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Eric A. Severson Date

Mechanisms of outside-in JAM-A mediated signaling

By

Eric A. Severson
Doctor of Philosophy

Graduate Division of Biological and Biomedical Sciences
Genetics and Molecular Biology Program

____
Charles A. Parkos, M.D., Ph.D.
Advisor

____
Asma Nusrat, M.D.
Committee Member

____
Andrew Kowalczyk, Ph.D.
Committee Member

____
Judy Fridovich-Keil, Ph.D.
Committee Member

____
Carlos Moreno, Ph.D.
Committee Member

____
John Lucchesi, Ph.D.
Committee Member

Accepted:

____
Lisa A. Tedesco, PhD.
Dean of Graduate School

____
Date

Mechanisms of outside-in JAM-A mediated signaling

By

Eric A. Severson
B.S. University of Oklahoma, 2002

Advisor: Charles A. Parkos, M.D., Ph.D.

An Abstract of
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2009

Abstract

Mechanisms of outside-in JAM-A mediated signaling
By Eric A. Severson

JAM-A is a transmembrane component of tight junctions that regulates multiple processes, yet studies on the mechanism of JAM-A function are lacking. We hypothesized that the ability of JAM-A to dimerize was necessary for the mechanism of JAM-A function. Overexpression of dimerization-defective JAM-A mutants or treatment with a dimerization inhibiting antibody in 293T cells reduced cell migration across permeable filters. Analyses of cells expressing the JAM-A dimerization-defective mutant proteins revealed diminished β1 integrin protein. A functional link between JAM-A and β1 integrin was confirmed by restoration of cell migration to control levels after overexpression of β1 integrin in JAM-A dimerization-defective cells. To extend upon these results, we reported that JAM-A is physically and functionally associated with the PDZ domain containing signaling molecules Afadin and PDZ-GEF2, but not ZO-1, in the intestinal epithelial cell line SK-CO15. Both Afadin and PDZ-GEF2 were observed to co-localize and co-immunoprecipitate with JAM-A. Loss of JAM-A, Afadin or PDZ-GEF2, but not ZO-1 or PDZ-GEF1, similarly decreased cellular levels of activated Rap1, β1 integrin protein and the rate of epithelial cell migration. The effects observed were secondary to decreased levels of Rap1A since knockdown of Rap1A resulted in decreased β1 integrin protein and cell migration. These findings suggest that JAM-A dimerization leads to the close apposition of Afadin and PDZ-GEF2. The proximity of PDZ-GEF2 and Afadin results in activation of Rap1A. Active Rap1A stabilizes β1 integrin protein levels, which controls the rate of cell migration. These results illustrate a novel mechanism for JAM-A signaling based on its structural motifs.

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Abbreviations:

JAM-A	Junctional Adhesion Molecule A
DL1	dimerization-defective JAM-A mutant with deletion of the distal most immunoglobulin-like loop
PDZ	PSD-95/Discs-Large/ZO-1 domain
JAMs	Junctional adhesion molecules
CLMP	CAR-like membrane protein
CAR	Coxsackie-Adenovirus Receptor
Igsf11a	Immunoglobulin Superfamily member 11a
SIRP	Signal Inhibitor Regulatory Peptide
TNF-α	Tumor necrosis factor alpha
INF-γ	Interferon gamma
VEGF	Vascular Endothelial Growth Factor
ZO-1	Zonula Occludens 1
CHO	Chinese Hamster Ovary
MUPP-1	multi-PDZ domain protein 1
PICK-1	protein interacting with C-kinase-1
aPKC	atypical protein kinase C
TJ	Tight Junction
TER	Transepithelial Resistance
siRNA	small interfering RNA
AF6	Afadin
FGF	Fibroblast Growth Factor
MEK	mitogen-activated protein (MAK) kinase kinase
ERK	extracellular signal-regulated protein kinase
Ig	Immunoglobulin
DMEM	Dulbecco's Modification of Eagle's Medium
HBSS	Hank's Balanced Salt Solution
SEM	Standard Error of the Mean
6163	dimerization-defective JAM-A mutant E61A/K63A
293T	293T human embryonic kidney epithelial cells
FA	focal adhesion;
QRT-PCR	Quantitative real-time RT-PCR analysis
Scr	Scramble
JA	Junctional Adhesion Molecule A
PDZ-GEF	PDZ Guanine Nucleotide Exchange factor
PG	PDZ-GEF
MAPK	Mitogen Activated Protein Kinase
DIC	Differential interference contrast microscopy
D1	Membrane Distal JAM-A Ig-like Loop
D2	Membrane Proximal JAM-A Ig-like Loop

Introduction

Junctional Adhesion Molecule A is a transmembrane component of epithelial tight junctions that has been proposed to play a role in regulating cell migration; however, the relationship between JAM-A structure, its functional domains and subsequent signaling events is incompletely understood. As outlined in the overview of the JAM family member proteins in Chapter 1, the expression of JAM-A has been implicated in many cellular functions. Explanations for how JAM-A regulates these cellular functions have remained elusive.

To investigate the structural elements of JAM-A mediated cellular function, we initially tested the hypothesis that structural components of JAM-A, specifically the dimerization domain and the PDZ binding motif, are necessary for JAM-A mediated cellular functions. The results stemming from this work are presented in Chapters 2 and 3. These chapters present evidence for a novel mechanism of JAM-A mediated cell migration, in which JAM-A dimerization promotes the close apposition of two JAM-A intracellular PDZ binding domains. Scaffolding proteins bind to the JAM-A PDZ binding domains and thus JAM-A dimerization promotes the association of Afadin and PDZ-GEF2. This interaction of PDZ-GEF2 and Afadin activates of Rap1A. Active Rap1A stabilizes β1 integrin protein levels, which in turn increases the rate of cell migration. Overall, these results present the first concrete example of a JAM-A mediated signaling cascade, and provide insight into the potential mechanisms for other JAM-A regulated functions. A more detailed discussion of these findings, their significance and potential therapeutic utility is related in the conclusions in Chapter 4.

<u>Chapter 1</u>

Structure and function of JAM proteins

Material from this chapter has been published in a review chapter in *Adhesion Molecules: Function and Inhibition*, **2007.** Dr. Charles A. Parkos is a co-author of the review chapter.

1.1. Introduction

Junctional adhesion molecules (JAMs) are immunoglobulin superfamily (IGSF) members that are variably expressed in a number of cell types, most notably at cell-cell contacts in endothelial and epithelial cells and in a variety of hematopoietic cells. Three of the best studied members termed JAM-A, B and C contain two extracellular Ig-like domains, a single type I transmembrane segment and a cytoplasmic tail ending in a PSD-95/Discs-Large/ZO-1 (PDZ) type II binding motif at the carboxy terminus. JAM proteins play important roles in diverse cell biological functions including regulation of barrier/permeability, cell adhesion/migration, angiogenesis and development of cell polarity. Furthermore, members of this protein family have been shown to function as receptors for certain viruses. This review focuses on the current understanding of the three most studied members of the JAM protein family, JAM-A, B and C and highlights structural features, protein interactions and the functional significance of such *in vivo*.

1.2 Nomenclature

The Immunoglobulin Superfamily (IgSF) is a large class of proteins that includes the JAM family of proteins. The current nomenclature for JAM members designates the first three described JAM proteins as JAM-A, JAM-B, JAM-C. Two other related proteins have been reported that have not been included in the standard nomenclature and are termed JAM-4 and JAM-L (AMICA). In earlier studies, numerical designations were used to define JAM proteins according to the timing of initial characterizations. However, this early nomenclature led to confusion in terminology for JAM-B and C due to the timing in which human and murine JAM-B and JAM-C were reported. To avoid

confusion, this review will use current nomenclature exclusively, as proposed originally by Muller, regardless of the designation given in the original reports(1).

