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Establishment of cell polarity by afadin during the formation of embryoid bodies

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Abstract

Afadin directly links nectin, an immunoglobulin-like cell-cell adhesion molecule, to actin filaments at adherens junctions (AJs). The nectin-afadin complex is important for the formation of not only AJs but also tight junctions (TJs) in epithelial cells. Studies using afadin-knockout mice have revealed that afadin is indispensable for embryonic development by organizing the formation of cell-cell junctions. However, the molecular mechanism of cell-cell junction disorganization during embryonic development in afadin-knockout mice is poorly understood. To address this, we took advantage of embryoid bodies (EBs) as a model system. The formation of cell-cell junctions including AJs and TJs was impaired in afadin-null EBs. The proper accumulation of the Par complex and the activation of Cdc42 and atypical PKC, which are crucial for the formation of cell polarity, were also inhibited by knockout of afadin. In addition, the disruption of afadin caused the abnormal deposition of laminin and the dislocalization of its receptors integrin α_6 and integrin β_1. These results indicate that afadin organizes the formation of cell-cell junctions by regulating cell polarization in early embryonic development.

Introduction

The organized formation of cell-cell junctions plays an important role in the embryonic development. The mechanisms of cell-cell junctions are rigorously studied in polarized epithelial cells that comprise several specialized junctional apparatuses such as tight junctions (TJs) and adherens junctions (AJs), which are typically aligned from the apical to the basal side along the cell-cell adhesion sites (Farquhar & Palade 1963). Two neighboring cells are completely sealed at TJs medicated by TJ strands composed of integral components, such as claudin, occludin, and ZO proteins (Tsukita *et al.* 2001). Thus, TJs strictly regulate the paracellular transport of solutes and water (barrier function) and prevent the intermingling of the cell surface proteins and lipids between the basolateral and the apical regions (fence function). On the other hand, AJs are the sites of cell recognition and mechanically connect adjacent cells to resist strong contractile forces and to maintain cell-cell junctions (Gumbiner 1996). At AJs, cadherin functions as a key Ca^{2+}-dependent cell-cell adhesion molecule and is linked to the actin cytoskeleton through many peripheral membrane proteins, including α-catenin, β-catenin, vinculin, and α-actinin, by binding to the cytoplasmic tail of cadherin (Takeichi 1991; Gumbiner 2000). All of these molecules participate in the increase in the cell-cell adhesion strength of AJs and facilitate the formation and maintenance of AJs.

Our recent studies have newly revealed that an emerging CAM nectin and its linker protein afadin specifically localize at AJs and play an essential role in the formation of not only AJs and TJs (Takai & Nakanishi 2003; Ogita & Takai 2006). Nectin is a Ca^{2+}-independent immunoglobulin-like CAM and the nectin family comprises four members: nectin-1, nectin-2, nectin-3, and nectin-4. On the other hand, afadin interacts with all of these nectin members between the PDZ domain of

afadin and the C-terminal four amino acids of nectin and also binds to actin filaments (F-actin) to link nectin to the actin cytoskeleton (Mandai *et al.* 1997; Takahashi *et al.* 1999). In the process of the formation of cell-cell junctions, the nectin-afadin complex first assembles at the initial cell-cell contact and then recruits the cadherin-catenin complex to the nectin-based cell-cell adhesion sites, leading to the formation of AJs. Thereafter, the claudin-based TJs are formed at the apical side of AJs in the action of a variety of molecules including the nectin-afadin complex, the Par polarity protein complex, and several actin binding proteins.

Afadin interacts with various proteins including α-catenin, ponsin, ADIP, LMO7, Rap1, and ZO-1 (Yamamoto *et al.* 1997; Mandai *et al.* 1999; Boettner *et al.* 2000; Tachibana *et al.* 2000; Pokutta *et al.* 2002; Asada *et al.* 2003; Su *et al.* 2003; Ooshio *et al.* 2004; Hoshino *et al.* 2005). The interaction of afadin with α-catenin, ponsin, ADIP, and LMO7 physically connects between the nectin-afadin and cadherin-catenin systems and promotes the recruitment of the cadherin-catenin system to the nectin-based cell-cell adhesion sites and the formation of AJs (Mandai *et al.* 1999; Tachibana *et al.* 2000; Asada *et al.* 2003; Ooshio *et al.* 2004). Afadin also forms a complex with Rap1 small G protein, which is activated by the *trans*-interaction of nectin, and this afadin-Rap1 complex enhances the adhesion activity of E-cadherin and prevents the endocytosis of E-cadherin cooperatively with $p120^{ctn}$, positively supporting the formation and maintenance of AJs (Hoshino *et al.* 2005; Sato *et al.* 2006). In addition, the association of afadin with ZO-1 or the Par complex is necessary for the formation of TJs in MDCK cells (Yamamoto *et al.* 1997; Ooshio *et al.* 2007). Therefore, afadin is likely to have multiple roles in the formation of cell-cell junctions.

Physiological significance of afadin has been clearly proved by the *in vivo* studies using afadin-null embryos (Ikeda *et al.* 1999; Zhadanov *et al.* 1999). Afadin-null embryos show developmental defects at stages during and after

gastrulation. In the absence of afadin, gastrulation itself appears to occur, because the mesoderm can be generated in afadin-null embryos, but the migration of mesodermal cells is suppressed and the ectoderm is disorganized due to the impaired cell-cell junctions. This causes loss of somites and other structures derived from both the ectoderm and the mesoderm, eventually resulting in embryonic lethal in afadin-knockout mice. Therefore, afadin is indispensable for embryonic development. However, as for the impaired cell-cell junctions in afadin-null embryos, we evaluated the localization of only the limited members of CAMs such as E-cadherin. The molecular mechanism by which cell-cell junctions in afadin-null embryos are disorganized is largely unclear, because the relationship between afadin and other CAMs including nectin was not certified when we made a study of afadin-knockout embryos (Ikeda *et al.* 1999). We used here embryoid bodies (EBs) generated from embryonic stem (ES) cells to investigate that molecular mechanism. EBs are a useful model system for analysis of early embryonic development *in vitro*, because EBs can reproduce the development of two-layered epithelial structures and subsequent mesoderm induction from the inner layer. We then investigated in more detail the localization of not only various CAMs but also the Par complex proteins, which is essential for the formation of cell polarity, and analyzed the activation of Cdc42 and atypical PKC (aPKC), which critically regulate the function of the Par complex, in wild-type and afadin-null EBs.

