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Chloe Yang March 24, 2022

Examining the role of STRIPAK in the tight junctions of epithelial cells

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An abstract of

a thesis submitted to the Faculty of Emory College of Arts and Sciences

of Emory University in partial fulfillment

of the requirements of the degree of

Bachelor of Sciences with Honors

Neuroscience and Behavioral Biology

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Abstract

Examining the role of STRIPAK in the formation of tight junctions in epithelial cells

By Chloe Saebin Yang

Background: Striatin family proteins, striatin, SG2NA, and zinedin, serve as scaffolds for striatin interacting phosphatase and kinase (STRIPAK) complexes, which play a role in several cellular processes, including signaling, cell cycle regulation, vesicular trafficking, cell polarity, vascular development, and cardiac functioning. In addition to core components, including striatin, protein phosphatase 2A, Mob3, CCM3, a GCKIII kinase, and STRIP1 or 2, different STRIPAK complexes can also contain additional components. Tight junctions (TJs) maintain cell polarity and protect organisms from their external environment. Striatin was recently shown to be present in tight junctions and to be important for their formation or stability, but what other components of STRIPAK that might be in tight junctions is not known. Moreover, how striatin is targeted to tight junctions is unknown. To define the STRIPAK complex present in TJs and to begin to study how STRIPAK is localized to junctions and what its role is in TJ formation, remodeling, and stability, we studied the localization of striatin and other STRIPAK components in human epithelial cells at steady-state and during remodeling.

Methods: The localizations of different STRIPAK components and TJ proteins were visualized using immunofluorescence (IF) in human colorectal cancer cells to characterize the STRIPAK complex present at tight junctions before and during TJ remodeling. In addition, stable cell lines expressing $PP2A^-$ or $CCM3^-$ striatin mutants were developed to help probe the relevant protein-protein interactions that are important for TJ formation and stability.

Results: We demonstrated that STRIPAK core proteins striatin, CCM3, GCKIII kinases Mst3 and Mst4, Mob3, and STRIP1 localize to tight junctions in epithelial cells. We also showed that of two mutually exclusive additional STRIPAK components, SLMAP and CTTNBP2/NL, only SLMAP is present in tight junctions. STRIPAK components CCM3, striatin, and STRIP1 were found to co-localize with junctional proteins during TJ remodeling, but SLMAP did not. $PP2A^-$ striatin mutant-expressing cells demonstrated an internalization of TJ scaffolding protein ZO-1.

Conclusions: Striatin-scaffolded core STRIPAK complexes containing SLMAP likely play a role in vesicular trafficking of TJ proteins to and/or from the membrane. This may be regulated in a PP2A-dependent manner. These findings provide insight into the regulation of TJ formation and CCM disease.

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Acknowledgements

First and foremost, I would like to thank Dr. Pallas for continuously supporting and guiding me through my undergraduate research and honors thesis experience.

I would like to thank Benjamin Cai for his contributions and valuable mentorship during his years at Pallas Lab.

I would like to thank Priscilla Lin and Victor You for their valuable contributions to this project. I would also like to thank Ryan Donovan, Ashley Zhu, and Frances Chiu for their support and contributions.

I would like to thank the other members of Pallas Lab, past and present, for their continuous support and help.

Finally, I would like to thank my friends and family for their uplifting words and emotional support during this journey.

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Introduction

Tight Junctions

Tight junctions (TJ), in conjunction with adherens junctions and desmosomes, maintain the integrity of epithelial and endothelial cells that protect organisms from their external environment (Zihni et al., 2016). These junctions, which are at the apical end of the overall junctional complex, are comprised of several protein complexes (Figure 1) that work in conjunction to maintain separate tissue compartments and enable cell polarity (Denker and Nigram, 1998). Dysfunction of tight junctions is associated with several neurological disorders, including Alzheimer's Disease, Autism Spectrum Disorder, and Cerebral Cavernous Malformation (Yamazaki et al., 2019, Fiorentino et al., 2016, Wei et al., 2020).

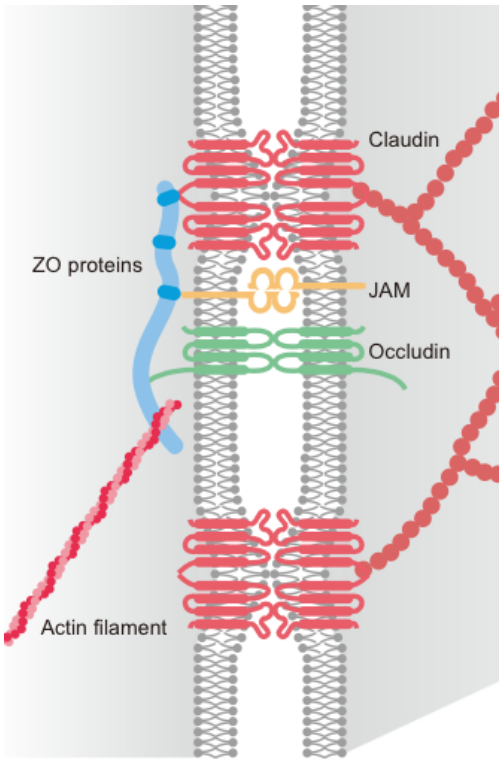

Figure 1. A model of tight junction structure
Adapted from "Tight Junction Structure and Function Revisited" by Tetsuhisa Otani and Mikio Furuse, 2020, *Trends in Cell Biology*, 30(10), p. 806

TJ proteins are characterized into two categories: integral transmembrane and scaffolding proteins. Integral transmembrane proteins include the TJ-associated MARVEL proteins occludin, tricellulin, and MARVELD3, the claudin family of tetraspan proteins, and junctional adhesion molecules (JAM). Together, they form a regulated permeability barrier that separates the apical and basolateral cellular domains (Denker and Nigam, 1998). The other key junctional protein group is scaffolding proteins, namely ZO-1, 2, and 3 (Zihni et al., 2016). These proteins function as adaptors that contain several protein-protein interaction domains that bind to junctional proteins and cytoskeletal components to maintain junctional integrity (Van Itallie and Anderson, 2017, Schneeberger and Lynch, 2004).

Tight Junction Assembly

Tight junctions assemble in a Ca2+ dependent manner (Gonzalez-Mariscal et al., 1990). In the absence of Ca2+, junctional proteins are synthesized, but fail to localize to the outer surface of the cell; consequently, these cells lack cell-cell contact, intercellular junctions, and cell polarization (Gonzalez-Mariscal et al., 1990, Denker and Nigam, 1998). Restoration of Ca2+ to the growth media restores junctional fibers in as little as 15 minutes in MDCK cells and restores TJ in 4-5 hours (Gonzalez-Mariscal et al., 1990). Calcium levels may also alter phosphorylation levels of perijunctional actin, resulting in changes to paracellular permeability (Kitamura et al., 1991).

During TJ remodeling, proteins are transported to and from the membrane in a process known as intracellular trafficking. The vesicles that transport the proteins are regulated by specialized GTPases known as Rab proteins (Segev, 2001). When proteins are internalized from the membrane during endocytosis, they are transported via early endosomes that are bound to

Rab5, a member of the Rab family (Zhu et al., 2004). Rab5 recruits rabaptin-5, which helps early endosomes bind to one another and facilitates membrane fusion via SNARE proteins (Zhu et al., 2004; Grant and Donaldson, 2009). As the endosome matures, the proteins are recycled or degraded (Maxfield and McGraw, 2004). Another notable Rab protein is Rab7, which targets its bound endosomes to lysosomes for degradation (Yap et al., 2018). At the tight junction, proteins are continuously recycled via endocytosis, which is essential for the proper function and structure of TJ (Ivanov et al., 2004). The Rab proteins have been found to be important for the transport of junctional proteins; previous studies have found that occludin co-localizes with Rab5-positive early endosomes during junctional remodeling (Ivanov et al., 2004). When epithelial layers are wounded or disrupted, Rab5-positive endosomes help transport occludin into the cell, which enables proper wound healing (Fletcher et al., 2012).

Phosphorylation, which is the addition of a negatively charged phosphate group to a protein by a kinase, alters the strength of protein-protein interactions. The assembly and disassembly of junctions are caused, in part, by the phosphorylation and dephosphorylation of serine and threonine residues (Itallie and Anderson, 2017). For claudins, the tyrosine phosphorylation within a specific binding motif decreases binding to ZO-1 and is thought to play a regulatory role in claudin localization by altering affinity or promoting binding specificity (Tanaka et al., 2005, Itallie and Anderson, 2017); however, phosphorylation of claudins outside of this binding motif result in enhanced TJ formation (Nunbhakdi-Craigh et al., 2002). Phosphorylation of occludin results in localization to tight junctions, while dephosphorylation results in barrier loss and localization to the lateral membrane (Sakakibara et al., 1997). Several kinases and phosphatases have been implicated in the regulation of TJ protein localization, including protein kinase C (PKC), Rho kinase, and protein phosphatase 2A (PP2A) (Dorfel and

Huber, 2012).

PP2A

Protein phosphatase 2A (PP2A) is a major heterotrimeric serine/threonine phosphatase (Janssens and Goris, 2001). PP2A plays a key role in many cellular processes, including DNA replication, transcription, translation, and the progression of the cell cycle (Janssens and Goris, 2001). It is composed of three subunits: an A scaffolding subunit, a C catalytic subunit, and a B regulatory subunit (Figure 2).

The A and C subunits each have α and β isoforms encoded by separate genes, while the B subunit has four different families, each with several isoforms, which allow PP2A to have high substrate specificity (Janssens and Goris, 2001).

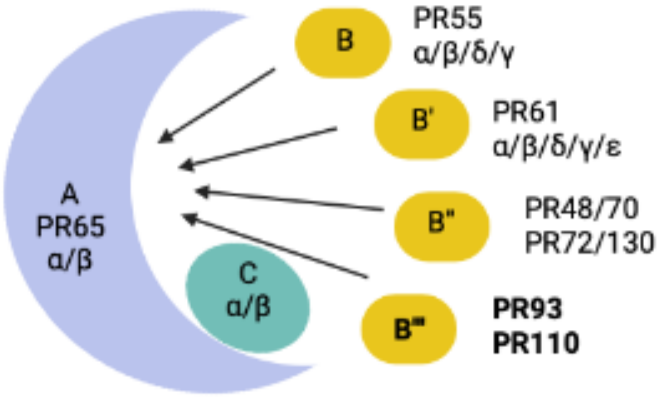

Figure 2. The structure of PP2A
Made with BioRender.com

PP2A functions in a number of cell signaling pathways and has notably been found to play a critical role in TJ homeostasis and cell polarity. PP2A has been found to concentrate around cell-cell contact sites during junctional assembly and interact with junctional proteins such as ZO-1, occludin, and claudin-1 in vitro, but not adherens junction proteins such as E-

cadherin (Nunbhakdi-Craig et al., 2002). Enhanced PP2A activity through the overexpression of the C subunit results in the dephosphorylation of TJ proteins and increased junctional permeability, suggesting that PP2A's dephosphorylating effects promote TJ disassembly (Nunbhakdi-Craig et al., 2002). Moreover, PP2A inhibition via fostriecin attenuates the TJ-disrupting effects of acetaldehyde incubation, diminishing the resultant cytosolic localization of occludin and ZO-1. (Dunangan et al., 2012). These findings provide compelling evidence that PP2A serves as a negative regulator for TJ assembly.

