Microtubules are required for efficient epithelial tight junction homeostasis and restoration

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Microtubules are required for efficient epithelial tight junction homeostasis and restoration. Am J Physiol Cell Physiol 307: C245–C254, 2014. First published June 11, 2014; doi:10.1152/ajpcell.00336.2013.—Epithelial tight junctions are critical for creating a barrier yet allowing paracellular transport. Although it is well established that the actin cytoskeleton is critical for preserving the dynamic organization of the tight junction and maintaining normal tight junction protein recycling, contributions of microtubules to tight junction organization and function remain undefined. The aim of this study is to determine the role of microtubules in tight junction homeostasis and restoration. Our data demonstrate that occludin traffics on microtubules and that microtubule disruption perturbs tight junction structure and function. Microtubules are also shown to be required for restoring barrier function following Ca²⁺ chelation and reppletion. These processes are mediated by proteins participating in microtubule minus-end-directed trafficking but not plus-end-directed trafficking. These studies show that microtubules participate in the preservation of epithelial tight junction structure and function and play a vital role in tight junction restoration, thus expanding our understanding of the regulation of tight junction physiology.

microtubules; tight junctions; dynein; kinesin

EPITHELIAL TIGHT JUNCTIONS (TJs) form barriers to limit free exchange of water and solutes across the paracellular space in a charge- and size-selective manner. It is well established that TJs are highly dynamic structures, maintained by vesicular trafficking and protein diffusion within the TJ, diffusion between the TJ and basolateral membrane, and exchange between the cytosol and the TJ (40, 54). These findings suggest that TJ structure is closely regulated in the resting state to prevent TJ dysfunction. Interruption of TJ homeostasis could impede ongoing TJ preservation and restoration, thus prolonging barrier dysfunction in disease states.

The actin cytoskeleton is actively involved in maintaining TJs. Actin depolymerization causes TJ disruption through caveolar-dependent endocytosis of the transmembrane TJ protein occludin (53). Our understanding of how other cytoskeletal elements, such as microtubules, contribute to TJ organization remains rudimentary. Prior publications suggest that microtubules may be involved in TJ regulation. Nocodazole-mediated microtubule disruption prevents apical junction complex disassembly by calcium chelation, and pharmacological inhibition of the plus-end-directed microtubule motor kinesin impedes disassembly, supporting a role for microtubules in apical junction regulation (28). Particle-tracking analysis of intracellular enhanced green fluorescent protein (EGFP)-occludin fusion protein revealed that nocodazole-induced microtubule disruption significantly reduced the movement of occludin-containing vesicles (56). However, neither the role of microtubules in the maintenance of TJs under steady-state conditions nor the role in the recovery of TJ structure and function following injury has been addressed.

In epithelial cells, microtubules are oriented apicobasally, with their minus ends directed toward the apical side of the cells; however, a new population of microtubules was recently described oriented in the apical plane (71). This population was found to associate end on with the TJ complex, an interaction directly mediated by cingulin and dependent on AMP-activated protein kinase. Knockdown of cingulin produced 3D epithelial cell cultures with distorted morphology compared with the spheroids of the wild-type culture (71). These data suggest that microtubule-TJ interactions play an important role in the apical configuration of epithelial sheets. Such findings also suggest that microtubules may participate in other aspects of TJ function, including TJ restoration after insult.

Numerous disease states are characterized by TJ barrier disruption including colitis, asthma, and hypomagnesemic hypercalciuria with nephrocalcinosis (19, 23, 42, 47, 59, 66, 67). Determining the cellular processes involved in TJ restoration could lead to the development of recovery-enhancing therapeutics, thus abbreviating disease courses. The hypothesis of this study is that microtubules are essential for the complete and efficient recovery of TJ structure and function. The aim of this study is to determine whether microtubules are required for TJ maintenance and reconstruction. Herein, we provide data that support a crucial role for microtubules, not only in the maintenance, but also in the restoration of TJ structure and function. Moreover, we provide compelling evidence that dynein-dependent, minus-end-directed trafficking is required for this process.