1.3 Relationship to other IgSF family members

JAM-A, JAM-B and JAM-C are the three most closely related family members, having 32-33% amino acid identity, as highlighted in the phylogram in *figure1*. JAM-4 and JAM-L are more closely related to each other than they are to either JAM-A, B, C or other IgSF proteins. Furthermore, JAM-A, B, and C have a C-terminal PSD-95/Discs-Large/ZO-1 (PDZ) type II binding motif and a conserved R-EWK dimerization motif, both of which are lacking in JAM-4 and JAM-L. JAM-L has been reported to contain a dimerization motif, however this domain is more similar to the CAR dimerization motif than it is to those on JAM-A, B and C (2). The dimerization motif for JAM-4 has not yet been identified. Thus, there are significant differences in the protein sequences of regions on JAML and JAM-4 that, as will be discussed below, likely have important functional consequences for JAM A, B and C. This review will highlight
putative functional relevance of conserved structural elements on JAMs and will focus on JAM-A, JAM-B and JAM-C.

1.4 Structure of JAM-A, JAM-B and JAM-C.

The JAM-A, B and C pro-protein structures are similar with each containing an N-terminal secretory signal peptide of 20-25 amino acids that targets the protein to the endoplasmic reticulum during synthesis after which cleavage occurs. As mentioned above, mature JAM proteins consist of two extracellular Ig-like domains followed by a single type I transmembrane domain and a cytoplasmic tail that terminates in a type II PDZ binding motif at the carboxy end. The conserved dimerization motif lies within the

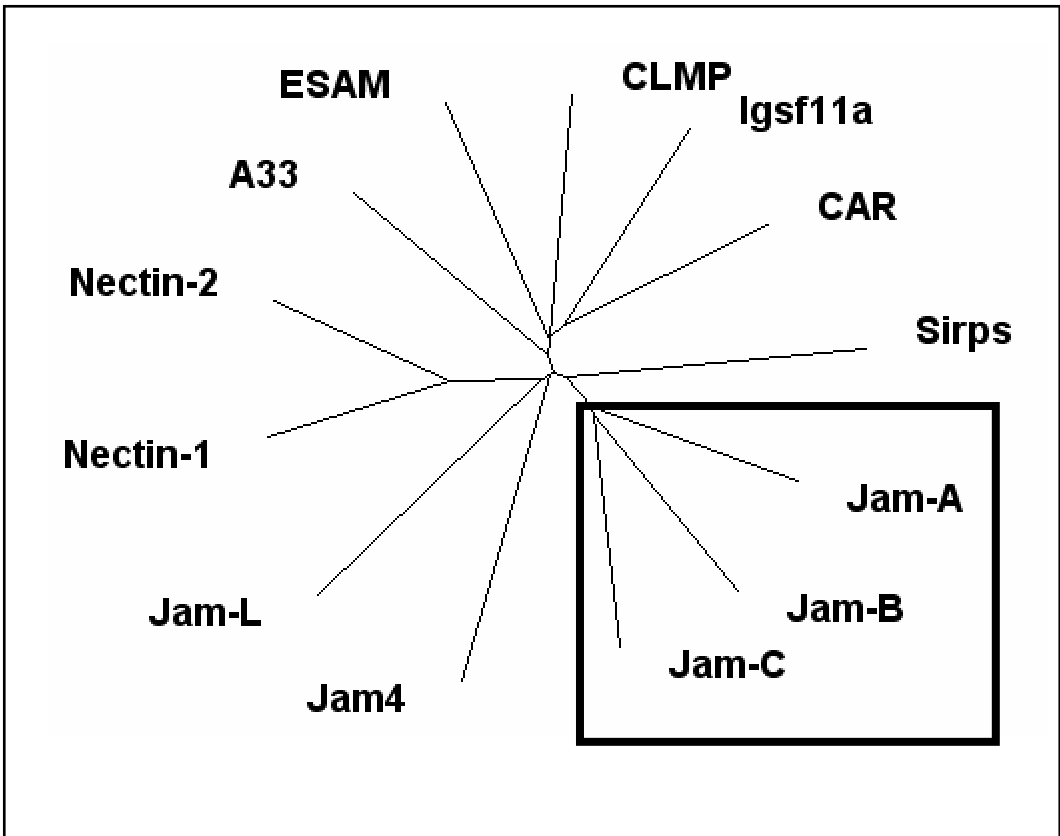


Figure 1.1: Phylogram tree generated with treeview from a CLUSTALW (http://www.ebi.ac.uk/clustalw/) alignment of various IgSF members. The relationship between JAM-A, JAM-B and JAM-C is highlighted in the box.

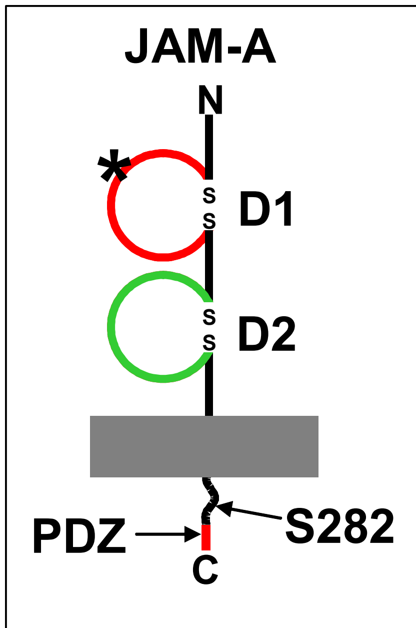


Figure 1.2: Structural schematic of the JAM-A protein. Key structural features include: D1, D2 - Ig loops; * - putative dimerization motif comprised of R-EWK in JAM-A, B and C; Tm – single pass transmembrane domain. PDZ - PDZ binding motif. S282 – Serine 282, a phosphorylation site. Adapted from (3).

membrane-distal Ig loop. In addition, the cytoplasmic tails of JAM-A, B, and C have potential phosphorylation sites. These structural features are highlighted in *figure 2*. Specific regions on JAMs have been linked to important protein functions. In particular there is a cis dimerization interface on the membrane-distal Ig loop composed of an R-EWK motif (the asterisk in *figure 2*)(4-6), a carboxy-terminal PDZ binding motif(4,7) and a phosphorylation site at S282 in both JAM-A(8) and JAM-C(9). Other functionally important regions remain to be determined, such as additional phosphorylation sites and additional structural requirements that mediate interactions of JAM proteins between cells.

<u>1.5 Tissue and Cellular Expression/Localization</u>

JAM proteins are expressed in a variety of tissues and cell types; however, the expression pattern for each family member differs. Such differences in expression patterns implies distinct functions for different JAMs. JAM-A is broadly expressed in endothelial cells (10), epithelial cells (10), fibroblasts (11) and hematopoietic cells(12,13), therefore it is not surprising that JAM-A expression has been reported in nearly every organ. JAM-B is expressed exclusively in vascular and lymphatic endothelium and was originally termed vascular-endothelial JAM to reflect this restricted distribution(14). Similar to JAM-A, JAM-C has a wider distribution with expression on endothelial cells(15), in lymphatics (15), leukocytes, (16), platelets(17), fibroblasts(11) and epithelial cells(18).

