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# ZO-1 and ZO-2 Independently Determine Where Claudins Are Polymerized in Tight-Junction Strand Formation

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[9] This paper is dedicated to Shoichiro Tsukita, who wrote the original version of the manuscript, asked Sachiko Tsukita to correspond with *Cell*, and would have been the corresponding author had he lived to see the paper's publication.
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## SUMMARY

A fundamental question in cell and developmental biology is how epithelial cells construct the diffusion barrier allowing them to separate different body compartments. Formation of tight junction (TJ) strands, which are crucial for this barrier, involves the polymerization of claudins, TJ adhesion molecules, in temporal and spatial manners. ZO-1 and ZO-2 are major PDZ-domain-containing TJ proteins and bind directly to claudins, yet their functional roles are poorly understood. We established cultured epithelial cells (1(ko)/2(kd)) in which the expression of ZO-1/ZO-2 was suppressed by homologous recombination and RNA interference, respectively. These cells were well polarized, except for a complete lack of TJs. When exogenously expressed in 1(ko)/2(kd) cells, ZO-1 and ZO-2 were recruited to junctional areas where claudins were polymerized, but truncated ZO-1 (NZO-1) containing only domains PDZ1–3 was not. When NZO-1 was forcibly recruited to lateral membranes and dimerized, claudins were dramatically polymerized. These findings indicate that ZO-1 and ZO-2 can independently determine whether and where claudins are polymerized.

## INTRODUCTION

Tight junctions (TJs) constitute the epithelial and endothelial junctional complex together with adherens junctions (AJs) and desmosomes and are located in the most apical part of the complex (Farquhar and Palade, 1963). TJs have dual roles as a barrier and a fence, which are essential for the development and maintenance of multicellular organisms. They create the primary barrier to the diffusion of solutes through the paracellular pathway and are thought to maintain cell polarity as a boundary between the apical and basolateral plasma membrane domains (Anderson et al., 2004; Balda and Matter, 1998; Tsukita et al., 2001). On ultrathin-section electron microscopy, TJs appear as a series of discrete sites of apparent fusion involving the outer leaflet of the plasma membranes of adjacent cells (Farquhar and Palade, 1963). On freeze-fracture electron microscopy, TJs appear as a set of continuous, anastomosing intramembranous particle strands (TJ strands) (Staehelin 1974). Each TJ strand associates laterally with another TJ strand in the apposing membranes of adjacent cells to form ''paired'' TJ strands (Tsukita et al., 2001).

The molecular architecture of TJs has been unraveled rapidly in recent years. Two distinct types of integral membrane proteins, occludin and claudins, have been identified as constituents of TJ strands (Furuse et al., 1993, 1998a). Both occludin and claudins bear four transmembrane domains but do not show any sequence similarity. Claudins, but not occludin, are thought to constitute the backbone of TJ strands (Tsukita and Furuse, 1999).

Claudins comprise a multigene family consisting of at least 24 members in humans and mice (Morita et al., 1999; Tsukita et al., 2001; Turksen and Troy, 2004). In addition to claudins and occludin, tricellulin was recently identified as a TJ strand protein whose localization is largely biased to the tricellular junction (Ikenouchi et al., 2005). Furthermore, another type of integral membrane protein, JAM (junction adhesion molecule), has also been reported to be concentrated at TJs (Martin-Padura et al., 1998). This molecule does not appear to constitute TJ strands per se but rather appears to laterally associate with strands (Itoh et al., 2001).

Now that our knowledge of the molecular architecture of TJs is improved, the most pressing questions concern the molecular mechanisms by which the polymerization of claudins, i.e., the formation of TJ strands, is regulated temporally and spatially during epithelial polarization: How are claudins allowed to polymerize exclusively at the junctional complex? From this perspective, proteins underlying the cytoplasmic surface of TJs have been thought to be important, and three closely related proteins called ZO-1, ZO-2, and ZO-3 have been identified as constituting the plaque structures of TJs (González-Mariscal et al., 2000; Mitic and Anderson, 1998) together with cingulin, the Par-3/Par-6/aPKC complex, ZONAB, GEF-H1/Lfc, etc. (Balda and Matter, 2000; Benais-Pont et al., 2003; Citi et al., 1988; Hurd et al., 2003).

ZO-1 was first identified as an ~220 kDa antigen for a monoclonal antibody raised against a junction-enriched fraction from the liver (Stevenson et al., 1986). ZO-2 was then identified as a 160 kDa protein that was coimmunoprecipitated with ZO-1 from cell lysates (Gumbiner et al., 1991). A phosphorylated 130 kDa protein was also found in the ZO-1 immunoprecipitate (Balda et al., 1993) and is now called ZO-3. Cloning and sequencing of cDNAs encoding these molecules showed that all have three PDZ domains (PDZ1–3), one SH3 domain, and one GUK domain in this order starting from their N termini (Haskins et al., 1998; Itoh et al., 1993; Jesaitis and Goodenough, 1994; Willott et al., 1993), indicating that ZO-1, ZO-2, and ZO-3 belong to the so-called MAGUK (membrane-associated guanylate kinase-like homologs) family (Anderson, 1995; Kim, 1995; Woods and Bryant, 1993). Among the three PDZ domains of ZO-1, ZO-2, and ZO-3, PDZ1 was shown to bind directly to the C termini of claudins (Itoh et al., 1999a), suggesting that these TJ MAGUKs are possible candidates for regulators of the polymerization of claudins in epithelial cells. However, irrespective of intensive study, our knowledge of their functions is limited.

One of the difficulties with the functional analysis of TJ MAGUKs has been their possible functional redundancy. In a database analysis of the mouse/human genome, no other TJ MAGUKs were found. Thus, one of the best ways to examine the functions of ZO-1, ZO-2, and ZO-3 would be to establish an epithelial cell line lacking the expression of all of these TJ MAGUKs. In recent years, the technology of RNA interference has enabled us to selectively suppress the expression of certain gene products (Paddison et al., 2002), but, as many researchers will attest, it is difficult to simultaneously suppress the expression of more than two gene products, especially structural proteins with a relatively high expression level. Set against this background, using mouse Eph4 epithelial cells in which ZO-3 was not expressed (Umeda et al., 2004), we successfully generated Eph4 cell clones lacking ZO-1 expression (*ZO-1*$^{-/-}$ cells) by homologous recombination (Umeda et al., 2004). In the present study, clones with suppressed ZO-2 expression were established from the *ZO-1*$^{-/-}$ cells by stably expressing short interfering RNAs. As a result, we obtained Eph4 cell lines which mostly lacked the expression of ZO-1, ZO-2, and ZO-3. In the confluent state, these cells were, interestingly, well polarized in terms of the differentiation of the apical/basolateral membranes and formation of AJs but lacked TJs completely. Here, by using a reverse genetics approach with this newly established cell line, we examined the functions of TJ MAGUKs in detail, with special attention to the regulation of the polymerization of claudins.

## RESULTS

### Establishment of Epithelial Cell Lines Lacking the Expression of TJ MAGUKs

A mouse epithelial cell line (Eph4 cells) lacking the expression of ZO-1 was previously established by homologous recombination (Umeda et al., 2004). Here, the parental Eph4 cells and the ZO-1-deficient cells are referred as to 1(w)/2(w) and 1(ko)/2(w) cells, respectively. We next attempted to knock out the *ZO-2* gene in Eph4 cells. However, in situ hybridization analyses suggested that there were at least four alleles for the *ZO-2* gene in Eph4 cells (data not shown), in contrast to the two alleles for the *ZO-1* gene, making it technically difficult to delete the *ZO-2* gene by homologous recombination. We then knocked down ZO-2 by stably expressing short interfering RNAs in 1(w)/2(w) and 1(ko)/2(w) cells. In these cells, called 1(w)/2(kd) and 1(ko)/2(kd) cells, respectively, ZO-2 expression was stably suppressed by more than 95% as determined by Western blot analysis (Figure 1A). Among 1(w)/2(w), 1(ko)/2(w), 1(w)/2(kd), and 1(ko)/2(kd) cells, no significant difference was detected in the expression levels of other TJ proteins such as claudin-3 (Figure 1A), occludin, JAM, nectin, and cingulin or in those of AJ proteins such as E-cadherin and afadin (Nagafuchi, 2001; Takai and Nakanishi, 2003) (data not shown). On transwell filters, these four cell lines grew at the same rate until confluent condition (Figure 1B). Immunofluorescence microscopy showed that 1(ko)/2(w), 1(w)/2(kd), and 1(ko)/2(kd) cells retain the epithelial morphology and that, in these cells, ZO-1, ZO-2, and ZO-1/ZO-2 appeared to simply disappear from cell-cell contacts (TJs), respectively, with no gross morphological changes in cell shape or size (Figure 1C).

