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Ana C. Monteiro

Mechanisms of JAM-A Dependent Regulation of Barrier Function

By

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Doctor of Philosophy

Biochemistry, Cell, and Developmental Biology
Graduate Division of Biological and Biomedical Sciences
Laney Graduate School, 2015

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Mechanisms of JAM-A Dependent Regulation of Barrier Function

By

Ana Carolina Costa Monteiro

B.Sc. Georgetown University, 2007

Advisor: Charles A. Parkos, MD, PhD

An abstract of a dissertation submitted to the faculty of the Graduate School of Emory University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Biochemistry, Cell, and Developmental Biology

Graduate Division of Biological and Biomedical Sciences

Laney Graduate School, 2015

Emory University

Abstract

Mechanisms of JAM-A Dependent Regulation of Barrier Function
By Ana Carolina Costa Monteiro

The intestinal epithelial barrier is a critical homeostatic component as it selectively controls the passage of nutrients across mucosal surfaces while deterring the passage of pathogens and toxins. Intestinal barrier function is regulated by epithelial tight junctions (TJs), structures that control paracellular permeability. Junctional Adhesion Molecule-A (JAM-A) is a TJ-associated protein that regulates epithelial proliferation, migration and barrier function, however mechanisms linking JAM-A to epithelial permeability are poorly understood. We first report that JAM-A associates directly with ZO-2 and indirectly with afadin, and this complex, along with PDZ-GEF1, activates the small GTPase Rap2c. Supporting a functional link, siRNA-mediated downregulation of the above regulatory proteins resulted in enhanced permeability similar to that observed after JAM-A loss. JAM-A deficient mice and cultured epithelial cells demonstrated enhanced paracellular permeability to large molecules, revealing a potential role of JAM-A in controlling perijunctional actin cytoskeleton in addition to its previously reported role in regulating claudin proteins and small-molecule permeability. Further experiments suggested that JAM-A modulates activity of RhoA and phosphorylation of non-muscle myosin, both implicated in actomyosin contraction.

Moreover, previous reports suggest that JAM-A homodimerization on the same cell surface (in cis) mediates JAM-A dependent signaling. However, there is accumulating evidence suggesting that homophilic interactions between JAM-A molecules on adjacent cells (trans) are necessary for mediating cell-cell contacts. Site directed mutagenesis along with cell-transfection and in-vitro protein interaction studies revealed that JAM-A dimerizes in both cis and trans at distinct sites on the distal-most immunoglobulin-like loop. These experiments also indicate that trans-dimerization is a low affinity interaction that likely requires the avidity supplied by JAM-A cis-dimers for assembly. The role of JAM-A trans-dimerization in cell signaling was also investigated. Cells expressing trans-dimerization null mutants displayed lower Rap2 activity compared to controls, and Rap2 activity was enhanced with cell confluence. These results suggest that JAM-A trans-dimerization may act as a barrier-inducing molecular switch that is activated when cells become confluent. Together, the studies in this thesis indicate that JAM-A multimerization may be important in recruiting a large complex of scaffold proteins to cell contacts so as to regulate epithelial barrier function.

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Content

Chapter 1: Introduction

Intracellular Mediators of JAM-A Dependent Epithelial Barrier Function

Material from this chapter has been published as a review in *Annals of the New York Academy of Sciences,* **2012**. Dr. Charles A Parkos is a co-author of the review article.

Abstract

JAM-A is a critical signaling component of the apical junctional complex, a structure composed of several transmembrane and scaffold molecules that control the passage of nutrients and solutes across epithelial surfaces. Observations from JAM-A deficient epithelial cells and JAM-A knockout animals indicate that JAM-A is an important regulator of epithelial paracellular permeability, however the mechanism(s) linking JAM-A to barrier function are not understood. This introduction highlights recent findings relevant to JAM-A–mediated regulation of epithelial permeability, focusing on the role of upstream and downstream signaling candidates. We draw on what is known about proteins reported to associate with JAM-A in other pathways and on known modulators of barrier function to propose candidate effectors that may mediate JAM-A regulation of epithelial paracellular permeability. Further investigation of pathways highlighted in this thesis may provide ideas for novel therapeutics that target debilitating conditions associated with barrier dysfunction, such as inflammatory bowel disease.

Introduction

The epithelial barrier is a critical component of tissue homeostasis as it selectively controls the passage of nutrients and solutes across mucosal surfaces while deterring the passage of pathogens and toxins. Functional regulation of the epithelial barrier is determined by a collection of tight junction (TJ) and adherens junction (AJ)-associated transmembrane and scaffold proteins termed the apical junctional complex (AJC). One transmembrane component of the TJ is Junctional Adhesion Molecule-A (JAM-A). Evidence suggests that JAM-A does not directly regulate barrier by forming a "seal" between cells but rather as a signaling molecule with divergent downstream target proteins (Liu et al., 2000; Laukoetter et al., 2007). Indeed, recent reports have implicated JAM-A–mediated signaling events in regulating a diverse array of epithelial functions including epithelial proliferation, migration and barrier function (Nava et al., 2011; Severson et al., 2009a; Laukoetter et al., 2007). While the evidence linking expression of JAM-A to TJ regulation and barrier maintenance is accepted, insights into downstream mechanisms linking JAM-A to regulation of barrier are limited. In this introduction, we summarize current findings that are relevant to how JAM-A might control barrier function, focusing on the role of upstream and downstream signaling components linking JAM-A to paracellular permeability. We draw on what is known about JAM-A effectors from other pathways to speculate on attractive candidate molecules that regulate the epithelial barrier.

JAM-A expression affects epithelial permeability

The importance of JAM-A in regulating barrier function is best illustrated by reported observations in JAM-A deficient intestinal cell lines and knock-out (KO) animals (Laukoetter et al., 2007; Mandell et al., 2004). In cell lines, it has been clearly shown that siRNA mediated loss of JAM-A expression results in enhanced permeability as determined by transepithelial resistance (TER) and paracellular flux of labeled dextran. Such observations have been confirmed in multiple epithelial and endothelial cell types (Mandell et al., 2004; Haarmann et al., 2010; Liu et al., 2000; Mandell et al., 2006; 2007), including primary rat alveolar epithelial cells, which exhibit decreased TER after treatment with JAM-A shRNA (L.A. Mitchell and M. Koval, personal communication). In vivo, JAM-A deficient mice have a leaky colonic epithelium (Laukoetter et al., 2007). Additionally, JAM-A deficient mice are more susceptible to dextran sulfate sodium (DSS) induced colitis compared to control mice, presenting with a higher disease activity index and more severe weight loss. Intriguingly, JAM-A KO mice also present with increased mucosal infiltration of leukocytes in the colonic mucosa and altered levels of pro-inflammatory cytokines (Laukoetter et al., 2007). This finding is consistent with current views that link impaired barrier function with increased susceptibility to mucosal inflammation. Future studies should determine whether enhanced permeability and leukocyte infiltration are also observed in other epithelial compartments of JAM-A deficient mice.

Epithelial barrier function is established by a complex series of poorly understood signaling events that culminate in the formation of mature TJs. Evidence

suggests that JAM-A is important in early events required for TJ assembly. Such early events begin with emergence of nascent puncta containing the AJ proteins E-cadherin and nectin, providing initial points of cell-cell contact that recruit other junctional proteins necessary to establish a mature apical junctional complex. JAM-A appears to be an early mediator of this process since it is recruited along with occludin immediately after puncta are established (Cereijido et al., 2008). These observations are consistent with our findings demonstrating that antibody blockade of JAM-A in cultures of subconfluent epithelial cells delays the development of a tight barrier, as determined by TER measurements (unpublished observations).

Dimerization of JAM-A is necessary for regulation of barrier

JAM-A is composed of a short cytoplasmic tail, a single-pass transmembrane region and two extracellular Ig-like loops (Fig. 1). A number of reports, including crystallography data (Prota et al., 2003), indicate that JAM-A forms functionally significant homodimers through ionic interactions within a conserved motif in its distal most Ig loop (Fig. 1, asterisk on D1) (Mandell et al., 2004; Severson et al., 2008a). These reports suggest that such ionic interactions within the D1 loop of JAM-A mediates homodimerization in a cis configuration (on the surface of the same cell), however structural data from murine JAM-A (Kostrewa et al., 2001) suggests that JAM-A also homodimerizes in trans, or across cells, at distinct but yet undefined site(s) in the same D1 loop. We observed that mutagenesis of residues promoting cis-dimerization of JAM-A or treatment with a monoclonal antibody that binds to the dimerization domain results in attenuated

JAM-A dependent regulation of epithelial cell migration (Severson et al., 2008a). Interestingly, the same dimerization disrupting antibodies delay barrier development in monolayers of epithelial cells with disassembled AJC after transient calcium depletion (calcium switch). These observations suggest that JAM-A homodimerization is necessary for assembly of functional TJs (Mandell et al., 2004; Liu et al., 2000). Studies on JAM-A mediated effects on cell migration eventuated in a model of JAM-A function involving dimerization-mediated activation of a signaling module that leads to cell migration. While mechanistic insights detailing how JAM-A dimerization leads to regulation of paracellular permeability are not understood, some clues are provided by the above model and from reports implicating known JAM-A interacting molecules with the regulation of barrier function. From these observations, we can begin to assemble a potential model by which JAM-A dimerization controls epithelial barrier function.

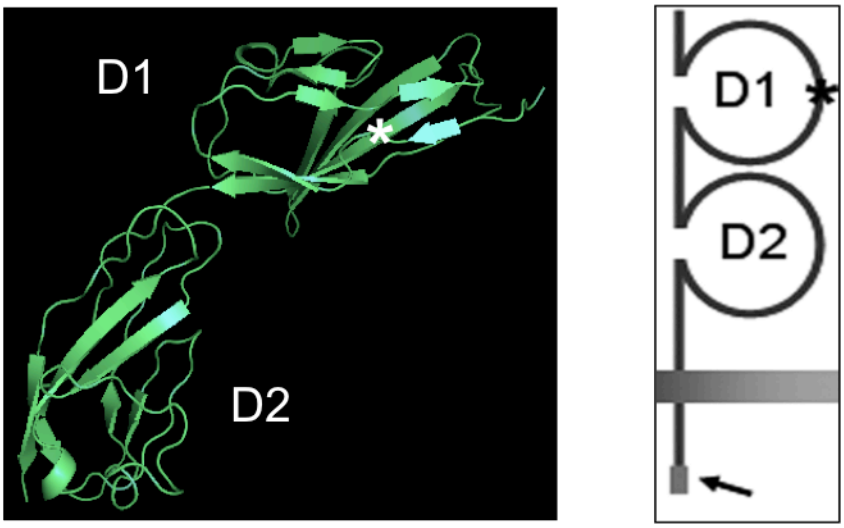


Figure 1: JAM-A is a single pass transmembrane protein with two extracellular immunoglobulin-like loops and a short, 40aa cytoplasmic tail. The ribbon structure of extracellular JAM-A (left) and the cartoon of the full-length molecule (right) highlight the site of JAM-A dimerization on the surface of the same cell (in cis) by an asterisk. The cartoon of full length JAM-A also indicates the c-terminal region containing the PDZ motif (arrow), allowing for interaction with PDZ-domain containing scaffold proteins.

Several stimuli that alter epithelial barrier also affect JAM-A expression

While there are limited studies on the signaling pathways that link JAM-A to regulation of barrier, there are several recent reports describing paracrine and autocrine cues that affect junctional integrity and alter JAM-A expression and localization. Such studies underscore the fluidity of the AJC, which is constantly restructured to accommodate physiological events such as leukocyte transmigration across epithelia and varying demands of fluid and nutrient absorption in the gastrointestinal tract.

Cytokine-mediated internalization of epithelial TJ proteins exacerbates inflammatory conditions by maintaining an open entryway for leukocytes to the inflamed region and enhancing leukocyte exposure to luminal antigens. Inflammatory cytokines such as INF-γ and TNF-α enhance permeability of endothelial and epithelial barriers by inducing internalization of JAM-A and other AJC proteins (Bruewer et al., 2003; Capaldo and Nusrat, 2009), while local administration of TGF-β and TNF-α to the blood testis barrier also induces clathrin-dependent internalization of AJC proteins that include JAM-A, occludin and N-cadherin (Xia et al., 2009). Conversely, cues that enhance barrier may be protective against chronic inflammation, again by altering the AJC. For example, estrogen, thought to have anti-inflammatory effects in the gut (Houdeau et al., 2007; Harnish et al., 2004), was reported to reduce the permeability of the intestinal epithelium in vivo and in vitro by upregulating TJ protein levels of JAM-A and occludin (Braniste et al., 2009). CD-24, a ligand for p-selectin implicated in epithelial restitution in mouse models of inflammatory bowel disease (IBD), was also reported to enhance

barrier function of the oral epithelium by upregulating JAM-A and claudins 4 and 15 in a src-kinase dependent manner (Ye et al., 2011). These examples indicate a potential reciprocal influence of inflammatory signals on mucosal permeability, which may act to perpetuate a pathological inflammatory response.

Other studies implicating a role of paracrine signaling in JAM-A expression provide mechanistic insights into JAM-A recruitment to TJs, which may be important for JAM-A stability and function. Studies using immortalized primary pancreatic duct cells (Yamaguchi et al., 2010) revealed that inclusion of fetal bovine serum (FBS) after serum starvation enhanced the expression and TJ localization of several TJ proteins including JAM-A, occludin, ZO-1 and several claudins in a PKC-dependent manner. Cells formed no functional barrier during serum starvation but develop a functionally tight barrier after the addition of serum. Interestingly, inhibition of PKC reduced JAM-A expression and TER to that of serum-free levels. In serum free media, addition of TPA, a DAG pharmacomimetic that activates typical PKCs, enhanced levels and TJ localization of ZO-1, ZO-2 and occludin, however JAM-A expression and TER remained unchanged. The study does not further explore the pathway regulating JAM-A expression, but it is tempting to speculate that JAM-A recruitment to TJs may be dependent on an atypical PKC, one not activated by TPA/DAG. This is consistent with observations of JAM-A association with aPKC (Ebnet et al., 2001) in the context of cell polarity. An understudied aspect of JAM-A is related to the multiple potential phosphorylation sites on the relatively short cytoplasmic tail that may be important for JAM-A recruitment and function, five of which are likely targets for PKC, as determined by ntePhosK analysis. Notably, JAM-

A has been shown to be phosphorylated by PKC in platelets (Ozaki et al., 2000). Furthermore, during the final editorial review of this manuscript, Ebnet and colleagues published a study demonstrating that the cytoplasmic tail of JAM-A is indeed phosphorylated by aPKCζ at serine 285 to affect tight junction assembly and epithelial barrier function(Ozaki et al., 2000; Iden et al., 2012). Further investigation of JAM-A phosphorylation by aPKC may provide additional insights on mechanisms controlling the stability and localization of JAM-A to the TJ, which may be an important event in the transition from nascent to mature TJ formation leading to a stable epithelial barrier.

Studies of other barrier forming pathways have clearly demonstrated that cytoskeletal dynamics play an important role in barrier function. One study has provided a potential link between JAM-A, cytoskeletal dynamics and barrier function. Mice lacking Guanylyl Cyclase C (GCC), a transmembrane receptor to endogenous ligands that modulates epithelial chloride conductance, demonstrate an intriguingly similar phenotype to JAM-A KO mice and have altered phosphorylation of actin-associated proteins. Compared to wild-type mice, GCC-null animals have a more permeable gut mucosa, increased levels of pro-inflammatory cytokines and large amounts of lymphocytes in the intestinal epithelial compartment, suggesting a concomitant inflammatory phenotype (Han et al., 2011). Importantly, GCC-null mice and GCC-deficient colonic epithelial cells have decreased levels of JAM-A and claudin-2 with increased phosphorylation of myosin light chain (pMLC), suggesting that the barrier deficiency observed in the GCC-null mice may be related to the loss of JAM-A and claudin-2 as well as the phosphorylation of MLC. Notably, MLC

phosphorylation has been implicated in increased epithelial permeability by inducing contraction of the epithelial acto-myosin ring and the enlargement of the intercellular space, thereby enhancing epithelial leak (Nusrat et al., 2000). However, Han et al. propose instead that pMLC is important for TJ assembly by recruiting JAM-A and other proteins to the AJC. Future studies are required to clarify the relationship between JAM-A signaling and acto-myosin contraction to better understand the role of cytoskeletal dynamics in JAM-A dependent regulation of epithelial permeability. The findings from these studies have been summarized in figure 2.

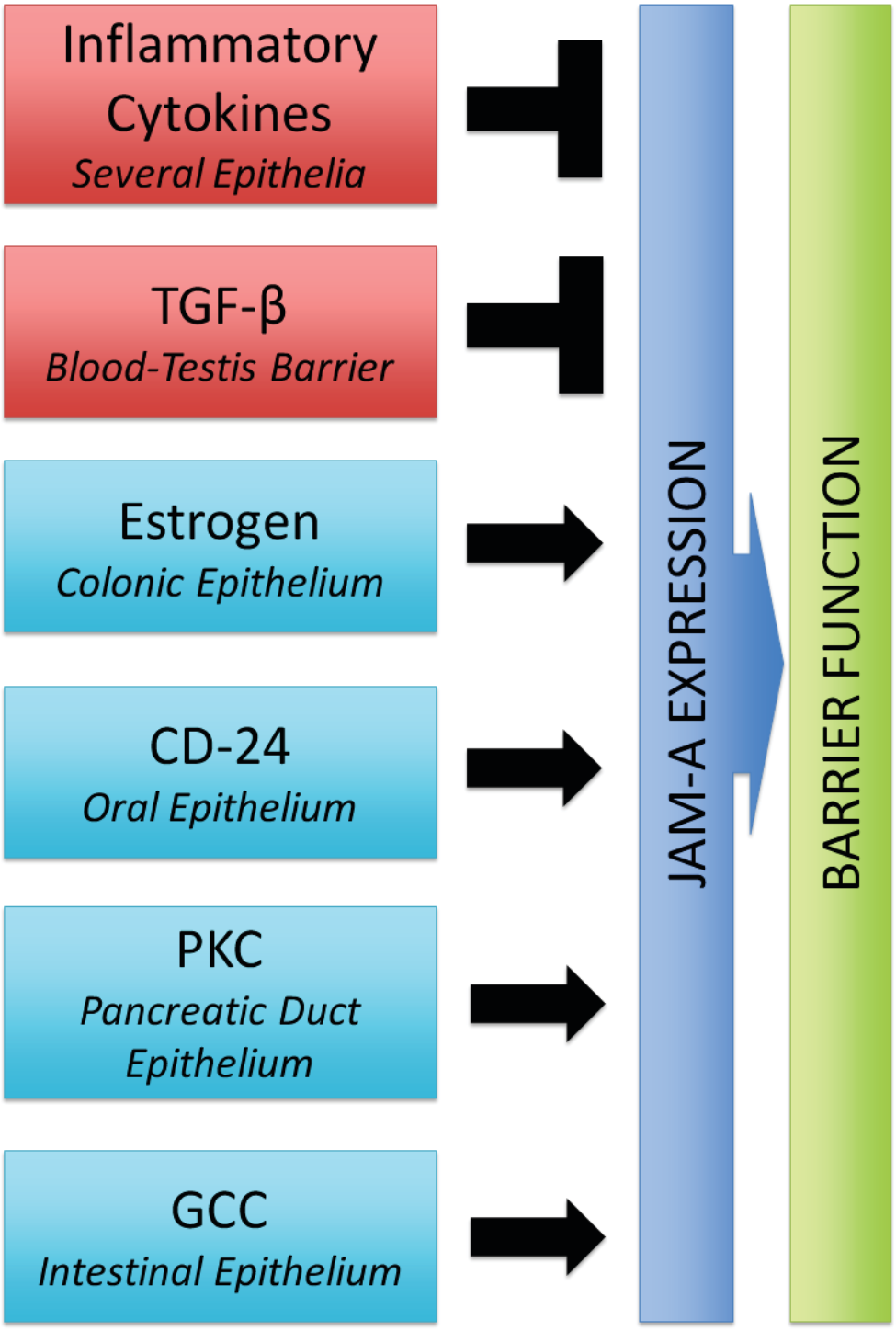


Figure 2 Examples of cues that affect epithelial barrier function and alter JAM-A expression.

Putative signaling effectors downstream of JAM-A that regulate barrier

While the importance of JAM-A in endothelial and epithelial barrier function is appreciated (Mandell et al., 2007; 2006; 2004; Liu et al., 2000), downstream pathways linking JAM-A to paracellular permeability are unknown. As mentioned above, signaling pathways regulating JAM-A dependent cell migration have been described (Severson et al., 2009a). From these studies, it is reasonable to postulate that similar pathway(s) may regulate barrier function. In migration studies, it was found that JAM-A associates with the scaffold protein afadin and the guanine nucleotide exchange factor PDZ-GEF2 resulting in the activation of the small GTPase Rap1a, stabilization of β1 integrins and enhanced cell migration. While these effector molecules have not been reported to directly affect barrier in epithelia, afadin, PDZ-GEFs and Rap1 have been widely implicated in regulation of endothelial barrier, as will be discussed below.

Afadin, a large PDZ-containing scaffold protein shown to associate with JAM-A (Severson et al., 2009a), has been strongly implicated in the regulation of barrier function. Mice with intestinal epithelial-targeted loss of afadin have increased intestinal permeability (Tanaka-Okamoto et al., 2011) and a phenotype similar to that observed with JAM-A KO mice. JAM-A KO mice have normal intestinal mucosal architecture but a leaky colonic epithelium, increased mucosal lymphoid follicles and enhanced susceptibility to acute injury-induced colitis (Laukoetter et al., 2007). While complete genomic deletion of afadin is lethal, mice with intestinal epithelial targeted loss of afadin (cKO) are viable, have a similar increase in gut permeability, and exhibit seemingly normal intestinal morphology. Similarly, afadin

cKO mice show enhanced susceptibility to acute injury-induced colitis. Although the above parallels between JA KO and afadin cKO animals are consistent with afadin regulation of barrier function downstream of JAM-A, afadin has also been regarded as a cadherin-associated scaffold that mediates outside-in signaling after nectin-driven nascent junctions are initiated (Takai and Nakanishi, 2003). While the latter observation might implicate afadin in controlling barrier downstream of nectin, mice lacking nectins-2 and -3 in the intestinal epithelium have no increase in intestinal permeability compared to wild type counterparts (Tanaka-Okamoto et al., 2011). Furthermore, the intestinal epithelium of such nectin-deficient mice has normal localization of afadin. These findings suggest that intestinal barrier function and junctional localization of afadin can occur by nectin-independent mechanism(s).

In addition to afadin, the guanine nucleotide exchange factor PDZ-GEF2 has been reported to associate with JAM-A and mediate β1 integrin dependent epithelial cell migration (Severson et al., 2009a), presumably through activation of the small GTPase Rap1a. Despite this observation, the role for PDZ-GEF2 or the closely related PDZ-GEF1 in regulating epithelial barrier is not understood. Loss of PDZ-GEF1/2 in epithelial and endothelial cells has been shown to affect the composition and architecture of the AJ so that its morphology resembles nascent puncta (Dube et al., 2008; Pannekoek et al., 2011), suggesting that PDZ-GEF1/2 may be important in the maturation of initial puncta into functionally developed AJCs. On the other hand, the differences in AJ architecture described in these reports were only detectable when cells were re-cultured at low confluence, suggesting that the role of PDZ-GEFs in barrier function may be limited to early events in junction formation. Additionally,

loss of PDZ-GEF2 in epithelial cells did not affect the localization of the TJ proteins occludin or ZO-1 (Dube et al., 2008), supporting the idea that altered AJ morphology does not necessarily translate to defects in TJ composition. In endothelial cells, Pannekoek et. al reported that PDZ-GEF1/2 depletion resulted in decreased transendothelial impedance, a proxy measure of barrier function (Pannekoek et al., 2011), but no analogous functional observations were reported for epithelial cells. Such observations support the concept of distinct regulatory mechanisms governing endothelial and epithelial barrier, which may be explained in part by the fact that the AJC in endothelial cells differs from that of epithelial cells by having a less defined separation between AJs and TJs (Schulze and Firth, 1993). It is therefore important to confirm the functional importance of PDZ-GEFs in epithelial barrier function so as to determine whether they may act downstream of JAM-A to affect epithelial permeability.

As is the case for PDZ-GEFs, the small GTPase Rap1 has been implicated in downstream signaling events from JAM-A mediated regulation of cell migration. Interestingly, Rap1a/b have been widely implicated in regulation of endothelial barrier function (Bos, 2005) but much less is known regarding the role of Rap1 as an effector of barrier in epithelial cells. In epithelial cells, Rap1a has been implicated in mediating trans-dimerization of E-cadherin (Price et al., 2004) and in organization of E-cadherin along cell-cell contacts (Hogan et al., 2004; Dube et al., 2008). However inactivation of Rap1 by the guanine nucleotide activating protein RapGAP does not affect the localization of ZO-1 to cell-cell contacts (Hogan et al., 2004), suggesting that TJ formation does not require Rap1 in epithelial cells.

Furthermore an in vivo study on the role of the oxidized phospholipid OXPAPC in lung epithelial permeability reported no changes in transepithelial resistance after downregulation of Rap1 (Birukova et al., 2011). Additionally, Rap1 null *c. elegans* display normal epithelial architecture of the epidermis and gut (Frische et al., 2007). Notably, most studies claiming a role for Rap1 in regulating endothelial and epithelial barrier are in fact describing functions of proteins known to alter the activation of Rap1, such as EPAC, RapGAP and PDZ-GEF1/2 (Pannekoek et al., 2011; Hogan et al., 2004). Since these mediators lack specificity for Rap1 (Roscioni et al., 2008; Janoueix-Lerosey et al., 1992; Kuiperij et al., 2003; De Rooij et al., 1999), the possibility of involvement of other small GTPases has not been excluded. The paucity of data directly relating Rap1 to functional measures of epithelial permeability leaves more questions than answers regarding a link between Rap1 and JAM-A dependent regulation of barrier function.

ZO-1 is an important TJ-associated scaffold protein and one of three zonula-occludens proteins. ZO-1 has three PDZ domains (PDZ1-3), a Src homology 3 domain (SH3) and a guanylate kinase homology domain (GUK) and has been reported to associate with JAM-A (Bazzoni et al., 2000). A recent crystallography study identified PDZ3 as the putative binding pocket in ZO-1 responsible for JAM-A association, and that this association required the presence of the SH3 domain (Nomme et al., 2011). Interestingly, mice lacking ZO-1 or ZO-2 do not survive, suggesting that both proteins are required for embryonic development (Xu et al., 2008; Katsuno et al., 2008). ZO-3 null mice, however, have little or very subtle phenotypic differences compared to their wild-type counterparts, suggesting a

redundant role of ZO-3 in epithelial function (Xu et al., 2008). Cell culture studies have further defined barrier-inducing roles of ZO proteins through simultaneous silencing of ZO-1, 2 and 3 followed by their replacement one at a time (Umeda et al., 2006; Yamazaki et al., 2008). Epithelial cells lacking all three ZO proteins have no TJs, highlighting the importance of these scaffold proteins to barrier formation. Interestingly, addition of either ZO-1 or ZO-2 to ZO-null cells is sufficient for establishing TJs. Additionally, it was found that protein levels of JAM-A, claudins and occludin are unchanged in ZO-depleted cells, suggesting that ZO-1 or 2 are likely necessary and sufficient for recruitment of TJ components to the AJC during barrier formation, but that synthesis of TJ proteins occurs independently of ZO-1/2 expression (Tsukita et al., 2009). ZO-1 has also been shown to associate with claudins via PDZ1, occludin through its GUK domain, afadin via its SH3 domain, and with other ZO proteins via PDZ2, allowing it to cluster several scaffold proteins, potentially leading to TJ maturation. It is possible that ZO-1, afadin, PDZ-GEFs and other PDZ containing scaffold proteins associate with transmembrane PDZ motif-containing proteins such as JAM-A to direct maturation and maintenance of barrier function. Ebnet and others have reported co-association between JAM-A and ZO-1 from cell lysates (Ebnet et al., 2000; Bazzoni et al., 2000) and Nomme et. al have shown in-vitro direct association of these proteins via crystallography studies (Nomme et al., 2011). However, there are no reports showing a direct association of JAM-A and ZO-1 in cells. Additionally, there is limited data on whether ZO-2 can associate with JAM-A or participate in the same signaling module that ZO-1 and JAM-A are reported to share. While the role of ZO-1 and -2 proteins in barrier

formation are well appreciated, the mechanisms linking ZO-1 and -2 to JAM-A mediated regulation of barrier function require further elucidation.

