Rho Signaling and Tight Junction Functions

Tight junctions are heteromeric protein complexes that act as signaling centers by mediating the bidirectional transmission of information between the environment and the cell interior to control paracellular permeability and differentiation. Insight into tight junction-associated signaling mechanisms is of fundamental importance for our understanding of the physiology of epithelia and endothelia in health and disease.

In multicellular organisms, epithelia consist of specialized cells that form selective barriers controlling the passage of water, ions, and other small molecules between different body compartments and the external environment. This requires cells to be polarized, that is, to form distinct membrane domains: an apical side, facing the lumen of a body cavity or the outside environment, and a basal side, adjacent to the underlying tissue. For instance, the epithelial cells lining the intestinal lumen are at the interface between the internal and external environments, regulating the absorption of nutrients and water, as well as ion homeostasis. In addition, these cells provide a protective barrier against commensal and pathogenic microorganisms. Epithelial cells have to adhere tightly to each other to form sheets and are connected to each other via a series of intercellular junctions, known collectively as the epithelial junctional complex (30, 49). This complex is subdivided into tight junctions (TJs), adherens junctions (AJs), and desmosomes. These structures consist of heteromeric protein complexes and are linked to different cytoskeletal elements. They mediate cell-cell adhesion to different extents and play crucial roles in the biogenesis and maintenance of epithelial barriers (FIGURE 1).

In epithelial cells, the distribution of TJs and AJs is generally spatially well defined: TJs are typically located at the apical end of the basolateral membrane, whereas AJs and desmosomes are located more within the basolateral membrane (31, 49). In contrast, the distribution of junctional complexes in endothelial cells is less defined, with TJs and AJs being more intertwined with each other (17, 135). TJs and AJs are both linked intracellularly to the actin cytoskeleton, which is often seen to co-localize with junctions to form a “belt like” ring of filaments encircling the perimeter of the cell, sometimes referred to as “perijunctional actin”. In contrast, desmosomes are linked to intermediate filaments. TJs at the resolution of transmission electron microscopy (TEM) appear as sites where there is close apposition of adjacent plasma membranes between neighboring cells (49). In freeze-fracture replicas, these close contact sites are seen as networks of intracellular strands that encircle the cells below the apical surface (35, 54, 58) (FIGURE 1).

TJs function as dynamic barriers, or gates, to selectively regulate the diffusion of water, ions, and other small molecules through the paracellular space between neighboring cells (121). They also help to maintain cell polarity by acting as a molecular fence, thus restricting the diffusion of apical and basolateral membrane components. Aside from these classical gate and fence functions, TJs have emerged recently to act as dynamic heteromeric signaling complexes. The signaling at TJs is bi-directional, such that signals are transmitted from the cell interior to forming or existing TJs to regulate its assembly and function, whereas TJs coordinate and modulate gene expression at TJs.

Multiple TJ components interact with the actin cytoskeleton. These interactions are known to be important to regulate the permeability properties of TJs and for processes that require junctional reorganization during assembly of junctions. Many studies have implicated both actinmonosin contractility and actin dynamics, such as filament polymerization and turnover, as being important mechanisms in the formation, maintenance, and regulation of the permeability properties of TJs (19, 27, 65, 87, 95, 106, 115, 118, 134, 145). TJs also recruit signaling proteins that regulate gene expression and, subsequently, proliferation and differentiation (13). In this review, we will first provide a brief overview of TJ composition since there are several excellent reviews for more comprehensive discussions regarding their molecular structure (2, 31, 54, 65, 107, 133). As important cytoskeletal regulators and modulators of gene expression, RhoGTPases have been identified as being major signaling components associated with TJs. We hence focus this review mainly on RhoGTPase signaling in the regulation of paracellular permeability and gene expression at TJs.

Molecular Composition of TJs

At the molecular level, TJs consist of multimeric protein complexes, having a similar architectural principle as other cell-cell adhesion complexes (11, 56, 65, 101). The TJ complex is composed of integral
membrane proteins linked to a network of adaptor proteins that form a cytoplasmic plaque. The transmembrane proteins are known to mediate cell-cell adhesion as well as to form the paracellular barrier. The adaptors of the cytoplasmic plaque act as linkers between transmembrane proteins and the cytoskeleton, and as scaffolds, to recruit different types of signaling proteins.