The expression of ZO-3 was undetectable in parental Eph4 cells by immunoblotting and immunofluorescence microscopy (Umeda et al., 2004) (Figure 1D). When exogenously expressed in 1(ko)/2(kd) cells, mouse ZO-3 was

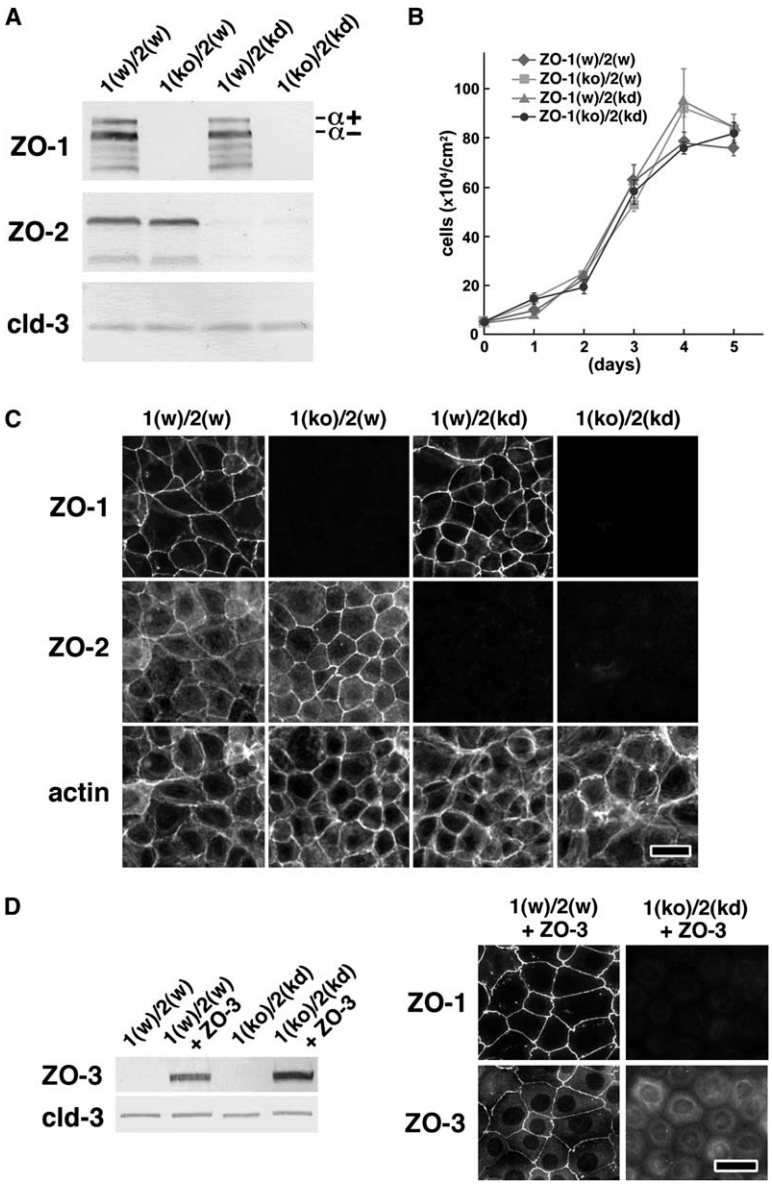


**Figure 1. Generation of TJ MAGUK-Deficient Cells**

(A) Immunoblotting of the total cell lysate of various Eph4 cell mutants (1(w)/2(w), 1(ko)/2(w), 1(w)/2(kd), and 1(ko)/2(kd) cells) with anti-ZO-1 mAb, anti-ZO-2 pAb, and anti-claudin-3 pAb. In 1(ko)/2(w) and 1(ko)/2(kd) cells, neither the α+ nor α− isotype of ZO-1 was expressed.

(B) Cell growth curve. 1(w)/2(w), 1(ko)/2(w), 1(w)/2(kd), and 1(ko)/2(kd) cells were plated on transwell filters 12 mm in diameter at $5 \times 10^4$ cells/well. Cell growth was measured at 1, 2, 3, 4, and 5 days by making duplicate counts of these cells with a hemocytometer. In this and in all other figures, error bars represent standard deviations.

(C) Immunofluorescence microscopy of 1(w)/2(w), 1(ko)/2(w), 1(w)/2(kd), and 1(ko)/2(kd) cells fixed with 3% formalin and triply stained with anti-ZO-1 mAb, anti-ZO-2 pAb, and Alexa Fluor 568 phalloidin (to stain actin filaments). Scale bar = 10 μm.

(D) ZO-3 was expressed exogenously in 1(w)/2(w) and 1(ko)/2(kd) cells (left panel). These cells were double stained with anti-ZO-1 mAb and anti-ZO-3 pAb (right panel). Scale bar = 10 μm.

not recruited to TJs but was diffusely distributed in the cytoplasm. By contrast, in 1(w)/2(w), 1(ko)/2(w), and 1(w)/2(kd) cells, exogenously expressed ZO-3 was exclusively recruited to TJs. These findings indicated that, distinct from ZO-1 and ZO-2, ZO-3 has no ability to target TJs by itself and requires ZO-1 or ZO-2 for its localization. Therefore, if a trace amount of endogenous ZO-3 is expressed in 1(ko)/2(kd) cells, it will not be recruited to TJs, indicating that 1(ko)/2(kd) cells can be regarded as TJ MAGUK-deficient cells.

## TJ MAGUK-Deficient Epithelial Cells Are Well Polarized

Phase-contrast microscopy revealed that, once confluent, all of the 1(w)/2(w), 1(ko)/2(w), 1(w)/2(kd), and 1(ko)/2(kd) cells showed the typical cobblestone-like appearance of epithelial cells (data not shown). These cells grown on transwell filters were then observed by ultrathin-section electron microscopy (Figure 2). 1(w)/2(w) cells showed typical simple epithelial characteristics with microvilli-bearing apical membranes (left inset) and closely apposed lateral membranes (Figure 2, upper panel). When the expression of ZO-1 (Umeda et al., 2004) or ZO-2 (Figure 2, middle panel) was suppressed, these characteristics were completely retained. Furthermore, interestingly, even in the absence of ZO-1 and ZO-2, 1(ko)/2(kd) cells still appeared to keep these epithelial characteristics (Figure 2, lower panel).