Cellular localization studies have revealed that JAM-A, B and C are expressed on the cell surface and concentrate at cell-cell junctions. In endothelial cells all three proteins localize to cell-cell junctions(10,14,15). This localization suggests that JAM-A,

B and C mediate adhesive interactions and could serve as potential ligands for migration of leukocytes. Interestingly, the distribution at endothelial cell-cell contacts is altered after treatment with inflammatory mediators. In particular, JAM-A is internalized from epithelial cell-cell contacts after treatment with cytokines such as TNF-α and IFN-γ(19), whereas JAM-C has been reported to redistribute from microvessel endothelial cell-cell contacts as a result of histamine and VEGF treatment(20). Interestingly, in polarized epithelial cells, which have well defined tight junctions, adherens junctions and desmosomes, JAM-A localizes primarily to the tight junction with some localization along the lateral cell border (see *figure 3*). JAM-C also localizes to intercellular junctions and has been reported to co-localize both with desmosomes and tight junctions in polarized intestinal epithelia(6,9,18).

From the above reports, it is apparent that JAM-A, B and C have overlapping but distinct expression and localization patterns. Indeed these observations would predict that JAMs are functionally distinct *in vivo*. Currently, it is not known how expression of JAMs is regulated in different tissues. Furthermore, little is known about how JAM proteins are targeted to various regions of the cell.

1.6 Homophilic Extracellular Interactions

JAM-A, B, and C all contain an R-EWK motif in the membrane-distal Ig loop, which has been reported to mediate homophilic dimerization in cis (4-6,14). Evidence for cis-dimerization is based on crystal structure data, cross-linking studies, functional studies from JAM-A dimer mutants(4,21,22), purified protein-cell binding for JAM-B (14) and functional data for JAM-C dimerization mutants in the R-EWK motif(6).

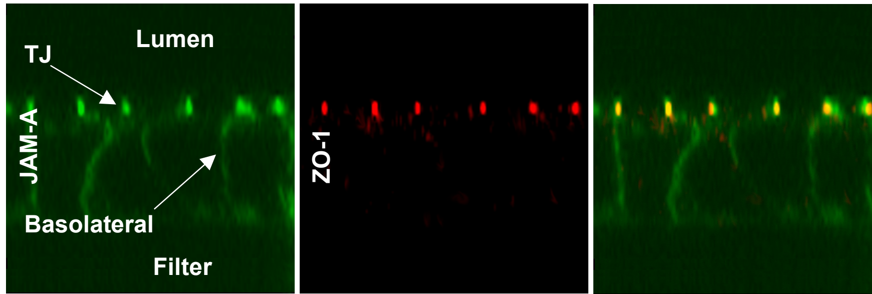


Figure 1.3: XZ reconstructed confocal immunofluorescence of JAM-A in T84 intestinal epithelial cells. JAM-A is shown in green and ZO-1 in red. Both are merged in the right panel. Note the concentration of JAM-A at TJ's as demonstrated by co-localization with ZO-1. Some lateral staining of JAM-A can be visualized. Adapted from (23).

Interestingly, there are no published reports demonstrating that the R-EWK motif is able to mediate interactions between cells (in trans).

Several lines of evidence indicate interactions between cells (in trans) occur between JAMs. For instance JAM-A overexpression in endothelial cells has been shown to mediate interactions with JAM-A on platelets(24). JAM-C interactions between cells has also been reported to mediate interactions between an epithelial tumor cell line and endothelial cells(6). In purified protein binding assays with JAM-A, B, and C, all mediate binding to CHO cells transfected with the corresponding JAM protein but do not mediate binding to untransfected CHO cells (6,25,26). Lastly, the murine JAM-A crystal structure(5) predicts that JAM cis dimer structures may interact in trans forming tetramers and higher order structures.

Despite the fact that the crystal structures of both murine and human JAM-A predict that R-EWK mediates cis dimerization between two JAM molecules (5,21), if two JAM-A molecules are aligned to interact in trans, the positive charge from arginine and lysine and the negative charge from glutamic acid in the R-EWK domain would form repulsive electrostatic interactions to inhibit trans dimerization. Thus, molecular mechanism(s) by which JAM molecules might interact in trans remains to be defined. Furthermore the above observations do not provide the stoichiometry for trans-interactions or conclusively demonstrate the existence of homophilic trans interactions in cells.

1.6 Heterophilic Extracellular Interactions

Currently, nearly all reports of extracellular binding interactions involving JAM-A, B and C have implicated the membrane-distal Ig loop with the exception of a single

report describing involvement of the membrane-proximal Ig loop in ligand binding. Perhaps the best documented reports of heterophilic interactions involve JAM-B and JAM-C(27). This interaction is sufficient to mediate disruption of JAM-C dimers by soluble JAM-B(28). Furthermore, immobilized JAM-B was actually used to purify JAM-C as an unknown ligand on leukocytes before the latter was identified. It is now apparent that JAM-B/JAM-C interactions are dependent upon the membrane-distal Ig loop for both binding partners. In addition, JAM-B/JAM-C dimers have been reported to interact with the leukocyte integrin $\alpha_m\beta_2$/Mac-I (28), while JAM-B has been reported to bind $\alpha_4\beta_1$/VLA-4(27) integrins; an interaction blocked by the presence of soluble JAM-C. Furthermore, in the testis, JAM-C has been demonstrated to directly bind to CAR presumably to maintain germ-line cell polarity(29). Additionally, through a yeast two-hybrid screen, JAM-A was reported to bind to the leukocyte integrin $\alpha_L\beta_2$/LFA-1. The authors further demonstrated binding of the membrane-proximal Ig loop of JAM-A to the ligand binding I domain of $\alpha_L\beta_2$/LFA-1(30,31) and reported that such binding interactions mediate leukocyte transendothelial migration, which will be discussed further below. There is also a report of association with $\alpha_v\beta_3$ integrins, but it is not known what domain of JAM-A is necessary for this interaction(32). There are likely more heterophilic interactions for the JAM proteins that are yet to be reported as the mechanisms for many JAM functions remain incompletely defined.

1.7 Intracellular Protein-Protein Interactions

The intracellular domains of JAM-A, B and C have been shown to mediate functional responses through interactions that are mainly mediated by the PDZ binding motif at the carboxy terminus (see *figure 1*). The PDZ binding motifs for JAM-A, B and

C are SSFLV-COOH, KSFII-COOH, and SSFLI-COOH respectively. However, not all of the intracellular protein interactions that have been reported appear to be dependent on these PDZ binding motifs suggesting that there may be, as of yet, undefined domains or phosphorylated residues in the cytoplasmic tail necessary for intracellular interactions. As shown in table 1, many cytoplasmic proteins have been reported to interact either directly or indirectly with JAM-A, B, or C (*table 1a, b or c respectively*). Of note, most of the cytoplasmic protein binding partners for JAMs are scaffolding proteins containing PDZ domains that serve to connect the plasma membrane to actin or microtubules and thus mediate signaling through the assembly of protein complexes. Specifically, such events may play a role in the regulation of cell polarity, to mention one example.

1.8 Cellular Function Mediated by JAM-A, JAM-B and JAM-C.

1.8.1. Determination of cell polarity.

Transport of substances across epithelia and endothelia requires the differential compartmentalization of proteins in the basal and apical aspects of the cell membrane. These different membrane domains are separated by the junctional complex in epithelia and endothelia and are determined by the polarity of the cells. Cell polarity is important for separating these membrane regions (basal vs. apical) as well as determining the direction of cell migration in response to chemotactic gradients in leukocytes. Interestingly, JAM-A and JAM-C have been implicated in the regulation of cell polarity in a variety of cell types.

