

**Phosphorylation of the Tight Junction Protein Occludin Regulates Epithelial
Monolayer Proliferation and Maturation**

by

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The self-made man is a myth. Hard work, perseverance, and independence are all important of course, but everyone who has achieved anything in life has been helped along the way. In my case, this list of individuals is long and can't be included in its entirety here. There are a few key individuals who must be mentioned though.

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LIST OF ABBREVIATIONS

ABAM	Anti-Biotic Anti-Mycotic
ABMH	4-amino-5-(bromomethyl)-2-methylpyrimidine
ABR	Actin Binding Region
AJ	Adherens Junction
AJC	Apical Junctional Complex
ANOVA	Analysis of Variance
ATCC	American Tissue Culture Collection
BEST-1 Cre	Bestrophin-1 Cre
bTJ	Bicellular Tight Junction
CAM	Cell Adhesion Molecule
CaMKII	Calcium/Calmodulin Dependent Kinase 2
CAR	Coxsackie Adenoviral Receptor
CIP	Contact Inhibition of Proliferation
CMV	Cytomegalovirus
DVL	Disheveled
ECIS	Electric Cell-substrate Impedance Sensing
EMT	Epithelial to Mesenchymal Transition
EV	Empty Vector
FBS	Fetal Bovine Serum
GJ	Gap Junction
GRK	G protein-coupled Receptor Kinase

GRK Inhib 22	G protein-coupled Receptor Kinase Inhibitor 22
GSK	Glycogen Synthase Kinase
GuK	Guanylate Kinase
IF	Immunofluorescence
IP	Immunoprecipitation
kD	Kilodalton
KD	Knockdown
KO	Knockout
LATS1/2	Large Tumor Suppressor 1 and 2
MAGUK	Membrane Associated Guanylate Kinase
MARVEL	MAL and Related Proteins for Vesicle Trafficking and Membrane Link
MDCK	Madin-Darby Canine Kidney
MET	Mesenchymal to Epithelial Transition
MST1/2	Mammalian Sterile 20-Like 1 and 2
NMR	Nuclear Magnetic Resonance
PBS	Phosphate-Buffered Saline
PDZ	PSD/DlgA/ZO-1
PLK	Polo-like Kinase
S471A Occ	Serine to Alanine Mutant Occludin
S471D Occ	Serine to Aspartic Acid Mutant Occludin
S471N Occ	Serine to Asparagine Mutant Occludin
SAV1	Salvador

TAMP	Tight Junction-Associated Protein
TAMRA	Tetramethylrhodamine
TAZ	Transcriptional Co-Activator with PDZ Binding Motif
TBS	Tris-Buffered Saline
TER	Trans-epithelial Electrical Resistance
tTJ	Tricellular Tight Junction
TJ	Tight Junction
TROSY	Transverse Relaxation-Optimized Spectroscopy
WT Occ	Wild Type Occludin Overexpression
YAP	Yes-Associated Protein
ZO-1, 2, 3	Zonula Occludens-1, 2, 3
ZONAB	ZO-1 associated Nucleic Acid Binding Protein

ABSTRACT

Barriers against the external environment are crucial for sustaining life in multicellular organisms. In mammals, epithelial barriers form following convergent growth and development of cell-cell junctions. The characteristics of these junctions greatly influence the attributes of the tissues they compose, and vary considerably based on the location and function of the tissue. At least four types of epithelial cell-cell junctions exist, the most apical of which is known as the tight junction (TJ). Barrier forming tissues are often semi-permeable in nature owing to TJ architecture and the organization of the over 40 involved proteins. These can be transmembrane or cytosolic, and are involved in cell signaling in addition to their barrier functions. A specific transmembrane protein known as occludin is highly phosphorylated on its C-terminal coiled-coil, and certain sites have been found to regulate specific aspects of TJ function, including the response to certain cytokines.

Previously, our lab discovered a novel phosphosite at serine 471 that is located at a contact site with an important scaffolding protein and central organizer of the TJ, zonula occludens-1. Phosphoinhibitory, serine to alanine (S471A) occludin point mutant MDCK cell lines demonstrate that S471A monolayers are poorly organized with mislocalized TJ proteins, increased small solute flux, and decreased electrical resistance compared to WT occludin (WT Occ) or phosphomimetic, serine to aspartic acid (S471D) lines. Additionally, S471A monolayers are composed of fewer, larger cells than controls, and exhibit proliferative arrest almost immediately following confluency, in contrast to control lines, which go through at least one additional round of proliferation. This phenotype including larger, less numerous cells and a low resistance barrier can

be recapitulated with a cell cycle inhibitor, demonstrating that confluent proliferation or cell packing is necessary for barrier maturation. G-protein coupled receptor kinase (GRK) was confirmed to be an S471 kinase by inhibitor experiments from a bioinformatically compiled candidate kinase list, and GRK inhibitors were able to recapitulate the disrupted monolayer and low resistance barrier of S471A lines.

Finally, S471A expression perturbed purified coiled-coil stability as determined by NMR. Modeling of inter-coil interactions identified several possible hydrogen bonds in addition to the negative charge of the phosphate that differ between the phosphorylated and non-phosphorylated forms. Expression of S471N (asparagine) transgenic occludin *in vitro* demonstrated highly organized border organization similar to WT Occ and in stark contrast to S471A, despite the lack of a negative charge at the S471 position. This result suggests that the border organization of p-S471 is not due to the negative charge at S471, and may be the result of differential intra-coil hydrogen bonding. In conclusion, cell packing is necessary for barrier maturation, and is regulated by the novel phosphosite, occludin S471. S471 is an important contributor to confluent proliferation, monolayer maturation, and barrier resistance, and plays a role in the barrier regulatory function of occludin.

CHAPTER I

Introduction

Multicellular organisms are by definition composed of multiple cells working together to accomplish processes necessary for survival of the organism. Multicellular organismal composition can be advantageous in that it allows for division of labor among cells or groups of cells, which in turn can lead to morphological specialization that assists in completion of a specific task. Groups of morphologically and occupationally similar cells can be composed into tissues, which can accomplish a dizzying array of functions throughout the organism. In the case of epithelial cells, examples include regulating solute and water balance in the tubules of the kidney, facilitating absorption of water, nutrients, and electrolytes in the intestine, or activating site specific dermal proliferation in response to injury to close a wound in the skin.

For individual cells to be able to coordinate their actions as part of a tissue, it is imperative that cell-cell signaling mechanisms for communication be present, and in the case of barrier forming tissues, a physical connection between adjacent cells must exist. Barrier forming tissues bestow obvious advantages on an organism by excluding potentially pathogenic material from the internal environment. In many cases however, it is crucial that these barriers be semi-permeable, allowing passage of certain substances while blocking others. This is true in the kidney tubule, in which various segments are more permeable either to solutes or to water, allowing the establishment and maintenance of an interstitial concentration gradient by which urine can be concentrated, allowing for reabsorption of valuable solutes and regulating water loss

(Vize et al., 2003). Others occur in immune-privileged environments such as the mammalian testis, where adjacent sertoli cells form a barrier that excludes immune and macromolecules while allowing small molecule diffusion, or in the eye, in which the cells of the retinal-pigmented epithelium allow passage of oxygen and nutrients from choroidal blood vessels to the metabolically active outer retina while preventing passage of potentially deleterious solutes (Frey and Antonetti, 2011; Mruk and Cheng, 2015). While multiple types of cell-cell junctions exist and are important for cell and tissue functionality, the current project focuses primarily on tight junctions (TJs), which are classically known to regulate paracellular flux, and are increasingly implicated in additional roles such as cell signaling. The current dissertation introduces the structure and relevant background of the four main cell-cell junctions, followed by two chapters of experimental data, and ending with discussion of the implications of the work and identification of limitations and future directions.

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1.1 Aim and Scope

TJs are composed of many different proteins, some of which are cytosolic while others are transmembrane. A specific transmembrane protein known as occludin plays an integral role in TJ regulation, and modification of its activity is associated with disease (further discussed in chapter II). The current project is based on previous work demonstrating the existence of specific phosphorylation sites that contribute to occludin-mediated regulation of the TJ (Murakami et al., 2009; Raleigh et al., 2011; Sundstrom et al., 2009), (further discussed in chapter III). Proteomic experiments performed in the Antonetti Laboratory identified a number of novel phosphorylation sites (Sundstrom et

al., 2009). The current study aims to investigate the role of a specific site, serine 471 (S471). S471 is a unique and particularly interesting site in that it sits at the center of an interaction domain between occludin and a known organizer and regulator of the TJ, the scaffolding protein zonula occludens-1 (ZO-1). As a result, we postulated that S471 may contribute to TJ integrity (such as permeability to ions, small molecules, or larger molecules), and might influence TJ morphology. Furthermore, I examined kinases that might phosphorylate S471 and probed what interactions at the molecular level might provide mechanistic understanding of any effects. Given the nature of the questions under investigation, a simple, *in vitro* system was needed and was used in the described experiments, supplemented by structural work using peptides and protein fragments.

1.2 Study Overview

The current dissertation opens with a review of relevant literature broken into two parts: the first includes topics such as the overview, structure and function of the main cell-cell junction types and important proteins present at these junctions. The second introduces the concepts of confluent proliferation, cell transitions, the role of biophysical forces, and other factors important in epithelial monolayer formation and maturation. This material is presented in chapter 2: Literature Review.

Original data presentation centers on characterizing the effects of occludin S471 phosphorylation and determining the mechanisms at the cellular and molecular levels by which these effects occur. Characterization of S471 phosphorylation was accomplished by expressing phosphoinhibitory (S471A) or phosphomimetic (S471D)

occludin in an immortalized epithelial cell line and observing the effects on TJ protein organization and barrier function. This approach is informative in that serine and alanine are structurally identical except that serine has an ethanol side chain that can be phosphorylated, while alanine's side chain terminates in a methyl group that cannot. Aspartic acid (D) has a carboxylic acid side chain that carries a permanent negative charge similar to a phosphate group, and mimics constitutive phosphorylation. In the course of these experiments, it was unexpectedly discovered that inhibition of S471 phosphorylation has dramatic effects on high confluency cell proliferation and monolayer maturation, leading to numerous studies measuring cell proliferation rate in the various mutants to further explore this effect. These experiments led to identification of the necessity of cell packing for monolayer maturation and identification of a novel regulatory role for occludin in this process. Since S471 is a recently identified site about which very little was known, substantial effort was devoted to identifying the kinase or kinases that phosphorylate this site. This was done initially through a screen of bioinformatically identified putative kinases, and later confirmed with multiple inhibitors to kinases of interest. These studies form the bulk of the dissertation, and are reported in chapter 3: Occludin S471 Phosphorylation Contributes to Tight Junction Formation and Cell Packing at Confluence.

As a result of the findings of chapter 3, structural experiments were performed in collaboration with colleagues at Pennsylvania State University School of Medicine, John Flanagan and Maria Bewley, to investigate specific interactions at the molecular level that might underlie the *in vitro* effects. Nuclear magnetic resonance (NMR) experiments were carried out by our collaborators, which suggest that peptides expressing occludin

S471A or S471D mutations shift the structure of the occludin coiled-coil.

Phosphorylation of serine changes the charge of the residue from neutral to negative, and adds a side chain phosphate group that is sterically larger, potentially leading to additional hydrogen bonding and other interactions with neighbors. To examine the role of the negative charge apart from size and other factors, WT Occ, S471A, or S471N occludin was transiently transfected into MDCK cells followed by immunofluorescence (IF) to evaluate organization of TJ proteins at the border. S471N mutant occludin retains the ability to hydrogen bond with nearby amino acids and is structurally very similar to S471D, but lacks the negative charge of the S471D or phosphorylated S471. These studies indicate that negative charge at S471 is not necessary for organized TJ protein localization at the border, suggesting that the effect of phosphorylation is through hydrogen bonding, or some other factor. These results are reported in chapter 4: Negative Charge at Occludin S471 is Not Necessary for Proper Localization and Organization of Tight Junction Proteins at the Border.

Following presentation of the results of chapter 4, the dissertation closes with a discussion of important findings and overall conclusions. Methodological limitations are discussed, and areas for future investigations and broader application of the presented findings are identified. These discussions make up chapter 5: Conclusions, Limitations, and Future Directions.

CHAPTER II

Literature Review

2.1 Epithelial Cell-Cell Interactions

Tissues in multicellular organisms are composed of many cells that act in a coordinated manner to adapt tissue characteristics in ways that are vital for survival. This requires individual cells to be connected to facilitate communication and coordinated action as a single tissue sheet or organ rather than a conglomeration of many individual cells. These connections can be specialized for specific and diverse functions ranging from propagating electric signals in cardiomyocytes to regulating water and solute permeability in kidney tubule cells, or restricting movement of blood-borne solutes across the outer blood retinal barrier into the predominantly immunoprivileged eye. In epithelial cells, four basic cell-cell junctions exist.

2.1.1 Gap Junctions

Gap junctions (GJs) are basolaterally located and specialize in enabling cell-cell signaling (Fig. 2.1). GJs are channels that span both membranes of adjacent cells and facilitate the flow of ions or other signaling molecules, allowing for rapid signal transduction between cells (Harris, 2001). Classically, they are composed of members of a tetraspan transmembrane protein family known as connexins, which are arranged in a cylindrical pattern to form a hemichannel with an aqueous pore at the center (Herve and Derangeon, 2013). Six connexins form a hemichannel on each side of the membrane that connects to a hemichannel on an adjacent cell to form a mature,

junction-spanning channel. GJs are ubiquitously expressed, and play an especially pivotal role in cardiomyocytes, where they assist in action potentials transmission between cells, leading to coordinated contractile activity (Stroemlund et al., 2015). Alterations in connexin expression or organization are associated with cardiac arrhythmias and decreased contractile function (Severs et al., 2008). Other electrically active cell types, including neural cells, can also be affected. Mutation of the GJ protein, connexin 32, in Schwann cells of the peripheral nervous system leads to X-linked Charcot-Marie-Tooth Disease and distal limb weakness (Nualart-Marti et al., 2013). Additionally, murine KO models of various connexins suffer from a range of symptoms ranging from arrhythmias (connexin 40) to cardiac related embryonic lethality (connexins 43 and 45), stressing the importance of GJs in highly conductive tissues (London, 2004).

2.1.2 Desmosomes

Just apical to GJs are desmosomes, cell-cell contacts involved in cell adhesion and traditionally described as “spot welds” (Getsios et al., 2004). Desmosomes develop soon after cell-cell contact, and are composed of several proteins including transmembrane members of the cadherin superfamily, desmoglein and desmocollin, along with cytosolic desmoplakin, plakoglobin (also known as γ -catenin), and plakophilin (Nekrasova and Green, 2013). Desmosome formation is associated with the adherens junction (AJ), specifically expression of E-cadherin (Lewis et al., 1997). Desmosomes are unable to form in E-cadherin KD cell lines, but are rescued by re-

expression of E-cadherin along with plakoglobin. Plakoglobin hepatic KO in mice compromises desmosome composition, rendering the animals more susceptible to tissue injury following insult compared to controls (Zhou et al., 2015). Similarly, humans with the autoimmune disease pemphigus vulgaris express anti-desmoglein antibodies in keratinocytes, which disrupts desmosomes (Sajda et al., 2016). This leads to decreased cell-cell adhesion and dermatological disruption, including chronic blistering. The association of desmosomal dysfunction with additional diseases including cardiomyopathy and cancer further indicates the importance of desmosomes in maintaining overall tissue integrity (Notari et al., 2015; Otsubo et al., 2015).

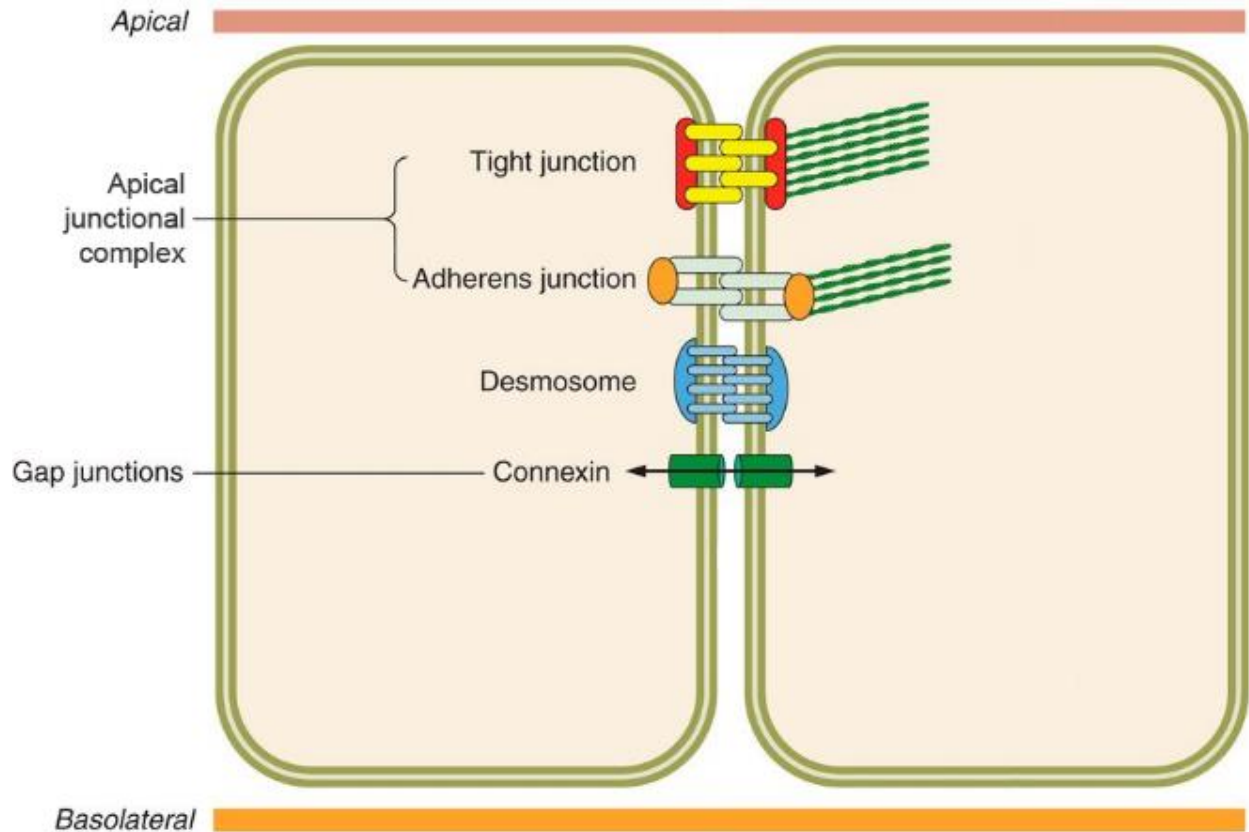


Fig. 2.1: Epithelial cell-cell interactions. (A) Schematic of the four main epithelial junctions: Gap junctions, desmosomes, adherens junctions, and tight junctions. Adherens junctions and tight junctions make up the apical junctional complex (AJC). (Image modified from Rossier et al., 2015).

2.1.3 Adherens Junctions

The apical junctional complex (AJC) is located apical to GJs and desmosomes, and is composed of AJs, and TJs (discussed in a later section) (Anderson et al., 2004; Farquhar and Palade, 1963; Tsukita et al., 1992). As the name would suggest, AJs (also known as zonula adherens) contribute to cell-cell adhesion, but also establish a paracellular barrier to large solutes. AJs are present in both vertebrates and invertebrates (Oda and Takeichi, 2011), and are thought to be the first junction to form

following cell-cell contact (Sajda et al., 2016). Recent studies have identified an increasingly broad AJ role that includes maintaining differentiation and discouraging tumorigenesis (Berx and van Roy, 2009; Stockinger et al., 2001).

AJs are composed of multiple proteins including a transmembrane protein known as E-cadherin, which has an intracellular, transmembrane, and extracellular domain (Shapiro and Weis, 2009). E-cadherin serves as a major AJ cell adhesion molecule (CAM), and forms homophilic dimers in *cis* and *trans* in a Ca^{2+} dependent manner (Pertz et al., 1999). Cadherin-cadherin *trans* dimers are an important form of initial contact between converging cells that facilitates monolayer formation, and *trans* binding is sufficient to decrease proliferative rate, even in subconfluent cells (Kim et al., 2011; Perrais et al., 2007). The importance of E-cadherin *in vivo* is further illustrated by the embryonic lethality of E-cadherin KO mutant mice (Larue et al., 1994). Finally, E-cadherin expression is inversely correlated with many types of cancer (Giroldi et al., 1994), and is a known tumor suppressor (Jeanes et al., 2008). E-cadherin loss is associated with dedifferentiation and epithelial to mesenchymal transition (EMT), and is correlated with increased tumorigenesis and metastasis (Berx and van Roy, 2009, See section 2.2).

Intracellularly, E-cadherin interacts with β -catenin, which in turn binds to α -catenin, which binds to F-actin, functionally connecting the AJ to the cytoskeleton (Gottardi and Gumbiner, 2001; Huber and Weis, 2001). α -catenin deficient cells exhibit decreased aggregation (Watabe et al., 1994), and α -catenin KO has a similar effect *in vivo* (Vasioukhin et al., 2001). The role of α -catenin as a linker between β -catenin and the cytoskeleton has become controversial however, leaving the possibility that

additional proteins may be involved (Watabe-Uchida et al., 1998; Yamada et al., 2005). A potential contributor is p120-catenin (also known as CTNND1), which interacts with E-cadherin and stabilizes it in the cell membrane. Expression of mutant p120 leads to increased E-cadherin internalization, but this effect is rescued by WT p120 (Liu et al., 2007). E-cadherin-catenin complex-based AJs contribute to AJ formation and development (Halbleib and Nelson, 2006), and together with the nectin-afadin complex plays a prominent role in cell adhesion (For discussion of nectin-afadin, see last paragraph of this section).

In addition to connecting E-cadherin to the cytoskeleton via α -catenin, β -catenin is necessary for the regulation of proliferation by cadherin-cadherin *trans* interactions (Perrais et al., 2007). Free β -catenin can also translocate to the nucleus and serve as a transcriptional co-activator (MacDonald et al., 2009). In this way, β -catenin serves as an important component of the Wnt signaling pathway (MacDonald et al., 2009). In the absence of Wnt signaling, β -catenin is bound by a complex that includes glycogen synthase kinase (GSK), ubiquitinated, and marked for proteasomal degradation, while Wnt binding to its receptor inhibits GSK and allows β -catenin to translocate to the nucleus and transcribe genes involved in proliferation (Fig. 2.2, Baron and Kneissel, 2013). While progress has been made, additional work is needed to identify the subtleties of β -catenin's diverse cellular functions (Gottardi and Gumbiner, 2001; Shapiro, 2001).

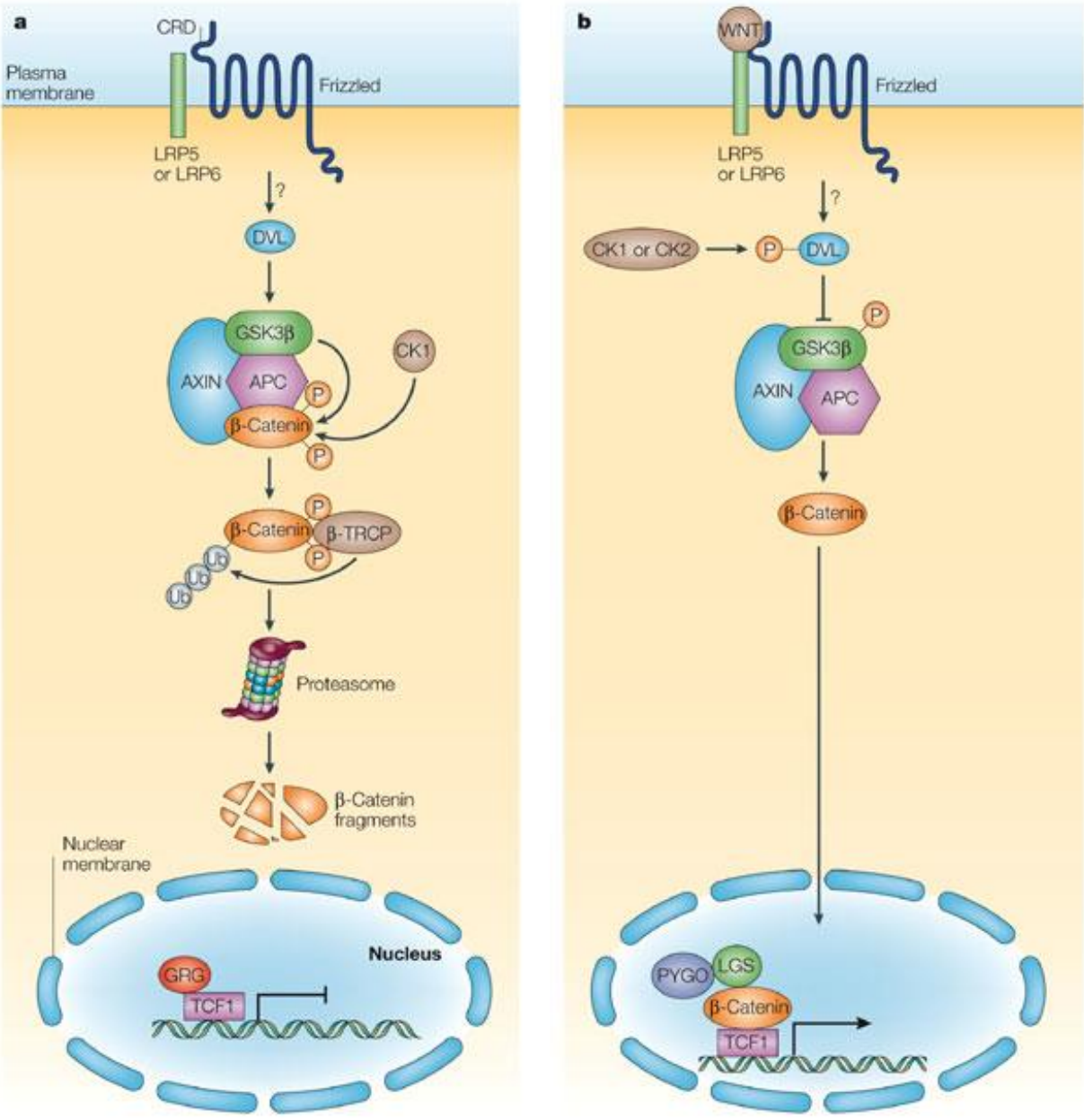


Fig. 2.2: Wnt signaling pathway. The Wnt signaling pathway mediates proliferation through regulating the cellular localization of β -catenin. When Wnt is inactive, β -catenin is sequestered in the cytosol, bound by the β -catenin destruction complex, and degraded. When Wnt is active, binding of Wnt to its receptor releases β -catenin from the complex and allows it to translocate to the nucleus, where it drives transcription of pro-proliferative genes as a transcriptional co-activator. (Image from Staal and Clevers, 2005)

Nectin is a second transmembrane protein CAM that forms *trans* homodimers, and plays a role in cell adhesion similar to E-cadherin, but acts independent of Ca^{2+} (Fig. 2.3, Takahashi et al., 1999). Nectin is connected to the cytoskeleton by binding to the PDZ domain of the linker protein, afadin, which connects to the cytoskeleton through its F-actin binding domain (Mandai et al., 1997). The nectin-afadin complex seems to be associated with the E-cadherin- α , β -catenin complex, as nectin and afadin colocalize with E-cadherin in cells that do not express tight junctions (TJs), and nectin helps localize E-cadherin at the junction (Mandai et al., 1997; Tachibana et al., 2000). Furthermore, microbead-bound E-cadherin extracellular domain binds the catenins, but also localizes the nectin-afadin complex to cell-bead junctions (Honda et al., 2003). Inhibition of the nectin complex also inhibits E-cadherin AJs (Honda et al., 2003); however, studies presented in this dissertation demonstrate that E-cadherin and β -catenin can be localized at the border even when afadin is mislocalized (see chapter 3). Murine afadin KO impairs both ectoderm organization and mesoderm migration (Ikeda et al., 1999). Paracellular permeability is also increased, as is susceptibility to tissue injury induced by dextran sulfate sodium (Tanaka-Okamoto et al., 2011). Furthermore, there is evidence that the nectin-afadin complex is involved in AJ and TJ formation. TJ proteins such as claudin, occludin, and zonula occludens-1 (ZO-1) localize to the junction in nectin-expressing MDCK cells, and ZO-1 initially binds near the nectin-afadin complex, then migrates apically (Fukuhara et al., 2002a; Fukuhara et al., 2002b). Microbeads bound to the nectin extracellular domain also localize with ZO-1 (Fukuhara et al., 2002a). More recent studies indicate that following its temporary interaction with afadin, ZO-1 moves on and interacts with the TJ protein, junctional adhesion molecule

(JAM, see next section) (Ooshio et al., 2010). Afadin KD in MDCK cells prevents both AJ and TJ formation, while KD of the TJ protein ZO-1 only affects TJs (Ooshio et al., 2010). Finally, inhibition of nectin dimerization prevents localization of occludin or claudin at the junction (Fukuhara et al., 2002b), but nectin-afadin complexes and TJs are formed in the absence of the cadherin complex (Yamada et al., 2006). While the exact protein-protein interactions and mechanistic sequence of events remains elusive, these findings strongly suggest that the nectin-afadin complex makes important contributions to AJ and TJ formation.