MATERIALS AND METHODS

Reagents and plasmids. GFP-tubulin was the generous gift of Dr. Vladimir Gelfand (Northwestern University). Nocodazole (Sigma, St. Louis, MO) was used at a concentration of 5 µg/ml in electrophysiology studies. Erithro-9-(2-Hydroxy-3-nonyl)adenine hydrochloride (EHNA; Torcis Bioscience, Minneapolis, MN) and adenylyl-imidodiphosphate (Roche, Indianapolis, IN) were used at concentrations of 500 µM (28). Dynemin/dynactin, kinesin heavy chain, and scrambled siRNA constructs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell culture. Caco-2 cells (ATCC, Manassas, VA) were maintained and propagated according to ATCC guidelines. Briefly, cells were passaged every 4 days in Dulbecco’s Modified Eagle’s Media (Invitrogen, Grand Island, NY) supplemented with 15 mM NaHCO₃ and...
20% fetal bovine serum. Caco-2BBE cells were maintained similarly. Stably transfected Madin-Darby canine kidney (MDCK) cells [monomeric red fluorescent protein (mRFP1)-occludin] were the generous gift of Dr. Jerrold Turner (University of Chicago). MDCK cells were passaged every 4 days in Dulbecco’s Modified Eagle’s Media (Invitrogen) supplemented with 15 mM NaHCO₃, 15 mM HEPES and 10% fetal bovine serum. All cell lines were cultured in 5% CO₂ at 37°C.

**Live-cell microscopy and analysis.** mRFP1-occludin-expressing MDCK transfectants were plated on 35-mm glass-bottom microwell dishes (MatTek, Ashland, MA). At 50–70% confluence, cells were transfected with GFP-tubulin-expressing plasmid using Lipo-Genion Instruments, Sarasota, FL). The baseline TER of Caco-2 monolayers ranged from 2,000 –5,000 ·cm²·m⁻¹ using Transwell permeable supports (Corning, Tewksbury, MA) and trans-well recovery media.

**Calcium switch assay.** Caco-2 or MDCK cells grown on Transwell permeable supports were incubated in 1 mM EDTA (Sigma) in calcium-free, magnesium-free PBS, pH 7.4, for 10–20 min at 37°C. TER was measured at baseline and followed after chelation. Once resistance had fallen by ~85–90%, monolayers were washed with warm cell culture medium, and medium was replaced with calcium-containing medium. TER was measured every hour. For monolayers that had been pretreated with nocodazole, 5’-adenylimidodiphosphate (AMP-PNP), or EHNA, these reagents were also added to the recovery media.

**RESULTS**

**Occludin traffic on microtubules.** Although previously published data suggest that intracellular occludin movement involves microtubules, the direct role of microtubules in occludin trafficking has not been definitively established (1, 28, 56). To determine whether microtubules participate in occludin transport, MDCK cells stably expressing mRFP1-occludin were transiently transfected with an EGFP-tubulin expression vector (53). Movement of mRFP1-occludin-containing vesicles relative to EGFP-tubulin-positive structures was captured by live-cell imaging (Fig. 1). These images show mRFP1-occludin-containing vesicles moving along EGFP-tubulin-positive microtubules (Fig. 1A and Supplementary Video S1; supplemental material for this article is available online at the American Journal of Physiology Cell Physiology website). Particle-tracking studies determined the average velocity of occludin-containing vesicles in control cells to be 1.59 ± 0.07 μm/s. To investigate the role of microtubules in occludin trafficking, we disassembled these structures using cold exposure (4°C) and adding nocodazole to the culture media before returning the temperature to 37°C to prevent microtubule reformation (3, 7, 63). After we warmed the cells to 37°C to permit protein trafficking, we reduced the average velocity of occludin in the absence of microtubules to 0.66 ± 0.13 μm/s. The average total distance traveled over 15 s in control cells was 24.3 ± 1.08 μm (Fig. 1, B and E) and decreased to 13.6 ± 1.67 μm total over 15 s in the absence of microtubules (Fig. 1C).

