Regulation of epithelial wound closure and intercellular adhesion by interaction of AF6 with actin cytoskeleton

Mihaela Lorger and Karin Moelling*

Institute of Medical Virology, University of Zurich, Gloriastrasse 30, CH-8006 Zurich, Switzerland

*Author for correspondence (e-mail: moelling@immv.unizh.ch)

Accepted 28 April 2006
Journal of Cell Science 119, 3385-3398 Published by The Company of Biologists 2006
doi:10.1242/jcs.03027

Summary

AF6 is a human multi-domain protein involved in signaling and organization of cell junctions during embryogenesis. Its homologue in rat is called afadin. Three different AF6 transcripts are known, but only isoform 1 (AF6i1) has been characterized as protein. We focused on the AF6 isoform 3 (AF6i3), which differs from the AF6i1 by an additional C-terminal F-actin-binding site. Knockdown of AF6i3 in epithelial cells, which express only this isoform, resulted in impaired E-cadherin-dependent intercellular adhesion due to concomitantly reduced association of E-cadherin with F-actin and p120-catenin. Impaired intercellular adhesion also accelerated wound closure due to increased directionality of cell migration and delayed de novo formation of cell junctions. In contrast to AF6i3, the AF6i1 displayed a reduced association with the actin cytoskeleton and did not stabilize intercellular adhesion. Therefore, we propose that the AF6i3 protein stabilizes E-cadherin-dependent adhesion during dynamic processes, such as wound closure and formation of cell junctions, by linking the E-cadherin–catenin complex to the actin cytoskeleton via its F-actin-binding site.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/119/16/3385/DC1

Key words: AF6 isoform 3, F-actin-binding site, E-cadherin, Cell-cell adhesion, Collective cell migration, Directionality

Introduction

AF6 is a tumor-suppressor-like protein that has been first described as a fusion partner of the acute lymphoblastic leukemia gene *MLL* (also known as *ALL-1*), which arises by a chromosomal translocation t(6;11) (Prasad et al., 1993). AF6 is a multi-domain protein with two Ras-association (RA) domains and one PSD-95–Dlg-1–ZO-1 (PDZ) domain (Schneider et al., 1999) (Fig. 1A). Three different human AF6 transcripts generated by alternative splicing have been identified (Saito et al., 1998) (Fig. 1A). The AF6 protein transcribed from the shortest transcript has been termed AF6 isoform 1 (AF6i1; P55196-1) or simply as AF6. The two longer AF6 transcripts, termed AF6 isoform 2 (AF6i2; P55196-2) and isoform 3 (AF6i3; P55196-3) (Fig. 1A) harbor additional C-terminal regions, which differ between these two isoforms in respect to their sequence; their functions, however, are unknown.

Human AF6 protein shares 92% amino acid identity with its rat homologue afadin (Mandai et al., 1997). The shorter of the two afadin isoforms, s-afadin, corresponds to the human AF6i1 (Fig. 1A). The longer isoform, l-afadin, contains an additional C-terminal region, which harbors an F-actin-binding site (Mandai et al., 1997) (Fig. 1A). The biological function of this domain has so far not been investigated.

AF6-knockout mice have impaired cell junctions during embryogenesis (Ikeda et al., 1999; Zhadanov et al., 1999). AF6 is localized at epithelial adherens junctions (Mandai et al., 1997; Buchert et al., 1999). They consist of two adhesion systems: the nectin-afadin and the E-cadherin–β-catenin–α-catenin system (for reviews, see Nagafuchi, 2001; Takai and Nakanishi, 2003; D'Souza-Schorey, 2005; Perez-Moreno et al., 2003). E-cadherin-dependent adhesion is stabilized by its association with the actin cytoskeleton through α-catenin and β-catenin (Imamura et al., 1999) and by its interaction with p120-catenin (Thoreson et al., 2000; Reynolds and Carnahan, 2004). AF6 promotes the latter interaction in a Rap1-dependent manner (Hoshino et al., 2005). AF6 and afadin further interact via their PDZ domains with the C-terminus of nectin (Takahashi et al., 1999; Miyahara et al., 2000), and nectin has also been shown to promote the E-cadherin-dependent cell-cell adhesion in epithelial cells (Honda et al., 2003a; Honda et al., 2003b; Peng et al., 2002; Tanaka et al., 2003).

Human AF6 shares 51% identity with its *Drosophila* homologue canoe (Miyamoto et al., 1995) (Fig. 1A). Canoe is required for the proper dorsal closure in embryos (Takahashi et al., 1998; Boettner et al., 2003), a process in which collective cell migration is involved (for reviews, see Nabeshima et al., 1999; Lee and Gotlieb, 2003; Friedl, 2004; Friedl et al., 2004). Collective cell migration is characterized by the maintenance of dynamic intercellular contacts between strongly cell-cell adhesive cells that exhibit coordinated movement (Nabeshima et al., 1995; Nabeshima et al., 1997; Farooqui and Fenteany, 2005). A possible involvement of AF6 or afadin in this process has so far not been investigated.

In this study we focused on AF6i3 and analyzed its function

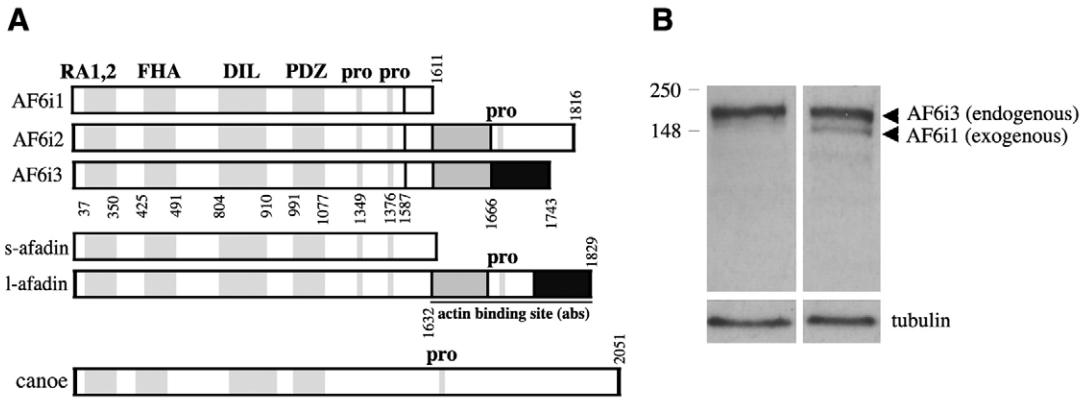

Fig. 1. Domain structure of AF6, afadin and canoe. (A) Domain structure of the three AF6 isoforms (AF6i1, AF6i2 and AF6i3), the two isoforms of its rat homologue afadin (long l-afadin and short s-afadin) and its *Drosophila* homologue canoe. Aa positions of individual AF6 domains are annotated. RA1,2: Ras-associating domain 1 and 2; FHA: forkhead associated domain; DIL: dilute domain; PDZ: PSD-95–Dlg-1–ZO-1 domain; pro: proline-rich domain. (B) Western blot analysis of HEK293 whole-cell lysate, using anti-AF6 antibody, showing the endogenous AF6i3 protein (left) and the endogenous plus the overexpressed AF6i1 protein (right).

by small hairpin RNA (shRNA)-mediated knockdown in epithelial cells. AF6i3 knockdown impaired E-cadherin-dependent intercellular adhesion by two mechanisms. By reduced association of E-cadherin with (1) p120-catenin and, (2) the actin cytoskeleton. This resulted in accelerated epithelial wound closure and delayed de novo formation of cell junctions. Rescue experiments with the AF6i1 and different AF6i3 deletion mutants identified the F-actin-binding site as the major domain involved in stabilization of intercellular adhesion during these processes.

Results

Cloning of AF6i3 protein

AF6i1 protein overexpressed in HEK293, HeLa and human mammary epithelial MCF10A cells, was running at a lower molecular weight than the endogenous AF6 protein (Fig. 1B and data not shown), indicating that it corresponded to a distinct isoform. To identify this AF6 isoform, the endogenous AF6 protein was immunoprecipitated from HEK293 whole-cell lysate and submitted to protein sequencing by using matrix-assisted laser-desorption ionization-time-of-flight (MALDI-TOF) mass spectrometry. The resulting peptide sequences (supplementary material Fig. S1A) were aligned against the protein sequences from the NCBI database. Two of the sequences were specific for the AF6i3 (supplementary material Fig. S1A, red boxes) and absent from AF6i1 and AF6i2. This defined AF6i3 as the major isoform expressed in HEK293 cells.

AF6i3 contains an additional 132 residue-long C-terminal region downstream of aa 1587 (Fig. 1A). In comparison to the rat l-afadin (Mandai et al., 1997), the AF6i3 C-terminus (AF6i3 CT) lacks a region of 64 aa that harbors the third prolin-rich domain (Fig. 1A). The rest shows 84% identity to the C-terminus of l-afadin.

The C-terminal region of AF6i3 interacts with filamentous actin

The C-terminal region of the rat l-afadin (aa 1632 to 1829; Fig. 1A) binds to F-actin (Mandai et al., 1997). To test this for the AF6i3 CT, we performed an F-actin co-sedimentation assay with GST-fusion proteins (GST-AF6i3 CT; Fig. 2Aa). Indeed, the longer form of GST-AF6i3 CT co-sedimented with F-actin (Fig. 2Ab). By testing two smaller regions of the AF6i3 CT (Fig. 2Aa,c), we were able to show that only the very C-terminal part of AF6i3 (aa 1667-1743) co-sedimented with F-actin. Thus, this sequence alone was necessary and sufficient for the binding to F-actin.

To test whether the AF6i3 CT interacted with F-actin also within living cells, HeLa cells were transfected with the hemagglutinin (HA)-tagged AF6i3 CT constructs (Fig. 2Aa) and analyzed by immunofluorescence using anti-HA antibody (Fig. 2B, green). F-actin was detected with phalloidin-TRITC (Fig. 2B, red). As can be seen in merged pictures (Fig. 2B, right panel), the colocalization with F-actin occurred for the two constructs containing the extreme C-terminus of AF6i3 (aa 1667-1743), whereas no colocalization was observed with AF6i3 CT (aa 1588 to 1666). Thus, the F-actin binding is mediated by the very C-terminal sequence of AF6i3 in vitro and in vivo.

Endogenous AF6 protein and the overexpressed full-length myc-tagged AF6i3 (myc-AF6i3) displayed similar localization predominantly at cell junctions (Fig. 2C). In contrast to the AF6i3 CT, the full-length protein colocalized with F-actin only at cell-cell contacts (Fig. 2C), indicating that their interaction occurred only in this compartment.

Effect of AF6i3 knockdown on epithelial wound closure

Canoe, the AF6 homologue in *Drosophila*, is required for the proper dorsal closure in the embryo (Takahashi et al., 1998; Boettner et al., 2003). In this process, collective migration of epithelial cells as a cell sheet is involved. Since the involvement of AF6/afadin in this process has not been studied so far, we decided to address this possibility. Focusing on the AF6i3 protein, we investigated its role during collective migration of the strongly cell-cell adhesive human mammary epithelial cell line MCF10A by AF6i3 knockdown. By western blot analysis, we confirmed that AF6i3 is the major AF6 isoform expressed in this cell type (data not shown). MCF10A cells that stably express the shRNA specific for all AF6 protein isoforms (AF6 shRNA) and cells that express