The GEFS and scaffold proteins discussed above are attractive candidate mediators regulating JAM-A dependent barrier function. However, potential distal signaling elements downstream of JAM-A that are intimately associated with regulation of epithelial permeability merit careful consideration. In particular, cytoskeletal dynamics and claudin composition of TJs directly affect paracellular permeability (Fig. 3) (Overgaard et al., 2011; Shen et al., 2011; Furuse and Tsukita, 2006). The tetraspan TJ forming claudins are classified as either leaky or tight and dimerize across the apical intercellular space to form channels that control the permeability of monolayers (Overgaard et al., 2011; Amasheh et al., 2011; Furuse and Tsukita, 2006). It was previously reported that JAM-A null mice and JAM-A depleted intestinal epithelial cells demonstrate enhanced levels of the leaky claudins 10 and 15 (Laukoetter et al., 2007), but not of claudin 2 or occludin, suggesting that JAM-A affects the claudin composition of TJs. It is not known how JAM-A does this, however previous studies indicate that JAM-A affects levels of β1 integrin by maintaining its stability at the cell surface (Severson et al., 2009b). Given the likely overlap in function of some of the signaling elements discussed above, it is reasonable to hypothesize that JAM-A may regulate barrier function through effects on the stability of certain claudin family members at the TJ. Clearly, further studies should help to answer this important question.

JAM-A associated AJC scaffold proteins such as afadin and ZO molecules have actin binding domains that allow for communication between the AJC and the

apically positioned actin-myosin ring, a critical component of barrier integrity (Nusrat et al., 2000). The association of the AJC with cytoskeletal components is necessary for maintaining AJC structure (Fanning et al., 1998; Mandai et al., 1997). An attractive potential mechanism for JAM-A regulation of barrier would involve signaling through effectors such as afadin to induce cytoskeletal changes that control paracellular flux and epithelial permeability. JAM-A modulates epithelial cell migration, a process dependent on dynamic restructuring of actin, and induces activation of Rap GTPases, which have important cytoskeletal regulatory properties (Severson et al., 2008a; Mandell et al., 2005). Analogous JAM-A dependent pathways that regulate barrier are therefore easily envisioned and require further exploration.

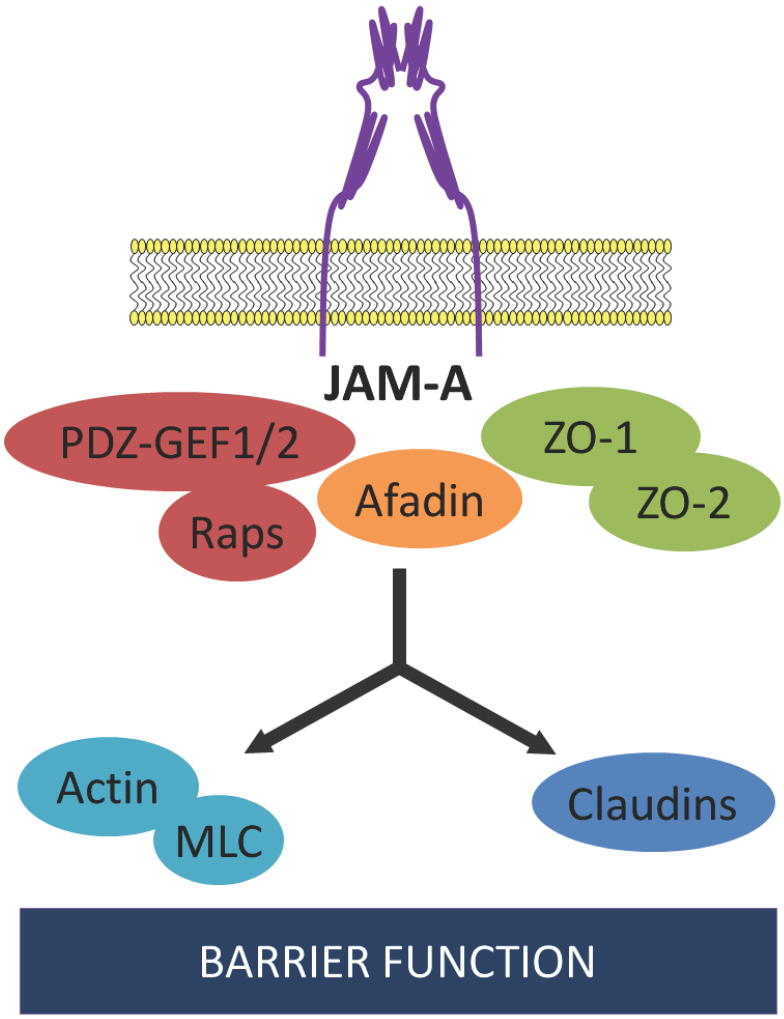


Figure 3: Possible downstream mechanisms linking JAM-A dimerization to barrier function. Although PDZ-GEF2, afadin, and Rap1 have been reported to associate with JAM-A, their potential roles in regulation of epithelial barrier require further elucidation. Other key components reported to affect epithelial barrier, including ZO-1, claudins, and the epithelial cytoskeleton, may also act downstream of JAM-A; however, association(s) with JAM-A have not yet been established.

Possible mechanisms that may differentiate divergent JAM-A signaling modules

A major challenge has been to understand how JAM-A mediates diverse cellular functions such as altered permeability (Mandell et al., 2005), cell migration (Severson et al., 2009b), and enhanced proliferation (Nava et al., 2011). It is assumed that functional specificity of JAM-A signaling is determined by the interaction of JAM-A with specific scaffold proteins, which may in turn be differentially distributed to sub-compartments of epithelial cells. Since previous observations have demonstrated that JAM-A concentrates at the AJC but also along the basolateral membrane (Liang et al., 2000), it is possible that JAM-A dimerization or phosphorylation may determine the localization of JAM-A in either compartment, allowing for specific activation of a particular JAM-A dependent signaling cascade. For example, dimerization of JAM-A in a cis but not trans configuration, as would be expected in spreading or subconfluent cells, might favor proliferation and/or migration. However trans interactions between cells, as would be expected in confluent epithelia, would favor close apposition of PDZ-bound molecules that promote barrier forming and perhaps even senescence-inducing cues (Fig. 4).

Likewise, JAM-A phosphorylation events may be important for determining sub-cellular localization. JAM-A localizes to nascent puncta with E-cadherin, nectin and ZO-1 to initiate AJC formation, however it also co-localizes with ZO-1 in mature TJs away from the AJ proteins E-cadherin and nectin. It is possible that JAM-A phosphorylation event(s) could facilitate movement of JAM-A towards TJ maturation after puncta establishment (Fig. 5). Strong support for this latter

proposed mechanism was very recently shown in a report published while this manuscript was in editorial review. In particular, aPKCζ-mediated phosphorylation of ser285 in the JAM-A cytoplasmic tail was shown to regulate JAM-A localization to mature TJs (Iden et al., 2012). It will be interesting to investigate whether such phosphorylation events alter binding affinities between JAM-A and different scaffold proteins, thereby determining activation of a specific JAM-A-dependent cascade in epithelial cells. These two proposed mechanisms may be key elements determining specificity of JAM-A dependent cues, and are attractive areas to be investigated as novel therapeutic targets may emerge that can target JAM-A dependent function.

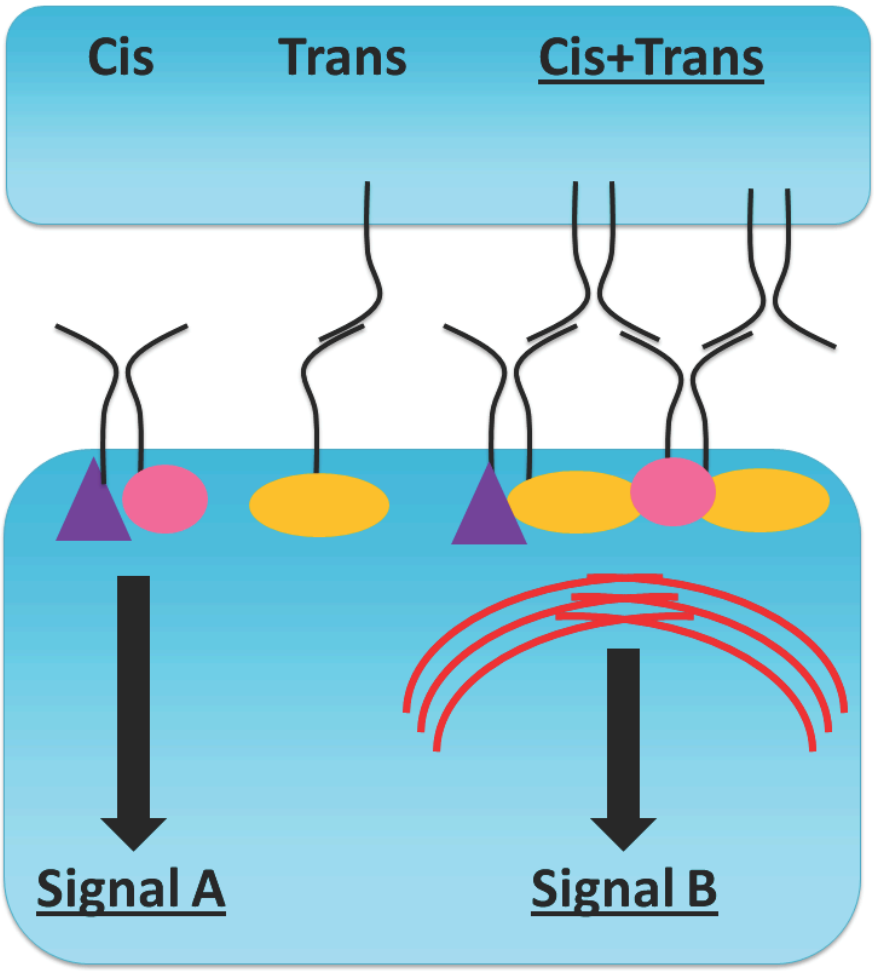


Figure 4: Model of how JAM-A may differentially induce di- vergent signaling modules based on JAM-A dimerization. When JAM-A homodimerizes exclusively in cis, as may be the case in subconfluent epithelial sheets, a proliferative or migratory path- way may be initiated. When JAM-A homodimerizes in cis and trans, as may be the case in confluent, fully polarized epithelial monolayers, JAM-A may associate with other scaffold proteins that promote barrier function and possibly senescence.

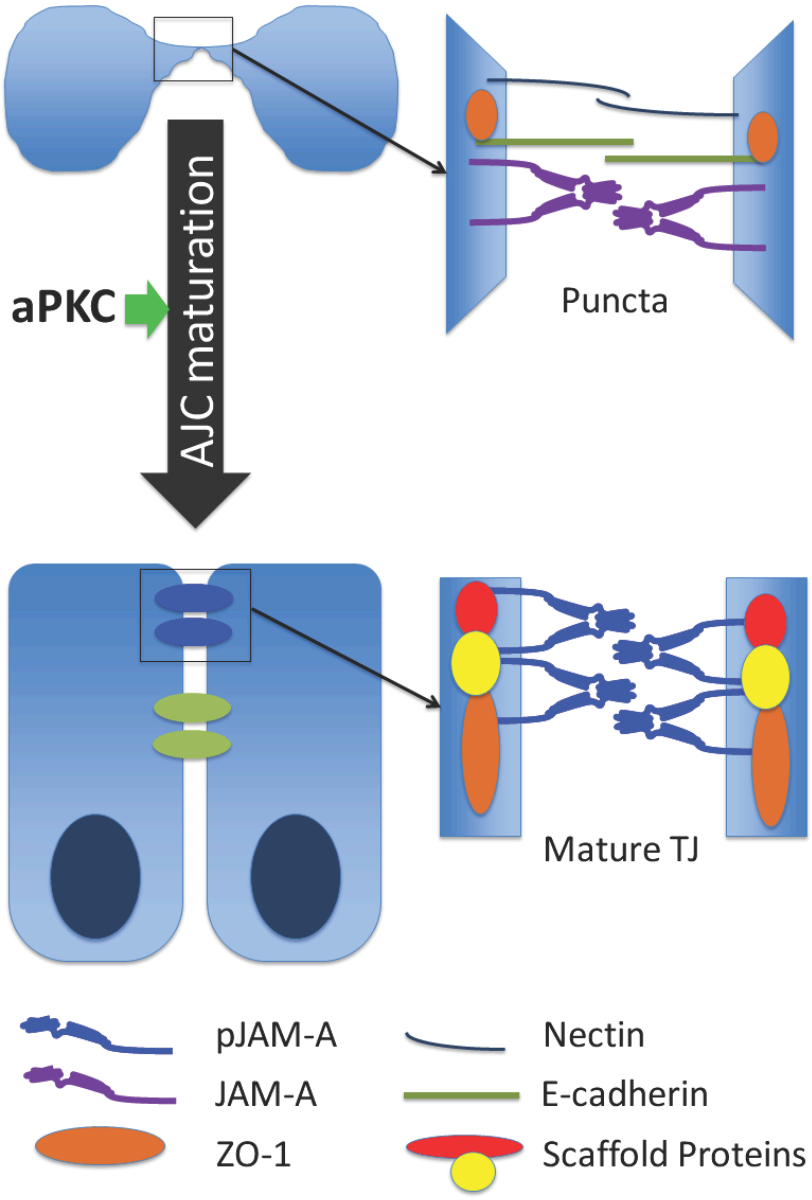


Figure 5: Model proposing how JAM-A may be recruited to differential subcellular compartments based on JAM-A phosphorylation. In the upper part of the figure, unphosphorylated JAM-A preferentially localizes to the basolateral membrane, where it can participate in events such as initiation of nascent puncta. JAM-A phosphorylation by an atypical PKC may then lead to its co-localization with TJ-associated proteins and maturation of the AJC (lower panels).

Conclusions

JAM-A is a transmembrane, TJ-associated Ig superfamily member that regulates epithelial barrier function, cell migration and proliferation. Current evidence indicates that JAM-A dimerization is necessary for functional regulation of barrier, however the mechanisms linking JAM-A to barrier function have not been elucidated. While it has been shown that JAM-A regulates cell migration through dimerization-dependent clustering of the cytoplasmic scaffold molecules afadin and PDZ-GEF2 resulting in activation of Rap1a to stabilize cell surface β1 integrin, the involvement of these pathways in regulation of barrier is unclear. Moreover, association of JAM-A with cellular components known to affect epithelial permeability, such as claudins, ZO-1/2 and the epithelial cytoskeleton remains to be established. Understanding mechanisms that regulate epithelial permeability downstream of JAM-A may be useful in identifying therapeutic targets for diseases associated with barrier dysfunction and may lead to novel approaches towards trans-epithelial drug delivery. Finally, future studies should aim to elucidate mechanisms that determine JAM-A expression and its subcellular localization, since such cues may be key to deciphering how divergent JAM-A-modulated signaling cascades are differentially induced. Identification of effectors that specifically modulate epithelial permeability downstream of JAM-A without affecting proliferation, migration or other pathways could lead to the development of specific pharmaceutical interventions with fewer off-target effects.

This thesis will describe our recent findings elucidating how JAM-A localizes a large protein complex to cell contacts in order to regulate epithelial

barrier function. It will also provide evidence for how JAM-A may simultaneously homodimerize across cells (in trans) and along the surface of the same cell (in cis) to form oligomers important for regulating epithelial permeability. Finally, this thesis will discuss new questions that have arisen from these studies, and propose important future directions for the field.

Chapter 2

JAM-A associates with ZO-2, Afadin and PDZ-GEF1 to activate Rap2c and regulate epithelial barrier function.

Material from this chapter has also appeared in an article in *Molecular Biology of the Cell,* **2013**. Contributing authors of this article were: Ronen Sumagin, Carl R. Rankin, Giovanna Leoni, Michael J. Mina, Dirk M. Reiter, Thilo Stehle, Terence S. Dermody, Stacy A. Schaefer, Randy A. Hall, Asma Nusrat, Charles A. Parkos.

Abstract

Intestinal barrier function is regulated by epithelial tight junctions (TJs), structures that control paracellular permeability. Junctional Adhesion Molecule-A (JAM-A) is a TJ-associated protein that regulates barrier, however mechanisms linking JAM-A to epithelial permeability are poorly understood. Here we report that JAM-A associates directly with ZO-2 and indirectly with afadin, and this complex, along with PDZ-GEF1, activates the small GTPase Rap2c. Supporting a functional link, siRNA-mediated downregulation of the above regulatory proteins resulted in enhanced permeability similar to that observed after JAM-A loss. JAM-A deficient mice and cultured epithelial cells demonstrated enhanced paracellular permeability to large molecules, revealing a potential role of JAM-A in controlling peri-junctional actin cytoskeleton in addition to its previously reported role in regulating claudin proteins and small-molecule permeability. Further experiments suggested that JAM-A does not regulate actin turnover, but modulates activity of RhoA and phosphorylation of non-muscle myosin, both implicated in actomyosin contraction. These results suggest that JAM-A regulates epithelial permeability through association with ZO-2, afadin and PDZ-GEF1 to activate Rap2c and control contraction of the apical cytoskeleton.

Introduction

The colonic epithelium facilitates selective absorption of nutrients while precluding the passage of toxins and pathogens into the body. This selective permeability is regulated by tight junctions (TJs), which are complex, dynamic structures that localize to the apical contacts between epithelial cells. It is now well appreciated that TJs are composed of a diverse array of structural and signaling proteins that include Junctional adhesion molecule-A (JAM-A). JAM-A is a transmembrane protein constituent of TJs that has been shown to regulate epithelial barrier function in addition to other homeostatic properties such as epithelial cell migration and proliferation (Ye et al., 2011; Laukoetter et al., 2007; Severson et al., 2008a; Nava et al., 2011).

There are several reports linking JAM-A to regulation of epithelial barrier function. For example, JAM-A knock-out mice have enhanced colonic permeability, and epithelial cells lacking JAM-A have decreased transepithelial electrical resistance (TER) and enhanced paracellular flux of small molecular weight dextran in vitro (Yamaguchi et al., 2010; Laukoetter et al., 2007). Previous studies linking JAM-A structure to cellular function have indicated that JAM-A forms homodimers on the surface of the same cell (in cis) at its membrane-distal Ig domain (Ebnet et al., 2001; Prota et al., 2003; Kostrewa et al., 2001) and that cis-dimerization is required for epithelial cell migration and barrier function (Ozaki et al., 2000; Severson et al., 2008a; Iden et al., 2012; Liu et al., 2000; Mandell et al., 2004). JAM-A has been reported to associate with signaling molecules such as the scaffold proteins ZO-1 and afadin as well as the guanine exchange factor PDZ-GEF2 via its cytoplasmic type

II PDZ-binding motif (Nusrat et al., 2000; Ebnet et al., 2000; Severson et al., 2009b). Close apposition of the latter two signaling components in dimerized JAM-A appears to be necessary for activation of the small GTPase Rap1a, stabilization of β1 integrin, and regulation of cell migration (Mandell et al., 2007; Severson et al., 2009b; Mandell et al., 2006; 2004; Liu et al., 2000). Despite these findings linking JAM-A mediated signaling to cell migration, the signaling events linking JAM-A to regulation of epithelial permeability are not known.

It is well appreciated that interactions of TJ-associated transmembrane proteins with large scaffold proteins and the actin cytoskeleton mediate regulation of paracellular permeability in a highly dynamic manner. Paracellular permeability to small molecules is directly determined by tetraspan claudins, which cluster as homodimers across cells to form channels of varying permeability to specific ions (Severson et al., 2009a; Furuse, 1998; Nitta et al., 2003; Furuse, 2010). Intriguingly, JAM-A-deficient cell lines and mice, which have enhanced intestinal permeability, also have altered expression of claudins 10 and 15, which have been shown to regulate permeability to small solutes (Severson et al., 2009a; Laukoetter et al., 2007; Van Itallie et al., 2003; 2006; Colegio et al., 2003). However, the mechanisms defining how JAM-A regulates claudin 10/15 are not known, neither is it understood whether this observed alteration in claudin composition is sufficient to account for the enhanced permeability observed in JAM-A deficient animals. On the other hand, paracellular permeability to larger molecules is regulated by the TJ-associated apical cytoskeleton that responds to extracellular cues by expanding and contracting via actomyosin interactions (Tanaka-Okamoto et al., 2011; Madara and

Pappenheimer, 1987) (Laukoetter et al., 2007; Bruewer et al., 2004; Nusrat et al., 1995). The contractile tone of the apical cytoskeleton is critical for maintaining a functional, polarized epithelium, and further stimulation of contraction has been shown to enhance paracellular flux of larger molecules by expanding the paracellular space (Takai and Nakanishi, 2003; Ivanov et al., 2007; Shen et al., 2006). Despite an abundance of evidence showing intimate interactions between TJ proteins and the apical cytoskeleton (Tanaka-Okamoto et al., 2011; Nusrat et al., 1995; Fanning et al., 1998; Itoh et al., 1999; Madara and Pappenheimer, 1987) the relationship between JAM-A and the apical cytoskeleton is not understood.

However, it is well appreciated that transmembrane TJ proteins communicate with the apical cytoskeleton through interactions with cytoplasmic scaffold or plaque proteins. Interestingly, JAM-A has been reported to associate with actin binding scaffold proteins ZO-1 and afadin (Severson et al., 2009a; Bazzoni et al., 2000; Ebnet et al., 2000; Severson et al., 2009b), both of which are implicated in the regulation of barrier. ZO-1 and its closely related family member ZO-2 regulate TJ assembly and play important roles in controlling epithelial permeability (Dube et al., 2008; Van Itallie et al., 2009; Pannekoek et al., 2011). Likewise, mice with intestinal epithelial-targeted loss of afadin demonstrate enhanced intestinal permeability (Dube et al., 2008; Tanaka-Okamoto et al., 2011) with a phenotype similar to that observed in JAM-A deficient mice, strengthening a functional link between JAM-A and afadin. Importantly, mice deficient in nectin, another afadin-associated adherens protein, did not demonstrate altered intestinal permeability (Pannekoek et al., 2011; Tanaka-Okamoto et al., 2011), suggesting that afadin may

regulate barrier function downstream of JAM-A in a nectin-independent manner. Scaffold proteins such as afadin and the ZO proteins have several functional binding regions such as PDZ domains that associate with transmembrane proteins, actin-binding domains, and RA domains that can serve as binding sites for small GTPases (Schulze and Firth, 1993; Van Itallie et al., 2009; Yamazaki et al., 2008; Mandai et al., 1997). Given the above observations, it is reasonable to assume that PDZ-dependent interactions between JAM-A and certain scaffold proteins may play important role(s) in regulating epithelial barrier function. However, the nature of such interactions and identity of signaling elements linking JAM-A to regulation of epithelial permeability remain unclear.

In this study, we utilized in vitro and in vivo techniques to better define mechanisms that link JAM-A to the regulation of epithelial barrier function. Our results suggest that JAM-A forms a complex with PDZ-containing scaffold proteins that regulate contractility of the apical cytoskeleton which, in turn, fine tunes epithelial permeability. Notably, we report that the tight junction scaffold protein ZO-2 directly interacts with JAM-A and is necessary for mediating indirect interactions between JAM-A and afadin. We also show that JAM-A and afadin mediate activation of Rap2c, a GTPase previously uncharacterized in the context of epithelial barrier function. Taken together, these findings provide new insights into the regulation of epithelial barrier function by JAM-A.

Results

JAM-A-binding reovirus σ1 protein induces JAM-A internalization and enhances permeability in vivo and in vitro

JAM-A-deficient mice and JAM-A deficient intestinal epithelial cells display reduced TER and increased flux to 3-4 kDa dextrans (Bos, 2005; Laukoetter et al., 2007), however mechanisms defining JAM-A regulation of epithelial permeability are not understood. To better understand the link between JAM-A and barrier function, we performed experiments comparing the role of JAM-A during TJ assembly/barrier formation versus maintenance of a stable barrier. For these studies, we used a recombinant form of reovirus σ1 protein, which has been shown to bind to the membrane-distal D1 domain of JAM-A and disrupt JAM-A homodimerization (Price et al., 2004; Guglielmi et al., 2007; Kirchner et al., 2008; Zhang et al., 2010). Addition of WT σ1 to subconfluent monolayers of model intestinal epithelial cell lines inhibited barrier development in comparison to cells treated with σ 1_G381A mutant protein, which is deficient in JAM-A binding (Hogan et al., 2004; Kirchner et al., 2008; Dube et al., 2008) (Fig 6a). Additionally, incubation of confluent monolayers of SKCO-15 (Fig 6b) or T84 cells (Fig 6.1a) with σ1 (20ug/ml, 1 hour for SK-CO15 cell and up to 3 hours for T84 cells) resulted in a significant reduction in TER in comparison to confluent cells treated with σ1_G381A, suggesting that JAM-A regulates both assembly and maintenance of the epithelial barrier. Immunofluorescence labeling and confocal microscopy revealed that cells exposed to σ1 had reduced levels of TJ-associated JAM-A when compared to cells incubated with σ1_G381A (Fig 6c), however localization of E-cadherin was

unaffected (Fig. 6.1b), suggesting that effects observed were specific to JAM-A and that epithelial architecture remained intact. To assess the in-vivo significance of the above in-vitro findings, we examined the effect of σ1 on intestinal permeability in anesthetized mice. Using an intestinal loop model, administration of σ1 into the intestinal lumen for 2 hours resulted in a four-fold increase in permeability to 3kDa dextran compared to treatment with σ1_G381A (Fig. 6d). These findings suggest that reduction of TJ-associated JAM-A after σ1 exposure compromises TJ barrier function.

Since exposure of intestinal epithelial cells to σ1 resulted in a reduction of TJ-associated JAM-A and concomitant barrier defects similar to that observed in knock-out mice, we initiated experiments to better define JAM-A-dependent mechanisms regulating barrier function using epithelial cell lines deficient in JAM-A. As can be seen in figure 6.1c, stable IECs deficient in JAM-A displayed delayed development of TER compared to control non-silenced (NS) IECs.