The transmembrane proteins of the TJ are often grouped according to the number of times they span the plasma membrane. There are single-pass membrane proteins such as JAMs (junction adhesion molecules) (7, 44, 88) and CAR (Coxsackievirus-adenovirus receptor) (36), three-pass membrane proteins such as BVES (blood vessel/epicardial substance) (105), and four-pass membrane domains such as the claudins (24 family members) (53, 54, 133), occludin (15, 130), MarvelD3 (122), and tricellulin, a protein enriched at cell-cell contact points between three adjacent cells (71). Although most of the transmembrane proteins seem to have at least some adhesive abilities, it is the tetraspan membrane proteins that seem to be more directly linked to epithelial barrier functions (54, 82, 121, 133).

The claudins are thought to be the major components of TJ strands and seem to underlie the different barrier properties seen by different types of epithelia.
due to the tissue-specific expression of different claudins (38, 79). Apart from being a structural component of the junctional barrier, claudins are responsible for the ion selectivity of the paracellular pathway, a function that is thought to involve the formation of ion-selective pores or channels (6, 82, 90, 133). However, the molecular structure of these paracellular pores is still unclear.

The role of occludin in barrier permeability is controversial because of the discrepancy between the occludin mouse knockout model and the many in vitro studies. Although the occludin knockout mouse model exhibited complex phenotypic defects in a range of epithelia, TJs themselves did not appear to be affected morphologically, and, as far as examined, the barrier function of the intestinal epithelium was normal (114, 117). However, data from cells in culture suggest an important role for occludin in the permeability properties of TJs (8, 15, 116). For example, overexpression studies of occludin in MDCK cells resulted in raised transepithelial electrical resistance (TER), a measure of the instantaneous ionic conductivity properties of an epithelial layer, but also in increased size-selective paracellular tracer permeability, a measure of slow diffusion across junctions (16, 90, 91). The observation that dominant negative occludin constructs inhibit paracellular tracer permeability suggests that occludin promotes permeability (8). The exact mechanisms behind these observations remain unclear, but these and similar observations suggest that ion conductance and tracer permeation are mediated by distinct molecular mechanisms that can be differentially regulated (121). When occludin was depleted by RNA interference in MDCK cells, paracellular permeability was not affected, but a failure to activate the RhoGTPase RhoA in response to certain stimuli was observed, suggesting that occludin may also have a role in signaling (147).

Many of the transmembrane proteins interact with proteins in the cytoplasmic plaque via PDZ (P/D/S/DlgA/ZO-1)-binding domains present in their cytosolic domains and PDZ domains found on components of the plaque such as ZO-1, ZO-2, and ZO-3 (18, 67, 134, 139); PAR-3 and PAR-6 (74); Pals1 (123).

**FIGURE 2.** RhoGTPases and regulation of paracellular permeability

Different signaling proteins have been implicated in the regulation of TJ paracellular permeability. Left: how TJ permeability may be regulated by RhoGTPase signaling. The transmembrane proteins (dark green) represent claudins, occludin, MarvelD3, and tricellulin since these components are known to regulate paracellular permeability. The cytoplasmic adaptors proteins (purple) represent proteins such as ZO-1, -2, and -3, which act as linkers to the actin cytoskeleton. Stimulation of Rho by GEF-H1, a TJ-associated guanine nucleotide exchange factor, results in activation of Rho specific effectors such as Rho kinases (ROCKs). ROCKs phosphorylate and inactivate MLCP (myosin light chain phosphatase) but phosphorylate and activate MLC2 (myosin light chain II), leading to activation of actinomyosin contractility and increased paracellular permeability. A Rho effector that contributes to actin filament nucleation is mDia. LIM kinase (LIMK) is another RhoA/ROCK signaling effector that phosphorylates and inactivates cofilin and leads to barrier enhancement. Rac and Cdc42 have also been linked to actin cytoskeleton, cell polarity, and paracellular permeability regulation. Possible Rac and Cdc42 effectors in actin rearrangement and paracellular permeability are PAKs and N-WASP. Alternatively, ROCK can directly phosphorylate TJ proteins such as occludin and claudin-5. Right: different PKC isoforms are also known to phosphorylate TJ proteins and MLCK, which is known to contribute to the regulation of TJ permeability.
and PATJ (119), MUPP1 (64) and the MAGI proteins (43). However there are several adaptor proteins that do not contain PDZ domains. Examples are JACOP/paracin- gulin (63, 104) and cingulin, a protein known to interact with junctional membrane proteins; ZO-1, -2, and -3; as well as f-actin, myosin II, and GEF-H1, a junction-associated activator of RhoGTPases (3, 41).

