# Involvement of afadin in barrier function and homeostasis of mouse intestinal epithelia

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*Accepted 4 February 2011*
*Journal of Cell Science 124, 2231-2240*

*doi:10.1242/jcs.081000*

## Summary

Afadin interacts with the cytoplasmic region of nectins, which are immunoglobulin-like cell adhesion molecules at adherens junctions, and links them to the actin cytoskeleton. Afadin regulates activities of cells in culture such as directional motility, proliferation and survival. We used Cre-*loxP* technology to generate mice conditionally lacking afadin specifically in the intestinal epithelia after birth. The loss of afadin caused increased paracellular permeability in the intestinal mucosa and enhanced susceptibility to the tissue destruction induced by dextran sulfate sodium. The junctional architecture of the intestinal epithelia appeared to be preserved, whereas the deficiency of afadin caused the mislocalization of nectin-2 and nectin-3 from adherens junctions to basolateral membrane domains but not that of other components of apical junctions. By contrast, such phenotypic changes were undetected in mice lacking nectin-2, nectin-3 or both. These findings suggest that afadin plays crucial roles, independently of the role as the nectin–afadin module, in barrier function and homeostasis of the intestinal epithelia once the epithelial structure has been established.

**Key words:** Apical junctions, Actin cytoskeleton, Dextran sulfate sodium, Intercellular adhesion, Paracellular permeability

## Introduction

Polarized epithelial cell structure is formed and maintained in part by the apical junctions, which are composed of adherens junctions (AJs) and tight junctions (TJs). There are two main junctional components: transmembrane proteins including claudins, occludin, and junctional adhesion molecules (JAMs) at TJs, and cadherins and nectins at AJs. There are also intracellular adaptor proteins including zonula occludens (ZO) proteins, catenins and afadin that directly or indirectly link cell adhesion molecules to the actin cytoskeleton (Miyoshi and Takai, 2005; Miyoshi and Takai, 2008). Cadherins are prototypic cell adhesion molecules in a variety of cell types, whereas nectins and nectin-like molecules (Necls) have emerged as a family of $Ca^{2+}$-independent immunoglobulin-like cell adhesion molecules consisting of nectin-1 to nectin-4 and Necl-1 to Necl-5 (Takai and Nakanishi, 2003; Takai et al., 2008). Engagement of nectins on cell–cell contact sites initiates the formation of AJs in epithelial cells in culture by recruiting cadherins and other TJ components to establish epithelial polarity (Sato et al., 2006). Given that afadin is the only adaptor protein that binds nectins, elucidating the roles of afadin might hold the key to our understanding of the biological significance of nectins.

Afadin, a filamentous (F)-actin binding protein at AJs of apical junctions in polarized epithelia, is composed of multiple functional domains: two Ras-binding domains, a forkhead association domain, a diluted domain, a PDZ (postsynaptic density protein-95, *Drosophila* discs large, and zonula occludens-1) domain, and three proline-rich domains. Afadin interacts with the cytoplasmic region of nectins at the PDZ domain and connects nectins to the actin cytoskeleton directly through its own F-actin binding ability or indirectly through interaction with complexes of ponsin–vinculin, afadin DIL domain-interacting protein–α-actinin, or LIM domain only 7–α-actinin (Asada et al., 2003; Mandai et al., 1997; Mandai et al., 1999; Ooshio et al., 2004; Takai and Nakanishi, 2003). Afadin mediates Rap1 (Boettner et al., 2000), Rac1 and Cdc42 signaling pathways that are required for the formation of junctional structures in Madin-Darby canine kidney (MDCK) cells (Fukuyama et al., 2005; Sato et al., 2006). Downregulation of afadin by RNA interference resulted in the failure of TJ formation and impaired motility in MDCK cells and intestinal cells (Ooshio et al., 2010; Sato et al., 2006; Severson et al., 2009). When afadin is bound to nectins in epithelial cells, afadin interacts with Rap1 to inhibit endocytosis of E-cadherin molecules that are not engaged, thereby strengthening the binding of $p120^{ctn}$ to E-cadherin (Hoshino et al., 2005). When afadin is free and unbound to nectins in migrating cells, it is located to the leading edge and associated with activated Rap1 to modulate membrane activities through reorganization of the actin cytoskeleton (Miyata et al., 2009a; Miyata et al., 2009b). Moreover, the embryoid bodies derived from afadin-deficient embryonic stem cells showed enhanced apoptosis, indicating that afadin is involved in the platelet-derived growth factor (PDGF)-induced cell survival signaling through activation of the phosphoinositide 3-kinase (PI3K)–Akt pathway (Kanzaki et al., 2008). Thus, except for regulating the formation of apical junctions, afadin plays roles in cell motility, proliferation, and survival in culture.

The role of afadin has been elucidated in genetically modified mice. Mice lacking afadin showed developmental defects during gastrulation at embryonic day 7.5 (E7.5), accompanied by

disorganization of the ectoderm, impaired migration of the mesoderm, and loss of somites and structures normally derived from both the ectoderm and the mesoderm (Ikeda et al., 1999; Zhadanov et al., 1999). Canoe, the *Drosophila* afadin homologue, has been shown to be essential for the linkage of the actin cytoskeleton to AJs during morphogenesis such as apical constriction of mesodermal cells but not for AJ assembly or maintenance (Sawyer et al., 2009). Recently, conditional strategies using the Cre-*loxP* system to remove afadin from mouse tissues have disclosed the crucial roles of afadin in synaptic remodeling in the hippocampus (Majima et al., 2009), in neuronal layer organization in the neocortex (our unpublished data), and in angiogenesis induced by vascular endothelial growth factor and sphingosine 1-phosphate (Tawa et al., 2010). On the other hand, knockout studies on nectins have clarified the role of the nectin–afadin module. For example, the heterotypic engagement between nectin-1 and nectin-3 plays an important role in forming synaptic junctions between the mossy fiber and CA3 pyramidal neuronal cells in the hippocampus (Honda et al., 2006), as well as in apex to apex adhesion between the pigment and non-pigment cell layers of the ciliary epithelia (Inagaki et al., 2005). Heterotypic interaction between nectin-2 and nectin-3 is essential for Sertoli cell-mediated spermatid development in the testes (Inagaki et al., 2006; Mueller et al., 2003). These studies indicate the importance of the nectin–afadin module in forming stable junctions between varieties of heterotypic cells. However, it still remains unknown whether afadin simply acts as the adaptor protein of nectins or has additional roles in epithelial structures and functions.

Conceivably, the role of afadin as the nectin–afadin module might reflect one of its biochemical aspects, given that afadin has multiple binding domains that directly interact with components of apical junctions. Therefore, we have generated mice lacking afadin specifically in epithelial cells of the intestines by using transgenic mice expressing Cre recombinase driven by the promoter of the villin gene (villin-Cre) (Pinto et al., 1999). Villin-Cre-mediated disruption of the afadin gene enables us to circumvent embryonic lethality due to the complete loss of afadin and provides us with the opportunity to study the role of afadin in intestinal epithelia. Here, we describe phenotypes of the mice and demonstrate novel functions of afadin in maintaining mucosal permeability and homeostasis of the intestines, as well as in recruiting nectins to apical junctions of epithelial cells.