Next, these cells were stained with antibodies specific for apical markers (syntaxin 3 or moesin) (Low et al.,

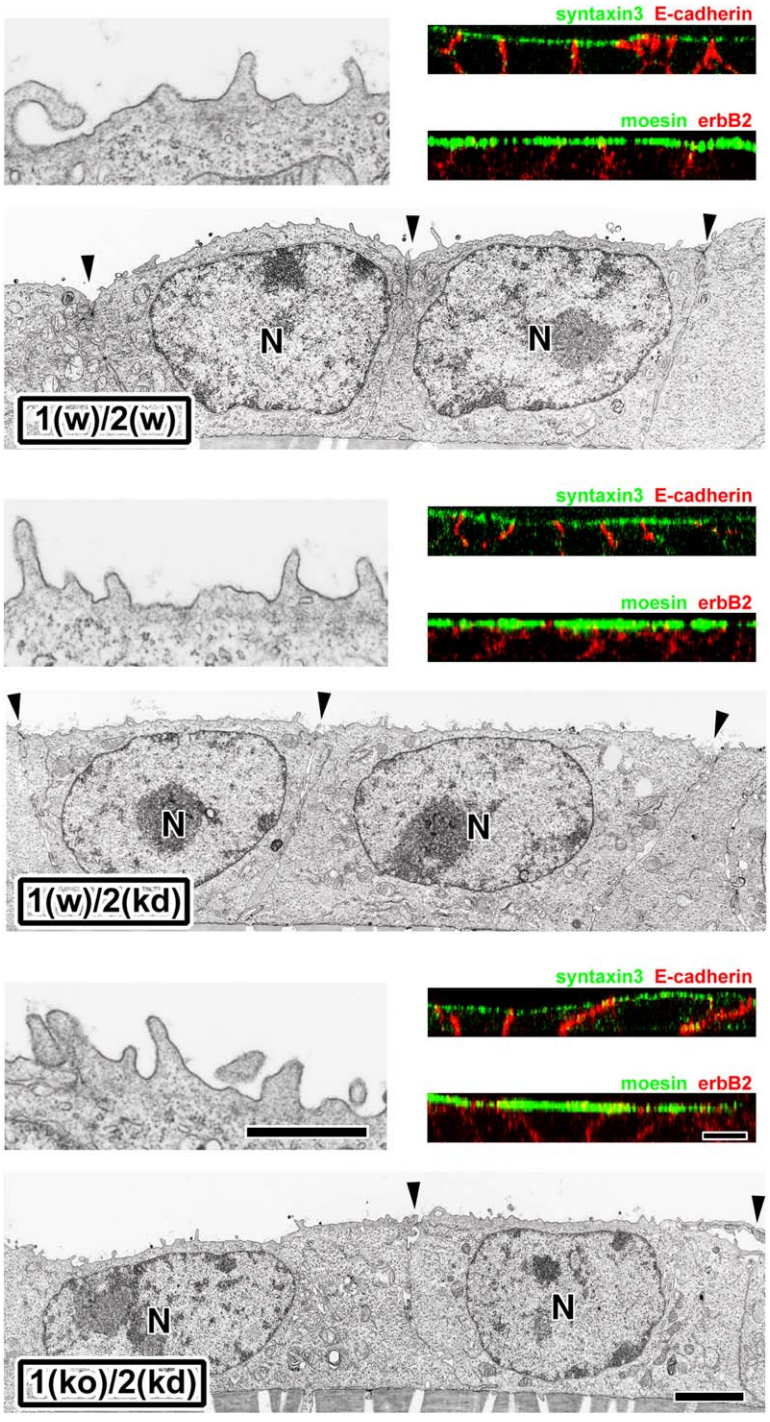


**Figure 2. Morphology and Polarity of TJ MAGUK-Deficient Cells**

Ultrathin-section electron microscopy and confocal microscopy (right panels) of 1(w)/2(w), 1(w)/2(kd), and 1(ko)/2(kd) cells. Lateral membranes were closely apposed (arrowheads). These cell lines were double stained for apical markers (syntaxin 3 or moesin, green) and basolateral markers (E-cadherin or erbB2, red) (right panels). Scale bars = 3 μm (bottom panel); 1 μm (left panel); 10 μm (right panel).

1996; Takeuchi et al., 1994) and basolateral markers (E-cadherin or erbB2) (Nelson et al., 1990; Borg et al., 2000), the distribution of which was then examined by confocal microscopy (Figure 2, right insets). In both 1(ko)/2(w) cells (data not shown) and 1(w)/2(kd) cells, these markers were distributed normally, and even in 1(ko)/2(kd) cells, the apical membranes appeared to be well differentiated from the basolateral membranes.

## TJ MAGUK-Deficient Epithelial Cells Lack TJs

We then focused on AJs and TJs in 1(w)/2(w), 1(ko)/2(w), 1(w)/2(kd), and 1(ko)/2(kd) cells. Among these cell lines, immunofluorescence microscopy identified no significant difference in the distribution and concentration of AJ components such as E-cadherin, afadin, and actin in the confluent state (Figure 3A, left panel). The cell aggregation assay quantitatively revealed that E-cadherin-based cell-cell

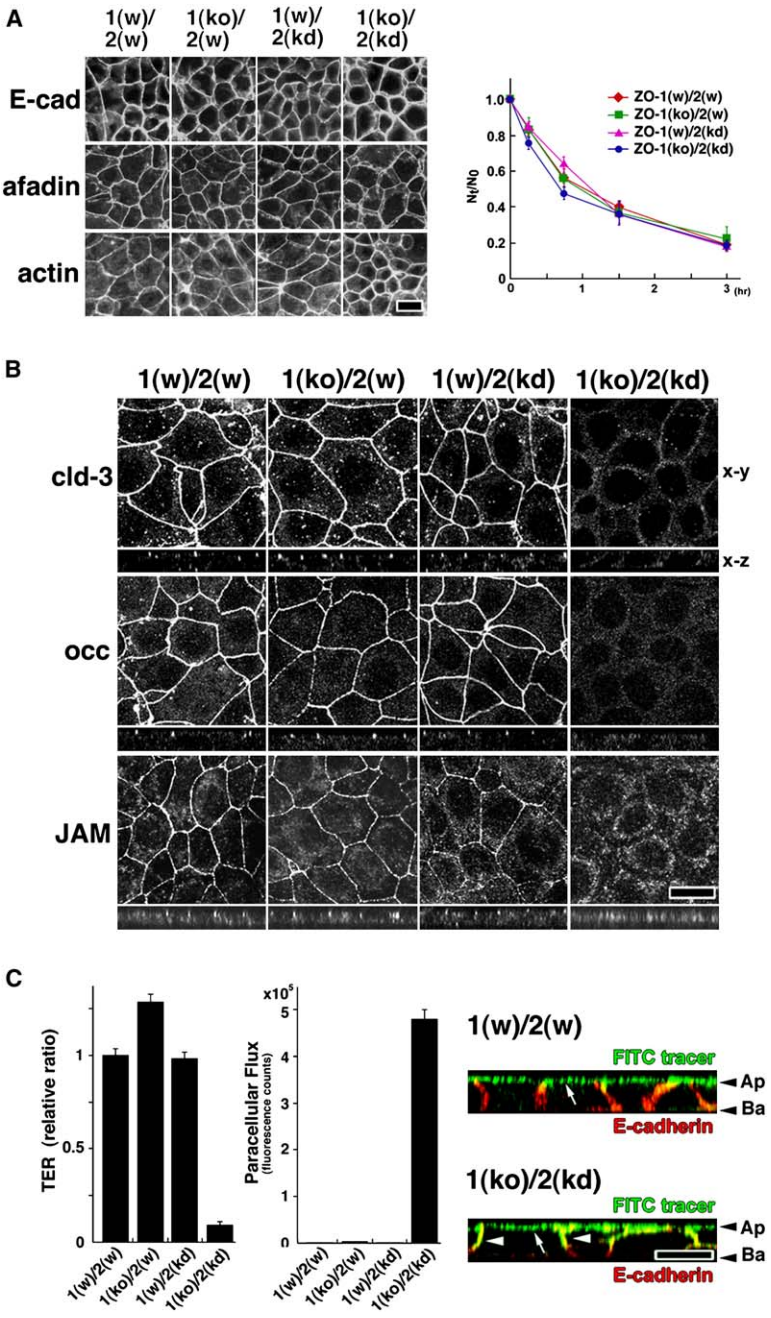


**Figure 3. Intercellular Junctions of TJ MAGUK-Deficient Cells**

(A) AJs in 1(w)/2(w), 1(ko)/2(w), 1(w)/2(kd), and 1(ko)/2(kd) cells. Cells were immunofluorescently stained with anti-E-cadherin mAb, anti-afadin mAb, and Alexa Fluor 568 phalloidin (left panel). Scale bar = 10 μm. In the right panel, the cell adhesion activity of cadherin was evaluated by cell aggregation assay with these cell lines quantitatively. The extent of cell aggregation was represented by the index $N_t/N_0$, where $N_t$ and $N_0$ are the total numbers of aggregates and cells per dish, respectively.