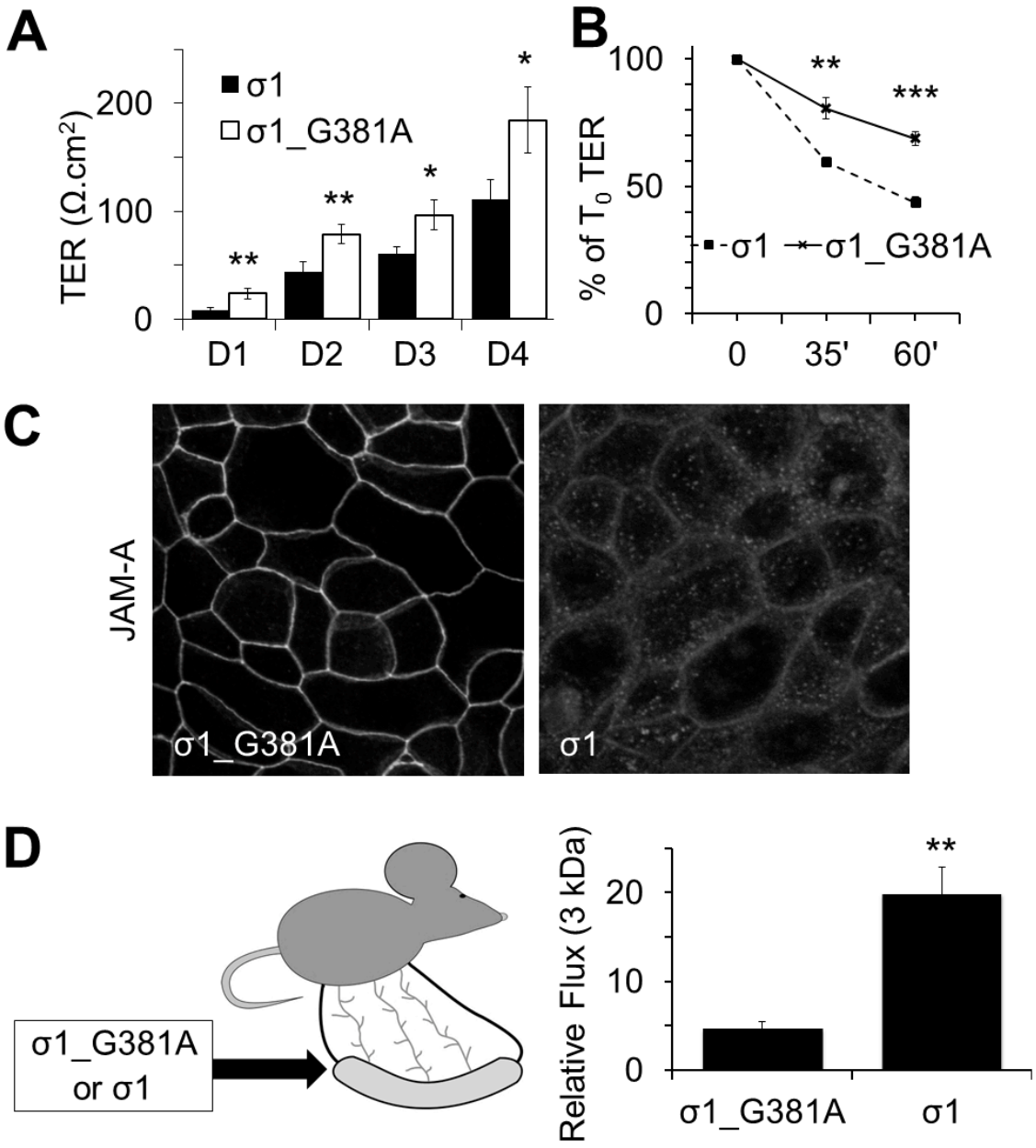


Figure 6: JAM-A regulates barrier function. Treatment of SK-CO15 with σ1 (10 μg/ml) on plating abrogates the formation of TER when compared to cells treated with mutant σ1_G381A (A, representative experiment with three independent samples. Mean +/- SD). Treatment of confluent SK-CO15 monolayers with σ1 (20 μg/ml) for 1 hour led to significant reduction in TER when compared to cells treated with σ1_G381A mutant (B, representative experiment with three independent samples. Mean +/- SD). Treatment of confluent SK-CO15 monolayers with σ1 (20 μg/ml) for 1 hour led to significant reduction in JAM-A expression at tight junctions (C). Administration of σ1 in vivo enhances permeability to small molecules. WT σ1 or σ1_G381A (100 μg/ml) was administered to intestinal loops of WT mice for 1 hour then assessed for 3 kDa dextran flux for another hour. (D, n=3 per group. Mean +/- SEM). For all experiments, *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ between groups at each time point per student's *t* test.

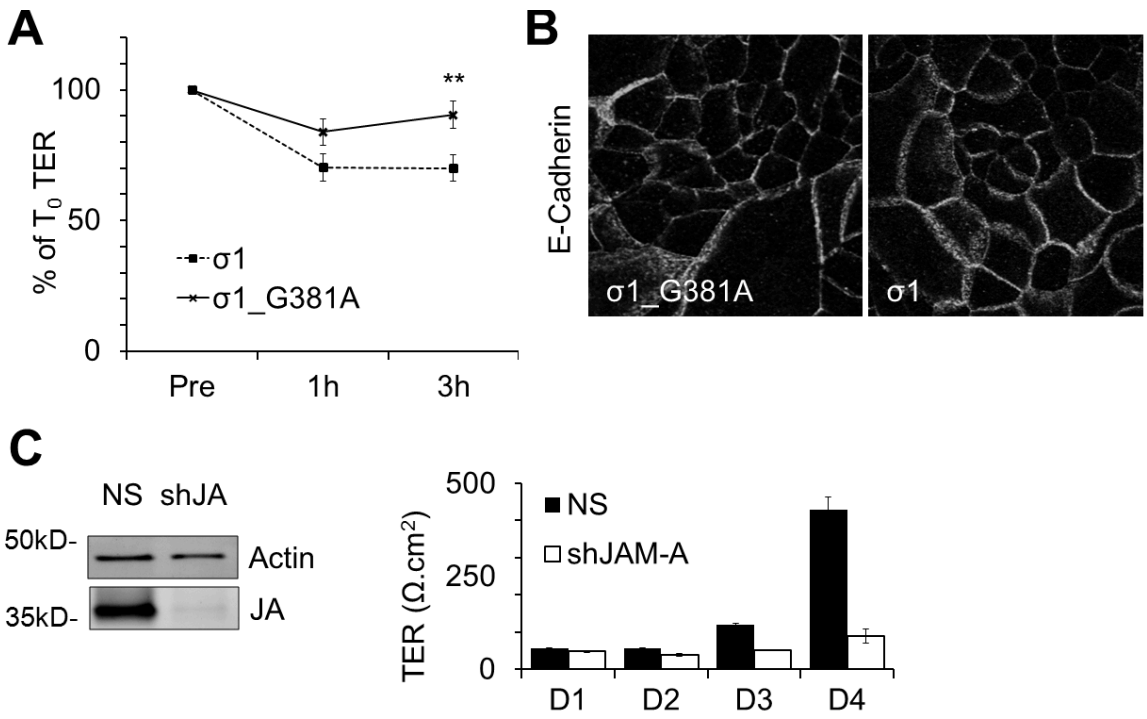


Figure 6.1: Treatment of confluent T84 monolayers with σ1 for 3 hours led to significant reduction in TER when compared to cells treated with σ1_G381A mutant. Either WT σ1 or σ1_G381A (20 μg/ml) was added to apical and basolateral compartments. TER was evaluated at 1 and 3 hours (A, representative experiment with three independent samples. Bars represent mean +/- SD). SK-CO15 cells stably expressing non-silencing shRNA (NS) or shRNA targeting JAM-A (shJAM-A) were verified by immunoblot. SK-CO15 cells stably downregulated for JAM-A (shJAM-A) do not develop trans-epithelial resistance (TER) after four days of plating when compared to SK-CO15 cells stably expressing non-silencing shRNA (NS) (B, representative experiment with three independent samples. Bars represent mean +/- SD).

JAM-A interacts directly with the TJ plaque protein ZO-2

Since loss of JAM-A at the apical junctional complex disrupted barrier development and maintenance, we sought to identify JAM-A-associated effector proteins that regulate epithelial permeability. JAM-A has been shown to interact with several scaffold proteins through its C-terminal PDZ binding motif (Hogan et al., 2004; Nomme et al., 2011). We screened for PDZ-dependent binding of the recombinant full length cytoplasmic segment of JAM-A (a.a. 261-300) using a proteomic array of 96 recombinant PDZ domains derived from 48 distinct scaffold proteins. Analyses of array results revealed binding of GST-tagged cytoplasmic tail of JAM-A to the second PDZ domain of ZO-2. Specificity for a PDZ-dependent interaction was confirmed by absence of ZO-2 binding to a GST-tagged JAM-A cytoplasmic tail mutant protein lacking the distal PDZ-binding motif (Fig. 7a). Interestingly, we observed in-vitro interactions of the full-length JAM-A cytoplasmic domain with the second PDZ domain of ZO-2 but not with any of the three PDZ domains of ZO-1, despite previous reports suggesting a direct interaction between JAM-A and the third PDZ domain of ZO-1 (Birukova et al., 2011; Nomme et al., 2011). To test whether full-length JAM-A and ZO-2 interact in epithelial cells, we performed co-immunoprecipitation assays for ZO-2 and JAM-A from lysates of polarized human IECs. Western blots of JAM-A immunoprecipitates prepared with buffers containing various detergents including NP40 alone or a mixture of Triton X-100, sodium deoxycholate and SDS (RIPA) revealed a prominent 160kDa band immunoreactive with ZO-2 antibodies (Fig. 7b and 7.1, respectively). siRNA-mediated knockdown of ZO-2 resulted in loss of the 160kDa protein band, confirming the identity of the co-

immunoprecipitating protein as ZO-2 (Fig. 7b). However, we were unable to detect JAM-A association with ZO-1 using the same co-immunoprecipitation procedure in SK-CO15 and T84 cells (Fig. 7b and 7.1), despite robust co-immunoprecipitation of ZO-1 with ZO-2 in the same experiment (Fig. 7.1). Finally, we investigated the localization of ZO-2 in epithelial cells treated with σ1, which decreased levels of junction-associated JAM-A in figure 6c. As shown in figure 7.1b, σ1 treatment also perturbed junctional localization of ZO-2, as assessed by confocal immunofluorescence imaging.

Given the array findings in figure 7a and results in figure 7b and 7.1b demonstrating association of JAM-A with ZO-2, we assessed whether downregulation of JAM-A and ZO-1/2 might have similar negative effects on barrier function. Transient downregulation of ZO-1 and ZO-2 in isolation or together resulted in decreased TER to levels similar to those observed after transient downregulation of JAM-A (Fig. 7c, d). The similar effects on TER observed after downregulation of either JAM-A and ZO proteins, along with results demonstrating JAM-A association with ZO-2 suggest that JAM-A and ZO proteins may be part of a common signaling pathway to regulate barrier function.

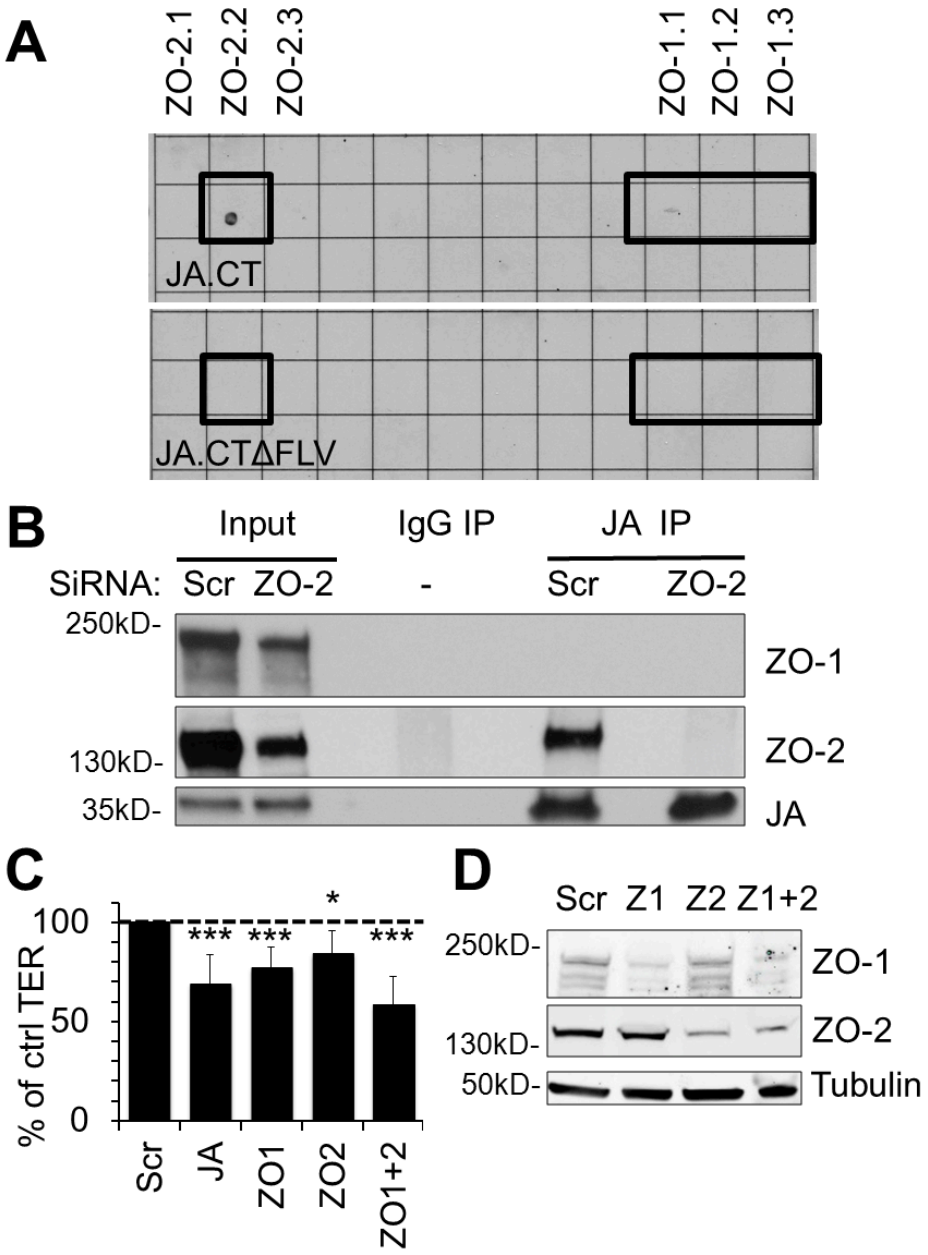


Figure 7: JAM-A associates with ZO-2, an important component of barrier function. JAM-A interacts with ZO-2 in vitro. A proteomic array containing 96 PDZ domains from 48 different proteins was used to screen for proteins interacting with the cytoplasmic PDZ-binding motif of JAM-A. The recombinant full-length cytoplasmic tail of JA (JA.CT) directly interacted with the second PDZ domain of ZO-2 on the array but did not detectably interact with any PDZ domains of ZO-1. In contrast, a mutant lacking the PDZ-binding motif on the cytoplasmic tail of JAM-A (JA.CTΔFLV) failed to interact with ZO-2 (A). JAM-A (JA) co-immunoprecipitates with ZO-2 but not ZO-1 in intestinal epithelial cells. JAM-A immunoprecipitates from cell lysates prepared with a NP40 based buffer revealed a 160 kDa ZO-2 immunoreactive band. siRNA downregulation of ZO-2 was used to confirm specificity of the detected band (B). Downregulation of ZO-1, ZO-2 or ZO-1 and -2 led to decreased TER in SK-CO15 cells at similar levels to downregulation of JAM-A relative to control cells (Scr) (C, n>5. Mean relative resistance with 95% confidence interval), as confirmed by immunoblotting (D).

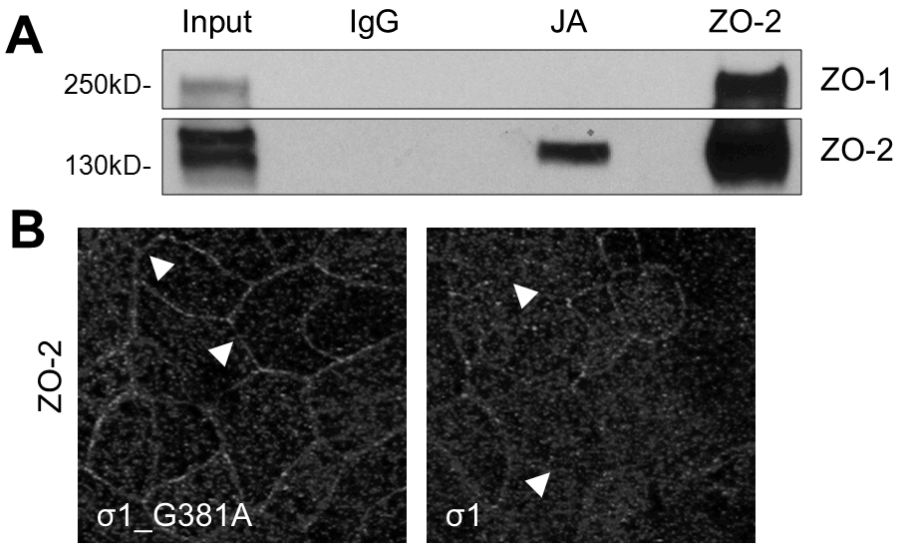


Figure 7.1: JAM-A (JA) co-immunoprecipitates with ZO-2 but not ZO-1 in intestinal epithelial cells. JAM-A immunoprecipitates from cell lysates prepared with a Triton X-100, Sodium deoxycholate and SDS based buffer (RIPA) revealed a 160 kDa ZO-2 immunoreactive band suggesting co-association between JAM-A and ZO-2, but did not reveal a 220 kDa ZO-1 immunoreactive band. Under the same conditions, ZO-2 immunoprecipitates revealed a 220 kDa ZO-1 immunoreactive band suggesting co-association between ZO-2 and ZO-1 (A). Treatment of confluent SK-CO15 monolayers with σ1 (20 μg/ml) for 1 hour led to significant reduction in ZO-2 expression at tight junctions (B).

Afadin and PDZ-GEF1, but not PDZ-GEF2 or Rap1, regulate epithelial barrier function

To identify other JAM-A effectors that regulate barrier function, we evaluated several signaling proteins that have been shown to play roles in JAM-A-mediated control of cell migration. Specifically, we tested whether afadin, PDZ-GEF2 and Rap1a, components of the pathway linking JAM-A to regulation of epithelial cell migration, could also affect barrier function (Frische et al., 2007; Severson et al., 2009b). Transient, siRNA-mediated downregulation of afadin caused a significant decrease in TER compared to cells transfected with scrambled siRNA. In contrast, siRNA-mediated downregulation of PDZ-GEF2 did not impair epithelial permeability (Fig 8a). However, downregulation of PDZ-GEF1, a closely related homologue of PDZ-GEF2, resulted in significantly decreased TER comparable to that observed after downregulation of JAM-A (Fig 8a). Experiments were then performed to examine the barrier-modulating roles of Rap1a or Rap1b, small GTPases that are known downstream target proteins of PDZ-GEF1/2 (Pannekoek et al., 2011; Hogan et al., 2004). Surprisingly, we observed that transient downregulation of Rap1a and Rap1b did not decrease TER in IECs (Fig 8a). Given the similar TER effects observed after downregulation of JAM-A and PDZ-GEF1, we performed co-immunoprecipitation experiments to see whether the two proteins are components of the same protein complex. PDZ-GEF1 immunoprecipitates from IEC lysates revealed a 37kDa protein band that was immuno-reactive with JAM-A antibody (Fig 8b), indicating that JAM-A is in a complex with PDZ-GEF1. Furthermore, using confocal microscopy and immunofluorescence staining, we observed that PDZ-GEF1

localized to epithelial junctions (Fig. 8c), as has been previously reported for afadin, another scaffold protein that has been shown to be in a complex with JAM-A (Roscioni et al., 2008; Severson et al., 2009b; Janoueix-Lerosey et al., 1992; Tanaka-Okamoto et al., 2011; Kuiperij et al., 2003; Ebnet et al., 2000; De Rooij et al., 1999). These results suggest that afadin and PDZ-GEF1 associate with JAM-A in the apical junctional complex.

We had previously reported an association between JAM-A and afadin (Bazzoni et al., 2000; Severson et al., 2009b) and confirmed that JAM-A is present in afadin immunoprecipitates of polarized IECs (Fig. 8b). Importantly, we observed that afadin immunoprecipitates revealed two protein bands of close molecular weight that were immunoreactive with JAM-A antibodies. It is likely that the JAM-A doublet observed in afadin immunoprecipitates represents two differentially phosphorylated forms of JAM-A, as has been recently described in the literature (Nomme et al., 2011; Iden et al., 2012). Based on this previous report, it is possible that the higher molecular weight species, which is of the same size as the single JAM-A band observed in immunoprecipitates of PDZ-GEF1 (Fig. 8b), represents phosphorylated, tight-junction associated JAM-A.

Interestingly, the same afadin immunoprecipitates also revealed a 160kDa band immunoreactive for ZO-2. Since our results suggest that afadin and PDZ-GEF1 are in a complex with ZO-2 and JAM-A, we performed experiments to gain further insight into which of these proteins might directly or indirectly interact with JAM-A. The in-vitro PDZ array results in figure 7a suggest that JAM-A might directly interact with ZO-2. Experiments were thus performed to determine whether the association

between JAM-A and afadin was dependent on ZO-2. Analyses of afadin immunoprecipitates from cells treated with either scrambled or ZO-2 siRNA revealed co-immunoprecipitation of JAM-A with afadin in control cells, but the protein association was greatly diminished in IECs after transient siRNA-mediated depletion of ZO-2 (Fig. 8d). In contrast, loss of ZO-2 had no effect on the co-immunoprecipitation of ZO-1 with afadin. Combined with the findings in figure 7, these results suggest that afadin association with JAM-A is dependent on ZO-2, whereas afadin association with ZO-1 is independent of ZO-2. These findings support a model where JAM-A binds directly with ZO-2 and indirectly associates with afadin and ZO-1.

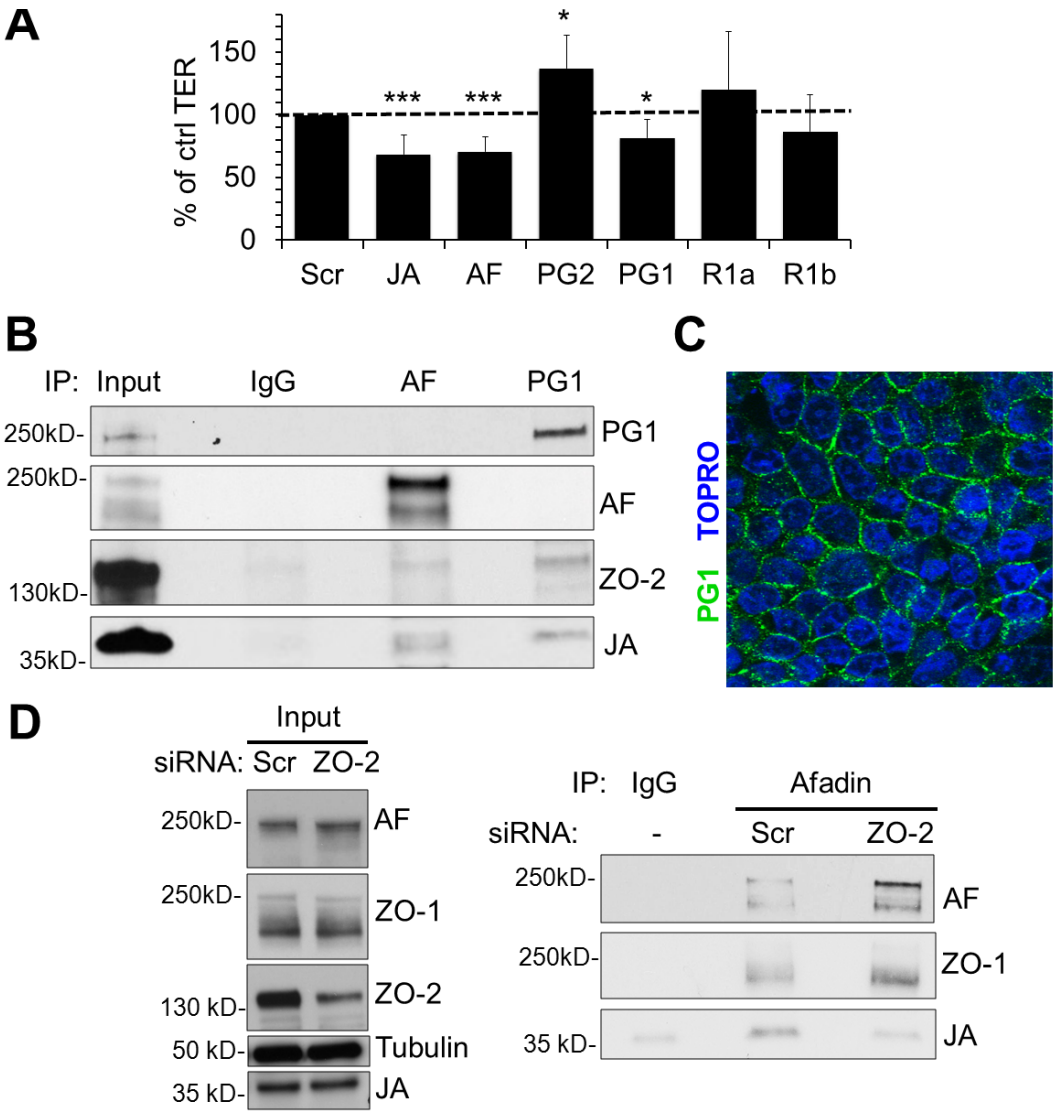


Figure 8: Downregulation of afadin and PDZ-GEF1, but not PDZ-GEF2 or Rap1a/b, leads to decreased resistance across IECs. Transient downregulation of afadin (AF) and PDZ-GEF1 (PG1) reduced intestinal epithelial TER to levels comparable to those observed after transient JAM-A downregulation (JA). Downregulation of PDZ-GEF2 (PG2), Rap1a (R1a) and Rap1b (R1b) did not affect intestinal epithelial TER (A, n>4. Mean relative resistance with 95% confidence interval. Data for JAM-A was reused from Fig 2). JAM-A (JA) and ZO-2 co-immunoprecipitate with PDZ-GEF1 (PG1) and afadin (AF) in IECs. Cell lysates were pre-extracted with an NP40-based buffer and pellets were resuspended in RIPA buffer before co-immunoprecipitation with PDZ-GEF1 or afadin (B). PDZ-GEF1 localizes to the peri-junctional region of IECs (C). JAM-A (JA) co-immunoprecipitation with afadin (AF) is disrupted after transient downregulation of ZO-2. Cell lysates were prepared with a Brij97-based buffer before co-immunoprecipitation with afadin (D).

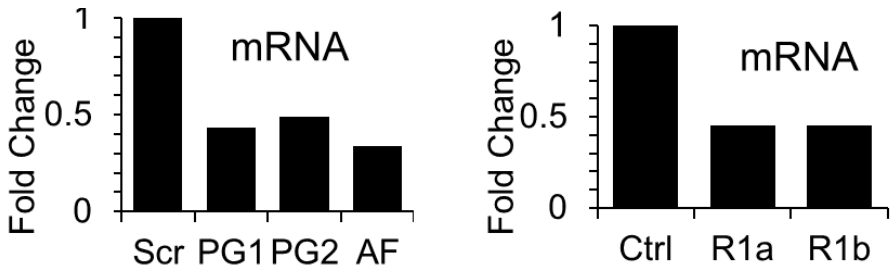


Figure 8.1: Sample qRT-PCR results verifying knockdown of targets in SK-CO15 cells used for in vitro permeability studies.

Rap2c localizes to apical cell contacts and regulates epithelial paracellular permeability downstream of JAM-A

Given the observed association between JAM-A and PDZ-GEF1 and the similar effects on permeability observed after siRNA-mediated downregulation of these two proteins, we performed experiments to identify putative barrier-regulating GTPases that might be activated by PDZ-GEF1 downstream of JAM-A. As shown in figure 8a, downregulation of Rap1a or Rap1b did not decrease TER in IECs, and thus other PDZ-GEF substrates were evaluated. Specifically, we assessed a role for Rap2, the only other known substrate for PDZ-GEF1 (Xu et al., 2008; De Rooij et al., 1999; Katsuno et al., 2008). PCR analyses revealed that SKCO-15 cells expressed mRNA for Rap2 subtypes Rap2b and Rap2c but not Rap2a, as confirmed by two sets of Rap2a primers (Fig. 9a and 9.1). To confirm protein expression, immunofluorescence staining and confocal microscopy was performed on SKCO-15 cells and demonstrated that Rap2 localized to apical cell-cell contacts (Fig. 9b). Additional immunofluorescence staining and confocal microscopy of native human colonic epithelium revealed that Rap2 localized along cell borders and co-localized with ZO-2 at apical cell junctions, consistent with TJ localization. Based on these results, we performed experiments to evaluate the role of Rap2b and c in regulating epithelial barrier function. Transient downregulation of Rap2c, but not Rap2b, using two separate siRNA targets for each gene, resulted in decreased TER similar to that observed after transient JAM-A downregulation (Fig. 9d). Further analyses of Rap2 expression revealed co-localization of Rap2 with JAM-A in cultured epithelial cells in vitro (Fig. 10a) and in native human colonic epithelium (Fig. 10b). Given that JAM-A

and Rap2 co-localized at junctions and had similar effects on TER, we hypothesized that Rap2c and JAM-A may share a common signaling pathway. To better define the role of Rap2c as a putative JAM-A effector protein that regulates barrier function, we performed double JAM-A, Rap2c knockdown studies. Simultaneous downregulation of Rap2c and JAM-A had no further additive effect on TER compared to downregulation of Rap2c or JAM-A in isolation (Fig. 10c). Given the observations suggesting that afadin and JAM-A are components of a protein complex that regulates TER and that afadin contains a GTPase binding site, we examined whether JAM-A and afadin also shared a common role in regulating the small GTPase Rap2. We thus assessed Rap2 activity using RalGDS binding assays in stable cell lines deficient in JAM-A and in cells transiently depleted of afadin. As shown in figures 10 d and e, Rap2 activity was reduced in JAM-A (Fig. 10d) and afadin (Fig. 10e) deficient cells. We next assessed whether Rap2 was part of a protein complex containing JAM-A and afadin. Since afadin contains a GTPase binding site, we probed immunoprecipitates of afadin from lysates of polarized IECs for Rap2, as shown in , 10f. Together with our findings demonstrating JAM-A co-localization with Rap2 (Fig. 10a), these results suggest that Rap2 is in a protein complex with JAM-A and afadin. Finally, given our findings implicating regulation of Rap2 activity by JAM-A and afadin as well as the observed co-immunoprecipitation of Rap2 with afadin, we performed experiments to determine whether the sub-cellular localization of Rap2c is also regulated by JAM-A. Confluent IECs transiently expressing flag-tagged Rap2c were incubated with reovirus protein σ1 to induce JAM-A localization away from TJs as highlighted in Figure 6c. Compared to incubation with JAM-A binding deficient

mutant σ1_G381A, IECs exposed to σ1 for 1 hour demonstrated a loss of junction-associated Rap2c (Fig. 10g). These findings suggest that JAM-A plays a role in mediating Rap2c distribution in cell junctions and that this complex is important for Rap2 activation.

