Visualizing the dynamic coupling of claudin strands to the actin cytoskeleton through ZO-1

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ABSTRACT The organization and integrity of epithelial tight junctions depend on interactions between claudins, ZO scaffolding proteins, and the cytoskeleton. However, although binding between claudins and ZO-1/2/3 and between ZO-1/2/3 and numerous cytoskeletal proteins has been demonstrated in vitro, fluorescence recovery after photobleaching analysis suggests interactions in vivo are likely highly dynamic. Here we use superresolution live-cell imaging in a model fibroblast system to examine relationships between claudins, ZO-1, occludin, and actin. We find that GFP claudins make easily visualized dynamic strand patches between two fibroblasts; strand dynamics is constrained by ZO-1 binding. Claudin association with actin is also dependent on ZO-1, but colocalization demonstrates intermittent rather than continuous association between claudin, ZO-1, and actin. Independent of interaction with ZO-1 or actin, claudin strands break and reanneal; pulse-chase-pulse analysis using SNAP-tagged claudins showed preferential incorporation of newly synthesized claudins into strand break sites. Although claudin strand behavior in fibroblasts may not fully recapitulate that of epithelial tight junction strands, this is the first direct demonstration of the ability of ZO-1 to stabilize claudin strands. We speculate that intermittent tethering of claudins to actin may allow for accommodation of the paracellular seal to physiological or pathological alterations in cell shape or movement.

INTRODUCTION
The paracellular seal between epithelial cells is formed by continuous cell–cell adhesive rows of the claudin (cldn) family proteins (Furuse et al., 1998). Although several different integral membrane proteins contribute to this seal, including occludin (Furuse et al., 1993), tricellulin (Ikenouchi et al., 2005), and junctional adhesion molecules (JAMs; Martin-Padura et al., 1998), cldns are the critical components that regulate paracellular ion and solute size selectivity (Van Itallie et al., 2008; Gunzel and Yu, 2013). Claudins are the primary constituents of the network of strands seen in freeze-fracture electron microscopy (FFEM) that forms a continuous seal around the apicolateral pole of epithelial cells (Furuse et al., 1998). The FFEM strands formed by cldns are stabilized by adhesive interactions between extracellular domains in the paracellular space; this interaction then facilitates lateral polymerization into strands (Piontek et al., 2008; Koval, 2013). The cytoplasmic amino acid sequence of most cldns ends with a C-terminal PDZ binding motif, which interacts with the first PDZ domain of the tight junction scaffolding proteins zona occludens (ZO) 1–3 (Itoh et al., 1999). The ZO proteins directly bind actin (Fanning et al., 1998) and numerous actin-regulating proteins (Rodgers and Fanning, 2011) and thus are hypothesized to form a stabilizing link between the seal and the cytoskeleton.

In spite of the well-demonstrated in vitro binding between cldns and ZO-1 and between ZO-1 and actin, the in vivo relevance, nature, and regulation of these interactions remain unclear. When expressed in epithelial or endothelial cells, cldns lacking the PDZ binding motif still localize to tight junctions (Ruffer and Gerke, 2004). ZO-1 knockout (Tokuda et al., 2014) in Madin–Darby canine kidney (MDCK) cells results in only minor effects on cldn and actin organization, although double knockdown of ZO-1 and ZO-2 decreases tight junction localization of some but not all cldns and also results in...
FIGURE 1: Cldns form strand patches when expressed in fibroblasts. (A) Endogenous ZO-1 in Rat-1 (top left) fibroblasts colocalizes with N-cadherin (top right) and a subset of actin (bottom left and merge) at cell contacts; bar, 20 μm. (B) Stable expression of GFP-tagged cldn2 results in strand patches that are associated with the cell borders (arrowhead); some patches have loose collections of cldn strands (arrow); bar, 10 μm. (C) Coculture of cells expressing GFP cldn2 (left) and mCherry cldn2 (middle) confirms that cldn strand patches form only between two cldn-expressing cells (merge); bar, 4 μm. (D) SIM analysis of GFP cldn2 strand patch shows a mixture of well-separated and condensed strands; bar, 2 μm. (E) Plot profiles of well-separated areas shows fluorescence intensity of single strand (purple) is half that of doubled strand (green).

accumulation of apical actin (Fanning et al., 2012). In contrast, ZO-1 knockout/ZO-2 knockout does result in loss of tight junction cldn organization (Umeda et al., 2006), but in these cells, adherens junction organization is also compromised (Yamazaki et al., 2008).

It has been known for many years that disruption of the actin cytoskeleton results in loss of tight junction organization (Meza et al., 1980), but because other cell junctions are disrupted as well, it is difficult to isolate the specific contributions of actin to tight junction organization. In addition, fluorescence recovery after photobleaching (FRAP) analysis of tight junction proteins demonstrates very different recovery kinetics for cldns, ZO-1, and actin, suggesting that their interactions at the junction are not static but are coupled in a highly dynamic manner (Shen et al., 2008).