JAM-A has been shown to interact with PAR-3/PAR-6/aPKC through its carboxy PDZ binding motif and the PDZ domain of PAR-3. PAR-3 and PAR-6 are highly conserved polarity proteins first described in *C. elegans*. They both contain PDZ

Table 1.1: Interactions of the JAM family cytoplasmic tails.

Table 1A	JAM-A
Protein	*Reference*
ZO family	(4,7)
Afadin	(7)
MUPP-1	(33)
PICK-1	(34)
PAR-3	(35,36)
CASK/LIN-2	(37)
Cingulin	(38)
Occludin	(38)

Table 1B	JAM-B
Protein	*Reference*
PAR-3	(39)
ZO-1	(39)

Table 1C	JAM-C
Protein	*Reference*
CAR	(29)
PAR-3	(39)
ZO-1	(39)

Table 1: Interactions of proteins with the intracellular tail of JAM-A (table 1a), JAM-B (table 1b), and JAM-C (table 1c) with references.

domains and function as scaffolding molecules that associate with atypical protein kinase C (aPKC). The proper cellular localization of this complex is involved in the establishment of cellular polarity through the action of aPKC. Recently, Rehder *et al.* reported that epithelial cells transfected with JAM-A lacking the PDZ binding domain had impaired cyst formation in 3D cell cultures, presumably due to a lack of interaction with PAR-3 and thus the PAR-3/PAR-6/aPKC complex(40).

JAM-C has been shown to interact with the PAR-3/PAR-6/aPKC complex in a similar manner, thus it is likely that JAM-C plays an important role in the maintenance of cell polarity as well. It has been reported that male JAM-C-/- mice are sterile due to the loss of polarization in spermatids, as defined by the absence of an acrosome and other polar structures. Presumably interaction of JAM-C in germ cells with JAM-B in sertoli cells is critical for development of polarity(41).

1.8.2. Barrier function

There are multiple lines of evidence that JAM-A plays a role in regulating barrier function as highlighted by the effects of its expression on trans- epithelial and endothelial monolayer resistance to passive ion flow (TER). TER is determined using Ohms law by assessing the potential generated across monolayers of cells cultured on permeable supports during the passage of a constant electrical current using specialized commercially available current/voltage clamps. Initially, overexpression of JAM-A was reported to increase resistance across transfected CHO cells(10). Furthermore, antibodies that bind near the cis-dimerization motif of JAM-A and inhibit JAM dimerization (23) as well as overexpression of the JAM-A intracellular domain(35) inhibit barrier recovery after disruption of intercellular junctions following transient calcium depletion(23,35). It

is unknown if antibodies that block cis-dimerization also block trans-dimerization. Interestingly, JAM-A dimerization blocking antibodies also enhance corneal swelling in rabbit eye preparations, suggesting that disruption of JAM-A dimerization inhibits barrier recovery(42). Based on the above evidence, it appears that cis-dimerization of JAM-A results in decreased cell monolayer permeability / increased TER. Additionally, treatment with TNF-α and IFN-γ(19) redistribute JAM-A from cell-cell contacts and cause increased permeability. Further evidence for a role of JAM-A in regulating barrier function is evident in siRNA studies where downregulation of JAM-A resulted in large increases in permeability of epithelial monolayers (3). Since altered permeability associated with manipulation of JAMs is well documented, it is possible that some of the effects of JAM-A manipulation on leukocyte migration could be indirect and related to altered cell permeability. Increased cell permeability could potentially enhance leukocyte transmigration by decreasing the integrity of the barrier through which the leukocytes must pass or by facilitating the diffusion of a chemotactic gradient. Thus, while JAMs have been implicated in the regulation of leukocyte transmigration, the mechanisms remain unclear.

1.8.3. Cell adhesion, integrin regulation and cell migration

JAM proteins have been reported to be involved in cell-cell adhesion, both directly and indirectly through interactions with other proteins such as integrins. JAM-A was first described having a role in adhesion of platelets to endothelial cells. Investigators observed that platelet adherence to activated endothelial cells was blocked by soluble JAM-A or peptide mimetics of the membrane-distal Ig loop suggesting direct binding of JAM-A (24). JAM-C has also been reported to directly mediate adhesion of a

tumor cell line to endothelial cells in culture(6). Adhesion of lymphocytes to endothelial cells has been shown to be mediated by interactions between JAM-B and JAM-C (43). The above reports serve as examples of direct binding of JAM proteins in mediating adhesive cell-based interactions; however, none define the exact stoichiometry of binding.

Indirectly, JAM proteins have been shown to modulate adhesion in a number of instances, usually through effects on integrin expression levels or activation. In endothelial cells, JAM-A siRNA has been reported to reduce adherence to vitronectin(44), and JAM-A-/- endothelial cells were shown to have decreased adhesion to fibronectin(45). Overexpression of JAM-A in endothelial cells results in increased adhesion to fibronectin that is mediated by αvβ3 integrin, that has been reported to directly interact with JAM-A(46). In epithelial cells, siRNA-mediated downregulation of JAM-A causes decreased adhesion of cells to collagen I, collagen IV, and fibronectin and this decrease is mediated by diminished β1 integrin protein expression(3). Our own observations indicate that interfering with JAM-A function through expression of dimerization-defective mutants causes decreased cell adhesion to fibronectin, suggesting a role for the dimerization interface in the regulating integrin protein expression levels (unpublished observations). Further examples of interactions of JAM proteins and integrins are highlighted by a JAM-B/JAM-C/αmβ2 complex that forms between endothelial cells and leukocytes during leukocyte transmigration(28). JAM-C has also recently been reported to regulate cell adhesion to fibronectin in an indirect fashion by activation of β1 and β3 integrins without changing integrin protein levels (9).

In some of the studies cited above, it was also observed that loss of JAM-A expression through siRNA-mediated downregulation or gene knockout resulted in decreased endothelial cell motility. Since cellular adhesion is an initial event in a number of cellular processes such as cell migration and angiogenesis, it is not surprising that JAM-mediated regulation of cell adhesion would also have an effect on cell migration. Indeed, depending upon the localization of active integrins, strong adhesive interactions could reduce cell migration, while strong interactions with an extracellular matrix substrate in cellular extensions could accelerate cell migration.

The mechanisms by which JAMs regulate cell adhesion are largely unknown. It is possible that JAM cis-dimerization may be required for the formation of a signaling complex through its interactions with scaffolding proteins. Cis dimerization is likely a necessary requirements for at least some of JAM-A functions since our unpublished studies indicate that interfering with JAM-A dimerization decreases cell migration. We propose that cis-dimerization is important for the formation of a signaling complex through increased spatial proximity of scaffolding proteins such as ZO-1 and Afadin by interactions with the JAM PDZ motif. Clues as to signaling events down stream of JAM-A come from studies that implicate the small GTPase, Rap1 (3,9), a closely related ras homologue that has previously been reported to regulate integrin levels(3,9). Specifically, our data indicate that Rap1a specifically is activated through interactions with scaffolding proteins in the presence of JAM-A and that active Rap1a protects β1 integrin from proteolysis. Other candidate elements of the JAM sigaling cascade include members of the MEK pathway where it has been reported that siRNA-mediated downregulation of JAM-A leads to decreases in pERK1/2 (44). From these observations

we can hypothesize that JAM-A dimerization may be necessary to assemble a signaling complex that includes multi-function scaffold proteins such as ZO-1 or afadin that in turn lead to activation of Rap1 and ERK. Such signaling events downstream of JAM-A, B and C are just beginning to be examined and may be the key to understanding mechanisms for the diversity of JAM functions detailed in this review.