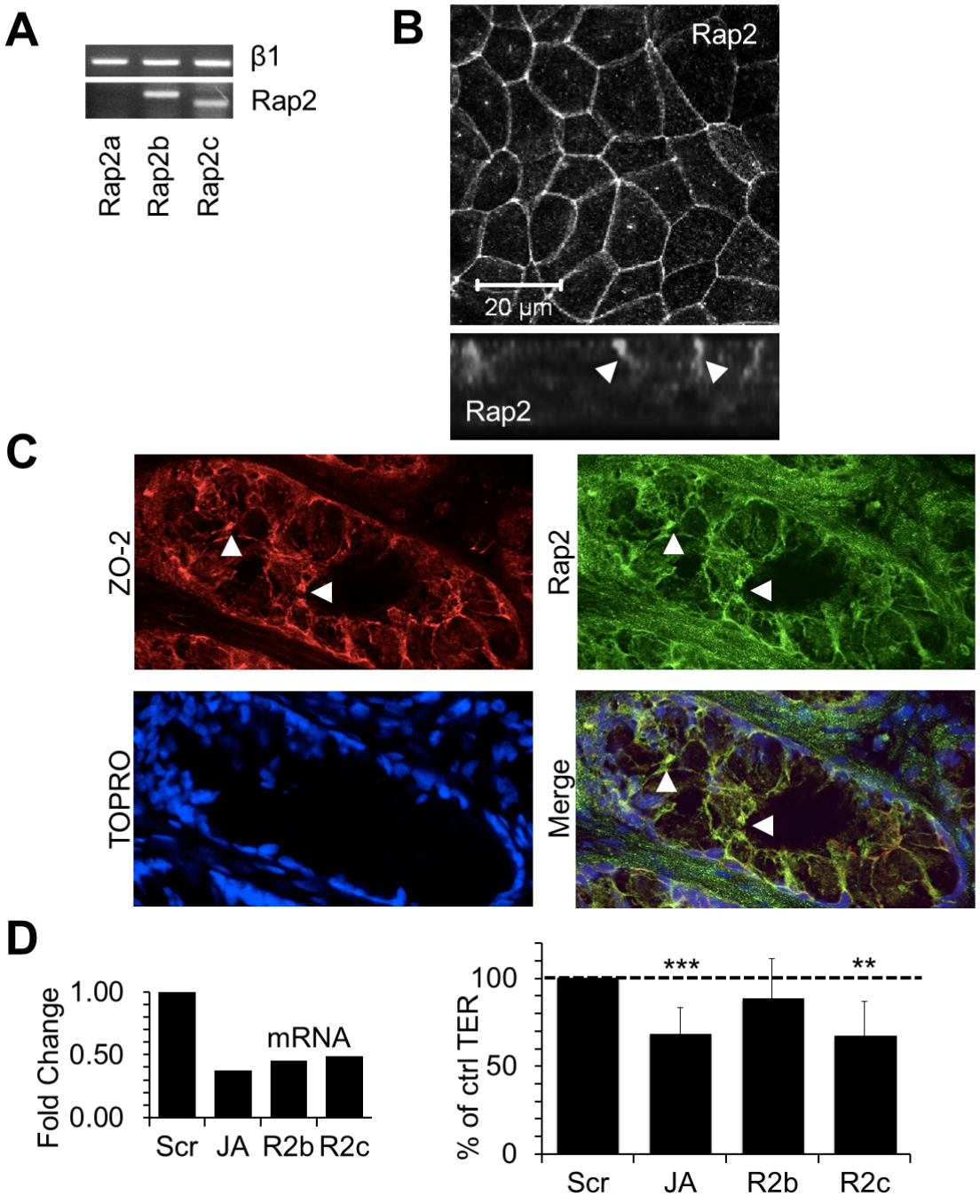


Figure 9: Rap2 is expressed in the apical spaces between IECs and Rap2c is involved in the regulation of intestinal epithelial barrier function. mRNA for Rap2 subtypes Rap2b and Rap2c but not Rap2a are present in SK-CO15 cells, as observed by PCR. RNA was extracted from confluent SK-CO15 cells and subjected to RT-PCR. A common cDNA template and PCR master mix was prepared and then subdivided before addition of Rap2a, Rap2b or Rap2c primers (A). Rap2 protein is present in apical intercellular junctions of SK-CO15 cells (B). Rap2 co-localizes with the tight junction marker ZO-2 in colonic mucosa from human pathology specimens. Tissue was pre-treated with 0.1% Triton X-100 before fixation (C). qRT-PCR-verified downregulation of Rap2c (R2c) but not Rap2b (R2b) led to significant reductions in colonic epithelial TER at levels comparable to those observed after transient JAM-A downregulation (JA) (D, n>5. relative mean resistance with 95% confidence interval. Data for JAM-A was reused from figure 2).

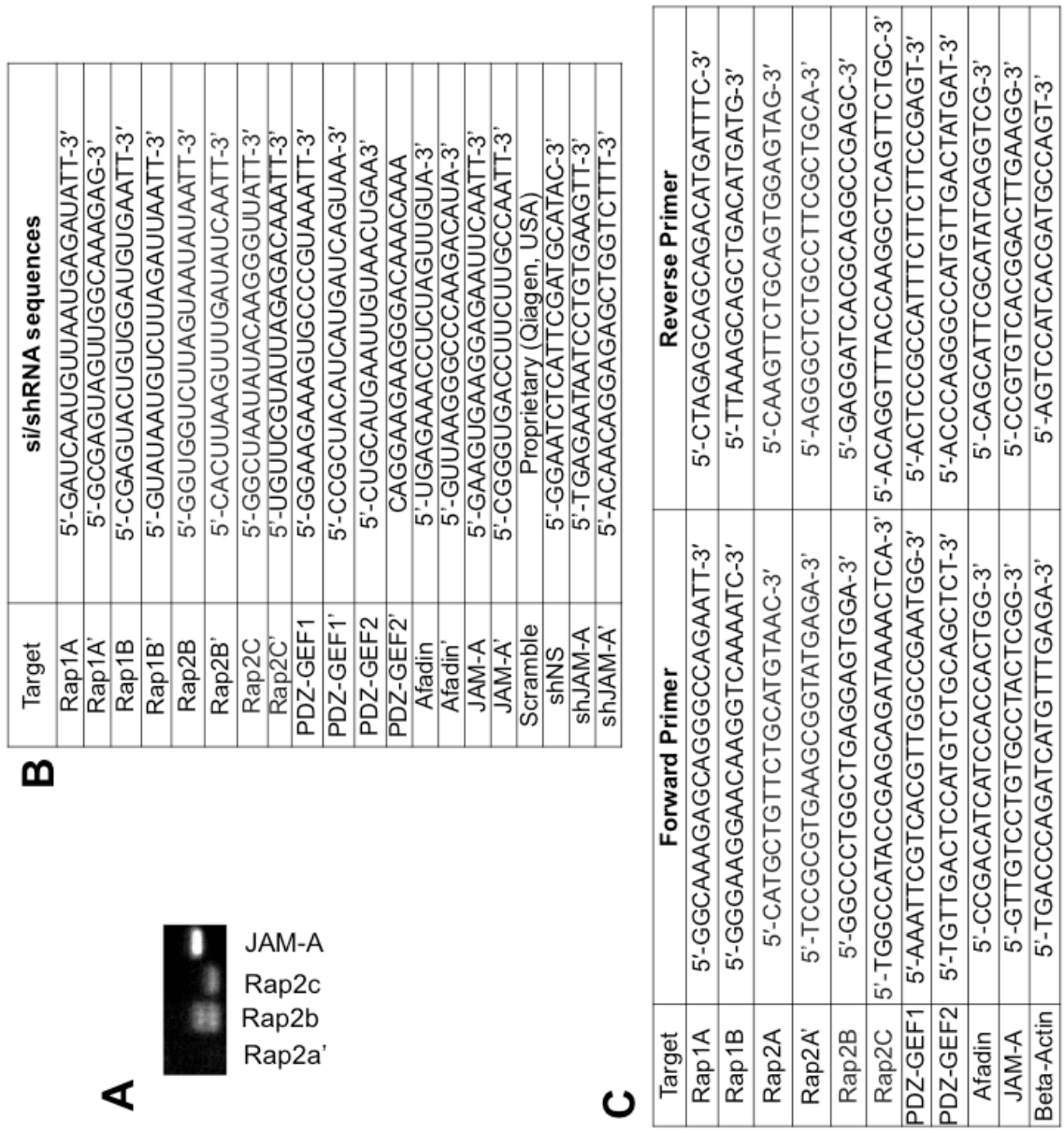


Figure 9.1: Additional PCR demonstrating lack of signal with alternate Rap2a primer pair. A common cDNA template and PCR master mix was prepared and then divided into four tubes containing specific primer pairs (A). Table outlining si/shRNA targets used in this study (B). Table outlining PCR primers used for PCR and qRT-PCR. β-actin was used as a housekeeping gene (C).

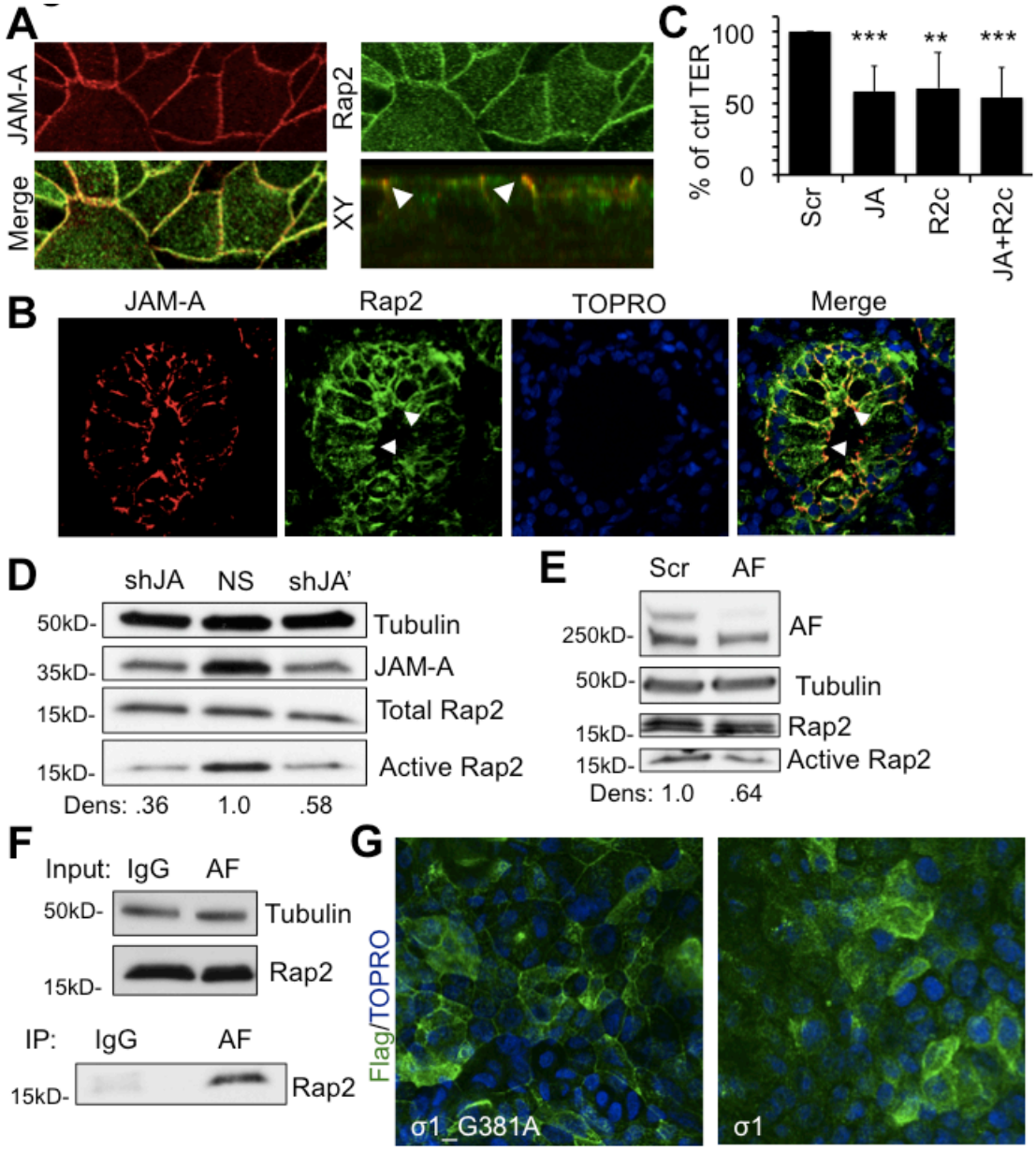


Figure 10: JAM-A regulates Rap2 activity and localization to tight junctions. Rap2 co-localizes with JAM-A (JA) in IECs (A) and in colonic mucosa from human pathology specimens (B). Cells and tissues were pre-treated with 0.1% Triton X-100 before fixation (A, B). Simultaneous downregulation of JAM-A (JA) and Rap2c (R2c) in SK-CO15 cells has no additive effect on TER when compared to isolated downregulation of JAM-A (JA) or Rap2c (R2c). (C, n=3. Mean relative resistance with 95% confidence interval). JAM-A (JA) deficient IECs from two independent JAM-A targets display decreased Rap2 activity when compared to control cells (NS), as assessed by Ral-GDS pull down (D, densitometry calculated as Rap2 pull-down signal over total Rap2 signal, relative to NS control). IECs transiently deficient in afadin (AF) have decreased levels of Rap2 activity compared to control cells (Scr) (E, densitometry calculated as Rap2 pull-down signal over total Rap2 signal, relative to Scr control). Rap2 co-immunoprecipitates with afadin from cell lysates solubilized with a Brij97-based buffer (F). Incubation of confluent SK-CO15 monolayers with σ1 or σ1_G381A (10 µg/ml) for 1 hour leads to decreased Rap2c localization at tight junctions. (G).

Loss of JAM-A increases epithelial paracellular permeability to high molecular weight molecules

Since afadin, ZO-1 and -2 have known actin binding sites (Mandai et al., 1997) and Rap2 activity has been implicated in cytoskeletal regulation in neurons and enterocytes (Ryu et al., 2008; Gloerich et al., 2012), we considered whether JAM-A dependent regulation of barrier function may involve Rap2c-mediated effects on the actin cytoskeleton. We first examined whether loss of JAM-A in vivo and in vitro resulted in barrier defects consistent with cytoskeletal deregulation. While permeability to small molecules is largely dependent on the composition and stability of claudin-forming pores (Nitta et al., 2003), paracellular passage of larger molecules is determined by expansion of the paracellular space secondary to regulation of the apical cytoskeleton (Nusrat et al., 1995; Jou et al., 1998; Nusrat et al., 2000; Shen et al., 2011). Previous studies have shown that JAM-A KO mice have increased intestinal epithelial permeability to small molecules (4kDa Dextran) (Laukoetter et al., 2007), however intestinal permeability to larger solutes, which would indicate a role for cytoskeletal regulation of barrier function, has not been assessed in these animals. We thus investigated the role of JAM-A in the regulation of permeability to large molecules in an intestinal loop model using anesthetized mice (setup illustrated in Fig. 6d). Introduction of 40kDa dextran to the intestinal lumen of JAM-A KO mice demonstrated a six-fold increase in intestinal permeability compared to values obtained in WT animals (Fig. 11a), suggesting a potential role for cytoskeletal regulation of JAM-A dependent barrier function. We also tested whether cell lines with stable JAM-A knockdown had increased permeability to high

molecular weight dextran (40kDa). As shown in figure 11b, we observed that in-vitro flux of 40kDa dextran across monolayers of JAM-A deficient cells was significantly increased compared to non-silenced cells. Given the observed association between ZO-2 and JAM-A and that loss of either protein elicited similar effects on TER, we also assessed the effect of ZO-2 downregulation on permeability to high molecular weight molecules. As shown in Figure 11c, transient downregulation of ZO-2 in IECs led to a significant increase in 40kDa dextran flux relative to scramble siRNA transfected cells, confirming a previous report that ZO-2 also plays a role in regulating permeability of high molecular weight molecules in epithelial cells (Hernandez et al., 2007).

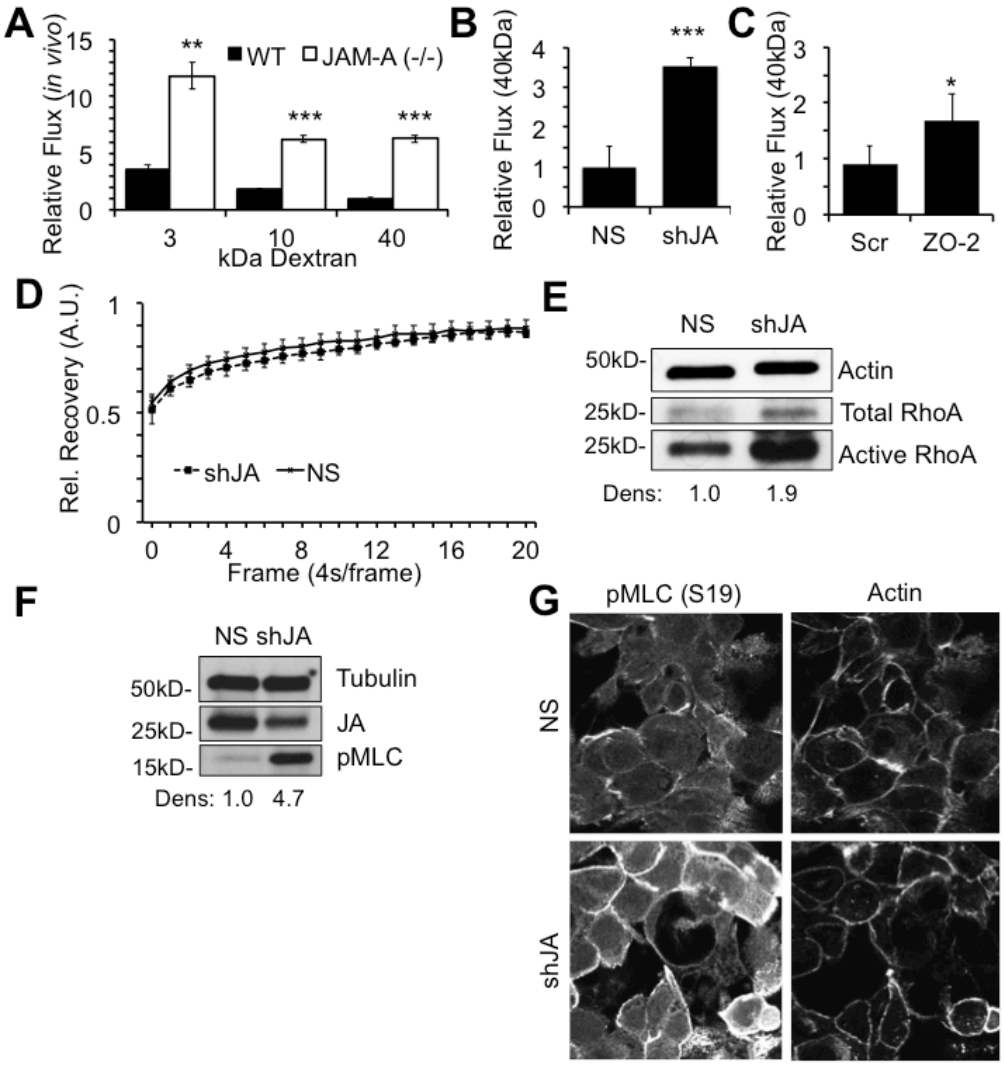


Figure 11: JAM-A downregulation enhances cytoskeletal contraction. JAM-A-/- mice exhibit increased intestinal permeability to 3, 10 and 40kDa dextran when compared to wild-type (WT) mice. (A, n=3. Bars represent mean +/- SEM). JAM-A deficient SK-CO15 cells (shJA) demonstrate enhanced flux to 40kDa dextran compared to control cells (NS). Dextran flux to the bottom chamber was assessed after 2 hours (B, representative experiment with three independent samples. Mean +/- SD). Transient downregulation of ZO-2 in IECs result in enhanced permeability of 40kDa dextran compared to control (Scr). (C, representative experiment with three independent samples. Mean +/- SD). Actin turnover rates in control (NS) or JAM-A deficient (shJA) cells are not statistically different as assessed by fluorescence recovery after photobleaching (FRAP). Stable control (NS) or JAM-A deficient (shJA) cells expressing actin-GFP and grown in chambered wells before assessment of FRAP for actin-GFP at junctions. (D, n=8. Mean +/- SEM). Stable downregulation of JAM-A (shJA) leads to enhanced levels of total and active RhoA as determined by Rhotekin pull-down assay (E, densitometry calculated as RhoA pull-down signal over total RhoA signal, relative to NS control). Stable downregulation of JAM-A (shJA) leads to enhanced levels of pMLC (S19) as determined by western blot (F, densitometry calculated as pMLC signal over tubulin signal, relative to NS control) and confocal immunofluorescence staining (G).

JAM-A downregulation leads to RhoA mediated cell contraction

Given the enhanced permeability to large molecules observed with in vivo and in vitro JAM-A deficiency, we investigated whether JAM-A played a role in the regulation of peri-junctional actin turnover or apical actomyosin contraction. To test whether JAM-A expression plays a role in actin turnover, we examined fluorescence recovery after photobleaching (FRAP) in IECs transfected with actin-GFP. In these experiments, segments of peri-junctional actin-GFP were photobleached by at least 50% in control and JAM-A deficient IECs followed by analysis of the rate of fluorescence recovery. As shown in Figure 11d, FRAP experiments revealed similar rates of recovery between control and JAM-A deficient cells, suggesting that JAM-A does not regulate epithelial permeability by affecting actin turnover. We next asked whether JAM-A loss results in altered actomyosin contractility. Given our findings linking JAM-A dependent barrier function to afadin (Fig. 8) and previous reports linking afadin to regulation of RhoA (Miyata et al., 2009), a small GTPase reported to enhance apical cytoskeleton contraction and increase permeability in IECs and endothelial cells (Nusrat et al., 1995; Hirase et al., 2001), we examined whether JAM-A deficient cells had altered RhoA activity. As shown in Figure 11e, JAM-A deficient cell lines exhibited increased RhoA activity when compared to NS controls. Since RhoA is implicated in regulation of acto-myosin contraction through phosphorylation of non-muscle myosin 2 (pMLC), we examined levels of pMLC in JAM-A deficient IECs by western blot. Compared to NS controls, JAM-A deficient stable cell lines exhibited higher levels of pMLC (Fig. 11f). To confirm that changes in pMLC observed in cell lysates reflected signaling at the level of the cortical acto-

myosin belt, we assessed localization of pMLC in JAM-A deficient stable cell lines by immunofluorescence staining. As shown in figure 11 g, JAM-A deficient IECs exhibited enhanced perijunctional pMLC staining when compared to NS cells. These observations suggest that JAM-A may regulate barrier function through RhoA mediated effects on the contractility of the actomyosin belt, without influencing mobility of peri-junctional actin. From these findings we propose a model highlighting a signaling module downstream of JAM-A that regulates epithelial barrier function (Fig. 12).

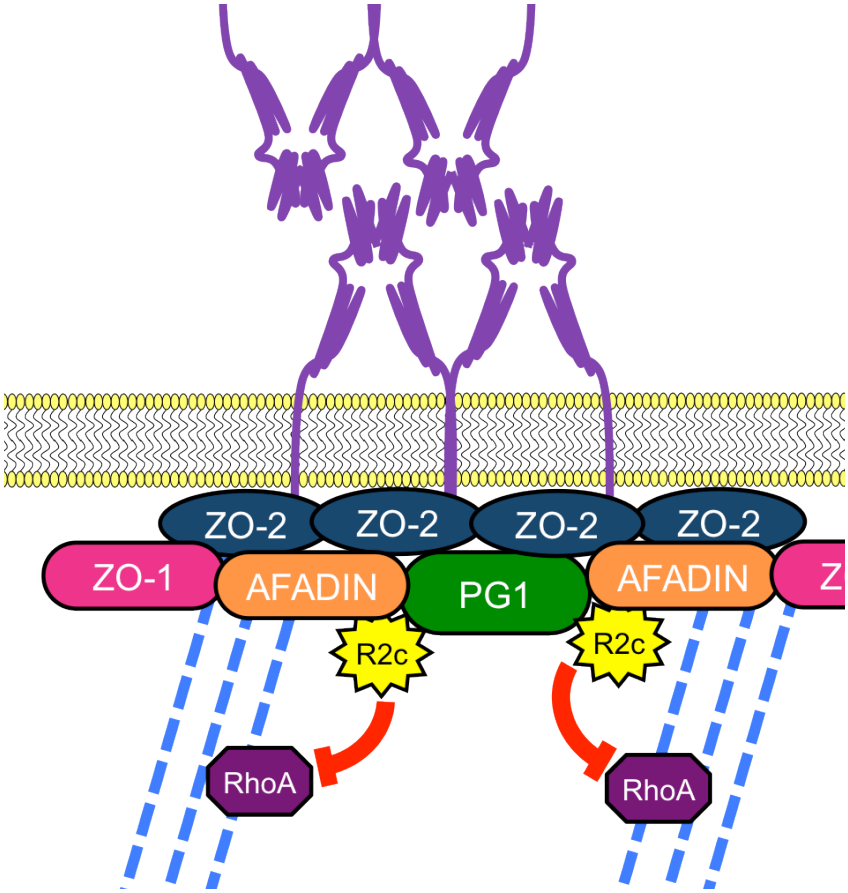


Figure 12: Model of JAM-A function. We propose that JAM-A is part of a complex composed of ZO-2, afadin and PDZ-GEF1 (PG1) that recruits and activates Rap2c (R2c) and controls acto-myosin contraction via RhoA activation to regulate epithelial barrier function.

Discussion

In this study, we provide new mechanistic insights into how JAM-A regulates epithelial barrier function. While previous reports have implicated JAM-A in the control of barrier (Liang et al., 2000; Laukoetter et al., 2007), the signaling pathways linking JAM-A to regulation of epithelial permeability have not been defined. We used a variety of in-vitro and in-vivo approaches to report that JAM-A is part of a complex containing ZO-2, afadin, and PDZ-GEF1 that regulates activation of Rap2c and actomyosin contraction via RhoA.