**Occludin movement**, however, did not always progress in one direction but rather traveled back and forth along microtubules. In the absence of microtubules, occludin-containing vesicles moved back and forth in a very limited space, which could not be reflected in the analysis of total distance traveled. Therefore, it was important to compare instead net distance traveled. Interestingly, the net displacement of occludin, distance moved in one direction only over 15 s, was dramatically reduced in the absence of microtubules from 12.0 ± 1.27 μm to 2.72 ± 0.79 μm (Fig. 1D). These studies demonstrate that intracellular occludin-containing vesicles move along microtubules and that the rate of movement depends on intact microtubule networks.
Microtubules are required for maintenance of TJ structure and function. Having definitively demonstrated that microtubules participate in occludin trafficking, we sought to determine their role in maintaining TJ structure in the steady state. TJ-associated occludin, but not zonula occludens (ZO)-1 or claudin-1, correlates with intact barrier function, so occludin was selected as a representative TJ protein (53). Nocodazole treatment of Caco-2 cells induced progressive disruption of microtubules over 4 h (Fig. 2A), which correlated with intracellular accumulation of occludin-containing vesicles in a dose- and time-dependent fashion (Fig. 2B).

Because microtubule disruption perturbs TJ structure, we examined the impact of microtubule disruption on TJ function. TER measurements of nocodazole-treated Caco-2 monolayers over 4 h (Fig. 2A), which correlated with intracellular accumulation of occludin-containing vesicles in a dose- and time-dependent fashion (Fig. 2B).

Microtubule disruption inhibits TJ recovery after calcium switch. The above data support a role for microtubules in TJ maintenance; therefore, we questioned whether microtubules are also involved in TJ restoration (Fig. 3). To this end, we employed the calcium switch assay, a well-established approach for studying TJ assembly (9, 29, 41, 48, 55). Chelation of extracellular calcium drives the internalization of TJ proteins, and barrier function is lost (10). Normalization of extracellular calcium concentration allows for the rapid recovery of TJ structure and function.

Caco-2 monolayers were treated with 5 μg/ml nocodazole for 1 h to disrupt microtubules but preserve TER; calcium switch assays were then performed. TER dropped ~90% from baseline readings within 10 min of calcium chelation as expected, at which point calcium was restored. TER was then
measured in control and nocodazole-treated monolayers. TER recovery was significantly reduced in monolayers lacking microtubules achieving only 40.8 ± 5% of baseline values, while the TER of control monolayers recovered to near baseline levels. Although low-dose nocodazole had little effect on TJ structure or function for short time periods, prolonged exposure to this drug may exert deleterious effects. To circumvent this possibility, we employed the well-established method of exposure to 4°C to disassemble microtubules in a synchronized fashion (3, 7, 63). Caco-2 cells were incubated at 4°C for 1 h, at which time, no intact microtubules were identified. Nocodazole was added to one group of monolayers before it was returned to 37°C to maintain microtubule disruption. Microtubules do not reform at 37°C in the presence of nocodazole (Fig. 4A). In the absence of nocodazole, microtubules began to reform as early as 10 min after return to 37°C and recovered completely by 1 h. In the absence of microtubules, occludin progressively accumulated within the cytosol over 5 h. These data suggest that TJ proteins utilize microtubules to maintain localization at the plasma membrane.

The role of microtubules in TJ reassembly was tested using the calcium switch assay (Fig. 4C). Caco-2BBE cells grown on Transwells were brought to 4°C to disrupt microtubules, and nocodazole was added to selected monolayers to block microtubule reformation (Fig. 4B). All monolayers were rewarmed to 37°C for 1 h, and calcium-switch assays were performed. Control monolayers recovered to 80.6 ± 5.29% of baseline TER values by 4 h, whereas those harboring disrupted microtubules recovered to only 46.6 ± 5.01% of baseline values, similar to the level of recovery seen with nocodazole alone.

Efficient TJ restoration requires dynein but not kinesin function. Cargo transport along microtubules requires the motor proteins kinesin and dynein (22, 44). Kinesin is a plus-end-directed motor, and dynein is a minus-end-directed motor (52, 68). In polarized epithelial cells, microtubules are oriented with...
their minus ends toward the apical plasma membrane (4, 6, 39). Dynein therefore transports cargo in the apical direction and kinesin in the basal direction.