Scaffolding proteins also recruit many different types of signaling proteins to the TJ. These include various protein kinases such as different isoforms of protein kinase C (PKC); protein and lipid phosphatases; small monomeric G proteins such as Rho, Rab, and Ras GTPases and their regulators; as well as heterotrimeric GTPases (14, 31, 59, 80, 107, 108, 112). In addition, transcription factors are also recruited to junctions via scaffolding proteins, which is thought to be important for the regulation of gene expression (13).

Similarly to AJs, TJs are linked to the actin cytoskeleton (FIGURE 2). As discussed below, regulation of the actin cytoskeleton and its interactions with TJ components plays a major role in the regulation of epithelial barrier properties. Great efforts have hence been put into identifying f-actin binding junctional components. Most of these proteins are adaptor proteins (i.e., ZO-1/2/3, cingulin, and JACOP/paracin-gulin); however, an f-actin binding activity has also been identified in the COOH terminal cytoplasmic domain of occludin (2, 48, 65, 104, 140). Despite the availability of these biochemical data, the functional roles of specific interactions between junctional proteins and f-actin are not well understood. However, it is generally assumed that these interactions function as anchors that provide junctional stability as well as connectors that transmit force during junctional remodeling and cell shape changes.

Some junctional proteins interact not only with actin but also with other important actin-associated proteins and proteins that regulate actin dynamics, suggesting that they represent functional scaffolds that link signaling pathways to actin dynamics. One such example is cingulin, which, in addition to f-actin, also binds nonmuscle myosin II as well as Rho signaling components that regulate myosin activity (3, 41). However, the functional significance of such a cingulin-based module in the regulation of the actinomyosin cytoskeleton is not well understood. Another interesting example is Shroom2, an actin-binding protein that binds ZO-1 as well as myosin-VIIa, suggesting that junctional adaptors can also function as linkers to actin and unconventional myosins (47). Another Shroom family member, Shroom3, has been demonstrated to mediate junctional recruitment of Rho kinases, an upstream regulator of myosin II (102). Hence, actin-binding adaptor proteins can function as scaffolds that not only link junctions to the actin cytoskeleton but also actin-associated motors and upstream regulatory mechanisms.

Work performed by a large number of different laboratories has thus led to the identification of a large number of different TJ-associated components that seem to form actin-bound, multimeric protein complexes, interconnecting neighboring cells, that receive and send signals and thereby regulate epithelial barrier functions as well as epithelial proliferation and differentiation.

**RhoGTPases and Their Regulation in the Assembly and Function of TJs**

RhoGTPases are molecular switches that cycle between active (GTP-bound) and inactive (GDP-bound) states. When activated, the RhoGTPase undergoes a conformational change enabling the recruitment of effector proteins that mediate downstream effects. Cycling between active and inactive states is catalyzed by guanine nucleotide exchange factors (GEFs) and GTPase activating Proteins (GAPs). In mammals, the Rho family of GTPases contains ~20 members (46). The number of GEFs and GAPs largely outnumbers the number of Rho proteins since over 70 GEFs (55) and GAPs (21) are encoded by the human genome. It is thought that the large number of Rho regulators enables spatially and temporally restricted control of Rho signaling pathways.

RhoGTPases were first identified as being major regulators of the actin cytoskeleton (111). They are also important regulators of gene expression and contribute to mitogen-activated protein kinase cascades and inflammatory signaling (39, 77, 97, 110). A role for small GTPases in junction formation was first demonstrated using a non-hydrolysable GTP analog, GTPγS, which inhibited junction formation in calcium-switch experiments (10). The calcium-switch technique is a method to study de novo junction formation. It involves culturing cells in a medium that contains low calcium, preventing formation of cell-cell junctions; addition of calcium to the medium then triggers junction formation (32). The use of C3 transferase, which inactivates RhoA-C (referred to as Rho), but not Rac or Cdc42, was also shown to inhibit junction assembly as well as to affect the permeability properties of TJs; similar observations were made when mutant Rho GTPases were expressed in epithelial and endothelial cells (28, 75, 103, 127, 141). In rat brain endothelial cells, C3 transferase was reported to inhibit lymphocyte transmigration, indicating that Rho is required for junction dynamics (1). Overall, these studies suggest that activities of Rho family GTPases need to be finely balanced to obtain optimal junction integrity.