To identify JAM-A associated scaffold proteins that may play a role in regulating epithelial permeability, we screened a library of PDZ domain containing scaffold proteins for interaction with recombinant cytoplasmic JAM-A. We observed direct binding of the cytoplasmic region of JAM-A to the second PDZ domain of ZO-2, and confirmed this interaction by demonstrating co-immunoprecipitation of ZO-2 with JAM-A from cell lysates derived from polarized human IECs. This is the first report of an association between JAM-A and ZO-2. Interestingly, our results did not demonstrate an interaction between JAM-A and ZO-1, in contrast to earlier reports (Ebnet et al., 2000; Nomme et al., 2011). As can be seen in figure 7 and 7.1, there was robust co-immunoprecipitation of ZO-2 with JAM-A using lysates from two polarized, non-transfected, human IECs (SK-CO15 and T84) under different detergent conditions. Although these findings suggest a direct interaction between JAM-A and ZO-2, given the conserved nature of PDZ-dependent interactions, it is not surprising that ZO-1 has been reported to associate with JAM-A. For example, a recent crystallography study reporting a direct interaction between JAM-A and ZO-1 was

based on experiments using micromolar concentrations of cytoplasmic segments of JAM-A and the third PDZ domain of ZO-1 (Nomme et al., 2011). In comparison, our observations were based on in-vitro interactions between nanomolar concentrations of cytoplasmic JAM-A segments and the second PDZ-domain of ZO-2. Since sequence alignment between the third PDZ domain of ZO-1 and the second PDZ domain of ZO-2 shows greater than 40% identity (Altschul et al., 1997; 2005), it is not unreasonable to expect that a cytoplasmic segment of JAM-A that directly binds to ZO-2 could also associate with ZO-1 at higher concentrations. However, the most plausible explanation for our results using polarized IECs is that JAM-A directly interacts with ZO-2, while indirect interactions between JAM-A and ZO-1 may be mediated through known associations between ZO-1 and ZO-2 (Gumbiner et al., 1991).

We performed further experiments to define JAM-A effectors involved in regulating barrier from insights obtained from previous studies on JAM-A regulation of cell migration. We observed that transient downregulation of afadin but not PDZ-GEF2 or Rap1a/b reduced TER. Although we were not able to show a role for PDZ-GEF2 in barrier maintenance, we observed that loss of the closely related PDZ-GEF1 resulted in enhanced permeability similar to what was observed with JAM-A loss. Since the association with PDZ-GEF1 has not been previously defined, we performed co-immunoprecipitations that demonstrated interaction between JAM-A and PDZ-GEF1. It is noteworthy that a direct interaction between JAM-A and PDZ-GEF1 was not observed in the proteomic PDZ array, even though the PDZ domain of PDZ-GEF1 was probed, suggesting that the association between PDZ-GEF1 and JAM-A is likely indirect and/or transient in nature.

Immunoprecipitation of afadin not only confirmed association between afadin and JAM-A but also revealed an association between afadin and ZO-2. Given the observed interactions between JAM-A and PDZ-GEF1, afadin, and ZO-2, and previously reported interactions of ZO-1 with ZO-2 (Gumbiner et al., 1991) and afadin (Takahashi et al., 1998), we sought to further define the order of association between proteins in this complex. Based on findings from the PDZ array and co-immunoprecipitations indicating a potential direct interaction between JAM-A and ZO-2, we tested whether the association of afadin with JAM-A was dependent on ZO-2 expression. Transient downregulation of ZO-2 followed by afadin immunoprecipitation revealed that IECs deficient in ZO-2 demonstrated decreased association between JAM-A and afadin. This suggested that JAM-A, ZO-2 and afadin were in a complex and the interaction between afadin and JAM-A required the presence of ZO-2. These results, which support an indirect association between afadin and JAM-A are inconsistent with a previous study supporting a direct interaction between afadin and JAM-A (Ebnet et al., 2000). We attribute these diverging interpretations to differences in experimental models employed. The earlier study was largely based on overexpression of proteins in yeast that were confirmed using recombinant protein-based pull-down assays, whereas the results of our proteomic screen were supported through antibody-based analyses of lysates from polarized human IECs containing endogenous levels of JAM-A, ZO-2 and afadin. The former study also demonstrated co-immunoprecipitation of afadin with JAM-A in endothelial cell lysates (Ebnet et al., 2000) which is consistent with our previous observations in lysates of IECs (Severson et al., 2009b), however these co-immunoprecipitation

results do not distinguish between direct or indirect interactions. Considering the present finding that ZO-2 depletion in IECs attenuates association between JAM-A and afadin, our results suggest that JAM-A associates directly with ZO-2 and indirectly with afadin. The above experiments also revealed that association between afadin and ZO-1 was not altered upon ZO-2 depletion, suggesting that ZO-2 is not required for the interaction between afadin and ZO-1. Given these observations and previous reports on ZO-1 interacting partners, we predict that ZO-1 may indirectly associate with JAM-A via interactions with afadin and/or ZO-2.

It was surprising to find that downregulation of Rap1a/b did not enhance epithelial permeability. Rap1 is important in the regulation of endothelial tight junctions (Kooistra et al., 2007; Pannekoek et al., 2011). However, the role of Rap1 in epithelial barrier has not been clearly defined (Chapter 1). While others have shown a role for Rap1 effectors such as EPAC and RAPGAP in the regulation of the apical junctional complex in epithelial cells, such effectors have not been reported to be specific for Rap1, and, in fact, have been observed to modulate Rap2 signaling (Roscioni et al., 2008; De Rooij et al., 1999; Tsygankova et al., 2010). Here we report that Rap2c, a previously uncharacterized GTPase in epithelial cells, mediates JAM-A regulation of epithelial permeability.

Rap2a, but not Rap2c, was recently reported to play an important role in brush border formation in small intestinal enterocytes (Gloerich et al., 2012). Moreover, Rap2 modulation has been shown to be required for ordered formation of neuronal dendritic spines (Ryu et al., 2008). Such reports suggest that Rap2 is important in regulating actin architecture, as has been observed for other members of the Ras

superfamily of GTPases (McLeod et al., 2004; Noda et al., 2010). Interestingly, mRNA for Rap2b and Rap2c but not for Rap2a was present in SKCO-15 cells, which helped to define a role for Rap2c in IECs. We observed that Rap2 co-localizes with JAM-A at the apical junctional complex and that Rap2 activity is dependent on JAM-A expression. In addition, PDZ-GEF1, shown in this report to associate with JAM-A, is an established activator of Rap2 (De Rooij et al., 1999; Kuiperij et al., 2003). We also performed experiments testing whether Rap2 activity was regulated by afadin, another JAM-A associated plaque protein. We found that transient downregulation of afadin led to decreased activity of Rap2 in IECs (Fig. 10c), and, along with co-immunoprecipitation results in figure 8b, these data collectively suggest that JAM-A forms a complex with ZO-2, afadin and PDZ-GEF1 to regulate Rap2 activity, as highlighted in the model proposed in figure 12.

We performed a series of experiments to determine how the proposed signaling complex in figure 12 regulates epithelial permeability. We previously observed decreased TER and increased flux of 4kDa dextran in JAM-A null mice and demonstrated that JAM-A null mice exhibited increased protein levels of claudins 10 and 15 (Laukoetter et al., 2007). In this study, we report that JAM-A deficiency also results in an enhanced permeability to large molecules, suggesting that additional mechanisms, including altered regulation of the actin cytoskeleton, may be important in JAM-A mediated regulation of barrier function. We observed that stable cell lines deficient in JAM-A had enhanced activity of RhoA and increased phosphorylation of myosin light chain. These observations complement previous reports indicating that afadin deficient cells exhibited enhanced RhoA activity (Miyata et al., 2009) and that

epithelial cells deficient in ZO-1/2 revealed enhanced pMLC at the AJC compared to control cells, as assessed by immunofluorescence confocal microscopy of actin and myosin light chain (Fanning et al., 2012).

The findings in this study have significant physiological relevance. Transient and incomplete downregulation of JAM-A, ZO-1/2, afadin, PDZ-GEF1 and rap2c in vitro led to TER decreases of ~ 15-45% compared to control groups. These relatively small in-vitro differences observed in JAM-A deficient cells translated to 3 fold (small molecules) and 6 fold (large molecules) increases in intestinal permeability in JAM-A KO relative to WT mice. Similarly, afadin cKO mice were reported to exhibit a 3-fold increase in permeability to small molecular weight dextran, though permeability of large molecular weight solutes was not reported. While in vivo permeability data is not available for all protein targets that we studied in vitro, given that transient downregulation of JAM-A, afadin, PDZ-GEF1 and Rap2c led to statistically similar effects on TER in vitro, we predict that mice with epithelial-targeted deficiency of PDZ-GEF1 or rap2c may have barrier defects similar to those observed in JAM-A and afadin deficient mice. There are significant physiologic consequences secondary to the permeability defects observed in JAM-A and afadin deficient mice. Mice lacking intestinal epithelial afadin (Tanaka-Okamoto et al., 2011) or JAM-A (Laukoetter et al., 2007) have higher susceptibility to DSS colitis. Moreover, we have also shown that JAM-A deficient mice develop immune-compensatory mechanisms to protect them against the development of spontaneous colitis secondary to enhanced antigen exposure (Khounlotham et al., 2012). Such studies highlight the physiological ramifications of JAM-A and afadin deficiency in vivo.

Collectively, these data therefore support a model where the transmembrane protein JAM-A is part of a complex containing afadin, ZO-1/2 and PDZ-GEF1 that induces activation of the small GTPase Rap2c and, through RhoA, controls contraction of the junction-associated apical cytoskeleton to maintain a functional and selectively permeable epithelial barrier. This model may provide new ideas for therapeutic targets that allow for the modulation of intestinal barrier function in health and disease.

Chapter 3:

JAM-A homodimerizes across cells at a distinct motif from JAM-A cis-dimerization to regulate Rap2 activity

Material from this chapter has been submitted to *Molecular Biology of the Cell*. Contributing authors of this article are: Anny-Claude Luissant, Caroline Lai, Ronen Sumagin, Volker Spindler, Franziska Vielmuth, Oskar Laur, Mattie Wolf, Terrence Dermody, Asma Nusrat, Charles Parkos.

Abstract

Junctional Adhesion Molecule A (JAM-A) is a tight junction associated signaling protein that regulates epithelial cell proliferation, migration and barrier function. JAM-A homodimerization on the same cell surface (in cis) mediates JAM-A dependent signaling. However, there is accumulating evidence suggesting that homophilic interactions between JAM-A molecules on adjacent cells (trans) are necessary for mediating cell-cell contacts. Site directed mutagenesis along with cell-transfection and in-vitro protein interaction studies revealed that JAM-A dimerizes in both cis and trans at distinct sites on the distal-most immunoglobulin-like loop. These experiments also indicate that trans-dimerization is a low affinity interaction that likely requires the avidity supplied by JAM-A cis-dimers for assembly. The role of JAM-A trans-dimerization in cell signaling was also investigated. Cells expressing trans-dimerization null mutants displayed lower Rap2 activity compared to controls, and Rap2 activity was enhanced with cell confluence. These results suggest that JAM-A trans-dimerization may act as a barrier-inducing molecular switch that is activated when cells become confluent.

Introduction

Junctional Adhesion Molecule-A (JAM-A) is a tight junction associated signaling protein that regulates epithelial cell proliferation, migration and barrier function (Nava et al., 2011; Mandell et al., 2005; Monteiro et al., 2013). As a single-span transmembrane protein, JAM-A initiates cytoplasmic signaling mechanisms by associating with scaffold proteins through its C terminal PDZ binding motif (Severson et al., 2008b; Mandell et al., 2005; Monteiro et al., 2013; Ebnet et al., 2000). The N-terminal extracellular segment of JAM-A is composed of two extracellular immunoglobulin (Ig) loops, the distal most of which (D1) mediates dimerization of JAM-A in cis (on the same cell surface), as confirmed by structural and biochemical studies. Interestingly, crystallographic studies on murine JAM-A suggest that JAM-A may also dimerize in a trans conformation (across cells) at a site distinct from that which mediates dimerization in cis (Kostrewa et al., 2001). Additional evidence of JAM-A trans-dimerization comes from a study demonstrating JAM-A dependent adhesion between platelets and endothelial cells (Babinska et al., 2002). Trans interaction between JAM-A protein is also supported by observations from cells overexpressing JAM-A that demonstrate accumulation of JAM-A at contacts between transfected cells (Mandell et al., 2004). Despite the above supportive evidence, crystallographic studies on human JAM-A do not show evidence of trans interactions. In addition to the debate of whether JAM-A dimerizes in trans, the molecular basis and functional consequences of this interaction are not known.

Although definitive molecular evidence for JAM-A trans-homodimerization is lacking, there is substantial evidence documenting dimerization in cis. Mutagenesis studies have defined the charged amino acids arginine 61 and glutamate 63 on the distal most Ig loop as two key components that interact to form a salt bridge that mediates cis-interactions between two JAM-A molecules (Severson et al., 2008b). Expression of JAM-A mutants where arginine 61 and glutamate 63 have been switched to alanines (termed 6163) or where the entire distal most Ig loop has been deleted (termed DL1) demonstrated important functional roles for cis-homodimerization in epithelial cells. JAM-A regulates cell migration and does so through association with the Rap exchange factor PDZ-GEF2 and the scaffold protein afadin to activate the small GTPase Rap1 and subsequently stabilize β1 integrin levels. However, overexpression of cis-null JAM-A mutants 6163 or DL1 in cells leads to decreased β1 integrin levels and inhibited cell migration (Severson et al., 2009b). Finally, cells expressing a JAM-A dimerization null mutant lacking the distal most Ig loop (DL1) exhibit enhanced proliferation compared to cells overexpressing wild-type JAM-A (Nava et al., 2011).

JAM-A cis-dimerization disrupting reagents such as function blocking antibodies and JAM-A binding viral proteins have provided important insights into potential roles of JAM-A dimers in epithelial cell function. For example, an anti-human mAb (J10.4) targeting a motif involving N117 on the D1 Ig loop of JAM-A disrupts JAM-A cis-homodimers and potently inhibit barrier formation after calcium switch, while a mAb that binds to another unrelated motif on the D1 Ig loop (1H2A9) has no effect (Mandell et al., 2004). Treatment of HEK-293T cells with

J10.4 or 1H2A9 prior to in vitro cell migration studies confirmed that JAM-A dimerization was important for epithelial migration (Severson et al., 2009b). Another cis-dimer disrupting reagent, the reoviral attachment protein σ1, when applied to confluent high resistance monolayers of intestinal epithelial cells, acutely disrupts barrier function (Chapter 2).

While previous studies have focused on the functional importance of JAM-A cis-dimerization, the observation of accumulation of exogenous JAM-A at cell contacts (Mandell et al., 2004) suggests that trans-dimerization may also be functionally important. However, expression of a cis-dimerization defective mutant of JAM-A (6163) resulted in diffuse JAM-A accumulation (Mandell et al., 2004), suggesting that cis-dimerization may be required for trans dimerization of JAM-A across cells. Thus, it is feasible that simultaneous cis- and trans- homodimerization of JAM-A form oligomers that mediate signal transduction through clustering of associated proteins into large complexes. Indeed, epithelial permeability and migration studies have suggested that JAM-A dimerization is important in bringing together a large complex of proteins to the apical junctional complex ((Monteiro et al., 2013; Severson et al., 2009b). JAM-A expression is important for bringing afadin and PDZ-GEF2 to close apposition (Severson et al., 2009b), and as mentioned above, overexpression of dimerization-null JAM-A mutants DL1 JAM-A and 6163 JAM-A results in decreased cell migration (Severson et al., 2009b). In epithelial permeability studies of Chapter 2, JAM-A was shown to interact directly with ZO-2 and indirectly with afadin and PDZ-GEF1 to activate Rap2c and regulate the dynamics of apical F-actin. Finally, exposure of confluent monolayers of epithelial

cells to the dimer-disrupting reoviral attachment protein σ1 results in loss of JAM-A at junctions and mislocalization of JAM-A associated proteins away from cell contacts in parallel with enhanced epithelial permeability (Monteiro et al., 2013). Although σ1 disrupts cis-homodimerization of JAM-A (Babinska et al., 2002; Guglielmi et al., 2007), it is not known whether the enhanced epithelial permeability observed after exposure to σ1 is a direct result of disrupting JAM-A cis-dimerization or due to mislocalization of JAM-A away from junctions. However, such findings indicate a potential role for JAM-A dimerization in establishing protein complexes important for signaling events and that further studies are needed to test whether JAM-A cis- and/or trans-dimerization deficient mutants regulate JAM-A effectors implicated in epithelial function. Here we demonstrate that JAM-A trans-dimerization is observed in cells and in solution. Further, we identify putative motifs for trans-dimerization and suggest a functional role for these JAM-A trans dimerization events.

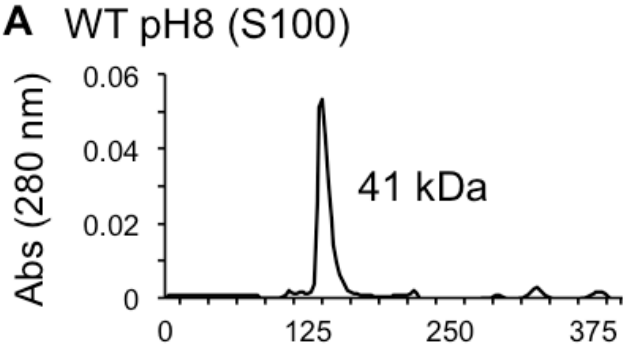
Results

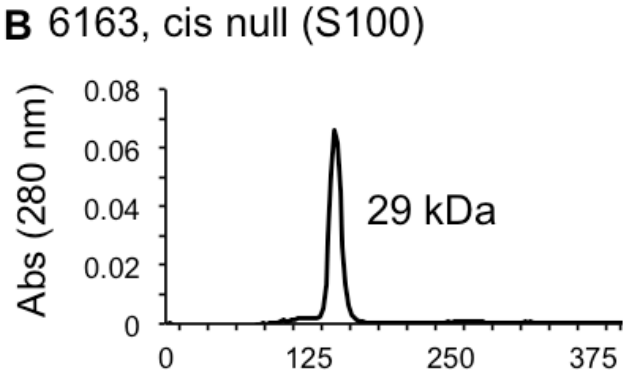
Recombinant soluble JAM-A homodimerizes in a pH-dependent manner

To investigate properties of JAM-A homodimerization, recombinant soluble JAM-A ectodomains comprised of the two extracellular Ig loops were analyzed by size exclusion chromatography to determine stokes' radius. Dimerization was assessed under several different buffer conditions with pH values ranging from 5 to 8. Size exclusion chromatography of soluble, full-length segments of extracellular JAM-A resuspended in Tris-based buffers at pH 8 revealed a prominent elution peak at 41kDa (Fig. 13a). As soluble JAM-A monomers have a predicted size of 25kDa and dimers have a predicted size of 50kDa based on SDS-PAGE analysis, the peak observed in size exclusion chromatography suggested that under native conditions JAM-A is a dimer as previously observed (Guglielmi et al., 2007). To confirm the Stokes' radius of monomeric JAM-A by size exclusion chromatography, we analyzed elution peaks for soluble, extra-cellular segments of a JAM-A mutant lacking the cis-dimerization motif (6163 JAM-A). Size exclusion chromatography of soluble extracellular segments of 6163 JAM-A in Tris buffer at pH 8 revealed a single peak with stokes' radius of 29kDa (Fig. 13b). Together, these results suggest that monomeric JAM-A has a stokes' radius of approximately 29kDa at pH 8 and that soluble, extracellular segments of JAM-A preferentially form dimers under native conditions.

Further experiments were performed to determine the pH dependence of JAM-A dimerization. Soluble extracellular segments of WT JAM-A were resuspended in pH 5 citrate buffer and analyzed by size exclusion chromatography. A single

elution peak at 25kDa (Fig. 13c) was observed, consistent with JAM-A monomers. Elution of JAM-A at pH 5.6 revealed a major peak at 28kDa (Fig. 13c), also consistent with monomeric JAM-A. Size exclusion chromatography of WT JAM-A in pH 6.9 buffer revealed a single peak at 50kDa, suggesting that disruption of JAM-A dimerization occurs between pH 5 and pH 6.9 (Fig. 13c). To ensure that disruption of JAM-A dimerization at low pH was not due to protein denaturation, we performed size exclusion chromatography of soluble extracellular JAM-A that was first incubated at pH 5 or 5.6 followed by neutralization to pH 8. Elution fractions corresponding to monomeric JAM-A that had been resuspended at pH 5 or 5.6 and neutralized to pH 8 before size exclusion chromatography revealed a peak of stokes' radius 45kDa (Fig. 13c), consistent with a dimeric form of JAM-A. These observations suggest that pH dependent disruption of JAM-A dimerization is reversible.





C

	Elution Vol. (ml)	Stokes Radius (kDa)	Status
WT pH8 (S100)	136.3	40.6	Dimer
6163_JAM-A (S100)	147.5	29.3	Monomer
WT pH6.9 (S100)	128.5	49.4	Dimer
WT pH5.6 (S300)	233.6	28.1	Monomer
WT pH5 (S300)	238.4	24.3	Monomer
WT ph5 to 8 (S300)	218.9	43.7	Dimer
WT pH5.6 to 8 (S300)	218.9	43.7	Dimer
DL1_JAM-A (S100)	173.7	17.7	Monomer

Figure 13: JAM-A homodimerizes in a pH dependent manner. Size exclusion chromatography of JAM-A in Tris-Buffered Saline pH 8 reveals an elution peak of stokes radius of 41kDa, suggesting a predominance of compact JAM-A homodimers in solution (A). Cis dimerization-null JAM-A mutants (6163_JAM-A) elute from size exclusion chromatography at a stokes radius of 29kDa, consistent with the predicted size of monomeric JAM-A (B). Size exclusion chromatography values for JAM-A and mutants eluted at different buffer conditions and column size (S100 or S300, as indicated). Elution volume, calculated stokes' radius and dimerization status are indicated. Notably, WT JAM-A eluted at pH 5 reveals a peak of stokes radius of 25kDa, suggesting a predominance of JAM-A monomers at low pH. Size exclusion chromatography of JAM-A eluates from pH 5 that were then neutralized to pH8 revealed a predominance of JAM-A at 45kDa (C).

Identification of JAM-A trans-dimerization sites by site directed mutagenesis

The chromatography studies in figure 13a-c indicate that JAM-A ectodomains form pH-sensitive homodimers, which is consistent with previous studies that have explored the functional role of JAM-A cis-dimerization. However, crystallographic analyses have raised the possibility of JAM-A trans-dimerization at a site distinct from where cis-dimerization occurs. Specifically, these studies identified a potential site for trans-dimerization on the protein surface opposite from where cis-dimerization occurs on the distal most Ig loop of JAM-A.

As the size exclusion chromatography studies highlighted above in panels A-C of figure 13 did not reveal any JAM-A complexes larger than dimers, we considered the possibility that trans-dimerization is a low affinity event. To further investigate whether JAM-A homodimerizes across cells (in trans), and to identify sites of JAM-A trans-dimerization, we performed mutagenesis of full length JAM-A followed by subcellular localization studies. Two novel constructs were designed containing alanine substitutions to a putative trans-dimerization region located on the D1 loop opposite from the cis dimerization motif (Fig. 14a), as predicted from the crystal structure of murine JAM-A and platelet adhesion studies (babinska et al., 2002; Kostrewa et al., 2001). The two putative trans-dimerization null mutants have alterations that correspond to distinct motifs on the distal most Ig loop, which combine to form a three-dimensional groove that would be a favorable site for trans-dimerization mediated by non-ionic interaction forces (Fig. 14 b). The NNP mutant protein contains alanine substitutions of two polar (glutamine 43, glutamine 44) and one non-polar (proline 45) residue of the predicted trans-dimerization

motif (Fig 14c). The KSV mutant protein contains alanine substitutions of one positively charged (lysine 97), one polar (serine 98) and one non-polar (valine 99) residue (Fig. 14d).

JAM-A trans-dimerization was assessed by overexpressing full length WT (Fig. 15a) and the mutant JAM-A constructs (Fig. 15b-e) in chinese hamster ovary (CHO) cells, which have no detectable levels of endogenous JAM-A. By immunofluorescence labeling, we evaluated whether the JAM-A mutant proteins accumulated at intercellular contacts. Surface expression of each of the mutants was confirmed by flow cytometry. Indeed, three independent transient transfections of CHO cells with plasmids encoding WT or mutant JAM-A led to similar levels of JAM-A surface expression as assessed by flow cytometry (Fig. 15.1). For confocal microscopy, transfected CHO cells were co-stained with a rabbit polyclonal antibody against the JAM-A cytoplasmic segment as well as with a murine monoclonal antibody against a three-dimensional epitope on the distal most Ig loop of JAM-A (J10.4, (Mandell et al., 2004; Severson et al., 2009b). Predictably, all JAM-A mutant proteins were detectable with the mAb J10.4 with the exception of DLI JAM-A, which lacks the distal most Ig loop and as such was only detectable with the pAb against the cytoplasmic segment of JAM-A. Consistent with previous observations (Mandell et al., 2004), we found that exogenous WT JAM-A accumulated at junctions between JAM-A expressing CHO cells (Fig. 15f, highlighted with arrows). However, DL1 JAM-A, which lacks the distal most Ig loop, did not distribute to junctions between JAM-A expressing cells, and instead dispersed around the cell surface in a uniform, diffuse fashion (Fig. 15g, arrowheads). This observed pattern of distribution suggested that

motifs located at the distal most Ig loop are important for concentration of JAM-A between cells. Interestingly, NNP and KSV JAM-A also distributed diffusedly on the surface of CHO cells and not at cell-cell junctions between transfected cells (Fig. 15h-i, arrowheads). Consistent with previous observations (Mandell et al., 2004), the JAM-A mutant lacking the 61st and 63rd amino acids involved in cis-dimerization (6163 JAM-A) similarly localized in a diffuse pattern around cells (Fig. 15j). Quantification of the frequency of JAM-A accumulation at contacts, calculated as the percentage of cells expressing JAM-A predominantly at junctions between two transfected cells, relative to the total number of cells expressing JAM-A, revealed that 46% (+/- 4% SEM) of cells expressing WT JAM-A displayed localization of JAM-A to contacts between JAM- expressing cells. In contrast, analyses of cells expressing DL1, 6163, NNP and KSV mutants revealed significantly reduced JAM-A distribution to cell contacts, at a frequency of 16% (+/- 4% SEM), 30% (+/- 5% SEM), 22% (+/- 3% SEM) and 25% (+/- 2% SEM), respectively (Fig. 15k). To ascertain that the results in figure 15 represented loss-of-function effects specific to the residues that had been altered, and that mutagenesis in itself was not non-specifically affecting the localization of JAM-A, we also quantified the rate of JAM-A targeting to cell contacts of cells expressing a JAM-A mutant protein containing cysteine substitutions at residues lysine 72 and serine 112 on the distal most motif. Importantly, this JAM-A mutant accumulated at junctions at a similar frequency as observed for WT JAM-A (Fig. 15k and 15.2), indicating that mutations of distinct residues in JAM-A dimerization regions specifically affect the accumulation of JAM-A

at cell contacts. These findings indicate that the motifs altered by 6163, NNP and KSV mutations are important for the interaction of JAM-A across cells.

