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Na,K-ATPase and epithelial tight junctions

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1. ABSTRACT

Tight junctions are unique organelles in polarized epithelial and endothelial cells that regulate the flow of solutes and ions across the epithelial barrier. The structure and functions of tight junctions are regulated by a wide variety of signaling and molecular mechanisms. Several recent studies in mammals, drosophila, and zebrafish reported a new role for Na,K-ATPase, a well-studied ion transporter, in the modulation of tight junction development, permeability, and polarity. In this review, we have attempted to compile these new reports and suggest a model for a conserved role of Na,K-ATPase in the regulation of tight junction structure and functions.

2. INTRODUCTION

Epithelial and endothelial cells line the surfaces in many organs to form the barrier between distinct compartments with defined but different fluid compositions. The transport of water, ions and other solutes across the epithelial barrier is a highly regulated process and occurs via the paracellular and transcellular pathways. Occluding junctions, such as the vertebrate tight junctions (TJs) and the invertebrate septate junctions (SJs), limit free diffusion of the paracellular pathway and function as permeability barriers by sealing the paracellular space between cells. As such, they allow for the regulated water and solute exchange between the compartments in response

to appropriate stimuli. Transcellular transport processes involve the channels and transporters in the apical and basolateral plasma membrane of epithelial cells. Na,K-ATPase generates the transepithelial electrochemical and osmotic gradients that drive the passive movement of solutes and has been linked to many cellular transport processes. While the coupling between the paracellular and transcellular transport processes by the Na,K-ATPase through the generation of ion gradients is evident, recent research suggests that Na,K-ATPase plays a more fundamental role in regulating TJs. Here, we have reviewed the recent findings on how Na,K-ATPase is involved in the regulation of TJs in vertebrates and non-vertebrates and suggest a conserved role for Na,K-ATPase in the regulation of TJ function.

3. TIGHT JUNCTIONS

Tight junctions (TJs) are the most apical components of the junctional complexes in epithelial cells that also include adherens junctions, desmosomes, and gap junctions. In transmission electron microsocopy, TJs appear as close contacts between adjacent cells, seemingly fusing the neighboring plasma membranes. At these sites of cell-cell contacts, the barrier is formed where strands of adhesive transmembrane proteins contact across the paracellular space, and it behaves as if perforated by pores possessing size and charge selectivity. The degree of sealing varies according to cell type, physiological stimuli, and pathological conditions and seems to be at least in part dependent on the pore number and the profile of claudins expressed (1). In addition to this gate function to regulate the passage of ions and small molecules, TJs also serve to maintain cell polarity by forming a fence within the plasma membrane that restricts the diffusion of proteins and lipids between the apical and basolateral surfaces. Recent evidence suggests that TJs have additional roles in cell signaling, regulating epithelial cell proliferation, differentiation, and gene expression (2, 3).

Tight junctions are multiprotein complexes composed of integral membrane proteins that mediate cell-cell adhesion and of cytoplasmic plaque proteins that serve as a bridge to the actin cytoskeleton, as a scaffold for the recruitment of signaling proteins, and as regulators of TJ assembly and function. A detailed description of the individual molecules and the regulation of TJ components by signaling pathways have been the focus of excellent recent reviews (2-5). In this review, we will highlight some of the main characteristics of TJs. Three families of transmembrane proteins of the TJs have been described, including claudins and occludin, which are both tetraspan proteins but do not share sequence homology, and the single transmembrane JAMs. Claudins (5, 6) are the major components forming the barrier of the TJ. They constitute a multigene family of at least 24 members in vertebrates ranging from 20 to 27 kDa (2, 5, 6) and have also been described in invertebrates such as zebrafish. Two claudin-like homologues in Drosophila SJs that are involved in forming the paracellular barrier have also been found (7, 8). Claudins mediate cell-cell adhesion independent of calcium and can associate homotypically or heterotypically with each other to form TJ strands in a tissue-specific manner (5). Together with the variability in the two extracellular loops that determine the paracellular barrier functions of the different claudin isoforms, this tissue-specific expression pattern is thought to be associated with the tissue-specific differences in TJ characteristics (5, 9-11). The C-terminal amino acids of claudins are highly conserved and, with the exception of claudin 12, end with PDZ-binding motifs, directly interacting with the PDZ domains of the zonula occludens scaffolding proteins ZO-1, ZO-2 and ZO-3 (12), as well as with multi-PDZ proteins (MUPP) (13, 14) and PATJ (15).

While it seems plausible that TJ functions are regulated through claudins, this is still an emerging field. Phosphorylation of claudins might be involved in regulating the paracellular barrier, and increased phosphorylation has been correlated with either decreased (e.g., claudin-3, 4) (16, 17) or increased (e.g., claudin-1) (18) barrier function. Treatment of intestinal epithelial cells with interferon (IFN)-γ induced the endocytosis of claudin-1 and increased the paracellular permeability (19). However, under these conditions, occludin is internalized as well (19, 20), making it difficult to confirm the specific role of claudins in the regulation of the paracellular permeability in this model. Regulation of claudin expression levels by growth factors has been reported frequently. In Madin-Darby canine kidney (MDCK) cells, epidermal growth factor receptor (EGFR) activation results in reduced claudin-2 and increased claudin-1, -3, and -4 expression, which is accompanied by an increase in transepithelial electrical resistance (TER) (21, 22). Hepatocyte growth factor (HGF) alters claudin expression and increases or decreases TER depending on the cell-type used in the study (23-26). Transforming growth factor (TGF)-β_3 downregulates the expression of claudin-11 in cultured Sertoli cells and inhibits the TJ permeability barrier (27). Expression of the transcription factor Snail during epithelial-to-mesenchymal transition (EMT) leads to downregulation of claudin and occludin expression and of the cell-cell adhesion molecule E-cadherin, and is associated with an increase in TJ permeability (28, 29). Apart from Snail, several transcription factors such as the β-catenin/Tcf complex (30, 31) and the hepatocyte nuclear factor (HNF)-1α (32) were reported to regulate a variety of claudin promoters. Although much circumstantial evidence suggests that claudin integration into TJs is one mode of regulating the TJ barrier, the specific contributions and the physiologically relevant molecular mechanisms remain to be defined.