Tight junction contacts must be both sufficiently stable to maintain a continuous seal and sufficiently dynamic to allow adaptation of this seal to the continuous motion relative to adjacent cells. Both FRAP and live-cell imaging studies (Van Itallie et al., 2015; Schlingmann et al., 2016) demonstrate this dynamic nature of tight junction contacts. However, the small size and the geometry of intact epithelial junctions limit our ability to dissect microscopically the influence of specific protein–protein interactions in modulating the balance between stable and dynamic behavior. Sasaki et al. (2003) demonstrated the utility of expressing green fluorescent protein (GFP) cldns in fibroblasts to study cldn strand dynamics. We turned to a similar reductionist system, using structured illumination microscopy (SIM; Gustafsson, 2000) for live-cell imaging of tagged cldns, ZO-1, actin, and occludin (ocln) in fibroblasts to directly investigate the role of ZO-1 and association with the actin cytoskeleton in cldn behavior.

RESULTS
Expression of GFP Cldn2 in Rat-1 fibroblasts results in tight junction–like strands at cell–cell contacts

Immunofluorescence imaging of Rat-1 fibroblasts demonstrated that endogenous ZO-1 is found at well-defined N-cadherin–based cell contacts, where actin filaments also accumulate (Figure 1A); these fibroblasts are flat, and the overlapping cell contacts are amenable to high-resolution imaging in the xy-plane. Expression of N-terminally tagged GFP cldn2 in these cells results in the formation of strand patches similar to those described by Sasaki et al. (2003). High-resolution confocal analysis of transfected cells using the Zeiss Airyscan detector demonstrated that these patches are mainly found at the cell periphery where cells overlap (Figure 1B) and consist of both tight, complex strand networks (Figure 1B, arrowhead) and loose associated strands (Figure 1B, arrow). As previously suggested (Sasaki et al., 2003), strand patches were composed of paired strands from adjoining cells; we confirmed this by coculture of cells expressing either GFP or mCherry cldn2 (Figure 1C), which showed complete strand overlap from the contiguous cells. That cldn strand patches formed exclusively between two cells is consistent with the primary requirement for transepithelial interaction to promote strand formation (Piontek et al., 2008; Koval, 2013).

When visualized by SIM, GFP cldn2 forms complex strand networks (Figure 1D) reminiscent of networks seen in FFEM; we observed similar strand organization in cells expressing GFP cldn1 and cldn11 (Supplemental Figure S1). Line profile analysis of the images of well-separated strands (Figure 1D, magenta and green lines) showed that the fluorescence intensity across strands increased in unitary steps (Figure 1E), which suggested that it was possible to recognize single, doubled, and occasionally tripled (unpublished data) strands, although the strand density in many patch areas was too high to allow further resolution. We assume that the well-separated fluorescent strands of unit fluorescence intensity correspond to individual FF strands visualized by EM (Sasaki et al., 2003). To
This page discusses the interaction of claudin (cldn) with ZO-1 and its effect on localization and strand dynamics. The authors demonstrate that the presence of a linker region improves binding of claudin to ZO-1, although the colocalization and behavior of GFP-tagged claudin with and without the linker region were similar. Live-cell imaging was used to compare claudin dynamics in cells expressing GFP-tagged claudin with and without the linker region. The authors also used pull-down assays to confirm these findings.

**Figure 2:** Interaction of claudin with ZO-1 affects localization and strand dynamics. (A) Both N-terminal GFP claudin2 (top) and claudin2(−3) (bottom) form strand patches in fibroblasts, but the PDZ binding motif is required for ZO-1 colocalization; bar, 10 μm. (B) Pull down of claudin2 constructs with myc-tagged ZO-1 (ZNA) (lysate; left) demonstrates that GFP linker claudin2 binds ZO-1 better than GFP claudin2, whereas GFP claudin2(−3) fails to bind ZNA (right). (C) Frames from live-cell imaging of GFP Linker claudin2 (top; Supplemental Figure 2C Video1) and GFP Cldn2(−3) (bottom; Supplemental Figure 2C Video2) demonstrates increased mobility in claudin2(−3) strand patches; yellow outline, patch shape at the start and end of 10-min movies (bar, 2 μm). (D) Variation in strand patch areas over 10 min is significantly greater in (−3)-expressing cells than in those expressing GFP linker claudin2 (p > 0.001; F test to compare variances).