Results

Morphological analysis of wild-type and afadin-null EBs

The external appearance of wild-type and afadin-null EBs were similar **(Fig. 1A)**, but histological analysis with hematoxylin and eosin (H-E) staining showed that the central cyst was full of cells and that the cavitation was remarkably impaired in afadin-null EBs compared with wild-type EBs **(Fig. 1B)**, as we previously reported (Ikeda *et al.* 1999). In addition, the formation of basement membrane was somewhat disorganized in afadin-null EBs. Ultrastructural analysis by transmission electron microscopy (TEM) revealed that in wild-type EBs, the cell-cell junctional complex including AJs and TJs was established between the cells, especially at the apical side of both the ectoderm and endoderm **(Fig. 1, C and D)**. In contrast, in the absence of afadin, cells in the ectoderm had only sporadic cell contacts on the cell-cell interface and the cell-cell junctional complex was not well developed **(Fig. 1E)**. Although the H-E staining did not exhibit the apparent difference in the endoderm between wild-type and afadin-null EBs, the precise observation with TEM depicted that some of the cells in the endoderm of afadin-null EBs had loose cell-cell adhesions **(Fig. 1F)**. Consistent with the result from the H-E staining, the basement membrane in afadin-null EBs was tortuous and often abnormally present within the endodermal and ectodermal layers **(Fig. 1G)**. These results confirms our previous observation that afadin plays an essential role in the formation of cell-cell junctions in early embryonic development.

Dislocalization of nectin in afadin-null EBs

The relationship between afadin and nectin was not certified at the time of our previous study using afadin-knockout embryos (Ikeda *et al.* 1999). Nectin is a binding partner of afadin and one of CAMs localized at AJs. We examined here

the localization of nectin in wild-type and afadin-null EBs. In wild-type EBs, the immunofluorescence signals for afadin and nectin-2 identically localized at the apical side of both the ectoderm and endoderm **(Fig. 2, A and B)**. However, in afadin-null EBs, the signal for nectin-2 was hardly detected in the ectoderm and was observed along the cell-cell contact sites, but not restricted at the apical side, in the endoderm **(Fig. 2B)**. The expression level of nectin-2 was similar between wild-type and afadin-null EBs **(Fig. 2C)**. These results indicate that afadin is necessary for the proper localization of nectin, which is involved in the formation of not only AJs but also TJs. In addition, the deposition of laminin was highly disturbed in afadin-null EBs **(Fig. 2A)**. This is likely to be consistent with the above result that the basement membrane of EBs was disorganized in the absence of afadin, because laminin is one of the extracellular matrix (ECM) proteins and a component of basement membrane.

Dislocalization of AJ and TJ components in afadin-null EBs

Since the formation of cell-cell junctions was impaired and the dislocalization of nectin was observed in afadin-null EBs as described above, we further examined the localization of other components of AJs and TJs in wild-type and afadin-null EBs. In wild-type EBs, the signal for β-catenin, an AJ marker, preferentially co-localized with that for F-actin along the cell-cell adhesion sites in the endoderm and was highly concentrated at the apical portion of the ectoderm, adjacent to the central cavity **(Fig. 3A)**. Although the signal for β-catenin in the endoderm of afadin-null EBs was also distributed along the cell-cell adhesion sites, its co-localization with that for F-actin was markedly reduced, suggesting the formation of weak and immature AJs, because AJs are generally undercoated with F-actin that enhances the function of AJs for connecting adjacent cells. The signal for β-catenin in the ectoderm was hardly observed in the absence of afadin. Essentially the same

results were obtained for other AJ markers E-cadherin and α-catenin (Data not shown). Next, the signal for ZO-1, a TJ marker, was concentrated at the outermost and innermost part of the endoderm and ectoderm, respectively, in wild-type EBs, whereas that for ZO-1 was more diffusely and weakly distributed along the cell-cell adhesion sites in the endoderm and was almost diminished in the ectoderm in afadin-null EBs **(Fig. 3B)**. Essentially the same results were also obtained for another TJ marker occludin (Data not shown). The expression levels of β-catenin and ZO-1 were similar irrespective of the presence or absence of afadin **(Fig. 3C)**. Taken together, these results indicate that the formation of AJs and TJs was impaired in the endoderm and almost disrupted in the ectoderm of EBs by knockout of afadin.

Impaired accumulation of the Par complex proteins in afadin-null EBs

We also examined the localization of the Par complex in each type of EBs, because the Par complex plays a crucial role in the formation of cell polarity and the cell polarization is prerequisite for the proper organization of cell-cell junctions (Ohno 2001). In addition, we reported that Par-3, one of the components of the Par complex, binds to nectin (Takekuni *et al.* 2003). The signal for Par-3 localized at the apical side of both the ectoderm and endoderm in wild-type EBs, whereas in afadin-null EBs, that for Par-3 was only detected as dot-like spots randomly localized in the ectoderm and was not observed in the endoderm **(Fig. 4A)**. Essentially the same results were obtained for Par-6, another member of the Par complex, although the signal for Par-6 in afadin-null EBs was not concentrated anywhere in either ectoderm or endoderm and even its dot-like spots were not observed **(Fig. 4B)**. However, the expression levels of these Par complex proteins were similar between wild-type and afadin-null EBs **(Fig. 4C)**, leading to the notion that in afadin-null EBs, these Par proteins diffusely and sparsely distributed

throughout the cytosol in both ectodermal and endodermal cells without the reduction of their expression levels. These results indicate that afadin plays a role in the proper localization of the Par complex during the EB formation, and that the impaired accumulation of the Par complex in the absence of afadin is at least partly one of the reasons why the formation of cell-cell junctions as well as polarization is disrupted in afadin-null EBs.

Reduced activation of Cdc42 and aPKC in afadin-null EBs

To further explore the molecular mechanism of impaired cell polarization by disruption of afadin, we investigated the activation of Cdc42 small G protein in afadin-null EBs, because the activation of Cdc42 was induced by the action of nectin and activated Cdc42 binds to Par-6 and regulates the function of the Par complex (Kawakatsu *et al.* 2002; Suzuki & Ohno 2006). When the amount of GTP-bound form of Cdc42 was determined by pull-down assay in wild-type and afadin-null EBs, it was apparently less in afadin-null EBs **(Fig. 5A)**. We next examined the activation of aPKC, which is induced by Cdc42 through Par-6. aPKC plays a central role in the formation of cell polarity by phosphorylating many proteins related to cell polarization (Etienne-Manneville 2004; Macara 2004). The phosphorylation of aPKC was actually less in afadin-null EBs than wild-type ones **(Fig. 5B)**. These results indicate that afadin is required for the activation of Cdc42 and aPKC, and suggest that the accumulation of the Par complex in afadin-null EBs is disrupted at least partly due to the suppression of the activation of Cdc42 and aPKC.