STRIPAK Complex

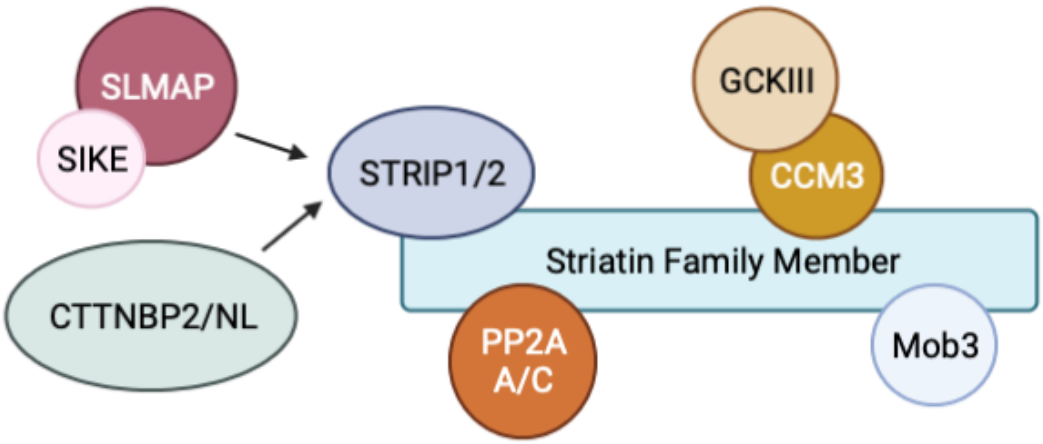

Figure 3. STRIPAK complex structure
Made with BioRender.com

Striatin-interacting phosphatase and kinase (STRIPAK) complexes are thought to play a role in several key cellular processes, including signaling, cell cycle regulation, vesicular trafficking, cell polarity, vascular development, and cardiac functioning (Hwang and Pallas, 2014). The core complex is comprised of a member of the striatin family, the A and C subunits of protein phosphatase 2A (PP2A), a member of the GCKIII subfamily of mammalian sterile 20-like (Mst) kinases, CCM3, and either striatin interacting proteins 1 or 2 (STRIP1/2) (Goudrealt et

al., 2009). STRIP1/2 either binds either sarcolemmal membrane-associated protein (SLMAP) and suppressor of IKK$_\varepsilon$ (SIKE) family member or a cortactin-binding protein 2 family member (CTTNBP2 or CTTNB2NL) to produce distinct STRIPAK complexes (Goudrealt et al., 2009).

Striatin Family

Members of the striatin family function as a B''' family of B-type regulatory subunits in PP2A (Moreno et al., 2000). In mammals, the striatin family consists of striatin (STRN), SG2NA (STRN3), and zinedin (STRN4). These proteins, especially STRN and STRN4, are highly expressed in the central nervous system and are thought to play key roles in brain function and development (Hwang and Pallas, 2014). STRN is highly expressed along dendritic spines within the striatum and has been found to be important for dendritic development and locomotion in rats (Li et al., 2018; Bartoli et al., 1999). Furthermore, STRN has been implicated in the development of intracranial aneurysm through the regulation of neuronal projection development (Wei et al., 2017). All members of the STRN family share a caveolin binding region, coiled coil domain, calmodulin binding region, and a C-terminal WD40-repeat domain (Figure 4).

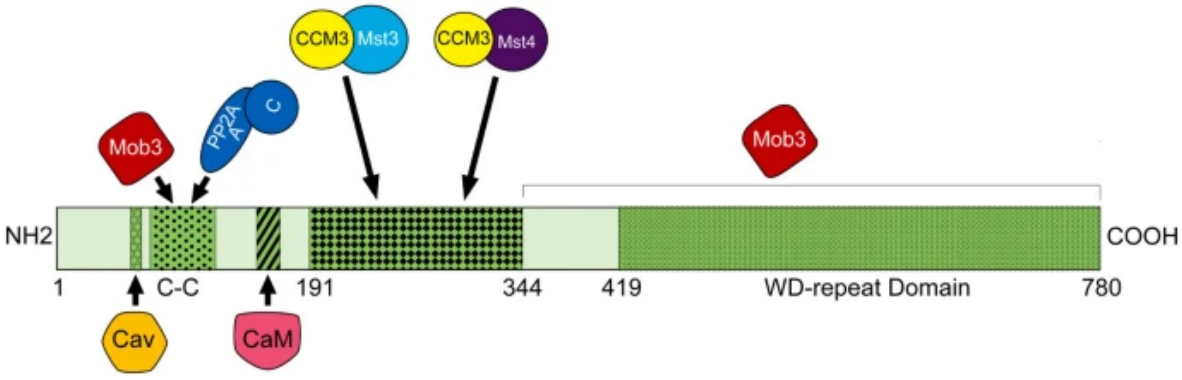

Figure 4. Striatin binding domains
Adapted from "Protein phosphatase 2a (PP2A) binds within the oligomerization domain of striatin and regulates the phosphorylation and activation of the mammalian Ste20-Like kinase Mst3" by Gordon et al., 2011, *BMC Biochemistry*, 12(54), p. 12

As the scaffolding protein in the STRIPAK complex, members of the striatin family have several protein-protein interaction domains that enable STRIPAK's diverse range of functions within the cell (Hwang and Pallas, 2014, Gordon et al., 2011). The coiled-coil domain, which is found between amino acids 70-116, enables oligomerization with other striatin family members, as well as their association with PP2A (Gordon et al., 2011). CCM3, which recruits Mst3 and Mst4 kinases, is thought to bind between residues 191-344 (Gordon et al., 2011). Interaction between striatin and PP2A is essential for proper STRIPAK functioning. Previous studies have discovered that the STRIPAK complex is comprised of several phosphoproteins which are reversibly phosphorylated and that the inhibition of PP2A using okadaic acid results in the increase in striatin-associated proteins (Goudreault et al., 2009; Moreno et al., 2001). Furthermore, a study using STRN mutants that were PP2A deficient found that Mst3 was hyperphosphorylated and more active, suggesting that within the STRIPAK complex, PP2A negatively regulates the activation of Mst3 kinase (Gordon et al., 2011).

GCKIII and CCM3

GCKIII kinases present in STRIPAK include MST3, MST4, and sterile 20/oxidant stress-response kinase 1 (SOK1/YSK1) (Zihni et al., 2016). They serve several functions within the cell, including golgi organization, cytoskeleton regulation, and cell polarity (Zalvide et al., 2013). They have also been found to play roles in programmed cell death, with Mst3 and YSK1 activation resulting in apoptosis, while Mst3 serves a protective role against accidental cell death due to oxidative stress (Pombo et al., 1996; Huang et al., 2002; Fidalgo et al., 2011). GCKIII kinases have been implicated in several pathologies, including Alzheimer's Disease and

Parkinson's Disease (Matsuki et al., 2012; Zach et al., 2010). Importantly, they can form a homodimer or a heterodimer with adaptor proteins such as CCM3 (Rual et al., 2005).

CCM3, also known as PDCD10, is a ubiquitously expressed protein that plays a role in cell death and is part of the core STRIPAK complex (Shi et al., 2016). It consists of an N-terminal dimerization domain and a C-terminal focal adhesion targeting-homology (FAT-H) domain (Li et al., 2010). The FAT-H domain of CCM3 forms complexes with either CCM2 or STRN in a mutually exclusive manner (Kean et al., 2011). CCM3 can form a homodimer or a heterodimer with one of the GCKIII kinases (Zhang et al., 2013, Ceccarelli et al., 2011). The interaction between GCKIII kinases and CCM3 is important for GCKIII kinase interaction with the STRIPAK complex, with CCM3 downregulation reducing GCKIII-STRIPAK binding and increasing GCKIII binding to GM130, a golgi matrix protein (Kean et al., 2011). Due to its interactions with both STRN and GCKIII, CCM3 acts as a bridge between the GCKIII kinase and PP2A.

Mob3

Mob3, also known as Phocein, is a core STRIPAK protein that plays a role in vesicular trafficking and cell fusion (Baillat et al., 2002; Maerz et al., 2009). It interacts with several proteins that are involved in vesicle trafficking, such as Eps-15, Dynamin-1, and nucleoside-diphosphate kinase (NDPK) (Baillat et al., 2002). It is thought to play a role in several neuronal functions, such as synapse formation, neurite growth, vesicular trafficking along axons, and microtubule organization in *Drosophila* (Schulte et al., 2010). Given that axonal transport dysfunction underlies several neurological disorders such as ALS and Huntington's Disease, understanding the role of Mob3 and thus STRIPAK in vesicular trafficking may help with the discovery of novel therapeutic targets for these debilitating disorders (Chung et al., 2018).

STRIP1/2 and associated proteins

STRIP1 and STRIP2, also known as FAM40A and FAM40B, respectively, are striatin-interacting proteins that play a key role in actin organization and cell migration (Bazzi et al., 2017). Studies have implicated STRIP1 and 2 as regulators of cancer cell mobility and metastasis by enabling PP2A to dephosphorylate and deactivate Mst3/4 within the STRIPAK complex (Madsen et al., 2014). STRIP1/2 bind mutually exclusively with either CTTNBP2/NL or SLMAP, which may target the STRIPAK complex to different subcellular locations.

CTTNBP2/NL

CTTNBP2 and CTTNBP2NL are homologous proteins that both interact with cortactin but are located at different subcellular locations and serve different functions within the cell (Chen et al., 2012). CTTNBP2 has been found to target STRN and PP2A to dendritic spines and plays a role in the growth of dendritic spines (Chen et al., 2012). It has been implicated in autism spectrum disorder (ASD), suggesting that CTTNBP2 plays a key role in neuronal function (Cheung et al., 2001, Iossifov et al., 2012). CTTNBP2NL, on the other hand, targets cortactin to stress fibers (Chen et al., 2012).