Sasaki et al. (2003) demonstrated the dynamic behavior of strand patches using claudins tagged on the C-terminus with GFP, which blocked interaction with ZO-1. Because ZO-1 binding might be likely to anchor claudins at the junction, we used live-cell imaging to compare claudin dynamics in cells stably expressing GFP Linker claudin2 with those expressing GFP claudin2(−3). As expected, we found that claudin strand patches in cells expressing GFP claudin2(−3) were much more mobile than strand patches composed of GFP Linker claudin2; we quantified this by measuring strand patch area at the start of imaging and 10 min later (Figure 2C, top, and Supplemental Figure 2C Video1). All strand patches translocated with the cells as the cells

**GFP Cldn2 strand dynamics is constrained by interaction with ZO-1**

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moved. However, the patch area and shape were relatively constant in cells expressing GFP Linker cldn2 but fluctuated in cells expressing GFP cldn2(−3). This meant that the final patch shape and size were often different in these cells compared with the initial organization (Figure 2C, bottom, and Supplemental Figure 2CVideo2; compare yellow outlines from the start of imaging). The behavior of the strand patches over time was quantified by comparing the variance in patch sizes in cells expressing GFP Linker cldn2 and GFP cldn2(−3). The small variance in area in cldn strands that could bind ZO-1 compared with the significantly larger variance in those that could not was consistent with the hypothesis that binding to ZO-1 is likely to stabilize cldn strands at the tight junction. The overall strand dynamics (independent of ability to interact with ZO-1) was not affected by administration of the Rho kinase inhibitor Y27632, the Arp2/3 inhibitor CK666, or the administration of blebbistatin (unpublished data).

**Phosphomimetic substitution of Cldn2 Y224 results in increased strand mobility**

We recently showed that cldn2 interaction with PDZ1 of ZO-1 was stabilized by a tyrosine at −6 (Y224) in the cldn tail (Nomme et al., 2015) and that this site can be phosphorylated in vivo (Van Itallie et al., 2012). Introduction of cldn2 with a phosphomimetic Y224E mutation strongly altered the transepithelial resistance and charge selectivity of MDCK monolayers (Nomme et al., 2015). To determine whether the phosphorylation of this residue might regulate cldn strand behavior, we examined strand dynamics in cells stably expressing GFP cldn2 with and without the phosphomimetic mutation Y224E. We first confirmed by pull-down assays using lysate from transfected HEK cells that GFP linker cldn2 Y224E had greatly reduced affinity for ZNA compared with wild-type GFP Linker cldn2 (Figure 3A). We then compared live-cell behavior of cells expressing GFP Linker cldn2 with and without the Y224E substitution and showed that, like cldn2(−3), strand patches composed of GFP Linker cldn2 Y224E showed larger variations in strand patch size compared with those of wild-type GFP Linker cldn2 (Figure 3B, Supplemental Figure 3BVideo3, and Supplemental Figure 3BVideo4; quantified in Figure 3C). The different behavior of the linker-containing constructs without and with the Y224E mutation suggests that tyrosine phosphorylation at the cldn2 −6 position might be a physiological mechanism for regulation of interaction with ZO-1 and thus changes in cldn strand behavior.

**GFP Cldn2 is associated with actin through binding to ZO-1**

ZO-1 binds to actin and many actin-binding proteins (Fanning et al., 1998; Rodgers and Fanning, 2011) and thus might constrain cldn strand dynamics by cross-linking cldn to the actin cytoskeleton. If this were true, cldn2 that could interact with ZO-1 might be oriented along actin filaments, but cldn2 strand patches that could not bind ZO-1 would not align with actin. We used the ImageJ plug in FibrilTool (Boudaoud et al., 2014) to test whether there were differences between GFP cldn2 with or lacking the PDZ binding motif and actin filaments by comparing relative orientation and anisotropy of GFP and actin signals. Figure 4A (top) shows two examples of the orientation of the GFP and actin signals as determined by FibrilTool in cells expressing GFP linker cldn2; the correlation of the orientation of GFP and actin in this cell clone (Figure 4B) was calculated to be 0.959. In contrast, the correlation between GFP and actin was much weaker in cells expressing GFP cldn2(−3) (Figure 4A, bottom); the correlation coefficient was 0.450 for cells expressing this construct (Figure 3C). To verify that this interaction was dependent on ZO proteins and not on other PDZ domain-containing proteins, we made clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9–mediated knockout (KO) ZO-1/2 Rat-1 cell lines (Figure 4D). Single KO of ZO-1 or ZO-2 had little effect on GFP cldn2 behavior (unpublished results), but double ZO-1/2 KO resulted in formation of fewer GFP cldn2 strand patches and sparse, scattered strands that had no clear association with the underlying actin cytoskeleton (Figure 4E); the...
The correlation coefficient for actin and GFP was −0.1534 (Figure 4F). Unsurprisingly, the anisotropy of the GFP Linker cldn2 was significantly higher than that of GFP cldn2(−3) or the GFP Linker cldn2 expressed in the ZO-1/2 double-KO cells. These findings are consistent with a role for ZO-1 in linking and orienting cldns to the actin cytoskeleton.

To better understand the relationships between actin, endogenous ZO-1, and GFP Linker cldn2, we compared their localization using SIM. This analysis demonstrated a very close relationship between the ZO-1 signal and that of the actin filaments (Figure 5), which is particularly evident in the ZO-1/actin overlay (bottom, middle). The GFP Linker cldn2 is associated with the ZO-1 signal, but it also forms a free network of strands that appear to be only intermittently associated with ZO-1 (arrow, top, middle). This is easier to see in a skeletonized image of the GFP cldn2/ZO-1 overlay (compare top two right images). This loose network of strands could be due in part to a mismatch in the levels of expression of scaffolding and cldn proteins, but it was a common feature of the claudin strand patches in multiple cell lines at every cldn expression level. The immunofluorescence signal for the GFP cldns was always continuous, whereas those for ZO-1 and actin was always intermittent (endogenous or tagged), consistent with intermittent and irregular rather than continuous and stoichiometric tethering of strands to ZO-1 and ZO-1 to actin.