## Results

### Generation of mice lacking afadin specifically in intestinal epithelia

To examine the functions of afadin in the intestinal epithelia, we generated conditionally knockout (cKO) mice by using the Cre-*loxP* system. Afadin-floxed mice generated as described (Majima et al., 2009) were mated with the villin-Cre transgenic mice that exclusively express Cre recombinase in epithelia of small and large intestines (Pinto et al., 1999). We analyzed the time course of villin-Cre-mediated disruption of the afadin gene by polymerase chain reaction (PCR) using DNA materials from the embryonic and neonatal intestines, and expression levels of afadin by reverse transcription PCR (RT-PCR) using mRNA materials (Fig. 1A,B). Cre-mediated deletion of the afadin gene was detected in newborn mice at post-neonatal periods, but not in embryos during any of the developmental stages. Expression of the afadin gene was almost undetectable by RT-PCR analyses, suggesting the nearly complete disruption of the afadin gene mediated by villin-Cre in newborn mice. Immunoblot analyses showed that a 205-kDa isoform of afadin (l-afadin) was predominantly expressed in the small and large intestines from the wild-type mice, whereas a 190-kDa isoform of afadin (s-afadin) was abundantly expressed in the

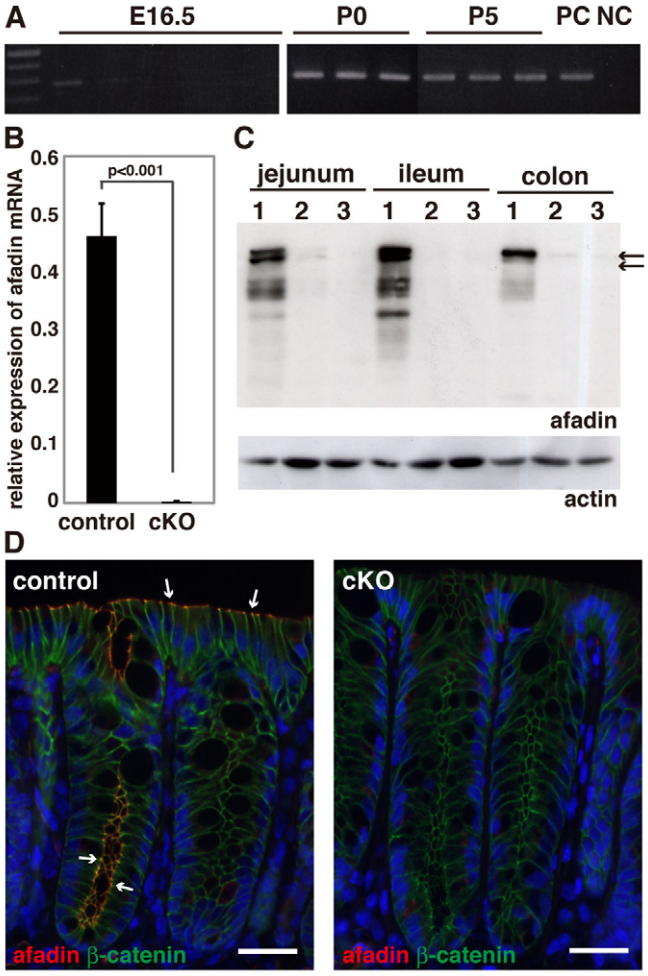


**Fig. 1. Villin-Cre-mediated disruption of the afadin gene in the mouse intestines.** (**A**) Detection of Cre-*loxP*-mediated DNA recombination by the PCR product that indicates the deletion of exon 2 of the afadin-floxed allele. DNA samples obtained from the intestines of five afadin cKO mice at E16.5, three afadin cKO mice at postnatal day 0 (P0) and P5, were analyzed by PCR. Samples obtained from adult afadin$^{flox/-}$ and wild-type mice were used as the positive (PC) and negative (NC) controls, respectively. PCR primers (5′-GAAGGAAGATCATAGTTGCA-3′ and 5′-GTCATTTCTGTGCCTGTAAC-3′) are located 5′ upstream and 3′ downstream to exon 2. (**B**) Expression of afadin-encoding mRNA in the intestines. Relative mRNA levels were quantified by real-time PCR and normalized to the level of control *GAPDH* mRNA. Three experiments were performed independently. Error bars indicate s.d. (**C**) Immunoblot analyses with an anti-afadin antibody using epithelial lysates from the intestines, as indicated. Samples were obtained from a control mouse (lane 1) and two afadin cKO mice (lanes 2 and 3). Arrows indicate position of l-afadin (upper) and s-afadin (lower) proteins. Smaller bands of the control mouse (lane 1) probably reflect degradation of afadin proteins during preparation (upper panel). Actin protein bands are shown as loading controls of each sample (lower panel). (**D**) Immunohistochemistry showing colonic epithelia doubly stained with anti-afadin (red) and anti-β-catenin (green) antibodies. The signals for afadin are located to the apical junctions (arrows), whereas β-catenin signals are located to the lateral surface of epithelial cells in the control mice. Scale bars: 30 μm.

jejunum and ileum but not in the colon. s-Afadin, also known as AF-6, is a splice variant lacking the F-actin binding domain in the C-terminus and thus mediates afadin signaling, except for direct binding to the actin cytoskeleton (Mandai et al., 1997). By contrast, the levels of afadin were undetectable in the small and large intestines from the afadin cKO mice (Fig. 1C). Moreover, immunohistochemical analyses showed the loss of signals for afadin at apical junctions of the epithelial cells throughout the intestinal sections, including the surfaces, villi and crypts, but not in other cell types, including endothelial cells in the afadin cKO mice (Fig. 1D). Nevertheless, mice homozygous for the afadin-floxed allele with the villin-Cre transgene were born at the Mendelian rate; they were viable and fertile with a life span comparable to that of the control afadin-floxed mice in an environment free of specific pathogens. Thus, the conditional strategy successfully circumvented embryonic lethality due to the complete loss of afadin, and generated mice with defective functions of afadin specifically in epithelia of the intestines.

### Architecture of the intestinal epithelia in mice lacking afadin

We examined histological and ultrastructural characteristics of intestines in the afadin cKO mice. We prepared the samples from the colon, jejunum and ileum, and then examined morphological architectures of the crypts, villi and epithelial surfaces. Colon sections from the afadin cKO mice, stained with hematoxylin and eosin, showed apparently normal morphology compared with the control mice (Fig. 2A). Sections of the small intestines also showed similar morphology as the colon sections (Fig. 2A). Selected specimens from the colon and jejunum were further processed for electron microscopy. Although there exist minor fluctuation in the electric density of the epithelial apical junctions of the colon surface, no critical change was detected in the ultrastructures of the colon and jejunum in the afadin cKO mice (Fig. 2B). These results suggest that the structure of the apical junctions is morphologically conserved in the absence of afadin, once established in the adult mouse.