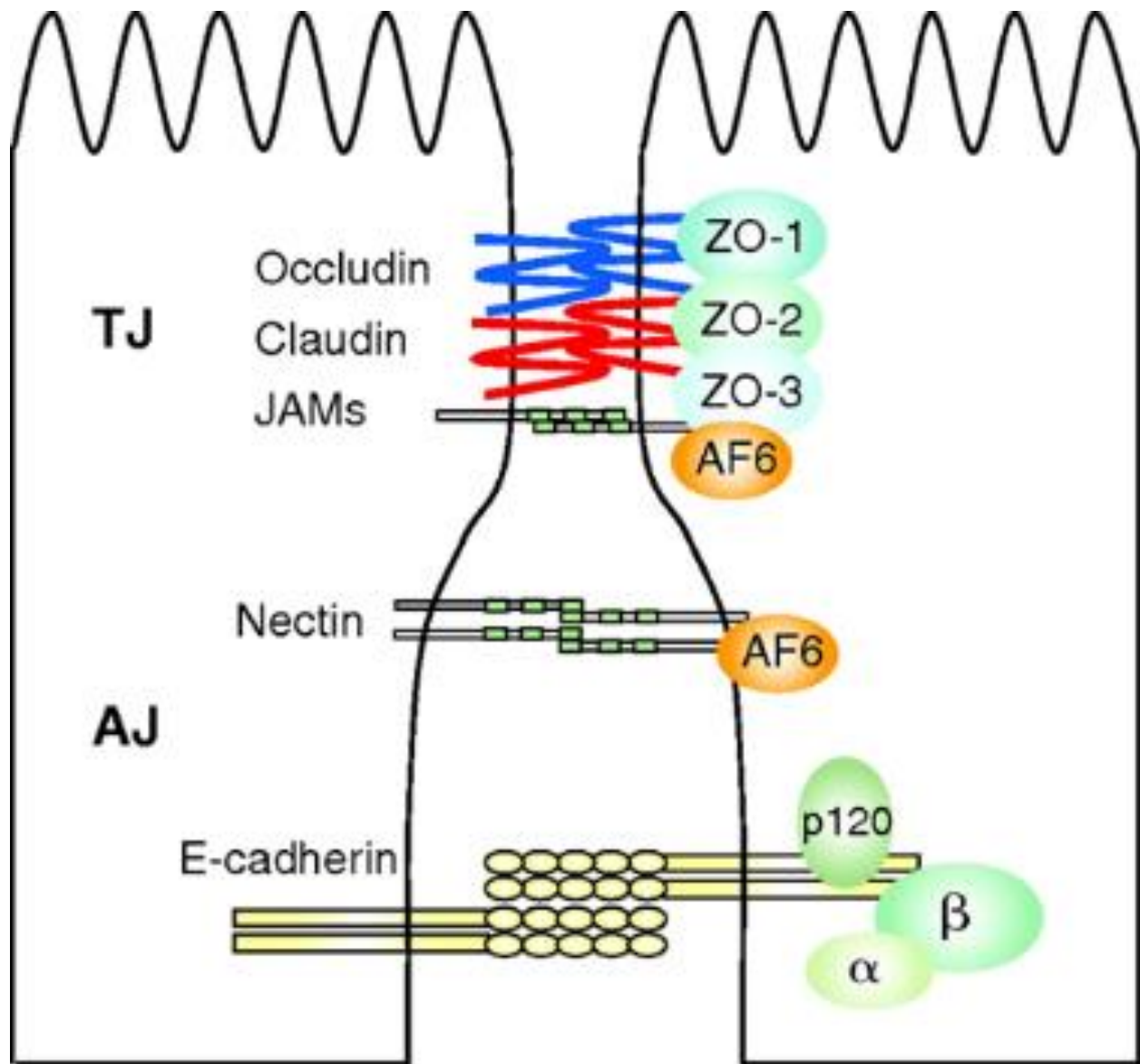


Fig. 2.3: Structure of the apical junctional complex (adherens and tight junctions). Adherens junction (AJ) and tight junction (TJ) between two adjacent epithelial cells, depicting relevant proteins. These include the transmembrane proteins occludin, claudin, JAM, nectin, and E-cadherin, and the cytoskeletal proteins ZO-1, 2, 3, afadin (AF6), and p120, α and β catenin. (Image from Kooistra et al., 2007).

2.1.4 Tight Junctions

Adherence of adjacent cells by establishment of AJs is followed by the formation of other specialized cell-cell contacts known as tight junctions (TJs). TJs are a feature of many different types of epithelial and endothelial cells in many species of vertebrates, and are homologous to the septate junctions found in *Drosophila melanogaster* (Willott et al., 1993). First described in 1963 (Farquhar and Palade, 1963), TJs traditionally have the roles of maintaining apical-basolateral polarity by restricting the movement of specific membrane proteins to the opposite side of the cell (the fence function, which has become controversial in recent years, (Umeda et al., 2006), and establishing selectively permeable paracellular barriers between adjacent cells (the gate function, D'Atri and Citi, 2002; Diamond, 1977). In the latter case, this barrier is vital for selective absorption of electrolytes and solutes in tissues such as the kidney and intestine, and also allows for establishment of blood barriers in immunoprivileged tissues including the brain, eye, and testes. Freeze fracture electron microscopy images show that TJs take the form of many strands laterally encircling the apical cell, interacting with each other and with TJ proteins on membrane leaflets of the adjacent cell (Fig. 2.4, Staehelin, 1973; Staehelin, 1974).



Fig. 2.4: Tight Junction Strands Visible Via Freeze-Fracture Microscopy.

Electron microcopy image of epithelial cell apical aspect. Adjacent cell membrane has been removed to expose the Intertwining TJ strands in the YZ plane (arrow, image modified from Furuse, 2010).

Transmembrane TJ Proteins

Over 40 TJ proteins have been identified to date including transmembrane proteins, which facilitate restriction of paracellular flux, and cytosolic proteins which serve as organizers of transmembrane proteins and connect them to the cytoskeleton (Schneeberger and Lynch, 2004). The result is a continuous junction that stretches from the cytoskeleton across the membrane to TJ proteins on adjacent cells (Fanning et al., 1998). The tetraspan transmembrane protein, occludin (also called MARVELD1, Furuse et al., 1993) is located in the membrane of mature epithelial monolayers,

forming homodimers in *cis* through its coiled-coil domain (Nusrat et al., 2000; Raleigh et al., 2011), and interacting homophilically in *trans* (Bellmann et al., 2014). Peptides encoding a central region of the second extracellular occludin loop (AA 210-228) bind occludin and other transmembrane TJ proteins, and interact with the TJ *in vitro* to the exclusion of endogenous TJ proteins (Nusrat et al., 2005). Occludin is an important contributor to the TJ as overexpression increases trans-epithelial electrical resistance (TER) in MDCK cells (Balda et al., 1996; Van Itallie et al., 2010), and partial deletion of either extracellular loop attenuates the overexpression-mediated TER increase (Balda et al., 2000). However, occludin KD/KO yields complex results. As the first transmembrane TJ protein discovered, occludin was originally assumed to physically “occlude” paracellular flux and was named accordingly. This notion was subsequently dismissed however when occludin KO mice were found to have TJs that were anatomically normal and did not exhibit increased permeability or decreased TER (Saitou et al., 2000; Schulzke et al., 2005). Similarly, occludin KD via siRNA in MDCK cells did not affect TER in the steady state or following a Ca^{2+} switch assay (Yu et al., 2005). Closer analysis revealed however that occludin KO animals exhibit a constellation of phenotypes consistent with barrier dysregulation including brain calcification, male sterility, maternal inability to nurse, and gastric epithelial hyperplasia (Saitou et al., 2000; Schulzke et al., 2005). These mice also undergo hair cell apoptosis in the inner ear, resulting in deafness several weeks after birth (Kitajiri et al., 2014). A possible explanation for the absence of increased TJ permeability in these animals is compensation by a related protein with structural similarities, known as tricellulin or MARVELD2 (discussed later in this chapter), which is normally present at tricellular tight

junctions (tTJs) but is found at bicellular tight junctions (bTJs) in these animals and after occludin KD *in vitro* (Ikenouchi et al., 2008; Kitajiri et al., 2014). These findings have contributed to the prevailing view that occludin acts as an important regulator of the TJ. Occludin is a multi-phosphorylated protein, particularly on its C-terminal coiled coil region (Sundstrom et al., 2009). Early studies found that truncation of the coiled-coil region leads to increased permeability and decreased TJ organization, and changes in TER are correlated with changes in phosphorylation (Balda et al., 1996; Clarke et al., 2000). Increasingly, accumulating evidence suggests that specific amino acids mediate the TJ response to specific factors or cytokines and represent a mechanism of occludin-mediated TJ regulation (see chapter 3 for further discussion).

Additional studies have identified an inverse relationship between permeability and occludin content across many tissues (Antonetti et al., 1998; Hirase et al., 1997; Wong and Gumbiner, 1997), and identified a role for occludin in diverse diseases. Occludin is downregulated in many inflammatory diseases, including Crohn's and ulcerative colitis (Bertiaux-Vandaele et al., 2011; Coeffier et al., 2010; Vivinus-Nebot et al., 2014). TJs present a physical obstacle for pathogen entry into cells, and occludin is frequently targeted by viruses including: Norovirus (Troeger et al., 2009), HIV (Nazli et al., 2010), and Dengue virus (Talavera et al., 2004), and bacterial (including *Campylobacter jejuni* (Elmi et al., 2015), *Porphyromonas gingivalis* (Nakajima et al., 2015), and *Pseudomonas aeruginosa elastase* (Nomura et al., 2014) pathogens. Hepatitis C viral infection is normally restricted to cells of primate origin, but infection of murine cells is possible by overexpressing occludin. Additionally, KD of occludin in normally permissive human cells attenuates infection, suggesting that occludin is

necessary for hepatitis C infection. (Ploss et al., 2009). Furthermore, certain viruses, including Coxsackie B and Adenoviruses, bind to a specific transmembrane protein known as the Coxsackie Adenoviral Receptor (CAR) to facilitate cellular entry (Coyne and Bergelson, 2006). Occludin is endocytosed along with Coxsackie B virus and is necessary for infection, demonstrating utilization of the TJ and occludin by pathogenic microorganisms (Coyne et al., 2007).

A new role for TJ proteins as regulators of proliferation and the cell cycle has recently been discovered, specifically for occludin loss as an emerging factor in the development of several types of cancer (Tobioka et al., 2004; Nemeth et al., 2009). Downregulation or loss of TJ proteins including occludin is associated with decreased epithelial character and epithelial to mesenchymal transition (EMT), associated with increased cancer risk (see chapter 3 for discussion.) These findings confirm the physiological importance of occludin for exclusion of inflammatory factors and pathological agents as well as for proliferative control, and demonstrate increased disease susceptibility when altered.

Occludin is a member of the myelin and lymphocyte (MAL) and related proteins for vesicle trafficking and membrane link (MARVEL) protein family, which specialize in membrane coherence and are especially prevalent in cholesterol rich regions (Sanchez-Pulido et al., 2002). In addition, occludin is one of three known members of the tight junction-associated MARVEL protein (TAMP) subfamily, which form selective barriers based on size (Raleigh et al., 2010). All three TAMPs have a similar tetraspan transmembrane structure and cellular roles that are at least partially unique (Raleigh et

al., 2010). The remaining two members of the TAMP family are tricellulin (also known as marvelD2), and the recently discovered marvelD3 (Steed et al., 2009).

As suggested by its name, tricellulin is typically found at tTJs, and is recruited there by the novel tTJ protein, LSR (Masuda et al., 2011). The convergence of three cells requires a specific junctional connection. The cells are assembled with a central tube at its center, a space approximately 10 nm in diameter and 1 μ m in length (Staehelin, 1973). Flux at this tube is regulated by tricellulin, and while the tTJ is a minor route for small ion flux, it is thought to play a significant role in the flux of macromolecules (Krug et al., 2009). Despite its tricellular localization, both tTJ and bTJ are poorly organized after tricellulin KD, indicating a tricellulin effect on bTJs as well (Ikenouchi et al., 2005). Tricellulin is found at bTJs in cases of occludin loss or tricellulin overexpression in animal and cell models, and may compensate for occludin (Ikenouchi et al., 2008; Krug et al., 2009). Tricellulin mutant or KO mice are viable, but exhibit deafness due to loss of K⁺ gradient in endolymph and subsequent apoptosis of hair cells in the organ of Corti of the inner ear (Kamitani et al., 2015; Nayak et al., 2013). Additionally, post-natal deafness observed in occludin KO animals is proposed to be due to tricellulin compensation via tricellulin reallocation from tTJs to bTJs, and the resulting lack of tricellulin at tTJs (Kitajiri et al., 2014).

MarvelD3 is a recently identified TAMP which localizes to bTJs, and colocalizes with occludin and the scaffolding protein ZO-1 (Raleigh et al., 2010). MarvelD3 does not interact homophilically in *trans* (on adjacent cells, and opposite sides of the paracellular space), but interacts with itself, occludin, and tricellulin in *cis* (on the same side of the paracellular space, Cording et al., 2013). TJ assembly as assessed by

maximum TER is delayed but not inhibited by marvelD3 silencing, and marvelD3 expression is unable to rescue this delay in the absence of tricellulin or occludin, suggesting at least partially non-redundant function (Raleigh et al., 2010). Instead, marvelD3 is involved in proliferative control, as KD increases proliferation in Caco-2 intestinal cells while re-expression in tumor cells decreases proliferation (Steed et al., 2014). MarvelD3 expression decreases phosphorylation of the MAPK family member, JNK, which is involved in the response to environmental stresses, and activates nuclear transcription factors controlling proliferation and apoptosis (Davis, 2000; Steed et al., 2014). This results in decreased JNK activity leading to decreased proliferation. Additionally, marvelD3 is downregulated during EMT in cancer cells (Kojima et al., 2011). This suggests that marvelD3 contributes to proliferative regulation, possibly by acting as a connector between JNK and the TJ.

In addition to the TAMPs, a second tetraspan transmembrane protein family, known as the claudins, is also found at TJs. Claudins regulate paracellular flux and are found at regions where adjacent cell membranes are so close that the paracellular space is undetectable by electron microscopy, known as kissing points (Tsukita et al., 2001). Claudin subtypes form homo and heterodimers in *cis* and interact with each other homophilically in *trans* (Cording et al., 2013; Piontek et al., 2011). There are currently 27 known members of the claudin family in mammals (Soini, 2011), some of which seal the barrier (1,3,5) while others, known as conductive claudins, form selective pores (Krug et al., 2014). Selectivity can be for anions (17), cations (15), or for water (2) (Gunzel and Fromm, 2012; Krug et al., 2014). Furthermore, the claudins can be classified as either classic (ex. 1-10) or non-classic (ex. 11-13) based on sequence

homology (Krause et al., 2008). *In vitro/in vivo* KD/KO of various members of the claudin family results in increased permeability (claudins 4 and 18, (Kage et al., 2014; Li et al., 2014), aberrant permeability regulation (claudins 2 and 10 (Breiderhoff et al., 2012; Muto et al., 2010) or lethality (claudin 1, (Furuse et al., 2002). Additionally, claudin perturbation is associated with many clinical pathologies including inflammatory bowel disease (Weber et al., 2008), Crohn's disease (Zeissig et al., 2007), ulcerative colitis (Oshima et al., 2008), breast and colon cancer (Kinugasa et al., 2010; Kulka et al., 2009), and the dermatological disease, ichthyosis (Hadj-Rabia et al., 2004), Taken together, these findings strongly suggest an important role for claudins in TJ functionality.

Finally, members of the third TJ transmembrane protein family are known as junction adhesion molecules (JAMs). The JAM family consists mainly of three members, JAMs A-C, which differ in amino acid sequence but share an intracellular sequence approximately 50 amino acids in length (Ebnet et al., 2004). A larger subfamily of proteins, including JAM4, are related but exhibit prominent differences, including length of intracellular tail. Epithelial cells express JAM-A, which forms homodimers in *cis* and interact with adjacent homodimers in *trans* (Kostrewa et al., 2001; Prota et al., 2003). Prevention of homodimerization disrupts localization and decreases TER following Ca^{2+} switch, suggesting a role for JAM-A in barrier assembly (Mandell et al., 2004). JAM-A KD decreases TER and increases solute flux, indicating a leaky barrier (Laukoetter et al., 2007). Additionally, genetic deletion of JAM-A increases intestinal permeability, and renders mice more susceptible to dextran sulfate sodium-induced tissue damage (Laukoetter et al., 2007). This is in agreement with studies

reporting that JAM-A is reduced in human inflammatory bowel disease (Vetrano et al., 2008), and is decreasingly localized at TJs by the inflammatory cytokine, interferon gamma (Bruewer et al., 2005). JAMs also interact with Par3 of the Par3/Par6/aPKC polarity complex and assist in localizing the complex to the TJ (Ebnet et al., 2001). While JAMs can be found very near TJ strands in MDCK cells, JAM expression in TJ null cells is not sufficient to form TJ strands (Itoh et al., 2001). This is in contrast to claudins and suggests that JAMs do not share the paracellular flux restricting role of claudins (Furuse et al., 1998). Occludin, the claudins, and the JAMs therefore have differing roles in the restriction, facilitation, and regulation of paracellular flux.

Scaffolding TJ Proteins

The cytosolic domains of occludin, the claudins, and the JAMs interact with a family of scaffolding proteins located in the cytosolic plaque near the cell membrane, known as the zonula occludens (ZO-1, 2, and 3) (Itoh et al., 1999; Li et al., 2005; Lye et al., 2010). Originally identified in 1986, ZO-1 is a central organizer of the TJ that has many binding partners including transmembrane, cytosolic, and cytoskeletal proteins, making it a linker between the cytoskeleton and the barrier (Fanning et al., 1998; Stevenson et al., 1986). All three ZO proteins contain 3 PSD/DlgA/ZO-1 (PDZ) regions with which they interact with transmembrane proteins like claudins and JAMs, and with each other (Lye et al., 2010). They are also members of the membrane associated guanylate kinase (MAGUK) protein family, and have the characteristic guanylate kinase-like (GuK) domain, which serves as a specialized Ser/Thr binding pocket and facilitates interaction with occludin (Tash et al., 2012; Zhu et al., 2011). In addition, ZO-1/2 have

an N-terminal actin binding region (ABR) with which they interact with the cytoskeleton (Fig. 2.5).

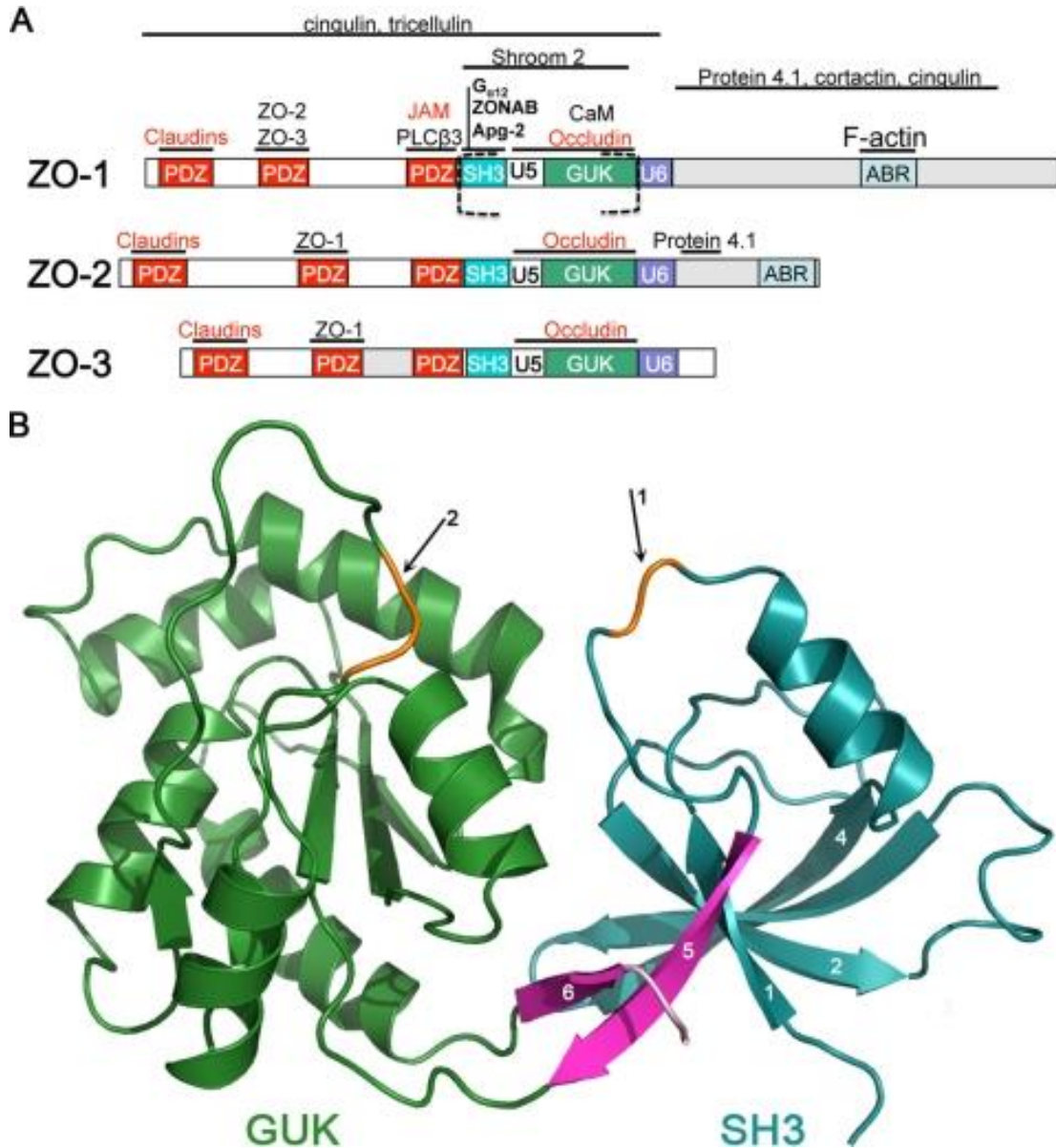


Fig. 2.5: Structure and domains of zonula occludens (ZO) proteins. ZO-1, 2, and 3 are scaffolding proteins that interact with many binding partners via their multiple binding domains. All three have three PDZ domains and one SH3, U5, GUK, and U6 domain. ZO-1/2 also have an N-terminal actin binding region (ABR) while ZO-3 does not. Proteins that interact with ZO at specific sites are noted above each domain. (Image from Lye et al., 2010).

Truncation and deletion experiments have determined that the first two PDZ domains, the SH3, and both the U5 and U6 domains are all necessary for ZO-1, claudin-2, and occludin localization at junctions (Rodgers et al., 2013). The interaction between ZO-1 and F-actin is not necessary for junctional localization of these proteins, but deletion of the ZO-1 ABR changes cell border morphology from wavy and tortuous to very straight, suggesting changes to cytoskeletal organization. While a TJ protein, ZO-1 also associates with proteins of other cell-cell contacts including AJs (α -catenin) and GJs (connexin) (Itoh et al., 1997; Kausalya et al., 2001). ZO-1/2 have at least partially overlapping roles leading to compensation in single KD experiments (Umeda et al., 2006). As a result, ZO-1/2 double KD cells are badly disorganized, fail to assemble the TJ, and exhibit reduced TER, but ZO-1 or ZO-2 single KD cells do not (Rodgers et al., 2013; Umeda et al., 2006). This compensation appears to be insufficient *in vivo* however, as both ZO-1 and ZO-2 single KO mutations are embryonic lethal (Katsuno et al., 2008; Xu et al., 2008). ZO-3 exogenously expressed in ZO-1/2 double KD, ZO-3 null cells fails to locate at the border, and ZO-3 KO mice are viable, suggesting distinct roles for ZO-1/2 and ZO-3 (Umeda et al., 2006; Xu et al., 2008).

Proliferation and AJC Proteins

Recent studies have demonstrated novel roles for the AJC in cell signaling and growth control in addition to canonical regulation of paracellular permeability/cell adhesion. In contrast to the seemingly static nature of AJC proteins in fixed images, high trafficking of some TJ proteins (Shen et al., 2008) and distinct protein localization, creates separate protein pools. Proteins such as p120 catenin, ZO-2 and β -catenin locate to the nucleus where they can influence transcriptional activity (Spadaro et al.,

2012; Zhang et al., 2011). In the case of β -catenin, the target is the Wnt pathway transcription factor, TCF/LEF, which transcribes pro-proliferative genes in response to β -catenin (MacDonald et al., 2009). β -catenin KO is sufficient to reduce proliferation-induced hepatic tumors (Singh et al., 2014), suggesting that the role of E-cadherin in proliferative control may simply be to sequester β -catenin at the membrane, preventing nuclear translocation (see section 2.2). In addition to stabilizing E-cadherin at the membrane, p120-catenin can also localize to the nucleus and influence transcriptional activity. Increased nuclear p120 catenin localization is implicated in breast cancer progression via antagonizing the transcriptional repressor, Kaiso (van de Ven et al., 2015), and p120 phosphorylation at specific sites is associated with increased lung cancer invasiveness (Zhang et al., 2011). Conditional KO mouse studies have demonstrated that p120-catenin also has tumor suppressive activity (Schackmann et al., 2013).

Occludin is present at multiple cellular locations, and plays a particularly important role in growth control. Occludin is located at centrosomes in cells and increases Ser490 phosphorylation upon proliferation (Runkle et al., 2009). Phosphorylation of occludin S471 is associated with confluent proliferation, and phosphorylation inhibition at this site results in proliferative arrest (see chapter 3). Loss of occludin is also associated with increased proliferation in cells and intestinal hyperplasia in occludin KO animals (Saitou et al., 2000; Wang et al., 2005). Crucially, occludin re-expression in Raf-1 transformed cells is sufficient to reverse the proliferative phenotype of these cells both in vitro, and upon implantation in mice (Wang et al., 2005). These findings indicate a role for occludin in proliferative regulation.