Occludin was the first transmembrane protein of the TJ to be identified and is one of the constituents of the TJ intermembrane strands (33). While over-expression of occludin increases TER in mammalian epithelial cells (34, 35), occludin per se is not required for the formation of TJ strands. Disruption of both occludin alleles in embryonic stem cells did not prevent their differentiation into polarized epithelial cells (36), and occludin null mice did not display obvious structural or functional TJ abnormalities (37). Although occludin does not seem to be essential for the TJ barrier function, recent evidence

suggests a likely role in regulating various signaling events to and from TJs (2). Occludin is a ~60 kDa tetraspan membrane protein and has two extracellular loops and cytosolic amino- and carboxy-terminal domains. The carboxy-terminal domain associates with ZO-1, ZO-2 and ZO-3 and binds to the actin-myosin binding protein cingulin. It is rich in serine, threonine, and tyrosine residues, which are targets for several protein and tyrosine kinases such as the nonreceptor tyrosine kinase c-Yes (38), the serine/threonine kinases casein kinase (CK) 2 (39), and atypical protein kinase C (aPKC)-ζ (38), as well as for the serine/threonine protein phosphatase 2A (PP2A) (40, 41). Occludin phosphorylation is further affected by a variety of signaling events that include growth factors such as vascular endothelial growth factor (VEGF) (42, 43) and TGF-β (27) and signaling molecules involved in actin organization such as Rho/ROCK (44), including the activity of Na,K-ATPase (45). Occludin further interacts with the regulatory subunit p85 of the phosphatidyl-inositol 3 (PI3) kinase (38), actin (46), the E3 ubiquitin ligase Itch (47), and the gap junction protein connexin-32 (48). Transcriptional regulation of occludin by tumor necrosis factor (TNF)-α (49) as well as Snail (29), which both also regulate claudin expression, has been reported. This array of mechanisms regulating occludin expression and function suggests that occludin could function to integrate a wide variety of signals and act as gate keeper to modulate TJ permeability.

The three members of the junctional adhesion molecule family, JAM-A, -B, and C have, unlike claudins and occludin, only a single transmembrane domain (50), and their extracellular domains contain two immunoglobulin-like motifs and dimerization motifs that play a role in their interactions (51, 52). The detailed role of JAMs in TJ function remains to be determined. Recent studies implicated JAM proteins in the epithelial barrier function, since inhibitory antibodies for JAM result in decreased TER and defects in TJ assembly (53). Like claudins and occludin, JAM proteins have a PDZ-binding motif at their C termini and interact with TJ scaffolding proteins that include ZO-1 (54). JAM-A interacts with MUPP-1 (13), afadin (54), the calcium/calmodulin-dependent serine protein kinase (CASK/Lin2) (55), MAGI-1 (56), PICK-1 (57) and Par-3 (58); these interactions appear to be important for proper TJ function (50).

The transmembrane TJ proteins, occludin, claudins, and JAMs, are linked to the actin cytoskeleton via the interaction of their intracellular domains with peripheral membrane proteins. Of these, the Zonula occludens proteins ZO-1 and its binding partners ZO-2 and ZO-3 are well-characterized (3, 59, 60). They are members of the membrane-associated guanylate kinase (MAGUK) family of proteins and are thought to regulate the kinetics of the assembly of TJs (61, 62). ZO-1, as with all MAGUK proteins, is characterized by its PDZ domain, SH3 domain, and the guanylate kinase homologous domain and, in addition, contains two nuclear localization signals and a carboxyl region with several proline-rich regions (60, 63). It has been proposed to be a scaffolding protein between transmembrane and cytoplasmic proteins of the TJs, as it can bind to occludin, claudins, and JAMs and F-actin, either directly or indirectly via actin-associated proteins such as cingulin and afadin (60, 63). In addition, ZO proteins interact with a multitude of other proteins. ZO-1 has been shown to bind to β- and α-catenin (64, 65), which are proteins associated with adherens junctions, and to transcription factors, such as ZONAB (ZO-1 associated nucleic acid binding) that regulates the promoter of erbB-2 (66). ZO-2 not only interacts with various nuclear proteins such as Jun, Fos, CCAAT/enhancer binding protein, and DNA-binding protein scaffold attachment factor B, but it also localizes to the nucleus, as does ZO-1, suggesting that they themselves could regulate transcription (67-71). As these are only a few of the factors that associate with ZO proteins, it is conceivable that the functions of these proteins and of TJs go well beyond a structural role. As more and more of these functions are being discovered, it is vital to determine the molecular factors that regulate TJs. While TJ function is affected by many growth factors, transcription factors, and other structural proteins, the regulation of TJs by ion channels and transporters is an emerging field and has been reviewed recently (72).

4. NA,K-ATPASE

4.1. Functions, subunits and isoforms

One of the better-known transporters that regulate TJ function is the Na,K-ATPase, a member of the family of cation transporting P-type ATPases. The Na,K-ATPase, also known as sodium pump, is found in the cells of all higher eukaryotes and transports 3 Na^+ out and 2 K^+ into the cell by hydrolyzing one molecule of ATP. In addition to maintaining the intracellular ion homeostasis, this pumping process generates a transmembrane electrochemical gradient that regulates other cellular activities such as secondary active transport of other ions, nutrients, and neurotransmitters, for maintaining intracellular pH, cell volume and size, and for electrical excitability. In most epithelial cells, the Na,K-ATPase is localized to the basolateral plasma membrane, and the gradients generated by Na,K-ATPase are involved in regulating directional transport of molecules across epithelial cells (73-75). Recent evidence suggests that Na,K-ATPase might have a more direct or indirect role in transport across the epithelial barrier by regulating TJ structure and permeability.

The functional Na,K-ATPase is a heterodimeric protein consisting of an α-subunit and a β-subunit (76, 77). A third tissue-specific regulatory γ-subunit, members of the FXYD family, has also been described (78-80). The α-subunit is the catalytic subunit of the Na,K-ATPase, and four distinct isoforms have been identified in mammalian cells (α_1, α_2, α_3 and α_4). Each has unique kinetic properties and a distinctive response to second messengers (81, 82). The α-subunit has a molecular mass of about 110 kDa with 10 transmembrane segments with 5 extracellular loops and both termini located intracellularly. It contains the binding sites for Na^+, K^+, ATP and cardiotonic steroids such as the specific inhibitor ouabain (83). The recently published crystal structure of the Na-K-ATPase revealed that the carboxy terminus of the α-subunit is contained within a pocket between the transmembrane helices as a regulatory

element controlling sodium affinity and that the β- and the γ-subunits are associated with the transmembrane helices αM7 /αM10 and αM9, respectively (84).