Coexpression of occln with GFP-cldn2 alters occludin FRAP recovery but not strand dynamics

Shen et al. (2008) used FRAP analysis to demonstrate differential recovery rates and mobile fractions for cldn1, occln, and ZO-1 in epithelial cells. Because Rat-1 fibroblasts might differ from epithelial cells in how they control dynamics of these proteins, we first documented as a baseline the FRAP parameters of GFP ZO-1, GFP occln, and GFP cldn2 with and without the PDZ binding motif or containing the Y224E mutation. Prebleach and postbleach images of representative areas for each of these proteins (Figure 6A) demonstrates quick recovery for GFP ZO-1 and occln but no measurable recovery of cldn2, cldn2(−3), or cldn2 Y224E. Overall these results are very similar to those described in epithelial cells. The lack of recovery of cldn that cannot bind ZO-1 was previously shown in fibroblasts by Sasaki et al. (2003). Second, we asked whether we could detect interactions between the transfected cldns and ZO-1 by performing FRAP analysis on cell lines stably coexpressing GFP ZO-1 and occln.

**Figure 4:** Cldn2 associates with the actin cytoskeleton through interaction with ZO-1/2.
(A) FibrilTool (ImageJ) was used to analyze relationships between cldn2 and actin. There was higher correlation between the orientation of the linker construct (top; B) and actin than that of the (−3) construct and actin (bottom; bar, 2 μm; C). (D) KO of ZO-1/2 resulted in loss of correlation between GFP Linker cldn2 and actin (E, F; bar 2 μm). (G) Analysis of anisotropy of GFP cldns shows significantly higher anisotropy values for Linker construct compared with (−3) or Y224E cldn2.

**Figure 5:** Colocalization of GFP cldn2 with endogenous ZO-1 and actin demonstrates good correlation between ZO-1 and actin localization (bottom, middle), whereas GFP cldn2 is associated with ZO-1 and actin but also forms independent loops (arrow; top right, skeletonized image of middle; bar, 1.5 μm).
ocln clearly concentrates at strand ends and strand-to-strand junction points. The molecular organization of cldns at sites at which two strands join is unknown, but the accumulation of ocln at these sites suggested that they might be structurally specialized or disorganized in a way that promotes interaction with ocln.

**GFP cldn strand breaks occur primarily at branch points; free ends show a propensity to reanneal**

Although Sasaki et al. (2003) demonstrated that individual strands within a cldn strand patch break and reanneal, because they used cldn that could not interact with ZO-1 in these experiments, it was unclear whether this behavior would be modified by interaction with ZO-1. To test whether ZO-1 binding affected strand breaks, we counted the number of breaks in equivalent-sized strand patches from cells expressing GFP cldn2 with or without the PDZ binding motif. Breaks were relatively rare (approximately two breaks per average patch size of 5 μm²), but we found that although the overall strand patch area is stabilized by interaction with ZO-1, the ability to interact with ZO-1 had no apparent effect on the pattern or number of strand breaks or joins (Figure 8A). However, it was clear that the majority of breaks occur at sites where two strands join at a T-junction (91%, n = 57); this pattern can be observed in still images in Figure 8B and time-lapse images in Supplemental Figure 8BVideo5; arrows mark the sites of breaks and joins. Often the T-junction will break, reanneal, and rebreak at another arm of the T (Figure 8B, top, arrows), suggesting that the junction point is more fragile at all join points, not just at the site at which the strand makes a 90° end-to-side attachment with a straight strand. In some cases, a strand end appears to translocate along a straight strand (Figure 8C, top, and Figure 8BVideo6). Occasionally, strand breaks occur midstrand (Figure 8C, bottom, and Figure 8CVideo6); in many cases (arrows), the breaks reanneal almost immediately. Strand ends behave as if their structure is unstable, in that they often quickly join and rebreak. Often, free ends (Supplemental Figure 8CVideo6) behave as if they are guided back to reanneal, as if there were as-yet-undetermined influences promoting strand continuity.

Strand breaks and rejoins are not associated with obvious changes in the underlying actin cytoskeleton (Figure 8D, arrows), which was consistent with our finding that breaks and reannealing behavior are not affected by the ability of the GFP cldn2 construct to bind ZO-1.