Additionally, involvement in proliferation has been observed in several other AJC proteins. JAM-A KO/KD increases cell proliferation, but this effect can be rescued through re-expression (Nava et al., 2011). This is suggested to be due to JAM-A antagonism of Akt, and the prevention of β -catenin activation and nuclear translocation, demonstrating TJ regulation of specific proliferation-associated signaling pathways (Nava et al., 2011). In addition, ZO-1/2 are both present at the nucleus in subconfluent cells and after wounding, but locate to the TJ upon confluence (Gottardi et al., 1996; Islas et al., 2002). ZO-2 is clearly involved in proliferative control, though reports conflict as to whether it increases or decreases proliferation. ZO-2 is reportedly associated with decreased cell proliferation (Gonzalez-Mariscal et al., 2009; Huerta et al., 2007) and increased apoptosis (Walsh et al., 2010), and with cancer when mRNA levels are decreased (Paschoud et al., 2007). However, ZO-2 KO mice exhibit decreased proliferation and increased apoptosis (Xu et al., 2008). This suggests that ZO-2 may act in a context specific manner. Finally, the ZO proteins interact with ZO-1-associated nucleic acid binding domain (ZONAB), a TJ-associated transcription factor that interacts with ZO-1 at its SH3 domain and regulates promoter activity (Balda and Matter, 2000). Cell proliferation and density are increased by ZONAB overexpression and decreased by depletion (Balda et al., 2003). Cytosolic sequestration of ZONAB decreases cell proliferation and nuclear localization of both ZONAB and cell division kinase 4, suggesting that ZONAB may contribute to proliferative control at least in part through nuclear trafficking of other proteins (Balda et al., 2003). These findings dispute the archaic model of the AJC as a static, monofunctional structure, and instead indicate that AJC proteins actively participate in cell signaling and growth control.

2.2 Cell Monolayer Formation

2.2.1 Cellular Transitions

Mammalian bodily tissues develop from one of the three germ layers that emerge following gastrulation: ectoderm (skin, hair), mesoderm (bones, blood vessels), and endoderm (liver, pancreas) (Gilbert, 2000; Solnica-Krezel and Sepich, 2012). The phenotype of differentiated cells is influenced by lineage. This is particularly evident in comparing mesoderm-derived mesenchymal cells, which are characterized by motility, invasiveness, and migratory ability, with epithelial cells which are stationary and express adhesion proteins that connect them to their neighbors (Kalluri and Weinberg, 2009; Thiery, 2002). Phenotypic plasticity is such that in certain situations during development or in pathology, cells of epithelial or mesenchymal origin will take on characteristics of and transition into the other type of cell (Nakaya and Sheng, 2013; Sheng, 2015; Yao et al., 2011; Yilmaz-Ozcan et al., 2014). This occurs in a process known as epithelial to mesenchymal transition (EMT), which is a natural part of embryogenesis and development, but is also implicated in cancer development and has been the focus of intensive studies (Kalluri, 2009; Kalluri and Weinberg, 2009).

The inverse and less studied process is known as mesenchymal to epithelial transition (MET). While MET has recently been implicated in re-differentiation of secondary tumors, it also plays a role in specific developmental events, most notably in the kidney (Rothenpieler and Dressler, 1993; Yao et al., 2011). During early kidney development, a mass of epithelium known as the ureteric bud is exposed to growth factors by surrounding mesenchyme that induces branching of the epithelium, forming

primitive structures that will eventually become nephrons (Herzlinger et al., 1992; Saxén, 1987). In most tissues, the surrounding mesenchyme contributes growth factors and influences development of the encircled epithelium, but does not itself become part of the mature organ. However, through MET, some of these nephrotic mesenchymal cells differentiate into epithelium and are incorporated into the mature kidney. The transition from densely packed mesenchyme to epithelium begins roughly 24 hours after induction, and is completed within the next 12 hours (Davies and Garrod, 1995). During this period, previously mesenchymal cells stop expressing mesenchymal proteins such as fibronectin and instead express AJ and desmosomal proteins such as E-cadherin and the desmoplakins in conjunction with polarization (Garrod and Fleming, 1990; Thiery, 2002; Vestweber et al., 1985; Yilmaz-Ozcan et al., 2014). Transitioning cells also lose mesenchymal characteristics such as high motility, linear proliferative rate, and inability to form cell-cell junctions, and take on epithelial characteristics including decreased motility, decreased proliferative rate, and mature cell-cell junctions that form a mature monolayer. This process of MET may be modeled in cell culture as cells reach confluency and differentiate into a mature epithelium (Puliafito et al., 2012).

2.2.2 Contact Inhibition

Contact inhibition of proliferation (CIP) is a characteristic of epithelia in which previously proliferative cells come into physical contact with each other, and greatly decrease or cease proliferative growth despite the continued presence of nutrients (McClatchey and Yap, 2012; Puliafito et al., 2012). Motility is also affected in highly confluent cells in a related process known as contact inhibition of locomotion (CIL, also called contact mediated inhibition of cell migration), defined as cessation of cell motility

in a specific direction following cell-cell contact (Abercrombie, 1979; Batson et al., 2013; Mayor and Carmona-Fontaine, 2010; Puliafito et al., 2012). Both processes have the obvious advantages of facilitating mature monolayer formation while discouraging tumorigenesis. CIL at low confluency often leads to motility in a different direction following a collision and thus cell scattering, but effectively prevents single cell motility in high confluency conditions (Aston et al., 2010; Puliafito et al., 2012). This is evident in developing epithelial monolayers and during wound healing, and loss of CIL is thought to be a contributor to metastatic potential (Astin et al., 2010; Middleton, 1972; Puliafito et al., 2012; Vermeulen et al., 1995). In MDCK, subconfluent cells exhibit certain mesenchyme-like characteristics such as linear proliferative growth, cell motility about the substrate, development of cellular protrusions, and lack of clear TJ border staining (Mishima et al., 2002; Puliafito et al., 2012). These characteristics are lost however in favor of differentiated epithelial characteristics following cell-cell contact, CIP, and CIL, leading to sweeping morphological changes, most prominently the loss of protrusions and acquisition of the familiar cobble-stone appearance. While CIP and CIL are both important processes for the MET-like changes that occur during monolayer formation, the mechanisms behind CIL remain largely elusive (reviewed in Mayor and Carmona-Fontaine, 2010). Accordingly, the following sections will primarily just consider the contributions of CIP.

2.2.3 Hippo/MST Pathway

An important contributor to CIP is the Hippo/MST Pathway, a cell membrane to nucleus signaling pathway originally discovered in *Drosophila melanogaster* that is involved in proliferation and cell survival, and alters transcription based on the

extracellular environment. Epithelial cells express the AJ protein E-cadherin, which has an extracellular domain that interacts homophilically when adjacent cells that come within close physical proximity. E-cadherin binding initiates a kinase cascade in the canonical hippo/MST pathway in which the central serine/threonine kinase, Mammalian Sterile 20-like 1 and 2 (MST1/2, known as Hippo in *Drosophila*), is bound to a scaffolding protein known as Salvador (SAV1), and phosphorylates the downstream kinases Large Tumor Suppressor 1 and 2 (LATS1/2, Warts in *Drosophila*) (Gumbiner and Kim, 2014; Johnson and Halder, 2014; Kim et al., 2011). LATS1/2 interacts with a scaffolding protein and co-factor known as MOB1 and phosphorylates Yes-Associated Protein 1 (YAP) and Transcriptional-Co-Activator with PDZ Binding Motif (TAZ, also known as WWTR1). YAP and TAZ are both transcriptional co-activators, and must associate with other transcription factors such as TEAD or SMAD to drive transcription. When the Hippo/MST Pathway is off, YAP and TAZ are dephosphorylated and free to locate to the nucleus where they interact with TEAD and SMAD and transcribe genes involved in cell proliferation and survival. When the Hippo/MST Pathway is activated, YAP and TAZ are phosphorylated by LATS1/2, excluded from the nucleus, and marked for ubiquitination and eventual degradation, preventing proliferative transcription (Fig. 2.6, Johnson and Halder, 2014).

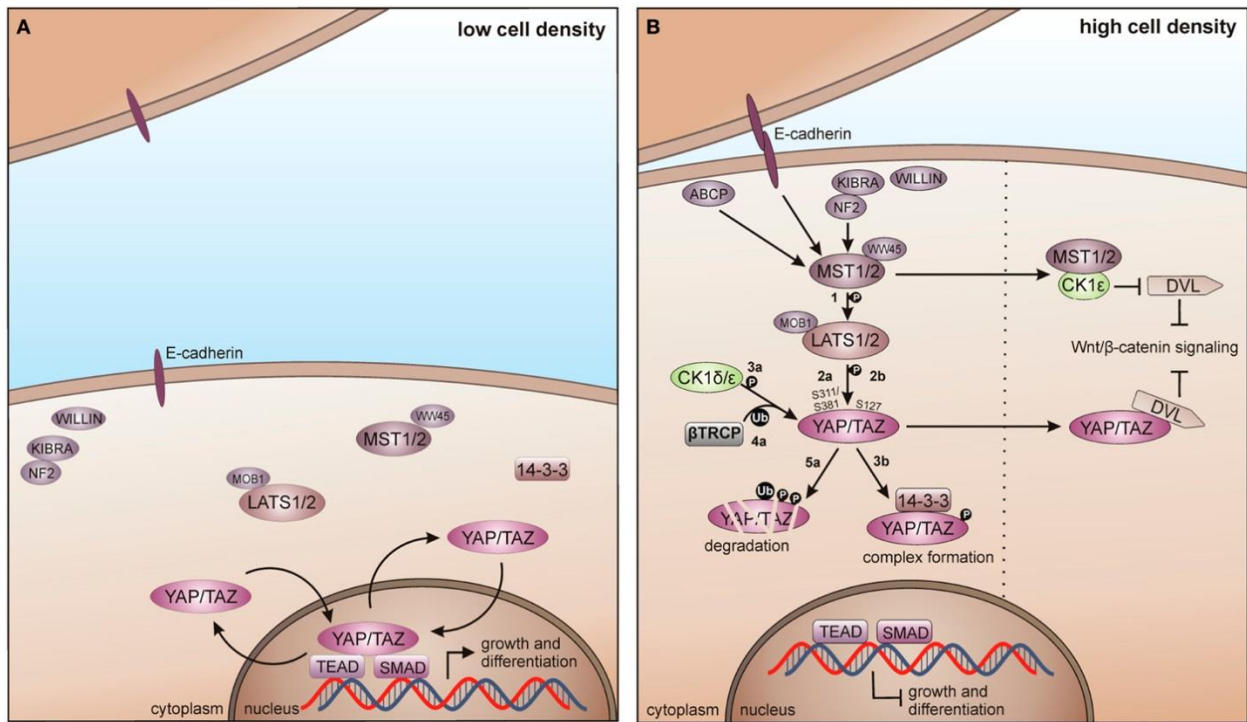


Fig. 2.6: Vertebrate Hippo/MST signaling. Schematics of (A) inactive and (B) active Hippo/MST signaling. When Hippo/MST is inactive, YAP/TAZ are not phosphorylated and free the transcriptional co-activators, YAP/TAZ, allowing their translocation to the nucleus where they are responsible for transcription of pro-proliferative genes. When Hippo/MST is activated through homophilic E-cadherin interaction as a result of high cell density, the kinase initiates a series of phosphorylation events that ultimately phosphorylates YAP/TAZ, excluding them from the nucleus and decreasing proliferation. (Image from Knippschild et al., 2014).

2.2.4 E-cadherin, β -catenin, and CIP

E-cadherin plays an important role in CIP and proliferative control, primarily by binding β -catenin of the Wnt signaling pathway (discussed in an earlier section) and initiating Hippo/MST signaling. Microbeads expressing E-cadherin extracellular domain are able to suppress MDCK proliferation in a manner dependent on β -catenin and Hippo/MST (Kim et al., 2011; Perrais et al., 2007). Expression of extracellular domain-deleted E-cadherin decreases proliferation, while intracellular domain-deleted E-

cadherin does not, suggesting that β -catenin/E-cadherin binding is necessary for this effect while E-cadherin homophilic binding is not (Sasaki et al., 2000). Consistent with this, exogenous E-cadherin expression rescues proliferative control in epithelial cells that have undergone EMT, but the intracellular β -catenin binding domain is necessary for this effect (Stockinger et al., 2001). β -catenin localization is a key determinant in carcinoma tumor cell characteristics, as nuclear β -catenin is associated with dedifferentiated, mesenchymal cells, while cytoplasmic and membranous β -catenin is typical of a more epithelial phenotype (Brabletz et al., 2001). These findings indicate that the E-cadherin/ β -catenin interaction is important in proliferative control, potentially by excluding β -catenin from the nucleus and preventing transcription of specific genes. Reports conflict however as to whether E-cadherin/ β -catenin binding decreases β -catenin transcriptional activity (Perrais et al., 2007; Stockinger et al., 2001). Finally, both Hippo/MST and Wnt contribute to proliferative control, and there is interaction between these pathways through their member proteins, YAP/TAZ of Hippo/MST pathway and β -catenin of Wnt pathway. When Hippo/MST is inactive and YAP/TAZ are cytosolic, YAP/TAZ interact with the upstream Wnt protein, disheveled (DVL), preventing its phosphorylation and subsequent Wnt signaling, or bind to the β -catenin destruction complex directly, excluding β -catenin from the nucleus (Imajo et al., 2012; Varelas et al., 2010). Conversely, activation of Wnt releases YAP/TAZ from this complex, and facilitates nuclear translocation and transcription of target genes (Azzolin et al., 2014). Thus, Hippo/MST activation discourages proliferation directly through nuclear exclusion of YAP/TAZ, and through inhibition of Wnt. Wnt encourages proliferation directly by preventing degradation of and allowing for nuclear translocation

of β -catenin, and indirectly by releasing YAP/TAZ from the β -catenin destruction complex, thus encouraging nuclear translocation (see Fig. 2.7).

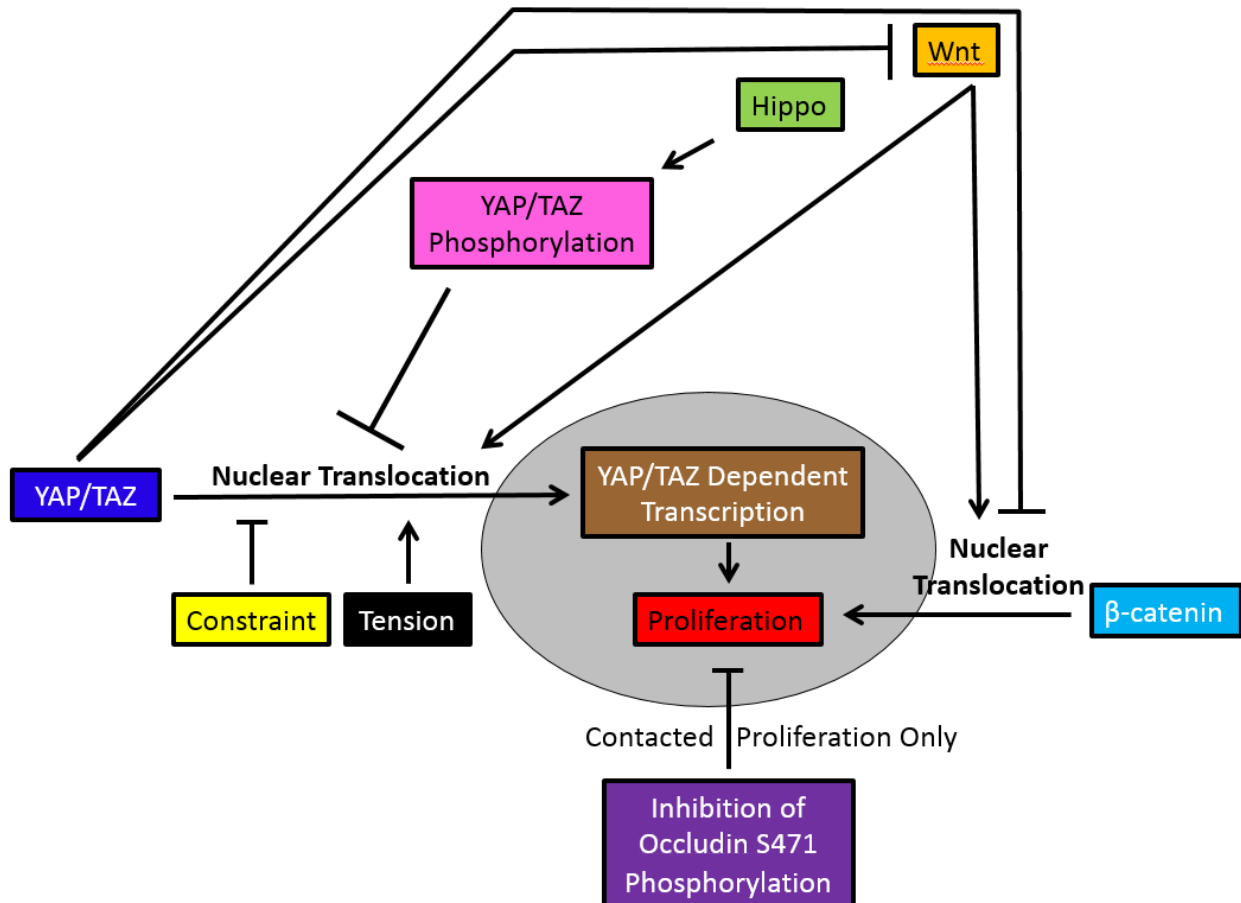


Fig. 2.7: Crosstalk between the Hippo/MST and Wnt signaling pathways. Hippo/MST discourages proliferation directly through YAP/TAZ phosphorylation and nuclear exclusion (nucleus in gray), and indirectly through cytosolic YAP/TAZ inhibition of Wnt and β -catenin nuclear translocation. Wnt encourages proliferation directly and indirectly by enabling nuclear translocation of β -catenin and YAP/TAZ, respectively.

2.2.5 Biophysical Forces and Confluent Proliferation

Recent studies have demonstrated a new, canonical Hippo/MST independent contributor to proliferative control: biophysical force. When cells proliferate in subconfluent conditions, mother cells divide, leaving two daughter cells with approximately half the area of the mother. These daughter cells then undergo hypertrophic growth until they are approximately the same size as the mother cell and can divide again (Puliafito et al., 2012). This process continues until contact with other cells is made, and is driven at least in part by biophysical forces experienced by cells due to hypertrophic and proliferative growth, extrusion, and locomotion. Cells that are not surrounded by neighbors on all sides such as those in low confluency culture or at the periphery of a proliferating colony experience tensile force as they migrate away from the colony and are simultaneously pulled towards it by adhesion to neighbors (Puliafito et al., 2012). Tensile force is known to promote nuclear localization of YAP and proliferation, and subconfluent cells subjected to stretching of the ECM proliferate at an increased rate and exhibit increased nuclear YAP percentage compared with controls (Aragona et al., 2013). Conversely, cells within a confluent environment such as those in confluent monolayers or at the center of a proliferating colony experience inward constraining forces from neighbors and relief of tensile force, resulting in decreased nuclear YAP and proliferation rate. LATS1/2 of the Hippo/MST pathway excludes YAP from the nucleus through phosphorylation at specific sites (Pan, 2010). Tensile relief from plating on a soft substrate decreases YAP/TAZ dependent transcription and nuclear localization, even in subconfluent conditions, in the absence of LATS1/2, or upon expression of LATS1/2 insensitive YAP, suggesting a Hippo/MST

independent mechanism (Aragona et al., 2013; Dupont et al., 2011). Similarly, proliferation and nuclear YAP/TAZ percentage progressively decrease as cells are plated on patterned substrates of decreasing area, and both ROCK (Rho-associated protein kinase) and YAP/TAZ are necessary for this effect (Dupont et al., 2011). Tensile relief via treatment with the Rho inhibitor, C3, ROCK inhibitor, Y27632, non-muscle myosin inhibitor, blebbistatin, or cytoskeletal inhibitor, latrunculin A, increase YAP/TAZ nuclear exclusion and decrease YAP/TAZ dependent transcription. Tensile restoration by siRNA KD of F-actin capping and severing proteins is sufficient to restore proliferative capacity and nuclear YAP localization, even on soft substrate or under dense plating conditions (Aragona et al., 2013). Taken together, these studies indicate that biophysical forces are sufficient for YAP/TAZ regulation and act in a Hippo/MST pathway, MST1/2 and LATS 1/2 independent manner.

Finally, cells do not become proliferatively quiescent immediately following cell-cell contact, but instead progress through additional rounds of confluent proliferation, which is size reductive in nature (Fig. 2.8, Aragona et al., 2013; Puliafito et al., 2012). Analysis of cell area following proliferation shows that newly divided cells in confluent environments do not decrease the area of existing cells, suggesting that they are not compressible. However, the constraint exerted by neighboring cells restricts normal post-proliferative hypertrophic growth on newly divided cells, resulting in size reductive proliferation. As cell area decreases due to successive divisions, a critical cell area is eventually reached below which proliferation is dramatically reduced. This is known as the transition point, and is marked by a sudden, dramatic decrease in proliferative rate leading to CIP (Puliafito et al., 2012). As previously noted, evidence suggests that the

gradual reduction in tension and increase in constraining force as confluency increases excludes YAP from the nucleus independent of Hippo/MST, and is a major contributor to CIP. These findings affirm the existence of confluent, size reductive division, and suggest that CIP is a complex behavior that is mediated in part by cell density through evolving biophysical forces. As reviewed in the previous sections, AJC proteins play an important role in cell-cell interactions, junctional assembly, and proliferation control, and despite recent milestone advances in determining the mechanism of cell packing, it remains unknown what, if any, influence AJC proteins have in this process. The current dissertation examines cell packing and monolayer maturation, and describes for the first time a specific role for occludin phosphorylation in facilitating these processes. Results presented in the following chapters add to our understanding of monolayer maturation, and to the increasingly diverse cellular role of the AJC.

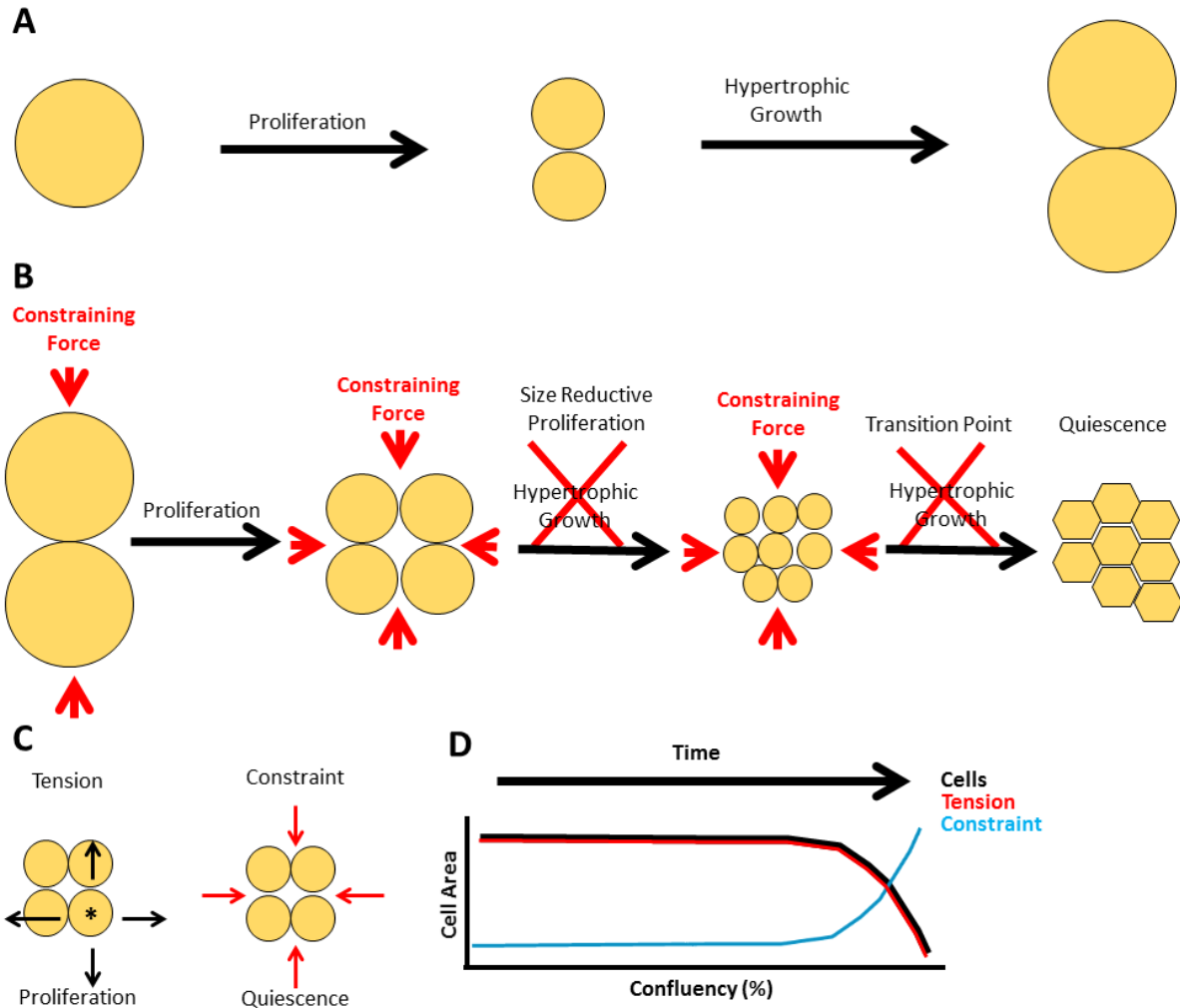


Fig. 2.8: Schematic of size reductive proliferation in MDCK cells. MDCK cells go through a process known as cell packing at high confluency, which is characterized by size reductive proliferation and regulated at least in part by biophysical force. (A) Subconfluent mother cells divide, yielding two daughter cells of approximately 50% size compared to the mother. The daughter cells then attain the size of the original mother through hypertrophic growth. (B) Following many rounds of subconfluent proliferation, increasing confluency leads to constraining force from adjacent cells. This inhibits hypertrophic growth following proliferation, leading to a 50% reduction in cell area at each division. This is termed size reductive proliferation. Ultimately, a critical cell area known as the transition point is reached, after which proliferation dramatically decreases, leading to quiescence. (C) Tension on an individual cell (asterisk) from simultaneous migration away from neighboring cells and adherence to them induces proliferation (Aragona et al., 2013). Constraint from neighboring cells at high confluency relieves this tension, and decreases proliferation. (D) As cells proliferate and become increasingly confluent, cell area and tension decrease, while constraint increases, leading to size reductive proliferation, cell packing, and ultimately, to proliferative quiescence.

CHAPTER III

Occludin S471 phosphorylation contributes to tight junction formation and cell packing at confluence¹

3.1 Abstract

Multiple organ systems require epithelial barriers for normal function, and barrier loss is a hallmark of diseases ranging from inflammation to epithelial cancers. However, the molecular processes regulating epithelial barrier maturation are not fully elucidated. After contact, epithelial cells undergo cell packing and tight junction maturation creating a dense, highly ordered monolayer with high resistance barriers. We provide evidence that the tight junction protein, occludin, contributes to regulation of epithelial packing upon phosphorylation of S471 in its coiled-coil domain. Overexpression of an occludin S471A mutant that cannot be phosphorylated prevents cell packing, proper tight junction protein localization, and electrical resistance in a dominant manner. Inhibition of cell proliferation in confluent but immature monolayers recapitulated this phenotype. A kinase screen identified G-protein coupled receptor kinases (GRK) as S471 kinases, and GRK inhibitors delayed epithelial packing and junction maturation. We conclude that cell packing is necessary for barrier maturation, and that occludin contributes to these processes in a phosphosite specific manner.