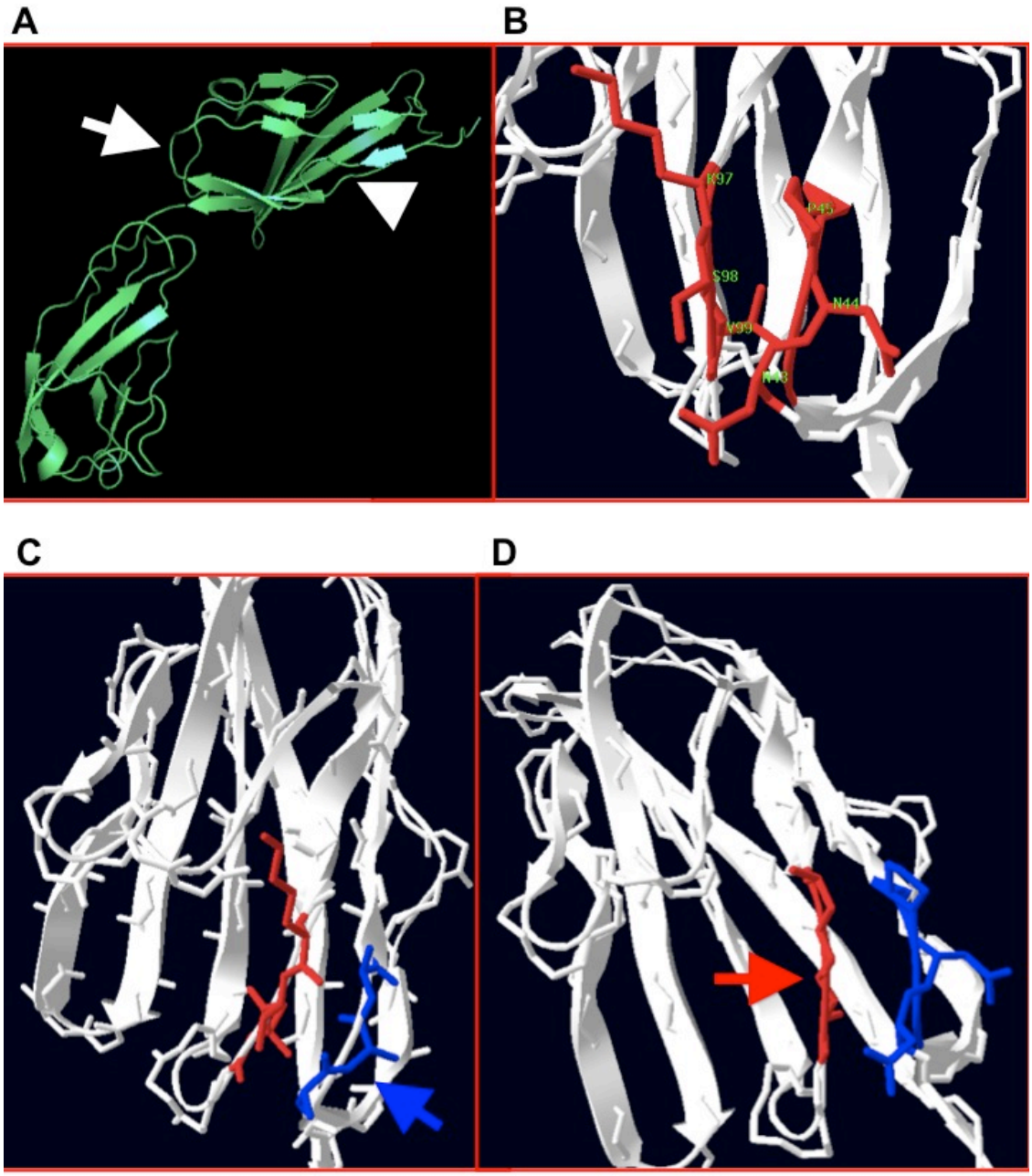
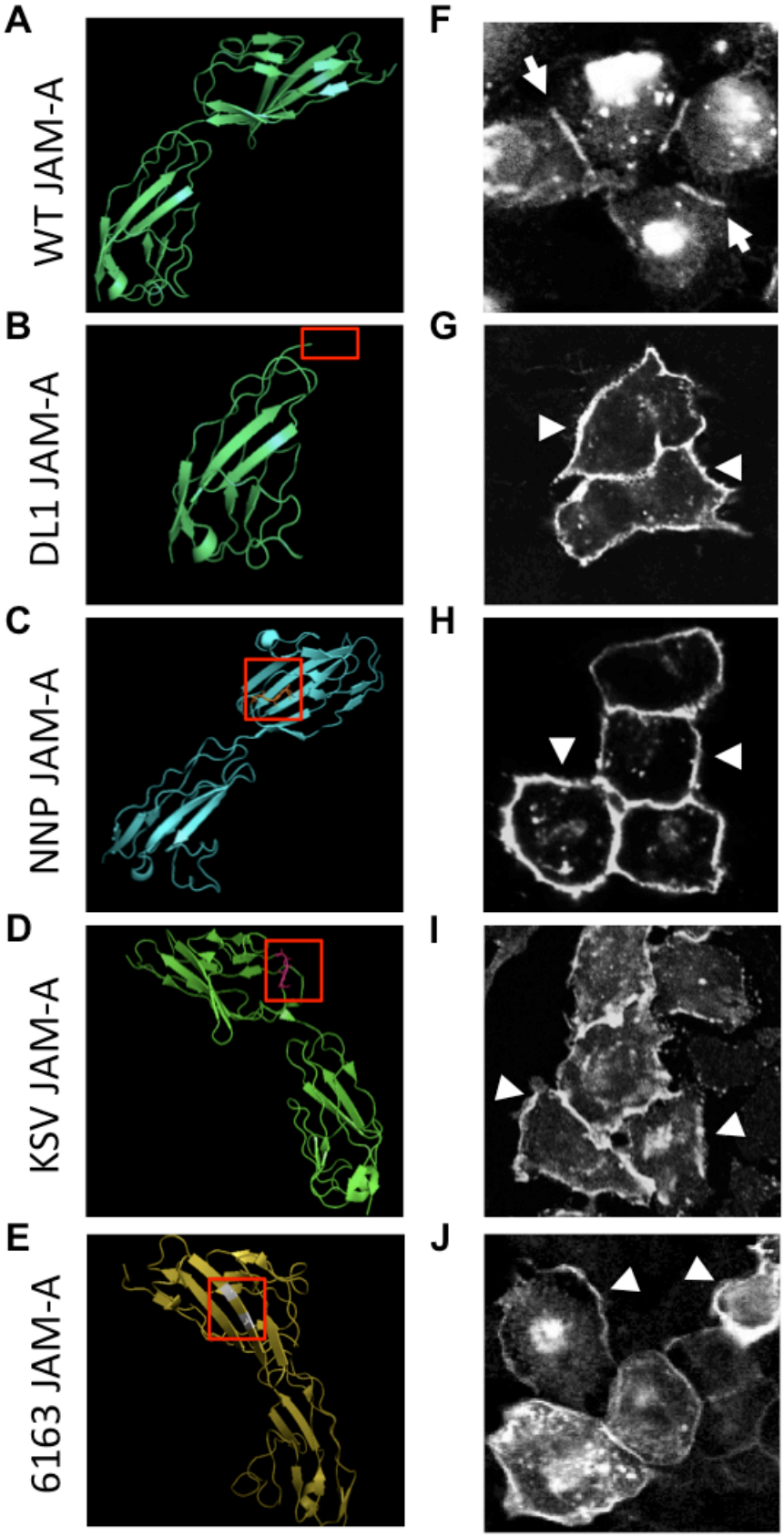


Figure 14: Two novel JAM-A mutants have null mutations to the putative trans-dimerization interface. JAM-A cis-dimerization occurs by ionic interaction between residues located on the second beta-pleated sheet of the D1 loop (arrowhead, A) while trans-dimerization is predicted to occur on the opposite face of the D1 loop (arrow, A). The predicted site for trans-dimerization involves two loops composed of residues N43-P45 and K97-V99, which combine to form a three-dimensional groove for low affinity interactions (B). The NNP mutant contains alanine substitutions to amino acids N43-P45 (blue arrow, C) and the KSV mutant contains alanine substitutions to amino acids K97-V99 (red arrow, D).


A
WT JAM-A
B
DL1 JAM-A
C
NNP JAM-A
D
KSV JAM-A
E
6163 JAM-A
F
G
H
I
J

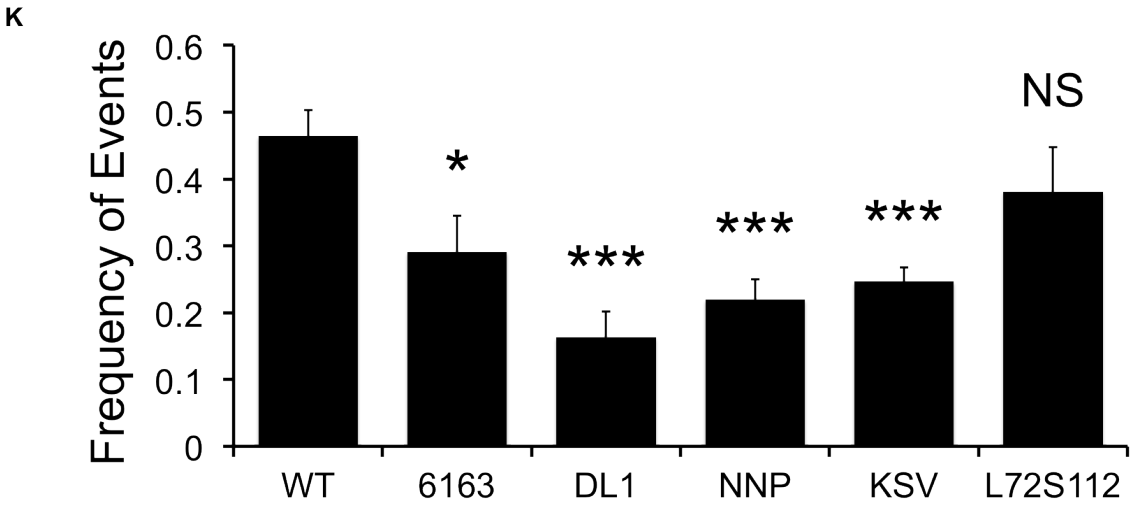


Figure 15: JAM-A mutagenesis reveals sites of trans-dimerization. JAM-A cis- and predicted trans- mutants were created utilizing site-directed mutagenesis (A-E). WT JAM-A is composed of two extracellular Ig loops, the distal most of which is involved in JAM-A homodimerization (A). DL1 JAM-A lacks the entire membrane-distal Ig loop and will be utilized as a negative control (B). NNP JAM-A has alanine substitutions to amino acids 43N, 44N and 45P, which are predicted to be involved in transdimerization (C). KSV JAM-A has alanine substitutions to amino acids 96K, 97S and 99V, which compose the second motif predicted to be involved in trans-dimerization (D). 6163 JAM-A has alanine substitutions to amino acids 61E and 63K, previously implicated in JAM-A cis-dimerization (E). CHO cells were transfected with WT and mutant JAM-A and fixed for immunofluorescence staining and confocal microscopy after 48 hours. Subcellular localization of exogenously expressed mutants elucidate sites on JAM-A important for trans-dimerization (F-J). WT JAM-A localizes primarily to contacts between JAM-A expressing cells (arrows) (F). DL1 JAM-A, however, does not accumulate at junctions and instead disperses diffusely around the cytoplasmic membrane, as highlighted by arrowheads (G). NNP and KSV JAM-A, like DL1 JAM-A, distribute diffusely along the cell surface (H-I). 6163 distributes as previously described, also diffusely distributing along the cell surface of CHO cells (J). Distribution of WT and mutant JAM-A expressed in CHO cells was quantified as ratio of cells with junction-localized JAM-A between JAM-A positive cells to total number of JAM-A positive cells per field. A JAM-A isoform with functionally irrelevant cysteine substitutions at Leu72 and Ser112 ("L72S112") distributed to junctions at a statistically similar rate to WT JAM-A (n>8 fields, collected >3 different transfections; mean +/- SEM; ***,$p<0.001$, *, $p<0.05$, NS, $p>0.2$) (K).

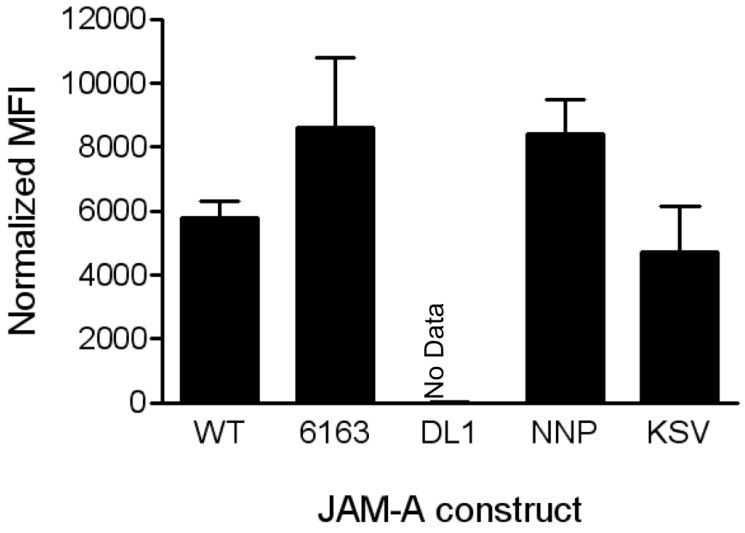


Figure 15.1: CHO cells were transfected with the JAM-A mutant plasmids shown. JAM-A expression was determined 48 h post-transfection. For surface expression analysis of CHO cells, cells were harvested and stained with a JAM-A-specific monoclonal antibody against the distal most Ig loop (J10.4). Mean fluorescence intensity of each sample was quantified using flow cytometry.

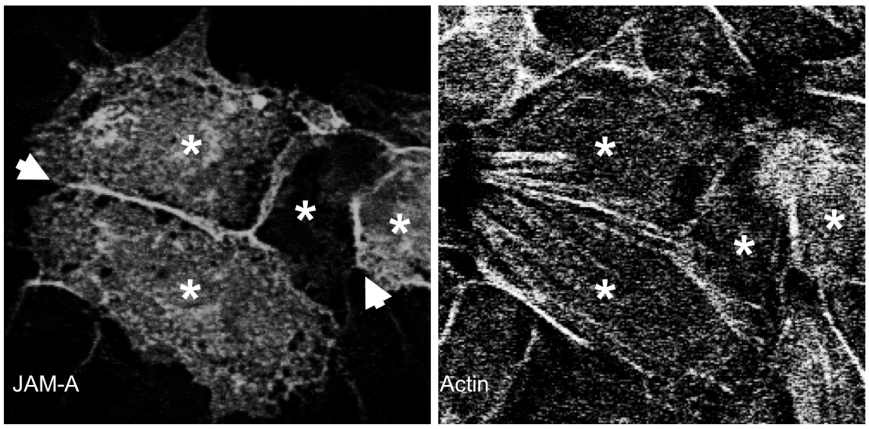


Figure 15.2: JAM-A mutant containing cysteine substitutions to Leu72 and Ser112 localizes to cell-cell contacts. CHO cells were transfected with a JAM-A mutant construct with point substitutions L72C and S112C then fixed for immunofluorescence labeling and confocal microscopy after 48 hours. L72C_S112C JAM-A localized to cell contacts between transfected cells. Phalloidin was utilized as a counter-stain to demonstrate surrounding untransfected cells.

Although analysis of the subcellular localization of WT and mutant JAM-A in figure 15 suggested that both cis and putative trans motifs are important for localization of JAM-A at cell-cell junctions, we performed further experiments to ascertain that mutation of the putative trans-dimerization motifs did not globally affect the tertiary structure of JAM-A. Recognition by mAb J10.4, which is immunoreactive to a three dimensional epitope on the distal Ig loop of JAM-A (Mandell et al., 2004), was retained in all JAM-A mutants with the exception of DLI_JAM-A, suggesting that different mutations did not globally affect the tertiary structure of JAM-A. We further assessed the mutant proteins for functional effects on reovirus infection, which is dependent on JAM-A binding events (Guglielmi et al., 2007; Antar et al., 2009). Specifically, the cis-dimerization interface of JAM-A mediates reoviral attachment and infection (Kirchner et al., 2008; Antar et al., 2009). Having confirmed expression of each of the mutants by western blot (Fig. 16a) as well as surface expression by flow cytometry (Fig. 15.1), we exposed CHO cells expressing 6163 or DL1 JAM-A to reovirus and observed that infection rates were significantly less than that observed in CHO cells expressing WT JAM-A (Fig. 16b) which is consistent with previous reports. Importantly, reovirus infection was not significantly different between CHO cells expressing WT or the trans-null mutants KSV or NNP (Fig. 16c). Taken together, the above results suggest that the alanine substitutions at the putative trans-dimerization interface did not globally alter the tertiary structure of JAM-A.

Immunofluorescence staining and confocal analysis of CHO cells overexpressing WT or mutant JAM-A suggested that cis- and trans-dimerization of

JAM-A is necessary for accumulation of JAM-A at cell-cell contacts. We consequently asked whether these findings could be substantiated in cell free systems where JAM-A dimerization could be analyzed in a manner that is independent of other cellular proteins. To do this, we utilized an in vitro bead-based clustering assay. Soluble, c-terminally his-tagged extracellular segments of each of the JAM-A mutants were generated by bacterial expression and protein purification. Purity of mutant proteins was confirmed by SDS-PAGE and protein stain (Fig. 17a). Soluble extracellular segments of His-tagged WT or mutant JAM-A were functionalized to 1μm nickel coated beads, allowing for controlled orientation of JAM-A with the distal-most, N-terminal Ig loop (D1) facing away from the beads. Using these JAM-A coated beads, trans-dimerization events would be expected to result in enhanced clustering compared to control uncoated beads as is observed by immunofluorescence labeling and confocal imaging in figure 17b. Clustering events were quantified by flow cytometric determination of forward and side scatter. Flow cytometric analysis of uncoated beads revealed that 70% of beads were not clustered, suggesting that uncoated beads non-specifically interact (cluster) 30% of the time. Interestingly, beads decorated with his-tagged WT JAM-A formed higher order clusters at a significantly higher rate than beads alone (48% vs. 30%, or 18% difference, $p < 0.001$, Fig. 17c). Intriguingly, His-tagged cis-dimer null mutant (6163) JAM-A coated beads did not cluster differently than observed with beads coated with WT JAM-A (54% vs. 30%, or 24% above beads alone, $p > 0.3$ when compared to WT), suggesting that the cis-dimerization motif is not required for trans interactions in vitro (Fig. 17c). Importantly, beads decorated with his-tagged NNP, one of the

trans-null mutants, failed to cluster with values similar to empty control beads (<1% above beads alone, $p>0.7$, $p<0.001$ when compared to WT), suggesting that the NNP residues are critical for JAM-A dependent bead clustering. Interestingly, beads loaded with the NNP mutant had lower rates of clustering than beads loaded with the KSV mutant (<1% vs. >13%, $p>0.05$). Clustering of beads loaded with the KSV mutant was also lower than than that observed for beads loaded with WT JAM-A; however, this difference was not statistically different (13% vs 18% of clustering above background). These observations indicate that although both the NNP and KSV residues are important for trans dimerization in the bead clustering assay, it is likely that the NNP residues play a more prominent role in JAM-A trans-dimerization in vitro than the KSV residues. To ensure that clustering effects were not caused by differential loading of proteins onto beads, flow cytometry was used to confirm that the total protein content of beads conjugated with NNP, KSV of WT JAM-A was not different (Fig. 17.1). As the 6163 mutation did not affect trans dimerization in the bead clustering assays (Fig. 17) and cis dimerization motif-dependent reovirus infection experiments in figure 17 showed no effect of NNP and KSV mutations on reovirus entry, these findings strongly suggest that trans- and cis-dimerization are mediated by distinct motifs that act independently of one another.

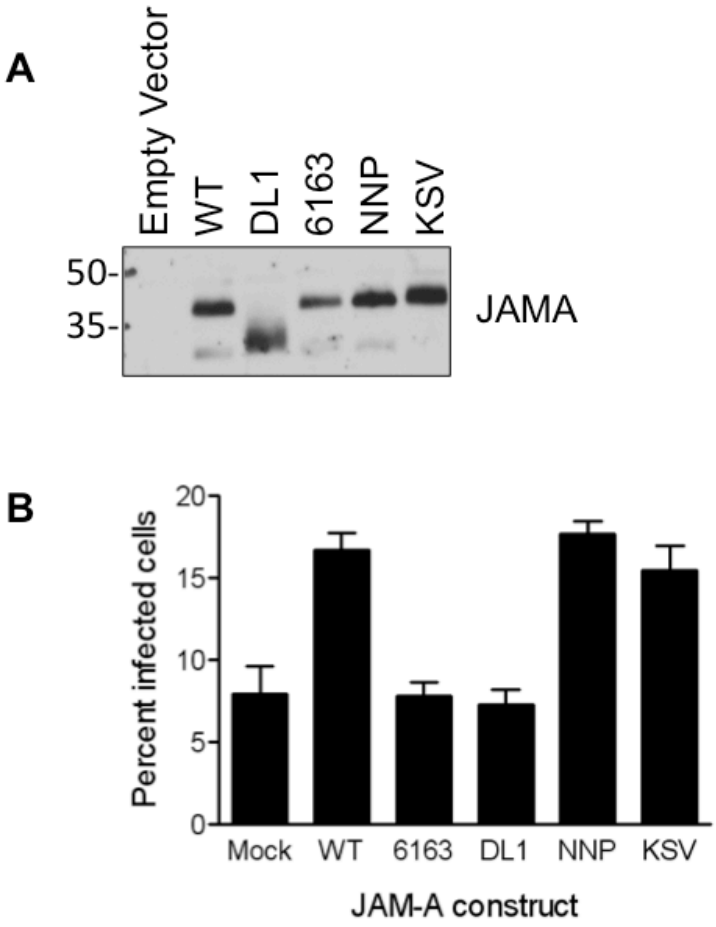


Figure 16: Alteration of JAM-A residues does not affect reovirus infectivity. CHO cells were transfected with the JAM-A mutant plasmids shown. JAM-A expression and reovirus infectivity in these cells was determined 48 h post-transfection. (A) For expression analysis, cells were harvested in RIPA buffer and stained with rabbit pAb against the cytoplasmic, C-terminal segment by immunoblot. (B) For infectivity assessment, CHO cells were adsorbed with T3 reovirus at an MOI of 100 PFU per cell at 37°C for 1 h, washed twice, and incubated at 37°C in fresh medium. After 20-24 h incubation, cells were harvested and stained with Alexa Fluor-conjugated reovirus-specific antiserum. The percentage of infected cells was quantified using flow cytometry. (N=3 independent experiments; mean +/- SEM)

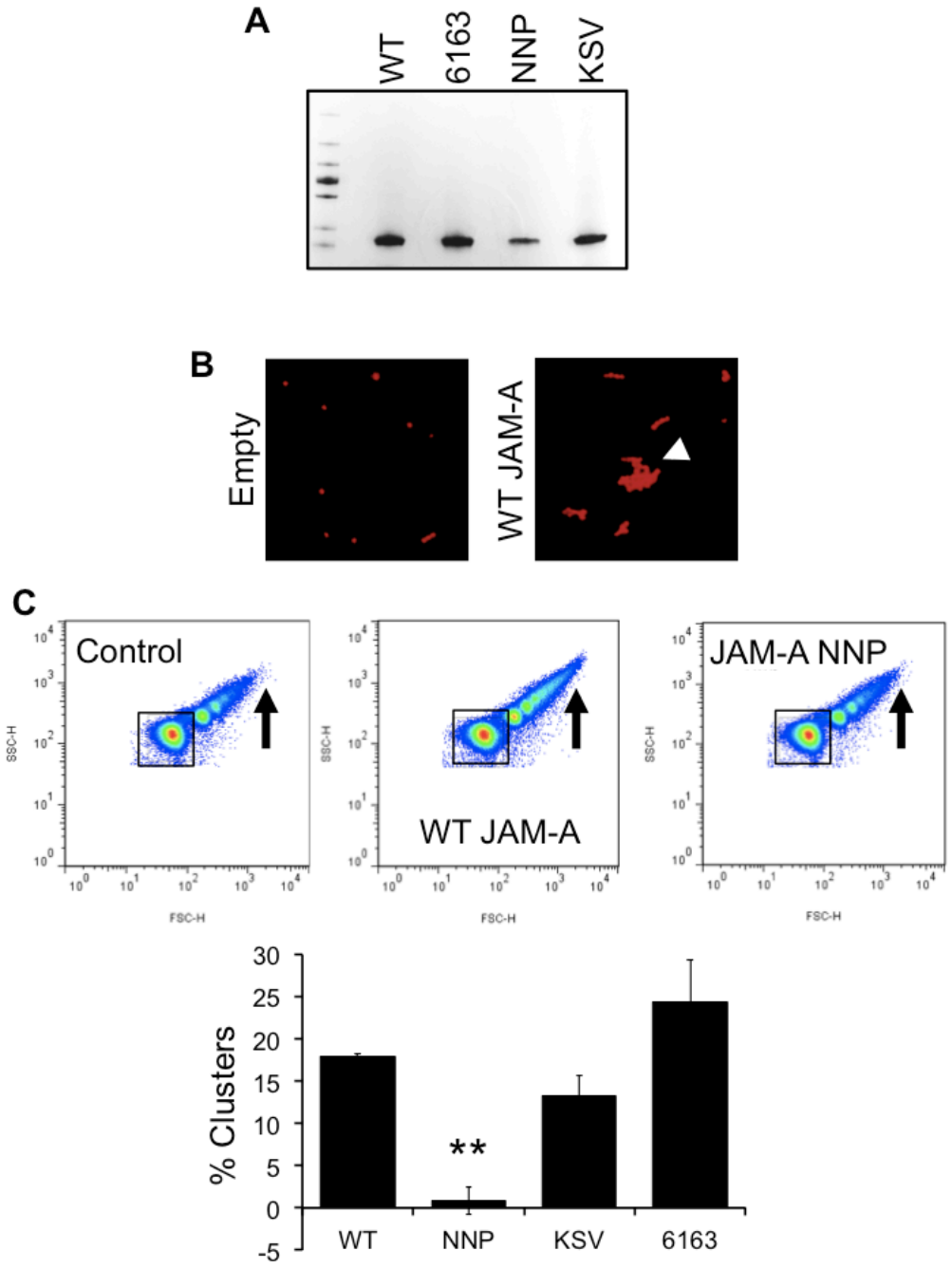


Figure 17: JAM-A conjugated beads cluster as determined by flow cytometry. (A) Bacterially expressed recombinant ectodomains of WT and mutant JAM-A with a c-terminal his tag were purified and analyzed by SDS-PAGE and protein stain. (B) His binding 1µm beads conjugated to his-tagged WT JAM-A clustered in solution (arrowhead) compared to unconjugated beads. (C) Bead clustering was quantified by assessment of side (SSC) and forward scatter (FSC) on flow cytometry, whereby frequency of unclustered beads (boxed) was measured as a function of total beads observed. Flow cytometry data were pooled and frequency of clustering was calculated by deducting frequency of unclustered beads from 100%. Baseline clustering observed in unconjugated beads (frequency= aprox. 30%) was considered to be background and was subtracted from the final readings observed in WT, NNP, KSV and 6163 JAM-A conjugated beads. (n>3 per group, mean +/- SEM. **, $p<0.01$ compared to WT).

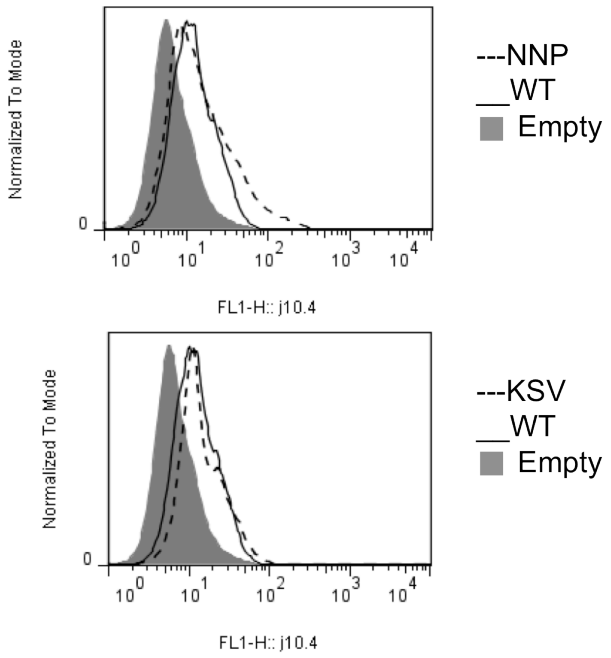


Figure 17.1: WT and Trans-null mutant JAM-A bind to beads with similar efficiency. Beads conjugated with WT, NNP and KSV JAM-A were stained with anti-JAM-A mAb J10.4 and assessed for immunofluorescence by flow cytometry. Beads conjugated with either WT or mutant JAM-A displayed similar fluorescence profiles.

Atomic Force Microscopy defines dimerization properties of JAM-A.