Impaired expression of integrins in afadin-null EBs

As described above, the deposition of laminin was highly disturbed in afadin-null EBs. This prompted us to investigate whether the localization and expression of

integrins that interact with laminin are affected by knockout of afadin. In wild-type EBs, the signals for integrin α_6 and integrin β_1 were clearly concentrated at the place of basement membrane and were also observed at the cell-cell adhesion sites of ectodermal cells **(Fig. 6, A and B)**. However, they did not exist at the innermost side of the ectoderm. In contrast, those for integrin α_6 and integrin β_1 were almost completely diminished throughout afadin-null EBs. The expression level of integrin α_6 was remarkably reduced by disruption of afadin **(Fig. 6C)**. As for integrin β_1, the amount of the matured form of integrin β_1 decreased in afadin-null EBs; instead, that of the immature form increased. Because the immature form of integrin β_1 preferentially accumulates in the intracellular precursor pool (De Strooper *et al.* 1991), this form of integrin β_1 does not seem to function as a receptor for laminin. Thus, these results indicate that afadin controls the expression and maturation of integrins, which may also lead to the establishment of cell polarity and cell-cell junctions.

Discussion

Although our and other groups reported that deficiency of afadin causes disorganization of cell-cell junctions and loss of cell polarity in mouse embryogenesis (Ikeda *et al.* 1999; Zhadanov *et al.* 1999), the molecular mechanism involved in such disorganization was poorly understood. To investigate the molecular mechanism, we took advantage of EBs that recapitulate the early stage of embryonic development *in vitro* (Keller 1995). Consistent with the results from afadin-knockout embryos, EBs derived from afadin-null ES cells showed the impaired formation of cell-cell junctions. Detailed analysis by TEM and immunofluorescence microscopy revealed that the cell-cell junctions in both the ectoderm and the endoderm are impaired in the absence of afadin and that the localization of all the AJ and TJ components observed were disturbed in afadin-null EBs, confirming the necessity of afadin for the formation of both AJs and TJs. However, the degree of abnormality in AJs and TJs was different between the endoderm and the ectoderm of afadin-null EBs: AJs and TJs were almost disrupted in the ectoderm, whereas the impairment of the formation of AJs was less severe than that of TJs in the endoderm. The exact reason why this discrepancy occurs in the endoderm of afadin-null EBs is unclear. An unknown rescue mechanism(s) against loss of afadin might exist in the endoderm for the formation of AJs, but this mechanism(s) might not be effective for the formation of TJs. The proper accumulation of the Par complex, which was crucial for cell polarization (Ohno 2001), was also inhibited in afadin-null EBs. Collectively, these results may provide a notion that impaired formation of cell-cell junctions in afadin-null EBs is caused by inability of cell polarization in which the Par complex is essentially involved. This would be supported by a recent study that shows the loss of AJs in conditional aPKCλ knockout mice (Imai *et al.* 2006).

To further explore the mechanisms of loss of accumulation of the Par complex, we examined the activation of Cdc42 and aPKC and showed that it is apparently reduced in afadin-null EBs. Rho family small G proteins such as Cdc42 are known to be important for the formation of cell polarization in epithelial cells in cooperation with the Par polarity complex (Joberty *et al.* 2000). The GTP-bound active form of Cdc42 directly binds to Par-6 and alters the intermolecular conformation of the Par-6-aPKC complex, resulting in the activation of aPKC (Macara 2004). Although we previously reported that afadin is not necessary for the activation of Cdc42 at the nectin-induced formation of cell-cell junctions (Kawakatsu *et al.* 2002), dislocalization of nectin in afadin-null EBs may attenuate the efficiency of the nectin-nectin *trans*-interaction and reduce the activation of Cdc42. The activation of aPKC preferentially induces the phosphorylation of its substrates and the formation of ternary complex with Par-3, Par-6, and aPKC and thus critically regulates the formation of apico-basal cell polarity at cell-cell adhesion sites. Moreover, since the interaction between nectin and Par-3 is reported (Takekuni *et al.* 2003), the abnormal localization of nectin in the absence of afadin would also contribute to the impaired accumulation of the Par complex. Taken together, Cdc42 of which activation is enhanced in the presence of afadin seems to be essential for the function of the Par complex and the consequent formation of proper cell polarity in EBs. However, the precise step of Cdc42 activation involved in the process of formation of cell polarity remains to be elucidated. The future study using Cdc42-null EBs would be helpful to clearly address these issues.

The deposition of laminin, one of the basement membrane components, was abnormal in afadin-null EBs, resulting in the turbulence of the formation of basement membrane. Since it is reported that the integrin expression was dependent on the ligand concentration (Condic & Letourneau 1997), the abnormal deposition of laminin in EBs lacking afadin may alter the expression pattern of

integrin α_6 and integrin β_1, both of which are the receptors of laminin. Integrin is crucial for the adhesion between cells and ECM and plays essential roles in many fundamental cellular functions including cell movement, cell proliferation, cell survival, and cell-cell junctions (Giancotti & Ruoslahti 1999; Geiger *et al.* 2001). Thus, such alteration in the expression pattern of integrins in afadin-knockout EBs itself might be enough to disorganize cell polarization and cell-cell junctions. Moreover, there is a report that integrin β_1 affects the localization of aPKC in the epidermis (Lechler & Fuchs 2005). Collectively, integrin of which expression is regulated by afadin seems to be involved in the organization of cell polarization and cell-cell junctions directly and/or cooperatively with the Par complex in the formation of EBs.

The mechanism how the expression of laminin receptors integrin α_6 and integrin β_1 is disturbed by depletion of afadin is also unclear. Previous studies demonstrated that the promoter region of integrin α_6 contains consensus binding sites for several transcription factors including SP1, AP1, AP2, GRE/PRE, and c-myc (Lin *et al.* 1997; Nishida *et al.* 1997). One of these studies suggested that the two transcription factors, SP1 and AP2, are probably major sites for the positive regulation of the integrin α_6 promoter (Lin *et al.* 1997). In addition, there is a report that transforming growth factor-β reduces the expression of integrin α_6 in epithelial cells (Lim *et al.* 2001). Although there is no direct evidence that link afadin to the regulation of the integrin expression, it is possible that afadin enhances transcription factors related to the transcription of integrins and induces their expression. On the other hand, the production of laminin, a component of basement membrane, itself was preserved, but its deposition was highly abnormal, probably leading to the inappropriate formation of basement membrane. Although the mechanism of the abnormal deposition of laminin is not well understood, this abnormality may additionally contribute to the impaired expression of integrins, because the

concentration of laminin affects the expression of integrin $\alpha_6\beta_1$ (Condic & Letourneau 1997), as mentioned above. In contrast, the disruption of integrin β_1 expression abrogates the formation of basement membrane in this mutant EBs (Aumailley *et al.* 2000). Taken together, the ECM protein laminin and its receptors integrins may mutually regulate their functions and expression during the formation of EBs.