3.2 Introduction

¹ Portions of this chapter are published in Bolinger et al., 2016. Molecular and Cellular Biology.

Cells characteristically form epithelial monolayers through logarithmic growth when subconfluent followed by cell to cell contact, and concluding with contact inhibition of proliferation (CIP), proliferative quiescence, and epithelial monolayer maturation including tight junction (TJ) formation (Li et al., 2004; Puliafito et al., 2012). CIP is an important step in monolayer maturation that is mediated in part by activation of the Hippo/MST pathway. Hippo/MST involves a signaling cascade with multiple mechanisms of regulation that may be initiated by homophilic interactions between extra cellular domains of the adherens junction (AJ) protein E-cadherin on adjacent cells, ultimately leading to exclusion of the transcriptional co-activator, Yes-associated protein (YAP), from the nucleus (Kim et al., 2011; Levine et al., 1965).

Puliafito et al. demonstrated however that cell-cell contact is not sufficient for CIP in Madin Darby canine kidney (MDCK) epithelial cells. In fact, proliferation continues at a near subconfluent rate even in confluent cells until a critical cell density, or transition point, is reached, after which proliferation diminishes until cells reach quiescence (Adam et al., 1982; Puliafito et al., 2012). Proliferation in confluent cells is accompanied by little or no hypertrophic growth. While subconfluent daughter cells ultimately attain nearly 100% of mother cell area, confluent cells remain at around 50%, indicating a nearly complete lack of hypertrophic growth in the densely confluent monolayer, consistent with the previously identified inverse relationship between individual cell size and density (Erlinger and Saier, 1982; Puliafito et al., 2012). This reduction in cell size acts as the major activator of YAP nuclear exclusion through reduction of cytoskeletal stress (Aragona et al., 2013; Rauskolb et al., 2014, see section 2.2.5). Indeed, forced reduction in cell size by growth on micropatterned fibronectin islands of defined area or

growth in a soft agar, to reduce cytoskeletal tension, leads to YAP nuclear exclusion in a manner dependent on F-actin capping/severing proteins but independent of cell contact (Aragona et al., 2013). Thus, subconfluent cells are subject to tensile forces on the cytoskeleton that, combined with lack of cell contact, promote YAP nuclear localization and proliferation (Aragona et al., 2013; Rauskolb et al., 2014; Trepap et al., 2009). Confluent, pre-transition point epithelial cells, while contacted, also maintain YAP nuclear localization due to continued cytoskeletal tension, promoting proliferation. However, the cells are exposed to constraining forces that discourage post-mitotic hypertrophic growth causing a period of size reductive proliferation ultimately reducing cytoskeletal stress and transitioning the cells to proliferative quiescence and finally monolayer maturation (Pulafito et al., 2012). This process sharply decreases cell area and increases cell density, resulting in increased uniformity of cell area and shape, and establishes a mature, packed, epithelial monolayer.

A mature epithelial monolayer possesses well-developed TJs, which are necessary to control fluid and solute flux. TJs form between adjacent cells apical to the AJ and create and maintain semi-permeable barriers to paracellular flux, and may contribute to maintaining cell polarity. Over 40 proteins have been identified at TJs (Anderson and Van Itallie, 2009) including occludin, the first transmembrane TJ protein to be discovered (Furuse et al., 1993). While occludin knockout mice failed to exhibit any increase in intestinal permeability, these animals presented with a constellation of complex phenotypes consistent with barrier dysregulation including male sterility, inability to nurse, and brain calcification (Saitou et al., 2000; Schulzke et al., 2005). Human patients expressing a recessive mutation in the occludin gene exhibit similar

brain calcification, as well as gross cranial malformation (O'Driscoll et al., 2010). Taken together, these studies support the prevailing view of occludin as a regulator of the TJ.

Recently, occludin has been increasingly implicated in non-barrier roles including regulation of cell proliferation. Occludin is present at centrosomes and regulates mitotic entry and cell proliferation in a phosphorylation dependent manner (Runkle et al., 2011). Occludin knockout mice exhibit intestinal cell hyperplasia (Saitou et al., 2000), and occludin down-regulation or loss has been implicated in cancers of the skin (Rachow et al., 2013), uterus (Tobioka et al., 2004) and breast (Martin et al., 2010), and is correlated with increased metastatic potential (Osanai et al., 2006). Furthermore, occludin re-expression rescues murine tumorigenesis after implantation of oncogenic, Raf1-transformed cells (Wang et al., 2005). The emerging importance of occludin in regulating cell proliferation suggests a role for occludin in cell packing.

Mass spectrometric analysis has identified several novel occludin phosphorylation sites, including the S471 site within the C-terminal coiled-coil domain (Sundstrom et al., 2009). Notably, this residue is located at the first turn of the coiled-coil, which has been established as an interaction point with the scaffolding protein, zonula occludens-1 (ZO-1, (Tash et al., 2012). ZO-1 interacts with and organizes many TJ proteins, and links the junction to the actin cytoskeleton (Lye et al., 2010), and members of the ZO family are necessary for assembly of TJs (Rodgers et al., 2013; Umeda et al., 2006). ZO-1 is a membrane associated guanylate kinase (MAGUK) protein and contains the typical catalytically inactive guanylate kinase (GuK) like domain. The GuK domain acts as a specialized P-serine/P-threonine binding pocket (Zhu et al., 2011), and the ZO-1 GuK domain interacts with the acidic head of the

occludin coiled-coil, including the S471 site, making it an intriguing site for potential functional regulation and further analysis (Tash et al., 2012).

In the current study, we present evidence that expression of occludin phosphoinhibitory mutant S471A prevents size-reductive proliferation and cell packing in MDCK cells. Expression of S471A has no effect on sub-confluent proliferation, but inhibits size reductive proliferation after contact and prevents bicellular and tricellular TJ maturation, creating a highly permeable monolayer that appears morphologically indistinguishable from an immature monolayer before cell packing. Furthermore, inhibition of cell cycle progression after cell contact yields a similar, immature monolayer, suggesting that cell packing is necessary for monolayer maturation and TJ formation. The S471 site is a target of members of the G-protein coupled receptor kinase (GRK) family, and inhibition of GRK 4-6 also inhibits size reductive proliferation and delays epithelial cell maturation. Further, the effect of the GRK inhibitors on monolayer maturation can be overcome by expression of the phosphomimetic S471D occludin. The data suggest that occludin phosphorylation at S471 contributes to control of size reductive proliferation after contact, and to epithelial maturation.

3.3 Methods

Cells

All reagents were purchased from Sigma Chemical (St. Louis, MO) unless otherwise noted. Madin Darby canine kidney (MDCK) cells were obtained from American Type Culture Collection (Manassas, VA) and cultured in minimum essential

media as previously reported (Runkle et al., 2011). All cells were kept at 37°C with 5% CO₂.

MDCK stable lines were generated via transfection with lipofectamine 2000 (Invitrogen, Carlsbad, CA) of empty vector, wild type human occludin, S471A mutant occludin, or S471D mutant occludin in a pmaxFP expression vector (Amara, Cologne, Germany) following manufacturer instructions and as previously described (Shen and Turner, 2005). Briefly, fluorescence-activated cell sorting was carried out following 2 weeks of culture in normal MEM with 2.5 mg/ml geneticin (Gibco, Carlsbad, CA). Single cells were plated in a 96-well plate with 2.5 µl/ml geneticin, grown out, and screened for GFP expression by western blot. W2, A3, and D2 lines were used unless otherwise indicated (Fig. 3.1). All reported experiments were repeated on separate days except for Fig. 3.4, which represents multiple platings. Cells were plated at a density of 162,500 cells/cm² unless otherwise noted.

Immunofluorescence (IF)

Cells were plated on chambered glass slides and fixed at 4 days post confluent unless otherwise noted with either 3.5% paraformaldehyde (PFA) for 10 minutes or 50% methanol/50% acetone for 20 minutes at -20°C (Thermo Scientific, Waltham, MA). PFA fixation was followed by 15 minutes permeabilization in TBS plus 0.25% triton-x 100 while methanol/acetone fixation did not require permeabilization. Cells were blocked in 10% goat serum (Life Technologies, Carlsbad, CA) with 0.25% triton-x 100 for 1 hour, then stained with indicated primary antibodies overnight at 4°C and 1:200 dilution in blocking solution, except for anti-turbo GFP (1:400, Evrogen, Moscow, Russia) and anti-

YAP (1:100, Santa Cruz, Dallas, TX). Secondary antibody (Alexa Fluor 488, 555, and 647, Life Technologies, Carlsbad, CA) exposure was 1 hour at room temperature in blocking buffer at a 1:1,000 dilution. Fluorescence was determined from Z-stacks of 0.5 μm slices taken on a Leica TCS SP5 confocal microscope (63x, 1.4 N.A oil objective, Wetzlar, Germany) with photomultiplier tube detectors, and using Leica Advanced Fluorescence software. Images were analyzed using Metamorph software (Molecular Devices, Sunnyvale, CA). In some cases, occludin and ZO-1 border staining was quantified by a semi-quantitative ranking score system based on a scale of 1 to 5: 1 for near complete loss of border staining (0-25%), 2 for 25-50% continuous border staining, 3 for 50-75% continuous border staining, 4 for 75-100% continuous border staining, and 5 for completely continuous border staining. Scoring was completed in a masked fashion by 3 impartial observers provided scoring standard images for comparison.

Adherens junction/cell height

Fixation was performed using 1:1 methanol/acetone or 3.5% PFA as previously described (AJ area), or 3.5% PFA in the absence of permeabilization (cell height). Area within E-cadherin border staining was determined from multiple 63x - 1x zoom confocal images using Metamorph software (Molecular Devices, Sunnyvale, CA). Cell height was determined from 63x - 1x zoom XZ confocal images of plasma membrane staining and Leica Advanced Fluorescence software (Wetzlar, Germany).

Cell counts

Nuclei were counted from monolayers stained with either Hoechst or PicoGreen nuclear stain (Life Technologies, Carlsbad, CA) using the ImageJ cell counter plugin

(NIH, Bethesda, MD). At least four microscopy fields were averaged for each cell line or treatment (63x objective, 5x zoom). 1,400 cells/cm² were also plated on 12 well plates, then trypsinized and counted at 1, 2, 3, and 4 days post-plating using a Mo-FLO cell sorter (Beckman Coulter, Brea, CA). Forward scatter data was collected to determine suspended cell size, and exhausted cell media from each time point was saved for quantification of floating cells.

DNA synthesis/cell proliferation assays

7,857, 100,000, or 185,714 cells/cm² were plated on eight chambered glass slides (Thermo Scientific, Waltham, MA) and allowed to grow overnight (7,857 and 100,000 cells/cm²) or for 4 hours (185,714 cells/cm²). Cells were incubated with 10 μM EdU (Click-It EdU kit, Life Technologies, Carlsbad, CA) for 4 (7,857 and 100,000 cells/cm²) or 24 (185,714 cells/cm²) hours according to manufacturer instructions. Cells were fixed with 3.5% PFA and stained with Hoechst or PicoGreen nuclear stain (Invitrogen, Carlsbad, CA). At least 500 cells per condition were examined using the ImageJ cell counter plugin (NIH, Bethesda, MD), and percentage of total cells expressing EdU was calculated.

Trans-epithelial resistance (TER)

TER was determined using the electric cell-substrate impedance sensing (ECIS, Applied Biophysics, Troy, NY) system at 500 Hz. Cells were plated on 8W10E+ ECIS plates and allowed to become confluent overnight. All treatments were added at the time of plating and replenished along with fresh media every 24 hours unless otherwise

noted. Electrical resistance was determined on the fourth day post-confluence unless otherwise noted.

Solute flux assay

Cells were plated on 12-well plates with polyester transwell inserts (0.4 μ M pore, Corning, Corning, NY) and allowed to become confluent overnight. Solute flux was determined on the fourth day post-confluence as previously described (Phillips et al., 2008). Briefly, 480 nM TAMRA (467 Da) was added to the apical chamber followed by sampling of 50 μ L from the basolateral chamber every 30 minutes for 3 hours. 10 μ L was also removed from the apical chamber at the last time point for apical concentration determination. Fluorescence was measured (ex. 560, em. 590) in a flat bottom black wall 96-well plate (Greiner, Monroe, NC) using a FLUOstar microplate reader (BMG Labtech, Ortenburg, Germany).

Cell cycle inhibitor assays

Cells were treated with 10 μ M roscovitine (Calbiochem, San Diego, CA) 24 hours after plating, and drug was replenished along with fresh media every 24 hours. TER measurements proceeded for two additional days (from 24-72 hours post plating using ECIS system), or cells were fixed for imaging at 60h post plating.

Determination of protein expression and immunoprecipitation experiments

Protein expression was determined via western blot as previously described (Phillips et al., 2008). Briefly, MDCK cells were harvested in Stuart's Buffer composed of 100 mM NaCl, 1% triton-x 100, 0.5% sodium deoxycholate, 0.2% SDS, 2 mM EDTA,

10 mM HEPES (pH 7.5), 1 mM NaVO₄, 10 mM NaF, 10 mM sodium pyrophosphate, 1 mM benzamidine, 10 μM microcystin (Cayman Chemical, Ann Arbor, MI), and complete mini protease inhibitor tablet (EDTA free, Roche, Indianapolis, IN). Following gel electrophoresis and blocking in 2% ECL prime (GE Healthcare, Little Chalfont, Buckinghamshire, UK), proteins were probed with indicated antibodies including occludin, claudin-1, and tricellulin (Invitrogen, Carlsbad, CA), β-catenin and afadin (Sigma, St. Louis, MO), turboGFP (Evrogen, Moscow, Russia), ZO-1 and GRK (Millipore, Billerica, MA), and E-cadherin (BD Biosciences, Franklin Lakes, NJ). Alternatively, MDCK cells were harvested for immunoprecipitation as previously described (Titchenell et al., 2012) in buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 1% NP-40 (USB, Cleveland, OH), 1 mM NaVO₄, 10 mM NaF, 10 mM sodium pyrophosphate, 1 mM benzamidine, complete mini protease inhibitor tablet (EDTA free, Roche, Indianapolis, IN), 1 μM microcystin (Cayman Chemical, Ann Arbor, MI), 10% glycerol (Fisher, Waltham, MA) and 2 mM EDTA (Lonza, Walkersville, MD). Following harvest, cells were rocked for 15 minutes and spun at 12,000 g for 10 minutes. Lysate containing 1 mg of protein was pre-cleared with 30 μl packed volume protein G sepharose beads (GE Healthcare, Little Chalfont, Buckinghamshire, UK), then incubated overnight with 10 μg turboGFP antibody (Evrogen, Moscow, Russia). Protein G beads were added to the antibody and lysate mix for one hour, then washed with buffer and prepared for western blotting as previously described.

Co-culture experiments

Subconfluent parental MDCK cells were transduced with Cell-Light mitochondria BacMam baculovirus (Invitrogen, Carlsbad, CA). The next day, transduced parental

MDCK were plated with untransduced S471 Occ cells at a ratio of 10%/90%, respectively. Cells were fixed in PFA and prepared for IF as previously described.

Cell cycle stage measurements

Four day- post confluent cells were stained in suspension with Hoechst, which was then quantified using an LSR II flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Cell cycle stage was determined by amount of nuclear material present.

Ion permeability

97,500 cells were plated on Biopore cell culture inserts (0.4 μm pore, Millipore, Billerica, MA) with fresh MDCK media provided daily. On the fourth day post confluence, inserts were loaded into an Ussing chamber (Harvard Apparatus, Holliston, MA), and both sides were gently filled with HEPES ringer solution composed of 135 mM NaCl, 5 mM KCl, 10 mM HEPES, 10 mM glucose, 1.8 mM CaCl_2 , and 1 mM MgCl_2 . Permeability to specific ions was determined by voltage or current clamp (EC-800 amplifier, Warner Instruments, Hamden, CT) recordings collected in the presence of ringers with the NaCl or KCl concentration in the apical chamber reduced by 75% (33.75 mM NaCl, 1.25 mM KCl), and original osmolarity restored by mannitol addition. Ion permeability ratio was calculated using the Goldman-Hodgkin-Katz equation:

$$\eta = -(\varepsilon - e^v) / (1 - \varepsilon e^v).$$

where η is the ratio of permeability of the monolayer to Na^+ over the permeability to Cl^- ($\eta = P_{\text{Na}}/P_{\text{Cl}}$), ε is the dilution factor ($\varepsilon = C_{\text{basal}}/C_{\text{apical}}$), and $v = eV/kT$ (V is the dilution potential, k is the Boltzmann constant, e is the elementary charge and T is the

temperature in Kelvins). Absolute permeability of sodium and chloride was calculated using the simplified Kimizuka-Koketsu equation:

$$P_{\text{Na}} = (G / C) * (RT / F^2) * (\eta / (1 + \eta))$$

$$P_{\text{Cl}} = (G / C) * (RT / F^2) * (1 / (1 + \eta)),$$

where G is the total membrane conductance calculated using Ohm's law ($V = I * R$ or $G = I / V$), C is the concentration of ion in solution, R is the gas constant, F is Faraday's constant, and η is the ratio of permeability of the monolayer to Na^+ over the permeability of Na^+ to Cl^- . All experiments were conducted at room temperature.

Cell viability

Cell viability was determined using a WST-1 assay (Roche, Indianapolis, IN). 31,250 cells/cm² were plated in a 96 well plate, allowed to grow overnight, then incubated with 5 μl WST-1 reagent for 1 hour. Absorbance was measured (770/410 nm) using a FLUOstar microplate reader (BMG Labtech, Ortenburg, Germany).

Statistical analysis

Data were analyzed using two-tailed student's t-test (2 groups), or one-way ANOVA with Bonferroni post hoc (3 or more groups) using Prism 5.0 (GraphPad Software, La Jolla, CA). Line graphs were analyzed using a two-way ANOVA (simple effect within rows) with Bonferroni post hoc. Data are expressed as mean \pm SD unless otherwise indicated.

3.4 Results

S471A Occ expression compromises TJ protein organization.

MDCK lines overexpressing human occludin with a phosphoinhibitory serine to alanine (S471A) or phosphomimetic serine to aspartic acid (S471D) mutation were generated along with wild type occludin (WT Occ) control lines to investigate the effect of S471 phosphorylation on TJ protein organization (Fig. 3.1 A and B). Both exogenous GFP tagged occludin (80 kD) and endogenous (55kD) occludin were detectable by western blot, and no appreciable changes were observed in expression of any tested TJ or AJ proteins in lines utilized for the current study (Fig. 3.1 C).

Cells expressing WT Occ had well-organized localization at the border for the TJ proteins occludin (GFP-occludin and total occludin), ZO-1, and claudin-1 based on immunofluorescence (IF) labeling (Fig. 3.2 A). Additionally, tricellulin appeared at tTJs. Phalloidin staining for F-actin revealed stress fibers basolaterally and crisp cortical actin staining apically, while afadin was present at the border (Fig. 3.2 B). Finally, the AJ proteins E-cadherin and β -catenin demonstrated strong cell border organization (Fig. 3.2 C). All results were consistent with known localization of these proteins in cells with high electrical resistance and low solute permeability. S471D Occ expressing cells were similar in their staining pattern to control lines, and in many cases were qualitatively better organized with increased uniformity in cell shape, and brighter, more focused border staining, particularly in regards to cortical F-actin (Fig. 3.2 A-C). In contrast, TJs were disorganized in S471A Occ lines with mislocalized or missing border staining in all examined TJ proteins. These studies were performed on day 4 after plating, and the TJ disorganization was maintained for at least 10 days after plating (data not shown). The TJ proteins occludin, ZO-1 and claudin-1 appeared as non-continuous aggregates, often co-localizing with each other, while tricellulin staining was largely absent (Fig. 3.2 A). Expression of S471A Occ did not, however, decrease the occludin-ZO-1 interaction as judged by co-immunoprecipitation experiments (Fig. 3.3 A) or alter endocytosis as assessed by colocalization with early endosome antigen 1 (endosome marker, Fig. 3.3 B), nor was there evidence that S471A mutant cells corrupted neighboring parental cell TJ organization (Fig. 3.3 C), suggesting a cell autonomous effect. The cell cycle stage of proliferatively quiescent WT, S471A, and S471D Occ lines were also similar, indicating that the mutations do not cause arrest in a

non G₁/G₀ cell cycle stage (Fig. 3.3 D-E). By contrast, F-actin staining was altered with fewer visible stress fibers and dim, broad, cortical actin organization. Afadin was also disorganized or lost at bicellular junctions (Fig. 3.2 B), but importantly, the AJ proteins E-cadherin and β -catenin appeared unaffected (Fig. 3.2 C). To quantify the extent of ZO-1 protein mislocalization, co-localization between ZO-1, a marker of TJ organization, and E-cadherin, an AJ protein unaffected by S471A Occ expression, was measured. The percent of E-cadherin colocalized with ZO-1 (indicating ZO-1 border staining) was significantly decreased in S471A Occ lines and increased in S471D Occ lines compared with WT Occ (Fig. 3.2 D and E). Together, these observations demonstrate that expression of S471A Occ compromises TJ organization in a dominant manner.

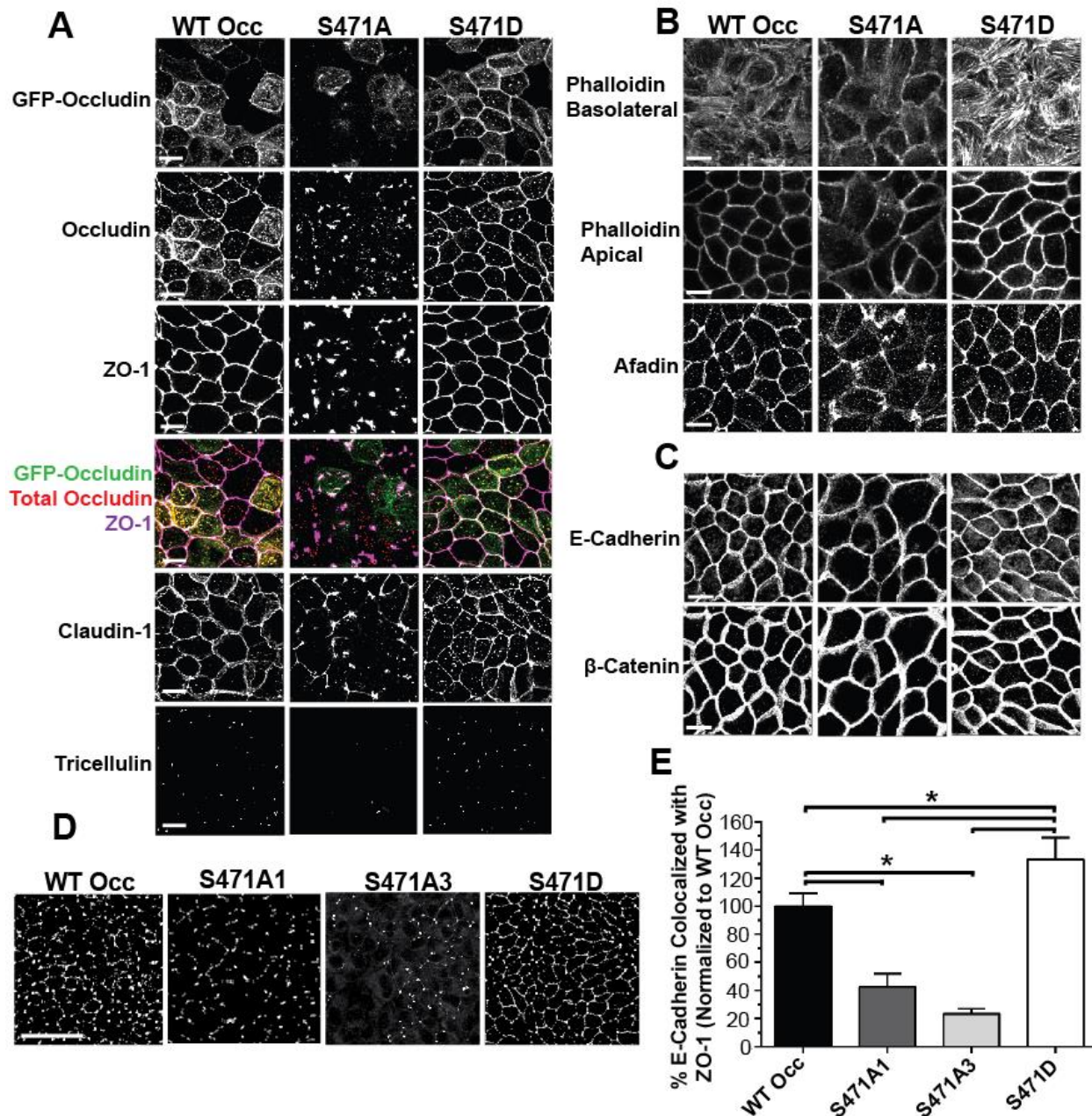


Fig. 3.2: Tight junction and cytoskeletal proteins are mislocalized in occludin S471A mutant cell lines. (A-C) Immunofluorescent maximum projected stacks (3 μm thick) or slices (phalloidin images, 0.5 μm thick) of indicated (A) TJ, (B) cytoskeletal staining and afadin, and (C) AJ proteins (scale bar = 10 μm). (D) Maximum projected colocalization of E-cadherin, used as a marker of junctional complex, with ZO-1 demonstrating loss of ZO-1 at junctions (scale bar = 50 μm). (E) Quantification of D (average of 4 images per cell line). Data are expressed as Mean \pm SD. * $P < 0.05$ compared to WT Occ or S471D Occ.