(B) TJs in 1(w)/2(w), 1(ko)/2(w), 1(w)/2(kd), and 1(ko)/2(kd) cells. In 1(ko)/2(w) and 1(w)/2(kd) cells, the TJ-specific localization of claudin-3, occludin, and JAM was observed by confocal microscopy. Scale bar = 10 μm.

(C) Transepithelial electric resistance (TER) measurements (left panel) and paracellular tracer flux assay (middle panel) in 1(w)/2(w), 1(ko)/2(w), 1(w)/2(kd), and 1(ko)/2(kd) cells. The value of TER in 1(w)/2(w) was defined as 1.0. Furthermore, we performed the paracellular tracer flux assay (fluorescence counts). In the right panel, to visualize the paracellular tracer flux, sulfo-NHS-LC-biotin was added to the apical compartment (arrow). The distribution of biotin was compared between 1(w)/2(w) and 1(ko)/2(kd) cells by confocal microscopy. Lateral membranes are marked in red by E-cadherin. Note that in 1(ko)/2(kd) cells, the biotin appears free to take the paracellular route over TJs (arrowheads). Ap, apical membrane; Ba, basolateral membrane. Scale bar = 10 μm.

adhesion activity was unaffected not only in 1(ko)/2(w) and 1(w)/2(kd) cells but also in 1(ko)/2(kd) cells (Figure 3A, right panel).

We then examined the distribution of claudin-3 (one of the most abundant claudins in Eph4 cells), occludin, and JAM in these cell lines by immunofluorescence microscopy (Figure 3B). As previously reported (Umeda et al., 2004), in confluent 1(ko)/2(w) cells, these TJ-related integral membrane proteins were concentrated at cell-cell contacts with the same pattern as in wild-type 1(w)/2(w) cells. Similarly, the distribution of these proteins did not appear to be affected in 1(w)/2(kd) cells. By contrast, in confluent 1(ko)/2(kd) cells, claudin-3, occludin, and JAM were not concentrated in junctional regions and were instead distributed diffusely along basolateral membranes and in the cytoplasm, suggesting that the formation of TJs was severely affected only in 1(ko)/2(kd) cells. The barrier function of TJs was then compared among 1(w)/2(w), 1(ko)/2(w), 1(w)/2(kd), and 1(ko)/2(kd) cells by measuring transepithelial electric resistance (TER) (Figure 3C, left panel) and the flux of a membrane-impermeable paracellular tracer (FITC-conjugated 40 kDa dextran) (Figure 3C, middle panel); clearly, the TJ barrier was only compromised in 1(ko)/2(kd) cells. In 1(ko)/2(kd) cells, even after

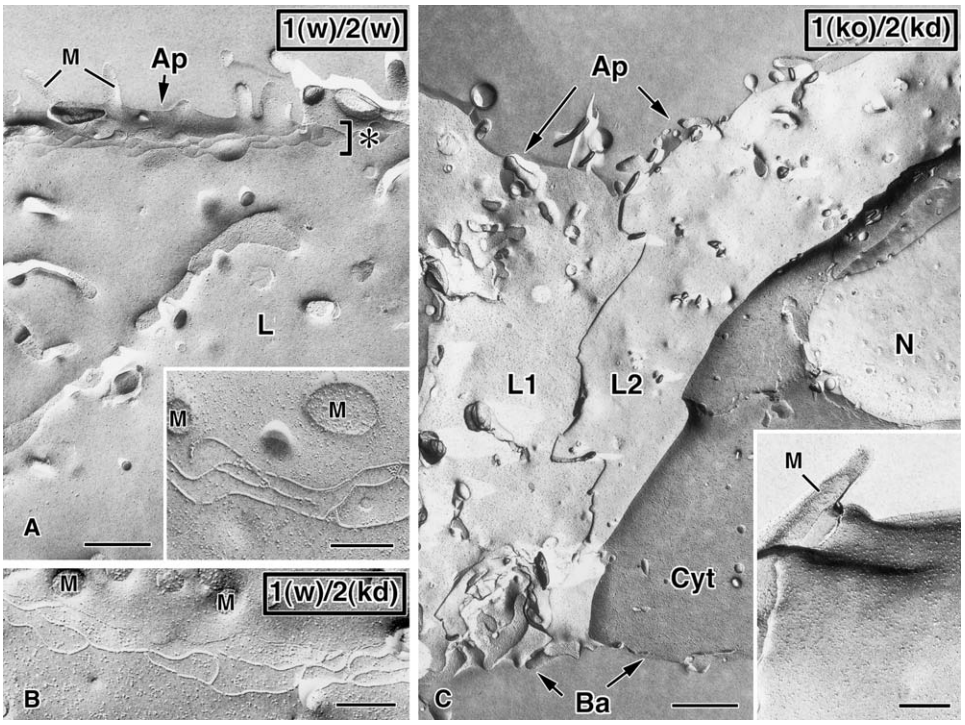


**Figure 4. Absence of TJ Strands from TJ MAGUK-Deficient Cells**

(A) Freeze-fracture replica electron microscopy of 1(w)/2(w) cells. TJs of 1(w)/2(w) cells were characterized by 2–4 continuous TJ strands (inset) and complementary grooves at the level of the junctional complex (asterisk). L, lateral membrane; M, microvillus; Ap, apical membrane. Scale bars = 1 μm; 0.3 μm (inset).

(B) Freeze-fracture replica electron microscopy of 1(w)/2(kd) cells. M, microvillus. Scale bar = 0.3 μm.

(C) Freeze-fracture replica electron microscopy of 1(ko)/2(kd) cells. L1 and L2 represent lateral membranes of two adjacent cells. Ap, apical edges; Ba, basal edges; N, nucleus; Cyt, cytoplasm. M, microvillus. Scale bars = 1 μm; 0.3 μm (inset).

6 day culture in the confluent state, the TER was not developed and, when challenged with FITC-dextran tracer, the paracellular barrier was severely affected. Furthermore, 1(w)/2(w) and 1(ko)/2(kd) cells were cultured on transwell filters in the confluent state, and a primary amine-reactive biotinylation reagent (443 Da), which covalently crosslinks to accessible proteins (Chen et al., 1997), was added to the medium in the apical compartment as a tracer. As shown in the right panel of Figure 3C, when the tracer was visualized with FITC-conjugated avidin by confocal microscopy, the tracer did not cross over to enter the paracellular routes in 1(w)/2(w) cells, whereas in 1(ko)/2(kd) cells, it freely passed through junctional areas into the basal compartment.

These findings were consistent with freeze-fracture replica electron microscopic observations (Figure 4). In 1(w)/2(w) cells, TJ strands were located exclusively in the most apical region of lateral membranes, and the network was usually composed of two to four strands (Figure 4, 1(w)/2(w)). As previously reported (Umeda et al., 2004), 1(ko)/2(w) cells also bore TJ strands at the normal position, and similarly, in 1(w)/2(kd) cells, TJ strands with a normal appearance and localization were formed (Figure 4, 1(w) /2(kd)). In contrast, in 1(ko)/2(kd) cells, the formation of TJ strands appeared to be completely suppressed: Freeze-fracture replica electron microscopy did not detect any strand-like structures in 1(ko)/2(kd) cells (Figure 4, 1(ko)/2(kd)). We concluded that TJ MAGUK-deficient epithelial cells completely lack TJs.