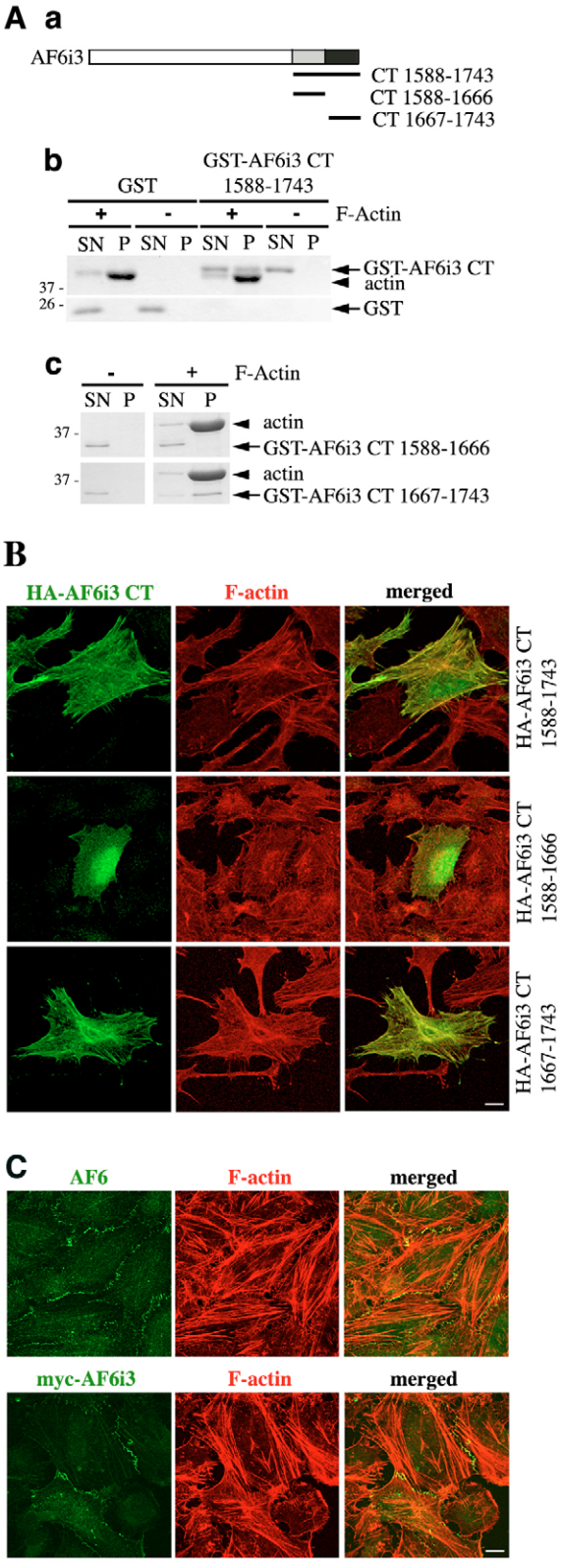

Fig. 2. Interaction of the AF6i3 C-terminus (AF6i3 CT) with F-actin. (Aa) Schematic representation of the AF6i3 CT constructs used in F-actin co-sedimentation assay (GST-tagged) and immunofluorescence (HA-tagged). (Ab,c) F-actin co-sedimentation assay performed with the GST fusion protein of (b) the whole AF6i3 CT (aa 1588-1743) or (c) the two C-terminal fragments (aa 1588-1666 and 1667-1743). F-actin-containing pellet (P) and the supernatant (SN) were analyzed on a denaturing protein gel and stained with Coomassie Blue. GST-fusion proteins or GST alone (arrows) and actin (arrowhead) are annotated. (B) Colocalization of the HA-tagged AF6i3-CT proteins (anti-HA antibody, green) and F-actin (red) in HeLa cells is depicted in merged pictures (right column). Bar, 20 μm. (C) Colocalization of endogenous (AF6; anti-AF6 antibody) and overexpressed full-length AF6i3 protein (myc-AF6i3; anti-myc antibody) (green) with F-actin (red). Bar, 20 μm.

control shRNA were generated. Expression of AF6 shRNA resulted in an approximately 90% reduction of endogenous AF6i3 protein levels (Fig. 3A).

We chose the MCF10A cell line because of its epithelial character. Confluent monolayers of these cells showed epithelial morphology with a prominent cortical actin cytoskeleton (data not shown) and predominant localization of junctional proteins AF6i3 (Fig. 3A), nectin-1, E-cadherin, α-catenin, β-catenin and claudin-1 to cellular junctions (data not shown).

Epithelial-cell-sheet migration was analyzed in a wound healing assay. Cells grown in the fully supplemented growth medium displayed strong scattering after wounding. We avoided this by starving the cells, which stabilized cell-cell adhesion. Migration was then initiated by wounding the confluent cell monolayer in epidermal growth factor (EGF)-containing medium (Fig. 3B). We measured the wound area that was covered by migrating cells within 13 hours after wounding, by subtracting the wound area at 13 hours from the wound area at the time point of wounding. After 13 hours, the wound area covered by AF6i3 knockdown cells was 30% larger than the wound area covered by control cells (Fig. 3B,C). Thus, AF6i3 knockdown accelerated the epithelial wound closure.

Mechanism involved in accelerated wound closure in AF6i3-knockdown cells

To further investigate the mechanism underlying faster epithelial wound closure in AF6i3-knockdown cells, time-lapse analysis of the cell movement during the wound healing assay was performed. Cells at the wound margin were tracked for 10 hours (Fig. 4A), resulting in corresponding trajectories (Fig. 4B). Surprisingly, cell velocity of cells within the epithelial cell sheet, defined by total path length of one cell over time, was not significantly altered in AF6i3-knockdown cells (data not shown). We therefore further determined the directionality of migration, defined by the ratio of the direct distance (D) from the cell-track start point to the end point, divided by total path length traversed by the cell (T), termed D/T ratio. In contrast to the cell velocity, the directionality of migration was increased by approximately 30% in AF6i3-knockdown cells (Fig. 4C). Thus, the main reason for accelerated wound closure of these cells was an increased directionality of migration.

We monitored cell proliferation by counting cell divisions using time-lapse video recordings (supplementary material Movies 1 and 2). Within 10 hours of wound closure we counted less than 2.5 divisions per hour in both AF6i3-knockdown and control cells. Therefore, proliferation did not significantly contribute to the wound closure under these conditions.

During migration, gaps appeared and disappeared between

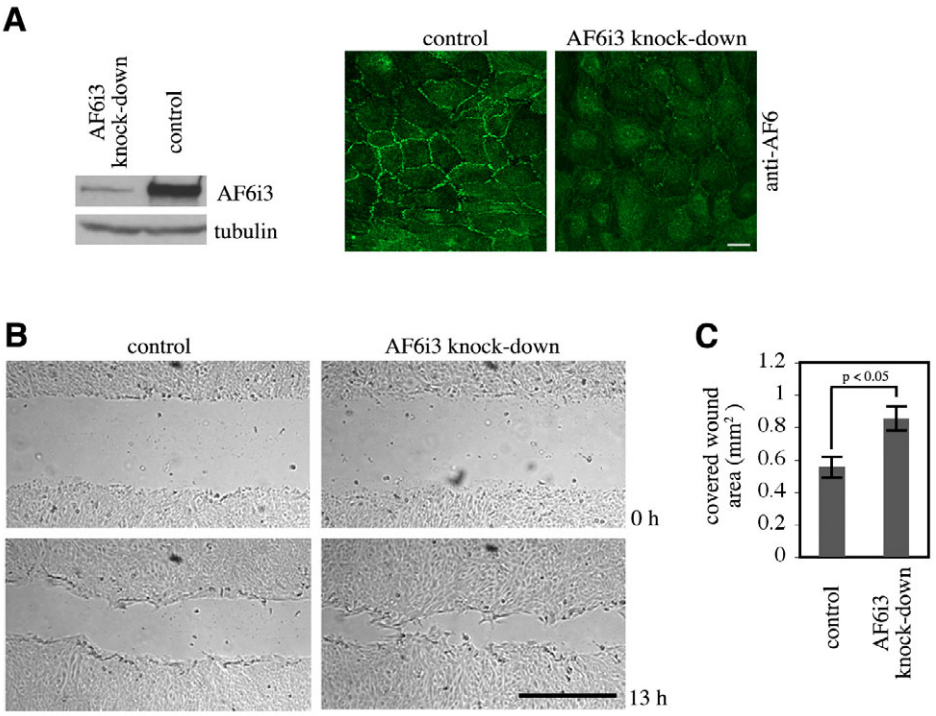

Fig. 3. AF6i3 knockdown in MCF10A cells accelerates epithelial wound closure. (A) (left) Western blot analysis of whole-cell lysate from MCF10A cells stably expressing AF6 shRNA (AF6i3 knockdown) or control shRNA (control). (Right) Immunofluorescence images of cell monolayers. Bar, 20 μm. (B) Light-microscopy images of control and AF6i3-knockdown cells immediately after wounding (0 h; top) and 13 hours later (13 h; bottom). Bar, 0.5 mm. (C) Graphic representation of the covered wound area 13 hours after wounding. Values for ±s.d. were derived from three independent experiments. Significant differences (p) were determined with the paired two-tailed Student's *t*-test.

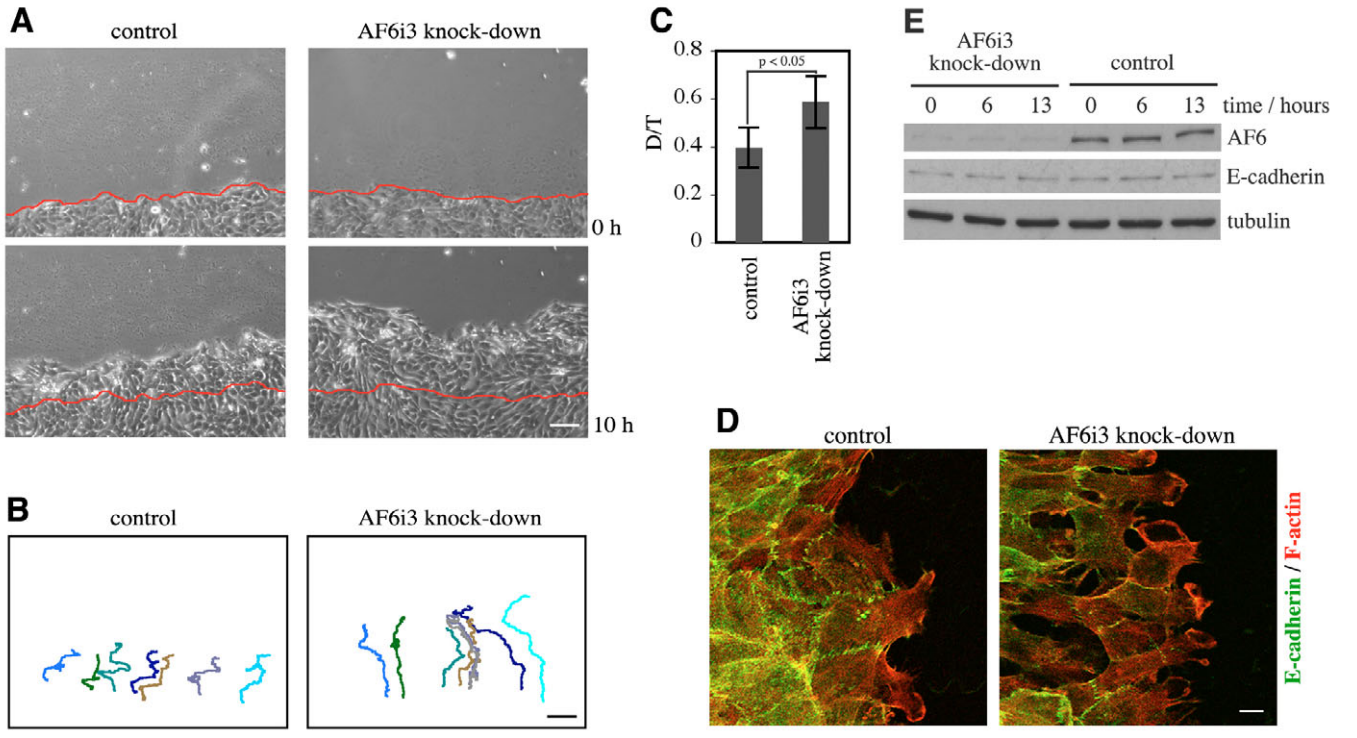

Fig. 4. Time-lapse analysis reveals increased directionality of migration and impaired intercellular adhesion in AF6i3-knockdown cells. (A) Time-lapse images showing the wound closure of AF6i3-knockdown and control cells at the time of wounding (0 h) and 10 hours later (see also supplementary material Movies 1 and 2). Red lines mark the boundary of the wound at time point 0 seconds. Bar, 0.1 mm. (B) Representative trajectories of cells during wound healing assay shown in A, followed from time 0 for 10 hours. Bar, 0.1 mm. (C) Graphic representation of the directionality of migration, defined by the D/T ratio, which represents the direct distance from the cell track start to the end point (D) divided by the total path length (T). Data are derived from three independent experiments. (D) Wound margin of migrating AF6i3-knockdown and control cells 10 hours after wounding. green, anti-E-cadherin antibody; red, phalloidin-TRITC. Bar, 10 μm. (E) Western blot analysis of whole-cell lysates during wound healing assay.