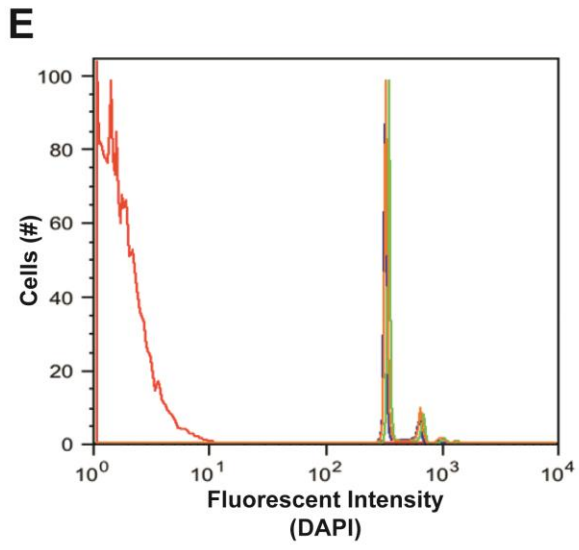
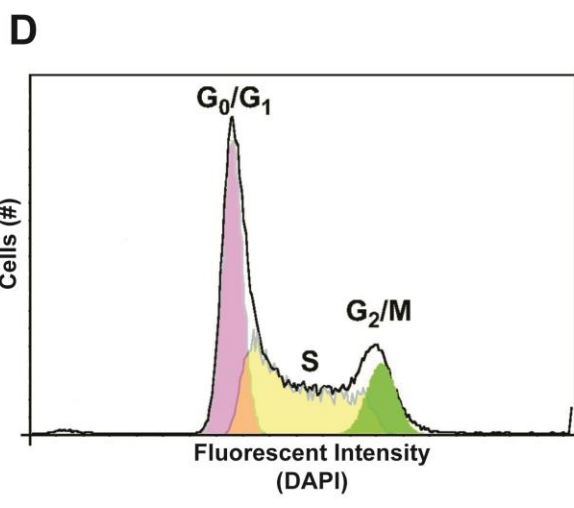
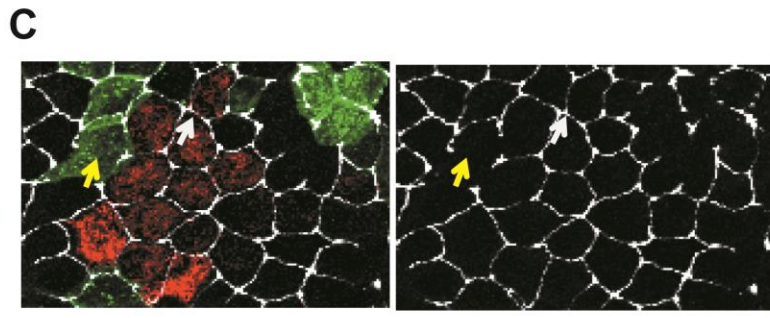
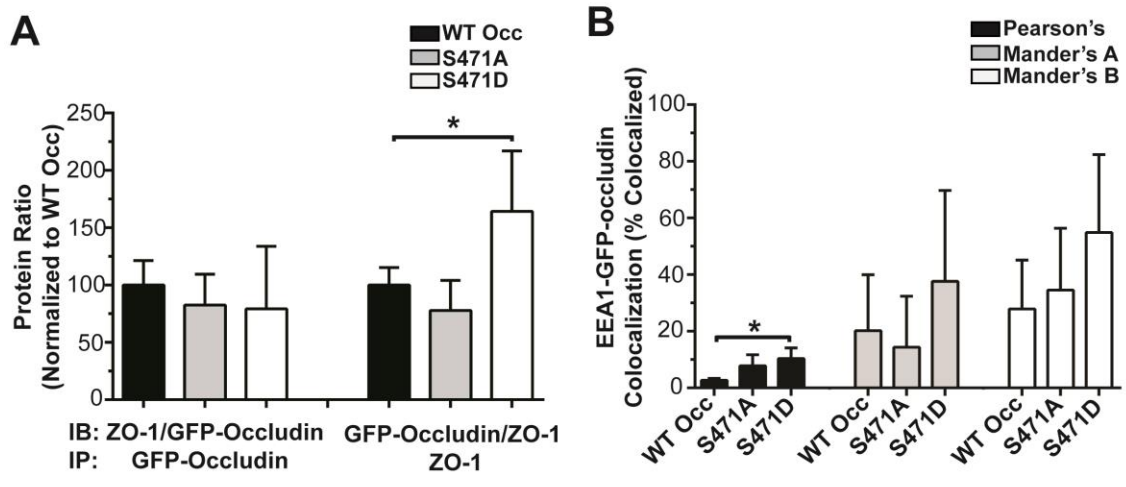
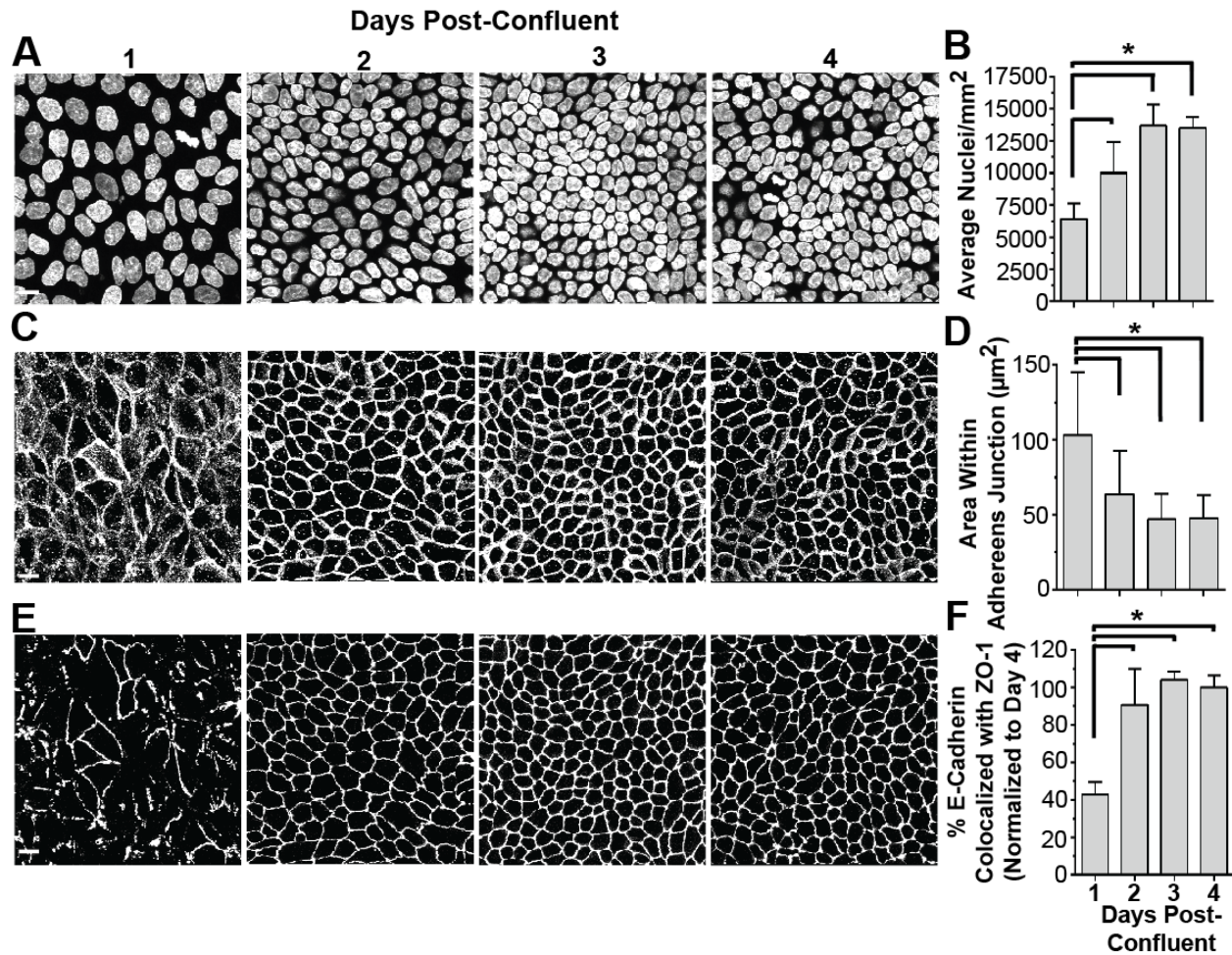


Fig. 3.3: S471 lines co-immunoprecipitation, colocalization and co-cultures. (A) Protein ratio of co-immunoprecipitated GFP-occludin and ZO-1 proteins, normalized to WT Occ protein ratio (n=5-6). (B) Colocalization of GFP-occludin and endosomal marker EEA1 including Pearson's, Mander's A (percent of EEA1 colocalizes with GFP), and Mander's B (percent of GFP that colocalizes with EEA1), average of 3-5 images per condition). (C) Parental cells labeled with Cell Light mitochondria BacMam baculovirus (red), co-cultured with GFP-tagged S471A Occ lines (green), and stained for ZO-1 (faux colored in white). Left, merged image, right ZO-1 alone, arrows indicating intact parental-parental (white) and disrupted S471A-S471A (yellow) borders. (D) Schematic of DAPI fluorescence intensity as an indicator of cell cycle (adapted from Henderson et al., 2013). (E) Fluorescence intensity of WT Occ (green), S471A (orange), S471D (blue), and unstained parental MDCK (red) cells stained with DAPI. Data are expressed as Mean \pm SD, *P<0.05 compared to WT Occ.

TJ formation occurs during size reductive proliferation.

As detailed in the introduction, size reductive proliferation is required for monolayer maturation. Here, the formation of TJs was examined across size reductive proliferation and epithelial maturation. MDCK cells were plated on 8 chambered slides at 162,500 cells/cm² (yielding confluency on the first day of plating). The cells underwent dramatic morphologic changes associated with size reductive proliferation in the first three days following confluency. Cell density increased by over 2-fold as indicated by nuclei counts (Fig. 3.4 A and B). Meanwhile, the AJs were partially completed by day 1 and largely formed by day 2, and area within the AJ decreased by half by day 3 (Fig. 3.4 C and D). Finally, TJ assembly assessed by ZO-1 staining was largely incomplete on day 1, but increased dramatically over days 2 and 3 such that colocalization with E-cadherin increased, indicating increased ZO-1 border localization (Fig. 3.4 E and F). Packing was complete by three days post confluency for each of the measured parameters, and no statistically significant changes occurred between days 3 and 4. The TJ organization observed in the S471A expressing lines appeared highly

similar to the early time point in epithelial maturation; therefore, cell size and proliferation were examined in the mutant lines.



Size reductive proliferation is attenuated by S471A Occ expression.

Cell monolayers expressing S471A Occ were composed of fewer and larger cells than WT Occ or S471D Occ monolayers (Fig. 3.5 A-C). Nuclei number was approximately half compared to cells expressing WT Occ or S471D, and the area within the AJ (E-cadherin staining) was increased in S471A Occ confluent monolayers by 2 to 4-fold compared to WT Occ or S471D Occ lines (Fig. 3.5 A and B). Cell height was not different however, suggesting an increase in cell volume in S471A Occ lines compared to WT Occ and S471D (Fig. 3.5 D). To determine if S471A mutants altered cell proliferation or size in suspension, cells were plated at low density (1,400 cells/cm²) and counted following trypsinization on days 2-4. S471A cell size in suspension was not different from WT Occ, indicating a strictly adherent cell effect (Fig. 3.5 E). Adherent cell number, as determined by flow analysis, was reduced in S471A Occ monolayers at higher confluences on days 3 and 4, but not on days 1 or 2 when confluency was low and there was little cell contact, suggesting loss of proliferation in a density dependent manner (Fig. 3.5 F). Non-adherent cells were counted to eliminate the possibility of increased cell extrusion in S471A Occ lines, and no difference was observed between any of the lines (Fig. 3.5 G). Finally, the nearby Y474 site participates in PI3K activation (Du et al., 2010), and its proximity to S471 represents a possible mechanism for proliferative differences amongst the mutants. Tyrosine phosphorylation of GFP-Occ from various S471 mutant lines was similar however, indicating that the use a Y474 phospho-specific antibody or other phosphorylation detection method that is Y474 specific may be necessary to evaluate this possibility (Fig. 3.5 H).

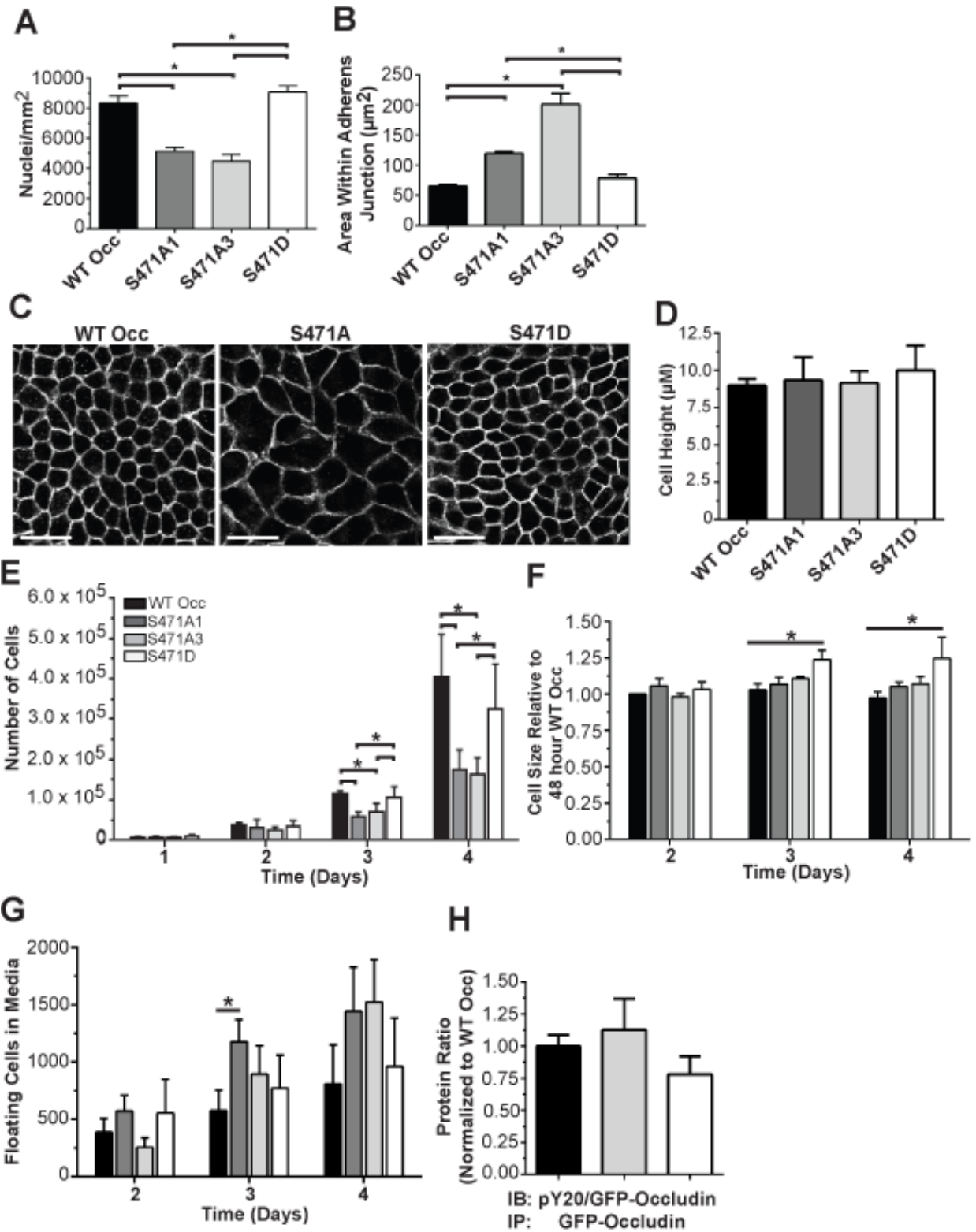


Fig. 3.5: Phosphoinhibitory (S471A) monolayers are composed of fewer, larger cells than controls. (A) Quantification of nuclei/mm² in various lines. (B) Quantification of area within AJ (E-cadherin staining) and (C) representative images (scale bar: 10 μm). (D) Average cell height. (E) Average suspended cell size at 2-4 days post confluent and (F) cell counts by flow cytometry 2, 3, or 4 days post plating. (G) Floating (extruded) cell counts 2-4 days post confluent. Floating cells were allowed to accumulate for 24 hours prior to counting. (H) Tyrosine phosphorylation of various lines following GFP-Occludin IP and pY20 IB. Data are expressed as mean ± SD, A-B and D represent the average of 4 images per condition, E-F, G-H represent average of 3 experiments). *P<0.05 compared to WT Occ or S471D Occ.

To examine the possibility of a density dependent decrease in S471A Occ proliferation, we measured the proliferative rate of WT Occ, S471A Occ, and S471D Occ lines at various confluences by DNA synthesis measurements (Click-IT EdU). At 7,857 (~10% confluent) and 100,000 (~55% confluent) cells/cm², the number of cells with active DNA synthesis was measured over a 4h period whereas when plating 185,714 (~100% confluent) cells/cm², the number of synthetically active cells was measured over 24h since the rate of overall synthesis dramatically slows as cells become more confluent. No difference in proliferation was observed at low confluence (Fig. 3.6 A), but became increasingly evident with increasing confluence (Fig. 3.6 B-C). When plated at ~ 100% confluence, fewer than 10% as many S471A Occ cells proliferated over the 24 hours (4-28 hours post plating) compared with WT Occ, while there was no difference between WT Occ and S471D Occ (Fig. 3.6 C). The proliferative rate was near zero in all cell lines from 28-52 hours post plating (Fig. 3.6 D), suggesting that when plated at high confluency, proliferation is restricted in MDCK to a size reductive or packing phase lasting no longer than 24 hours, followed by relative quiescence. However, expression of S471A Occ inhibits this packing phase. The transcriptional co-activator, YAP, promotes cell proliferation in the nucleus, and its

nuclear exclusion marks the end of the Hippo/MST signaling pathway and contributes to CIP (Gumbiner and Kim, 2014). Despite proliferative quiescence, YAP localization remained nuclear in S471A Occ lines compared to WT Occ at quiescence, consistent with the S471A mutant stalling size reductive proliferation, and preventing YAP nuclear exclusion (Fig. 3.6 E-F). Taken all together, these results indicate that size reductive proliferation is deficient in S471A Occ lines, and raises the possibility of a connection between defective packing and poor TJ assembly (Fig 3.2 A).

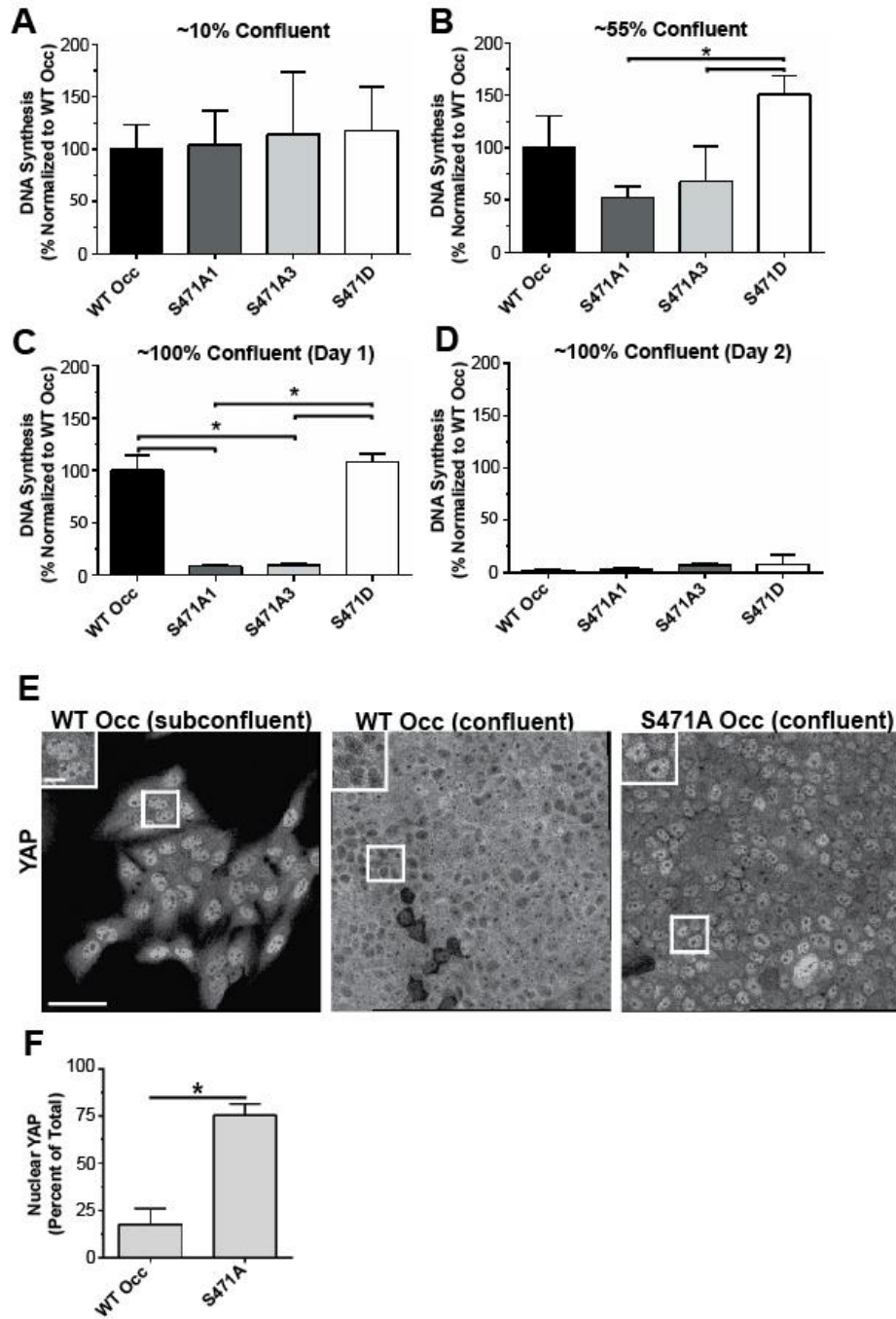


Fig. 3.6: Proliferation after contact was inhibited in S471A Occ lines. (A-D) EdU labeling was measured at the indicated confluences as described in Methods, and percent of cells demonstrating DNA synthesis was quantified (at least 500 cells examined per cell line, per condition). (E) IF images of YAP localization in subconfluent and confluent WT Occ, and confluent S471A Occ lines (scale bar: 50 μm , 10 μm for inset). (F) Quantification of percent nuclear YAP (average of 4 images per condition). Data are expressed as Mean \pm SD normalized to WT Occ. *P<0.05 compared to WT Occ.

Cell packing is necessary for barrier assembly.

To evaluate the necessity of cell packing alone for barrier assembly, we treated parental MDCK with the Cdk inhibitor, roscovitine, starting at 24 hours post plating to halt the cell cycle and size reductive proliferation. Nuclei number was decreased (Fig. 3.7 A) and area within the AJ was increased (Fig. 3.7 B) compared to controls, confirming that packing was significantly attenuated. Trans-epithelial electrical resistance (TER) was decreased on day 3 (Fig. 3.7 C), and there was a significant loss of ZO-1 border staining compared with continuous E-cadherin staining (Fig. 3.7 D-E), indicating disorganization of TJ proteins at the border. These studies demonstrate that inhibition of cell packing alone reduces TJ assembly and prevents high resistance barrier formation.

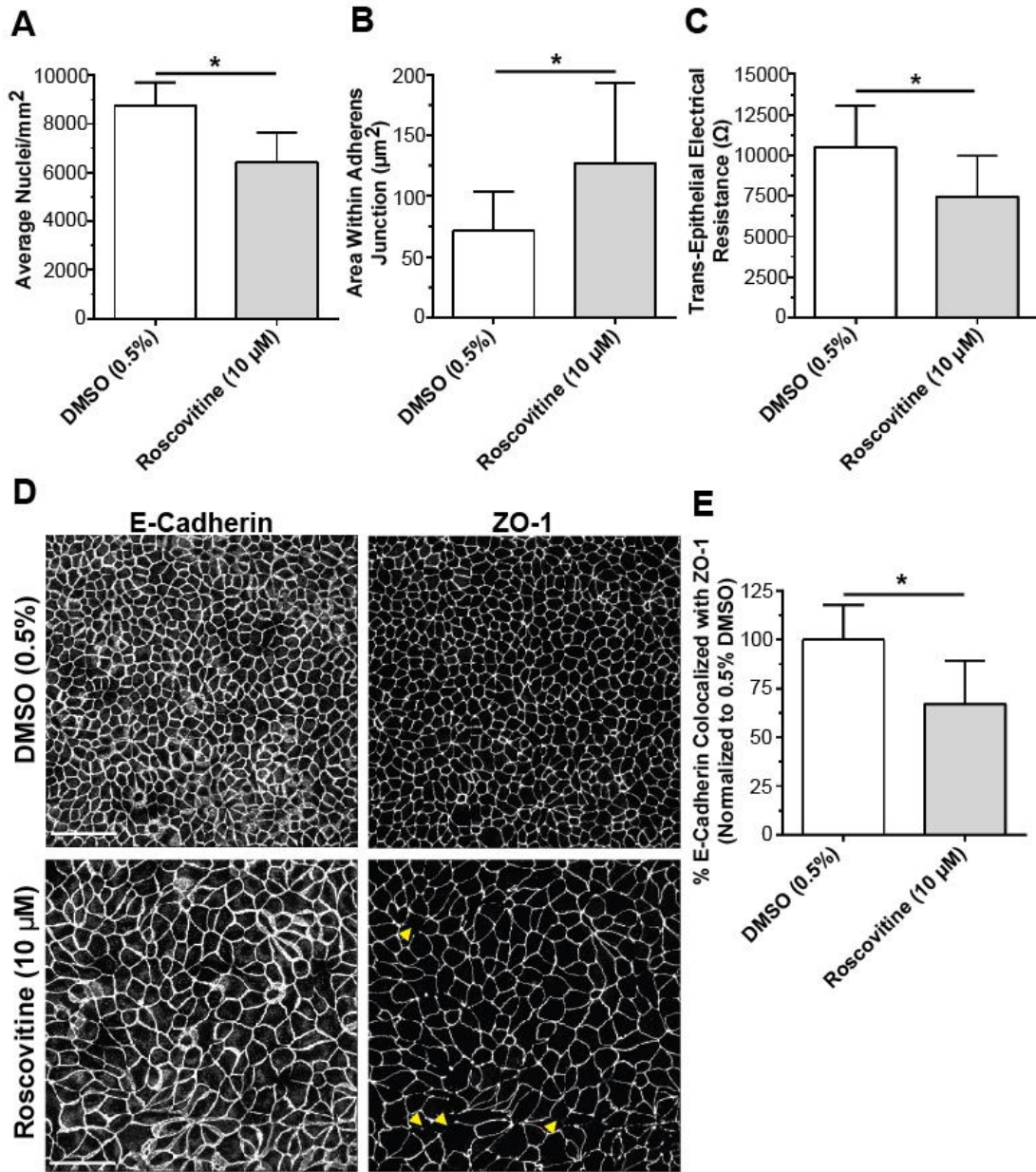


Fig. 3.7: Inhibition of size reductive proliferation delays peak TER and mislocalizes TJ proteins. (A) Nuclei counts and (B) AJ area in day 3 post confluent MDCK monolayers demonstrating inhibition of size reductive proliferation following treatment with the cell cycle inhibitor, roscovitine, added at 24h after plating. (C) TER of day 3 post confluent MDCK cells (n=10-11). (D) Representative images of ZO-1 (TJ marker) and E-cadherin (AJ marker) following treatment with roscovitine or DMSO control (scale bar: 50 µm). Yellow arrows highlight gaps in ZO-1. (E) Quantification of maximum projected colocalization of E-cadherin and ZO-1 as a measure of TJ organization. Data are expressed as Mean ± SD (average of 4 images per condition). *P<0.05 compared to 0.5% DMSO.

S471A Occ compromises barrier function.

A hallmark of epithelial sheets with well-formed TJ barriers is high TER. To determine whether S471A Occ expression affected TER, measurements were conducted in multiple cell lines. Loss of high resistance barriers was observed in S471A Occ expressing lines that had also shown stalled size reductive proliferation. WT Occ overexpression increased TER in mature (day 3, Fig. 3.8 A and day 4, Fig. 3.8 B) monolayers, as well as after 12 hours of reassembly following a Ca^{2+} switch experiment (Fig. 3.8 C) as previously reported (Balda et al., 1996; McCarthy et al., 1996; Van Itallie et al., 2010). No differences were evident between WT Occ and S471D Occ lines at any of the time points. In contrast, S471A Occ expression reduced TER at all time points in all lines tested, and permeability to Ca^{2+} , K^+ , and Cl^- ions was uniformly increased in S471A Occ mutants (Fig. 3.8 D-F). Permeability to the small fluorescent molecule tetramethylrhodamine (TAMRA, 467 Da) was also increased in S471A lines, and there was no difference between WT Occ, S471D, and parental lines (Fig. 3.8 G). These results indicate an increase in permeability to both ion and small molecule flux in S471A Occ lines.