## Exogenous Expression of ZO-1 and ZO-2 Restores TJ Strands in Junctional Complexes in TJ MAGUK-Deficient Cells

Next, we exogenously expressed ZO-1 or ZO-2 singly, or coexpressed them, into 1(ko)/2(kd) cells and obtained stable transfectants: 1(ko+)/2(kd), 1(ko)/2(kd+), and 1(ko+)/2(kd+) cells, respectively. As shown in Figure 5A, in these transfectants, the exogenously expressed ZO-1 and ZO-2 were specifically recruited to and concentrated in junctional-complex regions. Concomitantly, claudin-3 was also concentrated in junctional-complex regions not only in 1(ko+)/2(kd+) cells but also in 1(ko+)/2(kd) and 1(ko)/2(kd+) cells. Furthermore, TER measurements revealed that the exogenous expression of ZO-1 or ZO-2 sufficiently restored the barrier function of TJs (Figure 5B). Also, freeze-fracture replica electron microscopy revealed that in 1(ko+)/2(kd) (Figure 5C, left panel), 1(ko)/2(kd+) (data not shown), and 1(ko+)/2(kd+) cells (Figure 5C, right panel), TJ strands, i.e., claudin polymers, are reconstituted in the most apical part of lateral membranes. These

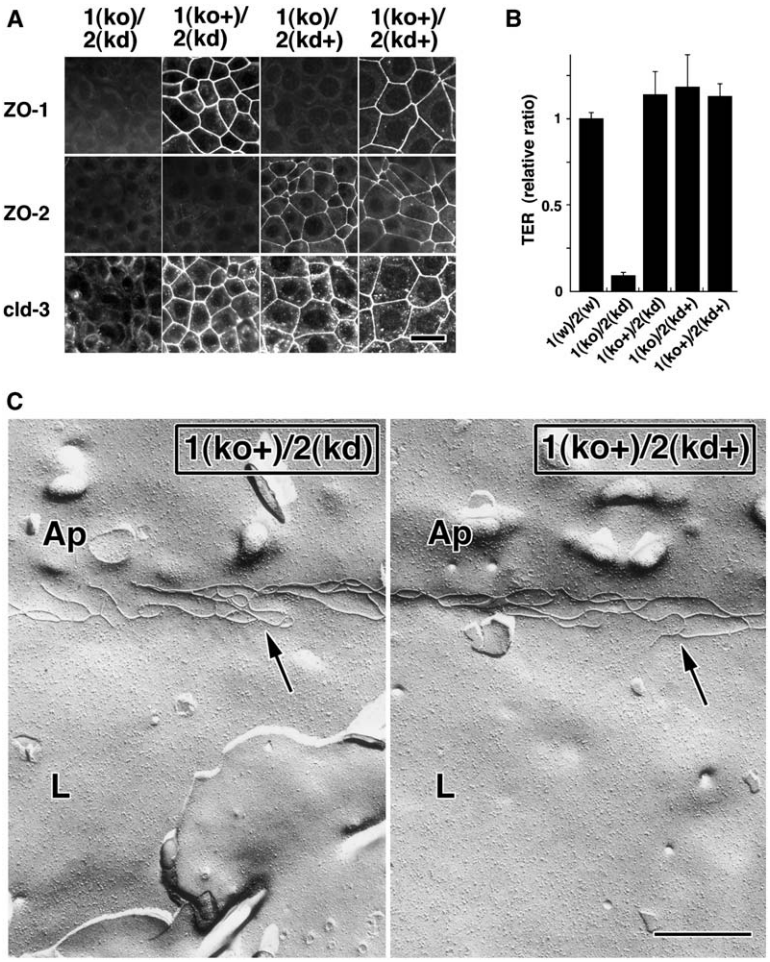


**Figure 5. Re-Expression of Full-Length ZO-1 and ZO-2 in TJ MAGUK-Deficient Cells**

(A) Immunofluorescence microscopy of 1(ko)/2(kd), 1(ko+)/2(kd), 1(ko)/2(kd+), and 1(ko+)/2(kd+) cells stained with anti-ZO-1 mAb, anti-ZO-2 pAb, and anti-claudin-3 pAb. Scale bar = 10 μm.

(B) TER measurements.

(C) Freeze-fracture replica electron microscopy of 1(ko+)/2(kd) (left panel) and 1(ko+)/2(kd+) (right panel) cells in a confluent state. TJs of these cells were characterized by 2–4 continuous TJ strands (arrow). Ap, apical membrane; L, lateral membrane. Scale bar = 500 nm.

findings confirmed that ZO-1 and ZO-2 are redundant in their role in the polymerization of claudins into TJ strands.

### Forced Recruitment of the Dimerized N-Terminal Half of ZO-1 to Plasma Membranes Induces Ectopic Formation of TJ Strands in Lateral Membranes of TJ MAGUK-Deficient Cells

The phenotype of 1(ko)/2(kd) cells, the absence of TJ formation, was rescued by recovery of ZO-1 or ZO-2, or both, leading us to conclude that at least one of ZO-1 and ZO-2 is essential for claudin polymerization around AJs. To further analyze the mechanistic role of ZO-1 in TJ formation, we attempted to specify the domains of ZO-1 that are responsible for claudin polymerization around AJs in 1(ko)/2(kd) cells. As the first step of analysis of the reason for claudin polymerization around AJs, N-terminal PDZ1–3 of ZO-1 (NZO-1), which directly binds to claudin, was introduced into 1(ko)/2(kd) cells, since we speculated that the claudin binding activity was the minimal requirement of ZO-1 for TJ formation. Unexpectedly, NZO-1 was localized in the cytoplasm, not in the membrane, and claudin did not polymerize, as revealed by immunofluorescence microscopy and freeze-fracture replica electron microscopy (Figures 6B and 6C). In contrast, when the longer construct consisting of PDZ1–3 and SH3/GUK, which bound to afadin/α-catenin, was introduced into 1(ko)/2(kd) cells, claudin polymerized around AJs. These results indicated that the SH3/GUK domain is involved in claudin polymerization around AJs. Thus, we further analyzed this domain. Previous studies suggested that the SH3/GUK domain of ZO-1 exhibits the following two types of functions. First, it directly binds to afadin/α-catenin and recruits ZO-1 to the proximity of plasma membranes around AJs (Imamura et al., 1999; Yamamoto et al., 1997), where ZO-1 binds claudins. Second, it plays a role in the dimerization of MAGUK proteins as reported for other MAGUK proteins, especially as shown by PSD-95 (McGee and Bredt, 1999; Shin et al., 2000; Tavares et al., 2001; McGee et al., 2001) and Dlg/SAP90/SAP102 (Kuhlendahl et al., 1998; Masuko et al., 1999). To analyze how these two functions of the SH3/GUK domain contribute to claudin polymerization, we used a sophisticated NZO-1-based construct (designated myrNZO-1-FKPBv): A myristoylation signal was added to the N terminus of NZO-1 to forcibly recruit NZO-1 to the membrane, and FKBPv was added to the C terminus to induce dimerization of NZO-1 by a homodimerizer (AP20187) (Spencer et al., 1993). Under non-FKBPv-activating conditions (monomer) in the