1.8.4. Angiogenesis

Since endothelial cell migration and permeability are critical determinants of angiogenesis, it is not surprising that the JAM family members are involved in angiogenesis. It is likely that JAM regulation of angiogenesis is secondary to altered cell migration and adhesion. Alternatively, angiogenesis could be altered due to signaling events initiated by JAM proteins. There are several reports implicating JAM-A in angiogenesis. In studies with JAM-$A^{-/-}$ endothelial cells it has been reported that bFGF mediated endothelial cell migration and angiogenesis are attenuated. Such effects may involve the MEK pathway, as pERK1/2 is decreased after bFGF treatment in cells with downregulated JAM-A(32). JAM-C also has been reported to play a role in angiogenesis, as antibodies specific to JAM-C have been observed to decrease angiogenesis and size of tumors in nude mice under conditions of high VEGF levels(47). Interestingly, there are no reports investigating the role of JAM-B in angiogenesis. Decreased or blocked JAM-A and JAM-C thus appears to inhibit angiogenesis in the above reported settings, however, it remains to be determined if such inhibition is due to activation of various signaling pathways, changes in cell adhesion/migration or other currently undefined mechanisms.

1.8.5. Role of JAM-A in leukocyte transmigration

There are numerous reports suggesting a role for JAM-A in regulating leukocyte transmigration. In an early report on JAM-A it was noted that the anti-JAM-A antibody BV12 inhibited transendothelial migration of murine monocytes *in vivo*(10). Paradoxically, JAM-A deficient dendritic cells in the skin are reported to have increased migration and random motility compared to wild-type dendritic cells, suggesting impaired polarization of dendritic cells with enhanced migratory ability(48). It is now apparent that JAM-A$^{-/-}$ neutrophils have a defect in polarization that most likely results in diminished capacity to migrate(49). In ischemia/reperfusion mouse models, there is decreased neutrophil transmigration in hepatic tissues in the absence of JAM-A(50). Finally, in a model of atherosclerotic endothelium, JAM-A$^{-/-}$, ApoE$^{-/-}$ mice have impaired macrophage recruitment compared to JAM-A$^{+/+}$, ApoE$^{-/-}$ mice.

The above studies are highly suggestive that JAM-A plays a key role in leukocyte transmigration. However, they do not mechanistically define the connection between JAM-A expression and leukocyte transmigration. There are a number of possibilities that may provide insight into how JAM-A regulates leukocyte transmigration. In particular, three well defined effects of JAM-A expression are decreased paracellular permeability, increased adhesion of leukocytes to endothelial cells and leukocyte polarization. JAM-mediated changes in paracellular permeability could alter leukocyte transmigration across endothelial and epithelial monolayers simply by tightening or loosening cell-cell contact through which the leukocytes must pass or by regulating the diffusion of chemotactic gradients. Likewise, altered cell adhesion is an early step in leukocyte transmigration and changes in both homophilic JAM interactions followed by altered integrin levels could lead to increases or decreases in leukocyte transmigration. Lastly, determination of cell

polarity in leukocytes in response to a chemotactic gradient is crucial for directed migration across epithelial and endothelial monolayers. As has been reported for JAM-A$^{-/-}$ neutrophils and dendritic cells, absence of JAM-A expression presumably leads to abnormal PAR-3/PAR-6/aPKC complex localization and hence defective cell polarization. There could be additional signaling events influenced by the absence or alteration of JAM-A protein levels that could influence leukocyte transmigration through as of yet undescribed mechanisms. A final possibility for the connection between JAM-A and leukocyte transmigration would be a JAM-A-leukocyte ligand interaction.

There are conflicting reports on the existence of a JAM-A-leukocyte ligand interaction. Initially, JAM-A encoded by a full-length cDNA was identified as binding to the leukocyte integrin LFA-1($\alpha 1\beta 2$) in a yeast two-hybrid assay using the $\alpha 1$ subunit as bait. It was reported that the membrane-proximal extracellular domain of JAM-A is a ligand for LFA-1 and migration of Jukrat T-cells across activated endothelial cells was inhibited by both anti-JAM-A mAb and anti-LFA-1 mAbs (31). A second report was published by the same group indicating that JAM-A binds to the I domain of LFA-1 through the membrane-proximal Ig loop of JAM-A (30,31). In contrast, another early report indicated that inhibition of JAM-A with multiple dimerization-blocking antibodies did not inhibit leukocyte transmigration across endothelial cells *in vitro*(51). Furthermore, while JAM-A is localized to cell-cell contacts where neutrophils cross the endothelial monolayer, it does not colocalize with LFA-1 as would be expected if CD11a/CD18 was a ligand for JAM-A(52). From these studies, it is clear that more studies are necessary to understand the in-vivo relevance of JAM-A binding to LFA-1.

1.8.6. Role of JAM-B/JAM-C in leukocyte transmigration

JAM-B has been demonstrated to mediate interactions with lymphocytes through a heterophilic interaction with JAM-C(43). In addition, inhibition of JAM-C interaction with JAM-B through a blocking antibody (H33) resulted in redistribution of JAM-C to the luminal aspect of endothelial cells monolayers that correlated with increased leukocyte transmigration (28). The authors hypothesized that interactions with junction associated JAM-B results in sequestration of JAM-C to cell-cell contacts. Under this scenario, inhibition of binding between JAM-B and JAM-C would "release" JAM-C to the cell surface for subsequent participation in endothelial-leukocyte interactions.

There are two reports demonstrating direct binding of JAM-C to the leukocyte adhesive integrin CD11b/CD18 (MAC-I)(17,18) . Leukocyte migration across endothelial and epithelial monolayers was inhibited with JAM-C inhibiting antibody treatment (Gi-11 or luca14) or soluble JAM-C(17,18). Furthermore, *in vivo* overexpression of JAM-C in endothelial cells has been reported to reduce circulating white blood cell levels, presumably due to increased JAM-C dependent migration into tissues(18,53,54) however it is not known if JAM-C plays a role in hematopoiesis or egress of leukocytes from the bone marrow. Finally, it has been reported that JAM-C expression is increased in endothelial cells during cerulean-induced pancreatits and the severity of disease is attenuated in parallel with reduction of leukocyte infiltration by anti-JAM-C antibodies(55). The above evidence suggests that JAM-C directly binds to CD11b/CD18 on leukocytes, and this interaction is important for leukocyte transmigration *in vitro* and *in vivo*. Details of specific regions on JAM-C that mediate such interactions remains to be determined. It will be intriguing to see if specific

mutations of JAM-C result in attenuated transmigration through altered ligand binding or if altered transmigration is secondary to other JAM-C/JAM-B mediated interactions.

1.8.7 Role of JAM-A in recovery from colitis and cellular proliferation

Physiologic and cellular changes in the colonic mucosa of JAM-A knockout mice were reported in 2007(56) and the findings confirmed by another group(57). It was shown that loss of JAM-A results in enhanced gastrointestinal permeability both *in vivo* and *ex vivo*. In the colon, JAM-A deficient animals were shown to have enhanced development of isolated mucosal lymphoid follicles and increased neutrophil infiltration, presumably secondary to leakage of lumenal bacterial products into the subepithelial space. The colonic epithelium of JAM-A knockout mice was also shown to have a "proliferative" phenotype when compared to wild-type animals as determined by Ki-67 staining. This intriguing proliferative phenotype suggests that there is a compensatory "balance" by apoptosis, necrosis or anoikis, since there is evidence of gross histologic hyperplasia(56).

JAM-A knockout mice have also been shown to be more sensitive than wild type animals to development of experimental colitis by administration of DSS.(56) Interestingly, mice lacking JAM-A in the hematopoietic and vascular lineage do not show increased sensitivity(57), which further supports the findings of Laukoetter *et al.* suggesting that the susceptibility to experimental colitis is secondary to loss of epithelial JAM-A expression.