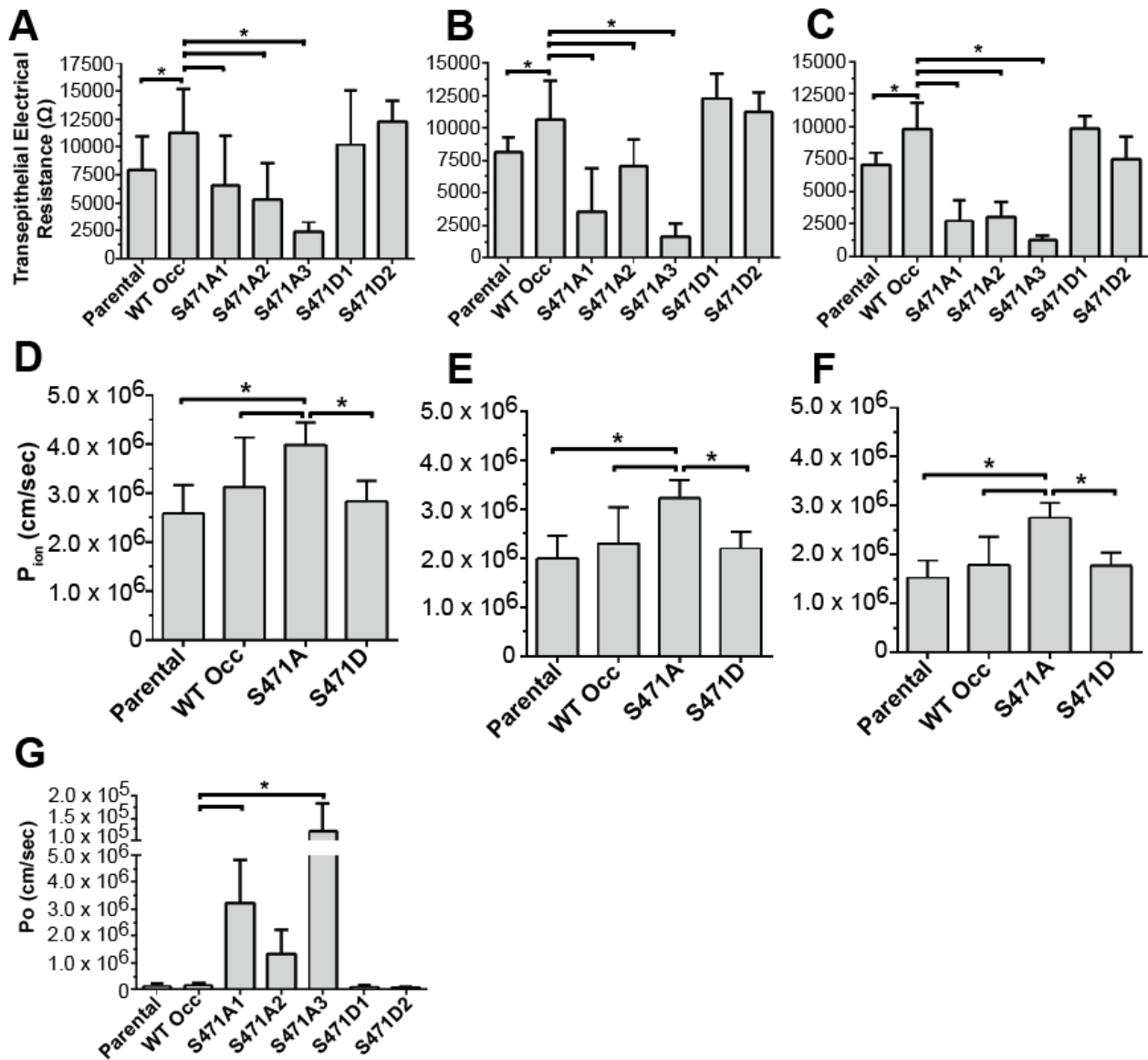


Fig. 3.8: Barrier permeability is increased in phosphoinhibitory S471A Occ lines compared to WT Occ or S471D. (A-C) Trans-epithelial electrical resistance (TER) 3 days (A) or 4 days (B) post confluence, or following 12 hours of recovery from a day 4 calcium switch experiment (C). (D-F) Ion permeability for (D) sodium, (E) potassium, and (F) chloride in parental and various S471 mutant MDCK lines. (G) Solute flux of TAMRA tracer (467 Da) 4 days post confluence. Data are expressed as Mean ± SD (n = 9-16 A-C, n = 9 D-F, n = 13-21 G). *P<0.05 compared to WT Occ (D-F courtesy of Aniket Ramshekar).

S471 is phosphorylated in vitro by G-protein coupled receptor kinase (GRK), and GRK inhibitors attenuate epithelial maturation.

Our previous studies suggest that S471 phosphorylation is important for cell packing and barrier formation. To screen potential cellular kinases for this residue, putative kinases were identified using three kinase prediction software programs, and kinases predicted by at least two were screened against a 21 AA peptide with S471 at the center (Fig. 3.9, see methods). This peptide was phosphorylated *in vitro* by members of the polo like kinase (PLK), calcium/calmodulin dependent kinase (CaMKII), and G protein coupled receptor kinase (GRK) families.

Kinase screen

A list of potential kinases was compiled by entering the sequence around S471 (KELDDYREESEEYMAAADE) into three online kinase prediction programs: GPS (Xue et al., 2008), KinasePhos (Huang et al., 2005), and NetPhos (Blom et al., 1999). Kinases predicted to phosphorylate S471 by at least two of the three programs were included in an *in vitro* P³² kinase assay (Millipore, Dundee, Scotland, UK) of the same peptide with two added lysines at the amino terminus to promote peptide capture on nitrocellulose filters (KKKELDDYREESEEYMAAADE, NeoBioSci, Cambridge, MA). GRK identified in this screen was inhibited in MDCK cells using the kinase-specific inhibitors CCG215022 (GRK Inhib 22, Homan et al., 2015), 4-amino-5-(bromomethyl)-2-methylpyrimidine hydrobromide (ABMH, Santa Cruz), and paroxetine (Toronto Research Chemicals, Toronto, ON, Canada). Kinase assays with all three inhibitors were performed as previously reported (Homan et al., 2015).

Kinase assays

GRK kinetic assays were conducted in a buffer containing 20 mM HEPES (pH 7.0), 2 mM MgCl₂, and 0.025% n-dodecyl-D-maltoside with 50 nM GRK and 500 nM tubulin in 5-minute reactions. Reactions were quenched with SDS loading buffer, separated via SDS-PAGE, dried, and exposed with a phosphoimaging screen prior to quantification via a Typhoon imager, as previously reported (Homan et al., 2015). Data were quantified using Image Quant and inhibition curves were then fit via GraphPad Prism with a three variable dose-inhibitor response curve with a fixed hill slope of 1.

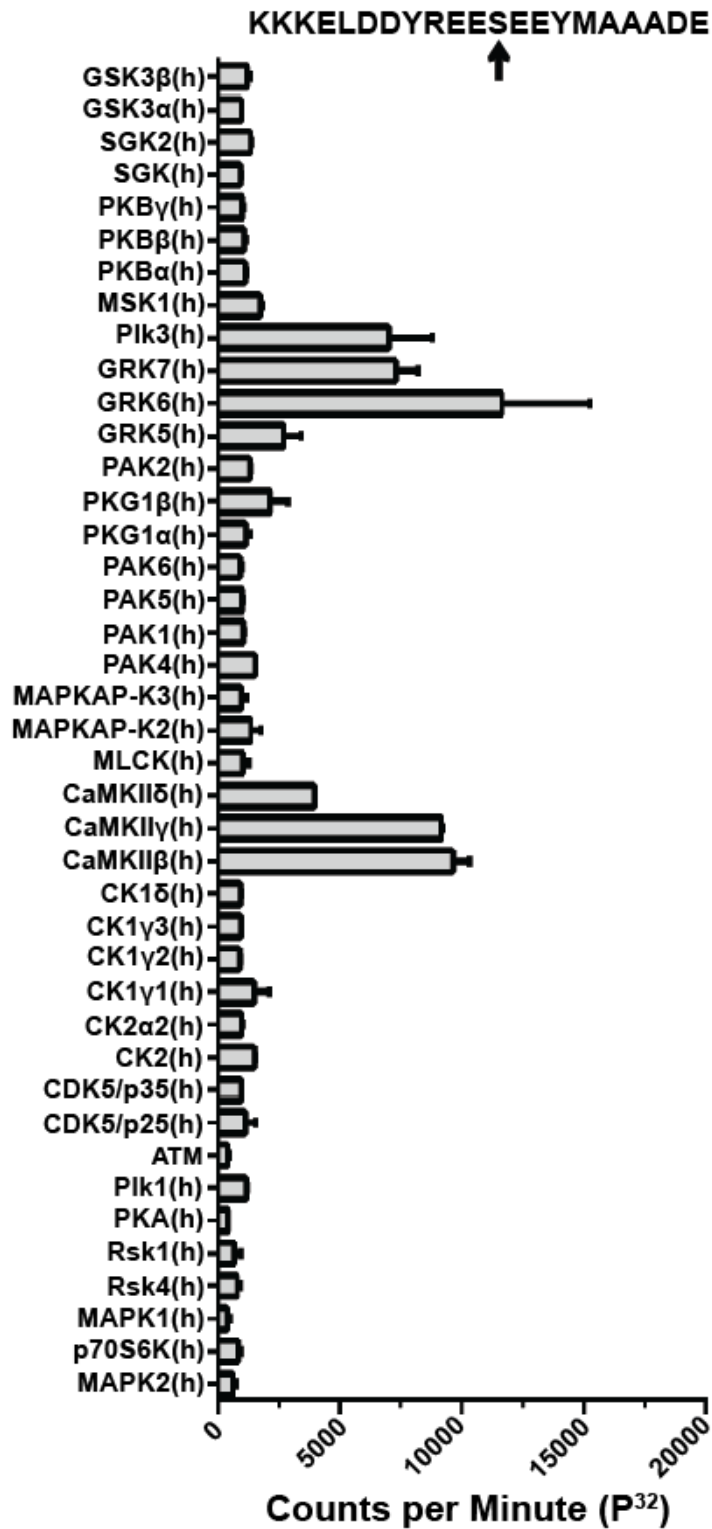


Fig. 3.9: S471 *in vitro* kinase screen. Phosphorylation of an S471-containing 21 amino acid peptide by various kinases quantified via P³² incorporation. Peptide sequence is given with location of S471 (arrow).

Since GRK has previously been implicated in cell size control (Chakraborty et al., 2014) and GRK isoforms were present in the MDCK cells, this kinase was targeted for cell packing and junction formation studies. siRNAs to GRK were unsuccessful in knocking down protein levels despite multiple attempts (data not shown). However, three chemically distinct pharmacological GRK inhibitors prevented epithelial maturation. GRK Inhib 22 (CCG215022), a potent GRK inhibitor (Homan et al., 2015), demonstrated inhibition of GRK6 with an IC₅₀ of 0.95 ± 0.12 μM, while 4-amino-5-(bromomethyl)-2-methylpyrimidine hydrobromide (ABMH) and paroxetine (Thal et al., 2012) inhibited GRK6 with IC₅₀s of 499 ± 144 μM and 78.80 ± 12.30 μM, respectively (Fig. 3.10 A).

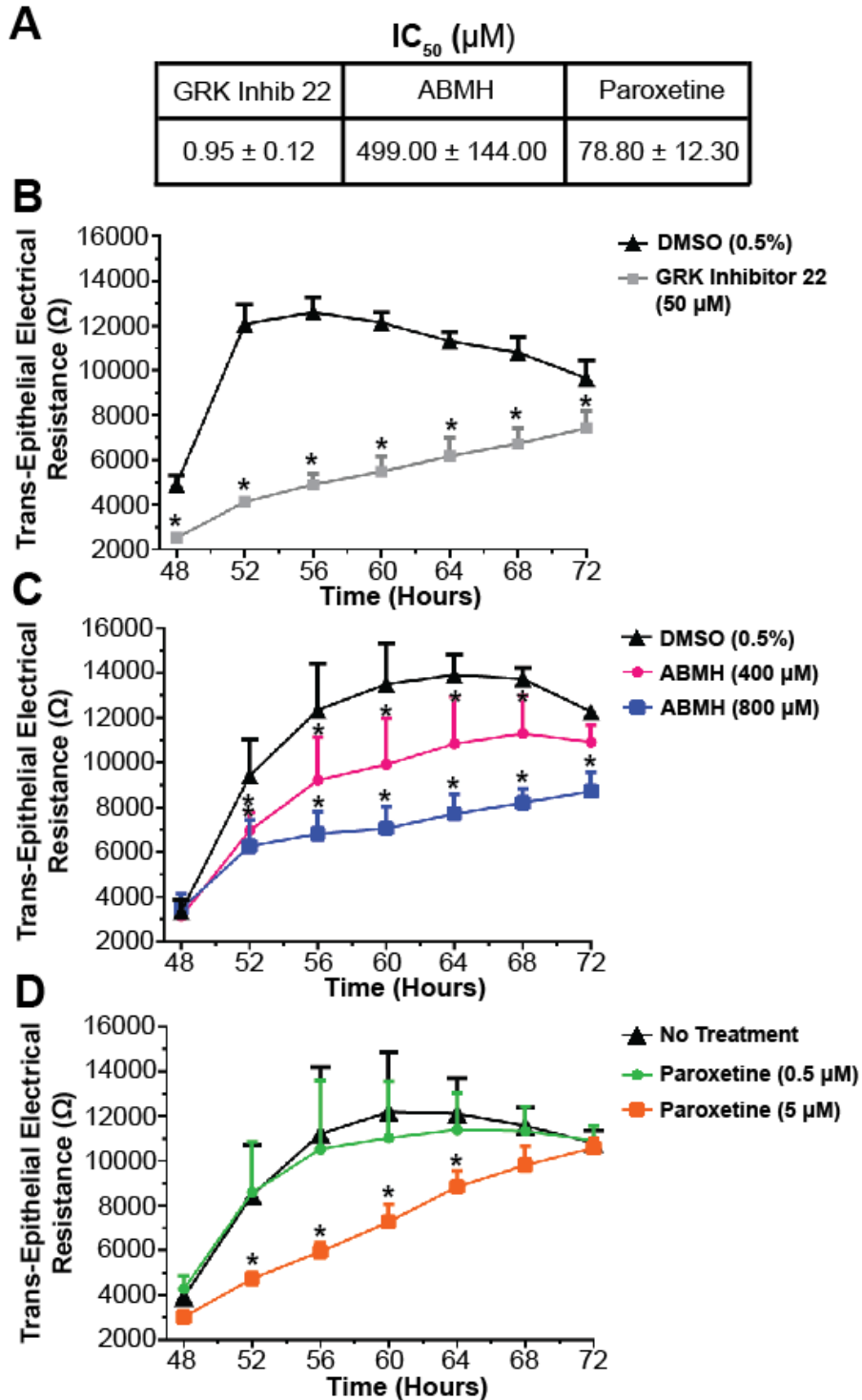


Fig. 3.10: GRK inhibitor activities and effects on TER. (A) *in vitro* IC₅₀s for GRK inhibitors. (B-D) Day 3 TER composite traces from ECIS measurements of various GRK inhibitors including (B) GRK inhibitor 22, (C) ABMH, and (D) paroxetine. Data are expressed as mean ± SD (n=6 A, n=4-8 B-D). *P<0.05 compared to DMSO (0.5%) or no treatment.

MDCK cells were plated at confluence and treated with GRK inhibitor upon cell feeding every 24h. Occludin and ZO-1 localization at the border was reduced on day 3 following treatment of parental MDCK cells with GRK Inhib 22 (Fig. 3.11 A and B) or ABMH (Fig. 3.11 A and C) compared with DMSO control. Cell packing was attenuated by both inhibitors, as demonstrated by a decrease in nuclei number (Fig. 3.11 D) and an increase in area within the AJ (Fig. 3.11 E). Cell viability was not decreased with any of the inhibitors (Fig. 3.12).

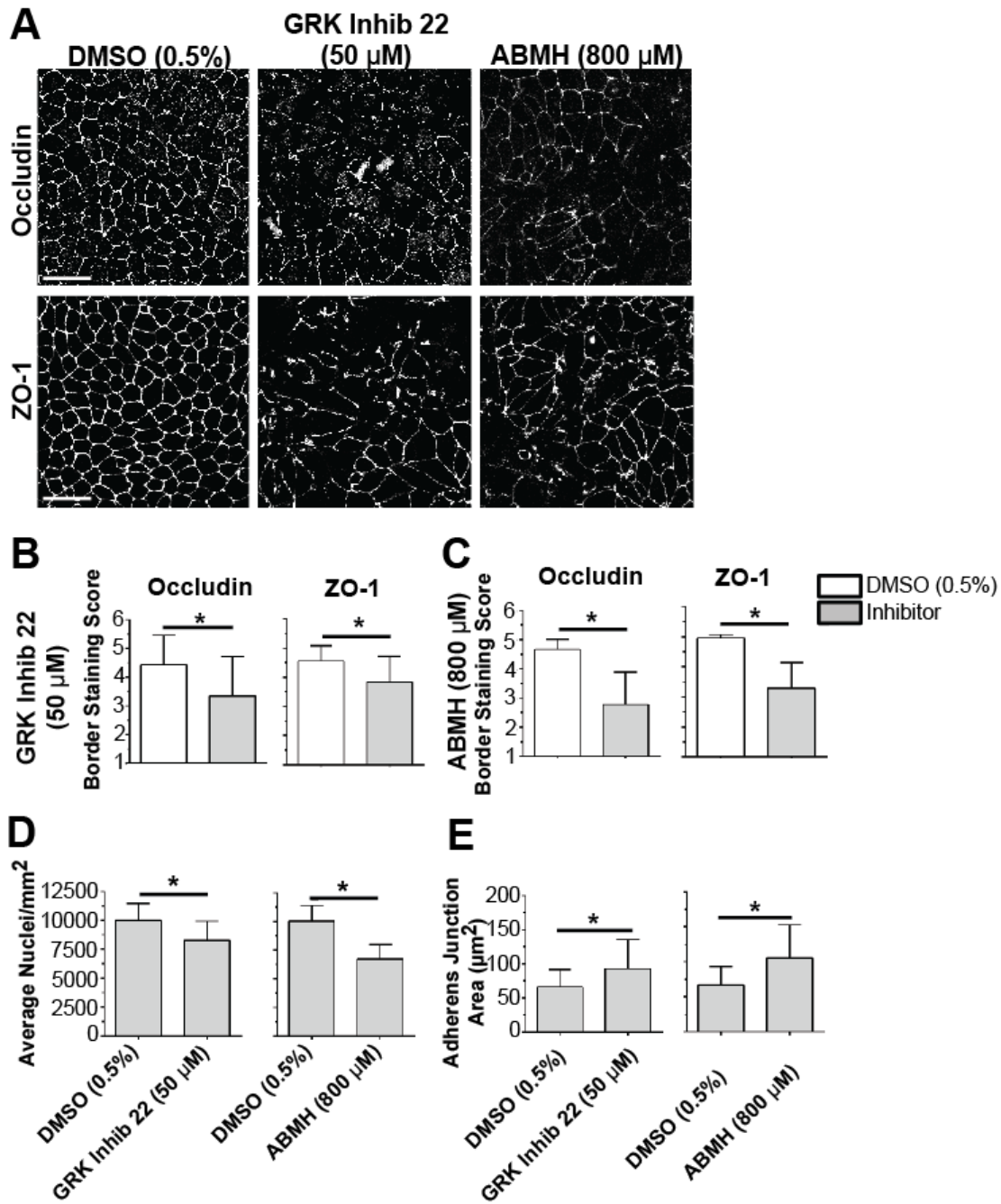


Fig. 3.11: GRK inhibitors decrease TJ border staining and cell number, and increase cell size. (A) IF maximum projected images of total occludin and ZO-1 in DMSO control, GRK Inhib 22, and ABMH-treated parental MDCK (scale bar: 50 μ m). (B-C) Scoring of occludin and ZO-1 treated with (B) GRK Inhib 22 or (C) ABMH. (D) Nuclei counts for DMSO control vs. GRK Inhib 22 and ABMH. (E) Quantification of area within the AJ for DMSO control vs. GRK Inhib 22 and ABMH. Drug was present from beginning of each run and replenished every 24 hours along with fresh media (average of 14 (B-C) or 4 (D-E) images per condition). *P<0.05 compared to WT Occ or DMSO control.

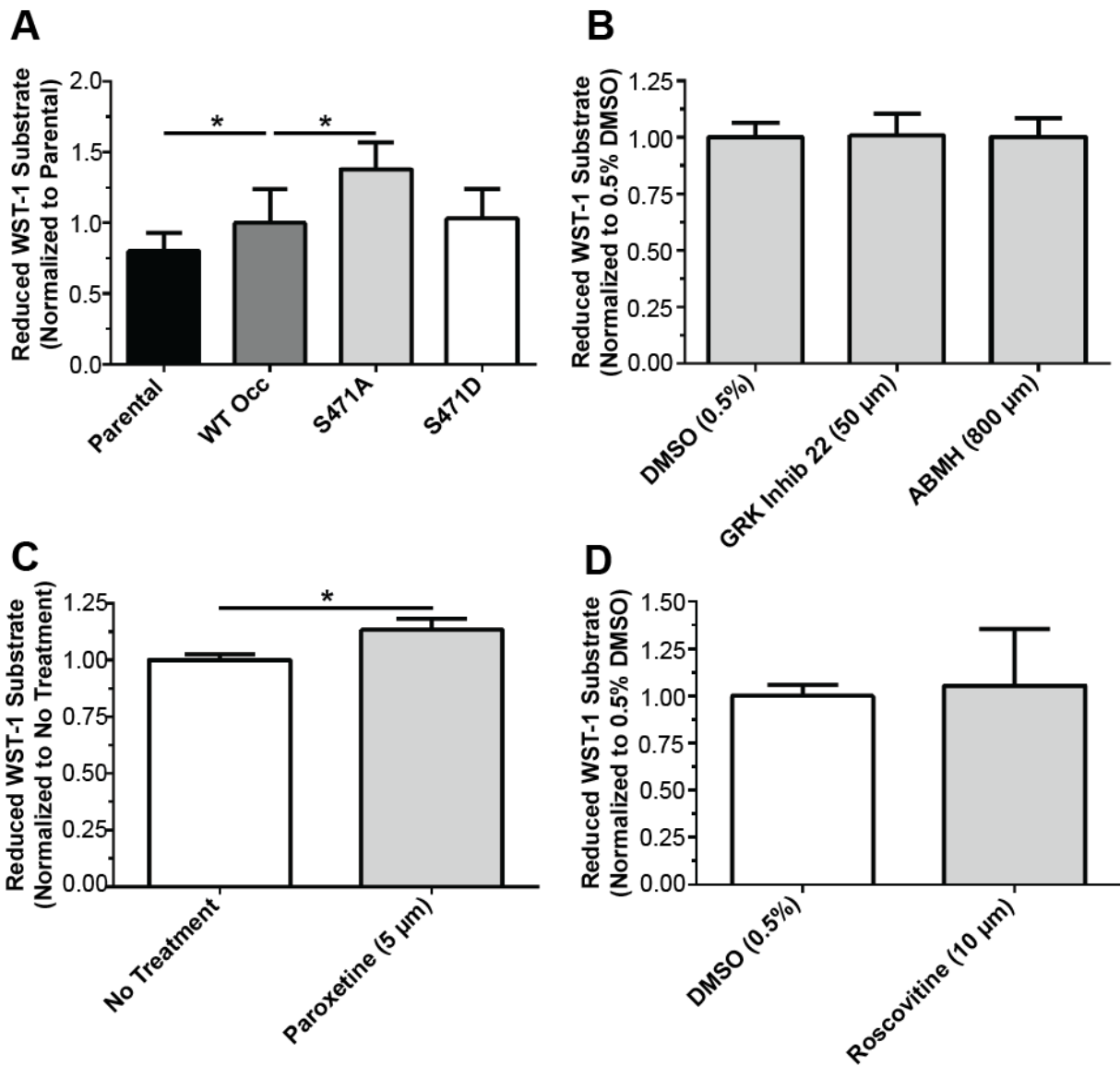


Fig. 3.12: Cell Viability is not decreased by occludin overexpression or tested pharmacological agents. Quantification of WST-1 reduction as a measure of viability for (A) parental and occludin overexpression cell lines and parental cells treated with (B) GRK Inhib 22 and ABMH GRK inhibitors, (C) paroxetine GRK inhibitor, or (D) roscovitine cell cycle inhibitor. Data are expressed as Mean \pm SD (n = 8). *P<0.05 compared to WT Occ, 0.5% DMSO, or no treatment.

TER was measured in parental cells treated with GRK inhibitors. Treatment with all three GRK inhibitors delayed barrier development in a dose-dependent manner yielding lower TER on day 3 (Fig. 3.10 B-D and 3.13 B-E). In contrast to the pan specific GRK Inhib 22, inhibitors specific to the GRK 2-3 subfamily (GRK Inhib 63 and 64) did not reduce TER (Fig. 3.13 B). Importantly, overexpression of S471D Occ was sufficient to attenuate the GRK Inhib 22-mediated TER decrease in MDCK cells while WT Occ expression tended to normalize TER but was not statistically significant (Fig. 3.13 F). These results strongly implicate GRK isoforms in phosphorylation of occludin S471, and that inhibition of GRK isoforms significantly decreases cell packing, TJ assembly, and peak TER, which may be attenuated by expression of the S471D Occ phosphomimetic.