The β-subunit of Na,K-ATPase is a type II single membrane-spanning protein of about 370 amino acids, and three mammalian isoforms have been described (β_1, β_2 and β_3). The molecular mass of the β-subunit is around 40-60 kDa and varies with tissue type and isoforms. There are 3 consensus N-glycosylation sequences in the extracellular domains of β_1 and β_3, and 7 in the β_2-isoform (85-87). The precise role of the β-subunit is still not known, and recent evidence suggests that it may have functions that are independent of its role in Na,K-ATPase enzyme activity. It is well-documented that the β-subunit is essential for the appropriate folding of the α-subunit in the endoplasmic reticulum and for the delivery of the α-subunit to the plasma membrane (88), as well as for the retention of the enzyme in the plasma membrane, which is dependent on the glycosylation of the β-subunit (89, 90). It has been suggested that the β-subunit may be more intimately involved in regulating the active transport function of the α-subunit as it is required for the occlusion of K^+ ions (88, 91). The recent crystal structure studies revealed that the β-subunit interacts with its Tyr 39, Phe42 and Tyr 43 with the α-subunit, and its conserved glycines in the GXXXG motif are exposed on the other side (84). It has been suggested that this motif is important for the homodimerization of the β-subunit and has a role in cell-cell adhesion (92, 93).

The γ-subunit of the Na,K-ATPase (now FXYD2) was originally identified as a component of Na,K-ATPase in sheep kidney (94), and subsequently, the third subunit of the Na,K-ATPase was identified in various other tissues including cancer tissues. These proteins belong to the family of FXYD proteins, and 7 members (FXYD1-7) have been reported to date. They are short polypeptides and are type I transmembrane proteins, except for FXYD3, which is a double-span protein. While it is now well accepted that FXYD proteins modify and fine-tune the transport properties of the Na,K-ATPase in a tissue- and isoform-specific manner (79, 80), it is not known whether FXYD proteins perform other cellular functions in addition to modulating the pump kinetics. For example, FXYD5 was originally identified as dysadherin and has been implicated in reduced E-cadherin expression, cancer progression, and metastasis (95, 96), but it remains to be determined whether this function is dependent or independent of its role in Na,K-ATPase enzymatic function.

4.2. Regulation and interaction with other proteins

Numerous mechanisms are involved in the regulation of the Na,K-ATPase to adapt to changing physiological demands. These include its own β- and γ-subunits as well as intracellular Na^+. Intracellular Na^+ is the limiting factor for the pump function of the Na,K-ATPase, and any change in intracellular Na^+ concentration affects its transport rate. A multitude of other factors such as endogenous cardiac glycosides (e.g., ouabain and its stereo or regioisomers and derivatives of bufadienolides) (97), corticosteroids (e.g., the mineralocorticoid aldosterone and the glucocorticoid dexamethasone), catecholamines (e.g., norepinephrine and dopamine), and peptide hormones (e.g., insulin, parathyroid hormone, angiotensin II) affect α- and β-subunit transcription as well as phosphorylation of the catalytic subunit (98). cAMP-dependent protein kinase A (PKA), cGMP-dependent protein kinases (PKG), Ca-phospholipid-dependent protein kinase (PKC), and atypical PKC-ζ, tyrosine kinases and protein phosphatases have all been reported to regulate Na,K-ATPase through post-translational modification of the α-subunit, ubiquitination, and endocytosis (98-100). Recently, phosphorylation of phospholemman (FXYD1) by PKA and PKC has been shown to regulate Na,K-ATPase in an isoform-dependent manner (101).

Na,K-ATPase subunits interact with a multitude of proteins including other ion transporters and structural and signaling proteins (72). The α-subunit has been shown to interact with cytoskeletal proteins such as the actin binding protein ankyrin, which is important for the trafficking and targeting of the Na,K-ATPase (102, 103), and cofilin (104). Other proteins that have been shown to associate with Na,K-ATPase and modulate its trafficking are arrestin, spinophilin, G-protein-coupled receptor kinases, and 14-3-3 ε (105). The α-subunit also interacts with proteins associated with the endocytic machinery such as the adapter protein AP-2, a clathrin adapter (106), and with caveolin (107), as well as with diverse other proteins such as polycystin-1 (108). Most importantly, recent evidence indicates that Na,K-ATPase associates with proteins involved in cell signaling, possibly forming a scaffolding platform (Figure 1). The α-subunit binds several signaling molecules such as phosphoinositide-3 kinase (109), src (110), PP2A (45), phospholipase C (PLC)-γ1 (111), and inositol 1,4,5-trisphosphate (IP3) receptor (111). As new functions of the β-subunit are being explored, new binding partners have also been identified, including the endoplasmic reticulum protein wolframin (112), annexin II (109), PP2A (45), retinoschisin (RS1) (113), and the small GTPases RalA and RalB (114).

4.3. Na,K-ATPase as a signaling molecule

The role of Na,K-ATPase as a modulator of cell signaling is becoming well accepted. Inhibition of Na,K-ATPase function by ouabain or by low K^+ concentration increases the expression of the proto-oncogenes c-fos and c-jun (115) and the transcription factor AP-1 in conjunction with an increase in hypertrophic growth (116). Later studies showed that inhibition of Na,K-ATPase by ouabain leads to the activation of Ras and p42/44 mitogen-activated protein kinase (MAPK) (117). Xie's group reported that Na,K-ATPase binds src kinase to inhibit src function and that addition of ouabain frees the kinase domain to activate src in a pump-independent manner (110). They further suggested that this mechanism is involved in the transactivation of EGF receptor and the activation of the Grb2/Ras/Raf/MEK extracellular signal-regulated kinase cascade (110, 118-120). Ouabain-treatment as well as expression of Na,K-ATPase β-subunit have been shown to activate PI3-kinase (109, 121, 122). Studies from our laboratory showed that inhibition of the