### Mislocalization of nectins in mice lacking afadin

We next performed immunohistochemical analyses to examine the localization of nectins at apical junctions because afadin is known to bind to the cytoplasmic region of nectins. Nectin-2 and nectin-3 were predominantly expressed in the colon of wild-type mice whereas nectin-1 and nectin-4 were hardly detected (Fig. 3A and data not shown). The signals for afadin were detected at the apical junctions in the mice lacking both nectin-2 and nectin-3 (Fig. 3A). By contrast, the signals for nectin-2 and nectin-3 were undetected at apical junctions of colon epithelial cells from the afadin cKO mice (Fig. 3B). These findings were further confirmed by immunohistochemistry of cross-sections of the villi of the colon (supplementary material Fig. S1). The signals for nectin-2 were not necessarily located to the apical junctions but were widely distributed throughout the basolateral membrane domains of the epithelial cells in the colon (Fig. 3B). This finding also appeared to be the case for nectin-3, although the signals were at lower levels (data not shown). These results suggest that localization of the nectins to the apical junctions depends on the presence of afadin in the mouse colon.

### Localization of other apical junction components in mice lacking afadin

We further addressed whether localization of other junctional proteins is affected or not by the absence of afadin once the epithelial structure has been established. We immunohistochemically analyzed localization of the TJ markers, occludin, JAM and ZO proteins, as well as the AJ markers, E-cadherin, and β-catenin and found no significant difference in the

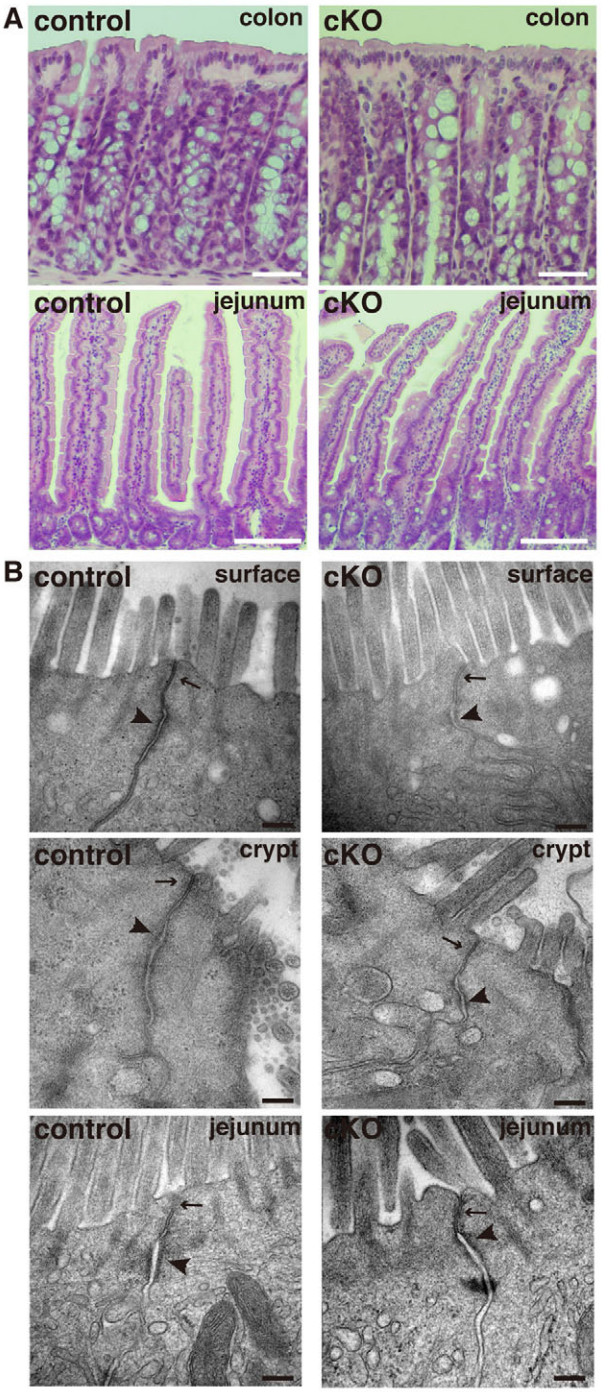


**Fig. 2. Preserved morphology of the colon epithelia lacking afadin.** (**A**) Colon and small intestine sections stained with hematoxylin and eosin. Scale bars: 20 µm (colon) and 150 µm (jejunum). (**B**) Electron micrographs of the epithelia from the surface area (upper panel) and the cryptic area (middle panel) of the colon, and those from the villus tips of the jejunum in the control and afadin cKO mice at the age of 13 weeks (lower panel). The TJs (arrows) and AJs (arrowheads) of normal electron density were observed in the colon and jejunum epithelia from the control and afadin cKO mice. Scale bars: 200 nm.

expression and localization of any of these proteins (Fig. 4A). We detected no change in expression and localization of claudins (data not shown), nor in the steady-state levels and localization of cortical F-actin (Fig. 4B). The immunohistochemical images of the colon sections with antibodies against nectins and α-catenin also showed the loss of colocalization of the nectins with α-catenin at the apical junctions (supplementary material Fig. S2). Although the knockdown study of afadin showed decreased levels of integrin β1 in the intestinal cultured cells (Severson et al., 2009), the levels of integrin β1 in the colon from the afadin cKO mice were almost equal to those of the control mice (data not shown). These results, as well as electron microscopy of the apical junctions (Fig. 2B), indicate that the loss of signals for nectin-2 and nectin-3 is not attributed to disorganization of the apical junctional complexes but to the mislocalization of nectins.

### Increased epithelial permeability of the intestines in mice lacking afadin

Although the intestines of mice lacking afadin showed no morphological abnormality, this finding does not necessarily imply