To determine whether dynein-mediated trafficking was required for TJ maintenance, Caco-2 cells were treated with the dynein inhibitor EHNA and stained for tubulin or occludin (11, 32). EHNA-treated monolayers displayed intact microtubule networks over 6 h (Fig. 5A). Despite intact microtubules, EHNA treatment induced progressive occludin accumulation in the cytosol compared with controls (Fig. 5B), suggesting that minus-end-directed trafficking is required to maintain TJ structure in the steady state.

The role of dynein in TJ restoration was tested using the calcium-switch assay. Caco-2 monolayers were treated with the dynein inhibitor EHNA for 1 h before chelation, which did not significantly affect TER. Following Ca\(^{2+}\) repletion, control monolayers recovered TER to 76.8 ± 3.29% of baseline. Dynein-inhibited monolayers, however, only reached 47.9 ± 3.94% of baseline TER by this time (Fig. 5C), suggesting that minus-end-directed trafficking is crucial for efficient TJ restoration.

To further address the specific role of minus-end motor molecules on TJ maintenance and recovery, dynein heavy chain and dynactin were knocked down using siRNA. Because inhibiting minus-end-directed trafficking disperses the Golgi apparatus, the number of cells exhibiting Golgi dispersal can be used to assess the effectiveness of dynein/dynactin siRNA knockdown, a standard measurement of this response (37, 45). Scrambled siRNA had no impact on Golgi morphology, whereas dynein/dynactin siRNA transfection induced Golgi dispersal in 50–60% of cells (Fig. 5D). Furthermore, dynein/dynactin knockdown also induced significant accumulation of occludin in the cytosol, indicating that minus-end motor proteins are required for maintaining TJ structure (Fig. 5E).

The role of dynein/dynactin in TJ recovery was assessed using the calcium-switch assay. Initial TER of cells transfected with scrambled siRNA was 1,670 ± 279 Ω·cm\(^2\); those transfected with dynein and dynactin siRNA had a baseline of 2,300 ± 267 Ω·cm\(^2\) (P > 0.05). MDCK monolayers treated with scrambled siRNA recovered to 97.2 ± 3.46% of baseline TER by 4 h following calcium replacement, whereas monolayers treated with dynein/dynactin siRNA recovered to only 71.5 ± 5.62% of baseline TER values at this time (Fig. 5F). Therefore, data with both pharmacological inhibition and siRNA knockdown of dynein/dynactin suggest that minus-end-directed trafficking is required for normal occludin localization and TJ restoration. Staining of MDCK monolayers for both occludin and dynein revealed significant colocalization of the two proteins, further supporting a role for dynein in occludin trafficking (Fig. 6).

In addition to lateral microtubule networks, other networks in polarized epithelia have a random orientation. Plus-end-directed trafficking along these networks may contribute to TJ recovery. The role of plus-end-directed trafficking in TJ restoration was investigated using the inhibitor AMP-PNP (28, 33, 58). Incubation with 500 μM AMP-PNP had no effect on microtubule structure compared with controls (Fig. 7A), nor did it impact TJ reassembly after calcium switch (Fig. 7B).

To more directly address the role of plus-end-directed trafficking in TJ maintenance and recovery, MDCK cells were transfected with either kinesin heavy chain or scrambled siRNA. Significant knockdown of kinesin, demonstrated by Western blot of kinesin heavy chain, had no impact on occludin localization, suggesting that plus-end-directed trafficking is not required for maintaining TJ structure (Fig. 7C). Similarly, there was no significant difference in the recovery of TER from calcium switch in MDCK monolayers treated with scrambled
siRNA vs. kinesin heavy chain siRNA (Fig. 7D). These findings demonstrate that plus-end-directed trafficking does not contribute to TJ restoration.

DISCUSSION

Investigations regarding the role of microtubules in the regulation of TJs are limited. The present study supports a vital role for microtubules in maintaining TJ structure and function and in the restoration of TJs following injury. To our knowledge, this is the first study exploring the role of microtubules in TJ reconstruction. We further show that dynein-dependent trafficking is essential for this process.