**Newly synthesized cldn is added to strands focally and preferentially at strand junction points**

The behavior of joins and breaks in the cldn strands suggested that these sites were less stable than the linear strands, and we speculated that they might be sites for addition of newly synthesized cldns. To

FIGURE 6: Tight junction proteins differ in FRAP dynamics. (A) GFP ZO-1, ocln, and cldn2 (wild type, −3, and Y224E) show distinct FRAP behaviors in Rat-1 cells. Images before and at the indicated time points after photobleaching. Brackets indicate bleach regions. (B) FRAP behavior after coexpression of GFP ZO-1 and ocln with mCherry cldn2; yellow signal, region of colocalization. (D) Mobile fraction and t½ of recovery for each protein calculated from the recovery curves in C; n = 10 for each cell line. Error bars represent SEM. (E) Analysis of strand patch size changes over 10 min in cells coexpressing ocln with cldns, showing no difference between patch area; F test to compare variances.

or GFP ocln with mCherry cldn2. We found that there was no effect of coexpression with cldn on the kinetics of ZO-1 recovery (Figure 6B, top), but that, in contrast, coexpression of cldn2 with ocln significantly decreased mobility of GFP ocln (Figure 6B, bottom). These results are quantified in Figure 6C and the mobile fraction and recovery T½ (Figure 6D) calculated from the recovery curves. These results are consistent with the finding that ocln can be incorporated into cldn strands in fibroblasts (Furuse et al., 1998). However, in spite of the interaction between ocln and cldn2, there was no clear effect of coexpression of ocln with cldn2 on strand patch mobility (Figure 6E).

Unsurprisingly, SIM images of Rat-1 cells coexpressing GFP cldn2 and Halo-tagged ocln (Figure 7) showed nonuniform incorporation of ocln into the cldn2 strands. The insets in Figure 7 highlight that although there were many patches of occludin along strands, Halo
although incorporation of new cldn increased as incubation time increased. These results suggest that (Figure 9D) addition of new cldns does not involve a molecular-level replacement of claudins along the strands but instead focal addition at special sites, such as breaks or ends. We were unable to see new cldn addition in live-cell imaging, but the sites of the appearance of new label were the same areas that were most dynamic in terms of breaking and reannealing in the earlier network analysis studies.

**DISCUSSION**

Formation and regulation of the tight junction seal are highly dependent on the state of perijunctional actin, and thus it is important to understand how cldn strands are coupled to actin and how these interactions are physiologically regulated and altered in pathology. We developed a reductionist model in fibroblasts to manipulate and visually study these links. Expression of cldns in tight junction–null fibroblasts results in flattened, spread strand patches that are accessible for superresolution live-cell imaging. This is not the case with intact epithelial tight junctions, where cldn strands are closely packed and arranged vertically on the apicolateral cell membrane; this configuration is difficult to image even with superresolution techniques, which have lower z-axis than xy-axis resolution (Schermelleh et al., 2010). Fibroblast cell models to study cldns were used previously to investigate cldn–cldn interactions (Cording et al., 2013) and dynamic behavior (Sasaki et al., 2003). Our use of a similar Rat-1 cell expression system allowed us to address the apparent contradiction between the concept that ZO-1 acts as a rigid molecular scaffold, linking cllds to the actin cytoskeleton, and the FRAP findings that ZO-1, cldns, and actin have very different motilities, suggesting that interactions among these components are intermittent and highly dynamic.

As mentioned previously, the importance of ZO-1 and ZO-2 as cldn scaffolds was demonstrated in mouse mammary epithelial

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**FIGURE 8:** Cldn strands break and reanneal, especially at T-junctions. (A) Frequency of cldn strand breaks in arbitrarily defined 5-μm strand patch areas is similar among GFP Linker cldn2, GFP cldn2(−3), and GFP cldn2Y224E. (B) Strand network reorganization in stills from live-cell imaging; arrows point to breaks (Supplemental Figure 8BVideo5). (C) Small still-image series shows apparent translocation of a strand end along another strand (top; Supplemental Figure 8CVideo6) and a breaking and rapid reannealing (bottom; Supplemental Figure 8CVideo7). (D) Immunofluorescence imaging of cldn and F-tractin (Supplemental Figure 8CVideo8) does not suggest association of breaks with changes in underlying actin; arrows point to breaking and reannealing (bar, 5 μm). Time in seconds.
FIGURE 9: Pulse-chase-pulse experiments indicate new cldn addition to strands is focal and concentrates at sites of strand breaks. (A) Diagram of pulse-chase-pulse method. (B) Immunofluorescence images of SNAP-tagged cldn labeled with TMR only (left), TMR, block, and after 3, 5, and 8 h with SNAP-Cell 647 (middle three consecutive panels), and with SNAP-Cell 647 only (right; bar, 5 μm). (C) Higher-magnification images from 3-h pulse-chase-pulse experiment; arrows point to sites of addition of newly synthesized cldns. (D) Models for possible mechanisms of cldn addition to new strands.