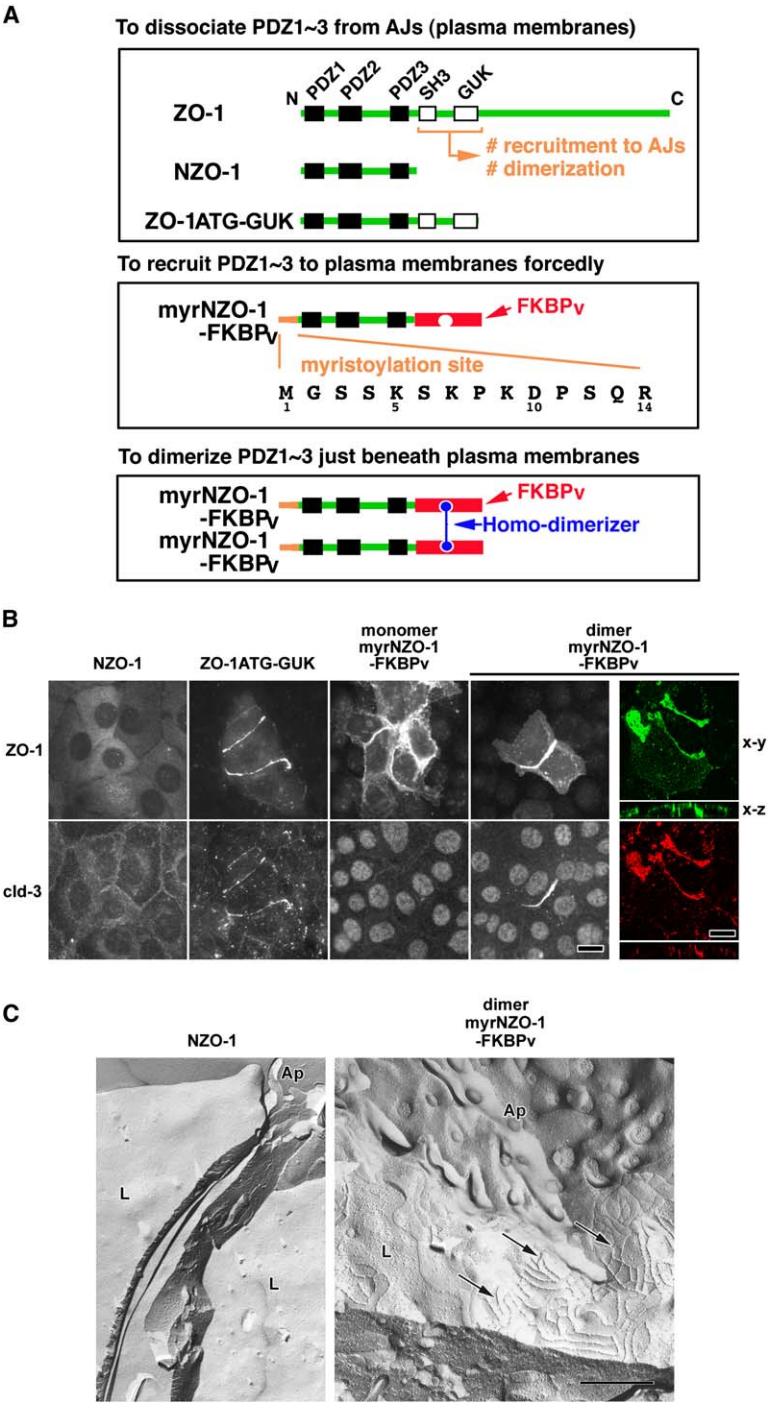


**Figure 6. Ectopic Formation of TJ Strands in TJ MAGUK-Deficient Cells by ZO-1 Mutants**

(A) Strategy and constructs. So that the ZO-1 mutants containing PDZ1 would not target the junctional complex, the AJ binding domain (SH3/GUK) and C-terminal half were deleted from full-length ZO-1 (NZO-1). As the SH3/GUK is known to dimerize, NZO-1 may lose not only its AJ-targeting ability but also the ability to dimerize. To rescue these two defects, a myristoylation sequence (14 aa), which was targeted to plasma membranes, was added to the N terminus of NZO-1, and FKBPv was fused to its C terminus (myrNZO-1-FKBPv). FKBPv can be dimerized by a homodimerizer (AP20187).

(B) Immunofluorescence-based localization of claudin in 1(ko)/2(kd) cells expressing NZO-1, ZO-1ATG-GUK, or myrNZO-1-FKBPv (in the absence and presence of AP20187). Scale bar = 500 nm.

(C) Freeze-fracture replica electron microscopy of TJ MAGUK-deficient cells expressing NZO-1 and myrNZO-1-FKBP. Only when AP20187 was added to myrNZO-1-FKBP-expressing cells to induce dimerization of NZO-1, a huge network of TJ strands appeared (arrows) in most of the lateral membranes. Ap, apical membrane; L, lateral membrane. Scale bar = 500 nm.

absence of AP20187 in the medium, this construct was recruited to the membrane due to the myristoylation signal, but no claudin polymerization occurred (Figure 6B). In contrast, when AP20187 was added to the medium to dimerize myrNZO-1-FKPBv, surprisingly, claudin polymerization was prominent throughout the lateral membrane, as revealed by immunofluorescence and freeze-fracture electron microscopy (Figures 6B and 6C). These findings favor the notion that dimerized ZO-1 (and probably also ZO-2) beneath the plasma membrane not only initiates the polymerization of claudins, i.e., the formation of TJ strands, but also determines the correct localization of TJ strands (Figure 7).

## DISCUSSION

ZO-1 was the first protein to be identified not only as a constituent of TJs (Stevenson et al., 1986) but also as a