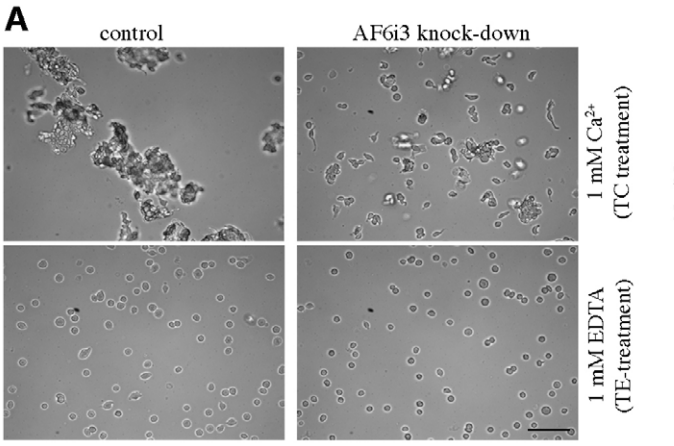

Fig. 5. AF6i3 knockdown reduces E-cadherin-dependent adhesion in dissociation assay. (A) Light-microscopy images of cells trypsinised in the presence of 1 mM Ca^{2+} (top) or 1 mM EDTA (bottom) and dissociated by pipetting. Bar, 0.125 mm. (B) Graphic representation of the extent of cell dissociation, represented by index N_{TC}/N_{TE}. N_{TC} and N_{TE} correspond to the total particle number after treatment of cells in the presence of Ca^{2+} or EDTA, respectively. Values for ±s.d. were derived from three independent experiments.

AF6i3-knockdown cells (supplementary material Movie 2), whereas the control cells retained the integrity of the cell layer (supplementary material Movie 1). This implied a weaker cell-cell adhesion in AF6i3-knockdown cells. In conjunction with this, interruptions of E-cadherin positive cell-cell contacts between migrating AF6i3-knockdown cells at the wound margin were revealed by immunofluorescence with an antibody against the junctional protein E-cadherin (Fig. 4D, green), whereas the control cells maintained intact intercellular contacts. Protein level of endogenous AF6i3 and E-cadherin stayed constant during the assay and E-cadherin amount did not differ between AF6i3-knockdown and control cells (Fig. 4E). This indicates that AF6i3 knockdown reduces the E-cadherin-dependent adhesion during migration, without affecting E-cadherin protein stability.

To elucidate this further, we performed a dissociation assay of confluent cell monolayers. When trypsinized in the presence of EDTA (TE-treatment), which disturbs E-cadherin-dependent adhesion by chelating Ca^{2+}, control and AF6i3-knockdown cells both dispersed to single cells after pipetting (Fig. 5A, bottom). By contrast, after trypsinization in the presence of Ca^{2+} (TC-treatment), which stabilizes existing E-cadherin-dependent adhesion, the control cells remained in large clusters, whereas the AF6i3-knockdown cells dispersed to single cells and smaller clusters (Fig. 5A, top). The extent of dissociation was represented by the N_{TC}:N_{TE} ratio, where N is the number of particles for TE and TC-treatment (Fig. 5B). This ratio was significantly decreased by AF6i3 knockdown, confirming a reduced E-cadherin-dependent adhesion.

To analyze whether there was a direct link between the directionality of cell migration and cell-cell adhesion, we analyzed whether cells that were not involved in cell-cell interactions, also displayed increased directionality as a consequence of AF6i3 knockdown. To hinder cells from forming cell-cell contacts, they were seeded at a very low density (Fig. 6A, top) and tracked for 2 hours after stimulation with EGF (Fig. 6A, bottom). At low density, the cells showed random migration. No difference in cell velocity or directionality of migration was observed between AF6i3-knockdown and control cells under these conditions (Fig. 6B). Thus, AF6i3-knockdown cells displayed an increased directionality only when they moved as cell sheets with close cell-cell interactions. Therefore, the directionality of migration appeared to be dependent on AF6i3-mediated intercellular adhesion.

Furthermore, we considered whether AF6i3 acts by destabilizing E-cadherin-dependent adhesion. We therefore blocked the E-cadherin trans-interaction with HECD-1 antibodies against E-cadherin (Pignatelli et al., 1992) (Fig. 6C) and performed time-lapse analysis of wound closure. Strikingly, we found that the inhibition of E-cadherin trans-interaction indeed accelerated wound closure (Fig. 6D) and increased the directionality of cell migration (Fig. 6E,F), similar to the effect seen when AF6i3 was knocked down. Thus, AF6i3 knockdown very likely increased the directionality of migration by reducing the E-cadherin-dependent adhesion.

In addition to intercellular adhesion, AF6i3 knockdown might alter other mechanisms involved in the regulation of cell directionality during collective cell migration, including actin polymerization and cell-matrix interactions. The small Rho GTPases Rac1 and Cdc42 are known modulators of actin polymerization and intercellular adhesion (Kaibuchi et al., 1999). We therefore analyzed their localization and activity. Immunofluorescence analysis revealed that Rac1 was recruited to the leading edge of migrating cells with similar kinetics in AF6i3-knockdown and control cells (supplementary material Fig. S3A). Rac1, furthermore, displayed similar activation levels and kinetics, as shown by Rac1-activation assay (supplementary material Fig. S3B) and equally unaltered protein levels during wound closure (Fig. S3D). By contrast, we were not able to detect activated Cdc42 in lysates of migrating cells (Fig. S3C) and Cdc42 was not markedly recruited to the leading edge upon stimulation (Fig. S3A), indicating that it was not significantly activated in MCF10A cells under these conditions. We also directly addressed the change in actin accumulation at the leading edge of migrating cells by staining F-actin with phalloidin (supplementary material Fig. S2A), but, again, could not detect any difference between AF6i3-knockdown and control cells.

To address the change in cell-matrix interactions, we analyzed the formation of focal adhesions by staining with anti-vinculin and anti-focal-adhesion kinase (FAK) antibodies (supplementary material Fig. S2B,C). We could not detect any change in the formation of focal-adhesion contacts in AF6i3-knockdown cells. Thus, AF6i3 knockdown did not seem to

affect the cell-matrix interactions, small Rho GTPases or the actin accumulation at the leading edge during migration. These findings correlate well with the lack of migratory phenotype in individually migrating AF6i3-knockdown cells. Therefore, we propose that there is a direct link between the reduced E-cadherin-dependent intercellular adhesion and increased directionality of cell migration, and that both are regulated by the AF6i3 protein.

Impact of individual AF6i3 domains on wound closure

Next, we characterized the involvement of individual AF6i3 domains in the regulation of wound closure. We constructed AF6i3-deletion mutants that lack either the Ras-associated domain 1 (RA1), which interacts with Ras and Rap1 (Linnemann et al., 1999) (AF6i3ΔRA1), or the PDZ domain, which interacts with junctional adhesion molecule 1 (JAM-1) and nectin (AF6i3ΔPDZ) (Fig. 7A). We used AF6i1, which lacks the F-actin-binding domain, to analyze the contribution of this domain. All constructs were made resistant to shRNA-mediated degradation by introduction of six silent point mutations within the targeted region; constructs were then stably expressed in AF6i3-knockdown cells, to allow for their analysis in the absence of endogenous AF6i3. Protein expression levels were adjusted to be similar, except for expression of AF6i3ΔRA1, which was expressed at a slightly lower level (Fig. 7A).

Re-expression of the full-length AF6i3 protein, as well as expression of the mutant AF6i3 lacking the RA1 or the PDZ domain, resulted in a decreased velocity of wound closure, similar to the wild-type phenotype (Fig. 7B,C). This confirmed the specific requirement for AF6i3 protein in the regulation of wound closure, and showed that the RA1 and the PDZ domains – and consequently the Ras or Rap1 signaling and interaction with nectin or JAM-1 – are not involved in this process. By contrast, expression of AF6i1 resulted in unchanged wound closure and thereby resembled the behavior of AF6i3-knockdown cells (Fig. 7B,C). Thus, the F-actin binding site is crucial for the regulation of wound closure.

AF6i1 and AF6i3 were expressed at comparable levels (Fig. 7A, bottom) and found to be indistinguishably located at cell-cell contacts within the confluent cell monolayer (Fig. 7D). Because the two isoforms differed in the presence of the F-actin-binding site, we analyzed their detergent solubility, because this is an established method for analyzing protein-cytoskeleton interactions (Stuart and Nigam, 1995). Confluent monolayers of AF6i3-knockdown cells reconstituted with AF6i1 or AF6i3 were extracted with 0.5% Triton X-100. The detergent-soluble supernatant (S) and the insoluble residue (R) were analyzed by western blotting (Fig. 7E). Detergent insolubility of proteins is an indication for their association with actin cytoskeleton.

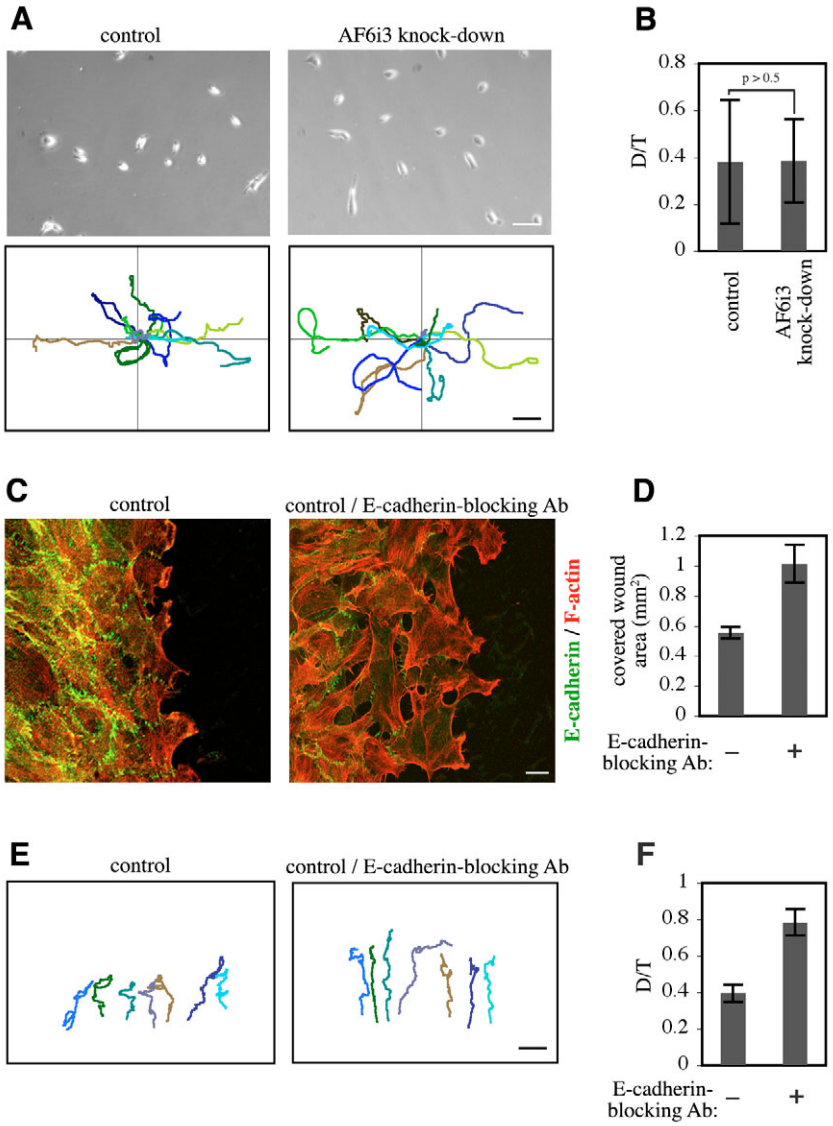

Fig. 6. Increased directionality of migration in AF6i3-knockdown cells is due to reduced intercellular adhesion. (A) AF6i3-knockdown and control cells were seeded at a low cell density (top), stimulated with EGF and tracked for two hours, resulting in corresponding trajectories (bottom). Start points of all trajectories were set to the same origin cross-point. Ten representative trajectories per sample are shown. Bar, 0.1 mm. (B) Graphic representation of the directionality of migration for cells shown in A, determined as described for Fig. 4C. Values for ±s.d. were derived from four independent experiments. (C-F) Wound healing assay of control cells in the presence or absence of HECD-1 antibodies, which block E-cadherin trans-interaction. (C) Wound margin 10 hours after wounding. Green, anti-E-cadherin antibody; red, phalloidin-TRITC. Bar, 10 μm. (D) Graphic representation of wound healing assay. Covered wound area 13 hours after wounding of control cell monolayers is depicted. Values for ±s.d. were derived from three independent experiments. (E) Representative trajectories of control cells in the presence or absence of HECD-1 antibody during wound healing assay, tracked for 10 hours. Bar, 0.1 mm. (F) Graphic representation of directionality of migration for cell tracks shown in E, determined as described for Fig. 4C.