cells, where ZO-1 knockout coupled with ZO-2 knockdown resulted in loss of cldn localization to cell contacts (Umeda et al., 2006). However, cldns lacking the PDZ binding motif localize appropriately to intact junctions (Ruffer and Gerke, 2004), suggesting that targeting is not solely dependent on PDZ interactions. In addition, cldns lacking not only the PDZ binding motif but almost the entire C-terminal intracellular domain can still form strand patches in L-cell fibroblasts (Furuse et al., 1999), so that strand organization does not depend on scaffold interactions. In the present study, we find strand patches made from cldns containing or lacking the PDZ binding motif form indistinguishable strand patches but that binding to ZO-1 (and ZO-2) has profound effects on strand movements. The finding that ZO-1 constrains the mobility of strands within patches is a direct demonstration that ZO-1 does not just localize to but also stabilizes tight junction strands. In addition, we found that mutation of cldn2 tyrosine 224, which contributes to PDZ-dependent ZO-1 binding (Nomme et al., 2015), both decreases affinity for ZO-1 and fails to restrain cldn strand mobility. This tyrosine is shared by 9 of 23 identified human cldns, and phosphorylation of cldn2 tyrosine 224 was previously identified in mass spectrometry analysis of cldn2 phosphopeptides (Van Itallie et al., 2012). Thus this finding suggests a potential tyrosine kinase regulatory mechanism for altering the stability of the strands.

Although it has long been speculated that ZO-1 forms a link between cldns and the actin cytoskeleton, the mutual dependence among these proteins is not well defined. For example, Kojima et al. (1999) showed that disruption of the actin cytoskeleton in primary cultured hepatocytes with an actin-depolymerizing agent resulted in displacement of occludin and ZO-1, but the tight junction strand organization was unaffected. Similarly, Shen and Turner (2005) demonstrated that actin depolymerization with latrunculin A resulted in changes first in occludin and then in cldn1 and finally ZO-1 localization; disruption of each of these proteins had distinct kinetics. The same group later showed that inhibition of myosin light chain kinase stabilizes junctional ZO-1 without affecting cldn1 or actin dynamics (Yu et al., 2010). These results suggest a complex relationship between junctional actin and the transmembrane proteins. However, in our reductionist fibroblast expression system, we found that cldn strand patches orient along actin strands only when they can interact with ZO-1. This is the first direct demonstration of the ability of ZO-1 to act as a scaffold linking cldns and actin.

Superresolution colocalization of ZO-1 and actin shows a complete overlay of the two proteins, whereas the continuous GFP cldn strands elaborate out from these structures. This implies that the cldn strands are only intermittently associated with ZO-1 and actin. Formation of intermittent interactions not only could stabilize the paracellular seal, but it might also allow for flexibility in coupling between the cldns, which must stay continuously coupled between cells to maintain the barrier, and the actin cytoskeleton, which must remodel and deform as cells move against each other in a monolayer. We speculate that limited coupling would also allow maintenance of paracellular barrier function in epithelial cells when the circumferential myosin and actin, and likely the associated scaffolding proteins, reorient, contract, or expand in response to a variety of physiological and pathological stimuli (Madara et al., 1986; Berglund et al., 2001; Ivanov et al., 2010).

The FRAP results in this study are consistent with more dynamic coupling between cldn2 and ZO-1 than between cldn2 and ocln. Unlike ZO-1, ocln was recruited to the cldn2 strands, and this interaction reduced the dynamic FRAP behavior of ocln, similar to what was reported previously (Furuse et al., 1998; Cording et al., 2013). We found that cldn strand networks show continuous rearrangement, breaking, and rejoining, as demonstrated previously (Sasaki et al., 2003), and, unexpectedly, this dynamic behavior is independent of...
interaction with ZO-1 or actin. Breaks and rejoining happen most often at strand junctions, the sites at which cldn also concentrates. Pulse-chase-pulse experiments demonstrated that these strand junctions are also preferred sites for addition of newly synthesized cldns, suggesting that the structure of the cldn strand at these sites may be less well organized and more dynamic than that in midstrand. It is possible as well that there are unidentified, associated proteins that might promote cldn polymerization and depolymerization; this will be investigated in future studies.

How do these findings translate into tight junction behavior in epithelial cells? Fibroblasts lack many critical tight junction proteins (e.g., tricellulin and JAMs), and even when stably expressing cldns, they do not form the linear, continuous belt-like junctions observed in epithelial cells. However, the similarity between our FRAP results and those described in well-polarized epithelial cells (Shen et al., 2008) suggests that the nature of the interactions between the tight junction proteins is shared in the two systems. The independent mobilities of cldns, ZO-1, and actin are consistent with a model for their intermittent interaction. Further, we expect network breaking and reforming to be a feature of normal tight junctions because their intermittent interaction. Further, we expect network breaking and reforming to be a feature of normal tight junctions because broken strands and complex meshes are features not only of our cldn strand patches, but also of epithelial junctions visualized in FFEM. The breaking and reforming of the cldn network has been suggested as contributing to epithelial paracellular flux of large compounds (reviewed in Anderson and Van Itallie, 2009). It also seems likely that, as we observe in fibroblasts, the strand junction points in epithelia could be sites for new cldn addition.