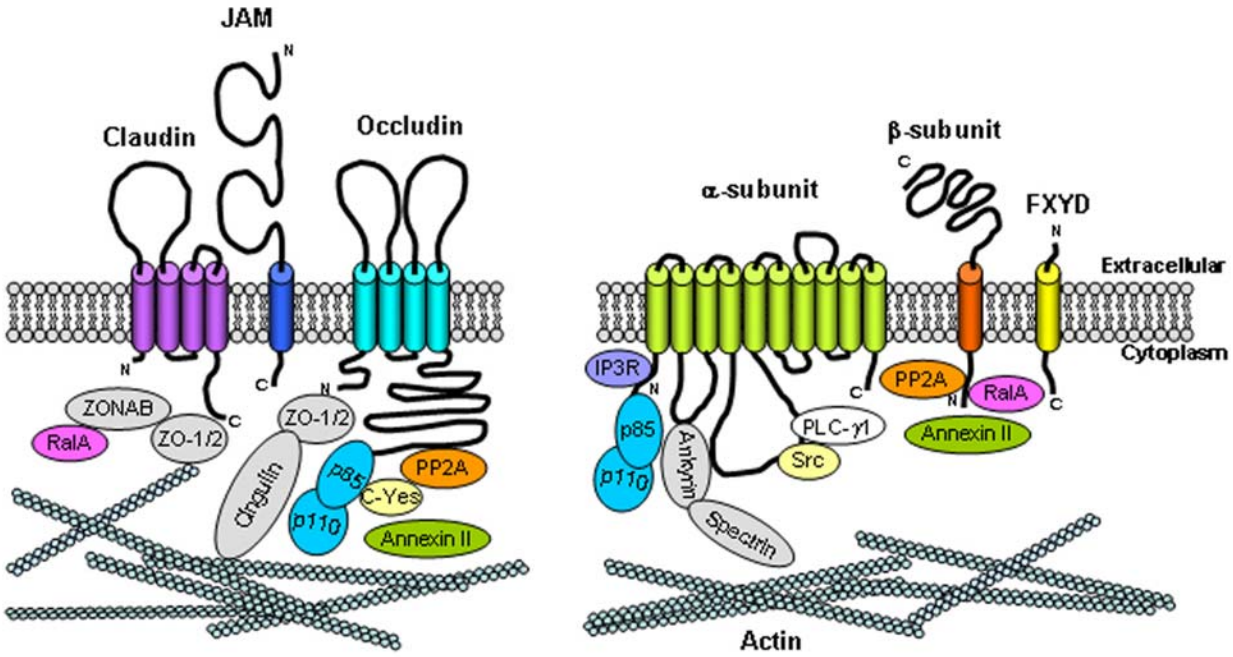

Figure 1. Na,K-ATPase forms a signaling scaffolding platform. Both, tight junctions (left) and Na,K-ATPase (right) form signaling scaffolds with their transmembrane proteins either linked directly or indirectly to the actin cytoskeleton. Some of the Na,K-ATPase associated signaling proteins are also found in the vicinity of tight junctions.

Na,K-ATPase activity with its concomitant increase in intracellular sodium inhibits the activity of RhoA, a small GTPase involved in the regulation of actin polymerization in epithelial and other cell types (123). As it is well-known that the physical and functional coupling between Na,K-ATPase and the Na^+/ Ca^{2+} exchanger regulates intracellular Ca^{2+} (124), this regulation also involves a more direct role of Na,K-ATPase via its interaction with PLC-γ_1 and IP3 receptor, which can be phosphorylated in a ouabain- and src-dependent manner (110). Further effects of ouabain include the inhibition of PP2A activity (45), the activation of Ral-GTPase (114), and the generation of reactive oxygen species (ROS) (125, 126). Inhibition of the Na^+/H^+ exchanger NHE3 (127) by ouabain seems to be due to regulation of NHE3 trafficking relayed by ouabain-induced Na,K-ATPase signaling (128). While some of the signaling functions appear to be due to alterations of ionic homeostasis following inhibition of Na,K-ATPase (such as inactivation of RhoA), others have been shown to be independent of Na,K-ATPase pump function and are rather due to interaction of Na,K-ATPase subunits with signaling molecules (e.g., src and MAPK signaling). Deciphering the specific contributions of pump-dependent and independent signaling pathways to the Na,K-ATPase signaling function and the interplay between these pathways remains a challenge.

5. NA,K-ATPASE AND TIGHT JUNCTIONS

Studies from our laboratory provided experimental evidence that the enzymatic function and subunits of Na,K-ATPase themselves have a role in the organization and permeability of TJs. Expression of the β_1-subunit of Na,K-ATPase in Moloney sarcoma virus (MSV) transformed MDCK cells that express E-cadherin, a calcium-dependent cell-cell adhesion molecule, induced functional TJs and epithelial polarity in this transformed cell line (129). MSV-MDCK cells express very low levels of E-cadherin (65, 129, 130). Exogenous expression of E-cadherin and Na,K-ATPase β_1-subunit was sufficient to induce an epithelial phenotype with functional TJs. We, therefore, suggested that Na,K-ATPase β_1-subunit functions synergistically with E-cadherin in the assembly and function of TJs. Interestingly, in these studies, expression of Na,K-ATPase β_1-subunit together with E-cadherin reduced the higher intracellular Na^+ level of MSV-MDCK cells to a more normal level as observed in MDCK cells. These low Na^+ levels were not observed when Na,K-ATPase β_1-subunit or E-cadherin was expressed alone, suggesting that low intracellular Na^+ levels are required for epithelial polarization (129). In subsequent studies using the specific inhibitor ouabain and K^+-depletion as independent methods to inhibit Na,K-ATPase activity, we further provided evidence for Na,K-ATPase enzyme activity in the regulation of TJs (45, 123, 131). Using MDCK cells in a calcium switch assay, we showed that inhibition of Na,K-ATPase activity prevented the formation of TJs. An increase in the intracellular Na^+ by Na^+ ionophores mimicked the effect of Na,K-ATPase inhibition on TJ formation, suggesting that epithelial cells require low intracellular Na^+ to establish TJs and polarity. RhoA GTPase, which has been implicated in the regulation of TJs in epithelial cells (132-134), was considerably inhibited following inhibition of Na,K-ATPase function or Na^+ ionophore treatment. Overexpression of wild-type RhoA GTPase significantly reduced the effect of Na,K

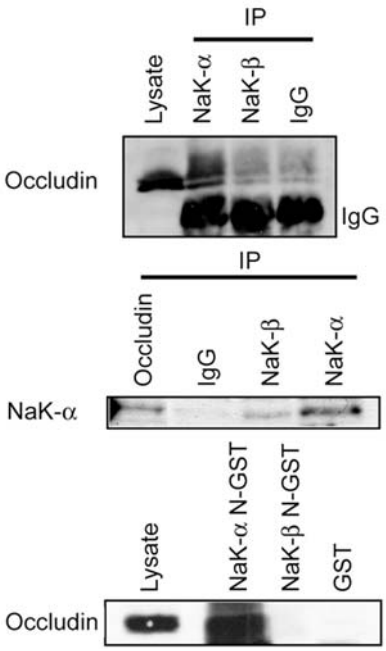

Figure 2. Association of Na,K-ATPase with occludin. Occludin and Na,K-ATPase are associated with each other as demonstrated by co-immunoprecipitation (upper and middle panel) and GST-pull down experiments (lower panel).