Only 25±6% of AF6i1 was detergent insoluble, whereas approximately 45±9% of AF6i3 remained in the insoluble fraction, further indicating its interaction with F-actin in vivo. We propose that this interaction stabilizes intercellular adhesion during migration.

Regulation of E-cadherin-dependent adhesion by AF6i3

The interaction between E-cadherin and p120-catenin inhibits E-cadherin endocytosis from the cell surface by a yet unknown mechanism, resulting in stabilization of E-cadherin-dependent adhesion (Thoreson et al., 2000; Reynolds and Carnahan, 2004). Stabilization of this interaction by AF6/afadin in a Rap1-dependent manner has recently been demonstrated (Hoshino et al., 2005). We therefore analyzed the interaction between p120-catenin and E-cadherin by co-immunoprecipitation. Indeed, 6 hours after wounding, the interaction between p120-catenin and E-cadherin was significantly decreased in AF6i3-knockdown cells in comparison to the control (Fig. 8C,D). However, the Rap1 inhibitor (Fig. 8E) and the deletion of RA1 domain in AF6i3 (Fig. 7B) had no effect on wound closure in our system, indicating a Rap1-independent stabilization of E-cadherin–p120-catenin interaction by AF6i3.

Association of E-cadherin with the actin cytoskeleton via α-catenin and β-catenin also stabilizes intercellular adhesion (Imamura et al., 1999). Our data suggested that the interaction between AF6i3 and the actin cytoskeleton also stabilized E-cadherin-dependent adhesion during migration. We therefore analyzed the effect of AF6i3 knockdown on the association of the E-cadherin–catenin complex with the cytoskeleton by

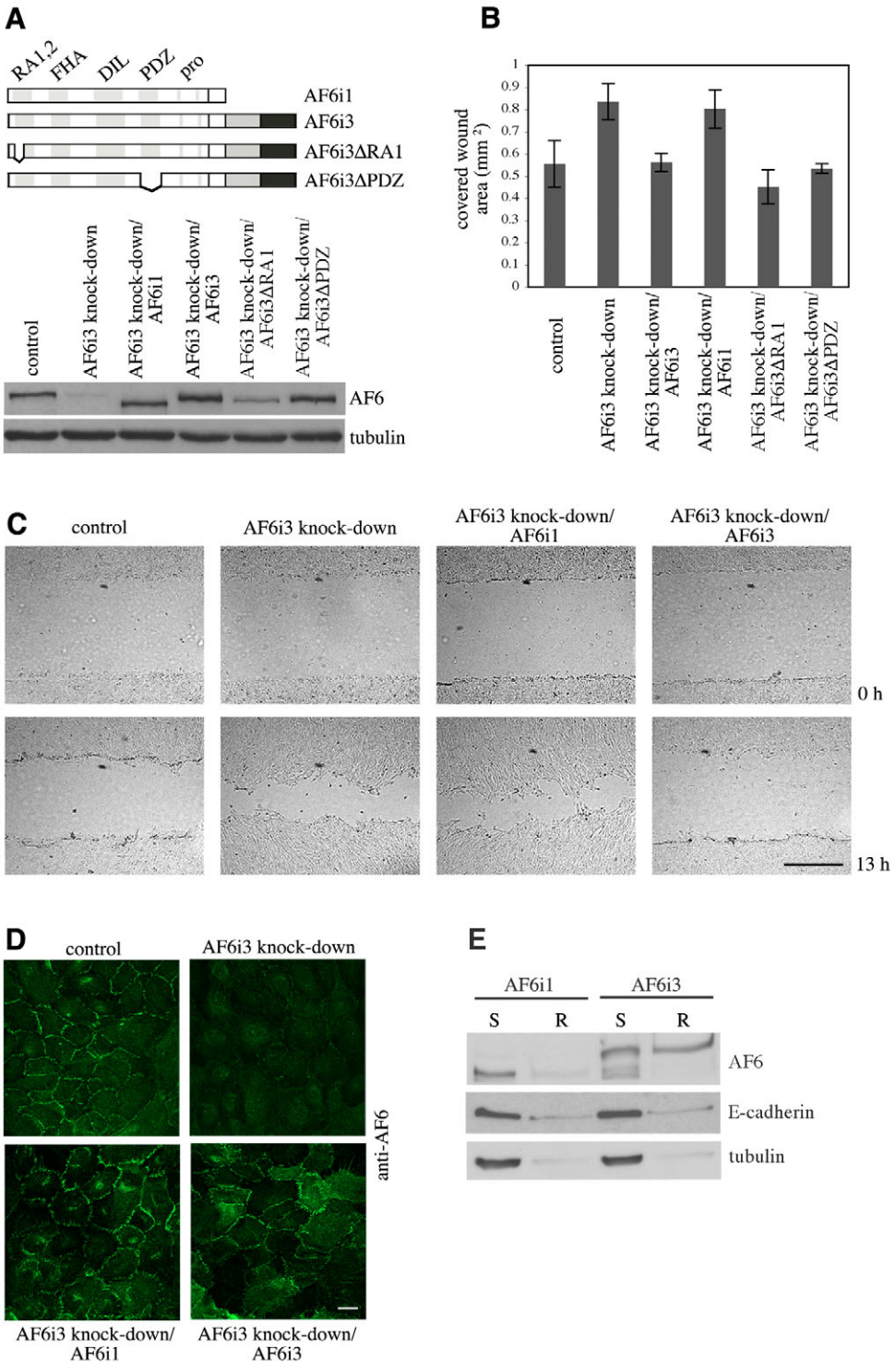

Fig. 7. AF6i3 deletion mutants in wound healing assay: a crucial role of the F-actin-binding site. (A) Schematic representation of AF6i1, AF6i3 and AF6i3 deletion mutants, AF6i3ΔRA1 and AF6i3ΔPDZ (top). Expression levels of shRNA-resistant AF6 constructs stably expressed in AF6i3-knockdown cells (bottom). (B) Graphic representation of wound closure 13 hours after wounding of AF6i3-knockdown cell monolayers reconstituted with mutant constructs as indicated. Values for ±s.d. were derived from three independent experiments. (C) Light microscopy pictures of control and AF6i3-knockdown cells without or with reconstitution by AF6i1 and AF6i3 immediately after wounding (0 h) and 13 hours later. Bar, 0.5 mm. (D) Immunofluorescence pictures of confluent cell monolayers of cells shown in C. Bar, 20 μm. (E) Western blot analysis of detergent-soluble supernatant (S) and the insoluble residue (R) of AF6i3-knockdown cell monolayers reconstituted with AF6i1 or AF6i3.

determining the detergent-solubility of E-cadherin and catenins. Prior to initiation of migration (0 hours) comparable amounts of E-cadherin and other analyzed proteins were present in detergent-insoluble fraction in AF6i3-knockdown and control cells (Fig. 8A,B). By contrast, 6 hours after wounding, the amounts of detergent-insoluble E-cadherin, β-catenin and p120-catenin were reduced in AF6i3-knockdown cells compared with control cells, whereas ZO-1 levels were not altered (Fig. 8A,B). Thus, AF6i3 knockdown resulted in reduced association of the E-cadherin, β-catenin and p120-catenin with the actin cytoskeleton, specifically during migration.

We further asked whether the Ras protein is involved in AF6i3-dependent intercellular adhesion, by using the inhibitors of its downstream targets Raf1 and PI 3-kinase. The Raf1 inhibitor abolished the migration of AF6i3-knockdown and control cells (Fig. 8E), but we did not find a difference in the degree of phosphorylation of ERK1/2 downstream of Raf1 during migration (Fig. 8F). The PI 3-kinase inhibitor reduced the wound closure in AF6i3-knockdown and control cells to a similar extent (Fig. 8E), indicating that this pathway is independent of AF6i3. Thus, although both the Raf1 and PI 3-kinase pathways downstream of Ras were essential for wound closure, they were independent of AF6i3. This is in agreement with our data obtained with the AF6i3ΔRA1 protein.

In conclusion, we showed that AF6i3 knockdown decreases E-cadherin-dependent intercellular adhesion by concomitantly destabilizing the association of E-cadherin with the actin cytoskeleton and with p120-catenin, resulting in accelerated wound closure due to increased migratory directionality.

Analysis of de novo cell-cell contact formation in AF6i3-knockdown cells

AF6 knockdown in mice results in improper organization of cell junctions during embryogenesis (Ikeda et al., 1999; Zhadanov et al., 1999). Furthermore, we demonstrated that E-cadherin-dependent adhesion was impaired in AF6i3-knockdown cells (Fig. 5). However, E-cadherin concentration

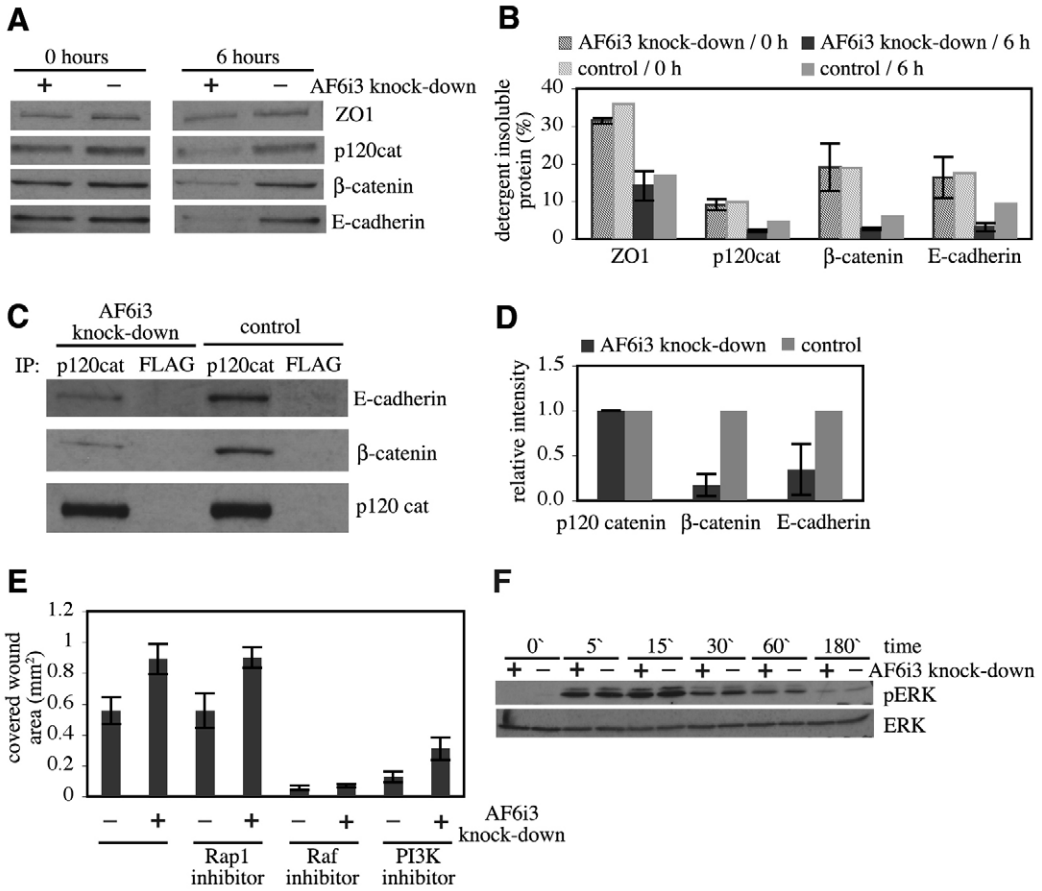

Fig. 8. AF6i3 knockdown results in reduced association of E-cadherin with the actin cytoskeleton and with p120-catenin during wound closure. (A) Western blot analysis showing the Triton-insoluble fraction of AF6i3-knockdown and control cells prior to migration (0 h) and 6 hours after wounding. (B) Graphic representation of western blot analysis shown in A. Values were normalized to the values for control cells. Values for ±s.d. were derived from three independent experiments. (C) Coimmunoprecipitation of E-cadherin and β-catenin with p120-catenin in AF6i3-knockdown and control cells 6 hours after wounding. Anti-Flag antibody was used as a negative control. (D) Graphic representation of coimmunoprecipitation shown in C. Values were normalized to the values for control cells. Values for ±s.d. were derived from three independent experiments. (E) Wound healing assay of AF6i3-knockdown and control cells in the presence of indicated inhibitors. Graphic representation of covered wound area 13 hours after wounding is depicted. Values for ±s.d. were derived from three independent experiments. (F) Western blot analysis of AF6i3-knockdown and control cell lysates during wound healing assay. One representative experiment out of three is shown.