Given the difficulty in imaging epithelial tight junctions at the resolution possible in fibroblasts, although the present observations made in fibroblasts might not fully capture epithelial tight junction behavior, they provide important insights into the interactions among cldns, ZO-1, actin, and ocn, with implications for epithelial barrier structure, function, and regulation.

MATERIALS AND METHODS

Cell culture and transfections

Rat 1 Tet-off (R1-R12) cells were obtained from the American Type Culture Collection and maintained at 37°C and 5% CO2 in high-glucose DMEM (4.5 g/l), supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin (pen-strep). Stable cell lines were made by cotransfecting tagged constructs with pSVZeo (Thermo Fisher) using Lipofectamine 2000 according to the manufacturer’s directions; cells were selected with 0.8 mg/ml Zeocin (Invivogen) for 14 d, and clonal lines were isolated and analyzed by immunoblot and immunofluorescence. Stable knockout cell lines have been previously described. Halo occludin was made by subcloning full-length occludin into pHalo C1 (kindly provided by John Hammer, National Institutes of Health, Bethesda, MD) into the Nhel/Sall sites. GFP cldn1 and cldn11 were made by direct cloning from previously described pTRE constructs (Van Itallie et al., 2003) into the EcoRi site of the EGFP N1 vector (Takara). TdTomato F-actin was kindly provided by Robert Fischer (National Institutes of Health; York et al., 2012).

Stable ZO-1, ZO-2, and double-knockout clones were made using the CRISPR/Cas9 system (Ran et al., 2013); two separate vectors targeting different exons were designed for both ZO-1 and ZO-2. Oligonucleotides were phosphorylated, annealed, and cloned into the Bbai site of pSpCas9(BB)-2A-Puro vector according to the Zhang laboratory protocols (MIT).

All constructs were verified by sequencing.

Antibodies

Mouse anti–ZO-1 (33-9100) and rabbit ZO-2 (38-9100) antibodies were from GE Healthcare, mouse Myc-Tag (9B11) antibody was from Cell Signaling Technology, rabbit anti-GFP (Ab290) and rabbit anti–N-cadherin (Ab76057) antibodies were from Abcam, and rabbit anti–β-catenin (C2206) antibody was from Sigma-Aldrich. Antibodies were validated by recognizing bands of the predicted size on immunoblots and by cellular immunolocalization where previously reported. Species-specific secondary antibodies for immunofluorescence (Cy2, Cy3, and Cy5 conjugated) and immunoblots (IR-labeled antibodies) were from Jackson Immunoresearch. Rhodamine phalloidin and Alexa Fluor 647 phalloidin were from GE Healthcare. Halo ocn was labeled using Janelia Fluor 646 (Grimm et al., 2015) at 1:200 in cell culture media plus serum, followed by four 10-min washes to allow free dye to diffuse out. Cells were otherwise fixed and stained normally.

Immunofluorescence microscopy

Rat 1 cells were cultured on uncoated glass coverslips, fixed with 1 or 4% paraformaldehyde in CSK buffer (10 mM 1,4-piperazinediethanesulfonic acid, pH 6.8, 100 mM KCl, 300 mM sucrose, 2 mM MgCl2, and 2 mM ethylene glycol tetraacetic acid) at room temperature for 20 min, permeabilized with 0.2% Triton X-100 for 10 min, quenched with 50 mM NH4Cl, and incubated in 2% normal goat serum in phosphate-buffered saline (PBS) for 60 min and in primary antibodies for 60 min. After washing, samples were incubated with fluorescent-labeled secondary antibodies; in some cases, rhodamine or Alexa Fluor 647–phalloidin was added with the secondary antibodies. In some cases, cells were fixed with 100% cold ethanol, washed twice with Dulbecco’s PBS, and blocked and incubated in primary and secondary antibodies as described. After washing,

DNA constructs

Sequences for all oligonucleotide primers used for cloning are shown in Supplemental Table S1. GFP cldn2 was constructed by cloning mouse cldn2 (Cologio et al., 2003) into enhanced GFP (EGFP) C3 vector (Takara) using EcoRi/Sall. GFP cldn2–3 was made from the GFP cldn2 using site-directed mutagenesis (Agilent Technologies) to introduce a stop after amino acid 227. GFP linker cldn2 was generated by site-directed mutagenesis (Agilent Technologies). GFP Linker cldn2 Y224E was made from this plasmid also using site-directed mutagenesis (Agilent Technologies). mCherry cldn2 was generated by exciting EGFP from GFP cldn2 and replacing it with mCherry (Takara). SNAP-tagged cldn2 was made by infusion cloning after PCR of cldn2 into the BamH1/Xhol sites in pSNAPf (New England Biolabs). GFP ZO-1 (Fanning et al., 2012), myc-tagged ZNA (Rodgers et al., 2013), and GFP occludin (Van Itallie et al., 2015) have been previously described. Halo occludin was made by subcloning full-length occludin into pHalo C1 (kindly provided by John Hammer, National Institutes of Health, Bethesda, MD) into the Nhel/Sall sites. GFP cldn1 and cldn11 were made by direct cloning from previously described pTRE constructs (Van Itallie et al., 2003) into the EcoRi site of the EGFP N1 vector (Takara). TdTomato F-actin was kindly provided by Robert Fischer (National Institutes of Health; York et al., 2012).