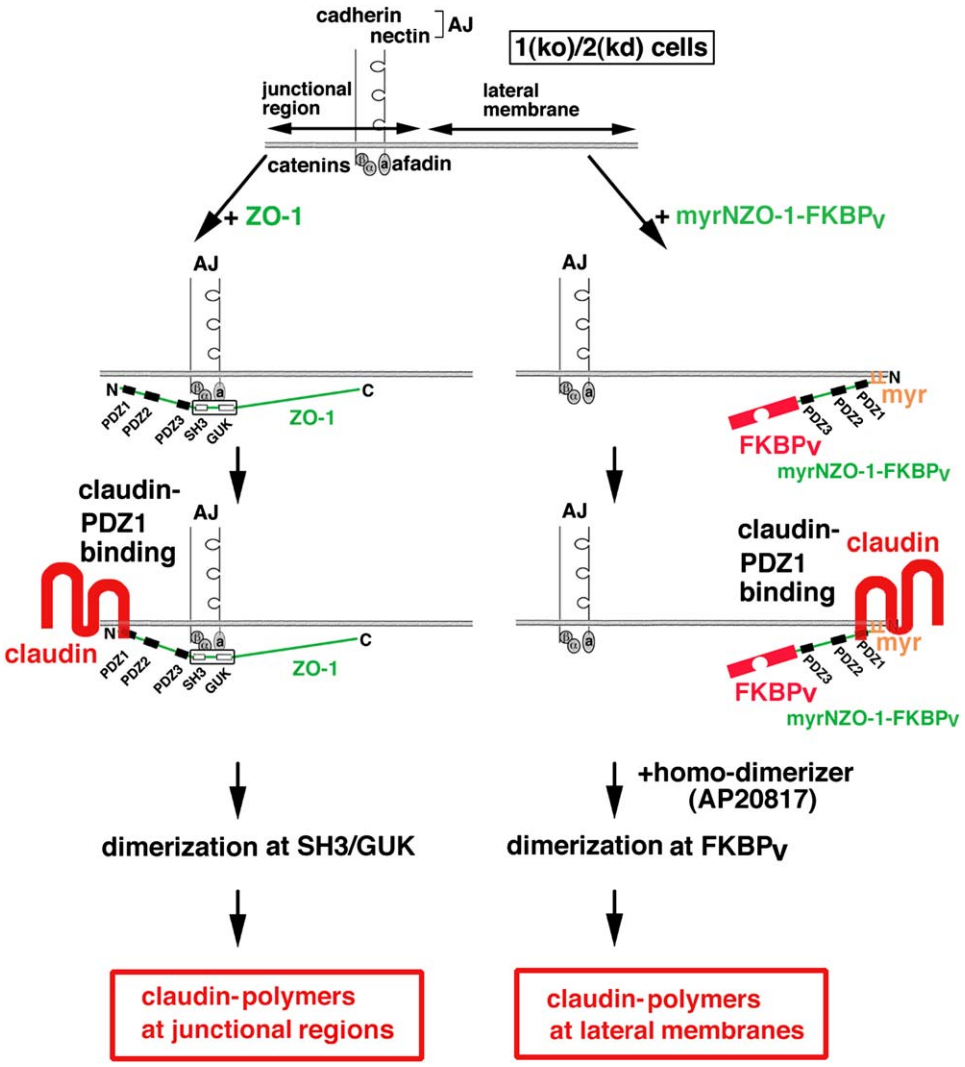


**Figure 7. Schematic Drawing of Our Interpretation**
See details in the text.

PDZ-domain-containing protein (Woods and Bryant, 1993). Afterwards, ZO-2 and ZO-3 were identified as TJ proteins sharing similar molecular domain structures, and these three proteins are now collectively called TJ MAGUKs (Anderson, 1995; Kim, 1995; Woods and Bryant, 1993). Because of their involvement in epithelial polarization, formation of TJs, and the barrier/fence functions of TJs, TJ MAGUKs have been attracting the increasing interest of cell biologists, but it is not easy to clarify their functions experimentally. In order to attack this issue head on, we attempted to perform reverse genetics using mouse cultured epithelial cells. As a first step, with a mouse epithelial cell line, Eph4, we previously succeeded in establishing ZO-1-deficient cells by homologous recombination (Umeda et al., 2004). These cells showed some curious phenotypes in that the formation of TJs was retarded during epithelial polarization and in that cingulin, a TJ plaque protein (Citi et al., 1988), disappeared from TJs, but in the confluent cell culture, the structure and functions of TJs as well as the epithelial polarization of these cells did not appear to be significantly affected. As the TJ MAGUKs are expected to be functionally redundant, in the present study, we attempted to establish Eph4 cells lacking the expression of all of three. Fortunately, as in the parental Eph4 cells, the expression of ZO-3 was undetectable (Figure 1D) (Umeda et al., 2004), and by suppressing the expression of ZO-2 in ZO-1-deficient cells with RNA interference, we successfully generated a novel Eph4 cell line, 1(ko)/2(kd) cells, that is mostly deficient in TJ MAGUKs.

The 1(ko)/2(kd) cells furnished clear and important information on the functions of TJ MAGUKs. These cells appeared to be normally polarized in terms of the distribution of apical and basolateral membrane markers and the

formation of AJs, except that they lacked TJs completely. Taking into consideration that the formation of TJs is often regarded as an important indicator of epithelial polarization (Cereijido et al., 2000; Roh and Margolis, 2003), these findings themselves were a big surprise. It is believed that TJ strands are formed by the linear polymerization of claudins within plasma membranes (Tsukita et al., 2001). This observation has directly indicated that the polymerization of claudins requires TJ MAGUKs. As the C terminus of claudins binds to the PDZ1 domain of TJ MAGUKs (Itoh et al., 1999a), it is safe to say that this binding directly initiates and/or facilitates the polymerization of claudins. Interestingly, when claudin-1 lacking the ability to bind to TJ MAGUKs due to deletion of its C terminus was overexpressed in mouse L fibroblasts, a huge network of TJ strands was reconstituted between adjacent cells (Furuse et al., 1998b). To examine the cell-dependency problems more closely, we generated F9[1(ko)/2(ko)] cells (see Figure S2 in the Supplemental Data available with this article online). It is well known that mouse teratocarcinoma F9 cells form cell aggregates and that their outer layer is differentiated by retinoic acid into polarized epithelial-like cells, visceral endoderm cells (Grover et al., 1983). After differentiating F9[1(ko)/2(ko)] cells by retinoic acid, the results on TJ formation and asymmetric distribution of membrane proteins were essentially the same between epithelial types of F9[1(ko)/2(ko)] and Eph4[1(ko)/2(kd)] cells (Figure S2). Therefore, distinct from the plasma membranes of fibroblasts, within the lateral membranes of epithelial cells, there must be some critical regulatory mechanism that allows claudins to be polymerized only when they bind to TJ MAGUKs.

This mechanism may restrict the site of polymerization in the most apical region of lateral membranes in epithelial cells, which is crucial for the physiological functions of individual epithelial cells. When exogenously expressed in 1(ko)/2(kd) cells, full-length ZO-1 was recruited around AJs; this was expected because ZO-1 has been reported to directly bind to major plaque proteins of AJs such as α-catenin and afadin at their SH3/GUK domains (Imamura et al., 1999; Yamamoto et al., 1997) and to actin filaments at their C-terminal domains (Itoh et al., 1997; Fanning et al., 1998). Then, only around AJs would claudins be polymerized into reconstituted TJ strands in a spatially regulated manner. Indeed, in 1(ko)/2(kd) cells, exogenously expressed NZO-1 was not recruited around AJs but was diffusely distributed in the cytoplasm without inducing the polymerization of claudins in any portion of the plasma membranes. In this study, we found that there are at least two requirements for NZO-1 to initiate the polymerization of claudins to constitute TJ strands. First, NZO-1 must be forcibly recruited to plasma membranes. This is important to direct the interaction of claudins with PDZ1 of NZO-1. Second, the claudin-associated NZO-1 must be dimerized. For unknown reasons, the dimerization of NZO-1 is required for claudins to be nucleated for polymerization. As shown in Figure 6C, this dimerization appears to be important for the polymerization of claudins. As it is generally believed that paired, but not single, TJ strands occur between apposing membranes (Furuse et al., 1999; Tsukita et al., 2001), myrNZO-1-FKBPv should induce the polymerization of claudins only at lateral membranes. These findings and our interpretation are summarized in Figure 7. We conclude that ZO-1 and ZO-2 are indispensable not only for initiating the polymerization of claudins but also for determining where claudins should be polymerized.

The present study with 1(ko)/2(kd) cells also provides crucial information on the complicated functional redundancy among TJ MAGUKs. We cannot discuss the functions of endogenous ZO-3 due to the lack of ZO-3 in parental Eph4 cells, but, as compared to ZO-1 and ZO-2, ZO-3 appears to be fairly different in its nature: When exogenously expressed in 1(ko)/2(kd) cells, ZO-1 and ZO-2 are recruited around AJs, but ZO-3 is not. As ZO-3 is recruited around AJs in 1(w)/2(kd) and 1(ko)/2(w) cells, ZO-3 cannot target junctional areas by itself but requires the preexistence of ZO-1 and/or ZO-2. Therefore, although the PDZ1 domain of ZO-3 as well as ZO-1/ZO-2 has been reported to directly bind to the C termini of claudins in vitro (Itoh et al., 1999a), we could not conclude that ZO-3 is functionally redundant with ZO-1 and/or ZO-2 within cells. The question then naturally arises as to the functional redundancy between ZO-1 and ZO-2. Our previous study with 1(ko)/2(w) cells suggested that ZO-1 and ZO-2 had their own specific functions (Umeda et al., 2004), but the present study revealed that, in terms of determining whether and where claudins are polymerized in epithelial cells, ZO-1 and ZO-2 appear to be functionally redundant. Therefore, through a combination of redundant and nonredundant functions, ZO-1 and ZO-2 may contribute to the barrier and fence roles of TJs.

In addition to its barrier functions, it has long been assumed that TJs act as a fence to prevent membrane proteins from freely diffusing between apical and basolateral membranes (Balda and Matter, 1998). However, in 1(ko)/2(kd) cells having no TJs, the normal segregation of membrane proteins takes place. Therefore, our study elucidates for the first time that TJs are not vital at least to the asymmetrical distribution of membrane proteins on cell membranes. Recent studies have revealed an important role of TJ complexes, such as Par3/Par6/aPKC and Crumbs/Pals1/Patj, for polarization in epithelial cells (Hurd et al., 2003). When we examined the distribution of Par3, aPKC, and Pals1 in Eph4[1(w)/2(w)] and F9[1(w)/2(w)] cells, these proteins concentrated in TJs; in contrast, in Eph4[1(ko)/2(kd)] and F9[1(ko)/2(ko)] cells, these proteins were localized in lateral membranes (Figure S3). Taken with the results from apical/basolateral markers in 1(kd)/2(kd) cells, these findings indicate that TJs might not be crucial for epithelial polarization. A paper by Hans Clevers (Baas et al., 2004) made a similar claim but was not convincingly documented. Now, the present paper strongly suggests that this is the case.