at cell junctions was reduced only during migration, whereas static monolayers of AF6i3-knockdown cells displayed intact cell junctions. Therefore, a major role for AF6i3 might be to stabilize E-cadherin-dependent adhesion during dynamic processes in which the remodeling of cell junctions is required. We elucidated this further by analyzing de novo formation of cell junctions during Ca^{2+} switch assays (Fig. 9). This assay is based on switch in Ca^{2+} concentration from normal (2 mM) to low (2 μm) and back to normal (2 mM). This results in disassembly and reformation of cell junctions, because Ca^{2+} is required for the E-cadherin trans-interaction (Takeichi, 1991). We first analyzed the endogenous AF6i3 protein by immunofluorescence microscopy (Fig. 9A). Four hours after the switch to low Ca^{2+} concentration the AF6i3 protein was

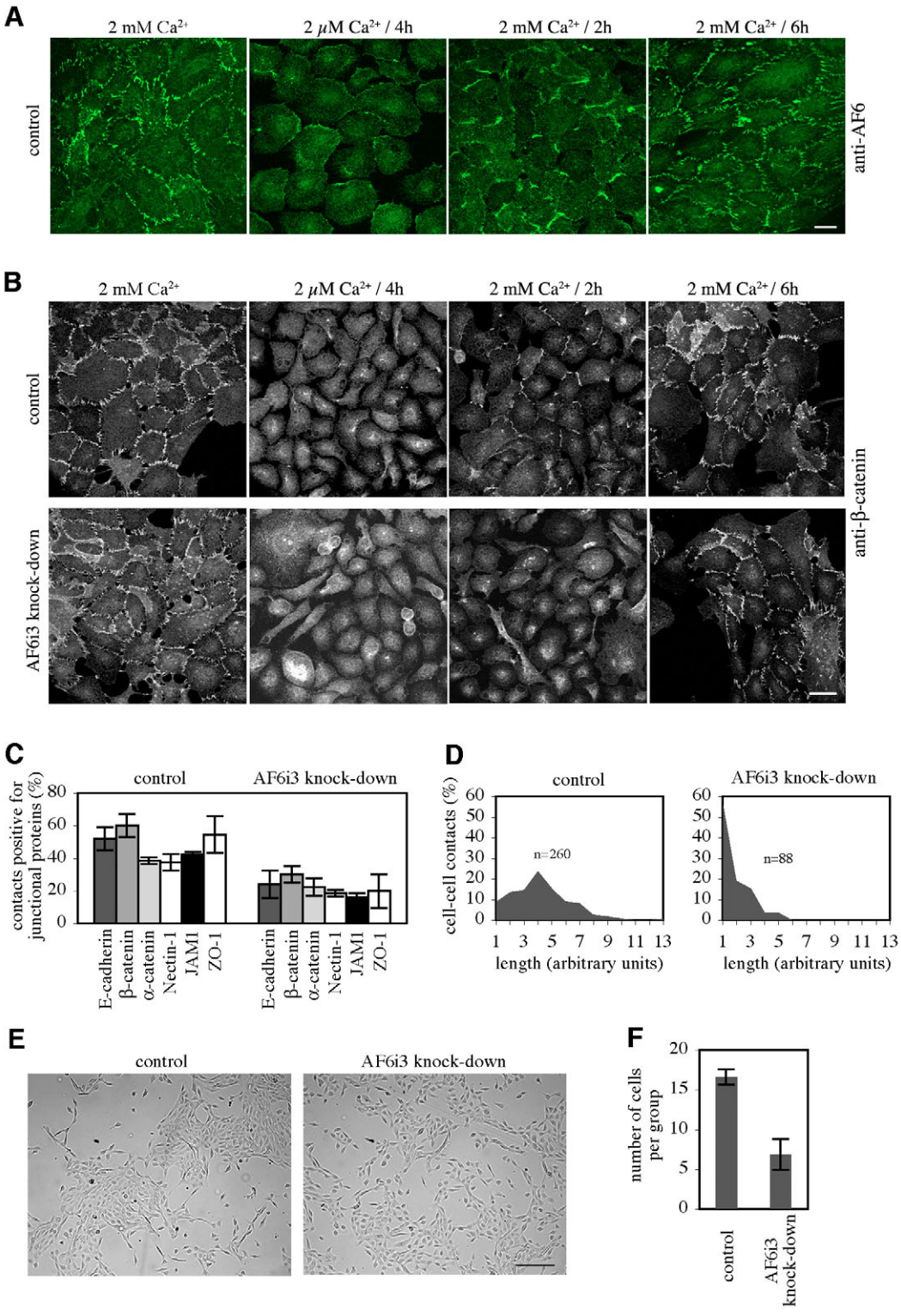

Fig. 9. AF6i3 knockdown decelerates cell-cell contact formation during Ca^{2+} switch assay. (A) Endogenous AF6i3 protein during Ca^{2+} switch assay. Cell monolayers (2 mM Ca^{2+}) were incubated at 2 μM Ca^{2+} for 4 hours to disturb cell junctions (2 μM Ca^{2+} / 4h). Reformation of cell-cell contacts 2 hours and 6 hours after switch to 2 mM Ca^{2+} is depicted. Bar, 20 μm. (B) β-catenin staining of AF6i3-knockdown (bottom row) and control cells (top row) during Ca^{2+} switch assay. Bar, 50 μm. (C) Graphic representation of the percentage of cell-cell contacts that stained positive for indicated junctional proteins 2 hours after switch to 2 mM Ca^{2+} in AF6i3-knockdown and control cells. Values for ±s.d.were derived from three independent experiments. (D) Length distribution of β-catenin-positive cell-cell contacts 2 hours after switch to 2 mM Ca^{2+} for cells shown in B. Lengths of all β-catenin-positive cell-cell contacts from three independent experiments were measured (n indicates the exact number) and grouped into 13 categories. Graphs represent the percentage of cell-cell contacts within each length category. (E) MCF10A AF6i3-knockdown and control cells grown in culture medium, 18 hours after seeding of singularized cells at equal sub-confluent density. Bar, 0.5 mm. (F) Graphic representation of Fig. 9E. The average number of cells per confluent cell group is depicted. Values for ±s.d. were derived from three independent experiments.

almost completely absent from cell-cell contacts and reappeared there after switch to a normal Ca^{2+} concentration. Six hours later its localization at cell junctions was completely restored.

Next we wanted to analyze the impact of AF6i3 knockdown on cell junction formation and stained the control and AF6i3-knockdown cells with antibodies against junctional proteins β-catenin (Fig. 9B), α-catenin, E-cadherin, nectin-1, JAM-1 and ZO-1 (Fig. S4A). Two hours after the switch to the normal Ca^{2+} concentration, AF6i3-knockdown cells displayed a significantly lower percentage of cell-cell contacts that stained positive for the tested junctional proteins (Fig. 9B,C and supplementary material Fig. S4A). Furthermore, computer-assisted measurement revealed that the cell-cell contacts were also significantly shorter (Fig. 9D). Six hours after switching to a normal Ca^{2+} concentration, cell junctions were completely restored and indistinguishable between AF6i3-knockdown and control cells considering the localization of all tested junctional proteins (Fig. 9B, right panel and data not shown). Western blot analysis confirmed that the amount of tested junctional proteins did not differ between the AF6i3-knockdown and control cells during the assay (supplementary material Fig. S4B), excluding reduced protein stability in AF6i3-knockdown cells. Thus, AF6i3 knockdown decelerated cell-cell contact formation, probably by delaying the translocation of junctional proteins to the newly formed cellular junctions.

At subconfluent density the control cells formed cell clusters. The size of cell clusters was reduced in AF6i3-knockdown cells, indicating a reduced intercellular adhesion (Fig. 9E,F). In summary, we demonstrated a negative effect of AF6i3 knockdown on cell-cell adhesion during three different dynamic processes: collective cell migration during epithelial wound closure, de novo cell-junction formation and cell growth at sub-confluent cell density.

Role of the AF6i3 F-actin-binding site in de novo formation of cell junctions

We showed that the F-actin-binding site is involved in stabilization of cell-cell adhesion during wound closure. We were wondering about its involvement in de novo formation of cell junctions. For that purpose we performed Ca^{2+} switch assays using AF6i3-knockdown cells reconstituted with AF6i1 or AF6i3 protein. Again, various cell junction-specific proteins (β-catenin, α-catenin, E-cadherin and nectin-1) were used as markers for cell-cell contacts. Two hours after switch to 2 mM Ca^{2+}, the percentage of cell-cell contacts within cell monolayer that were positive for the tested junctional proteins and the lengths of cell-cell contacts, were the same for the control and AF6i3-reconstituted knockdown cells (Fig. 10A-C). By contrast, after expression of AF6i1 in knockdown cells, they still displayed a reduced percentage of cell-cell contacts positive for junctional proteins (Fig. 10A,B). Strikingly, the cell-cell contacts were significantly shorter, reminiscent of the AF6i3-knockdown cells (Fig. 10C). Thus, AF6i3, which differs from the AF6i1 by its F-actin-binding site, was required for the efficient de novo formation of cell-cell contacts. This result illustrates the importance of the F-actin-binding site of AF6i3 protein.

Finally, we overexpressed the AF6i3 CT in control and AF6i3-knockdown cells and analyzed them in a Ca^{2+} switch assay (Fig. 11). During the assay the C-terminus showed similar localization as the endogenous full-length protein (Fig.

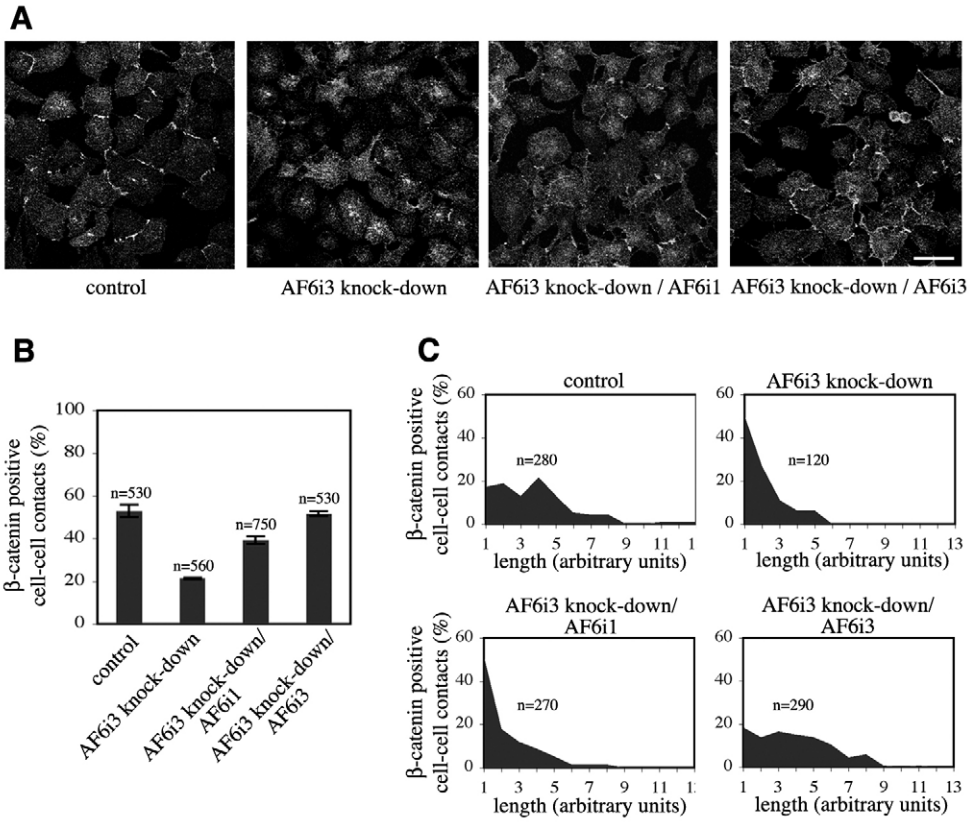

Fig. 10. AF6i1 and AF6i3 protein in Ca^{2+} switch assay: crucial role of the F-actin binding site. (A) Anti-β-catenin staining of control cells, AF6i3-knockdown cells and knockdown cells reconstituted with AF6i1 or AF6i3 construct, 2 hours after switching to 2 mM Ca^{2+}. Bar, 30 μm. (B) Graphic representation of the percentage of β-catenin-positive cell-cell contacts shown in A. Values for ±s.d. were derived from three independent experiments. Total numbers of counted cell-cell contacts are indicated (n). Similar results were obtained for E-cadherin, nectin-1 and α-catenin. (C) Length distribution of β-catenin-positive cell-cell contacts shown in A, determined as described for Fig. 9D.