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All constructs were verified by sequencing.

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Immunofluorescence microscopy

Rat-1 cells were cultured on uncoated glass coverslips, fixed with 1 or 4% paraformaldehyde in CSK buffer (10 mM 1,4-piperazinediethanesulfonic acid, pH 6.8, 100 mM KCl, 300 mM sucrose, 2 mM MgCl2, and 2 mM ethylene glycol tetraacetic acid) at room temperature for 20 min, permeabilized with 0.2% Triton X-100 for 10 min, quenched with 50 mM NH4Cl, and incubated in 2% normal goat serum in phosphate-buffered saline (PBS) for 60 min and in primary antibodies for 60 min. After washing, samples were incubated with fluorescent-labeled secondary antibodies; in some cases, rhodamine or Alexa Fluor 647–phalloidin was added with the secondary antibodies. In some cases, cells were fixed with 100% cold ethanol, washed twice with Dulbecco’s PBS, and blocked and incubated in primary and secondary antibodies as described. After washing,
samples were mounted with Mowiol containing 1% n-propyl gallate. Fixed samples were imaged on a Zeiss 710 2P Confocal microscope, using either 20×/numerical aperture (NA) 0.8 air or 63×/NA 1.4 oil objectives with 488-, 561-, and 633-nm laser lines of a Zeiss LSM 880 Airyscan in superresolution mode with a 63×/NA 1.4 objective. Raw data were processed using Airyscan processing with “auto strength” (mean ± SD = 5.5 ± 1.3) with Zen Black software, version 2.3

Superresolution images were taken using a GE OMX Blaze V4 Ultrafast Structured Illumination Microscope equipped with four scientific complementary metal-oxide semiconductor cameras using a 60×/1.42 NA lens using 488-, 561-, and 647-nm laser lines. Images were acquired using DeltaVision OMX software. For live-cell imaging, normal medium was supplemented with 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.4. Images were taken every 10–15 s for 10 min. Movies are maximum intensity projections of 0.8- to 1.2-μm stacks through the claudin strand patches. FRAP was performed on a Zeiss 780 confocal microscope using a 63×/NA 1.4 oil objective and heated stage in 5% CO₂. Bleaching of GFP signal was done with the 405-nm laser line (100% power for <10 s) in a defined region of interest. Subsequent images were taken at the same focal plane to monitor recovery of fluorescence; images were analyzed using ImageJ (National Institutes of Health, Bethesda, MD). Intensity measurements from flanking unbleached membrane regions were used as controls for background intensity changes with time. Subsequent analyses of recovery kinetics were performed using GraphPad Prism.

Contrast and colors were adjusted and figures made using Photoshop CS5.

Pull-down assays and immunoblotting
To test interactions between ZO-1 and cldn2 constructs, HEK293 tet-off cells were transfected with inducible myc-tagged ZO-1 N-terminal constructs (amino acids 1–887; ZNA) containing all three PDZ domains (Rodgers et al., 2013). HEK cells were also transfected with GFP alone, GFP cldn2, GFP cldn2(–3), or GFP Linker cldn2. Cells expressing myc-tagged and GFP-labeled constructs were lysed in 1% Triton X-100, 20 mM Tris, pH 7.5, 150 mM NaCl, and 1 mM EDTA with protease inhibitors. Insoluble material pelleted at 12,000 × g for 20 min, and supernatants were bound to GFP-trap beads (Chromotek) for 60 min at 4°C. Supernatant (precleared sample) from cells expressing myc-tagged ZNA was saved. GFP beads were washed and then incubated overnight at 4°C with precleared cell lysate from ZO-1–expressing cells as indicated. Beads were washed four times as before, and proteins were eluted with SDS sample buffer and subjected to SDS-gel electrophoresis, transfer, and immunoblot with GFP and myc antibodies.

Pulse-chase-pulse experiments
Rat-1 cells stably expressing SNAP-tagged cldn2 cultured on glass coverslips were incubated with 3 μM SNAP-Cell TMR-star (all SNAP-cell reagents from New England Biolabs) for 30 min in medium and rinsed three times, and then SNAP-Cell block (10 μM) was added for 30 min. Cells were again washed three times and incubated for various periods of time (2–8 h) before addition of SNAP-Cell 647 SIR and incubation for 30 min. Cells were again washed and incubated for an additional 30 min before fixation with cold ethanol, rinsed, and mounted with Mowiol. In some experiments, SNAP-Cell fluoros were reversed.

Statistical analyses
All comparisons with three or more conditions were compared by one-way analysis of variance, followed by Tukey’s or Dunnett’s tests. Comparison of two conditions was performed using Student’s t-test. p < 0.05 was set as the level for significant difference between groups. Statistics was performed using GraphPad Prism 6.

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