The findings displayed in this study using EBs provide a line of mechanistic evidence that afadin regulates the formation of cell polarity and cell-cell junctions during the early embryogenesis. Since the formation of EBs from ES cells includes the differentiation process (Weitzer 2006), it may be reasonable to consider that afadin is also involved in the regulation of the differentiation in embryogenesis. Although this issue is not investigated in this study, such investigation would be performed in the future to strengthen the importance of afadin for embryonic development.

Experimental procedures

Antibodies

The mouse monoclonal antibody (mAb) against afadin and the rat mAb against nectin-2 were prepared as described (Sakisaka *et al.* 1999; Takahashi *et al.* 1999). The following rabbit polyclonal Abs (pAbs) were purchased from commercial sources; anti-α-catenin (Sigma-Aldrich), anti-Par-3 (Upstate Biotechnology), anti-PKCζ (Santa Cruz Biotechnology), and anti-integrin α_6 (Santa Cruz Biotechnology). The mouse mAbs listed below were also purchased from commercial sources; anti-ZO-1 (Sanko Junyaku), anti-β-catenin (Santa Cruz Biotechnology), anti-integrin β_1 (BD Biosciences), anti-Cdc42 (BD Biosciences), and anti-phospho-PKCζ (Cell Signaling Technology). The rat mAbs listed below were also purchased from commercial sources; anti-laminin (Upstate Biotechnology), anti-integrin β_1 (BD Biosciences), and anti-integrin α_6 (BD Biosciences). The rabbit anti-Par-6 mAb was kindly supplied by Dr. S. Ohno (Yokohama City University, Yokohama, Japan). The horseradish peroxidase-conjugated and fluorophore-labeled secondary Abs were obtained from Chemicon and Molecular Probes, respectively.

EB formation

EBs were generated as described previously with some modifications (Ikeda *et al.* 1999). Briefly, 129/Sv RW4 wild-type and afadin-null ES cells were cultured without feeder cells on gelatin-coated dishes for 3 days in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal calf serum (FCS), 0.1 mM 2-mercaptoethanol (Sigma-Aldrich), 1,000 U/ml leukemia inhibitory factor (Chemicon), 0.1 mM nonessential amino acids (Invitrogen), 3 mM adenosine, 3 mM cytosine, 3 mM guanosine, 3 mM uridine, and 1 mM thymidine (Sigma-Aldrich).

EB formation was initiated by withdrawal of leukemia inhibitory factor, after ES cells were transferred to bacteriological dishes to grow in suspension culture in DMEM supplemented with 20% FCS. EBs were generated for 11 days in suspension culture with the replacement of the culture media every other day.

Histological analysis and TEM

For staining of EBs with H-E, EBs were fixed with 2% paraformaldehyde in PBS at 4˚C overnight, dehydrated in graded alcohols, embedded in paraffin, sectioned at 3 μm, and stained with H-E. For observation with TEM, EBs were fixed in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at 4˚C for 2 h. They were washed with 0.1 M phosphate buffer, and treated with 2% OsO_4 in the same buffer for 1 h, dehydrated in graded ethanol, and embedded in epoxy resin. Ultrathin sections were cut and then observed with JEOC JEM 1200EX electron microscope (JEOL).

Immunofluorescence microscopy

EBs were fixed with 4% paraformaldehyde solution on ice for 30 minutes and then suspended in 10% sucrose on ice, followed by replacement with 25% sucrose. Prepared EBs were frozen in OCT compound (Sakura Finetechnical) and sectioned at 10 μm thickness on a cryostat. Sectioned samples were mounted on glass slides, air-dried, washed with phosphate buffered saline (PBS), and permeabilized with 0.2% Triton X-100 at room temperature for 5 min. The samples were blocked in 1% bovine serum albumin (BSA)/PBS at room temperature for 10 min, and then were incubated with the primary Abs in 1% BSA/PBS at room temperature for 1 h. They were washed with PBS and then incubated with the secondary Abs in BSA/PBS at room temperature for 30 min. For integrin β_1 staining, the samples were blocked with 1% BSA/PBS and soaked in Blocking solution [PBS containing

20% BlockAce (Dainippon Pharmaceutical) and 0.05% saponin (Merck)] at room temperature for 15 min, respectively. The samples were incubated with the anti-integrin β_1 mAb in Blocking solution at room temperature for 1 h. They were washed with 0.005% saponin/PBS and then incubated with the secondary Ab in blocking solution at room temperature for 30 min. For integrin α_6 staining, the samples were permeabilized with 1% Triton X-100/10% BSA/PBS at room temperature for 10 min, blocked with 3% BSA/PBS, and then incubated with the anti-integrin α_6 mAb diluted in 3% BSA/PBS at room temperature overnight. They were then incubated with the secondary Ab for 30 min. The samples were analyzed using LSM510 META confocal microscope (Carl Zeiss).

Western blotting

EBs were washed twice with ice-cold PBS and suspended in Buffer A (20 mM Tris-HCl at pH 7.5, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1% Nonidet P-40, 3 μg/ml leupeptin, 5 μg/ml aprotinin, 1 mM APMSF). The samples were homogenized by sonication. The protein concentration of EB extracts was determined by BCA protein assay kit (Pierce). The samples were boiled in the SDS sample buffer for 5 min and were subjected to SDS-PAGE, followed by Western blotting with the indicated Abs.

Analysis of Cdc42 activation

EBs were washed with ice-cold PBS and suspended in Buffer B [50 mM Tris/HCl at pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 μM PMSF, 1 mM Na_3VO_4, 10 μl/ml Phosphatase Inhibitor Cocktail 1 (Sigma-Aldrich)] containing the glutathione-*S*-transferase (GST)-PAK-Cdc42/Rac interactive binding region (CRIB) fusion protein. GST-PAK-CRIB was overexpressed in *Escherichia coli* with an expression vector pGEX-PAK-CRIB

kindly supplied from Dr. T. Takenawa (Kobe University, Kobe, Japan) and purified according to the manufacturer's protocol (Amersham Biosciences). The samples were homogenized by sonication, followed by rotation at 0˚C for 30 min. The supernatant collected by centrifugation at 20,000 x *g* at 0˚C for 15 min was then incubated with glutathione-sepharose beads at 0˚C for 1 h. After the beads were extensively washed with Buffer B, the bound proteins were eluted from the beads by boiling in the SDS sample buffer for 5 min and were subjected to SDS-PAGE, followed by Western blotting with the anti-Cdc42 mAb.