The above experiments with cell and protein-based approaches helped to identify and distinguish motifs that mediate trans-dimerization of JAM-A. We sought to further characterize the biophysical profile of JAM-A homodimerization at the single-molecule level by performing atomic force microscopy (AFM). Soluble his-tagged extracellular segments of WT or mutant JAM-A proteins were functionalized to the AFM tip and substrate using amide linkage reactions. Amide linkage allowed for JAM-A immobilization in parallel and anti-parallel conformations that enabled both cis- and trans-dimerization events. By utilizing the spring characteristics of the AFM lever, force profiles were derived from application of Hooke's law. Force of binding between WT or mutant JAM-A homodimers was deduced from calculating unbinding force required to disrupt JAM-A interactions observed at different spring loading rates. Assessment of average binding force observed for all binding events at a particular loading rate revealed that WT JAM-A homodimerizes with greater force with higher loading rates, as has been observed for other junction associated proteins (Spindler et al., 2009; Baumgartner et al., 2000; Vedula et al., 2008; Zhang et al., 2010). At a loading rate of 5μm/s, homodimerization of WT JAM-A ectodomains was on average 133pN (Fig. 18). In comparison, at a loading rate of 5μm/s, force of interacting cis-null 6163 JAM-A was on average 110pN. Finally, ectodomains of trans null NNP JAM-A displayed an average interaction force of 116 pN at the same loading rate. These data suggest that at the level of the single molecule, cis null (6163) and trans null (NNP) JAM-A mutants interact in a significantly weaker ($p < 0.001$) fashion. Frequency of binding events between WT

or mutant JAM-A ectodomains was also assessed at each loading rate. Binding frequency analysis (data not shown) revealed that the cis null mutant (6163 JAM-A) displayed significantly less frequent homodimerization events compared to WT JAM-A. NNP JAM-A, which mediates trans-dimerization as determined by bead clustering assays, (Fig. 17) also showed lower binding frequencies compared to WT JAM-A, although frequency differences were not significant (data not shown). Pre-treatment of WT or NNP JAM-A coated surfaces with J10.4 Fab' fragment reduced binding events by 80%. These findings suggest that cis-dimerization occurs at higher detectable frequencies with AFM, but that trans-dimerization could also be observed as additional, less frequent events. These results are consistent with previous reports that cis-dimerization involves ionic interactions between charged residues, and support our own predictions that trans-interactions occur through low affinity non-ionic bonding between residues of the NNP motif. Interestingly, disruption of JAM-A homodimerization events after treatment with J10.4 Fab' fragments, which bind at residues surrounding glutamine 117 (Mandell et al., 2005), suggest that J10.4 antibodies indeed disrupt JAM-A cis dimerization, as has been previously reported (Severson et al., 2008b), but may also disrupt trans-dimerization due to steric hindrance. In sum, these AFM observations are consistent with previous conclusions that the NNP motif is important for JAM-A dimerization, however the previously characterized cis-dimerization interface is most prominently detected in conditions where both cis- and trans- dimerization events are possible. This supports a model where higher affinity cis-dimerization events

may supply the necessary avidity for JAM-A trans-dimerization, and as oligomers are formed, trans-dimerization events become more favorable.

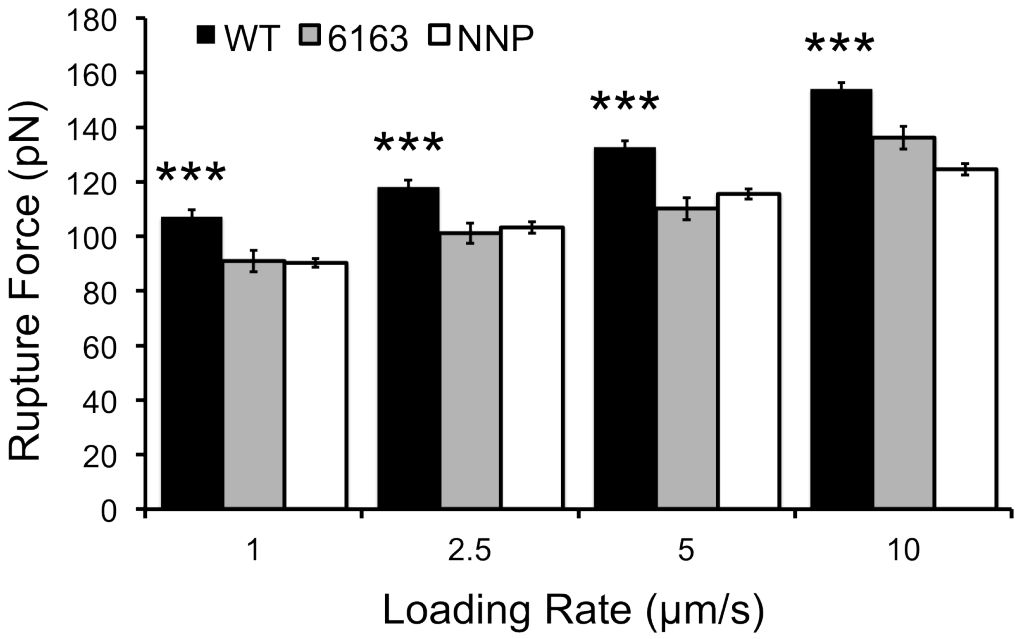


Figure 18: Atomic force microscopy reveals that JAM-A cis and trans-dimerization occur at the single molecule level. AFM tip and substrate were functionalized with WT, 6163 (cis-null) or NNP (putatively trans null) JAM-A by amide linkages and assessed for force required for rupture of homodimerizing ectodomains at different loading rates. (>500 force curves/condition, n>3).

Identification of JAM-A trans-dimerization dependent cell signaling events.

We considered whether trans-dimerization of JAM-A was important in the regulation of barrier function. As cis dimerization is necessary for JAM-A dependent regulation of cellular migration (Severson et al., 2009b). In chapter 2, we demonstrated that JAM-A interacts with a scaffold protein complex important for regulation of barrier function; however, it is not known whether this regulation of barrier function is dependent on dimerization of JAM-A. Given our results indicating that specific regions on the D1 domain of JAM-A mediate dimerization between cells in trans, we transiently transfected full length WT and dimerization mutant JAM-A in HEK-293T cells and compared the effects of disrupting cis and trans dimerization on JAM-A regulation of barrier function. As we had previously shown that loss of JAM-A in epithelial cells resulted in enhanced permeability linked to decreased Rap2 activity (Monteiro et al., 2013), we assessed whether Rap2 activity was altered with the expression of WT and mutant JAM-A. Intriguingly, HEK-293T cells over-expressing WT JAM-A demonstrated enhanced activity of Rap2 (Fig. 19a), which complements previously reported observations of decreased Rap2 activity in JAM-A deficient cell lines. Further, HEK-293T cells expressing the trans dimerization null mutants NNP and KSV JAM-A displayed lower Rap2 levels and activity compared to WT JAM-A transfected cells. Total Rap2 activity, assessed as the signal of active Rap2 standardized to the tubulin loading control, suggested that trans-dimerization is important in the activation of Rap2 (Fig 19a). In contrast, lysates of HEK-293T cells overexpressing 6163 JAM-A, which exclusively lacks the cis-dimerization motif, did not display reduced activity of Rap2 compared to HEK-293T cells expressing WT

JAM-A. These results suggest that JAM-A trans-, but not cis-, dimerization is important for enhancing Rap2 activity in cells. As JAM-A trans-dimerization events are, by definition, dependent on contacts between adjacent cells, we tested whether Rap2 activity was increased in confluent cell monolayers. As seen in figure 19b, Rap2 activity was almost 2 fold higher in confluent monolayers of epithelial cells compared to spreading epithelial cells.

Collectively, the findings presented suggest that trans dimerization occurs at a site distinct from that which mediates dimerization in cis and, despite being a lower affinity binding event, dimerization in trans mediates specific signaling events that regulate activation of Rap2. As illustrated in the model in figure 20, we propose that JAM-A on the surface of sub-confluent single cells do not activate barrier-inducing signals. However, JAM-A on confluent cells trans-dimerize to form JAM-A multimers, important for inducting signals that mediate barrier-function.

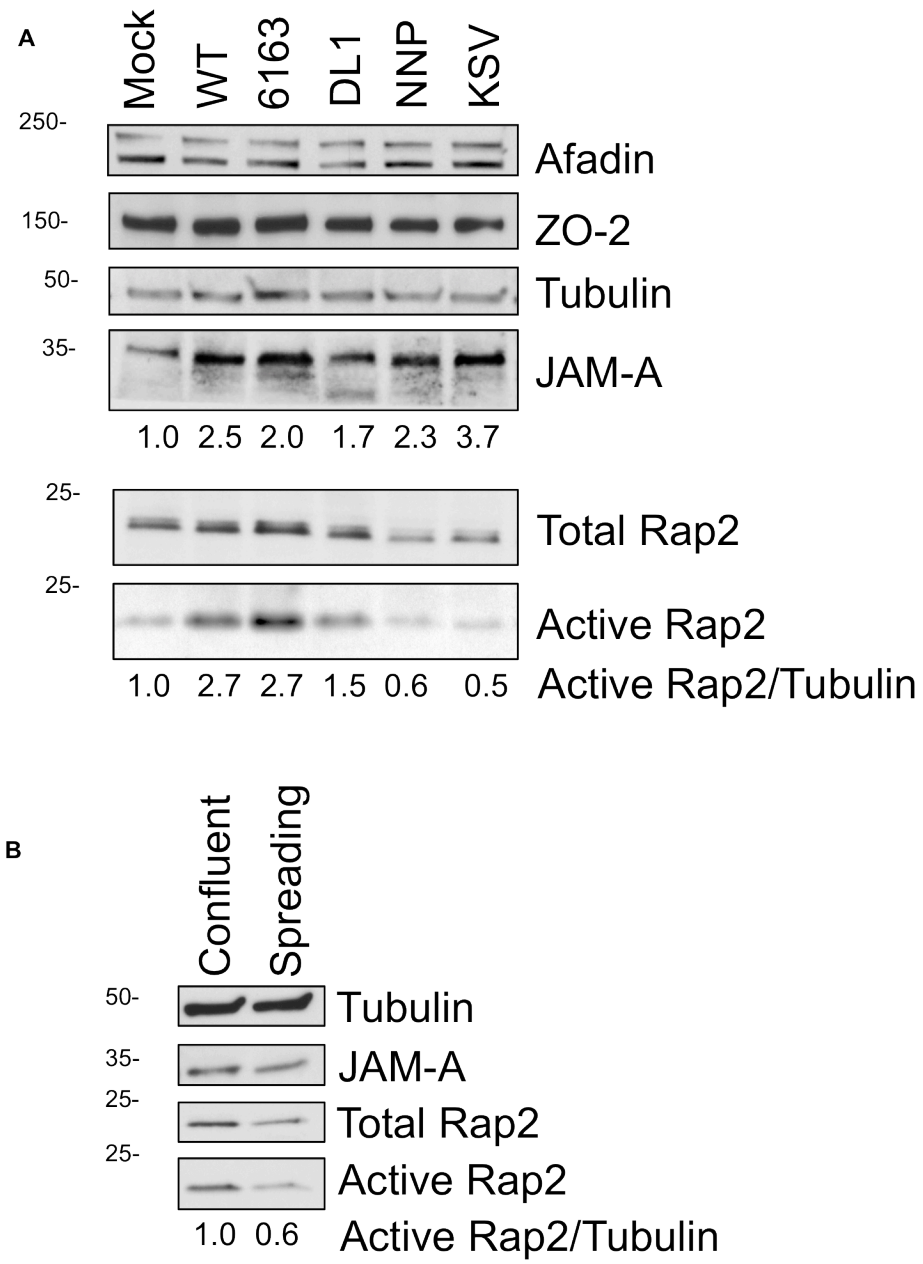


Figure 19: JAM-A trans-, but not cis-dimerization is important for Rap2 activity. (A) WT and mutant JAM-A were expressed in HEK-293T cells and assessed for Rap2 activity by Ral-GDS pulldown. Afadin and ZO-2 expression was not altered by overexpression of WT or mutant JAM-A. Overexpression of WT JAM-A (2.5 fold over control) resulted in enhanced Rap2 activity compared to mock transfected cells. Overexpression of cis-null, 6163 JAM-A resulted in similar increases in Rap2 activity. Overexpression of DL1 JAM-A by 1.7 fold resulted in lower Rap2 activity compared to overexpression of WT JAM-A. Overexpression of trans-null mutants NNP or KSV JAM-A led to lower levels of Rap2 activity compared to WT and mock transfected cells. (B) Assessment of Rap2 activity in confluent or spreading human intestinal epithelial cells (SK-CO15) revealed that both total and active levels of Rap2 were 40% lower in spreading cells.

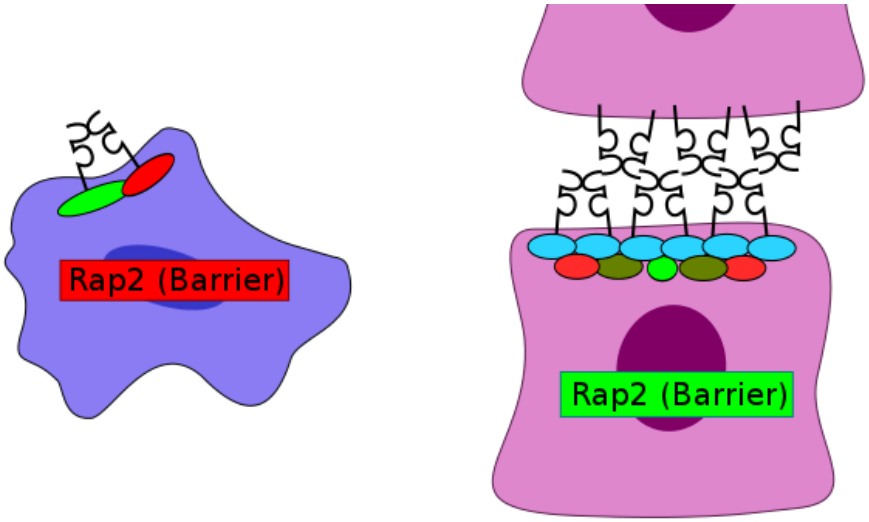


Figure 20: Model: JAM-A dimerization in cis may initiate different signaling modalities than that initiated by JAM-A multimerization, which is dependent on trans-dimerization. Left, Sub-confluent epithelial cells may engage JAM-A cis-homodimers, previously shown to be important in Rap1 activity which regulates epithelial migration. Right- Confluent cells may also engage in JAM-A trans-dimerization events, which we have observed to be important for Rap2 activity, previously implicated in barrier function signaling.

Discussion

In this study we demonstrate that JAM-A homodimerizes in trans at a distinct site from cis-dimerization. We report that transdimerization of JAM-A occurs through low affinity interactions in the distal most Ig loop at a site directly opposite to the cis-dimerization interface. Additionally, disruption of trans-dimerization by mutagenesis resulted in alterations to small GTPase signaling that was previously implicated in regulation of barrier function. From the observations presented in this manuscript, we propose a model where JAM-A multimers may initiate signaling events that are distinct from those initiated by JAM-A cis-dimerization (figure 20).

JAM-A trans-dimerization has been predicted by crystallographic studies of purified murine JAM-A and in studies of JAM-A dependent interactions between human platelets and endothelial cells. However, crystallographic studies of human JAM-A did not detect trans-dimerization, raising uncertainty about trans-dimerization of JAM-A in humans. By overexpressing WT and mutant JAM-A in CHO cells, we observed that expression of either cis- or trans-dimerization deficient mutants exhibited lower rates of JAM-A localization to cell-cell contacts, indicative of a role for JAM-A oligomerization at cell junctions (Fig. 15). We interpreted these findings to suggest that JAM-A cis-dimerization is required to provide the necessary avidity for trans-dimerization to occur. Indeed, in analogous studies, members of the cadherin family of proteins have been shown to trans-dimerize and accumulate laterally at junctions to form zipper-like cadherin multimers (Zhang et al., 2009; Baumgartner et al., 2000; Brieher, 1996; Takeda et al., 1999). Consistent with this idea, JAM-A cis-dimerization is predicted to occur at higher affinities than trans-

dimerization, since the charged residues involved in JAM-A cis dimerization establish a salt bridge between JAM-A monomers (Mandell et al., 2004), while JAM-A trans-dimerization would occur by lower affinity Van der Waals forces between mostly uncharged polar residues. These predictions are supported by chromatography results in figure 13 that reveal the existence of cis-dimers, but no higher order multimers, which were disrupted by the cis-null (6163) mutation. Additionally, AFM analysis of purified JAM-A ectodomains detected both cis- and trans- dimerization events, however cis-dimerization was detected more frequently, suggesting that cis-dimerization is a higher affinity interaction compared to trans-dimerization. On the other hand, results from in vitro bead-clustering experiments indicate that JAM-A cis-dimerization is not required for JAM-A trans-dimerization in that assay. Moreover, assessment of Rap2 activity in figure 19 also suggest that trans-dimerization may occur independently of cis interactions. Indeed, if cis-dimerization is a prerequisite for trans-dimerization in cells, any phenotype requiring trans-dimerization would also be affected by the disruption of cis-dimerization. However, our results in figure 19 indicate that Rap2 activation is reduced by overexpression of trans- but not cis-dimerization null mutants. As such, we cannot conclude that trans-dimerization requires the formation of cis-dimers, but that both cis- and trans-dimerization of JAM-A are required for its stabilization at cell-cell contacts.

The finding that JAM-A trans-dimerization may specifically affect the activity of Rap2 independently of cis-dimerization implies that JAM-A cis or trans-dimerization may act as a molecular switch, as outlined in the hypothetical model in

figure 20. In populations of subconfluent cells, isolated cis-dimerization of JAM-A do not initiate barrier-inducing signals, presumably because spreading cells lack the requisite cell contacts. Here we report that trans but not cis-dimerization is important for enhancing Rap2 activity (Fig. 19a), which has been previously implicated in the regulation of barrier function. Indeed, trans-dimerization of JAM-A would require cell contact with adjacent cells and would be more common in confluent cell populations. Moreover, assessment of Rap2 activity in confluent and spreading epithelial cell monolayers revealed that Rap2 activity is dependent on confluence (Fig 19b). These observations suggest that dimerization of JAM-A in either cis and/or trans at independent motifs may play a role as an epithelial sensor that signals towards barrier inducing phenotypes, depending on whether individual cells or a confluent monolayer is present. Future studies would be important in dissecting the specific roles of trans- and cis- dimerization as triggers of epithelial homeostasis.

Chapter 4: Discussion

Overview and Physiological Significance

The intestinal epithelium provides selective nutrient absorption and maintenance of a protective barrier against luminal pathogens, which are essential physiological functions for homeostasis. Maintenance of an intact mucosal barrier requires that epithelial defects are resealed by a combination of cellular proliferation, epithelial migration towards denuded areas and reformation of cell-cell junctions. Junctional Adhesion Molecule A (JAM-A) is an important transmembrane, junction-associated signaling protein that has been implicated in the regulation of cell migration, proliferation and barrier function (Chapter 2, (Nava et al., 2011; Severson et al., 2008b).

JAM-A was previously reported to homodimerize on the surface of a same cell (in cis) to initiate signaling mechanisms that regulate cell migration (Severson et al., 2009b). However, mechanistic understanding of the role of JAM-A in regulating epithelial permeability has been lacking. With these studies, we have determined the signaling cascade by which JAM-A regulates barrier function and have demonstrated a role for JAM-A dimerization across cells (in trans) as an important event that initiates barrier-regulating signals (Fig. 21).

Loss of barrier function has been implicated as an initial trigger for the complex pathophysiology of a number of chronic inflammatory disorders of the intestine. Human studies of inflammatory bowel disease (IBD) patients have revealed that individuals with IBD have enhanced intestinal permeability (D'Incà, 1999), and a study of intestinal permeability in Crohn's disease (CD) revealed that asymptomatic first degree relatives of CD patients also presented with increased

intestinal permeability (Thjodleifsson et al., 2003), suggesting that loss of barrier function may be the initial event that leads to the multifactorial pathology of IBD. Moreover, several studies indicate that the combined effect of enhanced epithelial permeability along with perturbed immune function contribute to the disease chronicity of IBD patients. Importantly, murine studies on the role of JAM-A in regulating in vivo barrier function revealed that JAM-A deficient mice developed enhanced mucosal permeability and were more susceptible to injury induced colitis (Laukoetter et al., 2007), however did not develop colitis spontaneously. Interestingly, these mice were found to develop remarkable adaptive immune compensatory changes that were protective against development of spontaneous colitis (Khounlotham et al., 2012). These findings underscore the complexity of IBD and support a multi-hit hypothesis where barrier dysfunction and additional immune system defects increase susceptibility to colitis.

Inflammation can further exacerbate barrier defects in IBD. High levels of in-situ pro-inflammatory cytokines induce the endocytic internalization of junction-associated proteins such as JAM-A (Bruewer et al., 2003), which further compromise epithelial barrier function. As such, therapies aimed at promoting epithelial barrier in the context of inflammation may mitigate the perpetual cycle of pathological barrier dysfunction and enhanced mucosal inflammation. Thus, further investigation is required to identify mechanisms to strengthen the epithelial barrier and protect from systemic exposure to luminal antigens and pathogens.

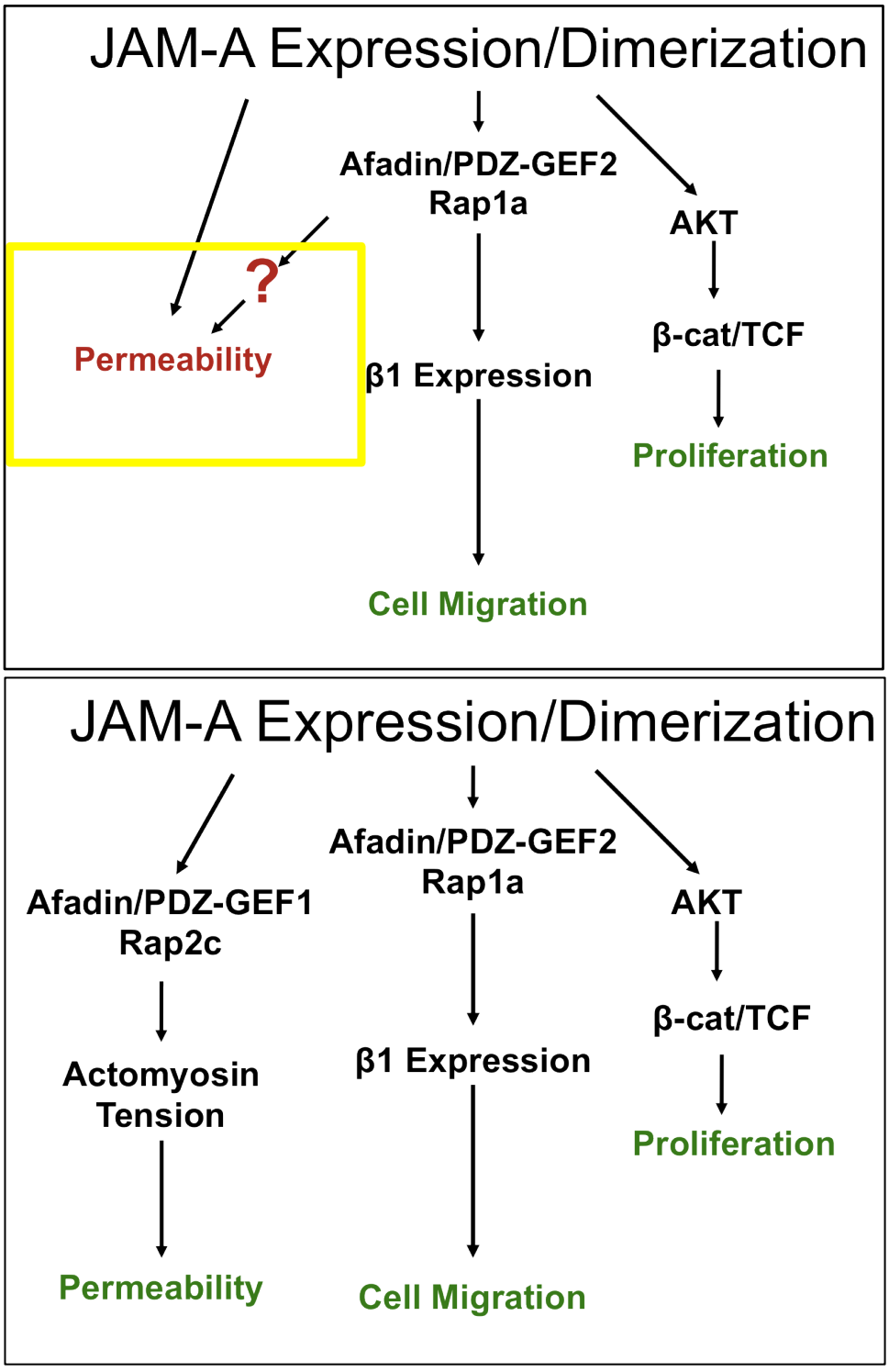


Figure 21: Understanding of JAM-A signal before (top) and after (bottom) completion of this thesis. JAM-A dependent mechanisms regulating cell migration and proliferation had been previously reported. The studies in this thesis reveal that JAM-A trans-and cis- homodimerizes and associates with Afadin, ZO-2 and PDZ-GEF1 to activate Rap2c and relieve acto-myosin contraction so as to regulate permeability epithelial permeability of large molecules.

JAM-A expression and dimerization in signaling

The work presented in this thesis provides insight on the functional significance of JAM-A as an important regulator of epithelial barrier function. Previous studies have shown that disruption of JAM-A signaling in vitro and in vivo results in enhanced intestinal permeability. In this study, we treated epithelial cells and murine intestinal loops with the reovirus attachment protein σ1, previously reported to disrupt JAM-A dimerization (Vedula et al., 2008). Using this approach, we observed that disruption of JAM-A dimerization enhances permeability of intact monolayers of epithelial cells by dislocating JAM-A away from cell contacts. These observations, along with co-immunoprecipitation, confocal microscopy and in vivo permeability studies indicated that JAM-A localizes a protein complex important for the regulation of barrier.

To further characterize the mechanisms by which the described JAM-A associated complex regulates epithelial permeability, we considered the role of the actin cytoskeleton in mediating barrier function. Permeability analyses in vitro and in vivo revealed that loss of JAM-A led to not only a leak to small molecules, attributed to changes in the claudin-mediated pore pathway, but also to the paracellular passage of large molecules (40kDa), which would imply the involvement of the junction-associated apical actin cytoskeleton. Indeed, further studies revealed that loss of JAM-A enhanced the activity of two known inducers of acto-myosin contraction- non-muscle myosin light chain-2 (MLC) and the small GTPase RhoA. These findings were used to suggest a model where JAM-A regulates

epithelial permeability by localizing the aforementioned complex to tight junctions and regulating tension of the apical acto-myosin belt.

Our studies on JAM-A dependent regulation of epithelial permeability and migration suggest that large complexes associate with JAM-A to initiate relevant signaling modalities. Given that JAM-A has a single cytoplasmic segment that spans 40 amino acids and contains a single PDZ-binding motif, no more than one PDZ-containing scaffold protein may associate with a single JAM-A molecule at one time. As such, models of JAM-A dependent regulation of cell migration have highlighted JAM-A homodimerization in cis (on the surface of a same cell) as a necessary event that brings JAM-A bound scaffold proteins in close apposition. However, the mechanistic role of JAM-A dimerization in the regulation of barrier function was not previously defined.

Disruption of JAM-A cis-dimerization by a variety of methods has been shown to disturb barrier function in epithelial cells. However, it is not known how dimerization affects barrier function. One possibility is that dimerization is directly responsible for initiating barrier-inducing signals by bringing different scaffold proteins into close apposition, as is the suggested mechanism for JAM-A regulation of cell migration. Alternatively, JAM-A dimerization maybe important for stabilizing JAM-A at intracellular junctions, so that disruption of dimerization displaces JAM-A away from the apical junctional complex either through JAM-A internalization into endocytic vesicles or by displacement into basolateral compartments, thereby disrupting barrier-inducing signals. As observed in figure 6 (chapter 2), confluent epithelial cells treated with reoviral protein σ1 display reduced levels of junction-

localized JAM-A and have enhanced epithelial permeability, suggesting that the latter mechanism is at least partially involved in barrier function signaling.