SLMAP

SLMAP (sarcolemmal membrane-associated protein) is a protein that interacts with STRIPAK via STRIP1/2. It has been found to play a role in intracellular trafficking; proteomic studies have found that SLMAP forms complexes with Rabaptin-5/RABEP1, which regulates endosomal fusion (Hauri et al., 2013; Stenmark et al., 1995). Previous studies have found that

SLMAP KO mice experienced reduced glucose uptake, further suggesting that it plays a role in endosomal trafficking within the cell (Chen and Ding, 2011)

Cerebral Cavernous Malformation

Cerebral cavernous malformation (CCM) is the most common type of vascular malformation with a prevalence of 1 in 200-250 individuals (Fischer et al., 2013). CCM is characterized by enlarged, blood-filled capillaries that lack supporting smooth muscle cells, ensheathing pericytes, and astrocytic foot processes that are essential for the maintenance of the tight junctions of the blood brain barrier (Clatterbuck et al., 2001, Brown et al., 2019). Due to the lack of tight junctions, there is often a leakage of red blood cells into the surrounding parenchyma, even in the absence of major hemorrhage (Clatterbuck et al., 2001). Consequently, these dilated blood vessels are prone to rupture and can lead to hemorrhages, stroke, seizures, or even death (Akers, et.al., 2009). CCM does not appear on angiograms and is instead detected using MRI; consequently, the condition often goes undetected with other imaging methods and may be misdiagnosed as lymphoma, epilepsy, or aneurysm (Kareem et al., 2021; Said et al., 2014)

CCM can be spontaneous or caused by loss of function mutations to Krit1, MGC4607, or PDCD10, which code for CCM1, CCM2, and CCM3, respectively (Fischer et al., 2013). However, mutations in CCM3, which account for 22% of hereditary CCM, put patients at a higher risk for more severe phenotypes, such as cerebral hemorrhage (Fauth et al., 2014). Interestingly, CCM3 mutations that cause CCM in human patients almost always impact the FAT-H domain, suggesting that interaction with either CCM2 or STRN is essential for the maintenance of a sufficient blood-brain barrier in endothelial cells (Li et al., 2010). Loss of CCM3 in both familial and sporadic CCM patients results in a reduction in protein expression of

ZO-1, occludin, and claudin-5 (Stamatovic et al., 2015). Furthermore, siRNA KD of CCM3 in vitro results in diminished interactions between structural junctional protein ZO-1 and claudin, occludin, JAM-A, and β-actin (Stamatovic et al., 2015).

Despite both sporadic and familial CCM resulting from loss of function mutations, the disease expresses as a focal lesion rather than a systemic defect (Snellings et al., 2021). This has been attributed to a two-hit model of the disease, in which a secondary, somatic mutation results in biallelic loss of CCM genes and triggers the formation of lesions (Akers et al., 2009). This finding explains why sporadic CCM presents as a single lesion while familial CCM patients suffer from multiple lesions, given their predisposition for biallelic loss from the so-called "second hit" (Snellings et al., 2021).

Recent studies have found that the endothelial junction disruption observed in CCM may be a result of dysfunctional signaling pathways. One proposed mechanism of CCM is deficiencies in Mst3 and Mst4, which give rise to vascular lesions in vivo and result in cellular abnormalities associated with CCM in vivo (Sartages et al., 2022). Yet another hypothesis is the the inability of Rho kinase (ROCK) to stabilize vascular stability due to loss of function (LOF) mutations in CCM genes (Stockton et al., 2010). ROCK hyperactivity, which has been found in both familial and sporadic CCM, results in an increase of myosin light chain phosphorylation, stress fiber formation, and vascular irregularities (Stockton et al., 2010). Preclinical studies have found fasudil, a ROCK inhibitor, reversed vascular leak in vivo (Stockton et al., 2010). Interestingly, PP2A has been found to negatively regulate RhoA in the maintenance of vessel lumen (Martin et al., 2013), which poses a potential role of STRIPAK in the maintenance of endothelial tight junction integrity.

Preliminary data and hypothesis

Previous research has shown that striatin colocalizes with junctional proteins ZO-1 and occludin, but not E-cadherin, suggesting that striatin localizes specifically to TJ and not to adherens junctions (Nunbhakdi-Craig et al., 2002). Co-immunoprecipitation studies revealed that STRN interacts with cingulin and occludin in mouse keratinocytes (R. Arbesfeld, unpublished data). In vitro STRN knockdown data has revealed that STRN is essential for the proper localization of ZO-1 to TJ (R. Arbesfeld, unpublished data). Furthermore, STRIPAK has been shown to play a key role in maintaining TJs in the inner ear that allow for proper hearing (Narad-Ponniah et al., 2020). However, it is not yet known whether these interactions and functions are directly mediated by striatin or mediated by other STRIPAK proteins. Moreover, the interactions between STRN and STRIPAK proteins that are important for junctional localization is unknown.

For this reason, we studied the importance of STRN and other STRIPAK proteins for TJ formation and integrity, to understand the overall role of STRIPAK in tight junctions. The purpose of our study was to characterize the specific STRIPAK complex present in TJs, and to assess the domains of STRN and protein-protein interactions that are essential for TJ formation by using STRN mutants that lack either PP2A or CCM3 binding sites. These studies have begun to address the hypothesis that striatin-scaffolded STRIPAK facilitates the vesicular transport of junctional proteins to and from TJ in a PP2A-regulated manner.

Materials and Methods

Table 1. Primary Antibodies Used

Antigen	Species	Catalog Number	Clone	Isotype	Working Solution	Source
Striatin	Mouse	610838	6	IgG2b	1:500	BD Bioscience
Striatin	Rabbit	RK16	n/a	IgG	1:100	Pallas Lab
ZO-1	Mouse	33-9100	ZO-1-1A12	IgG1	1:500	Invitrogen
ZO-1	Rabbit	40-2300	ZMD.437	IgG	1:500	Invitrogen
Occludin	Rabbit	71-1500	n/a	IgG	1:250	Invitrogen
CCM3	Goat	sc-67907	E-15	IgG	1:100	Santa Cruz Biotechnology
Mob3	Rabbit	RK130	n/a	IgG	1:100	Pallas Lab
STRIP1	Mouse	TA502314S	OTI7G7	IgG2a	1:100	OriGene
STRIP2	Rabbit	HPA019657	n/a	IgG	1:100	Novus
SLMAP	Mouse	sc-393336	B-9	IgG1	1:100	Santa Cruz Biotechnology
SIKE	Rabbit	HPA024177	n/a	IgG	1:50	Novus
CTTNBP2	Rabbit	HPA044654	n/a	IgG	1:100	Novus
16B12	Mouse	MMS-101R-500	HA.11	IgG1	1:1000	Covance

Table 2. Secondary Antibodies Used

Antibody	Target	Working Solution	Source
Goat Anti-Rabbit IgG DyLight 488 Conjugated	Rabbit IgG	1:1000	Bethyl
Goat Anti-Rabbit IgG DyLight 594 Conjugated	Rabbit IgG	1:1000	Bethyl
Goat Anti-Mouse IgG DyLight 594 Conjugated	Mouse IgG	1:1000	Bethyl
Goat Anti-Mouse IgG DyLight 488 Conjugated	Mouse IgG	1:1000	Bethyl
Rabbit Anti-Goat IgG DyLight 488 Conjugated	Goat IgG	1:1000	Bethyl

Table 3. Striatin constructs (Gordon et al., 2011)

Name	Protein Expressed
LIC-pLenti6/V5-Topo-2x-HA-4	None (Vector only)
LIC-pLenti6/V5-Topo-2x-HA-HA-hStriatin WT #6.2	WT striatin
LIC-pLenti6/V5-Topo-2x-HA-HA-hStriatin 100/101 #3.1	$PP2A^-$ mutant striatin
LIC-pLenti6/V5-Topo-2x-HA-HA-hStriatin 84/94/105 #3.2	$PP2A^-$ mutant striatin
LIC-pLenti6/V5-Topo-2x-HA-HA-hStriatin Δ191-344	$CCM3^-$ mutant striatin

Maintenance of Cell Lines

Caco-2 cells obtained from the Koval Lab (Emory University, Atlanta, GA) were plated on 60 mm tissue culture dishes in Caco-2 culture media, which consists of Minimal Essential Medium (MEM), 15% fetal calf serum, 1% nonessential amino acids, 1% penicillin/streptomycin, 1% L-glutamine supplement, and 1% sodium pyruvate supplement, in 5% CO2/95% air at 37ºC. For propagation, cells were rinsed with 0.05% Trypsin, and incubated 10 minutes in 0.25% Trypsin or until cells were largely present as single cells. Trypsin was neutralized with medium, and the cells were plated into new dishes or onto cover slips at different dilutions.

Immunofluorescence

Caco-2 cells were plated on 1.25 cm round glass coverslips in 35mm dishes, grown to confluence, and then processed for staining. Cells on coverslips were washed for 2 minutes in ice cold phosphate buffered saline (PBS) solution, then fixed in a 1:1 methanol-acetone solution for 2 minutes at room temperature. The coverslips were then washed in PBS, and permeabilized in 0.1% Triton x100/PBS for 10 minutes. They were then blocked to prevent nonspecific staining for 1 hour in a solution of 3% BSA with 10% horse serum in PBS. Dilutions of primary antibodies were prepared in 3% BSA and 10% horse serum in PBS. After blocking, 100 µL of the appropriate antibody dilutions (Table 1) were pipetted onto each of the coverslips, which incubated overnight at 4º C in a humidified environment. After incubating overnight, cells were washed in PBS for 15 minutes. Secondary antibodies were diluted in 3% BSA with 10% horse serum in PBS. 100 µL of the dilutions were pipetted onto the coverslips, which were incubated in

a dark, humidified environment at room temperature for 1 hour before being mounted using Dapi Fluoromount G on glass slides for imaging.

SDS/DTT Epitope Unmasking

A 1.3% SDS and 0.52% DTT aqueous solution was prepared and heated to 95 degrees Celsius in a heating block. After fixation and permeabilization, cells were heated in the heated SDS/DTT solution for 1 minute. Coverslips were washed in ice-cold Western Blot transfer buffer for 5 minutes, then washed in PBS for 5 minutes.

Calcium switch

Cell media was switched to 3 mmol calcium chloride in MEM with 15% fetal calf serum, 1% nonessential amino acids, 1% penicillin/streptomycin, 1% L-glutamine, and 1% sodium pyruvate solution for 10-12 hours to induce TJ disassembly. 5 μL EGTA was added to the media for varying times to further disrupt tight junctions by sequestering residual calcium. Cells were analyzed using immunofluorescence to assess protein localization at different time points.

Lentiviral Transfection and Infection

STRN mutant constructs (Table 3) were generated using the pLenti6/V5 Directional TOPO Cloning Kit (Thermo Fischer) and transfected into 293 FT cells at 50-80% confluence using the Virapower lentiviral expression system (Thermo Fischer). 293 FT cells were cultured in 10% FCS in Dulbecco's Modified Eagle Medium (DMEM) with 1% L-Glutamine. Media was changed to 10% DMEM with 1% L-Glutamine supplement and 1% penicillin-streptomycin supplement 24 hours after transfection. Media was changed to Caco-2 culture media 48 hours

after transfection. Lentivirus supernatant was then collected 24 hours and 48 hours after the media was changed to Caco-2 culture media. 10 μg/μL of polybrene was added to supernatant for a final concentration of 1 μL of polybrene per 1 mL of media prior to freezing at -80 degrees Celsius.