A previous study examined the impact of nocodazole on the movement of GFP-tagged occludin (56). Other work has demonstrated a new population of microtubules associated with the TJ in an end-on fashion, providing direct trafficking pathways to the apical membrane (71). Occludin movement was impeded in cells with disrupted microtubules, implying that these structures are required for trafficking. Our live cell imaging data demonstrate that occludin-containing vesicles use microtubules as “molecular highways.” Microtubule disruption significantly affected the total distance traveled by occludin, reducing it by 44%, supporting the previous finding that, in the absence of microtubules, occludin movement is severely limited. Total distance measures movement forward and backward, but trafficking involves the targeted movement of cargo in one direction. With nocodazole treatment, occludin movement was severely limited, traveling only short distances in both directions. The net displacement of occludin, distance traveled in one direction, captured the change in movement and was reduced by 77.3%. Together, these data suggest that microtubules play a vital role in occludin transport.

There is controversy regarding the role of microtubules in maintaining TJ structure. One publication reported that treatment of MDCK cells with nocodazole had no impact on TJ structure; however, only early time points, 10–90 min, were

**Fig. 5. TJ maintenance and efficient TJ restoration requires dynein. A: dynein inhibition using erythroc-9-(2-Hydroxy-3-nonyl)adenine hydrochloride (EHNA) has no effect on microtubule structure. Microtubules (red); nuclei (blue). B: in contrast, EHNA induces progressive occludin accumulation in the cytosol. C: EHNA treatment significantly impeded TJ recovery after calcium switch compared with untreated controls (n = 3 separate experiments, each condition in triplicate, *P < 0.001 chelated vs. EHNA and chelated, 1-way ANOVA/Bonferroni’s multiple-comparison test). D: Golgi-specific anti-giantin stain (green) of MDCK cells demonstrates successful knockdown of minus-end-directed trafficking using dynein/dynactin siRNA but not scrambled siRNA. E: siRNA knockdown of dynein/dynactin induces occludin (green) accumulation in the cytosol. F: initial TER measurements were taken at 0 h, and then calcium switch was immediately performed. siRNA knockdown of dynein/dynactin also significantly inhibits TJ recovery after calcium switch compared with scrambled siRNA controls, whereas scrambled siRNA had no effect (n = 3 separate experiments, each condition in triplicate, *P < 0.001 scrambled vs. dynein/dynactin, *P > 0.05 control vs. scrambled at 3–4 h, 1-way ANOVA/Bonferroni’s multiple-comparison test).**
examined (56). A separate study reported that prolonged perturbation of microtubules in polarized thyroid epithelium induced relocalization of ZO-1 and a significant drop in TER (72). One study reported no change in TER after an abbreviated nocodazole incubation, but an in vivo study demonstrated increased intestinal permeability in rat intestines after colchicine treatment (17, 38). Our results reconcile these controversial data by showing that the effect of microtubule disruption

Fig. 6. Dynein colocalizes with occludin-containing vesicles. Madin-Darby canine kidney cells were fixed and costained for dynein (red) and occludin (green). Insets: higher magnification and reveal significant colocalization (yellow).