ATPase inhibition on TJ assembly, indicating that RhoA GTPase is a key molecule affected by Na,K-ATPase during epithelial polarization (123).

Based on our results, we proposed a two-step model for the assembly of TJs in epithelial cells. According to this model, the first step involves E-cadherin-mediated signaling events that translocate TJ proteins to the plasma membrane, where they assemble and form discontinuous TJ strands. The second step is regulated by Na,K-ATPase, which involves polymerization of actin mediated by RhoA GTPase that is involved in the mobility and cohesion of discontinuous TJ strands to form continuous strands necessary for the establishment of functional TJs (135). Although this model suggests that E-cadherin and Na,K-ATPase are two major players of TJ formation and a polarized phenotype in epithelial cells, it is likely that other signaling mechanisms modulated by these or other proteins are involved in the process of epithelial polarization.

Na,K-ATPase function is necessary not only for the formation of epithelial TJs but also to maintain TJ function and structure. In polarized primary cultures of human retinal pigment epithelium (RPE) cells and in a polarized pancreatic cell line, HPAF-II, inhibition of Na,K-ATPase function reduced TJ membrane contact points and increased permeability to both ionic and non-ionic molecules (45, 131). In HPAF-II cells, the Na,K-ATPase β-subunit was associated with PP2A, a serine/threonine phosphatase localized to TJs. Inhibition of Na,K-ATPase activity considerably reduced PP2A activity, which correlated with increased phosphorylation of occludin and TJ permeability. Immunogold labeling and electron microscopy further confirmed that Na,K-ATPase β-subunit is localized to the TJ and adherens junction region in addition to the basolateral plasma membrane (45). Co-immunoprecipitation analysis in HPAF-II cells suggests that occludin is associated with the Na,K-ATPase α-subunit (Figure 2). These studies strongly suggest that Na,K-ATPase is also localized to the TJ and might be involved in the regulation of TJ structure, permeability, and signaling functions locally at the tight junction region. Whether Na,K-ATPase function regulates an ionic balance at the TJ region or modulates signaling via its interaction with other signaling molecules or the TJ proteins themselves requires future research.

6. NA,K-ATPASE IN THE DEVELOPMENT OF TIGHT JUNCTIONS

6.1. Na,K-ATPase in blastocyst development

During mammalian early development, the first epithelial structure that emerges is the trophectoderm epithelium covering the surface of the blastocyst and enclosing the inner cell mass (136, 137). The mouse trophectoderm emerges at the morula stage of an 8-cell embryo, as compaction becomes the first morphogenetic event of preimplantation development. This stage is characterized by increased cell-to-cell contact and the gradual assembly of adherens junctions followed by desmosomes, TJs, and cell polarization (138). As the outer blastomeres proceed to acquire complete epithelial characteristics to form the trophectoderm at the 32-cell stage, blastocyst formation (cavitation) is initiated following the establishment of ion gradients and osmotic fluid accumulation across the trophectoderm epithelium (139). The paracellular seal formed by TJs between adjacent trophectoderm is essential for the transepithelial transport processes, and the barrier function of the TJs in the trophectoderm is required for normal blastocyst formation (140).

It has been well documented that the Na,K-ATPase and the sodium gradient generated by its pump function provides the driving force for the vectorial transepithelial transport processes and promotes the osmotic accumulation of water across the epithelium (139, 141). Nevertheless, deletion of the Na,K-ATPase α-subunit gene (*Atp1α1*) did not prevent cavitation of the preimplantation mouse embryo but subsequently failed during the peri-implantation phase of development (142). Mouse embryos homozygous for a null mutation in the α_1-subunit gene were able to undergo compaction and cavitation, suggesting that other α-subunit isoforms are present that allow for the blastocyst formation to progress in the absence of the α_1 isoform. However, in subsequent studies by Violette *et al* (143) inhibition of the Na,K-ATPase enzymatic function allowed the mouse embryos to develop normally to the blastocyst stage (up to 6 hours) but affected the distribution of TJ proteins such as ZO-1 and occludin. The TJ barrier function was affected as demonstrated by their increased permeability to 4 kDA and 40 kDa FITC-dextran, suggesting that Na,K-ATPase is a key regulator of trophectoderm TJ function during murine

preimplantation development. These studies were further confirmed as mouse embryos injected with Na,K-ATPase β_1-subunit siRNA oligos failed to develop to the blastocyst stage (144). In addition, studies by Eckert *et al* (145) showed recently that inhibition of PKCζ delayed cavitation. Inhibition of cavitation with the a PKCζ inhibiting peptide was found to coincide with rapid internalization of the α-subunit of the Na,K-ATPase. These studies collectively demonstrated that Na,K-ATPase enzyme activity as well as its subunits play a role in blastocyst formation through the regulation of TJ formation and function during preimplantation development in mouse embryos as we have demonstrated using cultured cell lines (45, 123, 129, 131, 135).

6.2 Na,K-ATPase in zebrafish

In zebrafish, 9 α-subunits and 6 β-subunit genes have been identified (146-149). The zebrafish α1B1 subunit of Na,K-ATPase is encoded by the *heart and mind (had)* locus. In the developing heart, as the primitive heart tube grows, complex morphogenic events transforming sheets of cardiac precursors into a three-dimensional structure take place (150-152). Shu *et al* (153) identified a zebrafish mutant, *heart and mind (had)*, which caused severe abnormalities in primitive heart tube extension, cardiomyocyte differentiation, and embryonic cardiac function in an isoform-dependent manner, indicating a crucial role for the Na,K-ATPase α1B1 in zebrafish heart development. Inhibition of Na,K-ATPase α1B1 activity with ouabain produced the *had* mutant phenotype, and over-expression of α1B1 rescued the *had* cardiac phenotype, further supporting the hypothesis that mutations in α1B1 are responsible for the *had* phenotype. This group further suggested that Na,K-ATPase might regulate heart tube extension by rearranging the actin cytoskeleton and by regulating the polarity of cardiac cells as we have shown in cultured cells (123, 131).