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Figure legends

Fig. 1. Disorganization of cell-cell junctions in afadin-knockout EBs. (A) Light microscopic observation of external appearance of EBs. **(B)** Histological analysis EBs. EBs derived from wild-type and afadin-knockout ES cells were stained with H-E. **(C-G)** Ultrathin sections of wild-type and afadin-knockout EBs were subjected to TEM; **(C)** Innermost side of the ectoderm in wild-type EBs; **(D)** Endodermal layer of wild-type EBs; **(E)** Ectodermal cells of afadin-knockout EBs; **(F)** Endodermal layer of afadin-knockout EBs; **(G)** Basement membrane of afadin-knockout EBs. High magnified images in **(B)** and **(C)** correspond to areas surrounded by broken lines and arrowheads indicate the established junctional complex in the ectoderm and endoderm. Arrowheads in **(G)** indicate the ectopic localization of basement membrane in the ectoderm and endoderm layers. **cv**: cavity, **BM**: basement membrane, **ec**: ectoderm, **en**: endoderm. **Scale bars**, 100 μm **(A and B)**; 2 μm **(C-G)**. The results shown in this figure are representative of three independent experiments.

Fig. 2. Dislocalization of nectin-2 and abnormal deposition of laminin in afadin-knockout EBs. (A) Localization of afadin. Wild-type and afadin-knockout EBs were immunostained with the anti-afadin and anti-laminin Abs. **(B)** Localization of nectin-2. Wild-type and afadin-knockout EBs were immunostained with the anti-nectin-2 mAb. **(C)** Expression of afadin and nectin-2. The lysates of both types of EBs were subjected to Western blotting using the anti-afadin or anti-nectin-2 mAb. Actin was immunoblotted for the loading control. **WT**: wild-type, **KO**: knockout. **ec**: ectoderm, **en**: endoderm. **Scale bars**, 50 μm. The results shown in this figure are representative of three independent experiments.

Fig. 3. Dislocalization of AJ and TJ components in afadin-knockout EBs. **(A)** Localization of an AJ marker β-catenin. Wild-type and afadin-knockout EBs were immunostained with the anti-β-catenin mAb. F-Actin was counterstained with rhodamine-phalloidin. **ec**: ectoderm, **en**: endoderm. **(B)** Localization of a TJ marker ZO-1. Wild-type and afadin-knockout EBs were immunostained with the anti-ZO-1 mAb. F-Actin was counterstained with rhodamine-phalloidin. **(C)** Expression of β-catenin and ZO-1. The lysates of both types of EBs were subjected to Western blotting using the anti-β-catenin or anti-ZO-1 mAb. Actin was immunoblotted for the loading control. **WT**: wild-type, **KO**: knockout. **Scale bars**, 20 μm. The results shown in this figure are representative of three independent experiments.

Fig. 4. Impaired accumulation of the Par polarity complex proteins in afadin-knockout EBs. **(A)** Localization of Par-3. Wild-type and afadin-knockout EBs were immunostained with the anti-Par-3 pAb and anti-laminin mAb. F-Actin was counterstained with rhodamine-phalloidin. **(B)** Localization of Par-6. Wild-type and afadin-knockout EBs were triply immunostained with the anti-Par-6 pAb and anti-laminin mAb. F-Actin was counterstained with rhodamine-phalloidin. **(C)** Expression of Par-3 and Par-6. The lysates of both types of EBs were subjected to Western blotting using the anti-Par-3 or anti-Par-6 pAb. Actin was immunoblotted for the loading control. **WT**: wild-type, **KO**: knockout. **Scale bars**, 20 μm. The results shown in this figure are representative of three independent experiments.

Fig. 5. Reduced activation of Cdc42 and aPKC in afadin-knockout EBs. **(A)** Reduced amount of GTP-bound form of Cdc42 in afadin-knockout EBs. The

lysates from wild-type and afadin-knockout EBs were used for the pull-down assay and subjected to Western blotting with the anti-Cdc42 mAb. **(B)** Reduced phosphorylation of aPKC in afadin-knockout EBs. The lysates from wild-type and afadin-knockout EBs were subjected to SDS-PAGE, followed by Western blotting with anti-phospho-PKCζ and anti-PKCζ Abs. **WT**: wild-type, **KO**: knockout. The results shown in this figure are representative of three independent experiments.

Fig. 6. Suppressed expression of integrins in afadin-knockout EBs. (A) Localization of integrin α_6. Wild-type and afadin-knockout EBs were immunostained with the anti-integrin α_6 mAb. Nuclei and F-actin were counterstained with DAPI and rhodamine-phalloidin, respectively. **(B)** Localization of integrin β_1. Wild-type and afadin-knockout EBs were immunostained with the anti-integrin β_1 mAb. Nuclei and F-actin were counterstained with DAPI and rhodamine-phalloidin, respectively. **(C)** Expression of integrin α_6 and integrin β_1. The lysates of both types of EBs were subjected to Western blotting using the anti-integrin α_6 and integrin β_1 mAbs. Actin was immunoblotted for the loading control. An arrowhead and an asterisk indicate the matured and immature forms of integrin β_1, respectively. **WT**: wild-type, **KO**: knockout. **Scale bars**, 20 μm. The results shown in this figure are representative of three independent experiments.