Fig. 7. Kinesin is not required for TJ maintenance and restoration. A: incubation with the kinesin inhibitor 5'-adenylylimidodiphosphate (AMP-PNP) has no impact on microtubule structure (red). B: kinesin inhibition using 1-h pretreatment with AMP-PNP does not inhibit TJ recovery from calcium switch (n = 3 separate experiments, each condition in triplicate, P > 0.05 control vs. AMP-PNP at 2–4 h, P > 0.05 chelated vs. AMP-PNP and chelated at 2–4 h, 1-way ANOVA/Bonferroni’s multiple-comparison test). C: siRNA knockdown of kinesin heavy chain does not affect occludin (red) distribution despite substantial reduction in kinesin heavy chain (KHC) shown by Western Blot. D: initial TER measurements were taken at 0 h, and then calcium switch was immediately performed. siRNA knockdown of kinesin heavy chain does not significantly inhibit TJ recovery after calcium switch compared with scrambled siRNA controls (n = 3 separate experiments, each condition in triplicate, P > 0.05 scrambled vs. kinesin siRNA at 2–4 h, 1-way ANOVA/Bonferroni’s multiple-comparison test).
on TJs is time dependent, as incubation with nocodazole induced dose- and time-dependent changes in TJ structure and function (Fig. 2). It is also possible that alternative mechanisms, either actin based or as yet undetermined, compensate for the gradual loss of microtubules induced by lower concentrations of nocodazole at earlier time points. With higher concentrations, microtubule destruction is more rapid and functional compensation is impossible. The relationship between loss of TJ structure and function following microtubule disruption supports an essential role for microtubules in TJ homeostasis.

The role of protein recycling in TJ physiology is just beginning to be explored and may provide a framework for interpreting the time-dependent effect of microtubule disruption on TJs. Cell-surface biotinylation assays demonstrated that occludin is internalized continuously under homeostatic conditions with ~70% of internalized occludin being recycled back to the membrane within 5 min (40). A separate study imaged living cells and used fluorescence recovery after photobleaching to determine that occludin also diffuses laterally within the plasma membrane, whereas ZO-1 recycles from the cytosol (54). Claudin-1 also recycles (15, 16). We speculate that microtubules serve as conduits for the continuous recycling of occludin. With regard to the time-dependent effects of microtubule loss on TJs, in the absence of microtubules, intracellular pools of occludin may accumulate in the cytosol, unable to return to the membrane in an efficient manner. The extent of cytosolic occludin accumulation would depend on the recycling rate. At early time points following microtubule loss, the amount of occludin accumulated in the cytosol may be below detectable visual limits, thus explaining prior results. At later time points, we and others showed that microtubule loss resulted in significant TJ disruption and a corresponding loss of barrier function potentially induced by the inhibition of recycling (30, 72).

Although we have clarified an aspect of the relationship between occludin and microtubules, prior work suggests intriguing reciprocal relationships. In MDCK cells, occludin preferentially localized to the leading edge of migrating cells during wound healing (14). Knockdown of occludin using RNAi leads to derangement of the microtubule organizing center (MTOC), such that perpendicularly oriented networks, normally visualized during this process, were not observed. Occludin may recruit aPKC-Par3/PATJ, and the interaction may correctly orient the MTOC (14). Occludin has also been shown to associate with the centrosome during mitosis (49). Generation of an occludin mutant with a nonphosphorylatable alanine substitution prevents centrosome separation and significantly reduced cell proliferation (49). These data suggest that occludin may play an equally important role regulating microtubule networks as microtubules play in occludin dynamics at the TJ.

The cytoskeletal elements required for TJ protein recycling are currently not known; however, the movement of TJ proteins from the membrane to the cytosol has been shown to be microtubule dependent. In a previous study, the dissolution of microtubules prevented the movement of occludin and ZO-1 from the membrane to the cytosol in response to calcium chelation (28). The authors speculated that internalization required microtubule-based trafficking. The reverse process of TJ restoration, however, was not investigated. Nonetheless, taken together, several avenues of previously published data suggest an intriguing possibility. Escherichia coli cytotoxic necrotizing factor-1 induced internalization of occludin that was associated with Rab11-positive endosomes (25). Rab11 is involved in endosome recycling and utilizes microtubules for trafficking (8, 60, 64). These findings suggest that the recycling of internalized occludin to the membrane could hasten recovery of barrier function following insult.

In the homeostatic state, the mechanisms involved in occludin recycling are not well defined (40, 43, 54). The small GTPases Rab13, Rab11, and Rab8 have been identified as regulating occludin turnover (25, 40, 46, 60, 70). Also, occludin-containing endosomes have been associated with multiple markers including myosin II, caveolin, clathrin, and syntaxin-4 (29, 53, 65). However, these studies have focused on occludin endocytosis in response to stimuli like IFN-γ rather than on the homeostatic mechanisms that regulate occludin recycling.