Despite the clear importance of RhoGTPases in the assembly and function of TJs, how their activities are controlled in a TJ-specific context is less clear. Heterotrimeric G proteins have been implicated in Rho activation as well as in the regulation of TJs and might hence represent a functional link (10, 42, 94). For example, when the prostaglandin EP3β receptor,
which is coupled to Rho activation via heterotrimeric G proteins, was transfected and activated in MDCK cells, an increase in TER as well as an increase in paracellular tracer mannitol was observed (66). This suggests that Rho activation by heterotrimeric G proteins could be one of the mechanisms that regulate TJ permeability. However, the molecular mechanisms mediating G protein-induced Rho activation at TJs is not known. Several Rho GEFs can interact with heterotrimeric G proteins, which results in their activation, but none of them is known to localize to TJs.

“Regulation of myosin activity seems to play a major role during junction assembly and disassembly, as well as for the regulation of paracellular permeability.”

Currently, only two TJ-associated GEFs for RhoGTPases have been identified and functionally linked to TJs. Tuba, an activator for Cdc42, has only recently been shown to be recruited to TJs in a ZO-1-dependent manner and to regulate junction assembly in a Ca-switch experiment (106). Despite affecting junctional configuration and Cdc42’s importance for polarization, tuba does not seem to play an important role in establishing polarized epithelial cells in standard cultures. Since ZO-1 depletion only affects epithelial morphogenesis in three-dimensional cultures (120), it would be important to test the function of tuba in more complex tissue culture systems.

The second TJ-associated Rho GEF is GEF-H1, an activator of RhoA (19). GEF-H1 regulates paracellular permeability in epithelial and endothelial cells, as well as disassembly of junctions in response to calcium removal (19, 25, 115). At TJs, GEF-H1 interacts with cingulin, a junctional adaptor, resulting in inhibition of its exchange factor activity (3). However, cingulin depletion does not induce increased permeability despite increased Rho activation (3, 62), suggesting that compensatory mechanisms might counteract prolonged RhoA activation. Moreover, GEF-H1 also interacts with JACOP/paracin-gulin, which also affects junctional recruitment of GEF-H1 (63), suggesting that cingulin and JACOP/paracin-gulin may have overlapping functions. Nevertheless, the GEF-H1/cingulin complex represents an important Rho signaling pathway in epithelial cells since it stimulates epithelial proliferation by regulating G1/S phase transition and gene expression (3, 100).

GEF-H1 is known to interact with various different cellular components in different cell types, which includes microtubules and various protein kinases (23, 24, 29, 51, 148). In the context of TJs and regulation of epithelial permeability, most of these interacting proteins have not been analyzed in great detail. Some of them, however, are likely to play important roles in epithelia if analyzed under appropriate conditions since they also associate with epithelial junctions. Examples include 14-3-3 proteins, which bind GEF-H1 in a PAK-1-regulated manner (148), and Par1/MARK kinases, which are known to regulate epithelial morphogenesis (37, 100). Moreover, ERK1/2 phosphorylates GEF-H1, a necessary event for the induction of cellular responses including proliferation and motility (51). GEF-H1 is also a target of TNF-α signaling in endothelial and epithelial cells, resulting in Rho signaling and stress fiber induction (92, 100), and inhibition of ERK1/2 prevents TNF-α-induced stimulation of paracellular permeability (76). GEF-H1 may thus represent a Rho signaling activator that receives input from different types of upstream pathways to regulate assembly and disassembly of the junction, as well as junction function and gene expression.