WT Caco-2 cells were plated into a 12-well tissue culture plate the day prior to infection and allowed to reach 50-80% confluence. Growth media was replaced with 24-hour lentiviral supernatant. The multi-well plate was incubated at 37 degrees for 10 minutes in the incubator to equilibrate the pH, then centrifuged at 1000 g for 30 minutes to improve infection rates. Cells were reinfected with 48-hour supernatant 24 hours after initial infection. After an additional 24 hours, lentiviral supernatant was replaced with Caco-2 culture media with 3 μg/mL blasticidin for drug selection. Lines were carried in 1.5 μg/mL blasticidin thereafter.

Results

Novel epitope unmasking method reveals striatin localization to TJs

Our preliminary data showed that striatin localizes to tight junctions and co-localizes with ZO-1 (Figure 5) and occludin (Figure 13). However, the antibody used in these experiments recognize both striatin and zinedin. Consequently, it was essential that we demonstrate that striatin was present at TJs.

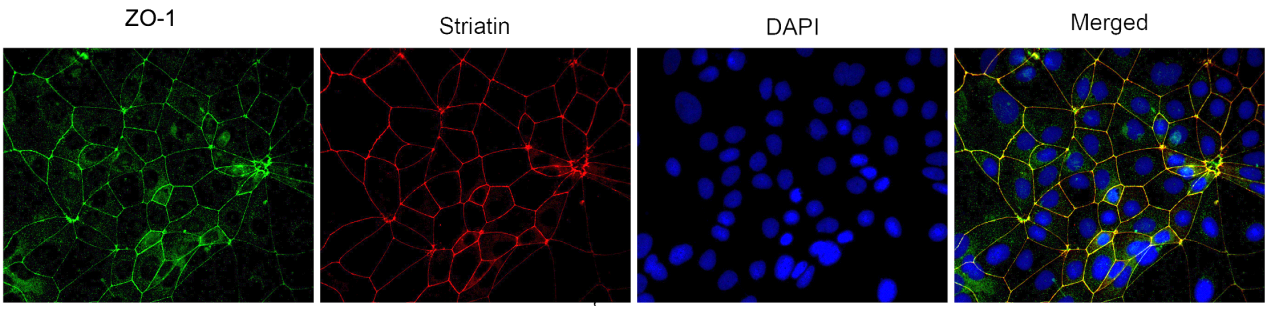

Figure 5. Striatin and/or zinedin co-localizes with ZO-1 at tight junctions
Caco-2 cells were fixed with a 1:1 methanol acetone solution, permeabilized in 0.1% 100xTriton in 1xPBS, and stained with anti-ZO-1 (green) and BD anti-striatin (red) antibodies.

Previously, we had unsuccessfully tried to visualize striatin at junctions using the RK16 anti-striatin antibody, which has a highly specific epitope recognition site. To counteract epitope masking, a phenomenon in which fixation methods that induce chemical cross-linking make epitopes less accessible for antibodies (Shi et al., 1997), we developed a novel, rapid unmasking method which involves heating coverslips in a sodium dodecyl sulphate (SDS) and dithiothreitol (DTT) buffer at 95 degrees Celsius for 1 minute after fixation with paraformaldehyde. This method has been shown to reduce background and improve staining for specific antibodies after paraformaldehyde fixation (Cai et al., unpublished). Using this method, we were able to partially recover striatin staining at TJ when compared to cells fixed with paraformaldehyde only (Figure 6).

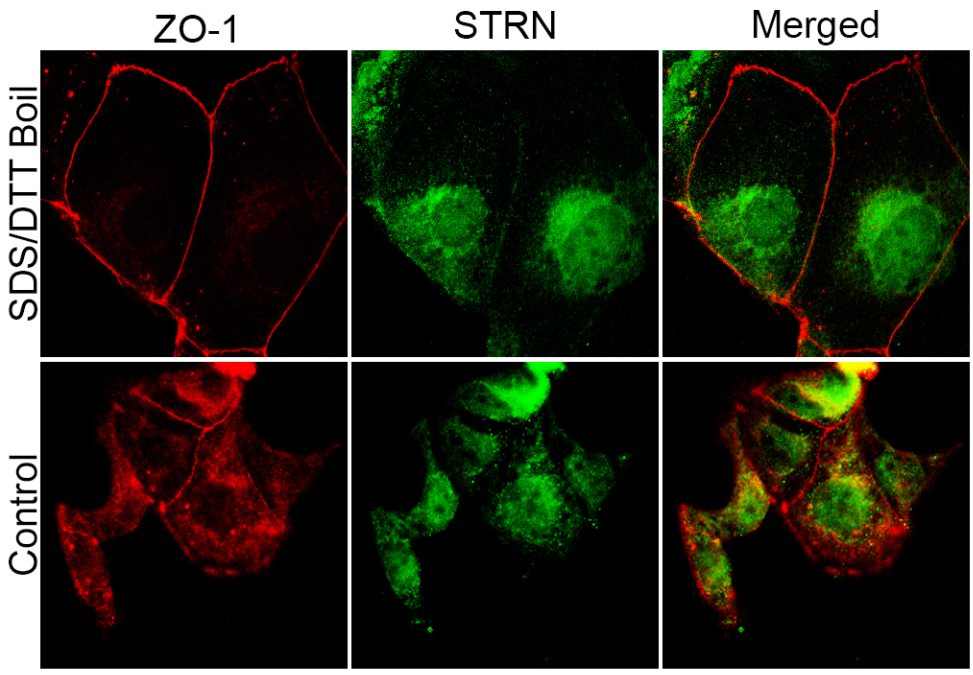

Figure 6. SDS/DTT epitope recovery method reveals striatin localization to TJ
Caco-2 cells were treated in an aqueous buffer containing 1.3% SDS and 0.52% DTT in a 95C water bath for 1 minute after 2% paraformaldehyde fixation but before blocking. Control cells were only fixed with 2% paraformaldehyde before blocking. Samples were then stained with anti-ZO-1 (red) and RK16 anti-striatin (green) antibodies.

Select STRIPAK component proteins localize at tight junctions

To characterize the STRIPAK complex present at tight junctions in epithelial cells, we probed Caco-2 cells with junctional and STRIPAK protein antibodies (Table 1). We found that, in addition to striatin, STRIPAK components STRIP1, SLMAP, MST3, MST4, and CCM3 localized to tight junctions (Figure 7). Mob3 faintly stained tight junctions (Figure 8), but primarily localized to the cytosol and around the nucleus, likely at least in part in intracellular membranes (Moreno et al., 2001).

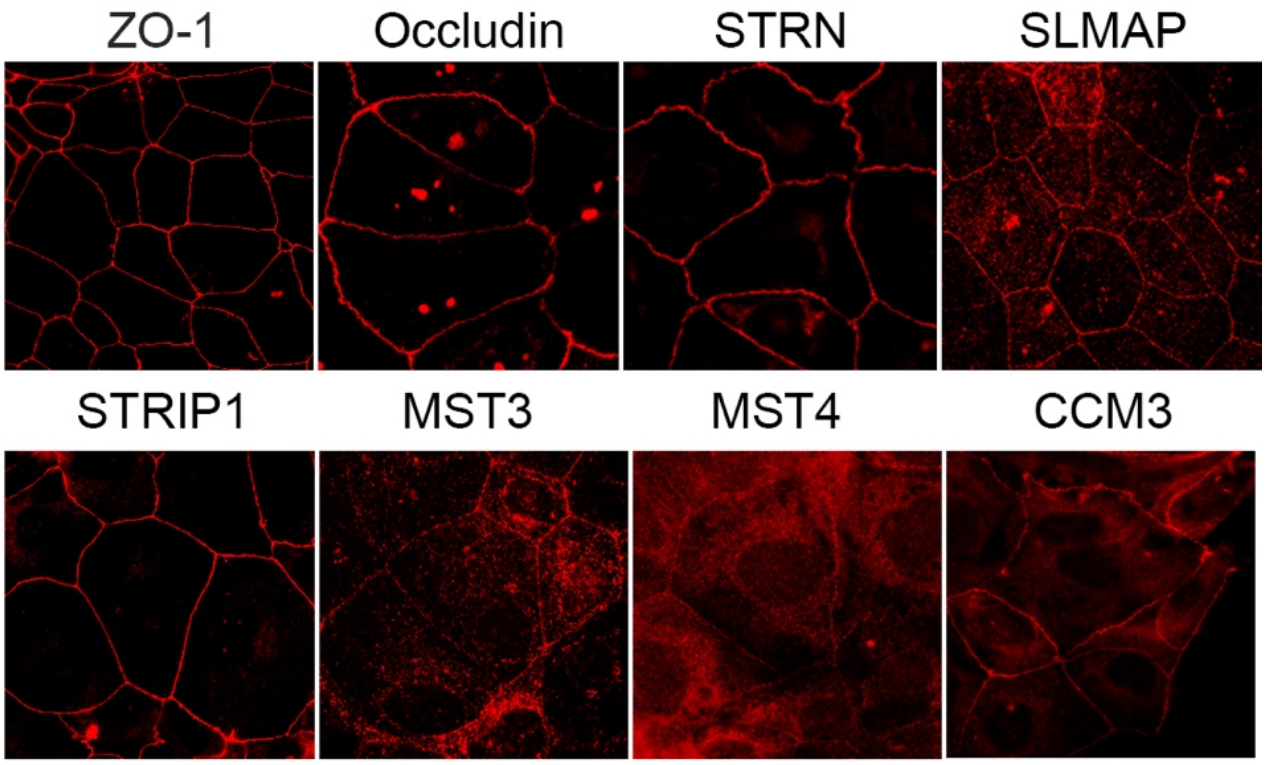

Figure 7. STRIPAK components localize to tight junctions in Caco-2 cells
Caco-2 cells were fixed with a 1:1 methanol acetone solution and probed with antibodies that recognize junctional and STRIPAK proteins (Table 1).

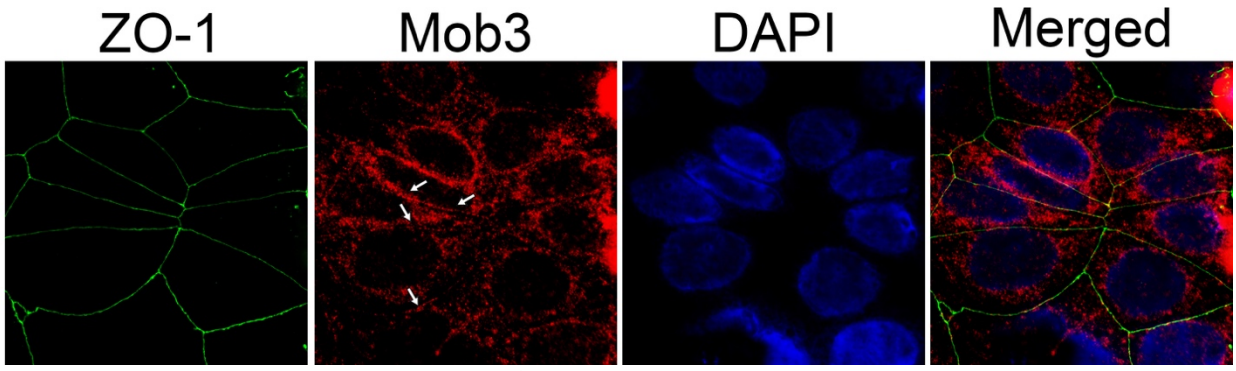

Figure 8. Mob3 partially co-localizes with ZO-1 in TJs
Caco-2 cells were fixed with a 1:1 methanol acetone solution and probed with anti-ZO-1 (green) and anti-Mob3 (red) antibodies. Mob3 was found to faintly localize to tight junctions, as illustrated by the white arrows.