Our study directly addressed the role of occludin recycling in TJ recovery using the well-described calcium-switch assay and monitoring TJ barrier recovery in the absence of microtubules. In the absence of intact microtubules, the recovery of barrier function is significantly inhibited (Figs. 3 and 4). We speculate that, without microtubules, occludin cannot recycle back to the membrane, thus impeding the structural and functional recovery of TJs. Our data support the concept of a process requiring microtubules that drives the constitutive reconstruction of TJs.

Although these data support a vital role for microtubules in TJ restoration, they also suggest the involvement of microtubule-independent mechanisms. Actin-based systems may account for the observed recovery of 40% of baseline TER, as actin is known to participate in maintaining TJ structure and function (53). Occludin is mobile without microtubules but travels significantly shorter distances (56). Simple diffusion of occludin-containing vesicles may contribute to TJ recovery in the absence of microtubules, but the observed plateau in TER recovery is not consistent with this possibility, as diffusion alone would result in a slow but continuous rate of recovery that could fully repair TJs. Microtubules, therefore, seem to play a critical role in TJ reconstruction that cannot be compensated for by other mechanisms.

Our data support a role for microtubules in TJ protein trafficking and restoration, implying that motor proteins are required for these processes. The impact of microtubule disruption on the velocity of occludin is interesting to view from the perspective of what is known about microtubule motors. Reductionist assays using purified dynein found the velocity of a single motor to be 800 nm/s (61). A single kinesin molecule has a velocity of 204 nm/s (58). Multiple motors of either type may pull a single cargo, generating speeds up to ten times greater than an individual motor (13, 20, 34, 36). Our velocity data, therefore, are consistent with published literature and suggest that occludin trafficking employs multiple microtubule-based motors.

There are limited data showing that kinesin is required for functional disassembly, but the role of microtubule motors in TJ reconstruction has not been explored (28). In polarized epithelial cells, microtubules are oriented longitudinally, with minus ends pointed apically and plus ends pointed basolaterally (4, 6, 39). Recent data has also identified a population of microtubules directly associated with the TJ structure in the apical plane (71). Kinesin depletion inhibits basolateral trans-
port, whereas microtubule disruption inhibits transport in both directions (35). Our TJ recovery data suggest that trafficking back to the membrane is mediated by dynein, further supported by the colocalization of occludin and dynein. We speculate that, without microtubules and hence without dynein-mediated trafficking, occludin cannot recycle to the membrane and thus remains in the cytosol, leading to prolonged barrier disruption. Our immunofluorescence data support this theory, as siRNA knockdown of dynein/dynactin induced occludin accumulation in the cytosol, whereas kinesin knockdown did not.

Other studies have demonstrated a link between dynein and Rab11 that supports our model of microtubule-dependent TJ reconstruction (26, 27). Rab11, which utilizes microtubules for trafficking and has been shown to participate in occludin trafficking, mediates its downstream effects via “family of Rab11-interacting proteins” (FIPs). FIP3 was identified as a primary recruiter of dynein to membranous vesicles, implying that a population of recycling proteins require microtubules and dynein for transport (26). Our data concur with these findings and strongly support a role for microtubules in TJ recycling and restoration. It is possible that, in the absence of microtubules, recycling occludin endosomes may be able to recruit dynein but are unable to reach the membrane and reconstruct the TJ.

TJ disruption plays a role in many disease states. In asthma, TJs of the tracheal epithelium are abnormal, potentially leading to inflammation after allergen exposure (2, 24, 69). Diabetes may result in compromised corneal TJ barrier function, leading to diabetic keratopathy (21, 31). Restoration of barrier function is associated with positive clinical outcomes. Inhibition of TNF improves intestinal epithelial barrier function in Crohn’s disease (5, 12, 51, 57). Severe allergic reactions in the eye may be ameliorated by restoring barrier function (18). Expanding our understanding of TJ restoration would lead to the development of barrier recovery-promoting strategies that would reduce morbidity associated with TJ disruption. Further investigation is needed to fully explore the potential clinical benefits.

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DISCLOSURES
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