RhoGTPase Effector Pathways and Regulation of Paracellular Permeability

Multiple RhoGTPase effector pathways target the actinomyosin cytoskeleton, which is thought to play a central role in the regulation of paracellular permeability (19, 25, 28, 52, 89, 92, 136, 142). On one hand, interactions between TJ proteins (such as occludin, cingulin, and the ZO proteins) and actin filaments are thought to stabilize the junction and might also provide a means to transmit force to alter protein-protein interactions within TJs, resulting in increased paracellular permeability (FIGURE 2). However, there is currently no experimental proof that any of these actin-binding functions indeed plays a direct role in the regulation of permeability. Nevertheless, the COOH terminal domain of occludin, which is important for the regulation of paracellular permeability (16), interacts via multiple adaptor proteins with F-actin (2, 65, 109, 116); hence, functionally important actin-binding proteins might be difficult to identify because of functional redundancy. Nonmuscle myosin heavy chain II isoforms seem to be major players in Rho-regulated junctional regulatory mechanisms. Myosin IIA and IIB have been linked to cell-cell adhesion in mouse knockout models (40, 85). Downregulation of myosin-IIA, but not of IIB or IIC, expression in SK-CO15 colonic epithelial cells resulted in profound changes of cell morphology and cell-cell adhesion (72). Myosin II associates with the actin cytoskeleton underlying the apical junctional complex and recruitment has been proposed to be mediated by ZO-1 and ZO-2 (145). However, the data supporting the importance of ZO proteins for junctional recruitment of myosin are controversial. In some cell types, depletion of ZO-1 is sufficient to inhibit junctional recruitment of myosin II, and in others it favors junctional accumulation (106, 134). It is thus unlikely that ZO proteins play a direct role in myosin II recruitment.
Regulation of myosin activity seems to play a major role during junction assembly and disassembly, as well as for the regulation of paracellular permeability. ROCKs (Rho-associated kinases) are Ser/Thr kinases that are important Rho effectors that regulate TJ permeability (68, 136). ROCKs are known to stimulate the activation of myosin by regulating the phosphorylation state of the regulatory light chain (MLC2) (5). ROCKs also phosphorylate and inactivate myosin phosphatase target subunit (MYPT), leading to inactivation of the phosphatase and, hence, increased myosin activity (45).

Myosin phosphorylation is also mediated by myosin light chain kinase (MLCK), and pharmacological inhibition of MLCK prevents both MLC2 phosphorylation and regulation of TJ barrier function in response to physiological and pathophysiological stimuli, such as Na+-nutrient co-transport, bacterial infection, or proinflammatory cytokines (84, 131, 132, 149). Overexpression of MLCK in mature monolayers further supports a role of MLCK in the regulation of permeability and suggests that MLC2 phosphorylation alone is sufficient to induce TJ regulation in the absence of any upstream stimuli (118). However, it has been suggested that, at least in endothelial cells, the gradual increase in permeability induced by TNF-α does not reflect contractile mechanisms mediated by Rho, ROCK, and MLCK but involves long-term reorganization of TJ proteins (92).

Apart from the non-muscle heavy chain myosin II pathway, TJ permeability may be controlled by other Rho/ROCK effectors such as the mammalian Diaphanous-related formin (mDia), which promotes actin nucleation and filament polymerization and is known to represent an opposing Rho effector to ROCK in the regulation of AJ formation and actin dynamics (113). In endothelial cells, for example, angiopoietin-1 prevents VEGF-induced endothelial permeability by sequestering Src through mDia (57). ROCK may also regulate TJ permeability by regulating actin filament stability. Another known substrate for ROCK is LIM kinase; activated LIM kinase phosphorylates and inactivates cofilin, an actin-depolymerizing factor, and therefore acts to stabilize the junctional actin cytoskeleton and, hence, paracellular permeability (78). ROCK may also regulate TJ permeability by regulating actin filament stability. Another known substrate for ROCK is LIM kinase; activated LIM kinase phosphorylates and inactivates cofilin, an actin-depolymerizing factor, and therefore acts to stabilize the junctional actin cytoskeleton and, hence, paracellular permeability (78).