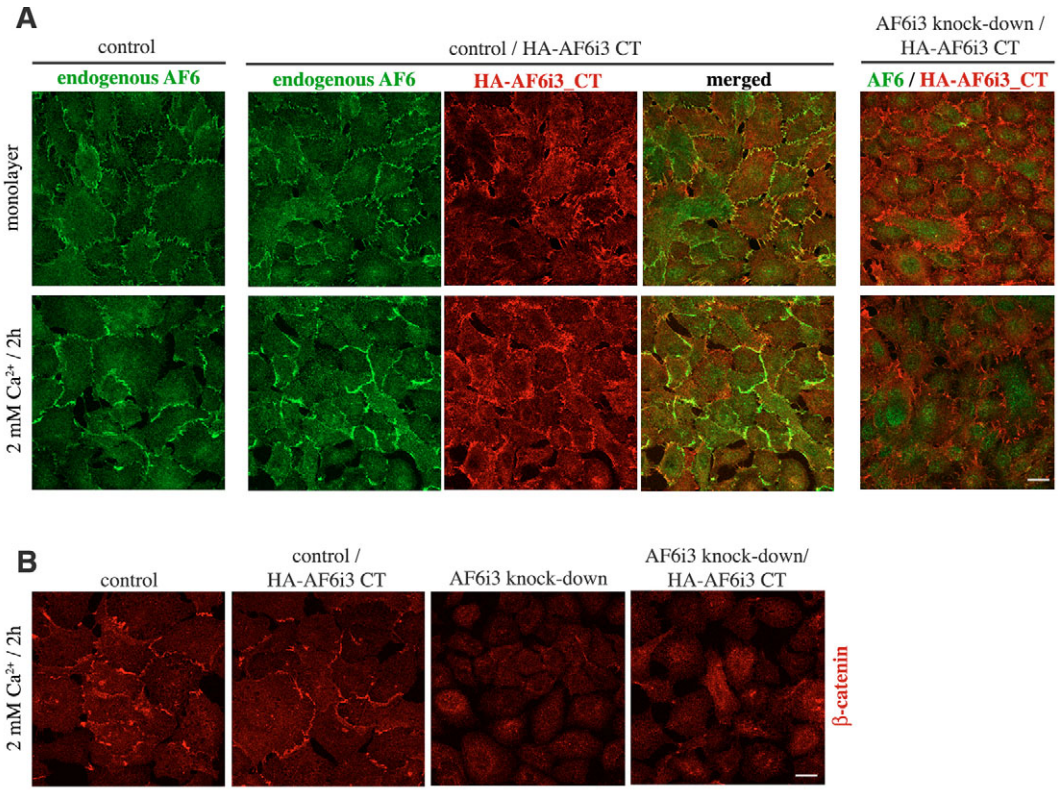

Fig. 11. AF6i3 C-terminus in Ca^{2+} switch assay. (A) Cell monolayers of the control cells and control or AF6i3-knockdown cells expressing the HA-tagged AF6i3 CT prior to assay (top) and 2 hours after switch to high Ca^{2+} concentration (bottom). Green, anti-AF6 antibody; red, anti-HA antibody. Bar, 20 μm. (B) Cells shown in A 2 hours after switch to high Ca^{2+} concentration, stained with anti-β-catenin antibody. Bar, 20 μm.

11A). Both proteins colocalized at cell-cell contacts in confluent cell monolayers and at the newly forming cell junctions two hours after the Ca^{2+} switch. Overexpression of the C-terminus alone in control cells did not alter the accumulation of endogenous AF6i3 protein (Fig. 11A) or β-catenin (Fig. 11B) at cell-cell contacts during the assay, nor did it promote the accumulation of β-catenin in AF6i3-knockdown cells (Fig. 11B). Thus, the AF6i3 F-actin-binding site promoted intercellular adhesion only in the context of full-length AF6i3 protein in cooperation with other AF6i3 domains. This indicates that concomitant interaction of AF6i3 with the actin cytoskeleton and junctional proteins is necessary for its function in intercellular adhesion and suggests its role as a linker between junctional adhesion complex and the actin cytoskeleton.

Discussion

In this study we are describing the biological function of so far uncharacterized isoform 3 of the AF6 protein in dynamic intercellular adhesion processes, with emphasis on the essential role of its F-actin-binding site. Although the existence of the F-actin-binding site in l-afadin (the rat homologue of AF6) was known (Mandai et al., 1997), the biological function of this domain has not been investigated so far. Here, we demonstrated that AF6i3 with its F-actin binding site is crucial for the stabilization of E-cadherin-dependent intercellular adhesion during dynamic processes, including collective cell migration during wound closure and de novo formation of cell junctions.

AF6i3-dependent intercellular adhesion regulates the directionality of migration

Accelerated wound closure in AF6i3-knockdown cells was due to reduced E-cadherin-dependent adhesion, as demonstrated by impaired E-cadherin-positive cell-cell contacts during migration and reduced cell clustering in the presence of Ca^{2+} in dissociation assay. Concomitantly, the directionality of migration was increased. We demonstrated that, the inhibition of E-cadherin cell-to-cell interaction with antibodies that block E-cadherin phenocopies the reduction of E-cadherin-dependent adhesion by AF6i3 knockdown regarding the increased directionality of migration. By that, we provided evidence for a direct link between the reduced cell-cell adhesion and increased directionality, and furthermore showed that both are regulated by AF6i3 protein.

Further evidence for the central role of intercellular adhesion in the AF6i3-mediated regulation of cell directionality was provided by analysis of individually migrating cells that were not undergoing cell-cell interactions. Under these conditions AF6i3 knockdown had no influence on the directionality of cell migration. In agreement with this, the activity and localization of Rho GTPases, actin concentration and the formation of focal adhesions at the leading edge of migrating cells were not altered by AF6i3 knockdown. Furthermore, signaling pathways known to regulate migration downstream of the EGF receptor (EGFR), including the Ras-Raf-MEK-ERK pathway (Singh and Harris, 2005), PI 3-kinase (Shien et al., 2004) and PLCγ (Mouneimne et al., 2004) (data not shown), as well as alteration of phosphorylation level of EGFR (data not shown), were not involved in AF6i3-knockdown-dependent phenotype. Alteration of these pathways should have resulted in altered directionality also in individually migrating AF6i3-knockdown cells, which was not the case.

AF6 is also known to negatively regulate Rap1-induced cell-matrix adhesion (Su et al., 2003; Zhang et al., 2005). However,

the deletion of the RA1 domain in AF6i3, which interacts with Rap1 (Linnemann et al., 1999), and the Rap1 inhibitor had no effect on migration, indicating that the increased directionality is independent of Rap1-induced adhesion. This finding is finally supported by the lack of migratory phenotype in individually migrating AF6i3-knockdown cells.

The gaps created between AF6i3-knockdown cells during wound closure might act as pseudo-free edges in the sub-marginal region and essentially allow for increased sub-marginal spreading in the direction of the wound. This would result in an increased directionality of migration and an increased overall rate of cell-sheet movement.

Role of the AF6i3 F-actin-binding site in intercellular adhesion

The full-length AF6i3 protein colocalized with F-actin only at cell-cell contacts, suggesting that this is the cellular compartment where the interaction occurs. In vivo interaction of AF6i3 with the actin cytoskeleton was supported by higher detergent insolubility of AF6i3 in comparison with AF6i1, which lacks the F-actin-binding site. Concomitantly, the presence of this domain in AF6i3 protein was essential for the stabilization of intercellular adhesion during wound closure and for efficient de novo formation of cell junctions. Therefore, we propose that AF6i3 protein interacts with F-actin via its C-terminal domain and through this interaction stabilizes the dynamic intercellular adhesion.

E-cadherin interacts with β-catenin, which then interacts with α-catenin, an actin-binding protein (Gumbiner, 2000). This association of the E-cadherin–catenin complex with the cortical actin cytoskeleton, which is known to stabilize intercellular adhesion (Imamura et al., 1999), was reduced in AF6i3-knockdown cells. Thus, AF6i3 might function as an additional linker between the E-cadherin and cytoskeleton. This might occur by direct interaction of AF6i3 and α-catenin (Tachibana et al., 2000; Pokutta et al., 2002), or their indirect association via afadin–DIL-domain interacting protein (ADIP)–α-actinin, LIM domain only (LMO7)–α-actinin or ZO-1 (Asada et al., 2003; Knudsen et al., 1995; Ooshio et al., 2004; Itoh et al., 1997; Yamamoto et al., 1997) (see supplementary material Fig. S5 for a model). AF6i3 protein, which harbors domains interacting with junctional proteins and the F-actin-binding site at the same time, promoted de novo formation of cell junctions. By contrast, neither AF6i1 lacking the F-actin binding site, nor the F-actin-binding site alone could fulfill this function. This suggests that concomitant interaction of AF6i3 protein with mentioned junctional proteins and actin cytoskeleton is necessary for stabilization of dynamic intercellular adhesion, supporting the linker function of AF6i3 protein.

AF6 interacts via its PDZ domain with further junctional proteins, nectin and JAM-1 (Takahashi et al., 1999; Ebnet et al., 2000). These interactions, however, were not essential for wound closure, because the deletion of the PDZ domain had no effect.

As an additional mechanism underlying the reduced E-cadherin-dependent adhesion in AF6i3-knockdown cells, we found also a reduced interaction between E-cadherin and p120-catenin (Thoreson et al., 2000; Reynolds and Carnahan, 2004). By contrast to data published by Hoshino et al. (Hoshino et al., 2005), this effect was independent of Rap1 in MCF10A cells, as already disscused. We were also not able to detect AF6i3 protein in p120-catenin–E-cadherin complexes. This suggests an indirect stabilization of p120-catenin–E-cadherin interaction by AF6i3.

In a recent study, Sato et al. found that the afadin knockdown affects exclusively E-cadherin recruitment to cell-cell contacts during a Ca^{2+} switch in MDCK cells (Sato et al., 2006). By contrast, in MCF10A cells the AF6i3 knockdown delayed a recruitment of all tested junctional proteins, including catenins. This discrepancy might be owing to different cellular backgrounds. The delayed recruitment of junctional proteins in MCF10A cells might be an indirect consequence of destabilized E-cadherin adhesion.

In summary, we demonstrated a crucial role for the AF6i3 protein and its F-actin-binding site in the stabilization of E-cadherin-dependent intercellular adhesion during processes in which the remodeling of cellular junctions is required, including collective cell migration of cell sheets during wound closure and de novo formation of cell junctions. We propose that the AF6i3 protein exerts this function by linking the junctional adhesion protein complex to the actin cytoskeleton (see supplementary material Fig. S5 for a model).

Materials and Methods

Sequencing of AF6 isoform 3 protein

HEK293 cells were lysed in lysis buffer [20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.5% Triton X-100, 0.5 mM DTT, 10 mM Na_3VO_3, 0.5 M NaF, 25 mM β-glycerolphosphate, trasylol and complete protease inhibitor cocktail (Roche)] and submitted to immunoprecipitation with the anti-AF6 antibody. The immunoprecipitate was separated by SDS-PAGE. The one dominant band running at ~200 kD was excised from the gel, digested with trypsin and subjected to the sequence analysis by MALDI-TOF mass spectrometry.

Plasmids and constructs

AF6i1-expressing vector pE(GFP)N2-AF6i1-flag was described elsewhere (Radziwill et al., 2003). AF6i3 C-terminal reverse primer (5′-TCACTTTG-TGTTCAGTTCATTC-3′) and AF6i1 specific primer (5′-GGGCCAGATGCGC-ACTCAGTCC-3′) were used to generate AF6i3 C-terminal fragment by PCR from a HEK293 cDNA library. *Pvu*I-digested PCR fragment was cloned into *Pvu*I/*Eco*RV-digested pE(GFP)N2-AF6i1-flag to generate pE(GFP)N2-AF6i3-flag. Plasmids harboring the myc-tagged or non-tagged AF6 constructs were generated by PCR.