However, certain STRIPAK components did not localize to TJ. SIKE did not localize to the membrane at all and instead appeared to stain punctate regions within the nucleus (Figure 9). CTTNBP2 localized to the membrane and appeared to localize proximal to some part of the junctional complex but did not localize to tight junctions specifically (Figure 10). It also did not co-localize with ZO-1, suggesting that both Mob3 and CTTNBP2 are not necessarily present at the TJ (Figure 6).

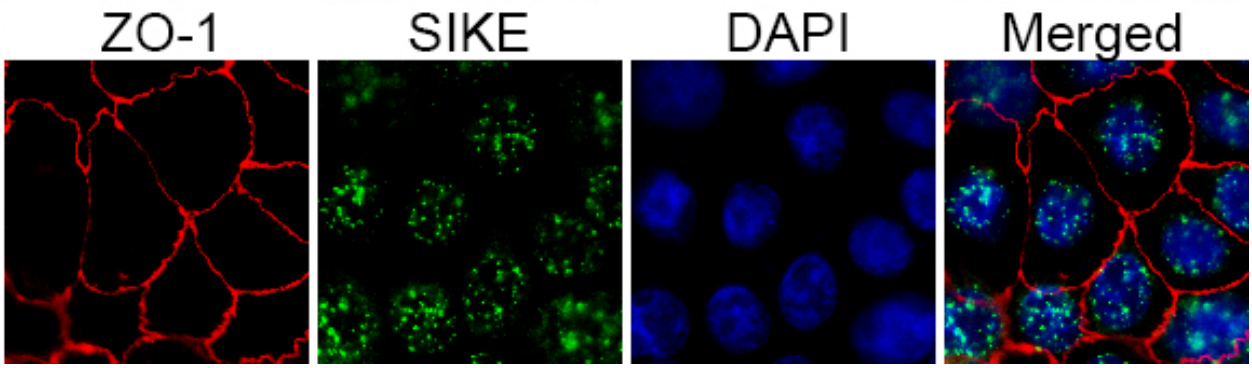

Figure 9. SIKE does not localize to tight junctions in Caco-2 cells
Caco-2 cells fixed with a 1:1 methanol acetone solution were probed with anti-ZO-1 (red) and anti-SIKE (green) antibodies.

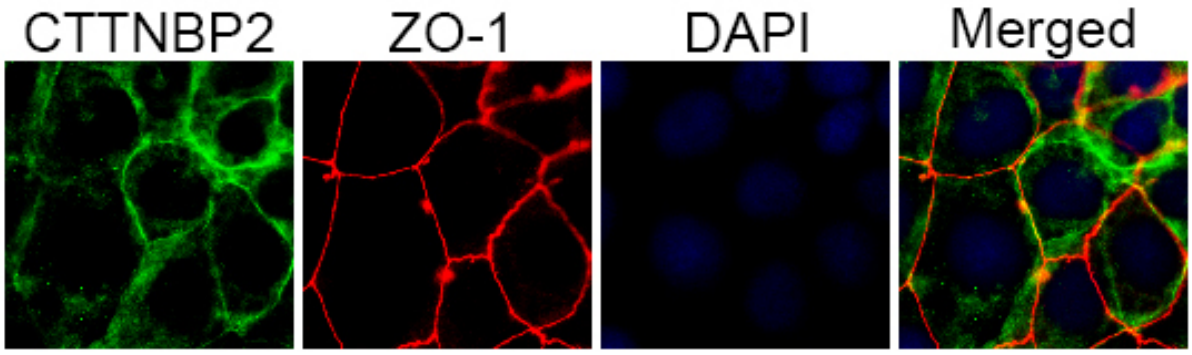

Figure 10. CTTNBP2 localizes to membrane, but not to tight junctions
Caco-2 cells were fixed with a 1:1 methanol acetone solution and probed with anti-CTTNBP2 (green) and anti-ZO-1 (red) antibodies.

Co-staining experiments were conducted to further characterize the STRIPAK complex present at epithelial TJ and to validate their co-localization with junctional proteins. As

previously demonstrated, striatin co-localized with junctional proteins ZO-1 (Figure 6). CCM3 was found to co-localize with striatin and ZO-1 at tight junctions (Figure 11 and 12). Other STRIPAK proteins, such as STRIP1 and SLMAP were also shown to co-localize with ZO-1 (Figure 13).

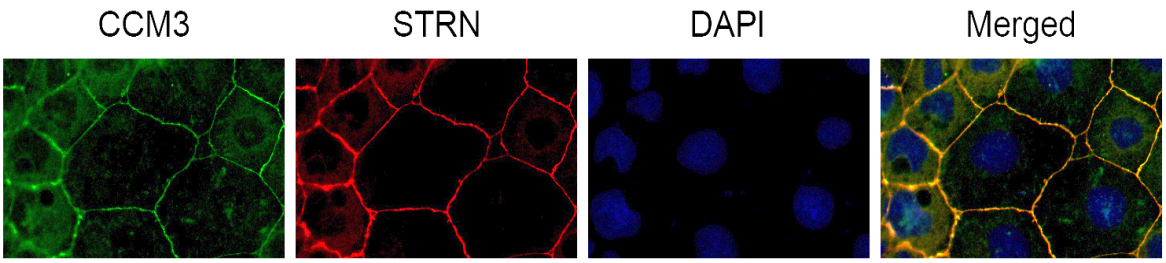

Figure 11. CCM3 co-localizes with striatin in tight junctions
Caco-2 cells were fixed with a 1:1 methanol acetone solution and probed with anti-CCM3 (green) and anti-striatin (red) antibodies.

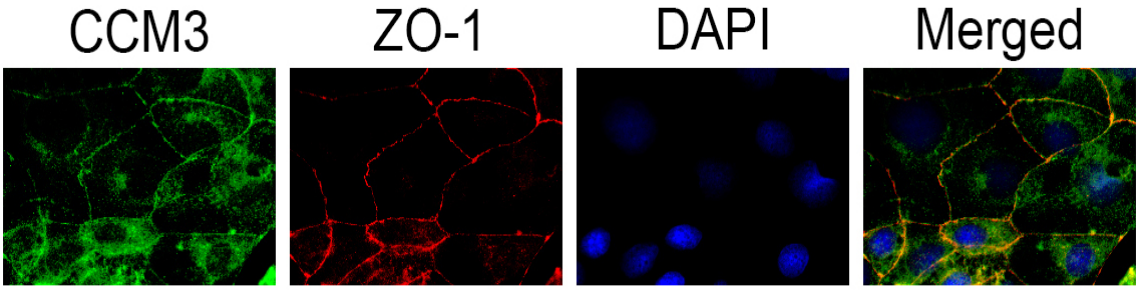

Figure 12. CCM3 co-localizes with ZO-1 in tight junctions
Caco-2 cells were fixed with a 1:1 methanol acetone fixation and probed with anti-CCM3 (green) and anti-ZO-1 (red) antibodies.

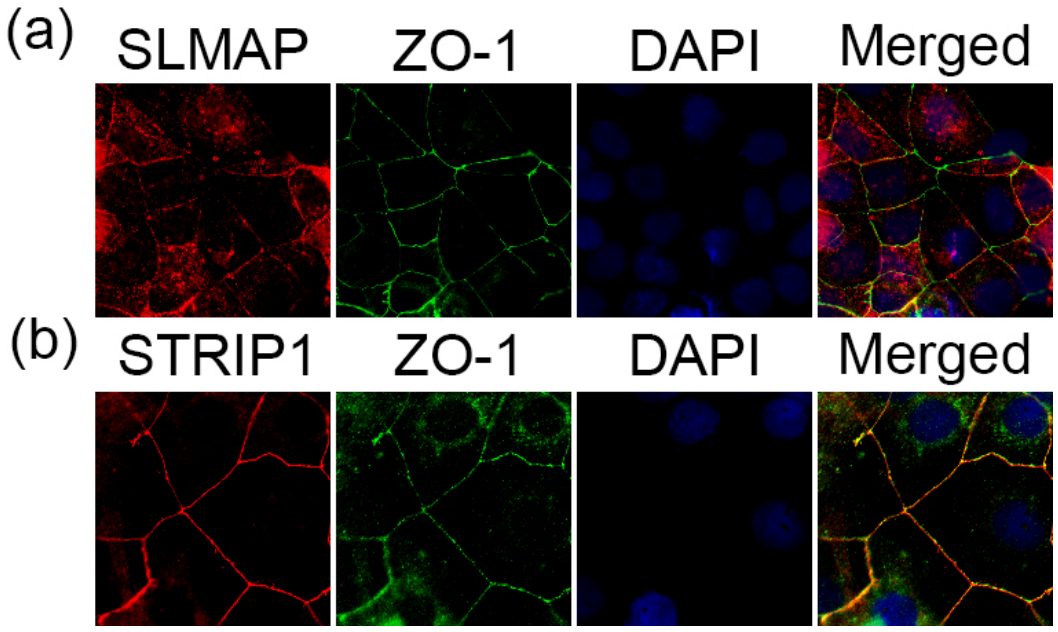

Figure 13. STRIP1 and SLMAP co-localize with ZO-1 in tight junctions
(a) Caco-2 cells were fixed with a 1:1 methanol acetone solution and stained with anti-ZO-1 (green) and anti-SLMAP (red) antibodies. (b) Caco-2 cells fixed with a 1:1 methanol acetone solution were stained with anti-ZO-1 (green) and anti-STRIP1 (red) antibodies.

STRIPAK proteins co-localize with junctional proteins during TJ remodeling

Upon characterizing the STRIPAK proteins present in epithelial TJ, we conducted Ca2+ switch experiments to examine protein localization during junctional remodeling. Confluent Caco-2 cells were incubated in low calcium media overnight, then incubated in EGTA to sequester residual calcium and break junctions fully. Striatin was shown to co-localize with occludin in the cytosol after the first 20 minutes of junctional remodeling (Figure 14).