The Par3/6-aPKC (atypical protein kinase C) polarity complex is known to cross-talk with the RhoGTPases Cdc42 and Rac during junction assembly and epithelial polarization (83, 86, 93, 126). PAR-6-PAR-3 mediates Cdc42-induced Rac activation through the Rac GEF Tiam1 (34, 93). Cdc42 and Rac, similar to Rho, are key regulators of cytoskeletal dynamics and have been linked to the regulation of paracellular permeability. However, the reported effects depend on the cell types analyzed and experimental approaches used to manipulate the activity of the GTPases (26, 28, 65, 81, 138, 141, 143). The Cdc42 or Rac effectors involved in the regulation of paracellular permeability are not well characterized. Nevertheless, there are interesting possible mechanisms that need further characterization. N-WASP (neuronal Wiskott-Aldrich syndrome protein), a Cdc42 effector, has been localized to AJ and TJs; and the assembly of these junctions was prevented by the N-WASP inhibitor wiskostatin (73). Additionally, depletion of N-WASP controls the shaping of cell junctions through the local activation of Cdc42 (106). Active PAK, an effector of cdc42 and Rac, can increase MLC phosphorylation, whereas dominant negative PAK has little effect, despite the increase in actin stress fibers (78). PAKs may also modify permeability by regulating Rho signaling since the TJ-associated Rho activator GEF-H1 has been shown to be phosphorylated by PAKs (29, 148). Future studies should characterize effectors of Cdc42 and Rac involved in actin polymerization and actinomyosin contraction that control the regulation of paracellular permeability.

An alternative mode of regulation of TJ by Rho may involve direct modifications of junctional membrane proteins. When a constitutively active form of RhoA was overexpressed in MDCK cells, occludin phosphorylation was increased, suggesting that Rho signaling can directly affect TJ proteins (60). When Rho was stimulated with either LPA or histamine in endothelial cells, phosphorylation of the carboxy-terminal domain of occludin increased, which is thought to regulate the interaction of occludin with the junctional actin cytoskeleton and, hence, paracellular permeability (68). Direct phosphorylation of occludin and claudin-5 by ROCK has been reported in brain endothelial cells, correlating with diminished barrier tightness and enhanced monocyte migration across BBB induced by human immunodeficiency virus-1 encephalitis (144). It thus seems that Rho signaling can affect paracellular permeability by different molecular mechanisms ranging from regulation of
actinomyosin contraction and actin polymerization to direct regulation of junctional membrane proteins (FIGURE 2).

**TJ and Regulation of Gene Expression by RhoGTPases**

Different molecular mechanisms that regulate gene expression have been associated with TJs (13). These include factors that may regulate chromatin structure (e.g., SAF-B), polyadenylation (i.e., symplekin), as well as various transcription factors that associate with junctional proteins (e.g., AP-1, Myc); generally, these associations are thought to be part of regulatory mechanisms (22, 70, 128).

Only one TJ-associated signaling pathway has thus far linked RhoGTPase signaling to gene expression. 

This mechanism is based on ZONAB, a Y-box transcription factor that binds to the SH3 domain of ZO-1 (FIGURE 3). ZO-1 binding inhibits ZONAB by cytoplasmic sequestration (12). The ZO-1/ZONAB pathway regulates cell proliferation and, in a three-dimensional culture system, epithelial morphogenesis (9, 120). ZONAB function can be activated by the heat shock protein Apg-2, which binds to ZO-1 and thereby stimulates ZONAB dissociation, which also influences proliferation and epithelial morphogenesis (4, 129). Furthermore, RaLa, a member of the Ras superfamily of small GTPases, inhibits ZONAB transcriptional activity in a cell density-dependent manner, and expression of oncogenic Ras alleviates transcriptional repression by ZONAB in a RaLa-dependent manner (50).

A second mechanism of ZONAB stimulation has recently been identified that is based on Rho signaling (100). ZONAB activity is Rho-dependent, and the transcription factor forms a complex with the TJ-associated Rho activator GEF-H1. Modulation of GEF-H1 activity by depletion or overexpression consequently inhibits or stimulates, respectively, transcriptional activity of ZONAB. Both GEF-H1 and ZONAB regulate G1/S phase transition, and GEF-H1-stimulated expression of cyclin D1, a cell cycle regulator and key target of RhoA signaling, is at least in part mediated by ZONAB (100).