AF6i3 C-terminal fragments (AF6i3 CT) harbored AF6i3 aa as indicated in Fig. 2Aa. Those fragments were cloned into pGEX-6P (Pharmacia) for GST-tagged constructs and into pCATCH (Georgiev et al., 1996) harboring a HA tag, for HA-tagged constructs.

For shRNA expression, following DNA oligonucleotides were ligated into pSUPER (Brummelkamp et al., 2002): 5′-gatccccGATGTAATCGAAACGCTC-GttcaagagaCGAGCGTTTCGATTACATCttttggaaa-3′ for AF6 shRNA and 5′-gatccccCGTACGCGGAATACTTCGAttcaagagaTCGAAGTATTCCGCGTACGttttt-ggaaa-3′ for GL2 shRNA used as a control (Gschwind et al., 2003). pSUPER fragments containing H1-RNA promoter and the shRNA construct were sub-cloned into pMSCVpuroΔ3′LTR vector (generated from the pMSCVpuro (BD Biosciences) by deletion of the 3′LTR, using the *Nhe*I and *Xba*I sites).

AF6i3 single-domain deletion mutants were generated by PCR. In AF6i3ΔRA1 aa 38 to 133 were deleted; in AF6i3ΔPDZ aa 998 to 1062 were deleted. AF6 shRNA-resistant constructs were generated by PCR by introducing point mutations at nucleotide positions 210, 213, 216, 219, 222 and 225 of the AF6i1 open reading frame (ORF) (U02478). For lentiviral transduction, all constructs were subcloned into pFUW vector (Lois et al., 2002).

Cell culture and retroviral transduction

HEK293T and HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS and MCF10A cells (ATCC, Rocville, MD) as previously described (Park et al., 2004).

MCF10A cells stably expressing shRNA specific for all AF6 protein isoforms (AF6 shRNA) or control shRNA were generated by retroviral transduction followed by selection with 2 μg/ml puromycin for 10 days. Recombinant retroviruses were obtained by co-transfection of HEK293T cells with shRNA-encoding pMSCVpuroΔ3′LTR, pVPack-GP and pVPack VSV-G (Stratagene), using Lipofectamine 2000™ (Invitrogen). AF6i3-knockdown cells stably expressing

reconstituted AF6 constructs were generated by lentiviral transduction without selection (transduction efficiency >90%). Recombinant lentiviruses were obtained from the co-transfection of HEK293T cells with pFUW plasmid encoding the corresponding AF6 construct, pCMV-ΔR 8.2 (Naldini et al., 1996; Lois et al., 2002) and pVPack VSV-G.

F-actin co-sedimentation assay

F-actin co-sedimentation assay was performed as previously described (Wu and Parsons, 1993). Briefly, polymerized F-actin (30 μg) was mixed with the fusion construct GST-AF6i3 CT (5 μg) and incubated for 1 hour at room temperature. The mixture was layered over 10% sucrose and centrifuged at 100,000 ***g*** at 4°C for 30 minutes.

Extraction of cells with detergent, coimmunoprecipitation, Rac1 and Cdc42 activation assay, and western blot analysis

Confluent cell monolayers were scratched multiple times and stimulated as described for wound healing assay. For Triton X-100 extraction, 35-mm tissue culture plates were extracted with 200 μl extraction buffer (lysis buffer containing 10 mM Tris, pH 7.5) at 4°C for 25 minutes on a rotating platform (soluble fraction, S). Insoluble residue (R) was recovered in 200 μl extraction buffer containing 0.5% SDS and 0.5% deoxycholate.

Coimmunoprecipitation was performed as previously described (Thoreson et al., 2000). Cell lysates were incubated with anti-p120-catenin antibody for 2 hours.

Rac1 and Cdc42 activation assay was performed using the Rac-Cdc42 activation assay kit (Cell Biolabs) following the manufacturer's instructions.

For western blot analysis, cells were lysed in lysis buffer containing 0.5% SDS and 0.5% deoxycholate. Antibodies against β-tubulin, E-cadherin, p120-catenin, β-catenin, α-catenin, nectin-1, JAM1 and Cdc42 were from Santa Cruz Biotechnology, anti-AF6 from Transduction Laboratories, anti-Rac1 from Upstate, anti-ZO-1 from Zymed and anti-pERK from Cell Signaling.

Immunofluorescence microscopy

Cells grown on glass coverslips were fixed with 4% PFA for 5 minutes and permeabilized with 0.5% Triton X-100 for 5 minutes. Stained cells were mounted in mounting medium containing 0.2 g/ml Mowiol 4-88 (Calbiochem). Immunofluorescence images were obtained using Leica confocal system TCS SP2 (Leica) and a microscope (DMIRBE, Leica) equipped with a 40× NA 1.25 oil immersion objective. Digital images were obtained using Leica confocal software 2.61 (Leica).

Anti-Claudin-1 (Zymed), anti-AF6 (Yamamoto et al., 1997), anti-nectin-1 CK41 (Krummenacher et al., 2000), anti-HA (Roche), anti-myc 9E10 and anti-FAK (Santa Cruz Biotechnology), anti-vinculin (Sigma) antibodies and antibodies described for western blotting were used for detection. Phalloidin-TRITC was from Sigma and TRITC or FITC-labeled secondary antibodies from Jacksons Immuno Research.

Dissociation assay

Dissociation assay was performed as previously described (Sato et al., 2006). Confluent cell monolayers were incubated with 0.1% trypsin in the presence of 1 mM EDTA or 1 mM Ca^{2+} for 1 hour at 37°C, followed by addition of 1 mg/ml collagenase D (Roche) for 30 minutes and dissociation by pipetting ten times.

Wound healing assay

Cell monolayers were cultured in non-coated 12-well plates (1×10^6 cells per well) for 24 hours and starved for 10 hours prior to wounding with a pipette tip and stimulation with 10 ng/ml EGF in DMEM/F-12 (1:1). Phase-contrast images were acquired with 4× NA 0.1 air objective, using an inverted microscope (DMIL, Leica), equipped with Leica DC 350FX digital camera. Digital images were acquired with Leica IM50 Image Manager software (Leica). Wound area was determined with Image Quant software (Molecular Dynamics).

HECD-1 antibody (Calbiochem) was used at 5 μg/ml, Rap1 inhibitor GGTI-298 (Calbiochem) at 10 μM, Raf1 inhibitor I (Calbiochem) at 15 μM and PI 3-kinase inhibitor wortmannin (Sigma) at 150 nM.

Ca^{2+} switch assay

MCF10A cells were seeded in 12-well plates (7×10^4 cells/well) on glass coverslips and grown for 48 hours. Cells starved for 10 hours were incubated in DMEM and F12 medium (1:1) containing 5 mM EGTA, for 4 hours (2 μM Ca^{2+}), followed by incubation in DMEM and F12 medium (1:1) alone (2 mM Ca^{2+}). Cells were fixed at different time points and stained with corresponding antibodies. The percentage of cell junction-protein-positive cell-cell contacts was calculated by dividing the number of contacts that stained positive for junctional proteins by total number of contacts. Length of cell-cell contacts that stained for β-catenin was measured using the Openlab software (Improvision).

Time-lapse analysis and cell tracking

Wound closure was followed by phase-contrast microscopy, using 10× NA 0.4 air objective on a microscope (DMIRBE, Leica) equipped with an environmental chamber (37°C, 5% CO_2) and a digital camera (ORICA-ER, Hamamatsu). Digital images were acquired at 3-minute intervals over 10 hours, using Openlab software (Improvision), which was also used to determine cell tracks.

For tracking of individual cells at low cell density, cells were plated in non-coated 35-mm tissue culture plates (9×10^3 cells/plate) and stimulated as described for wound healing.

Online supplemental material

Images for videos were collected at 3-minute intervals (as described for time-lapse analysis) using Openlab software (Improvision), which was also used to generate Quick Time movies at a display rate of 10 frames/second.

We thank G. Radziwill for generating and testing the AF6 shRNA expressing constructs. We thank M. Schwemmle and C. Urdieux for their contribution, T. Hoechli from EMZ, University of Zurich, for help with microscopy, K. Kaibuchi for kindly providing the anti-AF6 antibody, G. Cohen and R. Eisenberg for anti-nectin-1 CK41 antibody and D. Baltimore for pFUW and packaging plasmids. We also thank M. Baumgartner and G. Radziwill for very stimulating discussions and critical reading of the manuscript.

References

Asada, M., Irie, K., Morimoto, K., Yamada, A., Ikeda, W., Takeuchi, M. and Takai, Y. (2003). ADIP, a novel Afadin- and alpha-actinin-binding protein localized at cell-cell adherens junctions. *J. Biol. Chem.* **278**, 4103-4111.

Boettner, B., Harjes, P., Ishimaru, S., Heke, M., Fan, H. Q., Qin, Y., Van Aelst, L. and Gaul, U. (2003). The AF-6 homolog canoe acts as a Rap1 effector during dorsal closure of the Drosophila embryo. *Genetics* **165**, 159-169.

Brummelkamp, T. R., Bernards, R. and Agami, R. (2002). A system for stable expression of short interfering RNAs in mammalian cells. *Science* **296**, 550-553.

Buchert, M., Schneider, S., Meskenaite, V., Adams, M. T., Canaani, E., Baechi, T., Moelling, K. and Hovens, C. M. (1999). The junction-associated protein AF-6 interacts and clusters with specific Eph receptor tyrosine kinases at specialized sites of cell-cell contact in the brain. *J. Cell Biol.* **144**, 361-371.

D'Souza-Schorey, C. (2005). Disassembling adherens junctions: breaking up is hard to do. *Trends Cell Biol.* **15**, 19-26.

Ebnet, K., Schulz, C. U., Meyer Zu Brickwedde, M. K., Pendl, G. G. and Vestweber, D. (2000). Junctional adhesion molecule interacts with the PDZ domain-containing proteins AF-6 and ZO-1. *J. Biol. Chem.* **275**, 27979-27988.

Farooqui, R. and Fenteany, G. (2005). Multiple rows of cells behind an epithelial wound edge extend cryptic lamellipodia to collectively drive cell-sheet movement. *J. Cell Sci.* **118**, 51-63.

Friedl, P. (2004). Prespecification and plasticity: shifting mechanisms of cell migration. *Curr. Opin. Cell Biol.* **16**, 14-23.

Friedl, P., Hegerfeldt, Y. and Tusch, M. (2004). Collective cell migration in morphogenesis and cancer. *Int. J. Dev. Biol.* **48**, 441-449.

Georgiev, O., Bourquin, J. P., Gstaiger, M., Knoepfel, L., Schaffner, W. and Hovens, C. (1996). Two versatile eukaryotic vectors permitting epitope tagging, radiolabelling and nuclear localisation of expressed proteins. *Gene* **168**, 165-167.

Gschwind, A., Hart, S., Fischer, O. M. and Ullrich, A. (2003). TACE cleavage of proamphiregulin regulates GPCR-induced proliferation and motility of cancer cells. *EMBO J.* **22**, 2411-2421.

Gumbiner, B. M. (2000). Regulation of cadherin adhesive activity. *J. Cell Biol.* **148**, 399-404.

Honda, T., Shimizu, K., Fukuhara, A., Irie, K. and Takai, Y. (2003a). Regulation by nectin of the velocity of the formation of adherens junctions and tight junctions. *Biochem. Biophys. Res. Commun.* **306**, 104-109.

Honda, T., Shimizu, K., Kawakatsu, T., Yasumi, M., Shingai, T., Fukuhara, A., Ozaki-Kuroda, K., Irie, K., Nakanishi, H. and Takai, Y. (2003b). Antagonistic and agonistic effects of an extracellular fragment of nectin on formation of E-cadherin-based cell-cell adhesion. *Genes Cells* **8**, 51-63.

Hoshino, T., Sakisaka, T., Baba, T., Yamada, T., Kimura, T. and Takai, Y. (2005). Regulation of E-cadherin endocytosis by nectin through afadin, Rap1, and p120ctn. *J. Biol. Chem.* **280**, 24095-24103.

Ikeda, W., Nakanishi, H., Miyoshi, J., Mandai, K., Ishizaki, H., Tanaka, M., Togawa, A., Takahashi, K., Nishioka, H., Yoshida, H. et al. (1999). Afadin: A key molecule essential for structural organization of cell-cell junctions of polarized epithelia during embryogenesis. *J. Cell Biol.* **146**, 1117-1132.