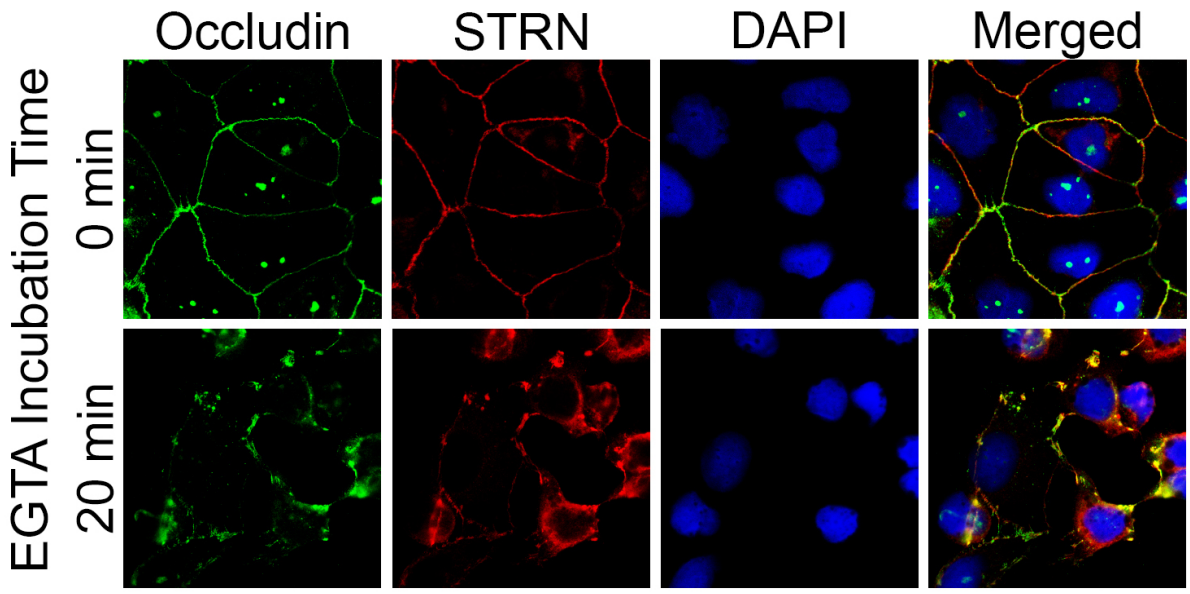

Figure 14. Striatin co-localizes with occludin during TJ remodeling
Caco-2 cells were incubated in low calcium Caco-2 growth media for 10-12 hours. After control coverslips were removed, cells were incubated in 0.25% EGTA in Caco-2 growth media for 20 minutes, allowing for TJ remodeling. Cells were fixed with 1:1 methanol acetone solution and stained with anti-occludin (green) and anti-striatin (red) antibodies.

We were unable to co-stain STRIPAK proteins with endosomal markers and Rab proteins because they were all mouse antibodies; instead, we did a side-by-side comparison to demonstrate that Rab-5 colocalizes with occludin in the cell (Figure 15), suggesting that striatin would also likely co-localize during TJ remodeling. CCM3 was also found to co-localize with striatin before and during junctional remodeling (Figure 16).

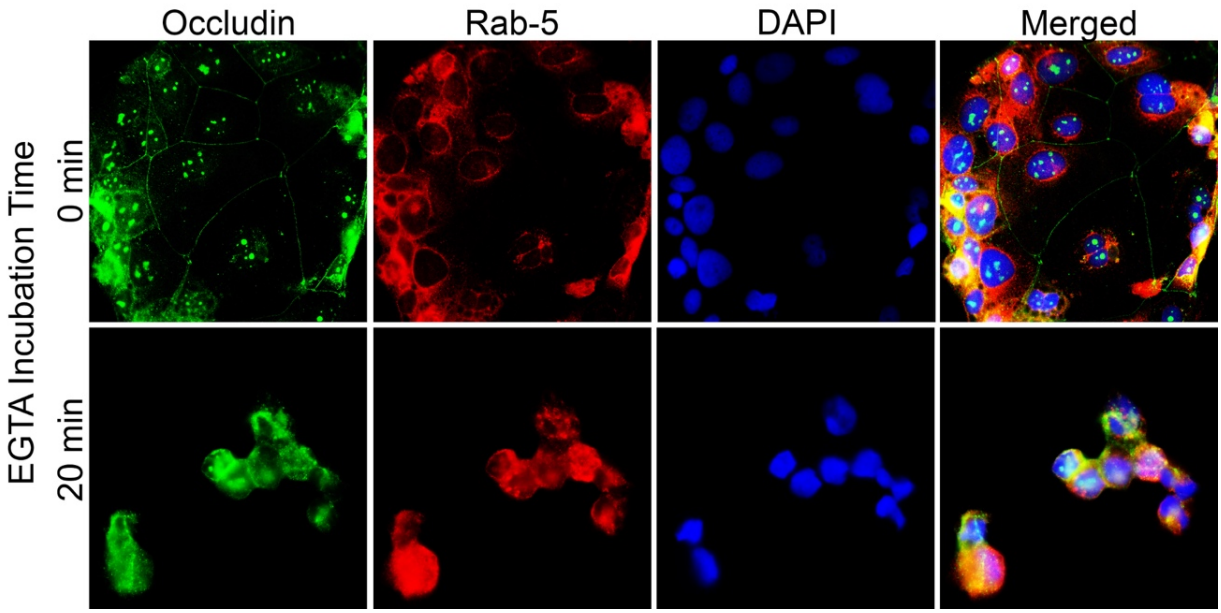

Figure 15. Occludin partially co-localizes with Rab5 during TJ remodeling
Caco-2 cells were incubated in low calcium Caco-2 growth media for 10-12 hours. Caco-2 cells were incubated in low calcium Caco-2 growth media for 10-12 hours. After control coverslips were removed, cells were incubated in 0.25% EGTA in Caco-2 growth media for 20 minutes, allowing for TJ remodeling. Cells were fixed with 1:1 methanol acetone solution and stained with anti-occludin (green) and anti-Rab5 (red) antibodies.

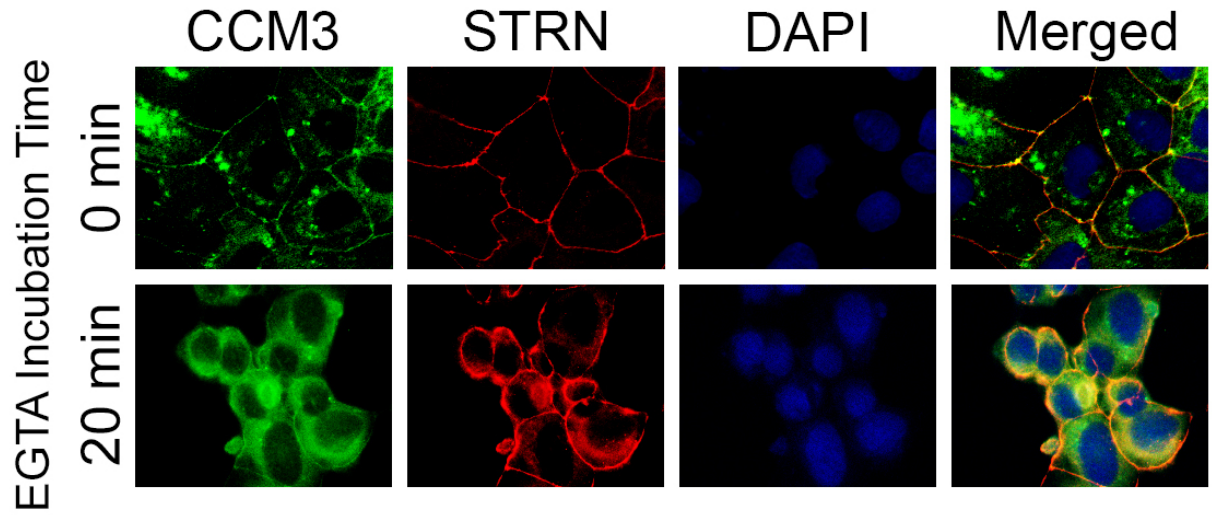

Figure 16. CCM3 co-localizes with striatin during TJ remodeling
Caco-2 cells were incubated in low calcium Caco-2 growth media for 10-12 hours. After control coverslips were removed, cells were incubated in 0.25% EGTA in Caco-2 growth media for 20 minutes, allowing for TJ remodeling. Cells were fixed with 1:1 methanol acetone solution and stained with anti-CCM3 (green) and anti-striatin (red) antibodies.

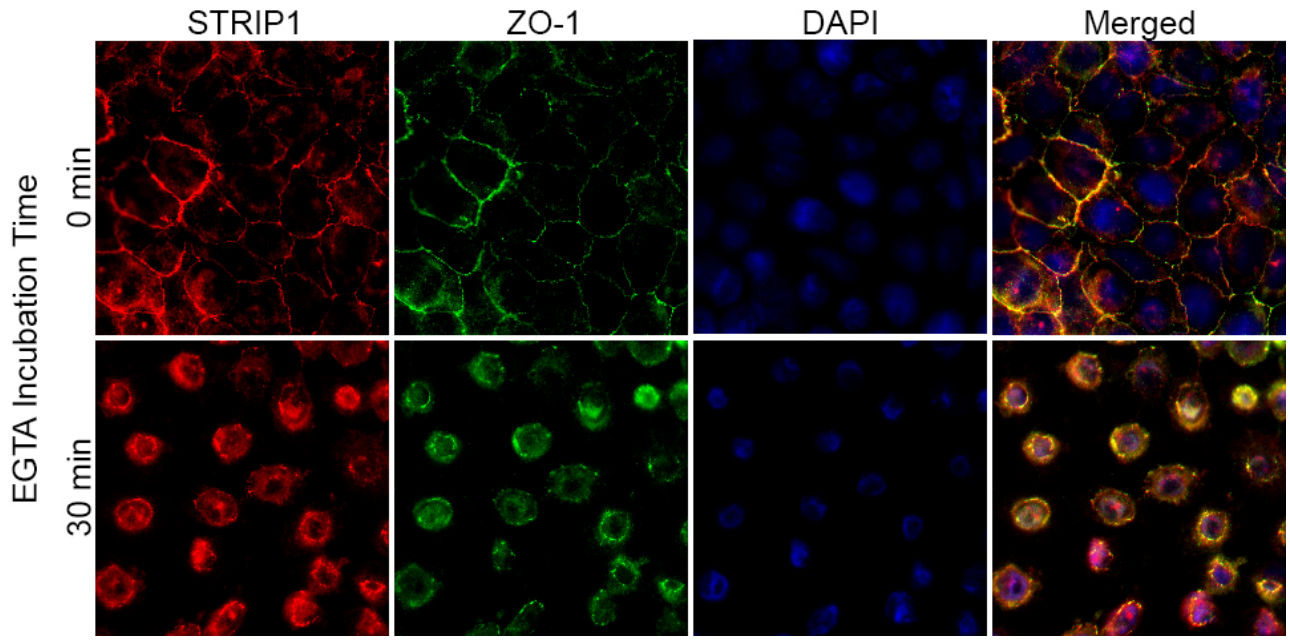

Figure 17. STRIP1 partially colocalizes with ZO-1 during TJ remodeling
Caco-2 cells were incubated in low calcium Caco-2 growth media for 10-12 hours. After control coverslips were removed, cells were incubated in 0.25% EGTA in Caco-2 growth media for 20 minutes, allowing for TJ remodeling. Cells were fixed with 1:1 methanol acetone solution and stained with anti-ZO-1 (green) and anti-STRIP1 (red) antibodies.