The above *in vivo* and *in vitro* observations serve to emphasize the important role of JAM-A in regulating colonic mucosal homeostasis. These findings provide ample

evidence for the importance of JAM-A and its functional *in vivo* effects but do not provide a unifying mechanism linking JAM-A expression and function.

1.9. The relationship of JAM function and human diseases: therapeutic implications.

JAM proteins have been linked to pathophysiology of several disorders in addition to being a reovirus receptor. It has been detailed how JAM-A and JAM-C have important roles in cellular adhesion and angiogenesis, thus their expression may be key in the growth of primary tumors, tumor metastasis and survival of cancer cells. Additionally, given the roles of the JAM proteins in cell permeability, cell polarization and heterophilic ligand binding, they may likely play key roles in a number of inflammatory conditions such as pancreatitis (for JAM-C(55)) and ischemia/reperfusion (for JAM-A(49)). Expression of JAM-A and JAM-C have also been implicated in atherosclerosis as wild-type endothelial cells lacking either protein have less severe atherosclerosis compared to endothelial cells deficient for JAM-A or JAM-C(58,59). Furthermore, the role of JAM-C as a polarity molecule makes it a potentially crucial determinant in male fertility, as disruptions in JAM-C inhibits spermatid polarization and cause infertility in mice(41). If further studies reveal that JAM-C deficiency is a cause of human infertility, gene therapy with JAM-C might be worth considering as a way to produce mature spermatids. Finally, the localization of JAM-A at intercellular junctions in mucosae and the vasculature is likely a reason that viral agents have evolved to exploit it to gain entry into the host. In the phylogram of figure 1, an interesting feature of several JAM-related IgSF proteins is their use as a receptor for a number of viral pathogens. In particular, CAR is a receptor for adenovirus and nectin is a receptor for

herpes virus. Similarly, JAM-A has been shown to serve as receptor for reovirus(60) and calcivirus(61).

Given the binding interactions reported for JAM-C and β2 integrin, JAM-C would be a potential anti-inflammatory target, as inhibition of such binding could potentially decrease pathologic leukocyte transmigration. Along similar lines, small molecule-mediated inhibition of dimerization of JAM-A could possibly be exploited to increase vascular or mucosal permeability for a number of applications in addition to inhibition of leukocyte migration.

1.10. Conclusions

JAM proteins are important mediators of cell polarity, paracellular permeability/barrier function, cell adhesion/migration and angiogenesis. The multiple functions of JAMs appear to be linked to key structural features shared by various family members. In particular, the dimerization motif within the membrane distal Ig loop might be an attractive target for development of therapeutics. Evidence discussed above indicates that disruption of this motif may attenuate a variety of pathologic conditions while also providing an avenue to enhance drug delivery across cellular barriers. Despite the growing list of reports on function of JAM-A, B and C, the precise mechanism(s) governing JAM function remain a mystery. A better understanding of signaling pathways influenced by JAM protein expression and ligand interactions will provide important insights into how this important protein family contributes to health and disease.

<u>Chapter 2</u>

Cis-dimerization mediates function of Junctional Adhesion Molecule A

Material from this chapter also appeared in an article in Molecular Biology of the Cell: 2008 May;19(5):1862-72. I am the first author and contributing authors of this article were: Liangyong Jiang, Andrei I. Ivanov, Kenneth J. Mandell, Asma Nusrat and Charles A. Parkos.

2.1 Abstract

JAM-A is a transmembrane component of tight junctions that has been proposed to play a role in regulating epithelial cell adhesion and migration, yet mechanistic structure-function studies are lacking. While biochemical and structural studies indicate that JAM-A forms cis-homodimers, the functional significance of dimerization is unclear. Here we report the effects of cis-dimerization-defective JAM-A mutants on epithelial cell migration and adhesion. Overexpression of dimerization-defective JAM-A mutants in 293T cells inhibited cell spreading and migration across permeable filters. Similar inhibition was observed with using dimerization-blocking antibodies. Analyses of cells expressing the JAM-A dimerization-defective mutant proteins revealed diminished β1 integrin protein but not mRNA levels. Further analyses of β1 protein localization and expression after disruption of JAM-A dimerization suggested that internalization of β1 integrin precedes degradation. A functional link between JAM-A and β1 integrin was confirmed by restoration of cell migration to control levels after overexpression of β1 integrin in JAM-A dimerization-defective cells. Lastly, we show that the functional effects of JAM dimerization require its carboxy-terminal PDZ binding motif. These results suggest that dimerization of JAM-A regulates cell migration and adhesion through indirect mechanisms involving post-transcriptional control of β1 integrin levels.

2.2 Introduction

Junctional adhesion molecule-A (JAM-A) is a transmembrane component of tight junctions in epithelial and endothelial cells. In addition, JAM-A is expressed on the surface of blood cells including leukocytes and platelets (12). JAM-A has been implicated in a diverse array of functions including intercellular junction assembly (23,25), cell adhesion (3), leukocyte transmigration (10,31,49,50,62,63), platelet activation (13,24,64,65), and angiogenesis (32,44). Additionally, JAM-A has been shown to be a receptor for reovirus (21,60,66,67). Structurally, JAM-A consists of an extracellular domain with two Ig-like loops, a membrane-spanning segment and a cytoplasmic tail containing a C-terminal PDZ-binding motif. The cytoplasmic tail of JAM-A has been reported to associate, either directly or indirectly, with PDZ domain containing proteins, such as ZO-1 (7,38), AF-6/Afadin (7) and Par3/ASIP (35,36), through characteristic hydrophobic residues (FLV) at the carboxy terminus. Evidence suggests that the cytoplasmic tail plays an important role in directing JAM-A localization to intercellular contacts (8), formation of tight junctions (40) and transduction of intracellular signaling events (3,45,46). The extracellular domain of JAM-A can form homodimers through its N-terminal Ig loop (21). Furthermore, the human JAM-A crystal structure predicts dimers forming between molecules on the same cell (in cis) (21), however, the murine protein crystal structure predicts tetramer formation between the extracellular loops between cells (in trans) (5). Despite these intriguing observations, mechanistic studies linking dimerization of JAM-A to these functions are lacking.

JAM-A is abundantly expressed in polarized epithelia, yet its role in epithelial cell migration has not been studied. In endothelial cells, controversy exists concerning the

functional role of JAM-A in the regulation of cell migration In a study by Bazzoni *et al*., the absence of JAM-A in endothelial cells enhanced spontaneous and random cell motility by reducing the stability of microtubules and impairing the formation of focal adhesions (45). Transfection of full-length JAM-A, but not a C-terminal PDZ-binding motif deleted JAM-A mutant restored random cell motility. Recently, JAM-A was shown to interact with integrin αvβ3 and enhance endothelial cell migration on vitronectin when overexpressed, as well as enhance phosphorylation of focal adhesion kinase (FAK) and mitogen-activated protein kinase (MAPK) (46). None of the above studies examined the role of JAM-A dimerization in mediating these effects on migration.

We recently reported that transient knockdown of JAM-A expression in epithelial cells resulted in decreased protein levels of β1 integrin that correlated with altered cell shape and decreased cell adhesion (3). This study suggested that JAM-A may regulate cell adhesion by increasing integrin protein expression, however the mechanisms for these JAM-A mediated effects were not investigated and is the topic of this report.