Fig. 1

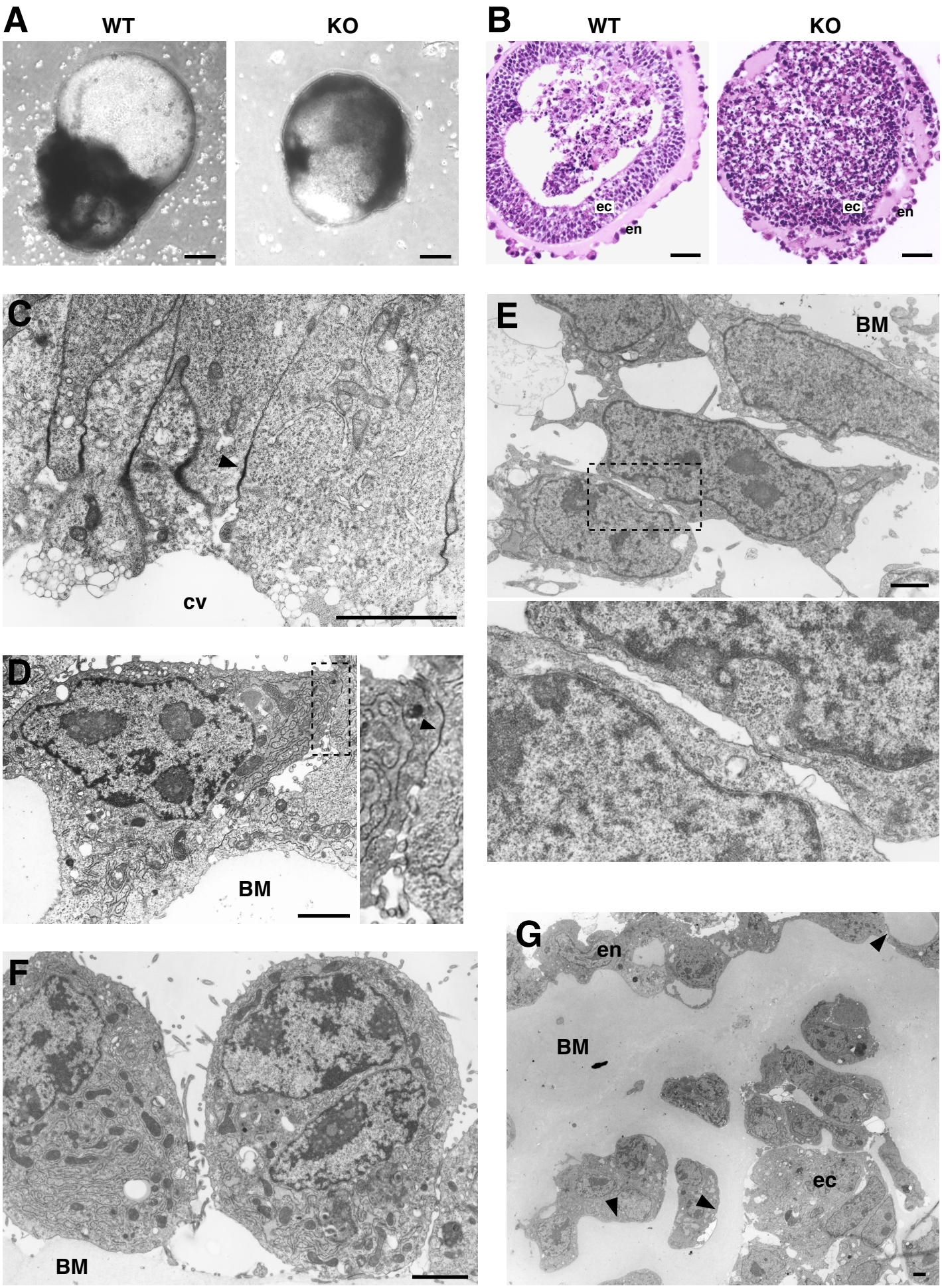
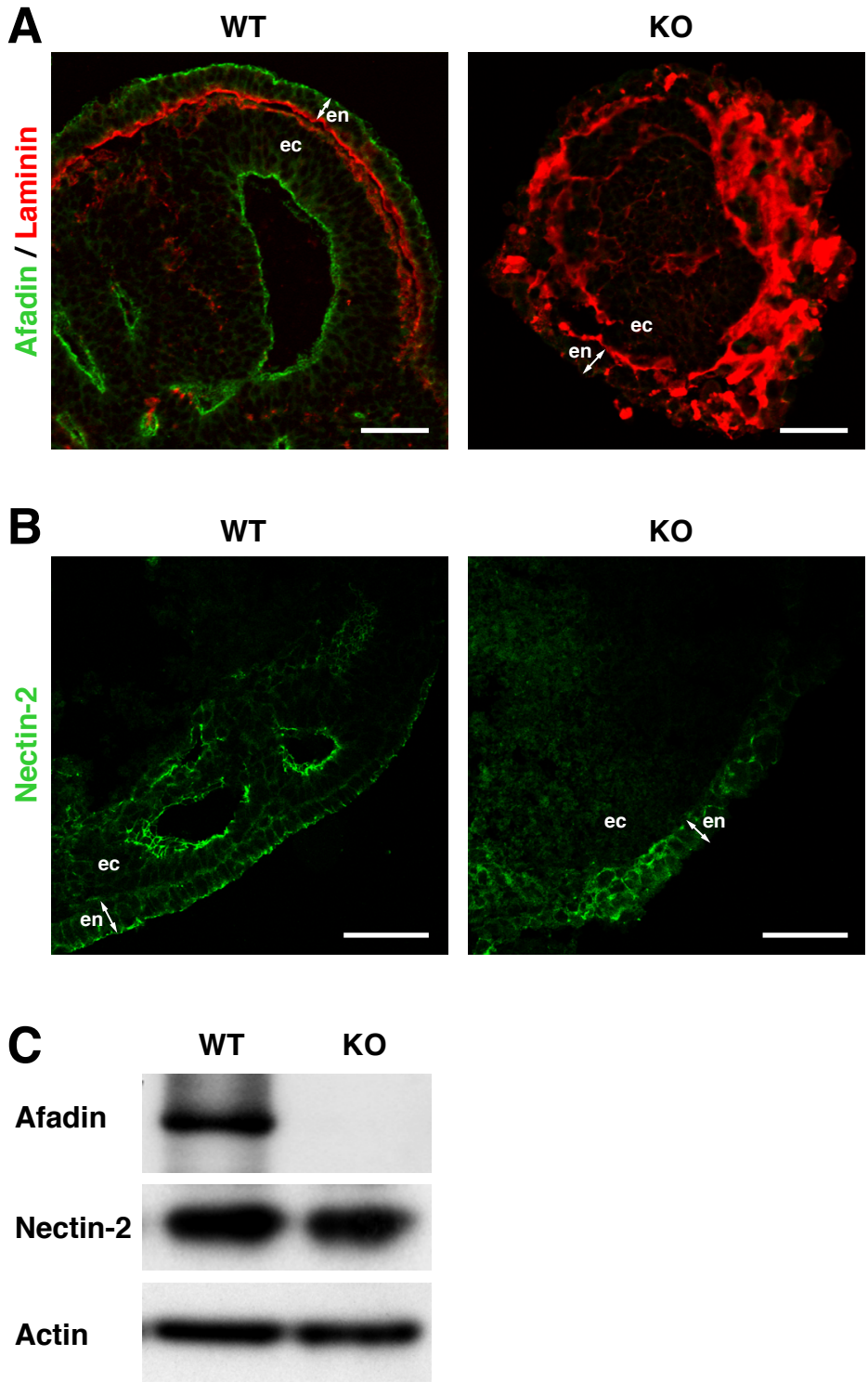