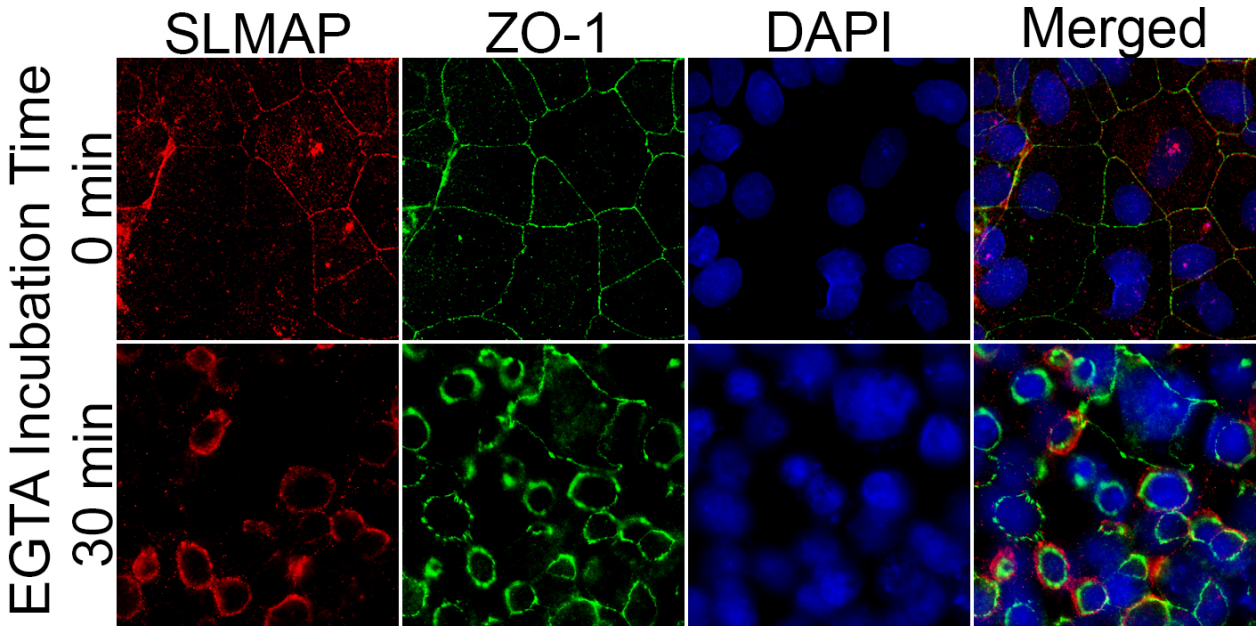

Figure 18. SLMAP does not co-localize with ZO-1 during TJ remodeling
Caco-2 cells were incubated in low calcium Caco-2 growth media for 10-12 hours. After control coverslips were removed, cells were incubated in 0.25% EGTA in Caco-2 growth media for 20

minutes, allowing for TJ remodeling. Cells were fixed with 1:1 methanol acetone solution and stained using anti-ZO-1 (green) and anti-SLMAP (red) antibodies.

STRIP1, which was previously found to localize to TJ, partially co-localized with ZO-1 before and during TJ remodeling with Ca2+ switch (Figure 17 and Figure 18). STRIP1 partially co-localized with ZO-1 within the cytosol, suggesting that they are transported together after junctions are broken. Interestingly, SLMAP did not localize with ZO-1 after 30 minutes of TJ remodeling (Figure 18).

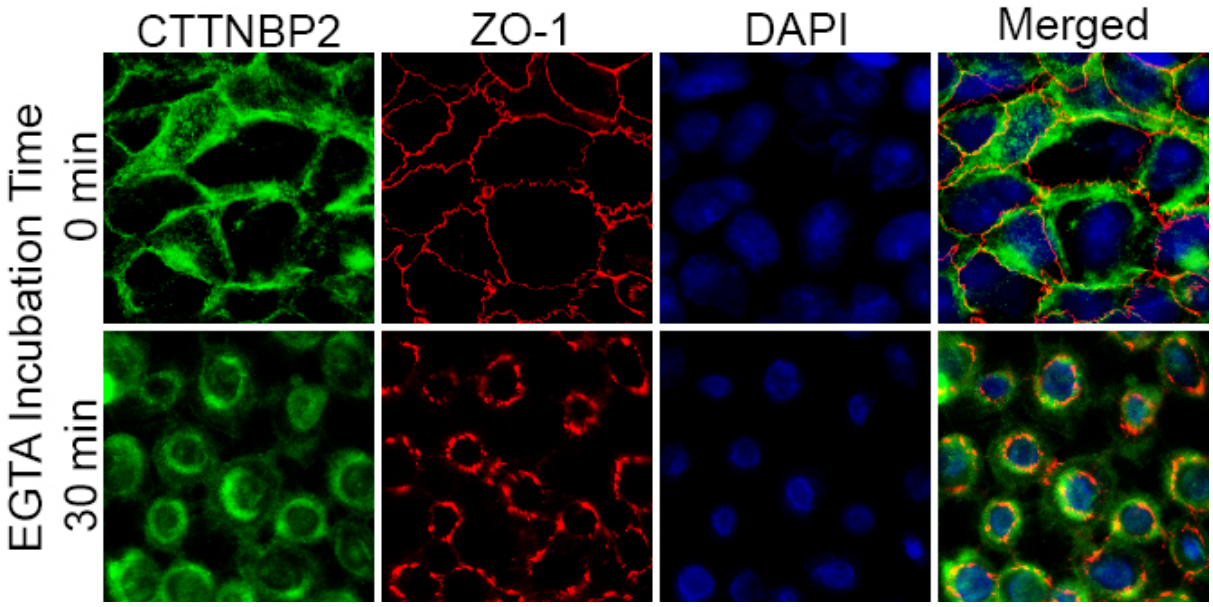

Figure 19. CTTNBP2 does not colocalize with ZO-1 during TJ remodeling
Caco-2 cells were incubated in low calcium Caco-2 growth media for 10-12 hours. After control coverslips were removed, cells were incubated in 0.25% EGTA in Caco-2 growth media for 20 minutes, allowing for TJ remodeling. Cells were fixed with 1:1 methanol acetone solution and stained using anti-CTTNBP2 (green) and anti-ZO-1 (red) antibodies.

As expected, CTTNBP2 did not co-localize with junctional proteins during TJ remodeling. Though it transported from the membrane into the cytosol during TJ remodeling, they did not specifically localize with ZO-1 (Figure 19).

Striatin Mutant Results

Caco-2 cells were infected with control lentivirus (empty vector) or lentivirus that expressed PP2A$^-$ (L84A/L94A/L105A) HA-tagged striatin mutant (Table 3). After selection for cells with integrated lentivirus using growth media containing blasticidin, the cells were grown on coverslips and analyzed via immunofluorescence. We validated the expression of the striatin mutants by staining both striatin mutant expressing cells and empty vector control cells with anti-HA tag antibody 16B12 and anti-striatin antibody RK16 (Figure 20). Empty vector cells expressed no HA tag and therefore showed no staining with the 16B12 antibody. Furthermore, the staining of striatin using the RK16 antibody and anti-HA antibody was identical, illustrating that the anti-HA tag antibody was indeed staining the PP2A$^-$ HA-tagged striatin (Figure 20). Immunofluorescence revealed that PP2A- mutant constructs failed to localize to TJ, though we cannot form any firm conclusions due to the absence of a WT STRN control (Figure 20).

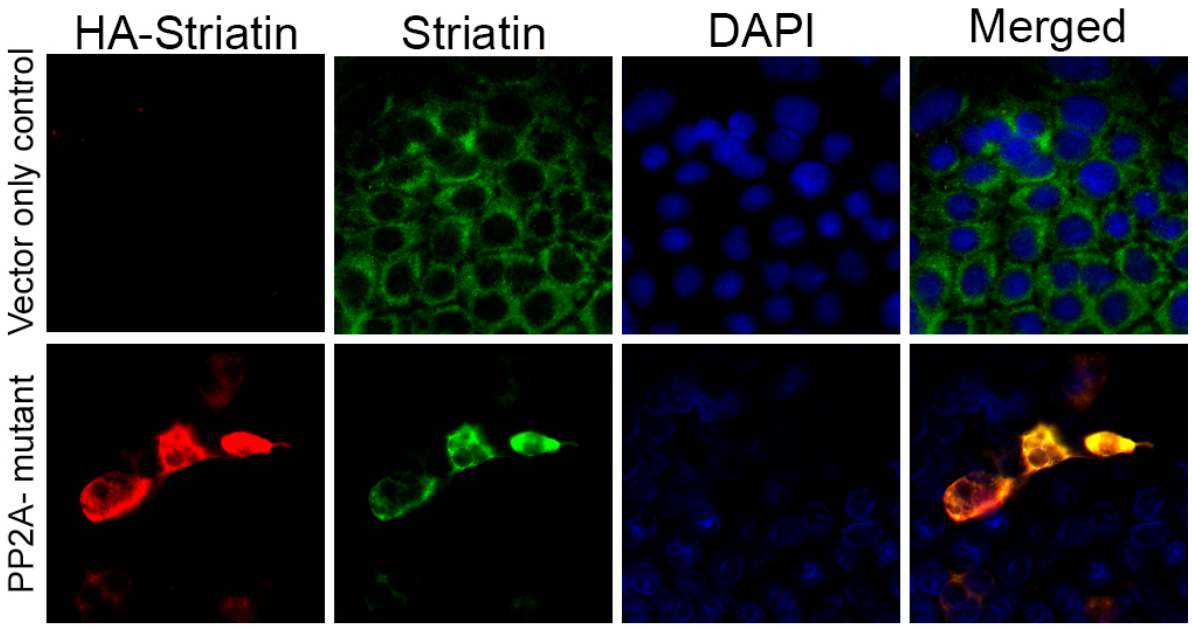

Figure 20. Infected Caco-2 cells express PP2A⁻ striatin mutant which does not localize to tight junctions
Caco-2 cells were infected with lentivirus with either no PP2A gene (vector only; top row) or PP2A⁻ (L84A/L94A/L105A) mutant striatin. Cells were fixed in a 1:1 methanol acetone solution and stained with anti-HA (red) 16B12 antibody and anti-striatin (green) RK16 antibody. Vector only cells expressed no HA tag and the PP2A⁻ mutant showed identical staining with HA-tag and striatin antibodies, suggesting that the anti-HA antibody recognized the striatin mutant.