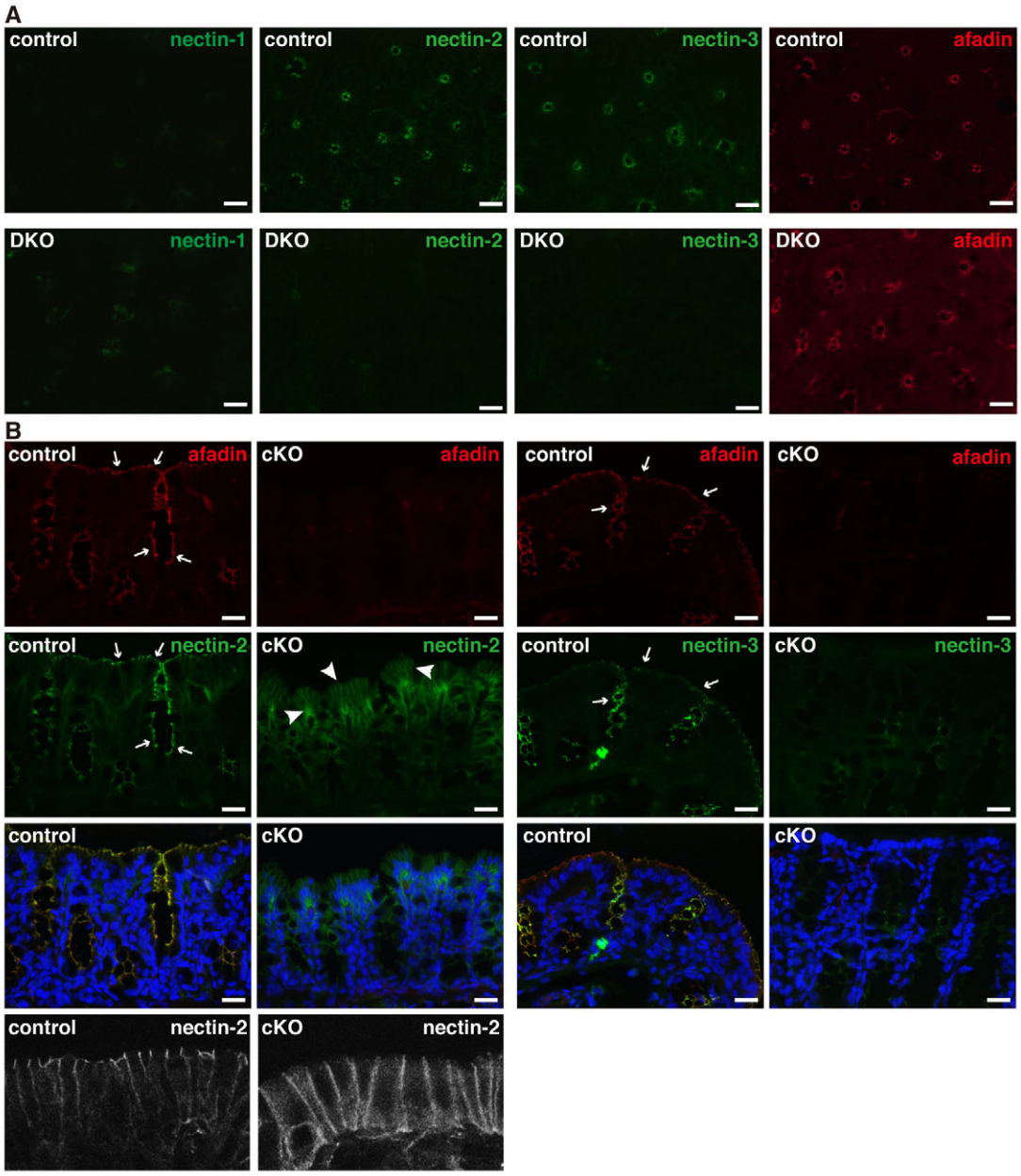


**Fig. 3. Localization of afadin and nectins in the colon from mice lacking afadin or nectins.** (**A**) Conserved localization of afadin in the colon epithelia obtained from a mouse heterozygous for nectin-2 and nectin-3 (control) and a mouse lacking both nectin-2 and nectin-3 (DKO). Horizontal sections of the intestines were immunostained with anti-afadin (red) and anti-nectin-2 (green) antibodies, as well as with anti-nectin-1 (green) and anti-nectin-3 (green) antibodies, respectively. (**B**) Mislocalization of nectin-2 and nectin-3 in the colon epithelia from mice lacking afadin. The bottom panels of nectin-2 staining represent high magnification of the epithelial cells in which nectin-2 disassembled from AJs to basolateral membrane domains. Arrows indicate the localization of afadin and nectins at AJs. Arrowheads indicate the localization of nectin-2 in the absence of afadin. The merged panels were counterstained with Hoechst 33258 to show the epithelial structure. Scale bars: 20 μm.

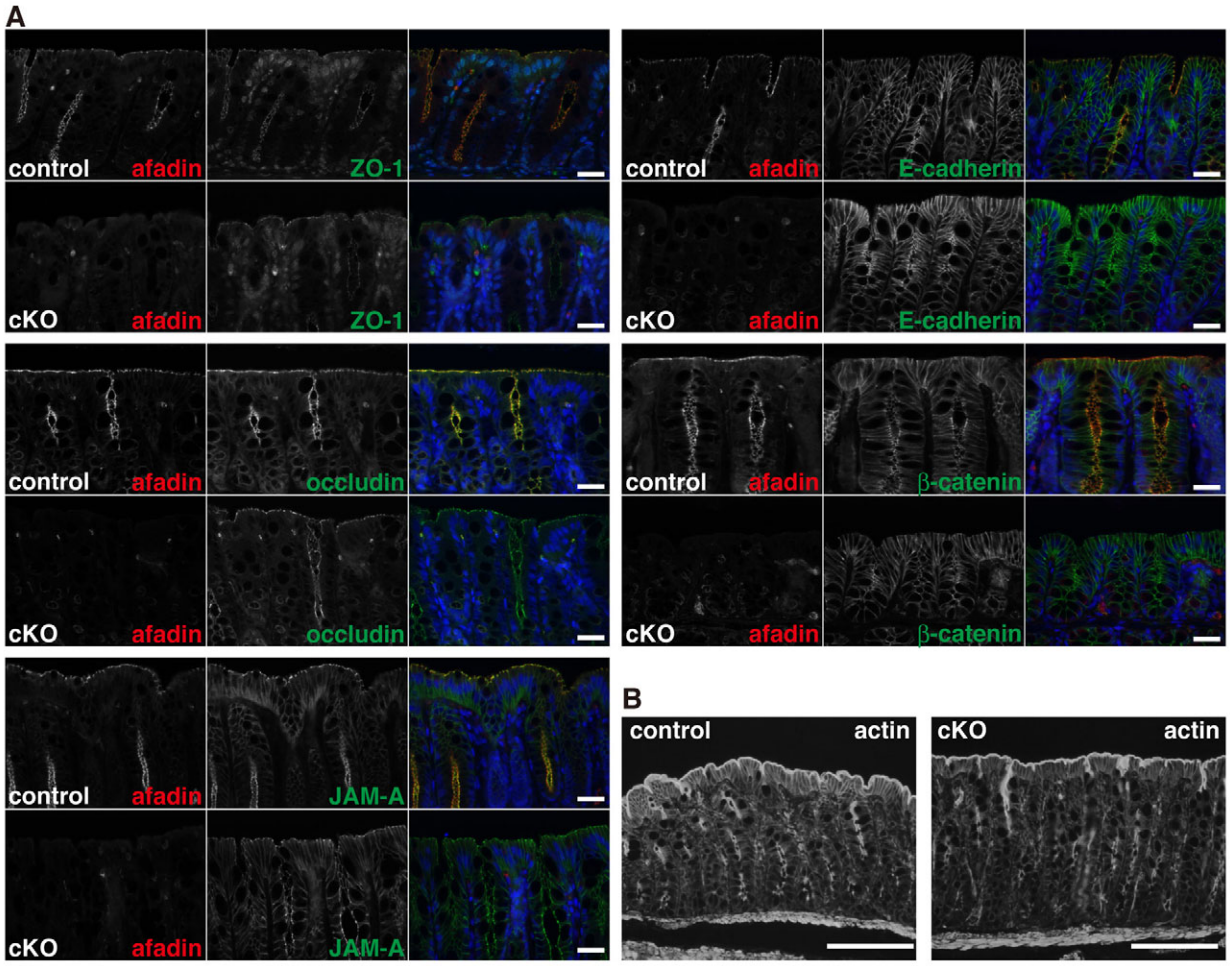


**Fig. 4. Localization of the junctional components at AJs and TJs.** (**A**) The signals for the AJ or TJ proteins are shown as indicated in each panel. Colon sections obtained from afadin cKO mouse littermates were immunostained with antibodies against the proteins as indicated. Scale bars: 30 µm. (**B**) Localization patterns of the actin cytoskeleton in the colon epithelia are compared between the control and afadin cKO mice. Scale bars: 200 µm.