In addition, recent studies in zebrafish suggest a role for Na,K-ATPase in myocardial cell junction maintenance (154). Mutations of *α1B1* cause heart tube elongation defects and other developmental abnormalities that are reminiscent of several epithelial cell polarity mutants (152, 155-157), suggesting a common defect underlying the loss of myocardial morphogenetic potential. Indeed, Cibrian-Uhalte *et al.* (154) demonstrated in zebrafish genetic interactions between Had and Nok, a TJ-associated scaffolding protein of the apical crumbs polarity complex involved in the maintenance of ZO-1-positive junction belts within myocardial cells, and that maintenance of ZO-1 junction belts required the Na,K-ATPase pump activity. As suggested by the authors, the correct ionic gradients modulated by Had may stabilize the integrity of the TJ and the paracellular diffusion barrier, which is consistent with our finding in polarized epithelial cells. Further, recent studies also suggest a role for Na,K-ATPase in otolith formation and semicircular canal development (147) as well as in single lumen development in the zebrafish gut (158). It remains to be determined whether the electrochemical gradient generated by the Na,K-ATPase or Na,K-ATPase subunits' interaction with other regulatory proteins are involved in the lumen development in the zebrafish gut.

6.3. Drosophila and septate junctions

The SJs in invertebrates are the functional equivalent of TJs in vertebrates (6) as they both are part of the paracellular transport pathway in epithelial tissues. Although TJs and SJs share the characteristic of being a permeability barrier, they are also distinct in various ways. While TJs appear as sites where the extracellular leaflets of the plasma membrane seem to fuse, SJs are characterized by a constant intercellular cleft of ~ 15-20 nm between adjacent cells. These clefts are either continuous or bridged with spaced bridges called septa (159). TJs are located at the most-apical pole of the lateral plasma membrane above the adherens junctions whereas SJs are found more basal below the adherens junction (160). Further, the vertebrate homologues of most SJ-associated proteins are not found in TJs, except for claudins (7, 8). Interestingly, we found that Na,K-ATPase β_1-subunit is localized to TJs and that the α-subunit associates with occludin in HPAF-II cells (Figure 2). In *Drosophila*, both subunits of Na,K-ATPase, α-(ATPα) and β- (Nrv2) are concentrated at the SJs (161-163). The significance of this is not known at this time but points to a conserved role for Na,K-ATPase in TJ/SJ function.

In *Drosophila*, there are two α-subunit loci, *ATPα* that produces at least 12 α-subunit isoforms and three β-subunit loci, *nrv1* and *nrv3,* which produce one isoform each, and *nrv2,* which encodes two isoforms, Nrv2.1 and Nrv2.2 (164). Data from immunoprecipitations and somatic mosaic studies suggest that ATPα and Nrv2 form an interdependent protein complex with Coracle (COR), Neurexin (NRX), Gliotactin and Neuroglian (NRG); some of which were previously known to localize to SJs (161). Mutations of both ATPα and Nrv2 were associated with a structural loss of the SJs accompanied by the disruption of the paracellular barrier function in the salivary gland. Analysis of the ultra structure of the SJs by transmission electron microscopy revealed that while the adherens junctions remained intact, the septae were disrupted, suggesting that Na,K-ATPase is necessary for establishing and maintaining SJs, the primary paracellular barrier in invertebrate epithelia (161). Besides the salivary gland, ATPα and Nrv2 but not other *Drosophila* Na,K-ATPase β-subunits were also found to be essential for the SJ function and epithelial tube size control in the *Drosophila* tracheal system and in epidermis (163). In a later study, the junctional activity of the Na,K-ATPase was found to be mediated by specific isoforms of the ATPα and by the extracellular domain of Nrv2. However, mutations predicted to block ion-pump activity had no effect on SJ formation, suggesting that the formation of SJs and the diameter of the tubes are independent of the pump function of the Na,K-ATPase (162).

The studies by both groups (161-163) pointed to a specific role for Na,K-ATPase in SJ function, as mutations of the Na,K-ATPase subunits did not cause the loss of adherens junctions. Immunofluorescence studies of

the adherens junction components E-cadherin (Shotgun) and β-catenin (Armadillo) showed that the localization and levels were unaffected (162, 163), but ultrastructural studies revealed the presence of SJs (161). Interestingly, we found that in the mammalian MSV-MDCK cells, expression of E-cadherin restored the assembly of adherens junctions but TJs were not induced. Nevertheless, expression of Na,K-ATPase β_1-subunit in these cells induced the formation of TJs (129). Similarly, inhibition of the Na,K-ATPase activity prevented the assembly of TJs but not adherens junctions (123). This suggests a specific role for Na,K-ATPase in the formation and regulation of the paracellular TJ barrier in both vertebrate cells and *Drosophila*.