Fig. 2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Actin

Fig. 3

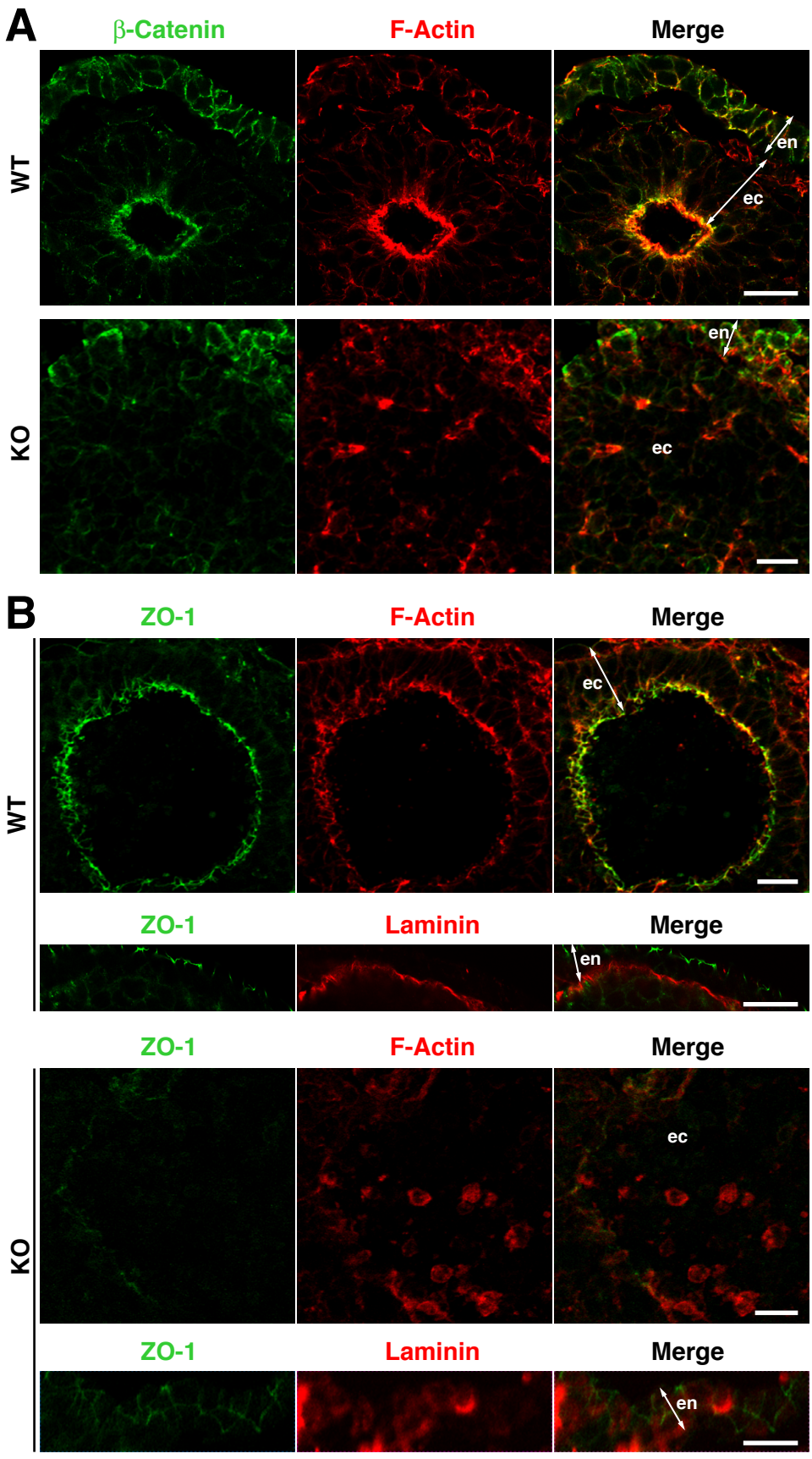
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Fig. 4

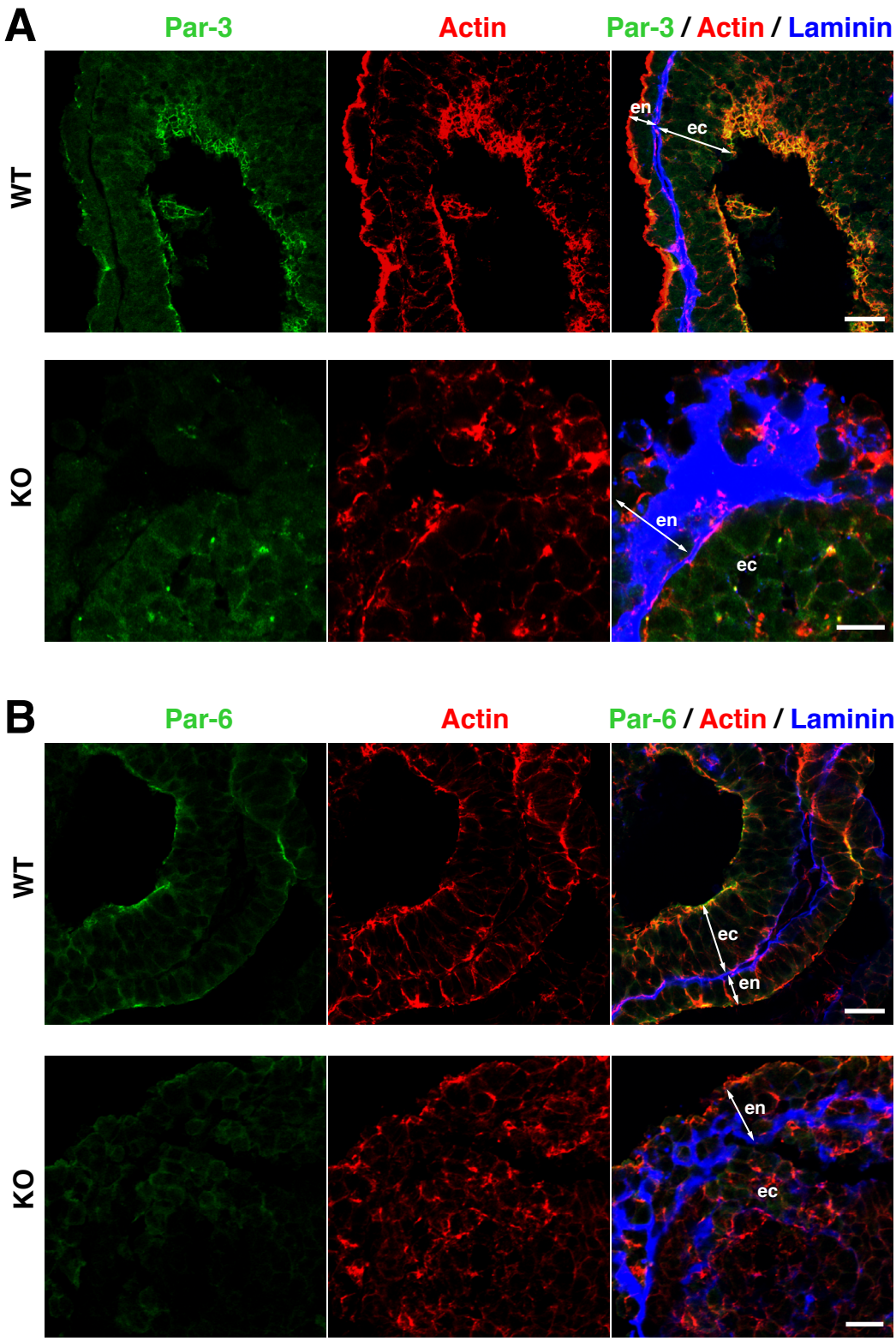
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Fig. 5