that they are functionally intact. Therefore, we examined barrier function of the intestinal mucosal membrane in the afadin cKO mice (Fig. 5). Intestinal permeability was studied by determining the serum concentration of fluorescently labeled dextran that was orally administered as described (Wang et al., 2007). Notably, the epithelial permeability in the afadin cKO mice was 2.7-fold higher than in control mice (Fig. 5A). To confirm the role of afadin in epithelial barrier function, we next compared this phenotype to that of complete KO mice lacking nectin-2 and nectin-3 in the same genetic background. In contrast to the afadin cKO mice, the mice lacking nectin-2, nectin-3 or both showed no significant change in the levels of intestinal permeability (Fig. 5B). Actually, the double-knockout mice lacking both nectins are not generated by the same conditional knockout strategy as the afadin cKO mice, even though they share the nearly identical genetic background. However, the expression and localization of the afadin protein were unimpaired in the mice lacking nectins (Fig. 3A), and protein levels of nectin-1 and nectin-4 were unchanged (data not shown). Therefore, it remains unknown whether or not other compensatory mechanisms modify the phenotype of the mice lacking nectins. These findings indicate that the loss of afadin was closely correlated with the impaired paracellular permeability of the apical junctions despite their seemingly normal structures, and that afadin might

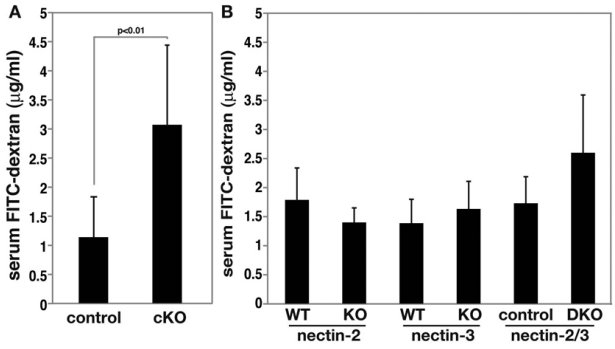


**Fig. 5. Alterations in epithelial permeability of the intestines of mice lacking afadin, nectin-2 and nectin-3.** (**A**) In vivo intestinal permeability was determined by serum concentration 4 hours after FITC-dextran gavage. Littermates from control ($n$=7) and afadin cKO mice ($n$=8) were analyzed; $P$<0.01. (**B**) Epithelial permeability in the intestines of control mice and mice lacking nectin-2 and/or nectin-3 was evaluated. Experiments were performed using wild-type (WT) littermates (WT, $n$=8; nectin-2 KO, $n$=4) (WT, $n$=7; nectin-3 KO, $n$=10). Control mice heterozygous for nectin-2 and nectin-3 ($n$=6), as well as double-knockout mice lacking both nectin-2 and nectin-3 (DKO) ($n$=10), were all littermates. No significant change was observed in the serum FITC-dextran levels between the three groups of mouse genotypes.

play roles in regulating epithelial permeability of the intestines, at least once the epithelial structure has been established.

### Enhanced susceptibility to treatment with dextran sulfate sodium in mice lacking afadin

Although the nectin–afadin module has been implicated in tissue morphogenesis, its role in the pathological context remains largely unknown. To address whether nectins and afadin act as a regulator in mucosal protection, we challenged the mice with the model for inflammation of colitis mediated by dextran sulfate sodium (DSS), a strong inducer of apoptosis in epithelial cells, as described (Okayasu et al., 1990). Increased susceptibility to DSS treatment often leads to deregulation of mucosal homeostasis. We examined whether or not the loss of afadin causes an increase in susceptibility to DSS in a dose- and time-dependent manner. As expected, the afadin cKO mice administered with 2.5% DSS for 14 days showed drastic loss of weight, atrophy of the colon, and a reduced survival rate as compared with the control mice (Fig. 6Aa–Ac). By contrast, no change in the susceptibility to DSS was observed in mice lacking nectin-2 or nectin-3 (data not shown), nor in mice lacking both nectin-2 and nectin-3 (Fig. 6Ac). Histopathology of the colon in the afadin cKO mice indicated severe degeneration of the epithelial structure, accompanied by infiltration of inflammatory cells, as compared with the control mice (Fig. 6B). We examined the effect of DSS on the small intestines for 5 days after the initiation of DSS treatment. As judged by hematoxylin and eosin staining, the distal part, but not the proximal part, of the small intestine in the afadin cKO mice showed disrupted epithelial structure similar to that observed in the colon (Fig. 6B). Moreover, we analyzed the time course of DSS treatment and found that the epithelial structure in the colon of the afadin cKO mice was disrupted early at the onset of DSS treatment as compared with control mice (supplementary material Fig. S3A). The afadin cKO mice showed increased lethality with disrupted mucosal structure