The large multi-protein complex suggested by our model of JAM-A dependent barrier function (Chapter 2, Fig. 12) implies that oligomerization of several JAM-A molecules, and not just JAM-A dimerization, is required for proper recruitment of a diverse array of scaffold proteins. We thus hypothesized that JAM-A oligomers are composed of a series of junction-associated cis-dimers that cluster together by simultaneous trans-dimerization with cis-dimers on adjacent cells. Trans-dimerization is predicted to occur in murine JAM-A at a second site on the distal most Ig loop (Kostrewa et al., 2001). However, prior to the studies reported in this thesis, it was unclear whether trans dimerization of human JAM-A was possible, nor was the putative trans-dimerization motif identified.

As discussed in chapter 3, the membrane distal domain of JAM-A was observed to dimerize in trans at a site that is distinct from where cis dimerization occurs. This conclusion was supported by observations from confocal microscopy, in vitro bead clustering assays and atomic force microscopy studies. To determine the functional significance of JAM-A trans dimerization, Rap2 activity was assessed in cells overexpressing WT or mutant JAM-A. Overexpression of JAM-A in HEK-293T cells revealed that compared to cells transfected with WT JAM-A, overexpression of trans-, but not cis-, dimerization null mutants decreased Rap2 activity, which was previously implicated in the regulation of epithelial permeability. These findings supported the hypothesis that JAM-A may simultaneously dimerize in cis and trans

to form higher order oligomers and that JAM-A multimerization maybe important for signals that regulate barrier function.

Our observations from chapter 3, figure 19 suggest that trans-dimerization is important for initiation of signals that regulate barrier function, presumably by enabling JAM-A oligomerization. As expression of JAM-A cis-null mutants did not decrease Rap2 activity, we suggested that cis and trans-dimerization may trigger disparate signaling events. Since by definition trans-dimerization involves interaction between two cells and is more likely to occur in a confluent cell monolayer than in spreading cells, we postulated that subconfluent cells, which contain cis-, but not trans- JAM-A homodimers, do not initiate barrier-inducing signals because barrier function is not relevant for isolated cells. In contrast, our findings suggest that trans dimerization of JAM-A, as observed in confluent monolayers of epithelial cells, triggers signals that maintain barrier stability, specifically through the activation of Rap2. In support of this model, we compared Rap2 activity between spreading cells, which predominantly have JAM-A dimerization in cis, and confluent monolayers, which engage in both cis- and trans-dimerization of JAM-A. Results from this experiment revealed higher activity of Rap2 in confluent monolayers compared to spreading cells.

From these studies, we propose a model where JAM-A oligomers composed of simultaneous cis and trans dimers are required for barrier inducing signals, as highlighted in the model in chapter 3, figure 20. Specifically, JAM-A oligomerization is proposed to be important for the recruitment of a complex composed of ZO-2, PDZ-GEF1 and afadin to junctions, leading to activation of Rap2c (Chapter 2, Fig.

12). Additionally, observations from previous studies have implicated the components of the aforementioned complex to the regulation of actin-myosin contraction (Gloerich et al., 2012; Miyata et al., 2009; Tsukita et al., 2009), which corroborate our own observations that JAM-A is important for regulating myosin light chain phosphorylation and RhoA activity. Together, these observations indicate that JAM-A oligomerization recruits a barrier-inducing complex to junctions, activating Rap2c and ultimately controlling tension of the apical cytoskeleton.

Future directions

Although the results from these studies have provided important insights on the physiological importance of JAM-A in regulating intestinal permeability, several new questions have been raised that warrant further investigation. For instance, as has been described in chapter 1, JAM-A has been observed to localize at tight junctions and at the basolateral membrane of epithelial cells (Liang et al., 2000), however it is not known whether JAM-A localized to these different pools initiate specific signals. As predicted in chapter 1 and corroborated by a subsequent study (Iden et al., 2012), phosphorylation of the cytoplasmic segment of JAM-A may determine whether JAM-A is localized to tight junctions or the basolateral membrane. However, it is not known if junction-associated, phosphorylated JAM-A is able to recruit different scaffold proteins and, as such, trigger specific downstream signals compared to non-phosphorylated JAM-A. By isolating phosphomimetic and phosphorylation-null JAM-A mutants exogenously expressed in epithelial cells, future studies could assess phosphorylated JAM-A-associated

complexes by mass spectrometry and compare results with those obtained from non-phosphorylated mutants.

In addition, the possibility that basolaterally localized JAM-A may serve as a reservoir for exchange with tight junction-associated JAM-A cannot be excluded. Indeed, FRAP studies of claudin-1 and occludin revealed that these proteins move from a mobile basolateral pool to an immobile, tight junction associated pool, albeit at different rates and based on phosphorylation states (Le Shen et al., 2008; Raleigh et al., 2011). FRAP based investigation of JAM-A turnover would be particularly useful in this regard, and may lend support to conclusions that tight junction-localized JAM-A may be primarily responsible for inducing signals that control epithelial permeability. As such, the mechanisms that regulate JAM-A phosphorylation may be critical for establishing junction localization of JAM-A, which in turn may regulate epithelial permeability. Therefore identification of kinases and phosphatases that regulate the phosphorylation status of JAM-A may reveal important molecular switches that determine which JAM-A-dependent signals are triggered.

Additionally, JAM-A dimerization status may be important in determining how JAM-A is phosphorylated, however this relationship has not yet been explored. For example, it is possible that JAM-A oligomerization may lead to recruitment of specific phosphatases and kinases not recruited during JAM-A cis-dimerization in isolation. Assessment of JAM-A phosphorylation status in spreading and confluent cells, or in cis- and trans-null JAM-A mutants expressed in epithelial cells, could provide insight into this important question. Overall, understanding mechanisms

that determine how the TJ-associated and basolateral JAM-A pools are regulated could provide opportunities for therapeutic control of epithelial barrier function downstream of JAM-A.

Though JAM-A surface expression has been assumed to be required for JAM-A dependent signaling, the regulation of JAM-A internalization and recycling is not understood. We learned from our study on barrier regulation that σ1-mediated disruption of JAM-A homodimers led to dissociation of JAM-A from cell contacts, possibly due to enhanced JAM-A internalization (Chapter 2, Fig. 6). Moreover, size exclusion chromatography studies of soluble JAM-A ectodomains have revealed that JAM-A dimerization is pH dependent, and that dimers are reversibly disrupted below pH 6 (Chapter 3, Fig. 14). It is still unclear whether JAM-A dimerization is required to stabilize JAM-A at the cell surface, but it would be reasonable to assume that once internalized, acidification of recycling endosomes would lead to disruption of JAM-A dimers. Whether it is dimerization status that determines JAM-A internalization or the other way round, mechanistic insight on JAM-A internalization may provide ways to silence or enhance JAM-A signaling.

Indeed, JAM-A internalization and subsequent disruption of epithelial barrier function may be an important mechanism in the pathophysiology of colitis. Previous studies have implicated pro-inflammatory cytokines and other molecules as external triggers of JAM-A internalization (Bruewer et al., 2005), however no mechanisms have been described. To gain insight on this pathway, assessment of whether cytokine mediated internalization of JAM-A involves regulation of JAM-A phosphorylation or dimerization status should be considered. For example, it would

be useful to determine whether covalently stabilized JAM-A dimers or phosphomimetic JAM-A mutants expressed on the surface of epithelial cells are more resistant to cytokine-induced internalization compared to dimerization null or phosphorylation null mutants. Likewise, identification of whether JAM-A internalization is dependent on clathrin, caveolin or another endocytic mechanism would provide insight into novel targets for controlling JAM-A internalization.

Therapeutic Implications

JAM-A is tight-junction associated transmembrane protein important for the regulation of cell proliferation, migration and barrier function. As such, understanding the mechanisms behind JAM-A function could lead to important therapies for manipulating multiple cell processes simultaneously. Targeting several signaling pathways at once could be an efficient way to treat multi-faceted conditions such as invasive carcinomas, which require enhanced proliferation and migration. Considering that JAM-A is also expressed in endothelial cells and leukocytes (Woodfin et al., 2007; Bazzoni, 2003), regulation of JAM-A signaling may also control inflammatory pathways by reducing migration of immune cells to sites of injury. However, early reports investigating the therapeutic potential of JAM-A in cancer cell progression have revealed that targeting several pathways simultaneously can lead to divergent effects. Though some studies reported a reduction in cancer cell invasiveness with the inhibition of JAM-A signaling (Murakami et al., 2011), others have shown just the opposite (McSherry et al., 2009; 2011; Gutwein et al., 2009), even when addressing the same malignancy. As such,

therapies that specifically target a single pathway may lead to more consistent clinical outcomes.

Intriguingly, the results in chapter 3 have indicated that JAM-A trans-dimerization may be a specific molecular switch that regulates epithelial barrier function. IBD and other inflammatory conditions may be treated with enhancement of barrier function, thereby reducing antigen exposure to leukocytes and mitigating subsequent pro-inflammatory mechanisms. Approaches that would enhance barrier function include increased Rap2 activity, which could be achieved by enhancing GEFs, which activate Rap2, or inhibiting GAPs that disrupt Rap2 activity. Additionally, mechanisms that inhibit TJ internalization in response to pro-inflammatory cytokines, as outlined in the sections above, should also be considered.

Alternatively, transient barrier disruption may be desired for targeted drug delivery through mucosal barriers. Specifically, gastrointestinal delivery of insulin therapies would spare diabetics from the painful and inconvenient injections they currently rely on. Small molecule inhibitors that specifically inhibit JAM-A trans-dimerization and transiently disrupt JAM-A multimers to enhance permeability could be designed for this purpose. These and other therapeutic strategies that target JAM-A dependent mechanisms provide novel and exciting future translational implications to the findings in this thesis.

Conclusions

In summary, the observations presented in this document provide important mechanistic insight into how JAM-A homodimerizes laterally (cis) and across cells (trans) to regulate epithelial barrier function. These findings have important therapeutic implications. Observations from chapter 3 suggest that JAM-A trans-dimerization affect specific signaling cascades independently of cis-dimerization and, as such, therapies that disrupt particular dimerization motifs may be designed to specifically disrupt epithelial barrier function. Future studies that stem from the findings presented in this document should assess how JAM-A localization to the cell surface, and specifically at tight junctions, may be manipulated so as to specifically enhance or silence JAM-A signaling. Further understanding of pathways governing JAM-A internalization, recycling and differential localization to basolateral or tight junction pools may lead to discovery of therapeutic targets against important pathological processes. Particularly, identification of ways to maintain JAM-A at junctions under conditions of decompensated inflammation may be a key approach to disrupting the multifactorial pathophysiology of chronic inflammatory diseases such as IBD.

Chapter 5: Experimental Methods

Cell Culture

Chinese Hamster Ovary cell (CHO), Human Embrionic Kidney Cells (HEK-293T), and SK-CO15 cells were grown in DMEM supplemented with 10% fetal bovine serum, 2 mM l-glutamine, 100 IU of penicillin, 100 μg/ml streptomycin, 15 mM HEPES, and 1% nonessential amino acids and were subcultured with 0.05% trypsin (Cellgro). For filter based studies, cells were seeded at a density of 1×10^5 cells/0.33 cm2. Transfections were performed with Lipofectamine 2000 (Qiagen) in Optimem (Invitrogen) per manufacturer's protocol. For Reovirus infection studies, CHO cells were cultured in F12 medium supplemented to contain 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 25 ng/ml amphotericin B. siRNA targets (Qiagen, Santa Cruz, outlined in Fig S4) were used at a total concentration of 100nM siRNA or less and knockdown was verified by western blot or qRT-PCR (Fig. S4). Cells were transfected with 1-3 ug of plasmid DNA containing wild-type or mutant JAM-A constructs per 1x10^6 cells using Lipofectamine (Invitrogen) according to the manufacturer's instructions. Cells were harvested for immunoblot, fixed for immunofluorescence staining or flow cytometry, infected with reovirus or assessed for barrier function 2-3 days after transfection.

Antibodies

The murine monoclonal anti-JAM-A antibodies 1H2A9, J10.4, and JF3.1 were purified as described (Liu et al., 2000). Other antibodies are commercially available:

polyclonal affinity-purified rabbit anti-JAM-A (Invitrogen), monoclonal mouse Rap2, monoclonal mouse anti-PDZ-GEF1, monoclonal mouse anti-afadin, monoclonal mouse ZO-1 and polyclonal affinity purified rabbit ZO-2 (BD Transduction Laboratories), polyclonal affinity purified rabbit anti-afadin 02246, monoclonal mouse anti-tubulin and polyclonal affinity purified rabbit anti-actin (Sigma), polyclonal affinity purified rabbit anti-Rap2c (Cell Signaling) and polyclonal affinity purified rabbit anti-RhoA (Santa Cruz). For immunoblots, HRP conjugated secondary antibodies were used (Jackson ImmunoResearch Laboratories). For Immunofluorescence, FITC and Alexa conjugated antibodies (Invitrogen) were used.

Expression and Purification of the JAM-A-binding Reovirus σ1 Protein

A cDNA fragment encoding amino acids 170-455 of the σ1 attachment protein of reovirus strain T3D was fused to a GCN4-pII trimerization domain (biomers.net) and a 10-amino-acid trypsin-cleavable linker. The resulting construct was inserted into a PQE-80L vector using BamHI and HindIII sites. A G381A point mutant in σ1 was engineered using the GeneArt® Site-Directed Mutagenesis System (Invitrogen) according to the manufacturer's protocol. Wild-type (WT) σ1 and σ1_G381A were expressed in DE3 *E. coli* by autoinduction and purified as described previously (Reiter et al., 2011).

Production of Lentiviruses and Stable Cell Lines

Lentiviruses were created in HEK293T TLA cells by transfection of viral plasmids (Open Biosystems) with lipofectamine 2000 (Qiagen). Lentivirus-containing media

were harvested 72hrs post-transfection. Titered supernates were then used at MOI of 4 to transduce SKCO-15 human colonic epithelial cells. Resulting polyclonal populations were purified into different clones by serial dilutions and grown in supplemented DMEM with puromycin (2 ug/ml). shRNA sequences are outlined in chapter 2, figure 9.1.

Immunoblots

Monolayers of epithelial cells were homogenized in radioimmunoprecipitation assay (RIPA) lysis buffer (20 mM Tris, 50 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1% sodium deoxycholate, 1% Triton X-100, and 0.1% SDS, pH 7.4) or 0.1% NP40 or 1% Brij 97 lysis buffer (10mM Tris HCl pH 8.0, 150mM NaCl, 1mM MgCl2, 1mM CaCl2) supplemented with protease and phosphatase inhibitor cocktails (Sigma). A bicinchoninic acid assay (Pierce) was used to determine lysate protein concentrations. Lysates were cleared by centrifugation and boiled in reducing SDS sample buffer. SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblots were performed by standard methods. Tubulin was used as a protein loading control.

Immunoprecipitation

SK-CO15 and T84 cells that were 80-90% confluent were harvested in 0.1% NP40 or 1% Brij 97 lysis buffer (10mM Tris HCl pH 8.0, 150mM NaCl, 1mM MgCl2, 1mM CaCl2) supplemented with protease and phosphatase inhibitor cocktails (Sigma). Lysates were incubated end-over-end for 20 minutes at 4C before being cleared at

14,000 rpm for 10 minutes. The supernatant (soluble fraction) was kept on ice while the pellet was resuspended in RIPA buffer, dounced and cleared at 14k rpm for 10 minutes (RIPA fraction). Each fraction was pre-cleared with 50uL sepharose for 30 minutes and incubated with 5ug antibody for 2 hours. Immune complexes were precipitated by 50uL of Protein A or Protein G sepharose for 1 hour. Immunoprecipitate pellets were washed three times in either lysis buffer or RIPA before being boiled in reducing sample buffer. Input and immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotting.

Immunofluorescence (IF) Microscopy

Cells were grown on .33 cm^2, 0.4-μm pore transwell filters (Corning Life Sciences) and human colonic tissue was flash frozen in OCT (Sakura) before being cryosectioned into 8 micron slices. Tissue and cells were pretreated for 5 minutes with 0.05% triton-x100 in HBSS+ at room temperature or directly fixed in 100% ethanol at −20°C for 20 min, and blocked in 5% BSA in HBSS+ for 1 h. Samples to be characterized for cytoskeletal components were fixed in 3.7% formalin for 10 minutes before being permeabilized with 0.1% triton at room temperature or ethanol at −20°C. Primary antibodies were diluted in blocking buffer and incubated with cells overnight at 4°C, fluorescently labeled secondary antibodies were diluted in blocking buffer and incubated with cells for 45 min at room temperature. Stained cells were washed in HBSS+ (Hanks Buffered Saline Solution, CellGro) and mounted in Prolong Antifade Agent (Invitrogen). A laser scanning microscope (LSM 510, Carl

Zeiss) was used to capture confocal fluorescence images. Image J and LSM software were used for image processing.

PDZ-Proteomic Array

The PDZ domain-containing proteomic array has been previously described (Fam et al., 2005; He et al., 2006). Briefly, nylon membranes were spotted with recombinant His/S-tagged PDZ domain fusion proteins at a concentration of 1 μg per bin. GST fusion proteins corresponding to the C-terminal 25 amino acids of JAM-A, or a mutant with residues comprising the C terminal PDZ binding motif (FLV) switched to alanines, were overlaid at concentrations of 100 nM in blotting buffer (2% nonfat dry milk, 0.1% Tween-20, 50 mM NaCl, 10 mM Hepes, pH 7.4) overnight at 4 °C. Arrays were washed three times with blotting buffer followed by incubation with horseradish peroxidase-conjugated anti-GST antibody (GE Healthcare). Interactions of GST fusion proteins and PDZ domains were visualized by chemiluminescence using an ECL kit (Pierce)

RhoA and Rap2 Activity Assay

RhoA and Rap2 activity assays were performed according to the manufacturer's instructions (Millipore and CellBio Labs). Briefly, cells were lysed in a Tris and Triton X-100 based lysis buffer provided at 4°C. Cell debris was removed by centrifugation, and 40 μl saved as input to determine total RhoA or Rap2 levels. Lysates containing equal amounts of protein for each sample (between .5 and 1.5 mg) were incubated at 4°C for 60 min with Rhotekin or Ral-GDS agarose beads to

bind active RhoA (Reid et al., 1996) or Rap2 (Knaus et al., 2007), respectively. Beads were washed three times with lysis buffer followed by boiling in SDS sample buffer. Entire samples were then analyzed by immunoblot with detection by RhoA or Rap2 Ab provided by the manufacturer.

Permeability Assays

Cells were grown on .33cm^2, .4-μm pore transwell filters (Corning Life Sciences, Lowell, MA) to confluence. Transepithelial resistance (TER) to passive ion flow was recorded using an EVOMX voltmeter with an STX2 electrode (World Precision Instruments). For dextran flux experiments, confluent monolayers grown on transwell filters were washed and placed in HBSS+ (Invitrogen) for 1 hour at 37C. 3, 4, 10kDa or 40kDa dextran labeled with FITC or rhodamine (sigma) were placed on the top chamber of transwells and incubated for 2 hours at 37C. Samples from the bottom chamber of the transwell were collected and fluorescence intensity was measured with a fluorescent plate reader (Fluostar).

In situ intestinal epithelial permeability was measured with a previously described intestinal loop model (Clayburgh et al., 2005) with slight modifications. Following overnight fasting, animals were anesthetized subcutaneously with a mixture of ketamine and xylazine at doses of 100 and 5 mg/kg, respectively. A midline abdominal incision was made to expose the small intestine, and a 4-cm loop was clipped at proximal and distal ends to isolate it from the rest of the bowel. For in-vivo σ1 treatment studies, 100ug/ml of σ1 in HBSS+ was administered directly into the loop lumen for 1 hour. Solutions containing FITC-dextran (3kd, 10kd or 40kd;

1mg/ml in HBSS) were then added to intestinal loops and, after 1 hour, fluorescence (an index of FITC-dextran absorption from the intestine lumen into the blood stream) was measured from whole blood obtained by cardiac puncture, using a fluorescence microtiter plate reader (Fluostar Galaxy; BMG LabTech). After cardiac puncture, all anesthetized mice were euthanized by cervical dislocation. All animal experiments were conducted according to protocols approved by the Institutional Animal Care and Use Committee at Emory University.

Fluorescence Recovery After Photobleaching (FRAP)

Stable non-silenced (NS) or JAM-A deficient (shJA) SK-CO15 cell lines were cultured on chamber slides (Thermo Fischer Scientific). At 50% confluence, cells were transduced with actin-GFP using a baculovirus delivery system (Cellight Bacmam 2.0, Invitrogen). Fluorescence recovery after photobleaching (FRAP) experiments were performed on a Nikon A1R TE 2000 inverted microscope with a 40x objective, and GFP fluorescence was imaged with a 488nm laser. All experiments were performed at 37°C with 5% CO_2 using a heating chamber. GFP fluorescence at cell contacts was bleached for 7s using a 404nm laser set at full power. To assess fluorescence recovery, images were acquired every 2 seconds over a period of 2 min. Fluorescence intensity data were corrected for overall loss in total fluorescence intensity as a result of fluorescence imaging and for loss of total cell fluorescence as a result of photobleaching. The fluorescence intensity of bleached regions over time was normalized to prebleached fluorescence intensity.

PCR and qRT-PCR

RNA was extracted from SK-CO15 cells with Trizol (Invitrogen) and further purified with an RNA extraction kit (Qiagen). For standard PCR, RNA was subjected to reverse transcription (Invitrogen) and PCR reaction using Taq Polymerase (Qiagen) and resulting DNA was assessed by agarose gel eletrophoresis. qRT-PCR was performed with one-step SYBR Green (Biorad). PCR and qRT-PCR were programmed as follows: 94°C for 3 min followed by 40 cycles of 94°C for 15 s, 57°C for 30 s, and 72°C for 30 s. qRT-PCR data were represented as fold-change, determined by applying the formula 2ΔΔ Ct, where ΔCt = Ct of target gene − Ct of loading control (β actin), and ΔΔCt = ΔCt of samples for target gene (siRNA treated) − ΔCt of the control for the target gene (scr siRNA treated). The primers used are outlined in chapter 2, figure 9.1.

Expression and Purification of recombinant JAM-A

WT, and cis-dimerization null mutants 6163 and DL1 JAM-A in pCDNA 3.0 were cloned as previously described (Severson et al., 2008b; Mandell et al., 2004). The NNP and KSV JAM-A point mutants were engineered from WT JAM-A in pCDNA 3.0 by overlap PCR. Initial amplification of WT JAM-A in pCDNA 3.0 was performed with primer pairs 5'-gctcggatccgccaccatggggacaaaggcgcaagt-3', 5'-atatctcgagtcacaccaggaatgacgaggtctg-3'. NNP and KSV point substitutions to alanines were introduced with amplification with primer pairs 5'-cctgaagtcagaattcctgaggctgctgctgtgaagttgtcctgtgcc-3', 5'-ggcacaggacaacttcacagcagcagcctcaggaattctgacttcagg-3' and 5'-

tgccaactggtatcaccttcgcggccgcgacacgggaagacactggga-3', 5'-tcccagtgtcttcccgtgtcgcggccgcgaaggtgataccagttggca-3', respectively. The inserts of trans-null mutants NNP and KSV were digested with BamHI and XhoI restriction enzymes and ligated into pDNA3.0 vectors. For bacterial expression of GST-tagged JAM-A, extracellular portions of WT, 6163, DL1, NNP and KSV JAM-A were amplified with primer pairs 5'-atatggatccggcattgggcagtgttacag-3' and 5'-atatctcgagctaattccgctccacagcttc-3' followed by restriction enzyme digest using BamHI and XhoI and ligation into pGEX vectors. For bacterial expression of his-tagged JAM-A, extracellular portions of WT, 6163, DL1, NNP and KSV JAM-A were amplified with primer pairs 5'-gtttaactttaagaaggagatatacatatgagtgttacagtgcactcttctgaa-3' And 5'-gcagcggtcggcagcaggtatttcattagtgatggtgatggtgatggtgat-3' followed by restriction enzyme digest using SLIC ndeI and ligation into pET22b vectors. All JAM-A constructs were expressed in DE3 *E. coli* by autoinduction, purified by gravity flow chromatography with Ni-NTA agarose (Qiagen) or glutathione agarose (Sigma) followed by dialysis in PBS.

Flow Cytometry of JAM-A Conjugated Beads

5uL of Dynabeads his tag (Invitrogen) measuring 1μm were incubated with 2ug of WT, 6163, NNP and KSV JAM-A in 500ul PBS for 10 minutes at room temperature. Magnetic racks were used to wash conjugated beads with PBS before resuspension in 500uL of PBS. JAM-A induced bead aggregation was examined by flow cytometry. Single beads were distinguished from doublets, triplets and larger aggregates by

size, as determined by light scatter. Both forward and side scatter were set to logarithmic scale.

To determine whether equivalent levels of JAM-A were functionalized to beads, conjugated beads were washed once with PBS, and incubated with FACS buffer (2% FBS in PBS) containing JAM-A-specific monoclonal antibody J10.4. After incubation at 4°C for 30 min with rotation, cells were pelleted, washed twice with FACS buffer, and incubated with FACS buffer containing an Alexa Fluor-conjugated secondary antibody. For each condition $1x10^5$ events were examined, and the percent of single beads out of the total events was measured using FlowJo software.

Reovirus Infection of CHO Cells

WT and mutant JAM-A was exogenously expressed in CHO cells and assessed for ability to associate with reovirus. JAM-A expression in transfected CHO cells was assessed by removing cells from tissue culture plates using CellStripper (Mediatech) and pelleted at 1000 x g. The mean fluorescence intensity (MFI) of each sample was determined using flow cytometry, as described above.

Reovirus infection of CHO cells transfected with wild type and mutant JAM-A was quantified following virus adsorption at a multiplicity of infection (MOI) of 100 plaque-forming units (PFU) per cell at 37°C for 1 h. Cells were washed twice with PBS, and fresh medium was added to each well. After incubation at 37°C for 20-24 h, cells were harvested with 0.05% trypsin-EDTA (Invitrogen) at room temperature and quenched with medium collected from each respective sample. Cells were pelleted, washed once with PBS, and incubated with FACS buffer containing Alexa Fluor-conjugated reovirus-specific antiserum. The percentage of reovirus antigen-

positive cells was determined using flow cytometry. All cell staining results were quantified using FlowJo software (Tree Star).

AFM

A Nanowizard III AFM (JPK Instruments, Berlin, Germany) mounted on an optical microscope (Axio Observer.D1, Carl Zeiss Microscopy, Jena, Germany) was applied to measure JAM-A interactions by force spectroscopy. Recombinant JAM-A proteins were coupled to flexible Si_3N_4 AFM cantilevers (MLCT probes, spring constant 0.03 N/m, Bruker, Calle Tecate, CA, USA) and mica sheets (SPI supplies, West Chester, PA, USA) via flexible polyethylene glycol spacers (acetal-PEG-NHS) essentially as described before (Wildling et al., 2011). In brief, AFM cantilevers and mica were functionalized with amino groups by ethanolamine treatment and then coupled to the *N*-hydroxysuccinimide ester group of the heterobifunctional linker. The linker's acetal-function was then converted to an aldehyde group by citric acid treatment to allow reaction with amino groups of JAM-A.

To measure JAM-A interactions, the AFM tip was lowered onto the mica surface and retracted again, and binding events were detected by constantly measuring the deflection of the cantilever as outlined before (Spindler et al., 2009). The AFM cantilever was moved in constant force mode with speed of 1 µm/s in a z-range of 300 nm, 0.1 s delay time on the mica and a retraction setpoint of 200 pN. At least 500 approach-retract cycles at 25 different positions on the mica were recorded for each cantilever/mica combination. Measurements were performed in Hank's buffered saline solution (HBSS) at 37 °C. J10.4 was incubated using 15 µg/ml for 30 min.

Statistics

Statistical differences between target vs. scrambled siRNA mediated knockdown groups were determined using a linear mixed effects modeling approach to control for random between-run differences in baseline TER, while accurately measuring the effects of siRNA-mediated knockdown relative to controls. Results were scaled to a baseline of 100% with 95% confidence intervals around the effect. Statistically significant results indicate a p-value < a type I error rate of 0.05. All statistical analyses were performed in the R statistical environment (R version 2.14, R Foundation for Statistical Computing. Vienna, Austria). For graphs representing three independent samples measured on a same day, a two-tailed Student's *t* test was used to determine *P* values between two experimental groups. $P < 0.05$ was considered significant.

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