7. FUTURE PERSPECTIVES

7.1. Na,K-ATPase, a member of the tight junction protein complex

The recent flurry of papers on the role of Na,K-ATPase in vertebrate TJ and *Drosophila* SJ structure and functions points to a conserved role of Na,K-ATPase in regulating the paracellular barrier in vertebrates and insects. The molecular aspects of this discovery are still in their infancy, and the next step will be to uncover the signals that are transmitted from the Na,K-ATPase to regulate the structure and function of these junctions. It is likely that the enzyme activity of the Na,K-ATPase and the intracellular ion homeostasis associated with it, as well as the α- and the β-subunits themselves independent of the ion transport function, regulate TJ function. As Na,K-ATPase has been found to be localized to TJs in vertebrates and SJs in insects, it is possible that the subunits directly or indirectly associate with TJ proteins to regulate structure and function. Density gradient centrifugation analysis of the epithelial apical junctional complex revealed that Na,K-ATPase co-distributed with the E-cadherin, β-catenin/α-catenin complex as well as with occludin and to some extent with ZO-1 and ZO-2 (165). We found that in HPAF-II cells, the Na,K-ATPase α-subunit and occludin were associated with each other as determined by co-immunoprecipitation and GST-pull down assays (Figure 2). Other proteins that are localized to the TJ complex or interact with TJ-associated proteins have as well been found to associate with Na,K-ATPase including annexin II (109, 166), IP3 receptor (111, 167), PP2A (40, 45), p85 subunit of PI3 kinase (38, 109), and Ral A (114, 168). It is also well known that Na,K-ATPase binds the actin-binding proteins ankyrin and spectrin (102, 103). In the case of the adherens junction protein E-cadherin, we found that expression of the Na,K-ATPase β-subunit in MSV-MDCK cells reduced the solubility of E-cadherin in Triton-X-100 extractions (129), and Vagin *et al.* (169) recently showed that the solubility of E-cadherin is increased when the glycosylation of the β-subunit is prevented. Together, these studies suggest that Na,K-ATPase strengthens E-cadherin's association with the actin cytoskeleton probably by recruiting more actin to the subplasma membrane region. Similarly, it is possible that Na,K-ATPase localized to the TJ region might recruit actin and actin-crosslinking proteins to further strengthen the association of TJ proteins with the actin cytoskeleton, which is involved in the regulation of the TJ permeability.

7.2. Na,K-ATPase β-subunit, a cell adhesion molecule

The integral membrane proteins of the TJs, claudins, occludin and JAMs have been found to have cell adhesion function through homotypic or heterotypic interactions. The Ca^{2+}-independent adhesion molecule on glia (AMOG) was originally identified as a molecule involved in cell adhesion as monoclonal AMOG antibody blocked neuron-glia adhesion (170). Gloor *et al.* (85) reported later that AMOG actually was the β_2-isoform of the Na,K-ATPase. More recently, the β_1-isoform has also gained attention as a molecule involved in cell-cell adhesion. In our initial studies, we showed that expression of the β_1-subunit in MSV-MDCK cells increased the cell-cell adhesion in a cell aggregation assay and suggested that the β_1-subunit might have a potential cell-cell adhesion function (129). Similarly, expression of the β_1-subunit in Chinese hamster ovary (CHO) fibroblast cells conferred adhesive properties when these cells were co-cultured with MDCK cells (89). Recent studies suggest that the transmembrane domain as well as glycosylation of the β_1-subunit confer adhesive properties. Work from our group provided a model in which the glycine zipper motif in the β_1-subunit transmembrane mediates β_1-β_1 oligomerization. Mutations in the GxxxG motif abolished the cell-cell aggregation in MSV-MDCK cells compared to cells overexpressing the wildtype β_1-subunit (92). The extracellular domain of the β_1-subunit contains three N-glycosylation consensus sites with all three being heavily glycosylated. Initial studies on the glycosylation pattern revealed that the predominant glycans of the β_1-subunit were a combination of the tetraantennary glycan form and the unusual glycan form of the tetraantennary with a limited number of repeating N-acetyl-lactosamine units (171). The glycan structures found in the β_1-subunit are processed to the same extent as adhesion molecules, and the authors concluded that the β_1-subunit may be related to an adhesion molecule. More recent studies found that the N-glycans of the β_1-subunit are indeed important for its cell adhesion activity (90, 169, 172, 173). Although these recent studies provided a basis for the β_1-subunit as a cell adhesion molecule, whether the cell-cell adhesion function is independent of the pump function of the Na,K-ATPase is still not conclusively demonstrated. However, we showed that the homodimerization of the β-subunit and its role in cell-cell adhesion could occur when the α-/β-subunit interaction was diminished by specific mutation of amino acid residues in the transmembrane domain of the β-subunit (92). It is not known whether a separate pool of β-subunit not associated with the α-subunit does exist and whether this pool would be involved in cell-cell adhesion, and this is a subject for future research. Towards this line, in a recent study, Xie and his coworkers identified a pool of non-pumping Na,K-ATPase (174), which might prove useful to address some of these questions in the future.

7.3. Na,K-ATPase, a signaling scaffold that regulates tight junctions

Experimental evidence suggests that the Na,K-ATPase could act as a signaling scaffold that might either be associated with the TJ complex or in the vicinity of the TJs. Treatment of cells with the glycoside ouabain is

involved in the activation of several signaling pathways; some of which seem to be activated independent of the pump function of the Na,K-ATPase (109, 120, 135, 175). Interestingly, some of the Na,K-ATPase-mediated signaling events seem to overlap with signaling pathways that have been shown to regulate TJs. For example, inhibition of Na,K-ATPase by ouabain or K^+ depletion leads to the inhibition of RhoA, an effect mimicked by the sodium ionophore gramicidin (123). The activity of the small GTPase RhoA is required for the formation of actin stress fibers (176) and regulates the barrier function of TJs (132-134). Na,K-ATPase can also form a signaling complex with src with ouabain treatment inducing the activation of Src and Erk1/2 independent of the pump function (110). The heterotrimeric G-protein $G\alpha_{12}$ has been shown to regulate MDCK TJs at least in part through the Src kinase signaling pathway (177, 178), and in Caco-2 cells, oxidative stress-induced disruption of TJs is mediated by the activation of c-Src (179). In ras-transformed MDCK cells, down-regulation of the MAPK pathway restored the TJ structure and barrier function (180). Likewise, activation of the Erk1/2 MAP kinase pathway induces TJ disruption in human corneal epithelial cells (181). In contrast to these studies, MAPK has also been suggested to mediate EGF-induced prevention of TJ disruption through its interaction with occludin (182). The polarity complex protein Par-3 regulates TJ assembly through EGFR signaling (183). Ouabain-treatment of LLC-PK1 has been suggested to transactivate EGFR in a pump-independent manner (118, 119). However, palytoxin down-modulates the epidermal growth factor receptor (EGFR) through a sodium-dependent pathway in Swiss 3T3 cells (184, 185). Palytoxin binds to the Na,K-ATPase, basically converting it to an open channel resulting in increased intracellular sodium (186, 187). As EGFR is a common element in the signaling pathways activated by cell volume changes in isosmotic, hyposmotic, or hyperosmotic conditions (188), it remains to be determined how tight junction regulation through EGFR activation by Na,K-ATPase is connected. Na,K-ATPase also plays a role in IP3 receptor, PI3 kinase, and PLCγ-1 signaling, all pathways that also have been implicated in the regulation of TJ function (2-5, 189). PP2A associates with both Na,K-ATPase and occludin and studies from our laboratory showed that inhibition of Na,K-ATPase enzyme activity inhibits PP2A activity, leading to the hyperphosphorylation of occludin and decrease in the TJ barrier in HPAF-II cells (45). The relative contributions of pump-dependent and -independent signaling by Na,K-ATPase in regulating tight junction permeability remain to be determined (190).