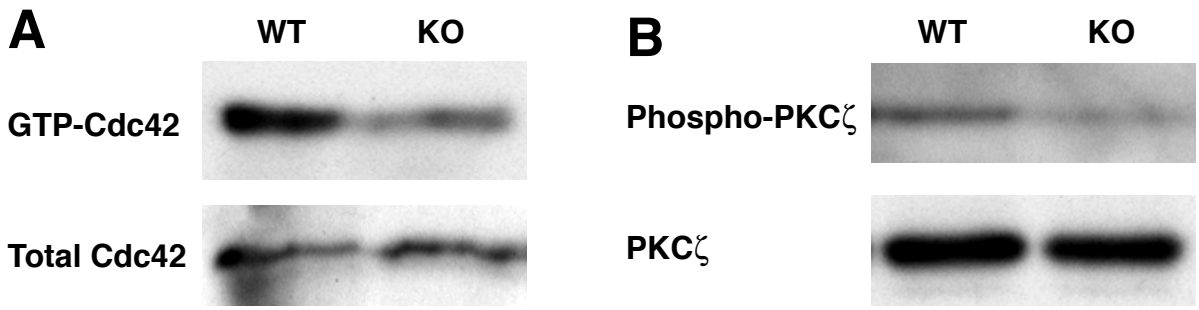
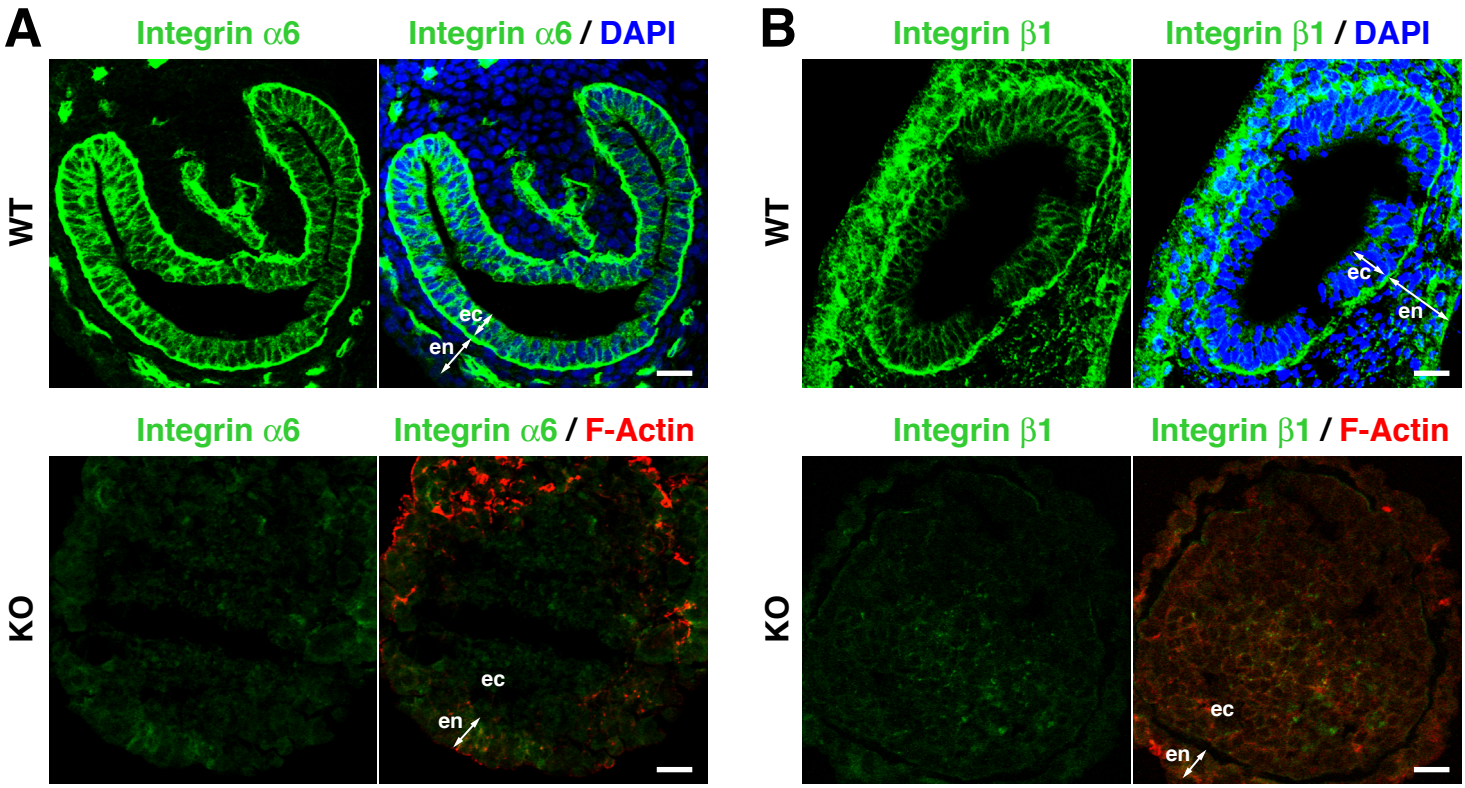


Fig. 6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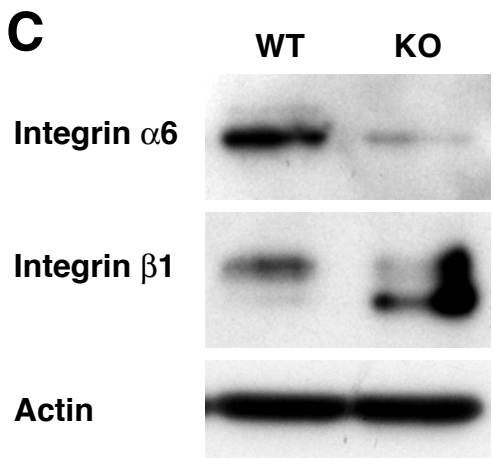
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