Imamura, Y., Itoh, M., Maeno, Y., Tsukita, S. and Nagafuchi, A. (1999). Functional domains of alpha-catenin required for the strong state of cadherin-based cell adhesion. *J. Cell Biol.* **144**, 1311-1322.

Itoh, M., Nagafuchi, A., Moroi, S. and Tsukita, S. (1997). Involvement of ZO-1 in cadherin-based cell adhesion through its direct binding to alpha catenin and actin filaments. *J. Cell Biol.* **138**, 181-192.

Kaibuchi, K., Kuroda, S. and Amano, M. (1999). Regulation of the cytoskeleton and cell adhesion by the Rho family GTPases in mammalian cells. *Annu. Rev. Biochem.* **68**, 459-486.

Knudsen, K. A., Soler, A. P., Johnson, K. R. and Wheelock, M. J. (1995). Interaction of alpha-actinin with the cadherin/catenin cell-cell adhesion complex via alpha-catenin. *J. Cell Biol.* **130**, 67-77.

Krummenacher, C., Baribaud, I., Ponce de Leon, M., Whitbeck, J. C., Lou, H., Cohen, G. H. and Eisenberg, R. J. (2000). Localization of a binding site for herpes simplex virus glycoprotein D on herpesvirus entry mediator C by using antireceptor monoclonal antibodies. *J. Virol.* **74**, 10863-10872.

Lee, T. Y. and Gotlieb, A. I. (2003). Microfilaments and microtubules maintain endothelial integrity. *Microsc. Res. Tech.* **60**, 115-127.

Linnemann, T., Geyer, M., Jaitner, B. K., Block, C., Kalbitzer, H. R., Wittinghofer, A. and Herrmann, C. (1999). Thermodynamic and kinetic characterization of the interaction between the Ras binding domain of AF6 and members of the Ras subfamily. *J. Biol. Chem.* **274**, 13556-13562.

Lois, C., Hong, E. J., Pease, S., Brown, E. J. and Baltimore, D. (2002). Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors. *Science* **295**, 868-872.

Mandai, K., Nakanishi, H., Satoh, A., Obaishi, H., Wada, M., Nishioka, H., Itoh, M., Mizoguchi, A., Aoki, T., Fujimoto, T. et al. (1997). Afadin: a novel actin filament-binding protein with one PDZ domain localized at cadherin-based cell-to-cell adherens junction. *J. Cell Biol.* **139**, 517-528.

Miyahara, M., Nakanishi, H., Takahashi, K., Satoh-Horikawa, K., Tachibana, K. and Takai, Y. (2000). Interaction of nectin with afadin is necessary for its clustering at cell-cell contact sites but not for its cis dimerization or trans interaction. *J. Biol. Chem.* **275**, 613-618.

Miyamoto, H., Nihonmatsu, I., Kondo, S., Ueda, R., Togashi, S., Hirata, K., Ikegami, Y. and Yamamoto, D. (1995). canoe encodes a novel protein containing a GLGF/DHR motif and functions with Notch and scabrous in common developmental pathways in Drosophila. *Genes Dev.* **9**, 612-625.

Mouneimne, G., Soon, L., DesMarais, V., Sidani, M., Song, X., Yip, S. C., Ghosh, M., Eddy, R., Backer, J. M. and Condeelis, J. (2004). Phospholipase C and cofilin are required for carcinoma cell directionality in response to EGF stimulation. *J. Cell Biol.* **166**, 697-708.

Nabeshima, K., Moriyama, T., Asada, Y., Komada, N., Inoue, T., Kataoka, H., Sumiyoshi, A. and Koono, M. (1995). Ultrastructural study of TPA-induced cell motility: human well-differentiated rectal adenocarcinoma cells move as coherent sheets via localized modulation of cell-cell adhesion. *Clin. Exp. Metastasis* **13**, 499-508.

Nabeshima, K., Asada, Y., Inoue, T., Kataoka, H., Shimao, Y., Sumiyoshi, A. and Koono, M. (1997). Modulation of E-cadherin expression in TPA-induced cell motility: well-differentiated human adenocarcinoma cells move as coherent sheets associated with phosphorylation of E-cadherin-catenin complex. *Lab. Invest.* **76**, 139-151.

Nabeshima, K., Inoue, T., Shimao, Y., Kataoka, H. and Koono, M. (1999). Cohort migration of carcinoma cells: differentiated colorectal carcinoma cells move as coherent cell clusters or sheets. *Histol. Histopathol.* **14**, 1183-1197.

Nagafuchi, A. (2001). Molecular architecture of adherens junctions. *Curr. Opin. Cell Biol.* **13**, 600-603.

Naldini, L., Blomer, U., Gallay, P., Ory, D., Mulligan, R., Gage, F. H., Verma, I. M. and Trono, D. (1996). In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science* **272**, 263-267.

Ooshio, T., Irie, K., Morimoto, K., Fukuhara, A., Imai, T. and Takai, Y. (2004). Involvement of LMO7 in the association of two cell-cell adhesion molecules, nectin and E-cadherin, through afadin and alpha-actinin in epithelial cells. *J. Biol. Chem.* **279**, 31365-31373.

Park, S., Mazina, O., Kitagawa, A., Wong, P. and Matsumura, F. (2004). TCDD causes suppression of growth and differentiation of MCF10A, human mammary epithelial cells by interfering with their insulin receptor signaling through c-Src kinase and ERK activation. *J. Biochem. Mol. Toxicol.* **18**, 322-331.

Peng, Y. F., Mandai, K., Nakanishi, H., Ikeda, W., Asada, M., Momose, Y., Shibamoto, S., Yanagihara, K., Shiozaki, H., Monden, M. et al. (2002). Restoration of E-cadherin-based cell-cell adhesion by overexpression of nectin in HSC-39 cells, a human signet ring cell gastric cancer cell line. *Oncogene* **21**, 4108-4119.

Perez-Moreno, M., Jamora, C. and Fuchs, E. (2003). Sticky business: orchestrating cellular signals at adherens junctions. *Cell* **112**, 535-548.

Pignatelli, M., Liu, D., Nasim, M. M., Stamp, G. W., Hirano, S. and Takeichi, M. (1992). Morphoregulatory activities of E-cadherin and beta-1 integrins in colorectal tumour cells. *Br. J. Cancer* **66**, 629-634.

Pokutta, S., Drees, F., Takai, Y., Nelson, W. J. and Weis, W. I. (2002). Biochemical and structural definition of the l-afadin- and actin-binding sites of alpha-catenin. *J. Biol. Chem.* **277**, 18868-18874.

Prasad, R., Gu, Y., Alder, H., Nakamura, T., Canaani, O., Saito, H., Huebner, K., Gale, R. P., Nowell, P. C., Kuriyama, K. et al. (1993). Cloning of the ALL-1 fusion partner, the AF-6 gene, involved in acute myeloid leukemias with the t(6;11) chromosome translocation. *Cancer Res.* **53**, 5624-5628.

Radziwill, G., Erdmann, R. A., Margelisch, U. and Moelling, K. (2003). The Bcr kinase downregulates Ras signaling by phosphorylating AF-6 and binding to its PDZ domain. *Mol. Cell. Biol.* **23**, 4663-4672.

Reynolds, A. B. and Carnahan, R. H. (2004). Regulation of cadherin stability and turnover by p120ctn: implications in disease and cancer. *Semin. Cell Dev. Biol.* **15**, 657-663.

Saito, S., Matsushima, M., Shirahama, S., Minaguchi, T., Kanamori, Y., Minami, M. and Nakamura, Y. (1998). Complete genomic structure DNA polymorphisms, and alternative splicing of the human AF-6 gene. *DNA Res.* **5**, 115-120.

Sato, T., Fujita, N., Yamada, A., Ooshio, T., Okamoto, R., Irie, K. and Takai, Y. (2006). Regulation of the assembly and adhesion activity of E-cadherin by nectin and afadin for the formation of adherens junctions in Madin-Darby canine kidney cells. *J. Biol. Chem.* **281**, 5288-5299.

Schneider, S., Buchert, M., Georgiev, O., Catimel, B., Halford, M., Stacker, S. A., Baechi, T., Moelling, K. and Hovens, C. M. (1999). Mutagenesis and selection of PDZ domains that bind new protein targets. *Nat. Biotechnol.* **17**, 170-175.

Shien, T., Doihara, H., Hara, H., Takahashi, H., Yoshitomi, S., Taira, N., Ishibe, Y., Teramoto, J., Aoe, M. and Shimizu, N. (2004). PLC and PI3K pathways are important in the inhibition of EGF-induced cell migration by gefitinib ('Iressa', ZD1839). *Breast Cancer* **11**, 367-373.

Singh, A. B. and Harris, R. C. (2005). Autocrine, paracrine and juxtacrine signaling by EGFR ligands. *Cell. Signal.* **17**, 1183-1193.

Stuart, R. O. and Nigam, S. K. (1995). Regulated assembly of tight junctions by protein kinase C. *Proc. Natl. Acad. Sci. USA* **92**, 6072-6076.

Su, L., Hattori, M., Moriyama, M., Murata, N., Harazaki, M., Kaibuchi, K. and Minato, N. (2003). AF-6 controls integrin-mediated cell adhesion by regulating Rap1 activation through the specific recruitment of Rap1GTP and SPA-1. *J. Biol. Chem.* **278**, 15232-15238.

Tachibana, K., Nakanishi, H., Mandai, K., Ozaki, K., Ikeda, W., Yamamoto, Y., Nagafuchi, A., Tsukita, S. and Takai, Y. (2000). Two cell adhesion molecules, nectin and cadherin, interact through their cytoplasmic domain-associated proteins. *J. Cell Biol.* **150**, 1161-1176.

Takahashi, K., Matsuo, T., Katsube, T., Ueda, R. and Yamamoto, D. (1998). Direct binding between two PDZ domain proteins Canoe and ZO-1 and their roles in regulation of the jun N-terminal kinase pathway in Drosophila morphogenesis. *Mech. Dev.* **78**, 97-111.

Takahashi, K., Nakanishi, H., Miyahara, M., Mandai, K., Satoh, K., Satoh, A., Nishioka, H., Aoki, J., Nomoto, A., Mizoguchi, A. et al. (1999). Nectin/PRR: an immunoglobulin-like cell adhesion molecule recruited to cadherin-based adherens junctions through interaction with Afadin, a PDZ domain-containing protein. *J. Cell Biol.* **145**, 539-549.

Takai, Y. and Nakanishi, H. (2003). Nectin and afadin: novel organizers of intercellular junctions. *J. Cell Sci.* **116**, 17-27.

Takeichi, M. (1991). Cadherin cell adhesion receptors as a morphogenetic regulator. *Science* **251**, 1451-1455.

Tanaka, Y., Nakanishi, H., Kakunaga, S., Okabe, N., Kawakatsu, T., Shimizu, K. and Takai, Y. (2003). Role of nectin in formation of E-cadherin-based adherens junctions in keratinocytes: analysis with the N-cadherin dominant negative mutant. *Mol. Biol. Cell* **14**, 1597-1609.

Thoreson, M. A., Anastasiadis, P. Z., Daniel, J. M., Ireton, R. C., Wheelock, M. J., Johnson, K. R., Hummingbird, D. K. and Reynolds, A. B. (2000). Selective uncoupling of p120(ctn) from E-cadherin disrupts strong adhesion. *J. Cell Biol.* **148**, 189-202.

Wu, H. and Parsons, J. T. (1993). Cortactin, an 80/85-kilodalton pp60src substrate, is a filamentous actin-binding protein enriched in the cell cortex. *J. Cell Biol.* **120**, 1417-1426.

Yamamoto, T., Harada, N., Kano, K., Taya, S., Canaani, E., Matsuura, Y., Mizoguchi, A., Ide, C. and Kaibuchi, K. (1997). The Ras target AF-6 interacts with ZO-1 and serves as a peripheral component of tight junctions in epithelial cells. *J. Cell Biol.* **139**, 785-795.

Zhadanov, A. B., Provance, D. W., Jr, Speer, C. A., Coffin, J. D., Goss, D., Blixt, J. A., Reichert, C. M. and Mercer, J. A. (1999). Absence of the tight junctional protein AF-6 disrupts epithelial cell-cell junctions and cell polarity during mouse development. *Curr. Biol.* **9**, 880-888.

Zhang, Z., Rehmann, H., Price, L. S., Riedl, J. and Bos, J. L. (2005). AF6 negatively regulates Rap1-induced cell adhesion. *J. Biol. Chem.* **280**, 33200-33205.