin-depleted A431 cells expressing GFP-tagged cadherin allowed us to diagnose their defects more precisely. Using traditional calcium switch experiments, we found that both control and afadin-depleted cells produce numerous cadherin junctional puncta almost instantly after the addition of calcium ions. The lack of afadin diminishes stability of these puncta that leads to the significant fluctuations in their fluorescence. In the sparse cell cultures, we found even more dramatic differences between control and afadin-depleted cells. The nascent adherens junctions are rapidly produced once two control cells touch one another, while all phases of this process is stagnant in cells lacking afadin. As a result, afadin-depleted cells require much more time to establish their intercellular contacts.

Altogether, our data clearly showed that afadin maintains cadherin puncta stability and/or facilitates their remodeling into the mature junctions. Several key questions concerning this effect have yet to be answered. The first one is whether these cadherin-related processes are regulated by nectin adhesion or solely by the absence of afadin. Previous experiments with nectin inhibitors, such as the recombinant HSV1 glycoprotein D fragment or the extracellular region of nectin-3<sup>31,41</sup> suggested

that adhesive interactions between nectin molecules are essential. By contrast, the defect in paracellular permeability of colon epithelia was shown to be induced by afadin knockout, but not inactivation of both nectin-2 and nectin-3 genes.<sup>34</sup> However, this double nectin knockout failed to suppress afadin localization in adherens junctions suggesting that a remaining nectin, apparently nectin-1, could compensate for nectin-2 and nectin-3 insufficiency. The work of Lörger and Moelling<sup>40</sup> showed that the nectin-uncoupled afadin mutant lacking the PDZ domain could restore adherens junctions in afadin-depleted cells. However, this result might be caused by the mutant overexpression that could compensate for the lack of its direct binding to nectin. Our own experiments rather support the first possibility. Using several different nectin-2-specific shRNAs, we were able to reduce this nectin expression, the major nectin of A431 cells, by about 50%. Even such relatively mild depletion of nectin-2 also slowed down adherens junction formation (not shown).

How does the lack of afadin destabilize cadherin clusters? The works performed in the Takai laboratory suggested that the afadin deficiency deregulates the clathrin-dependent cadherin endocytosis.<sup>29,30</sup> However, using biochemical and live-imaging approaches, we were unable to reveal any role of clathrin in adherens junction instability in cells lacking afadin. In brief, no differences were detected in the rate of cadherin endocytosis between control and afadin-depleted cells using surface-biotinylation assay. Imaging of the afadin-depleted cells co-expressing EcadGFP and Clc-mCherry showed that dramatic decrease in fluorescence of adherens junctions or even their rapid disappearance were not associated with clathrin. Finally, the total number of clathrin-dependent endocytic events within the cell-cell contact interfaces was very similar in the control and afadin-depleted cells.

Another mechanism that could destabilize cadherin clusters in afadin-depleted cells is abnormalities in the interaction between cadherin and the actin cytoskeleton. Indeed, afadin directly interacts with a key protein of the cadherin-catenin complex,  $\alpha$ -catenin,<sup>11,43</sup> and apparently, with p120.<sup>30,45</sup> In addition, the afadin mutant lacking the actin-binding domain was unable to rescue the adherens junctions in the afadin-depleted cells.<sup>40</sup> Therefore, it is possible that afadin provides an additional link between the cadherin-catenin complex and the actin cytoskeleton that is critical for the stability of nascent adherens junctions. However, our recent data showing that afadin interacts with the cadherin-catenin complex primarily outside the adherens junctions is inconsistent with the function of afadin as an additional linker between cadherin clusters and actin.<sup>1</sup> Furthermore, we recently showed that cadherin adhesive clusters could be rapidly assembled using the actin-binding domain of  $\alpha$ -catenin.<sup>24</sup> The available data therefore suggest that afadin stabilizes cadherin junctions using mechanisms more complex than the direct coupling of cadherin clusters to actin filaments. An attractive possibility is that afadin has a signaling or scaffolding function that is required for the rapid remodeling of the actin cytoskeleton immediately after two cells touch one another. Once formed, for example, nectin adhesive clusters could stabilize the associated actin bundles. This stabilization may, in

turn, facilitate more efficient assembly of cadherin clusters and increase their stability. More detailed studies of actin filament dynamics and remodeling during adherens junction assembly are critical to understand the role of afadin.