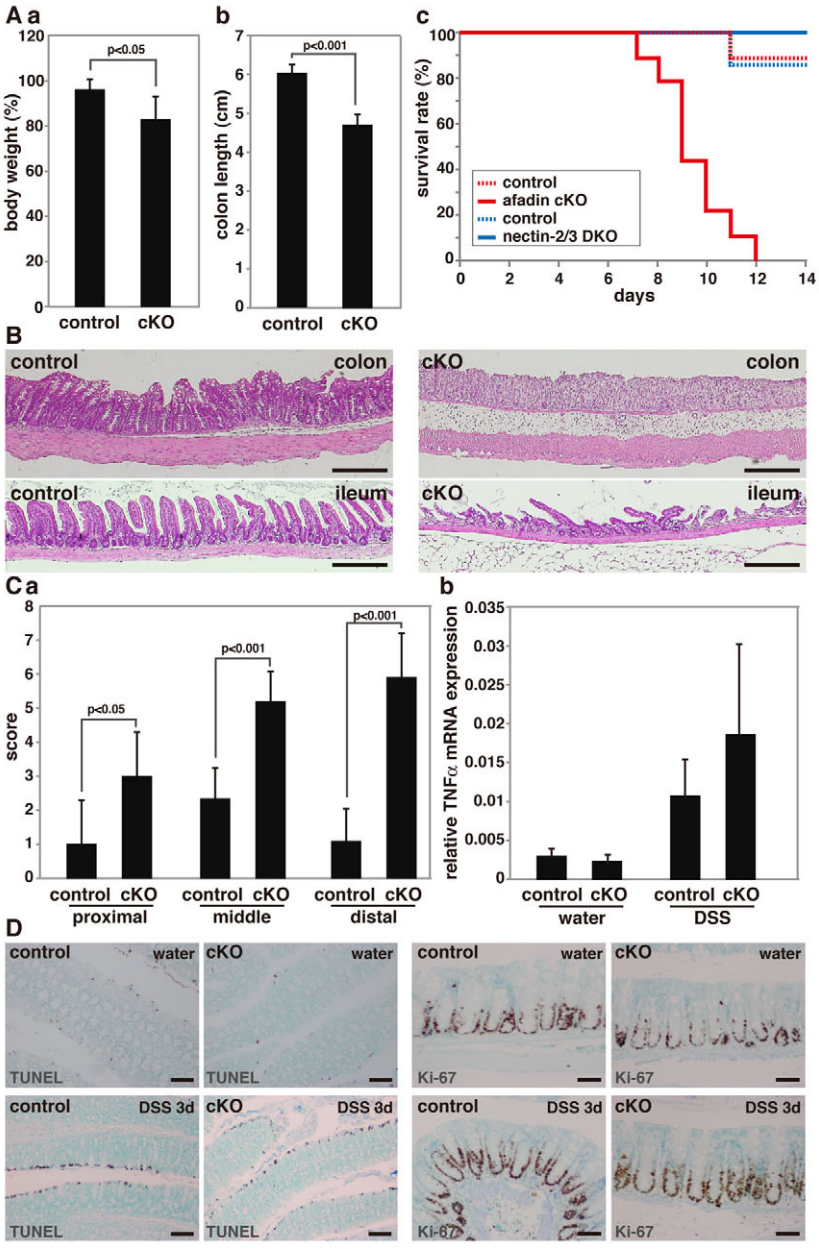


**Fig. 6. Enhanced susceptibility to DSS-induced epithelial injury in afadin cKO mice.** (**A**) Alterations in body weight (a), and colon length (b) in the control and afadin cKO mice 5 days after 2.5% DSS treatment. Differences in body weight and colon length between the control and afadin cKO mice were statistically significant ($P<0.05$ and $P<0.001$, respectively). Kaplan–Meier survival curves for the afadin cKO mice (c) and double-knockout mice lacking both nectin-2 and nectin-3 (DKO) (d). Two experiments were separately performed: first, the afadin cKO mice (red solid line, $n=9$) and the control mice (red dotted line, $n=9$) were administered 2.5% DSS for 14 days; and second, the DKO mice (blue solid line, $n=4$) and the control mice heterozygous for nectin-2 and nectin-3 (blue dotted line, $n=7$) were analyzed under the same conditions. The control mice were derived from littermates in each experiment. (**B**) Mucosal injury in the colon and ileum. Colon sections (upper panels) and ileum sections (lower panels) were stained with hematoxylin and eosin 7 or 5 days after DSS treatment, respectively. Compared with the control mice (left panels), prominent mucosal damage associated with inflammation, ulceration and loss of the crypts (right panels) was evident in the afadin cKO mice. Scale bars: 300 μm. (**C**) Histopathological scores for the mucosal damage. The scores for lesions at 5 days after DSS treatment were determined independently by three observers using a double-blind method. (a) Differences between the control and afadin cKO mice were statistically significant in the proximal part of the colon ($P<0.05$), the middle part ($P<0.001$) and the distal part ($P<0.001$). Error bars indicate s.d. (b) *TNFα* mRNA levels were evaluated by real-time PCR and normalized to the level of *GAPDH* mRNA ($n=6$). (**D**) Apoptosis and proliferation of residual epithelial cells during recovery from the mucosal injury. The TUNEL assay and immunostaining with an anti-Ki-67 antibody were performed using colon sections 3 days after DSS treatment. Scale bars: 100 μm (TUNEL); 50 μm (Ki-67).

in the intestinal epithelia. The phenotype was observed even when the duration of DSS treatment was reduced to 4 days, suggesting that the increased susceptibility to initial damage mediated by DSS might be the primary mechanism for the phenotype of the afadin cKO mice. Histological scores of mucosal damage and expression of inflammatory cytokines were significantly increased in the proximal to distal parts of the colon from the afadin cKO mice treated with DSS (Fig. 6Ca). The effect on the damage and extension scores is significant when they are separated (supplementary material Fig. S3B). The difference in TNFα expression was not statistically significant, although the averaged value of *TNF*α mRNA was higher than that of the control (Fig. 6Cb). On the other hand, the colonic segments from the afadin cKO mice were labeled at the same levels by staining with anti-Ki67 antibodies and by using the TUNEL method as compared with the control mice, indicating that afadin is not essentially required for proliferation and apoptosis of the residual cells after DSS treatment (Fig. 6D). Taken together, the loss of afadin impairs normal barrier function and homeostasis in the intestinal mucosa but affects neither proliferation nor apoptosis of the intestinal epithelial cells, whereas the nectin–afadin module plays no significant role in these conditions.

### Mechanistic implications of the deficiency of afadin in the mouse epithelia

Afadin contains multiple functional domains that regulate intracellular signaling, in addition to the PDZ domain that interacts with nectins. Importantly, afadin mediates activation of Rap1 small GTP-binding protein in AJ formation (Boettner et al., 2000; Fukuyama et al., 2005; Hoshino et al., 2005; Sato et al., 2006) and cell migration (Miyata et al., 2009a; Miyata et al., 2009b) in culture, whereas afadin transduces PI3K–Akt signaling downstream of the ternary complex composed of Necl, PDFGR and integrin αvβ3 (Miyata et al., 2009a; Miyata et al., 2009b; Kanzaki et al., 2008). To address these activities in mice, we investigated how the loss of afadin is implicated in signaling mediated by Rap1 activity and in PI3K–Akt signaling. We examined Rap1 activity by pulldown assay using the epithelia cells from the colon of the afadin cKO mice (Fig. 7A). The level of the GTP-bound form of Rap1 was reduced in the afadin cKO mice as compared with that of the wild-type mice, consistent with a previous report that activation of Rap1A was reduced in the afadin knockdown experiments using intestinal cells in culture (Severson et al., 2009). Afadin thus regulates the activity of Rap1 in the mouse intestines. To further evaluate the activity of the PI3K signaling during DSS treatment, we examined the phosphorylation of Akt in afadin cKO mice. We noted that the timing of the rapid increase in the phosphorylation of Akt coincides with the initiation of DSS treatment (Fig. 7B). The level of phosphorylated Akt tended to be higher in the afadin cKO mice than in the control mice, although the phosphorylation of Akt was increased in both of the mice during DSS treatment. Therefore PI3K signaling is likely to be activated in parallel with the extent of epithelial damage. These findings suggest that afadin regulates the barrier function of the mouse intestines and plays a protective role in DSS-mediated injury, at least in part, by activating Rap1 and regulating the PI3K signaling.

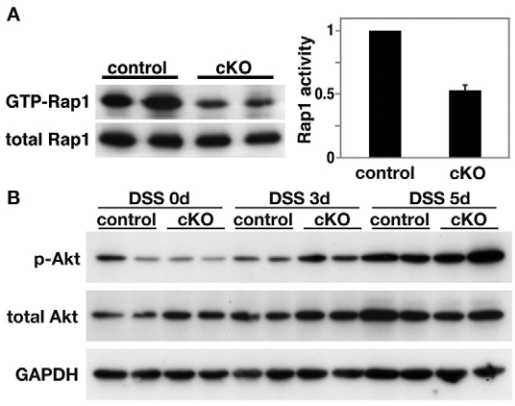


**Fig. 7. Alterations in the Rap1 and PI3K–Akt signaling in afadin cKO mice.** (**A**) Pulldown assay of Rap1 using the lysates from the colon of afadin cKO and control mice. The histogram shows the values of blots indicative of Rap1 activities ($n$=4). (**B**) Levels of phosphorylated Akt during the DSS treatment. Colon lysates were extracted with a cocktail of phosphatase inhibitors at days 0, 3, and 5 during the early course of DSS administration.