Based upon these observations, we hypothesized that cis-dimerization of JAM-A plays a key role in regulation of cell migration. To test this hypothesis, we stably overexpressed wild-type and JAM-A with mutations in the putative dimerization domain in 293T cells, a human epithelial cell line that expresses low levels of JAM-A. Dimerization of the extracellular domain is mediated by the predicted formation of salt bridges in the membrane distal Ig loop D1. One dimerization-defective JAM-A mutant we studied has point mutations at two residues (E61A/K63A) predicted by the crystal structure to be required for dimerization (6163) and both mutations have been shown to disrupt dimerization *in vitro* (22). Mutation of either residue has been shown to result in

JAM-A formation of only monomers as assessed by gel filtration (68). A second dimerization-defective construct that we tested consists of JAM-A with a deletion of the distal most immunoglobulin-like loop, which is necessary for dimerization (DL1). We observed that overexpression of both the 6163 and DL1 dimerization-defective mutants resulted in decreased 293T cell migration and spreading. We also determined that these cellular effects are mediated by decreased β1 integrin protein levels. Notably, for the dimerization-defective constructs to have an effect, we determined that there must be a carboxy terminal PDZ binding motif on JAM-A, suggesting that dimerization-defective mutants mediate their effects through sequestration of scaffolding proteins. These observations indicate that JAM-A dimerization indirectly regulates cell migration through signaling events that ultimately increase β1 integrin protein levels resulting in increased cell adhesion, spreading and migration.

2.3 Experimental Procedures

Cell Culture—293T human embryonic kidney epithelial cells were grown in Dulbecco's Modification of Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 IU of penicillin, 100 μg/ml streptomycin, 15 mM HEPES, and 1% nonessential amino acids (Cellgro). The cells were subcultured and harvested with 0.05% trypsin with EDTA in Hanks' balanced salt solution (Sigma). SK-C0-15 cells, a transformed human colonic epithelial cell line(3,69,70) were cultured as previously described in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM l-glutamine, 15 mM HEPES, 1% nonessential amino acids, 40 μg/ml penicillin and 100 μg/ml streptomycin, pH 7.4.

JAM-A mutant plasmid production—Production of the plasmids encoding full-length JAM-A, the JAM-A truncation mutant lacking the N-terminal Ig-like loop (DL1) and a JAM-A point mutant containing substitutions at residues 61 and 63 (E61R/K63E, 6163) have been described previously (22). Briefly, the coding region along with ~1.5kb of the 3'UTR was restriction enzyme digested and inserted into a pIRES-EGFP vector. Plasmids of all four constructs for transient transfections were amplified by PCR (5'-ATATGGTACCAGCCACCATGGGGAC AAA-3'; 5'-ATATCTCGAGTCACACCAG GAATGACGAGGTCTG-3') and digested with KpnI and XhoI before ligation into pCDNA3.0. A construct lacking both the DL1 and last three amino acids (FLV) was made from the DL1 mutant using PCR (5'-ATATGGTACCAGCCACCATGGGGACAAA-3'; 5'-ATATCTCGAGTCATGACGAGGT CTGTTTGAA-3'). PCR product was digested and ligated into pCDNA3.0 as described above.

Stable cell lines - 293T cells were transfected with constructs and empty vector (pIRES2-EGFP) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The transfected cells were enriched using flow-cytometric-based cell sorting by gating on EGFP fluorescence. Single clones were grown under G418 selection and expression of EGFP in colonies was verified. Cell lines were verified for expression of JAM-A constructs by western blot and immunofluorescence. Early passages were frozen in FBS and 10% DMSO. No cell lines were used for more than 10 passages from transfection in these studies, and expression of EGFP was monitored before each experiment to ensure that cells used remained stably transfected.

Transient Cell Transfections- For plasmid transfections, the 293T cells were transfected using Lipofectamine 2000 (Invitrogen) in Optimem I (Invitrogen) according to the manufacturer's protocol. For β1 integrin overexpression, constructs containing full length of β1 integrin cDNA in pCMV-XL6OriGene) or the empty pCMV-XL6 vector were used at a concentration of 1.0ug of plasmid per ml and assays were performed 48 hours after transfection. Smartpool siRNA targeted to β1 integrin was obtained from Dharmacon (Dharmacon). For transient overexpression of JAM-A or JAM-A mutants, pCDNA3.0 with the JAM-A protein coding sequence or empty vector (pCDNA3.0) were used in the same manner as the β1 integrin construct. SiRNA transfections were performed in Optimem I (Invitrogen) with HiPerFect (Qiagen) according to the manufacturer's protocol using either a Smart Pool for β1 integrin or siRNA for cyclophilin B (a control gene) and the final concentration of siRNA was 50nM. Three JAM-A siRNA sequences were used at a total concentration of 50nM: 5'-AGGGTCACATGCCAATAAA-3', 5'-CAGTCTATTTATTAACTTA-3', and 5'-

TCCCTTCTAAGTAGACAGC-3'. Experimental time points for DNA and siRNA transfections were 48 and 72 hours respectively.

Antibodies - The murine monoclonal anti-JAM-A antibodies J10.4 and 1H2A9 were previously described (22,23). All other antibodies were obtained commercially: Murine anti-β1 integrin (BD Pharmingen), Rat anti-β1 integrin (Mab13) (BD Pharmingen), rabbit anti-β4 integrin (Santa Cruz), rabbit anti-phospho-paxillin (Tyr118) (Cell Signaling), rabbit anti-Rap1 (Upstate) and murine anti-tubulin (Sigma). Horseradish peroxidase-conjugated secondary antibodies were obtained from Jackson Immunoresearch, and fluorescently labeled secondary antibodies were obtained from Molecular Probes.

Cell migration assays— Cells were washed in Hank's Balanced Salt Solution without Calcium (HBSS-) and then incubated in Cell Dissociation Buffer (Enzyme free, PBS-based) (Gibco) for 30 minutes to disrupt cell junctions. Cells were then washed in HBSS-, centrifuged, and resuspended in serum-free DMEM. 1×10^5 cells were added on the top side of 8 μm pore size Transwell (Costar) inserts that had been coated with fibronectin overnight (10 μg/ml.) on the underside of the transwell (See Figure 2A). Cells were allowed to migrate across inserts toward the fibronectin-coated side for three hours at 37°C. Inserts were then washed, fixed with ethanol, and stained with Phallodin. Confocal fluorescence microscopy was performed on the lower chamber side of inserts using a laser scanning confocal microscope (Axioplan2 Zeiss microscope equipped with LSM510 Meta). Cells were counted from two randomly chosen fields of view from three separate inserts and average counts with standard error of the mean (SEM) used to quantify the extent of migration. For the study on the effects of J10.4 and 1H2A9

antibodies on cell migration, cells were treated with 10 μg/ml J10.4 or 1H2A9 at 4°C for one hour prior to the addition of cells into Transwell inserts.

Cell Spreading — 5 × 10^4 cells were added on coverslips coated with fibronectin (10ug/ml) in 24-well plates and incubated for one hour at 37°C. Phase-contrast microscopy was used to capture images at 5x magnification for cell spreading assays. Two separate fields from each of three coverslips were averaged to assess the extent of cell spreading. Rounded cells with phase sharp edges and no protrusions were defined as rounded, and cells with protrusions and flattened morphology were defined as spreading. To study cell protrusion changes, 2.5 x 10^4 cells were incubated on fibronectin (10ug/ml) coverslips at 37°C for 48 hours and washed three times in Hank's balanced salt solution (HBSS+). Cells were then fixed in ethanol, blocked in 1% BSA in HBSS+ and stained with Alexa-488-phalloidin (1:1000) or topro-3 (1:1000). Confocal fluorescence images were captured using a Zeiss laser scanning microscope. The length of all cellular protrusions 30 high powered fields from three separate experiments were measured using the Metamorph imaging program (Zeiss) and reported as average length with standard error of the mean.