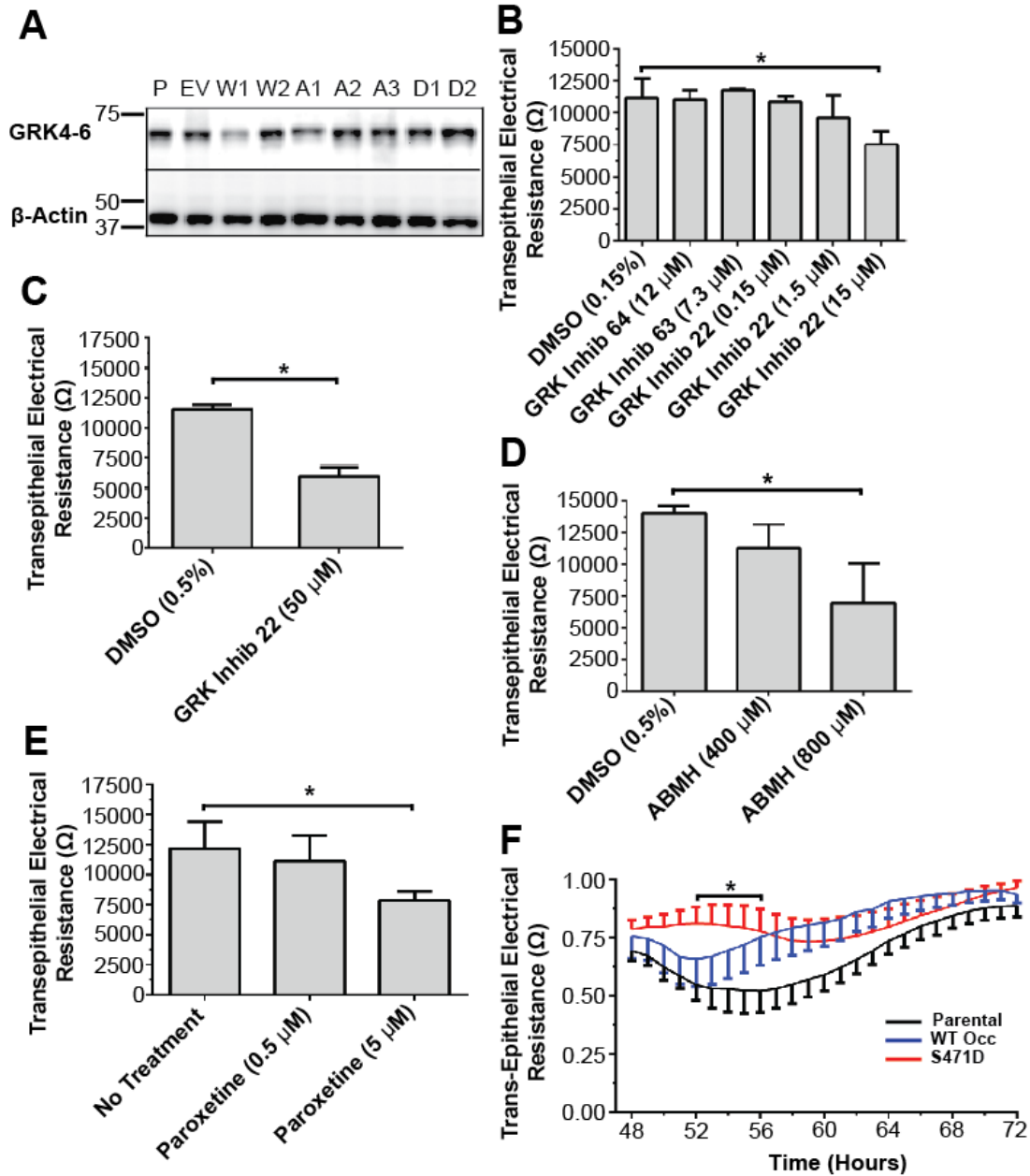


Fig. 3.13: GRK inhibitors reduce TER in a dose-dependent manner. (A) Western blot of all lines showing GRK subfamily 4-6 expression. (B-E) TER 3 days post confluence of MDCK treated from the beginning of each experiment with various GRK inhibitors including (B) GRK Inhibitors 64, 63, and 22 (15 μM), (C) GRK Inhibitor 22 (50 μM), (D) ABMH, and (E) paroxetine. Data are expressed as mean ± SD (n=4-8), *P<0.05 compared to control. (F) Comparison of TER from beginning to end of Day 3 post plating, showing ratio of DMSO control to GRK Inhib 22 treatment for each cell line. Data are expressed as mean ± SE (n=6-8) and compared using 2-way ANOVA with Bonferroni post-hoc test. *P<0.05 for parental vs. S471D at indicated times.

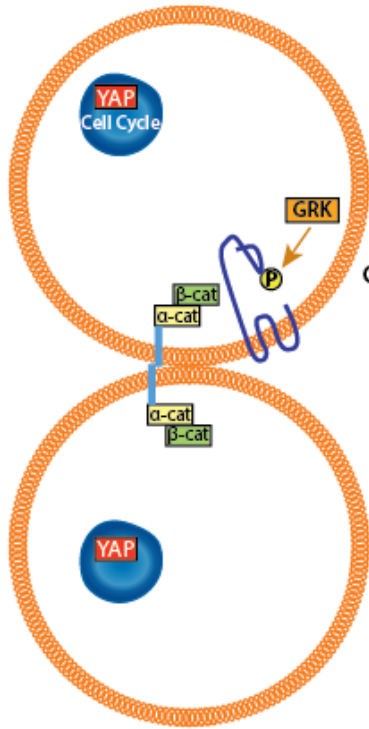
3.5 Discussion

Despite the importance of barrier formation and dysfunction in a variety of diseases, (Morgan et al., 2014; Murakami et al., 2012; Schmitz et al., 1999), the series of events leading to barrier maturation remain incompletely characterized. The present study demonstrates the necessity of size reductive proliferation or cell packing, for normal barrier maturation and identifies a role for the TJ protein occludin in this process. Previous studies have demonstrated that occludin contributes to proliferative control and cell cycle progression (Runkle et al., 2011; Wang et al., 2005), is altered in epithelial cancers (Gonzalez-Mariscal et al., 2007; Runkle and Mu, 2013), and may act as a tumor suppressor (Wang et al., 2005). Evidence is presented here demonstrating that phosphorylation of S471 of occludin regulates entry into size reductive proliferation after cell contact. Expression of an occludin S471 phosphoinhibitory mutant, Occ S471A, acts in a dominant manner to attenuate cell packing and subsequent TJ maturation, and formation of high resistance barriers. These effects can be recapitulated by treating confluent monolayers with a cell cycle inhibitor, demonstrating the necessity of cell packing for complete TJ assembly and monolayer maturation. Finally, Occ S471 was found to be a substrate for GRK, and three separate GRK inhibitors attenuated epithelial cell maturation. The data suggest that GRK phosphorylation of Occ S471 signals to allow size reductive proliferation. The reduced cell size then relieves cytoskeletal strain promoting Hippo/MST signaling for YAP nuclear exclusion (Aragona et al., 2013) and cellular quiescence with completed TJ formation. Inhibition of S471 phosphorylation or preventing cell cycle progression post-

confluency prevents size reductive proliferation leading to fewer larger cells with incomplete TJ formation (Fig. 3.14).

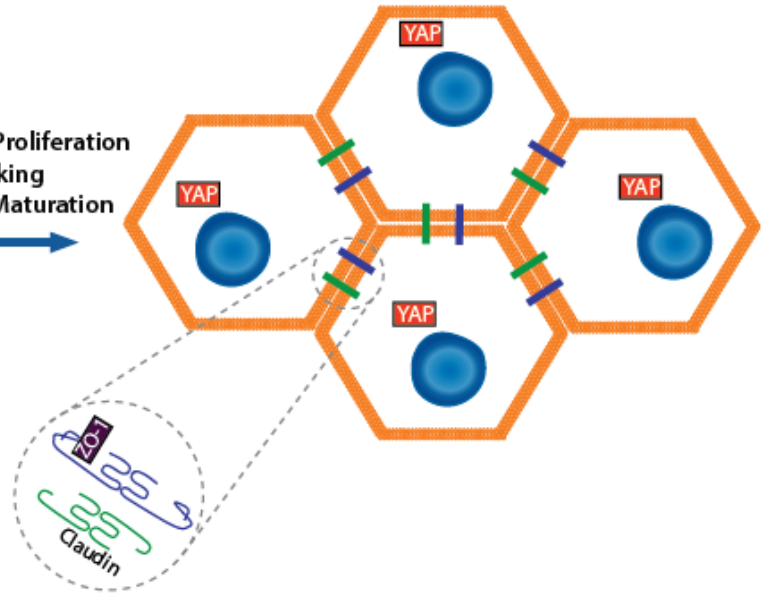
Monolayer Maturation (MDCK)

A Leaky Barrier

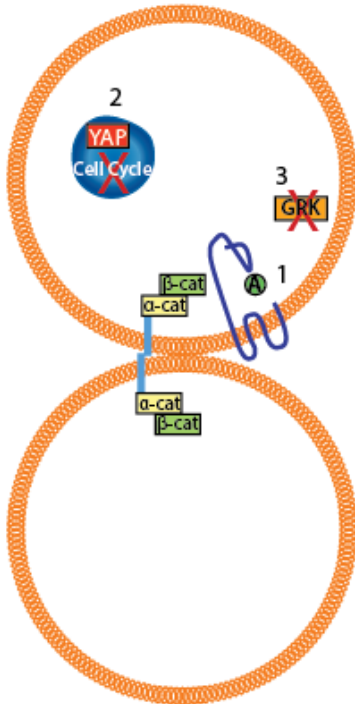


B Tight Barrier

Contacted Proliferation
Packing
Junction Maturation



C Leaky Barrier



Contacted Proliferation
Packing
Junction Maturation

D Leaky Barrier

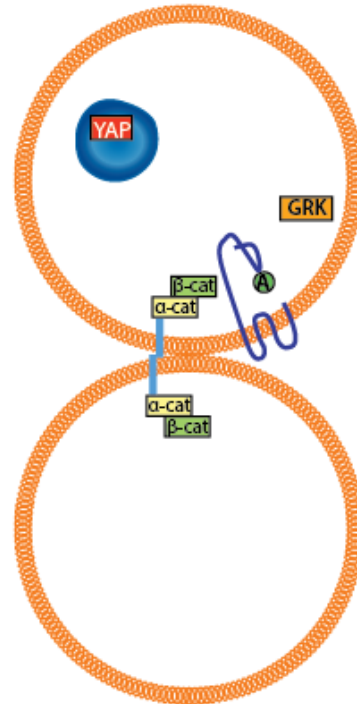


Fig. 3.14: Model of occludin S471 contribution to monolayer maturation. (A) Increasing confluency leads to contact by E-cadherin extracellular domains of adjacent cells (light blue bars). Cell confluency increases and occludin S471 is phosphorylated by GRK, allowing size reductive proliferation, decreasing cell area and increasing cell number. (B) As cell size decreases, YAP becomes excluded from the nucleus and junctional maturation proceeds, yielding a mature monolayer with high barrier resistance and proliferative quiescence. (C) Monolayer maturation may be perturbed by: (1) Ser to Ala mutation of occludin at S471, (2) cell cycle inhibition of newly confluent monolayers, or (3) inhibition of the S471 kinase GRK. (D) In all cases, size reductive proliferation is inhibited leaving an immature monolayer with complete AJ formation but poor TJ organization and low TER. Cells become quiescent despite nuclear localization of YAP and fail to undergo size reductive proliferation.

TJ barrier regulation by occludin is dependent on phosphosites which are particularly abundant in the C-terminal coiled coil (Cummins, 2012). Occludin dephosphorylation is associated with decreased electrical resistance and localization of occludin at the membrane, and C-terminal deletion increases solute flux and attenuates TJ organization (Andreeva et al., 2001; Balda et al., 1996). Specific phosphosites regulate the occludin-mediated response to various growth factors and cytokines. For example, phosphorylation at S490 by PKC β II in response to vascular endothelial growth factor treatment increases occludin ubiquitination leading to endocytosis and increased permeability (Murakami et al., 2009; Murakami et al., 2012). S490 phosphorylation also facilitates mitotic entry and increases proliferation (Runkle et al., 2011). Phosphorylation at S408 mediates interleukin-13-induced barrier loss, and inhibition of the S408 kinase, CK2, increases TER through altered inter-molecular complex formation within the TJ (Raleigh et al., 2011). Specific occludin phosphosites also regulate occludin's interaction with other TJ proteins, suggesting additional points of regulation (Dorfel et al., 2013; Elias et al., 2009; Suzuki et al., 2009). These studies support a role for specific occludin phosphosites in barrier regulation and suggest that novel sites may have important regulatory roles.

Overexpression of non-phosphorylatable S471A Occ disrupted cell packing and maturation of TJs but not AJs. AJs are formed prior to TJs following cell-cell contact, and while TJ and cytoskeletal organization were decreased in a dominant fashion in S471A Occ lines, AJ assembly was unaffected, revealing that AJ formation does not require cell packing as TJ formation does. Further, GRK inhibition did not alter AJ formation. Additionally, S471A Occ monolayers were composed of fewer cells with

increased area suggesting premature proliferative cessation. The Hippo/MST signaling pathway is an important determinant of cell and organ size, and nuclear exclusion of the co-activator, YAP, accompanies proliferative quiescence. While YAP is just one of several nuclear Hippo/MST proteins, proliferative cessation in S471A Occ cell lines despite continued nuclear YAP localization indicates a dominant effect of the mutant, leading to premature arrest of packing prior to completion of the Hippo/MST signaling pathway.

S471A Occ expression reduced TER despite similar TJ protein expression and Ca^{2+} , K^+ , and Cl^- ion permeability in all lines (Fig. 3.8 D-F), suggesting non-specific flux from gaps in the TJ rather than altered expression of claudins, or claudin pore formation to induce changes in permeability of a specific ion. Importantly, packing deficiencies evident in S471A Occ lines including mislocalized TJ proteins and reduced TER were recapitulated by pharmacological inhibition of packing alone, indicating that packing is necessary for establishing anatomically and functionally normal barriers. It is noteworthy that many TJ proteins appear at the cell border upon contact and before packing but that a continuous apical TJ is not completed until after size reductive proliferation and quiescence. How these downstream events are regulated remains an area for future investigation.

Structural analysis of occludin and ZO-1 binding suggests that occludin S471 is located within the acidic head of the coiled-coil that specifically binds the GuK domain of ZO-1 (Tash et al., 2012), raising the possibility that occludin S471 phosphorylation could affect the conformation of ZO-1 and thus epithelial maturation. The ZO family acts to organize cell junctions and links the junction to the actin cytoskeleton (Gonzalez-

Mariscal et al., 2000). Changes in localization of all the TJ proteins analyzed in the S471A Occ mutants as well as altered actin organization, particularly at the cortical ring, are consistent with an alteration in proper ZO-1 function at the cell membrane. Occludin expression is not necessary for TJ formation in the intestinal epithelia of mice or in MDCK cells (Schulzke et al., 2005; Yu et al., 2005), suggesting that the regulatory effect of occludin may eventually be compensated for in its absence. However, the effect of the S471A mutation was not transient, indicating that the presence of non-phosphorylatable S471 occludin inhibits packing and TJ formation in a dominant manner and revealing this site as a regulator of size reductive proliferation and epithelial maturation.

GRKs can phosphorylate S471 Occ, and inhibition of GRKs recapitulates results obtained with S471 Occ lines and cell cycle inhibition. GRKs contribute to signal transduction desensitization by phosphorylating G-protein coupled receptors and preventing coupling with cytoplasmic G-proteins. The sequence surrounding Occ S471 was tested in multiple kinase prediction software programs, and 41 kinases were selected for screening. GRK family members were able to phosphorylate the Occ S471-containing peptide consistent with recent studies demonstrating that non G-protein coupled receptor proteins including cytoskeletal (Chakraborty et al., 2014), nuclear (Martini et al., 2008), and membrane proteins (Dinudom et al., 2004), as well as transcription factors (Patial et al., 2010), can be GRK substrates. Seven GRK isoforms have been identified and are divided into three subfamilies based on sequence homology: isoforms 1 and 7 are tissue specific to the eye while 2 and 3 are ubiquitously expressed, as are isoforms 5 and 6 while isoform 4 has limited expression (Gurevich et

al., 2012). Importantly, three structurally distinct GRK inhibitors were able to recapitulate deficiencies in packing and TJ protein localization evident in S471A Occ lines in a dose dependent manner. In contrast to the pan-specific GRK Inhib 22, two inhibitors with greater specificity to the 2-3 subfamily did not decrease TER. This result along with a lack of GRK 1 and 7 expression in kidney and confirmed expression of the GRK 4-6 subfamily in MDCK cells by Western blot suggests that at least one member of the GRK 4-6 subfamily is an S471 kinase in MDCK cells. Silencing of GRK5 increases cell area, decreases tumor size (Chakraborty et al., 2014), and attenuates proliferation in various cancer lines (Kaur et al., 2013; Kim et al., 2012), demonstrating a regulatory role for the 4-6 subfamily in cell size and proliferation. While off target effects of GRK inhibitors used in the current experiment likely exist, the ability of three structurally distinct inhibitors to induce the same effect preventing epithelial maturation strongly suggests GRK as the target. Critically, the GRK inhibitor-mediated TER decrease observed in parental MDCK was significantly attenuated in the phosphomimetic S471D Occ overexpressing cells, implicating Occ S471 as a critical GRK target in epithelial maturation.

Taken together, these observations support a model in which Occ S471 phosphorylation contributes to the regulation of entry into size reductive proliferation after contact, followed by epithelial quiescence and assembly of TJs at the cell border leading to a high resistance barrier and a mature monolayer. These findings extend the current understanding of the role of occludin, and establish the importance of cell packing in barrier formation.

CHAPTER IV

Negative Charge at Occludin S471 is Not Necessary *in vitro* For Proper Localization and Organization of Tight Junction Proteins at the Border²

4.1 Abstract

Serine phosphorylation is a ubiquitous post-translational modification that acts as a molecular switch by which cells can turn various signaling pathways off and on to respond to internal or external conditions. Signaling can result from changes in protein-protein interactions mediated by phosphorylation status, as the addition or subtraction of a phosphorylation group can alter interactions within or between proteins. Previous experiments indicate that expression of a mutation at the S471 residue perturbs epithelial maturation and TJ formation. The current study examines the structural consequence of these mutations, and investigates the role of the negative charge carried by phosphorylation groups on the coiled-coil structure. WT Occ, S471A, and S471N occludin are transiently expressed in MDCK cells and compared with previous findings in WT Occ, S471A, and S471D lines. TJ proteins are well organized at the border in WT Occ but not in S471A lines, consistent with previous findings. S471N cells phenocopy WT Occ and previous S471D organization, despite asparagine (N) lacking a negative charge on its side chain. These results indicate that the negative charge is not necessary for TJ organization, and suggest that other molecular interactions provided by the phosphorylated serine are responsible. Modeling of S471 in phosphorylated and

² Portions of this chapter are included in a manuscript in preparation for a yet to be determined journal

non-phosphorylated forms suggest hydrogen bonding to Arg468 may stabilize the coiled-coil domain of occludin.

4.2 Introduction

Protein-protein interactions are important determinants of cell function and behavior, and contribute to cell-cell signaling, signaling cascades, and transcriptional control. Interactions between associated proteins occur at the molecular level and are influenced by factors such as polarity, charge, 3 dimensional structure (sterics), and proximity between specific protein residues. This is exemplified by the TJ protein, occludin, which binds to the scaffolding protein ZO-1. ZO-1 is a member of the MAGUK protein family, which is characterized by a catalytically inactive GuK domain that acts as a p-Ser/p-Thr binding domain (Lye et al., 2010; Zhu et al., 2011, See chapter 2). Characterization of other MAGUK and phosphorylated target binding interactions indicate important and separable binding domains for the interaction of both the p-Ser site, regulating the binding interaction and the nearby residues, yielding target specificity (Zhu et al., 2011). In these interactions, the MAGUK binding was dependent on phosphorylation while occludin and ZO-1 interaction do not require S471 phosphorylation; however, peptide binding studies reveal higher peptide binding with phosphorylation of S471 in an occludin peptide to the ZO-1 MAGUK (Tash et al., 2012). MAGUK contact sites include specificity binding region and phosphosite binding region. The specificity binding region restricts binding to specific substrate proteins while phosphosite binding region interacts with p-Ser/p-Thr (Zhu et al., 2011). In the case of the occludin-ZO-1 interaction, there is an electrostatic attraction between the relevant

protein domains conferring specificity. Specifically, the glutamic acid-rich and thus negatively charged C-terminal coiled coil head region of occludin interacts with ZO-1 at the face of its lysine rich, positively charged guanylate kinase (GuK) domain (Li et al., 2005; Tash et al., 2012), see chapter 3 for discussion). Previous studies suggest that glutamic acid residues flanking S471 on the coiled-coil interact with ZO-1 specificity sites, as negative to positive charge-reversal mutations at these residues decrease ZO-1 binding (Tash et al., 2012). In contrast, though S471 is a phosphorylatable residue, a charge-reversing S to K mutation at S471 at the center of the coiled-coil and interaction site minimally affects ZO-1 binding, indicating that S471 negative charge does not improve binding. Taken together, these findings suggest that occludin acts as a ZO-1 binding substrate, that residues E469-470 and E472-473 of occludin interact with ZO-1 GuK specificity sites, and that S471 interacts with an as yet unidentified phosphosite binding domain of the ZO-1 MAGUK and may be an important residue for mediating the effects of the occludin-ZO-1 interaction.

Occludin S471 is important for overall protein and TJ function, and overexpression of a phosphoinhibitory S471A form disrupts TJ assembly and function (see chapter 3 results and discussion). S471A expression does not, however, decrease the occludin-ZO-1 interaction as observed by Co-IP experiments, consistent with S471 interacting with a phosphosite binding domain that is not required for protein-protein interaction (Tash et al., 2012, Fig. 3.3). However, abolition of the occludin-ZO-1 interaction is not the only alteration that could alter function of either protein. The possibility remains that S471A could perturb the conformation/stability of the occludin

coiled-coil, leading to other, currently uncharacterized changes in ZO-1 interaction and function or interactions with other proteins.

The current study was part of a larger effort to investigate the effects of occludin S471 phosphorylation at the molecular level. Our collaborators John Flanagan and Maria Bewley at Pennsylvania State University found that expression of either S471A or S471D results in structural changes throughout the occludin coiled coil compared to non-phosphorylated WT. The changes observed upon S471D expression were surprising and in contrast to the hypothesized stabilizing effect of S471 phosphorylation. Serine phosphorylation changes the charge of the residue from polar uncharged to negative, but also changes the 3 dimensional structure by adding a phosphate group that is sterically larger and bulkier than unphosphorylated serine. Crystal structure and modeling data suggest that non-phosphorylated S471 interacts via hydrogen bond with Y474, and that phosphorylation enables hydrogen bonding with nitrogen both at the carboxyl backbone and near the side chain distal imine of R468. It remains unclear whether the contribution of S471 phosphorylation is due to the permanent negative charge carried by the phospho group or to other factors such as hydrogen bonding with nearby residues. The objective of the current study was to re-evaluate the *in vitro* effects of S471 phosphoinhibitory and phosphomimetic mutant occludin in TJ border localization, and to determine if the effects of S471 phosphorylation were due to charge or some other factor. Evidence is presented indicating that negative charge at S471 is not necessary for bTJ localization, implicating other, charge-independent factors.

4.3 Methods

TROSY NMR and molecular modeling

WT, S471A, and S471D mutant occludin coiled-coil domain was raised in *E. coli* vector and analyzed for structural differences using transverse relaxation-optimized spectroscopy nuclear magnetic resonance (TROSY NMR). Coiled-coil confirmation and hydrogen bonding was then modelled for WT, p-WT, S471D, and S471N coiled-coil using Chimera software (Biocomputing, Visualization, and Informatics, University of California San Francisco).

Cell culture

All reagents were purchased from Sigma Chemical (St. Louis, MO) unless otherwise noted. Madin Darby canine kidney (MDCK) cells were obtained from American Type Culture Collection (Manassas, VA) and cultured in minimum essential media as previously reported (Runkle et al., 2011). All cells were kept at 37°C with 5% CO₂.

Fusion protein constructs

Fusion protein constructs were generated by inserting cDNAs encoding WT human occludin or S471A mutant occludin into a pmaxFP expression vector (Amara, Cologne, Germany) following manufacturer instructions. An S471N construct was then generated using the WT Occ protein construct and Quik Change Site-Directed Mutagenesis Kit, following manufacturer instructions (Agilent Technologies, Santa Clara, CA). Mutagenesis primers were GGATGACTATAGAGAAGAAAATGAAGAGTACATGGCTGC (forward) and GCAGCCATGTACTCTTCATTTTCTTCTCTATAGTCATCC (reverse). Resulting

plasmids were sequenced to confirm serine to asparagine mutation. Images of amino acid structures were created in ChemDraw (PerkinElmer, Waltham, MA).

Transient transfections

WT Occ, S471A, and S471N were transiently transfected in to MDCK cells using lipofectamine 3000 and following manufacturer instructions. Cells were approximately 90% confluent at transfection.

Immunofluorescence

Cells were plated on chambered glass slides and fixed on the fourth day post transfection (three days post-confluent), with 3.5% paraformaldehyde (PFA) for 10 minutes (Thermo Scientific, Waltham, MA). Fixation was followed by 15 minutes permeabilization in TBS plus 0.25% triton-x 100. Cells were blocked in 10% goat serum (Life Technologies, Carlsbad, CA) with 0.25% triton-x 100 for 1 hour, then stained with GFP-occludin (Evrogen, Moscow, Russia), total occludin (Invitrogen, Carlsbad, CA), or ZO-1 (Millipore, Billerica, MA) primary antibodies overnight at 4°C and 1:200 (total occludin and ZO-1) or 1:400 (GFP) dilution in blocking solution. Secondary antibody (Alexa Fluor 488, 555, and 647, Life Technologies, Carlsbad, CA) exposure was 1 hour at room temperature in blocking buffer at a 1:1,000 dilution. Fluorescence was determined from Z-stacks of 0.5 µm slices taken on a Leica TCS SP5 confocal microscope (63x, 1.4 N.A oil objective, Wetzlar, Germany) with photomultiplier tube detectors, and using Leica Advanced Fluorescence software.

Tight junction width quantification and 3 dimensional projections

TJ width was quantified from confocal IF images using Metamorph software (Molecular Devices, Sunnyvale, CA). Border width was quantified by measuring GFP intensity along the border of adjacent cells both expressing GFP-occludin compared to borders of GFP-occludin expressing cells adjacent to non-GFP occludin expressing cells. IF staining was modeled by converted confocal IF files to 3 dimensional projections using the Imaris Software surface function (Bitplane, Belfast, UK).

Statistical analysis

Border width was normalized to WT Occ lines and compared in GFP-occludin-GFP-occludin borders as well as GFP-occludin non-GFP expressing borders via ANOVA followed by Bonferroni Post-hoc test (Prism 5.0 GraphPad Software, La Jolla, CA). Data are expressed as mean \pm SD, and $P < 0.05$ was considered statistically significant.

4.4 Results

S471 mutant occludin perturbs the occludin coiled-coil. To evaluate the effect of S471 phosphorylation status on the structure of the occludin coiled-coil, our collaborators performed transverse relaxation-optimized spectroscopy (TROSY) NMR experiments on WT, S471A, or S471D occludin coiled-coil. Both S471 mutations induced allosteric conformation changes within the coiled-coil (Fig. 4.1).