It has not been clear whether, during the formation of TJs, TJ MAGUKs concentrate around junctional areas

downstream or upstream of the polymerization of claudins. One idea was that claudins are first polymerized into strands, and then TJ MAGUKs are attracted to their cytoplasmic surfaces. However, the present study clearly shows that ZO-1 and ZO-2 are first recruited to the junctional area, which initiates and facilitates the polymerization of claudins. Of course, once the strands are formed, the possibility cannot be excluded that additional TJ MAGUKs are attracted to these newly formed strands. In general, it has been believed that the TJ plaque proteins, including TJ MAGUKs, play some important role in the intracellular signaling for epithelium- (or endothelium-) specific cell growth and differentiation (Matter and Balda, 2003; Schneeberger and Lynch, 2004; Tsukita et al., 2001). However, interestingly, 1(ko)/2(kd) cells completely lacked TJs and their plaque proteins but showed no detectable abnormalities in cell growth or epithelial morphogenesis. Detailed analyses using 1(ko)/2(kd) cells will be required to further evaluate the relationship between TJs and epithelial cell growth and differentiation.

The last issue we should discuss here is the relationship between ZO-1/ZO-2 and the functions of cadherins at AJs. ZO-1 and ZO-2 are widely expressed in various cells other than the epithelia/endothelia and are colocalized with cadherins. For example, in cultured fibroblasts, ZO-1 and ZO-2 are concentrated at P-cadherin-positive spot-like AJs, and in cardiac muscle cells, ZO-1 and ZO-2 are exclusively localized to intercalated discs that are disc-like AJs in which N-cadherin acts as the major cell adhesion molecule (Itoh et al., 1993, 1999b; Jesaitis and Goodenough, 1994; Volk and Geiger, 1984). As these cells of course lack TJs, it is expected that ZO-1 and ZO-2 probably have some cadherin-related functions in addition to TJ-related functions. Furthermore, in the initial phase of epithelial polarization, primordial spot-like AJs are formed between adjacent cells, where ZO-1 and ZO-2, but not claudins/occludin, are accumulated in large amounts together with E-cadherin and α-/β-catenin (Adams et al., 1996; Ando-Akatsuka et al., 1999; Yonemura et al., 1995). Therefore, it is reasonable to speculate that, also in these primordial spot-like AJs of epithelial cells, ZO-1 and ZO-2 are involved in cadherin-based cell adhesion. However, unexpectedly, 1(ko)/2(kd) cells showed the same cadherin-based cell aggregation activity as wild-type Eph4 cells and formed E-cadherin/afadin-positive belt-like AJs of normal appearance in the confluent state. Thus, the functional relationship between cadherins and ZO-1/ZO-2 remains a mystery, but 1(ko)/2(kd) cells will provide a valuable resource for further studying the complicated in vivo functions of TJ MAGUKs in molecular terms.

## EXPERIMENTAL PROCEDURES

### Cells and Antibodies

Mouse Eph4 epithelial cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Eph4 cells were a gift from Dr. E. Reichmann (Reichmann et al., 1989). Mouse anti-ZO-1 mAb (T8–754, Itoh et al., 1993), rat anti-occludin mAb (MOC37, Saitou et al., 1997), rabbit anti-JAM pAb (Komiya et al., 2005), and rat anti-moesin mAb (M22, Takeuchi et al., 1994) were raised and characterized previously. Rat anti-mouse E-cadherin mAb (ECCD2) and mouse anti-afadin mAb were provided by Dr. M. Takeichi (Center for Developmental Biology, Kobe, Japan) and Dr. Y. Takai (Osaka University, Osaka, Japan), respectively. Rabbit anti-claudin-3 pAb and rat anti-ZO-3 mAb were purchased from Sigma. Rabbit anti-ZO-2 pAb, rabbit anti-syntaxin 3, and rabbit anti-erbB2 were purchased from Santa Cruz Biotechnology, Synaptic Systems, and Nichirei, respectively.

### Generation of the 1(ko)/2(kd) Cell Line

ZO-1-deficient Eph4 cells were generated by gene targeting as described previously (Umeda et al., 2004). The oligonucleotides encoding shRNA targeting mouse *ZO-2* mRNA were designed according to the online program RNAi OligoRetriever (http://katahdin.cshl.org:9331/RNAi/html/rnai.html). The target sequence was *ZO-2* mRNA 946–977. The designed oligonucleotides were cloned into the vector pSHAG-1 (Paddison et al., 2002), which was a gift from Dr. G.J. Hannon (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, USA). For selection of stably expressed clones, the region containing the U6 promoter and shRNAi sequence was excised and subcloned into the upstream region of the puromycin resistance gene in pPGKpuro plasmid vector. The shRNAi vector was transfected into the cells using Lipofectamine 2000 (Invitrogen).

### Expression Vectors

To express full-length ZO-2 in ZO-2 knockdown cells (1(ko)/2(kd) cells), partially substituted ZO-2 mutants (C957A, T963C, and C966A) were generated using the QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene) without changing the amino acid sequence.

To express myrNZO-1-FKBPv, the following plasmid was constructed. The domains of ZO-1 PDZ1–3 (aa 1–522), cloned by PCR amplification, were subcloned into pC4M-Fv2E (Regulated Homodimerization Kit, ARIAD; www.ariad.com/regulationkits), providing an N-terminal myristoylation sequence, two tandem FKBPv domains, and hemagglutinin (HA) epitope sequence. The entire N-terminal myristoylation signal, PDZ1–3, two tandem FKBPv domains, and HA sequence was subcloned into pCAGIBsd. The pC4M-Fv2E plasmid and the dimerization reagent, AP20187, were kindly provided by ARIAD.

### Immunofluorescence Microscopy

Immunofluorescence microscopy was performed as described previously (Umeda et al., 2004). In brief, for ZO-1, ZO-2, claudin-3, occludin, E-cadherin, moesin, and erbB2 staining, cells cultured on transwell filters were fixed with 10% trichloroacetic acid for 30 min on ice. For afadin and actin staining, cells were fixed with 3% formalin in PBS for 15 min at room temperature. For JAM and syntaxin 3 staining, cells were fixed with 100% methanol for 3 min at −20°C. All fixed cells were incubated with Alexa 488-conjugated (Molecular Probes) or Cy3-conjugated secondary antibody (Jackson ImmunoResearch) for 30 min. For actin staining, Alexa Fluor 568 phalloidin (Molecular Probes) was added to the secondary antibody.

### Cell Aggregation Assay

The cell aggregation assay was performed as described previously (Umeda et al., 2004).

### Measurement of Transepithelial Electric Resistance (TER) and Analysis of Paracellular Tracer Flux

After 6 days of culture on transwell filters, TER was measured directly in culture media using a Millicell-ERS epithelial voltohmmeter (Millipore).

For the paracellular tracer flux assay, after 6 days of culture, FITC-dextran with a molecular mass of 40 kDa at a concentration of 2 mg/ml was added to the medium in the apical compartment. After 2 hr of incubation, a 100 μl aliquot of the medium was collected from the basal

compartment, and the paracellular tracer flux was measured as the amount of FITC-dextran in the medium using a fluorometer.

### Biotin Tracer Assay

The biotin tracer assay was performed using the cell-surface biotinylation method as described by Chen et al. (1997) with some modifications. Cells cultured on transwell filters were washed with HEPES-buffered saline (HBS; 25 mM HEPES-NaOH [pH 7.2], 137 mM NaCl, 5 mM KCl, 0.7 mM $Na_2HPO_4$, 6 mM dextrose, and 1.8 mM $CaCl_2$) and incubated with HBS supplemented with 1 mg/ml EZ-Link sulfo-NHS-LC-biotin (Pierce) in the apical compartment only. After 10 min incubation, cells were washed with DMEM and fixed with 100% methanol for 3 min at −20°C. Cells were then washed with PBS, blocked with 1% bovine serum albumin in PBS for 30 min, and incubated with rat anti-E-cadherin antibody for 1 hr. After a wash with PBS, they were incubated for 30 min with streptavidin and anti-rat antibody conjugated with Alexa 488 and Cy3, respectively.

### Ultrathin-Section and Freeze-Fracture Electron Microscopy

Cells were cultured on transwell filters 24 mm in diameter for 48–72 hr. Ultrathin-section and freeze-fracture electron microscopy were performed as described previously (Furuse et al., 1998a).

### Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, and three figures and can be found with this article online at http://www.cell.com/cgi/content/full/126/4/741/DC1/.

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