In summary, our experiments demonstrate that the nectin-binding protein afadin reinforces cadherin adhesive clusters. While the exact molecular mechanism of this reinforcement is not clear, this process is crucial for fast formation of adherens junctions at the sites of new cell-cell contacts.

## Material and Methods

### Cell Culture, Antibodies, and Plasmids

Transfection and growth of human A431 and A431D cells were done as described.<sup>21</sup> The plasmids encoding human E-cadherin tagged with GFP and mouse nectin-2 tagged with mCherry were described.<sup>8,21</sup> After co-transfection and antibiotic selection, the cells were sorted for transgene expression by FACS and only moderate-expressing cells were used. To generate afadin-depleted A431 or A431D cells, the cells were transduced with lentiviruses encoding afadin-specific shRNAs (V2LHS250765 and V2LHS250765, Thermo Fisher Scientific, Waltham, MA) and infected cells were selected with puromycin treatment (5  $\mu$ g/ml). The plasmid encoding Clc-mCherry<sup>39</sup> was obtained from Addgene (#27680, Cambridge, MA).

The following antibodies were used: mouse anti-E-cadherin (Zymed Laboratories, South San Francisco, CA); rabbit anti-afadin (Sigma, St. Louis, MO); mouse anti- $\beta$ -catenin (BD, Franklin Lakes, NJ); mouse antibodies specific to nectin-2 (clone B-C12 and clone R2.525), rabbit mAb anti-nectin-2 (abCAM, Cambridge, MA). To block dynamin-2 function, several commercially available Dynamin inhibitors (all from abCAM) were tested: Dynole-34-2 and Iminodyn-22 targeting dynamin GTPase activity as well as MiTMAB and OcTMAB targeting the lipid-binding domain of dynamin.

### Immunofluorescence and live-cell imaging

Live-cell imaging and regular wide-field immunofluorescence microscopy were performed essentially as described earlier.<sup>21,25</sup> Cells grown on glass coverslips were fixed with methanol-acetone (Figs. 2, 3 and 4). For live-cell imaging, cell suspension ( $\sim 1 \times 10^5$  cells) was plated into a homemade chamber built on cover glass. The next day, the culture media was replaced with imaging media (L-15 plus 10% FBS) and the chamber was imaged by the Eclipse Ti-E microscope (Nikon, Melville, NY) at 37 °C controlled with Nikon's NIS-Elements software. The microscope was equipped with an incubator chamber, a CoolSNAP HQ2 camera (Photometrics, Tucson, AZ), Plan Apo 60x/1.40 and Plan ApoVC 100x/1.40 lenses and halogen and mercury light sources. Time-lapse images were taken in both FITC and TRITC channels simultaneously using a beam splitter in one-second intervals using halogen light that minimized phototoxicity and photobleaching. All images were saved as Tiff files and processed using ImageJ software (National Institutes of Health). Structured Illumination Microscopy was performed as described previously (Indra et al., 2013). The images were taken using a Nikon



N-SIM system available in the imaging center of Northwestern University and were then analytically processed using Nikon element version 4 software. N-SIM-obtained movies were made using 100 msec image acquisition time. Green and red images in these sequences were obtained one after another.

#### Biotinylation of cell surface proteins

The endocytosis of biotinylated proteins was determined essentially as earlier described (biotin internalization assay, ref. 45). In brief, the cells of one 6-cm dish reaching near confluent growth were washed with ice-cold PBS containing 0.2 mM CaCl<sub>2</sub> (PBS-Ca). The plate was incubated at 4 °C with 2 ml of 0.5 mg/ml of sulfo-NHC-LC-SS-biotin (Pierce Chemical Co, Rockford, IL) in PBS-Ca for 10 min. The reaction was stopped by washing the cells in ice-cold glycine solution (200 mM glycine /200 mM Tris, pH 7.5). The cells were then incubated in standard medium (37 °C) for various durations to resume endocytosis. Noninternalized biotin was then stripped from the

surface by two 20 min-long washes with 50 mM glutathione at 4 °C. Remaining biotinylated proteins were then precipitated by streptavidin-agarose and analyzed by immunoblotting.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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