## Discussion

Our results reveal a novel role for afadin by using the conditional strategy for disruption of the afadin gene in the mouse intestine. The role annotates afadin as a regulator that recruits nectins to the apical junction of epithelial cells, and moreover underscores the importance of afadin in the functional integrity of epithelia in the intestines. Our previous study showed that tissue morphogenesis stops at around E7.5 in the afadin KO mice (Ikeda et al., 1999). The timing coincides exactly with the onset of the primary epithelial-to-mesenchymal transitions that generate the mesoderm and endoderm structures from the neuroectoderm (Acloque et al., 2009). In this study, villin-Cre recombinase initiates disruption of the gene encoding afadin in mice after birth, implying that afadin plays a role required for tissue morphogenesis during embryonic development. Afadin was not detected in the intestines of the 10-week-old cKO mice and, in concert, the signals for nectin-2 and nectin-3 were disassembled from the apical junctions and mislocalized to the basolateral domains of the epithelial cells. By contrast, the signal for afadin was localized to the apical junctions in mice lacking both nectin-2 and nectin-3. Accordingly, afadin seems to be essential for recruiting nectins to apical junctions, presumably AJs, in the colon. Nevertheless, the afadin cKO mice were viable and showed apparently normal architecture of the intestinal epithelia, suggesting that the nectin–afadin module is crucial to the morphogenesis of the epithelial structure but not essential to the physiological turnover of epithelial cells in the intestines once the epithelial structure has been established. These findings are consistent with our previous studies in cell culture (Sato et al., 2006), as well as the findings on Canoe in *Drosophila melanogaster* (Sawyer et al., 2009).

Phenotypic analyses of the mice revealed the consistent and crucial roles of afadin in barrier function and in homeostasis of the intestines. First, paracellular permeability of the colon epithelia was increased in afadin cKO mice as compared with control mice. Second, the direct functional role of afadin in the homeostasis of intestines was underscored by the enhanced susceptibility to DSS treatment. The AJ and TJ modules interact with each other and play roles cooperatively in the formation and functions of the apical junctions. Afadin has been reported to bind to JAM-A, a component of TJs, and JAM-A knockout mice showed a phenotype similar to the afadin cKO mice (Laukoetter et al., 2007). The impairment of intestinal barrier function presented here indicates

that the TJs in the intestinal epithelia of the afadin cKO mice are morphologically normal but functionally impaired. The phenotype is conceivably explained by such TJ-oriented roles of afadin.

The mislocalization of nectin-2 and nectin-3 might also play a causative role in the development of impaired barrier function and homeostasis. However, this possibility seems unlikely because complete genetic disruption of nectin-2, nectin-3 or both, had no influence on the degree of paracellular permeability or on DSS-mediated injuries. It is conceivable that compensatory mechanisms might have an impact on the phenotype of mice lacking nectins. However, the exact mechanism of compensation remains unclear because the expression levels of other nectin members were unchanged in the intestines. The afadin cKO mice notably showed a profound phenotype compared with the mice that lacked both nectin-2 and nectin-3, despite the differences in knockout strategy. Moreover, the afadin cKO mice showed decreased activities of Rap1 and enhanced PI3K signaling in the intestines, indicating the involvement of the regulatory mechanisms directly correlated with afadin. Therefore, it seems to be the absence of afadin, but not nectins, that mainly causes the phenotype of the afadin cKO mice. Nectins have not as strong adhesion force as E-cadherins (Tsukasaki et al., 2007), and less force is required to separate doublets of fibroblasts expressing nectin-1 or nectin-3 than to separate doublets of E-cadherin-expressing cells (Martinez-Rico et al., 2005; Vedula et al., 2007). Therefore, the main role of the nectin–afadin module is probably to organize mature apical junctions in the intestines of developing mice rather than to act as a major cell–cell adhesion molecule in barrier function and homeostasis. In other words, the nectin–afadin module is unnecessary for the functional maintenance of apical junctions.

We have proposed nectins and Necls as a family of $Ca^{2+}$-independent immunoglobulin-like cell adhesion molecules (Takai et al., 2008). The nectin/Necl family plays crucial roles in establishing the normal architecture of the nervous system, vascular system and epithelial structure in various organs. Afadin physically binds all of the nectins, but not Necls, implying that the main role of afadin in epithelial cells is directed towards the regulation of cell adhesion mediated by nectin engagement. However, recent studies have shown the involvement of afadin in Necl-mediated signaling initiated by the extracellular Necl–integrin–PDGFR complex (Miyata et al., 2009a; Miyata et al., 2009b). Moreover, the nectin/Necl family plays crucial roles in heterotypic cell adhesion between the epithelium and mesenchymal stroma, and contributes to boosting the invasive phenotype of tumor cells by activating a variety of genes that facilitate cell proliferation, dedifferentiation, migration and invasion (Fabre-Lafay et al., 2005; Ikeda et al., 2004; Kuramochi et al., 2001; Morimoto et al., 2008; Nakai et al., 2010; Raveh et al., 2009; Yu et al., 2005). The loss of afadin might conceivably alter the epithelial environment by impairing the interactions between epithelial cells and stromal fibroblasts or inflammatory cells. This could be achieved through the impaired interactions between nectin-2 and DNAM-1 expressed on T cells, NK cells, and monocytes (Bottino et al., 2003; Tahara-Hanaoka et al., 2004), as well as through loss of afadin-mediated regulation on the actin cytoskeleton and the impaired interaction with integrins. It remains unknown, however, which roles of afadin play an important part in our DSS-mediated injury model because the experimental conditions involve many biological aspects, such as acute tissue damage, inflammation and wound-healing processes. Our findings on the time course of the intestinal injuries indicate a role of afadin at the onset of DSS treatment, whereas we observed no increase in the number of apoptotic cells by TUNEL assays. Considering that TUNEL assays are unable to detect dead cells already removed from the epithelia, it seems difficult to elucidate the anti-apoptotic role of afadin at the initial phase of DSS treatment. We did not rule out the possibility that afadin also plays significant roles in inflammation and wound-healing processes. Unfortunately, our DSS injury model did not enable us to examine the role of afadin in the later steps subsequent to acute-phase reaction, although DSS actually induces chronic inflammation and carcinogenesis in the mouse colon (Abe et al., 2009). Because of dual function of the nectin/Necl family in tissue morphogenesis and remodeling, as well as in tumor progression, future investigations might seek to exploit afadin as a therapeutic target, especially in a combinatorial fashion with inhibitors of nectins, integrins and receptor tyrosine kinases.

## Materials and Methods

### Antibodies

All of the following antibodies were purchased from commercial sources: antibodies against afadin and nectin-3 (ABcam), nectin-1 (Santa Cruz Biotechnology), nectin-2 (Hycult), E-cadherin (R&D Systems and BD Biosciences), α-catenin (Sigma-Aldrich), β-catenin (BD Biosciences), ZO-1 (Sanko-junyaku, Tokyo, Japan), Ki-67 (Novocastra Laboratories, Newcastle upon Tyne, UK), JAM-A (R&D Systems), Akt, and phosphorylated Akt (Cell Signaling Technology). Anti-actin (C4) and anti-occludin antibodies, rhodamine-phalloidin, Hoechst 33258, and Alexa-Fluor-labelled secondary antibodies were purchased from Invitrogen.