When cells were co-stained with striatin and ZO-1, we observed that cells that had high levels of striatin mutant expression experienced an internalization of ZO-1 (Figure 21). The Unfortunately, several of the other striatin mutants, as well as the WT STRN construct were not ready for immunofluorescence analysis; however, this study is still ongoing, and the mutants will be analyzed in future experiments.

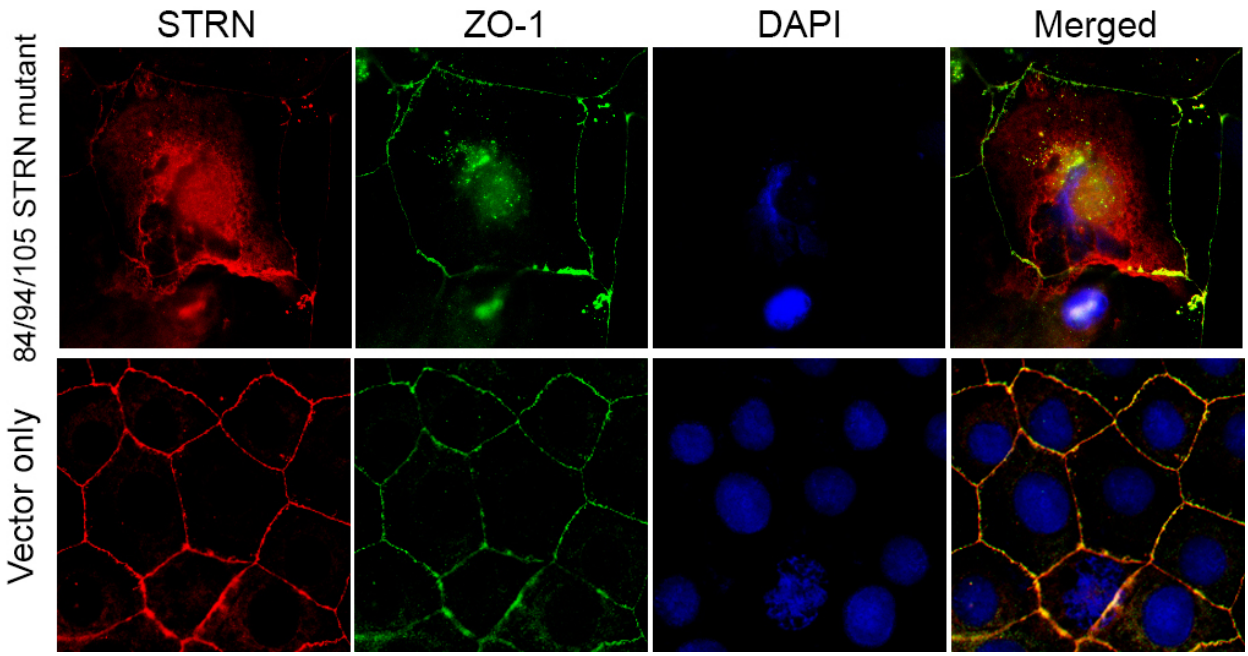

Figure 21. ZO-1 is internalized in cells that express PP2A⁻ striatin mutant
Caco-2 cells were infected with lentivirus with either no PP2A gene (vector only; bottom row) or PP2A⁻ (L84A/L94A/L105A) mutant striatin. Cells were fixed in a 1:1 methanol acetone solution and stained with anti-striatin (red) antibody and anti-ZO-1 (green) antibody.

Discussion

The results of this study help elucidate the specific STRIPAK complex that is present in human epithelial cells. A model of this complex is presented in Figure 21 below. There are several possible STRIPAK complexes, as presented in Figure 2, but we illustrated that, at colorectal (Caco-2 cells) epithelial TJ, STRIP1 and SLMAP are present instead of CTTNBP2 and CTTNBP2NL (Figure 7). CTTNBP2/CTTNBP2NL and SLMAP/SIKE bind to STRIP1/2 in a mutually exclusive manner, so this suggests that CTTNBP2/NL are not present in the TJ STRIPAK complex.

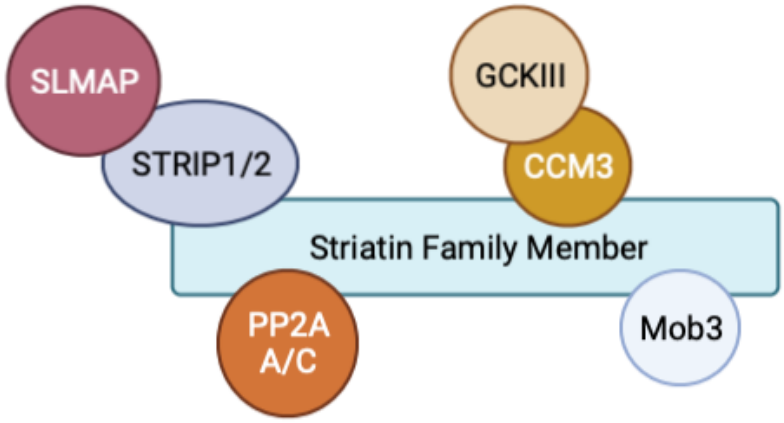

Figure 22. Proposed model of the STRIPAK complex at epithelial TJ
Made with BioRender.com

CTTNBP2 and CTTNBP2NL, as their names suggest, bind cortactin, which localizes to the membrane and to actin cytoskeletal fragments (Stamatovic et al., 2015). When CCM3 is knocked down *in vitro*, cortactin is significantly diminished and its interactions with ZO-1 and actin are diminished as well; this results in the hyperpermeability of endothelial tight junctions (Stamatovic et al., 2015). Hence, these cortactin-binding proteins may still be involved in the overall regulation of tight junctions, but not through direct interactions with the STRIPAK complex.

The results of the Ca2+ switch experiment further support our hypothesis that STRIPAK is involved in vesicular trafficking in epithelial cells. We successfully demonstrated co-localization of STRIPAK proteins striatin, CCM3, and STRIP1 with ZO-1 and occludin during TJ remodeling (Figure 13, 15, 16, and 17). These findings suggest that the same STRIPAK complex found in epithelial tight junctions help to traffick junctional proteins away from the membrane when TJ are disassembled. Furthermore, we were able to demonstrate co-localization of the junctional protein occludin and Rab-5 within the first 20 minutes of TJ remodeling induced by lowering calcium levels (Figure 14). The Rab proteins play a key role in regulating the destination of endosomes; Rab-5 is an early endosomal protein that regulates vesicular transport and early endosome fusion (Ivanov et al., 2004). Our side-by-side comparison between striatin, occludin, and Rab-5 co-localization helps illustrate that the STRIPAK complex, along with Rab proteins, likely traffick endosomes during junctional remodeling. SLMAP, however, did not co-localize with junctional proteins during TJ remodeling (Figure 18). This suggests that SLMAP may not be involved in the vesicular trafficking of TJ proteins; however, that is not to discount the role of SLMAP in junctional remodeling; SLMAP has been found to play a regulatory role in the initiation of early endosomal fusion (Dewan, 2016), which may explain why SLMAP was found to localize closer to the membrane than ZO-1 during TJ remodeling (Figure 18).

Interestingly, Mob3 was not visualized strongly at TJ (Figure 8). The Mob3 ortholog, dMob4, which is found in Drosophila, has been demonstrated to play a role in axonal transport of dense core vesicles in a STRIPAK-related and PP2A-dependent manner (Schulte et al., 2010; Neisch et al., 2017). Given its role in intracellular trafficking, an absence of Mob3 in the epithelial TJ complex would be surprising. However, faint staining of TJs for Mob3 were seen,

suggesting that it is likely present in some STRIPAK complexes at TJs. This does not rule out, however, the possibility that other STRIPAK components may help facilitate the transport of junctional proteins to and from the cell. SLMAP, which was found in this study to localize to the TJ complex in epithelial cells (Figure 7), is a likely second candidate for regulating these functions of STRIPAK. SLMAP interacts with syntaxin 3, which is important for the localization of apical membrane proteins (Huttlin et al., 2015, Sharma et al., 2006). It also interacts with proteins that regulate Rab5 (Huttlin et al., 2015), suggesting that SLMAP may target STRIPAK to early endosomes during TJ remodeling.

The PP2A$^-$ mutant shed valuable light on the potential role of PP2A on the formation and/or stability of tight junctions. As observed in Figure 21, ZO-1 was internalized in the PP2A-striatin mutant expressing cells. This illustrates that PP2A binding to striatin is necessary for the formation or maintenance of tight junctions. Though we observed that PP2A$^-$ mutants did not localize to tight junctions (Figure 20), we unfortunately cannot form any firm conclusions due to the lack of analysis on HA-tagged WT STRN expressing cells. Due to a low efficacy of the WT STRN lentivirus infection, very few cells remained after blasticidin selection and were not ready for analysis. This study is ongoing, and future analysis is required.

Some weaknesses of the current study are the limitations of the microscopy method used. Though the proteins appear to be localizing to junctions or co-localizing with junctional proteins using traditional fluorescence microscopy, a higher resolution microscopy method, such as confocal microscopy, is necessary for more careful analysis. Given that there are several junctional complexes along the membrane, the localization we observe may be lower down in the junctional complex at adherens junctions, which lies just below TJs. Confocal microscopy would enable us to observe localization in 3 dimensions, allowing for a more definitive

understanding of the results. Another notable limitation is that the Becton Dickenson (BD) anti-striatin antibody stains both striatin (STRN) and zinedin (STRN4). Though we demonstrated, using the RK16 antibody, that some striatin is present in tight junctions (Figure 6), the staining is very faint and not comparable to the staining we achieve using the BD anti-striatin antibody. For this reason, we cannot say for certain that co-localization observed with the BD antibody is striatin or zinedin or both. Further analysis is required to affirm the role of striatin in epithelial tight junctions.

There is still much work to be done in this study. Future experiments can utilize siRNA or shRNA methods to knock down STRIPAK proteins and examine localization of striatin within the cell, or vice versa. This would elucidate the protein-protein interactions within STRIPAK that are essential for TJ localization of STRIPAK complexes. As previously stated, further analysis using confocal microscopy is required to validate whether STRIPAK proteins are indeed localizing to tight junctions or to other portions of the junctional complex. Finally, co-immunoprecipitation assays should be conducted to examine and quantify the protein-protein interactions between STRIPAK proteins and junctional proteins when certain regions of striatin are deleted that are necessary for binding of specific STRIPAK components to striatin. This would encompass a structure-function study that would help answer how STRIPAK is targeted to the TJs. Studies such as these combined with my current results will provide important insight that may help elucidate the role STRIPAK has in regulating TJs and in CCM disease.

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