Many pathways that regulate TJ function also regulate Na,K-ATPase, such as IFN-γ (191), growth factors, PKC, and many more (98, 153). One of the best-known transcriptional regulators associated with the loss of TJ function in cancer cells is Snail. This transcription suppressor is induced in cells undergoing EMT and reduces the transcription of genes associated with junctional complexes in epithelial cells (28, 29, 192-194). Interestingly, Na,K-ATPase β_1-subunit is transcriptionally suppressed by Snail, whereas the α-subunit is not affected (195). It is possible that Snail targets a set of proteins associated with junctional complexes to accomplish EMT in cancer cells.

8. SODIUM/ION HOMEOSTASIS AND TIGHT JUNCTIONS

Recently, several ion transporters and channels have been identified as having a function to modulate TJ structure and paracellular permeability (72). As the function of Na,K-ATPase is crucial to maintaining the intracellular ion homeostasis, it is possible that some of the effects of other channels/transporters on TJ function are connected to the function of Na,K-ATPase in the paracellular barrier. For example, apical glucose uptake through the Na^+-glucose transporter SGLT-1 induces a drop in TER (196) and increases paracellular permeability in cultured Caco-2 cells as well as *in vivo* in rats and in healthy human subjects (197, 198). Also, the intestinal Na^+/H^+ exchanger NHE3 has been shown to regulate TER (199). We can envision that the signaling scaffolding complex of the Na,K-ATPase might sense changes in the intracellular ionic milieu, as the transport processes of SGLT-1 and NHE3 are probably associated with an increase in the intracellular Na^+ concentration. These changes in Na^+ concentration might then target the phosphorylation status of TJ proteins through signaling events involving the Na,K-ATPase leading to alterations in TJ structure and function. Recently, it has been shown that ouabain treatment of LLC-PK1 cells downregulated NHE3 activity and expression. Liu's group reported that activation of the Na,K-ATPase receptor complex by ouabain at concentrations that do not increase intracellular Na^+ regulates the trafficking of NHE3 (127, 128). It is possible that Na,K-ATPase might act to integrate changes in intracellular ionic milieu as well as signals obtained from the extracellular environment to regulate TJ permeability.

9. A ROLE FOR Na,K-ATPASE IN EPITHELIAL-MESENCHYMAL TRANSITION (EMT) AND CANCER

TJs are crucial for the normal structure and functioning of epithelial cells. In cancer, in the course of malignant cell transformation, TJs are generally lost (200). Coincidentally, changes in Na,K-ATPase function have been reported either as an increase in activity (201-203) or as inhibition (204). There is evidence that changes in Na,K-ATPase activity are already present at very early stage of tumorigenesis, even before gross tumors develop (204, 205). Changes in Na,K-ATPase subunit levels have also been reported in poorly differentiated cell lines (129, 195) and in tumors/tumor cell lines including kidney (206, 207), colon (208), prostate (209, 210), pancreas (211), and lung (212); in hepatic (211), breast (211), and bladder cancer (213), as well as neuroblastoma (211) and metastatic melanoma (214). In bladder and kidney cancer, the α- and β-subunit expressions predict recurrence and survival, respectively (207, 213). Other studies emphasized the presence of specific cancer-related FXYD proteins such as FXYD3 (Mat-8, a mammary tumor marker) in breast and prostate cancer and FXYD5 that is expressed in cancer tissues but only a few normal cell types (80, 215).

Together, these studies suggest that alterations in Na,K-ATPase function and expression might be associated with the loss of TJs in the process of tumorigenesis.

10. CONCLUSIONS

With our present understanding of Na,K-ATPase's role in TJ structure and function, it is quite possible that altered Na,K-ATPase function/expression might be a contributing factor in the development of cancer and other diseases associated with TJ malfunction. For example, mutations in the γ-subunit of Na,K-ATPase in kidney, FXYD2, have been linked to dominant renal hypomagnesemia (216). Interestingly, mutations in claudin16 (paracellin-1) were found in autosomal recessive hypomagnesemia (217). Whether FXYD2 and claudin-16 are functionally linked remains to be determined. Studies in inflammatory bowel disease, Crohn's disease, and ulcerative colitis indicated a decrease in Na,K-ATPase activity (218-220). Recent studies suggest a more direct role for Na,K-ATPase in these diseases, as pro-inflammatory cytokines inhibit Na,K-ATPase to downregulate the intestinal barrier function (191, 221). These studies suggest that Na,K-ATPase is a multifunctional protein, and changes in its function might be associated with many human diseases. Deciphering how Na,K-ATPase function is altered might provide insight into disease mechanisms as well as novel therapeutic approaches.

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Abbreviations: TJ: tight junction; SJ: septate junction; ZO: zonula occludens; MUPP: multi-PDZ protein; IFN: interferon; MDCK: Madin-Darby canine kidney; EGFR: epidermal growth factor receptor; TER: transepithelilal electrical resistance; HGF: hepatocyte growth factor; TGF: transforming growth factor; EMT: epithelial to mesenchymal transition; HNF: hepatocyte nuclear factor; CK: casein kinase; aPKC: atypical protein kinase C; PP2A: protein phosphatase 2A; VEGF: vascular endothelial growth factor; PI3: phosphatidyl-inositol 3; TNF: tumor necrosis factor; JAM: junctional adhesion molecule; MAGUK: membrane-associated guanylate kinase; ZONAB: ZO-1 associated nucleic acid binding; PKA: cAMP-dependent protein kinase A; PKG: cGMP-dependent protein kinase; PKC: Ca-phospholipid-dependent protein kinase; MAPK, mitogen-activated protein kinase; ROS: reactive oxygen species; MSV: Maloney sarcoma virus

Key Words: Na,K-ATPase, α-subunit, β-subunit, Tight Junction, polarity, FXYD, Occludin, Signaling, Review

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