### Mice

Animal care and experimental procedures were approved by the IACUC of the Osaka Medical Center for Cancer and Cardiovascular Diseases and of Kobe University. The targeting strategy for the disruption of the afadin gene was previously described (Majima et al., 2009). Afadin-floxed mice were mated with the transgenic mice expressing Cre-recombinase under the control of villin promoter (Jackson Laboratory, Bar Harbor, ME). Cre-expressing mice that were homozygous for afadin-floxed alleles were selected and maintained. All of the experiments were performed by using littermates obtained from intercrossing of afadin-floxed/floxed mice with or without the Cre transgene. The generation of mice lacking nectin-3 were previously described (Inagaki et al., 2005), whereas mice lacking nectin-2 were kindly provided by Eckard Wimmer, State University of New York, Stony Brook, NY (Mueller et al., 2003). All of the results presented here were obtained from genetically inbred mice of the same genetic background. Knockout mice of the first generation had a genetic background consisting of 129SV, C57BL/6 and DBA2 (50%, 25% and 25%, respectively). They were further mated with C57BL/6 mice or those expressing Cre recombinase. The final genetic background of the mice conditionally lacking afadin was 129SV:C57BL/6:DBA2=37.5:43.75:18.75 (%) whereas that of the mice conventionally lacking nectin-2 or nectin-3 was 129SV:C57BL/6:DBA2=25:37.5:37.5 (%).

### Isolation of epithelial cells

The mouse intestines were longitudinally cut and opened, and washed using Hank's balanced salt solution (HBSS). Intestinal epithelial cells were isolated as described (Clayburgh et al., 2005). Briefly, the tissues were incubated in calcium- and magnesium-free HBSS (CMF-HBSS) containing 10 mM dithiothreitol and 50 nM calyculin A (Calbiochem) for 30 minutes at 4°C, and then transferred to CMF–HBSS with 1 mM EDTA and 50 nM calyculin A and incubated at 4°C for 1 hour. The intestinal epithelial cells were desquamated by forcible pipetting and collected by centrifugation at 500 ***g*** for 10 minutes.

### Immunoblot analyses and pulldown assay

Cells and tissues were solubilized with RIPA lysis buffer: 50 mM Tris (pH 8.0), 150 mM NaCl, 1.0% Nonidet P-40, 0.5% deoxycholic acid, 0.1% SDS, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 20 μg/ml phenylmethylsulfonyl fluoride and phosphatase inhibitor. The lysate was clarified by centrifugation at 10,000 ***g*** for 10 minutes at 4°C, and protein concentration of clarified extracts was determined using a protein assay kit (Bio-Rad Laboratories, Hercules, CA). The applied extracts were resolved in SDS-polyacrylamide gels, and electrophoretically transferred to polyvinylidene difluoride membrane, followed by incubation with primary antibodies at 4°C overnight. The blots were subsequently incubated with horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit IgGs for 30 minutes, and further treated with ECL western blotting detection reagents (GE Healthcare, Little Chalfont, UK). For pulldown assay, lysates were prepared from the colon as described (Ishizaki et al., 2006), and incubated with Ral GDS-RBD agarose according to the manufacturer's instructions (Millipore Corporation). Finally, the membrane was incubated with immunoreaction enhancer solution (Toyobo, Osaka, Japan) for detecting Rap1 activities.

### Immunofluorescence microscopy

Mouse intestinal sections were fixed in 20% formalin neutral buffer solution, embedded in paraffin, and sectioned at 3–4 mm thickness. After deparaffinization, sections were occasionally treated with $H_2O_2$ solution for chemiluminescence detection. Antigens were retrieved by boiling with 10 mM sodium citrate buffer. After blocking with 5% skimmed milk and 0.005% saponin in phosphate-buffered saline (PBS), samples were incubated with primary antibodies for 1 hour and then with fluorescent or HRP-conjugated secondary antibodies for 30 minutes. Chemiluminescence or fluorescence images were recorded on a charge-coupled device camera (Keyence, Osaka, Japan), and processed using Vertical Horizon analyzer software and Adobe Photoshop.

### Transmission electron microscopy

Small pieces of colon and small intestine were fixed with 2% fresh formaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 2 hours at room temperature and kept at 4°C. After washing with 0.1 M cacodylate buffer (pH 7.4) three times (5 minutes each), they were postfixed with ice-cold 1% $OsO_4$ in the same buffer for 2 hours. The samples were rinsed with distilled water, stained with 0.5% aqueous uranyl acetate for 2 hours at room temperature, dehydrated with ethanol, and embedded in Poly/Bed 812. Ultra-thin sections were cut, doubly stained with uranyl acetate and Reynold's lead citrate, and viewed with a JEM 1010 transmission electron microscope (JEOL, Tokyo, Japan) at an accelerating voltage of 100 kV.

### TUNEL staining

The intestinal sections were deparaffinized and subjected to TUNEL assay as described in the manufacturer's instructions (Takara Bio, Otsu, Japan).

### DSS-induced colitis and histopathological analyses

Mice of 6 to 8 weeks old mice were treated with drinking water supplemented with 2.5% DSS (36,000–50,000, MP Biomedicals, Morgan Irvine, CA). The sections of proximal, middle and distal part of the colon were fixed in 20% formalin neutral buffer solution, embedded in paraffin, and stained with hematoxylin and eosin. The clinical scores of colitis were determined in a blinded fashion using a scoring system as described (Kitajima et al., 2000). Briefly, the samples were graded according to the extent of mucosal damage and extension of the lesion as follows: damage score (0, none; 1, loss of the basal 1/3 of the crypt; 2, loss of the 2/3 of the crypt; 3, loss of entire crypt but intact surface epithelial cells; 4, loss of both the entire crypt and the surface epithelial cells), extension score (0, none; 1, focal; 2, lesions involving 1/3 of the intestine; 3, lesions involving 2/3 of the intestine; 4, lesions involving the entire intestine).

### In vivo permeability assay

FITC-dextran (FD4, Sigma Aldrich) was used as an indicator to examine permeability of the intestines in 6- to 8-week-old mice as described (Wang et al., 2007). Briefly, FITC–dextran was administered through oral intake of 60 mg/100 g body weight for 4 hours. Fluorescence intensity of diluted samples was measured by ARVO MX (Perkin Elmer).

### Statistical analyses

The Student's *t* test was used to evaluate significance of differences between two mouse groups of different genotypes. $P<0.05$ was considered statistically significant. All calculations were performed using StatMate (ATMS). Data were analyzed using Kaplan–Meier estimates to determine survival curves for afadin cKO and wild-type mice.

This work was supported by grants-in-aid for Cancer Research from the Ministry of Education, Culture, Sports, Science, and Technology, Japan (to J.M., for the period 2004–2010). Deposited in PMC for immediate release.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/124/13/2231/DC1

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