GEF-H1 is regulated by different mechanisms, and some also affect regulation of gene expression, such as cingulin (3, 62). However, it is not known whether any of those genes are indeed ZONAB target genes. An additional complication is that not all mechanisms that stimulate GEF-H1 also activate ZONAB. For example, stimulation with TNF-α activates GEF-H1 (76, 100) but does not stimulate ZONAB activity (100). This suggests additional mechanisms that modulate ZONAB activity in different cellular and regulatory contexts. For example, RaLa, another small monomeric GTPase of the Ras superfamily, is known to be activated by TNF-α (124), and active RaLa binds and inhibits ZONAB activity (50); therefore, RaLa might inhibit ZONAB in response to TNF-α.

Although ZONAB represents a first direct mechanism that links Rho GTPase signaling at TJs to the regulation of gene expression, it is likely that different mechanisms exist, and it will be important to identify these pathways and determine how they interact with each other. [During the production of this review, GEF-H1 has also been identified as a TGF-β target gene and effector that regulates α-smooth muscle actin expression and cell migration (129a).] Moreover, there is still very little known about the pathological relevance of these mechanisms. ZONAB and GEF-H1 expression is deregulated in different types of tumors (24, 137, 146) and upregulation of GEF-H1 can be observed in migratory retinal pigment epithelial cells in retinal detachments (129a); however, whether this is a cause or a consequence of tumorigenesis is not known.

**FIGURE 3. Tight junction and regulation of gene expression by RhoGTPases**

Indicated are the main transcriptional pathways involving TJ-associated proteins linked to expression of genes that regulate proliferation, differentiation, and TJ permeability in response to Rho signaling. Arrows represent activation, and T-shaped lines represent inhibition. If such lines are in red, they represent a direct interaction. ZONAB forms complexes with both symplekin and CDK4, but it is not known whether these three proteins form a tripartite complex. ZO-1, by regulating ZONAB localization, has been linked to suppression of erbB-2 expression and increased expression of PCNA and cyclin D1. RaLa/ZONAB association in a GTP-dependent manner inhibits transcription of an erbB2 reporter promoter, which is also suppressed by symplekin via interaction with ZONAB. Symplekin and ZONAB have also been linked to the stimulation of cyclin D1 expression. ZONAB-dependent expression of cyclin D1 is also activated by GEF-H1-stimulated Rho activation. Cingulin regulates claudin-2 expression by a RhoA-dependent transcriptional mechanism. Cingulin also interacts with GEF-H1 and inhibits its activity; however, it is unknown whether GEF-H1 is involved in claudin-2 expression and whether cingulin regulates cyclin D1 expression. Although one might expect significant cross-talk between the different regulatory mechanisms that target ZONAB, how far that indeed occurs is not known. Note that not all mechanisms that target GEF-H1 also regulate ZONAB in a corresponding manner, suggesting that GEF-H1 may be part of different pathways or that other regulatory mechanisms can counteract Rho stimulation of ZONAB (e.g., inhibition by RaLa).
RhoGTPases are crucial components of signaling mechanisms associated with TJs. It is clear that they are major factors in regulating TJ permeability and in transmitting signals from TJs that regulate gene expression and thus control cellular behavior including proliferation and differentiation.

RhoGTPase-dependent regulation of the actin cytoskeleton is very important for processes requiring junction dynamics, such as assembly and the regulation of paracellular permeability. The available evidence suggests that a major pathway by which RhoGTPases regulate permeability of TJs involves GEF-H1 and RhoA, which lead to ROCK activation and regulation of non-muscle myosin II activity, resulting in actinomyosin contractility. It seems, however, that alternative RhoGTPase-dependent mechanisms that regulate actin filament dynamics may also play an important role in the regulation of TJ permeability. Activation of RhoGTPase signaling may also result in the direct modification of TJ transmembrane proteins by inducing phosphorylation and, thereby, resulting in altered permeability.

TJs can also regulate gene expression via RhoGTPase signaling. The interaction between activators of RhoA, such as GEF-H1, and the transcription factor ZONAB provide a mechanism by which TJs are linked to RhoA signaling and gene expression of proliferative genes. However, our knowledge about the spatial and temporal regulation of RhoGTPase activity and the interactions with different effector pathways is still limited. The identification of RhoGTPases regulatory and effector pathways in a TJ-specific context in response to physiological and pathophysiological stimuli to control cell behavior remain a crucial challenge for the future.

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