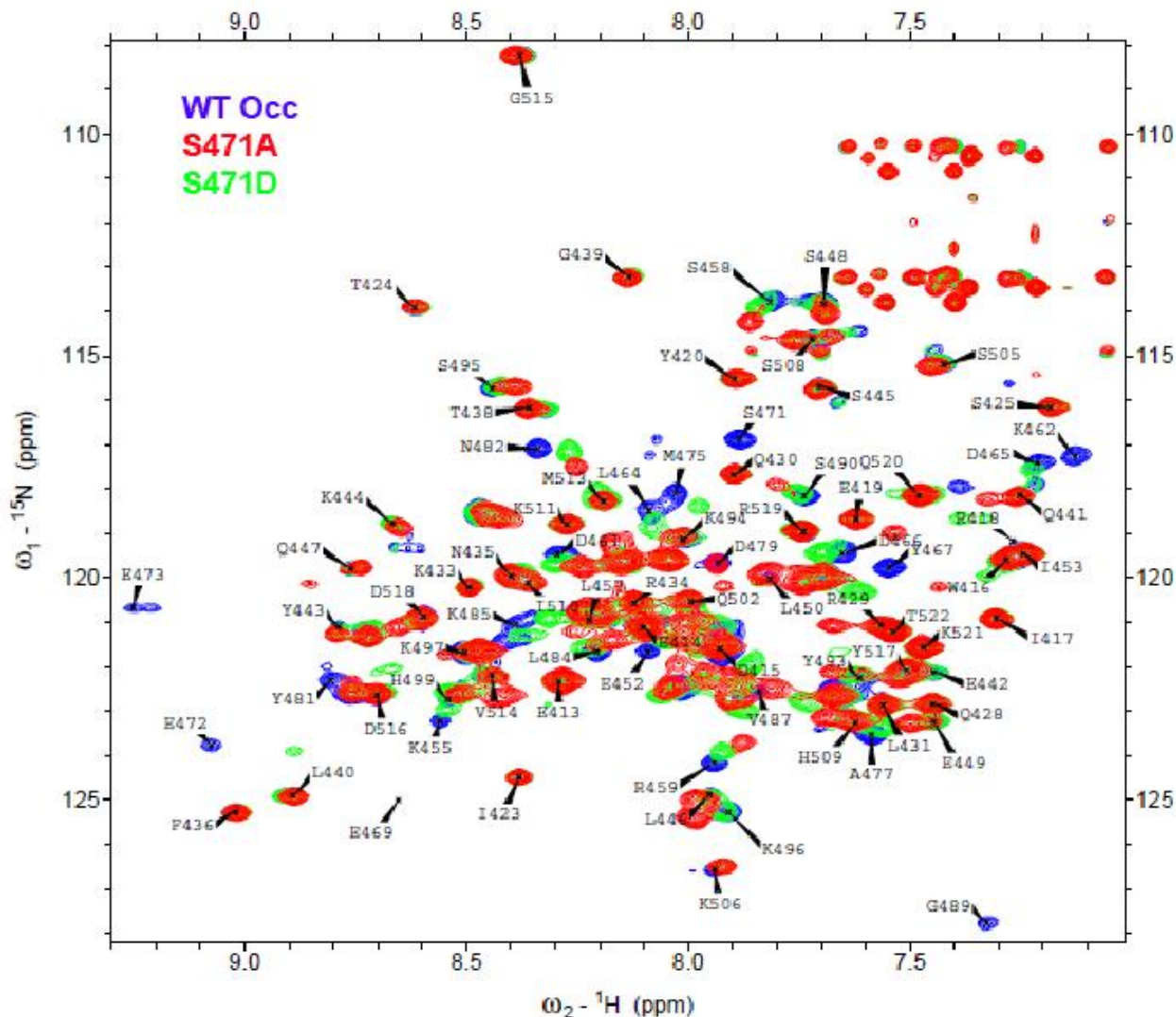


Fig. 4.1: S471 mutant occludin perturbs the occludin coiled-coil. Individual residues were resolved in WT (blue), S471A (red), and S471D (green) mutant occludin coiled-coil by TROSY NMR. Non-overlap of colors indicates a structural perturbation (Courtesy of Flanagan/Bewley Laboratory, Pennsylvania State University).

Phosphorylated S471 hydrogen bonds with neighboring residues.

This surprising result led us to investigate the effects of specific molecular interactions such as electrostatic attraction and hydrogen bonding in phosphorylated and non-phosphorylated S471. Our collaborators relied on known crystal structures and modeling to identify molecular interactions with nearby residues within the coiled-coil.

Phosphorylated and non-phosphorylated WT Occ as well as S471D and S471N mutant occludin are predicted to hydrogen bond from a side chain oxygen to a nitrogen on Y474 (Fig. 4.2), while S471A occludin is not (data not shown). Additionally, phosphorylated WT Occ, S471D, and S471N occludin are predicted to make two (p-WT Occ) or one (S471D and S471N) hydrogen bonds to R468 (Fig. 4.2). Thus, the phosphorylated S471 is predicted to form the most stabilized interaction with additional residues of the coiled-coil. The stabilized hydrogen bonding of S471N equivalent to S471D but without the negative charge addition allowed a direct assessment of the contribution of charge at Ser471 on organization of occludin into tight junctions.

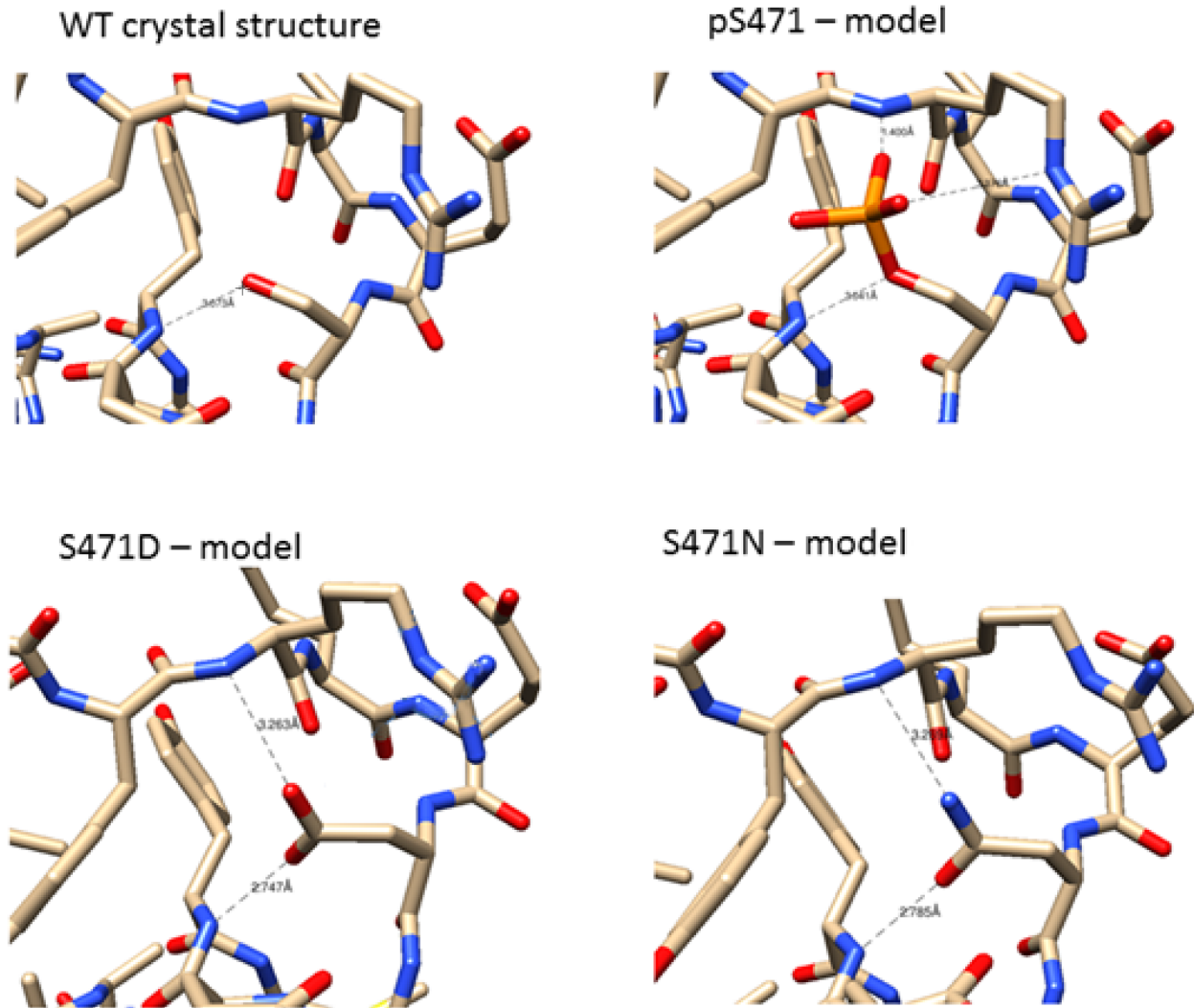


Fig. 4.2: Phosphorylated serine and phosphomimetic mutants hydrogen bond to R468. Crystal structure of WT Occ coiled-coil and models of p-S471, S471D, and S471N showing carbon (gray), oxygen (red), nitrogen (blue), and phosphorus (yellow). Confirmed and predicted hydrogen bonds are indicated by dotted lines with proximity given in angstroms (Courtesy of Flanagan/Bewley Laboratory, Pennsylvania State University).

S471 side chain negative charge is not necessary for in vitro TJ organization.

To evaluate the role of phosphorylation related negative charge on the occludin S471 sidechain in cells, MDCK cells were transiently transfected to overexpress GFP-tagged WT, S471A, or S471N mutant occludin. The alanine sidechain is uncharged

and lacks the necessary groups to hydrogen bond while asparagine is also uncharged, but is able to hydrogen bond and is structurally very similar to the phosphomimetic, aspartic acid (Fig. 4.3). WT Occ lines carry the native serine, which is presumably phosphorylated during monolayer maturation.

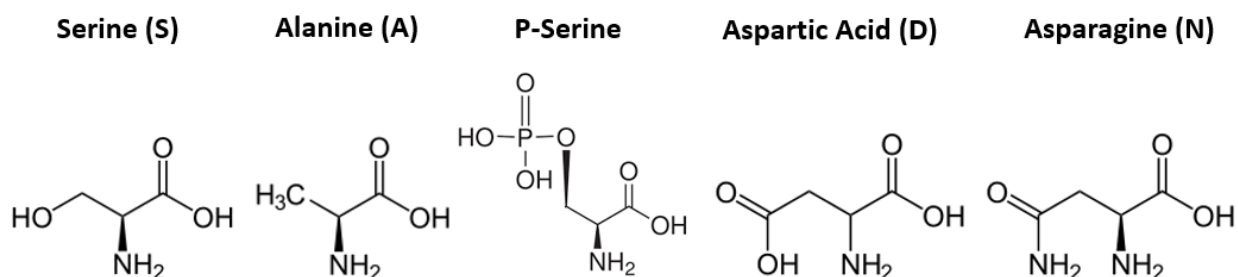


Fig. 4.3: Amino Acid Structures. Schematic of relevant amino acids. P-serine and aspartic acid carry negative charges on their side chains, while serine, alanine, and asparagine do not.

Analysis of borders between adjacent cells expressing WT exogenous GFP-occludin revealed crisp, well organized occludin and ZO-1 staining at the border, consistent with previous stable cell line experiments (Fig. 4.4 A-B, and see Fig. 3.2). In contrast, S471A staining for GFP and total occludin between two GFP-occludin expressing cells was broad and poorly organized at the border, again consistent with previous results (Fig 4.4 A-B, and see Fig. 3.2), while ZO-1 staining was conspicuously absent. S471N cell borders were well organized and very similar to WT in appearance for both occludin and ZO-1 (Fig. 4.4 A-B). Quantification of GFP-occludin border staining width for each occludin mutant confirmed disrupted protein localization in

S471A expressing cells as evidenced by broader GFP-occludin staining compared to WT Occ and S471N (Fig. 4.4 C). This effect was evident only in borders between two cells expressing the mutation, and no difference was observed between the various mutations in borders between a cell expressing exogenous occludin and one that did not (Fig. 4.4 D).

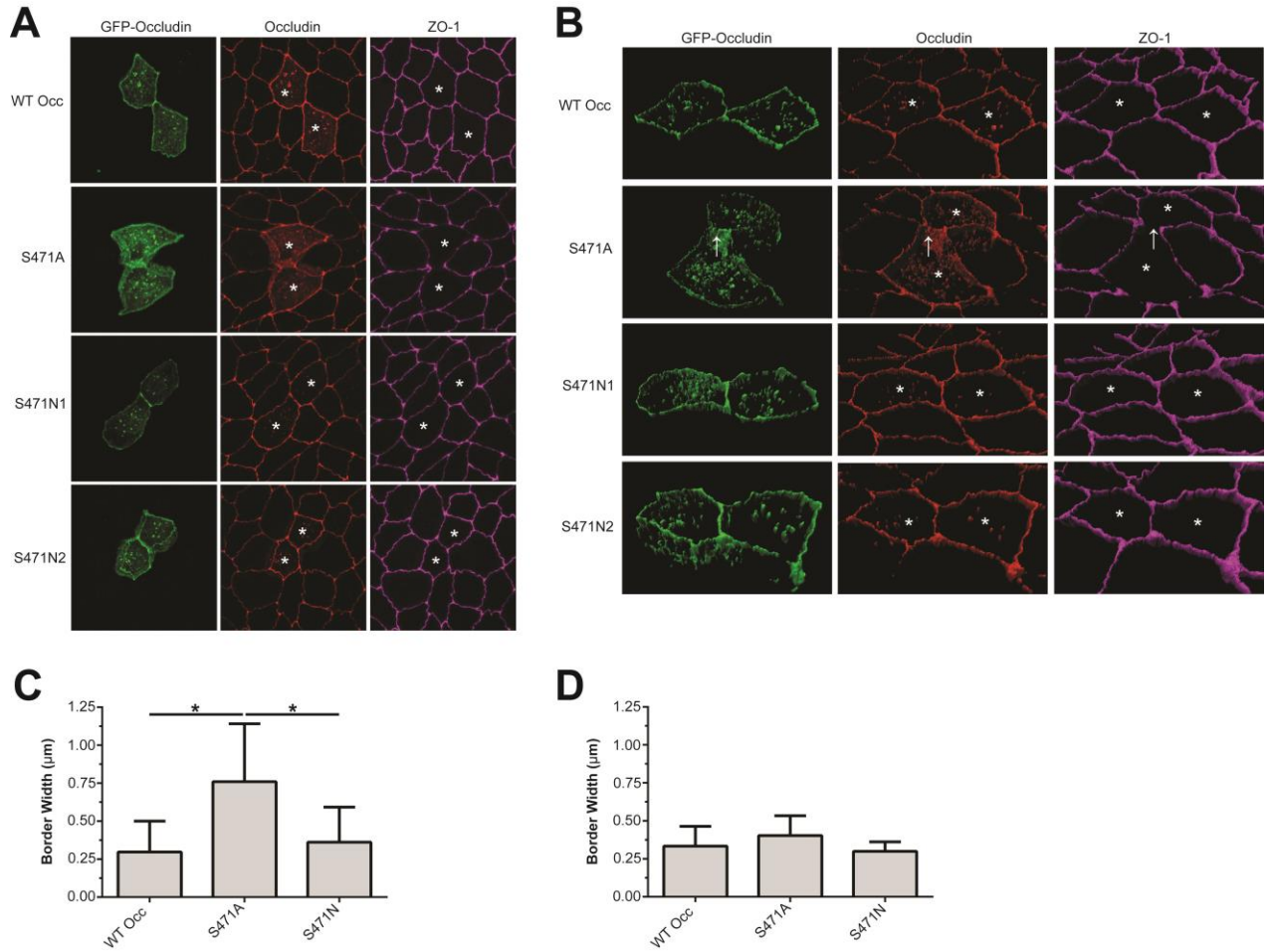


Fig. 4.4: WT Occ and S471N cell have well organized bicellular borders while S471A cells do not. MDCK cells transiently expressing exogenous GFP human occludin with no mutation (WT), S to A, or S to N point mutation at S471. (A) Immunofluorescence and (B) 3-dimensional modelling of indicated protein with white stars indicating cells expressing GFP-occludin and white arrows indicating poorly organized bicellular borders. Quantification of distance across the border of (C) two cells expressing GFP-Occludin as a measure of border organization, or (D) a control border between a GFP-Occludin expressing cell and a non-expressing cell. Data are expressed as mean \pm SD (n = 7-14). *P<0.05 compared to WT Occ.

4.5 Discussion

Amino acid phosphorylation is a post-translation modification that is crucial for signal transduction (Burnett and Kennedy, 1954; Thorner et al., 2014), and changes the amino acid side chain in at least two fundamental ways: it adds a negative charge, and a phosphate group that is sterically large. The phosphomimetic serine to aspartic acid point mutation effectively mimics phosphorylation electrically, but not sterically as the carboxylic acid side chain of aspartic acid carries a negative charge, but is not as bulky as a phosphate group. Phosphorylated serine hydrogen bonds with R468, and modelling of S471D indicates that a bond is still made with R468 despite the lack of bulk.

Previous studies with MDCK cells show that (presumably phosphorylated) WT Occ and S471D lines have well organized TJs at the borders, and transiently transfected WT Occ recapitulated this finding. TJ borders were also well organized in transiently transfected S471N cells. Since the asparagine side chain is uncharged, this result indicates that the negative charge found on aspartic acid or phosphorylated serine is not necessary for TJ organization, and the effect of phosphorylation is mediated by some other type of interaction. The identity of this factor remains elusive, but one possibility is hydrogen bonding. Importantly, non-phosphorylated WT Occ is not equivalent to S471A due to the lack of S471-Y474 hydrogen bonding in the later. This interaction is present in both phosphorylated and non-phosphorylated WT Occ however, as well as in S471D and S471N. Therefore, loss of Y474 hydrogen bonding would not account for the observed coiled-coil perturbation between non-phosphorylated WT Occ and S471D. A model is proposed in which occludin is phosphorylated at S471 at high

confluency, allowing for hydrogen bonding between S471 and R468 and stabilizing the coiled-coil in a confirmation favorable for initiation of TJ assembly. Given the dependence of TJ organization on packing through size reductive proliferation (chapter 3), this stabilized confirmation would presumably contribute to size reductive, confluent proliferation in some way, allowing for packing and TJ formation. S471 could then be dephosphorylated once a critical cell area (the transition point, see chapter 3) was reached, leading to loss of R468 hydrogen bonding, perturbation of the coiled-coil, and proliferative quiescence, or quiescence may be achieved through alternative signaling and S471 phosphorylation may be maintained. Additional studies will be necessary to evaluate the validity of this and alternative models.

CHAPTER V

Conclusions, Limitations, and Future Directions

5.1 Overall summary and conclusions

Recent findings concerning the participation of AJC proteins in non-canonical roles have changed the view of these junctions from static structures mediating only permeability/adhesion, to dynamic protein reservoirs that can participate through cell signaling in response to extracellular stimuli. The characterization of the novel occludin S471 site presented in this dissertation is consistent with this expanding role of AJC proteins, and with the already complex role of occludin. Previous publications in prestigious journals have already described in part the dynamics of epithelial cells at low and high confluency, the existence and size reductive nature of confluent proliferation, and the role of biophysical forces in this process (Aragona et al., 2013; Dupont et al., 2011; Puliafito et al., 2012). However, what was not appreciated, and is presented in the current dissertation, is the necessity of this monolayer maturation for barrier formation and the contribution of the occludin S471 site in this process. This dissertation links S471 phosphorylation to confluent proliferation, possibly as a checkpoint for its induction, as phosphoinhibitory (S471A) lines proliferate normally when subconfluent, but fail to initiate confluent proliferation. In this way, occludin influences the morphological characteristics of the monolayer and its constituent cells, but also affects TJ protein localization and the ultimate resistance of the barrier. The failure of S471A lines to undergo confluent proliferation and cell packing leaves monolayers with larger and less numerous cells at confluence, and as a result, poorly organized TJs and low resistance barriers. S471 phosphorylation may act as a

molecular switch that when phosphorylated, allows induction of confluent proliferation while preventing it when phosphorylation is prevented. Studies investigating the molecular consequences of phosphorylation within the occludin coiled-coil are presented and indicate that negative charge at S471 is unnecessary for TJ organization in cells. This suggests that some other consequence of S471 phosphorylation within the coiled-coil is responsible, potentially specific hydrogen bonding with R468, which is predicted to be present only when S471 is phosphorylated. S471 may also interact with the nearby occludin Tyr474 phosphosite, which has been identified as required for localization at the leading edge of migrating MDCK cells, and binds to PI3-kinase regulating cytoskeletal rearrangement (Du et al., 2010). In summary, the studies presented in this dissertation demonstrate roles for occludin S471 in proliferation, TJ organization, barrier resistance, and monolayer maturation. Additionally, an S471kinase is identified (GRK) and preliminary results of intra coiled-coil molecular interactions are reported. S471 is added to the list of confirmed, characterized occludin phosphosites including S408, S490, Y474 and S508, that mediate responses to specific stimuli, and facilitate the versatile and complex functions of the TJ protein, occludin.

5.2 Limitations

The approaches in this dissertation were sufficient to elucidate the importance of cell packing for monolayer maturation, the role of occludin in this process, and some of the atomic interactions within the occludin coiled-coil that may contribute to these effects. The studies were not, however, without limitations. The first is that the work was done *in vitro* and in cell lines. The nature of the questions under investigation

dictated that a simple model be used that would not have the complexity of animals or the time required for breeding. As with any *in vitro* study however, there may be limitations as to the external validity of our findings to other types of cells and to organisms. Additionally, the difficulty of transfecting or transducing MDCK cells led to the use of selected clonal cell lines. This carries with it the risk that a phenotype may be due to some unrelated mutation in a specific line rather than the mutation of interest, although multiple lines were used to mitigate this risk. Additionally, a key early finding was that S471A did not decrease the occludin-ZO-1 interaction. This conclusion was made based on Co-IP experimental results, but ZO-1 has contacts with many other TJ proteins, and is likely part of a large protein complex. Limitations of the Co-IP technique leave the possibility that S471A does decrease the occludin-ZO-1 interaction, but that they are both pulled down anyway due to a mutual binding partner or additional contact sites on the two proteins.

Finally, phosphoinhibitory and phosphomimetic mutations are mimics of phosphorylated or non-phosphorylated serine. Alanine and aspartic acid are good approximations of non-phosphorylated and phosphorylated serine, respectively on the basis of side chain charge, but both have key deviations in terms of 3D structure. Non-phosphorylated serine hydrogen bonds to the nearby Y474 backbone with its side chain hydroxyl group, a bond that cannot be made by the methyl side chain of alanine. How loss of this specific bond might affect coiled-coil stability is unknown. Aspartic acid differs from phosphorylated serine in that its side chain is a simple carboxylic acid, and therefore far less bulky than a phosphorylated serine. This difference has the potential to disrupt interactions that would be present with a phosphate group, particularly

hydrogen bonds, due to lack of proximity. In this case, our data suggests the S471D only makes one hydrogen bond with the nearby R468 rather than two like phosphorylated serine. No results were obtained at any point in these studies suggesting that S471D is anything less than a capable mimic of phosphorylated serine, but the effects of this reduction in bond number are unknown.

5.3 Future Directions

This dissertation describes many of the upstream events associated with S471 phosphorylation and its effects on monolayer maturation. These results provide some answers, but also raise many questions, and provide opportunities for further investigation. These include further studies of occludin S471 and broader questions of epithelial cell biology.

A logical addition to the findings in this dissertation would be the extension of applicability by testing GRK inhibitors or expressing S471A in other cell lines. This could include epithelial cells (Caco-2, HeLa), endothelial cells (BREC, HUVEC), or transgenic animals. Animal studies could include WT or mutant occludin knockin mice generated using emerging Crispr/cas9 technology. This dissertation also includes initial results from additional *in vitro* studies, which should be completed. Preliminary data indicates that the area of S471D cells is decreased compared to controls, even when subconfluent. This is a provocative result, and suggests that S471 phosphorylation is sufficient for size reductive proliferation. Additional experiments of cell area and

proliferative rates in subconfluent cells should be conducted to evaluate these initial findings.

A significant hurdle in explaining the downstream effects of S471A expression is the finding that S471A lines become proliferatively quiescent despite largely nuclear YAP (chapter 3). This is contrary to the prevailing view that YAP is nuclear only during proliferation, and indicates that this proliferative arrest is YAP independent. Furthermore, the normal subconfluent proliferation evident in these cells suggests that subconfluent and confluent (size-reductive) proliferation are separable processes, with subconfluent proliferation mediated by Hippo/MST, Wnt, biophysical forces, etc., and confluent proliferation mediated by an undescribed mechanism. If true, this dissertation describes a disruption in a pathway that is not yet known to exist. This adds significant complexity to understanding the S471 downstream mechanism, and dictates that further insights into the process of confluent proliferation may be necessary before the S471 mechanism can be fully appreciated. Conclusions can be made on the basis of this work that inhibition of S471 phosphorylation does not abolish the occludin ZO-1 interaction, does not result in endocytosis of occludin, and does not release any factor which disorganizes WT cell TJs (Fig. 3.3). Initial efforts at elucidating the true mechanism should focus on narrowing down potential areas of long term inquiry.

A good starting point would be to characterize subconfluent and confluent proliferation and cell area in occludin KD, or occludin/tricellulin/marvelD3 (TAMP) KD cells. Occludin KD cells have been generated with no reports of proliferative or packing abnormalities, but cell packing has not been specifically studied in these cells, and these experiments would answer questions about the necessity of occludin in confluent

proliferation. The documented compensation for occludin by TAMP family members may require that tricellulin and marvelD3 be silenced as well. Another worthwhile study would be to look for changes in overall or fractionated (nuclear, cytosolic, etc.) protein pools by Co-IP and mass spectroscopy, or of transcription-related changes by chromatin immunoprecipitation or electrophoretic mobility shift assays in control vs. S471A lines for both subconfluent and confluent conditions. A number of commercially available protein microarrays could also be used to probe for differences in protein expression or phosphorylation status. The goal of any of these experiments would be to identify proteins, phosphorylation events, or transcription factors/co-activators that differ between S471A and controls, and could help identify possible mechanisms.

Finally, the effects of S471A on proliferation suggest the involvement of a proliferative signaling pathway, and the contribution of marvelD3 to JNK signaling provides precedence for such an association in the TAMP family (Steed et al., 2014). The occludin Y474 site is a candidate for this type of association, as it is known to interact with and activate the p85 subunit of PI3K, allowing for organization of polarity proteins and the actin cytoskeleton (Du et al., 2010). Combined with known hydrogen bonding between Y474 and S471 (see chapter 4), this raises the tantalizing possibility that some connection such as facilitated phosphorylation may exist between Y474 and S471 in regulating PI3K, which could explain in part the effects of S471A mutants on proliferation. Preliminary studies examining tyrosine phosphorylation following GFP-occludin IP of WT or S471A Occ lines failed to demonstrate any differences in phosphorylation, but a general PY20 antibody was used (Fig. 3.5). Simple blots for

PI3K/Akt signaling could be performed followed by further trials with a PY474 specific antibody, which would better evaluate this possibility.

In terms of broader research directions, a conceptually simple yet promising research direction would be to determine the true role of TAMP family transmembrane proteins through multiple KD/KOs. Perhaps the largest confounder in the study of occludin over the last 15 years has been skepticism over its importance due to the perceived lack of phenotype of the occludin KO mouse (see chapter 3, (Saitou et al., 2000; Schulzke et al., 2005). Evidence has now emerged however showing that the TAMP family member, tricellulin, compensates at least in part for occludin (Ikenouchi et al., 2008; Krug et al., 2009), and it seems likely based on homology that the recently discovered family member, MARVELD3, may compensate as well. Knocking out all three proteins would prevent this compensation, and could show the true role of TAMPs.

Studies presented in this dissertation demonstrate the impact of monolayer maturation upon barrier functionality, providing justification for further investigation of maturation-associated mechanisms such as confluent proliferation. If an uncharacterized pathway for confluent proliferation exists, other components besides occludin are certainly involved. An approach for beginning to identify these components while further characterizing proliferation and monolayer maturation would be to employ a forward genetic screen in a model organism like *Drosophila melanogaster* or *Danio rerio* (zebrafish). A population could be screened following exposure to a mutagen, and individuals expressing proliferative or morphological abnormalities in cell monolayers could be further studied with the objective of identifying additional components of the

pathway. Any individual expressing monolayers that phenotypically resemble S471A cells would provide solid footing on which to start further investigations.

Finally, the ultimate goal of all biomedical research is to produce knowledge or treatments to improve human health, and questions of TJ and barrier biology are very relevant to the study of cancer. As previously described (chapter 2), AJC proteins play a significant role in proliferation and cancer development, especially in the context of cellular phenotypic transitions. Within this area, extensive study has been dedicated to the role of EMT in cancer including the loss of AJC proteins, the acquisition of a mesenchymal phenotype, and the resulting increase in metastatic potential. Very recently though, MET has been implicated in the development of secondary tumors following the arrival of metastasizing cells at their destination. Comparatively little study has been dedicated to MET, and opportunities exist in characterizing the phenotypic plasticity, AJC protein expression, and CIP/CIL expression of post-MET cells. This exciting area could apply knowledge of barrier biology and monolayer maturation to a significant and common pathology, and lead to future therapies to reduce or prevent metastasis.

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