Immunoblotting — Cells were homogenized in lysis buffer containing 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. Lysis buffer was supplemented with protease and phosphatase inhibitor cocktails containing 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF), pepstatin A, E-64, bestatin, leupeptin, aprotinin, microcystin LR, cantharidin, (-)-*p*-bromotetramisole, sodium vanadate, sodium molybdate, sodium tartrate, and imidazole (1:100 dilution; Sigma). Protein concentrations in lysates were quantified by

BCA assay. Lysates were cleared by centrifugation and immediately boiled in SDS sample buffer. SDS-PAGE and immunoblots were performed by standard methods. Immunoblots were probed for tubulin to ensure equal protein loading.

Immunofluorescence Microscopy — Cells were grown on transwell filters or glass coverslips, fixed in 3.7% paraformaldehyde for 20 minutes at 25°C, permeabilized with 0.2% triton X-100 for 10 minutes at 25°C, and blocked in 1% BSA in HBSS+ for 1 hour. Primary antibodies were diluted in blocking buffer and incubated with cells for one hour at 25°C. The cells were washed in HBSS+ and then incubated in fluorescently labeled secondary antibodies or Alexa fluorophore-conjugated Phallodin for 45 minutes at room temperature. Labeled cells were then washed and mounted in Prolong Antifade Agent (Molecular Probes). Confocal fluorescence images were captured using a Zeiss laser scanning microscope.

Chemical Inhibitors – MG-132 (Calbiochem) was used at 10μM final concentration, while MG-262 (Biomol) was used at 20μM final concentration. Cyclohexamide (MP Biomedicals) was used at a final concentration of 50ug/ml.

Real-time RT-PCR—Total RNA was extracted from cells using RNeasy® mini Kit (Qiagen) according to the manufacture's protocol. RT-PCR was performed by using iScript™ One-Step RT-PCR kit with SYBR Green (Bio-Rad) on iCycle iQ real-time PCR detection system (Bio-Rad) according to the manufacturer's instructions. A pair of PCR primers (5'-ATCCCAGAGGCT CCAAAGAT-3' and 5'-CTAAATGGGCTGGTGCAG-3') was used to amplify β1 integrin. Primer pair (5'-CGGCTACCACATCCAAGGAA-3' and 5'-GCTGGAATTACCGCGGCT-3') targeting 18S RNA was used as internal control.

Rap1 Activity Assay - Active Rap1 was detected using a pull-down procedure (71). Cells were lysed at 4C in lysis buffer (50mM Tris-HCl, pH 7.4, 0.5M NaCl, 1% Nonidet P-40, 2.5mM $MgCl_2$, and 10% glycerol) supplemented with protease inhibitor cocktails (1:100; Sigma). The lysates were clarified by centrifugation and then 200ug of protein from each lysate was incubated with 30ul agarose beads conjugated with Ral GDS-Rap-binding domain (Upstate) for 45 min at 4°C. The beads were washed four times in lysis buffer, resuspended in 2x reducing SDS sample buffer, and boiled for 15 min. The entire sample was then loaded into each well for separation by SDS-PAGE, and active Rap1 detected by immunoblot.

2.4 RESULTS

Expression of dimerization-defective JAM-A mutants inhibits migration of 293T cells

To investigate the role of JAM-A cis-dimerization in cell migration, we used 293T human embryonic kidney epithelial cells that normally express low levels of JAM-A protein. We generated 293T stable cell lines expressing two different JAM-A mutants that either lack the distal extracellular Ig loop (DL1) or contain mutations in a characteristic dimerization motif within DL1 (6163). The 6163 mutant contains two point mutations at residues 61 and 63 that are involved in the formation of JAM-A homophilic dimer salt bridges (fig 1A) (21,22). Both mutants have been reported to be unable to form extracellular homophilic dimers as reported by gel filtration (22). Furthermore, in western blots of JAM-A from cells expressing the 6163 mutant that were treated with a cell impermeable crosslinker, we observed no JAM-A dimerization in contrast to results from cells expressing only wild type protein (Supplemental Figure 1). We also generated two control cell lines, one that overexpresses wild type JAM-A and

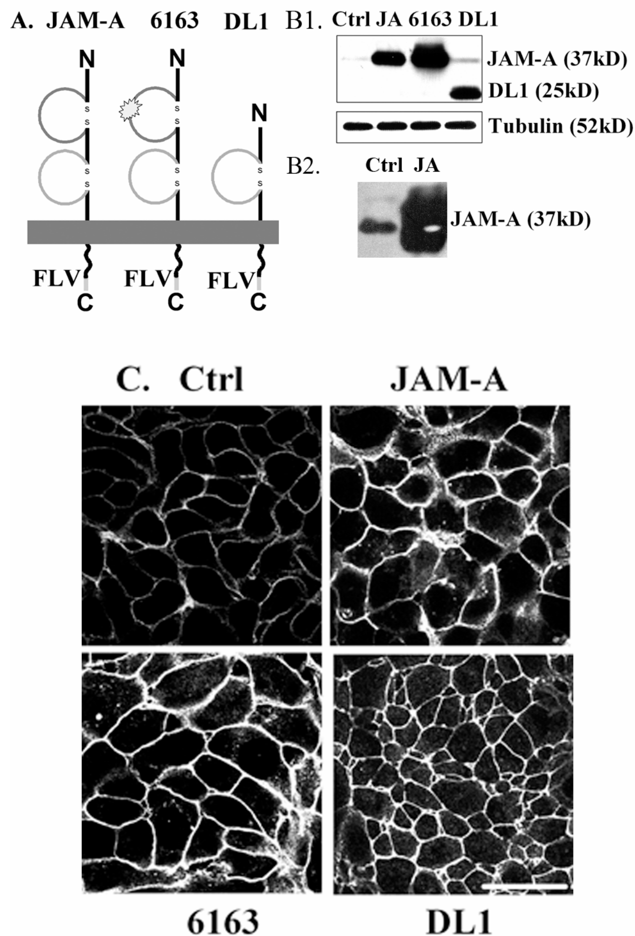
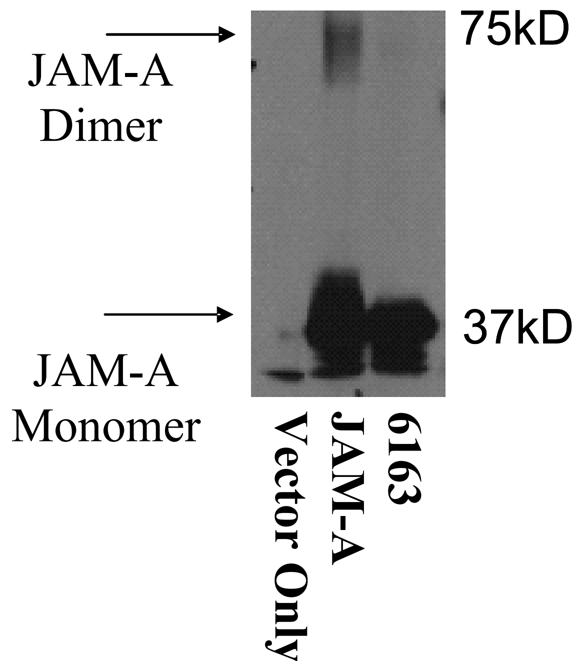


Figure 2.1. Stable expression of JAM-A mutants in 293T cells. A, The structure for endogenous JAM-A is shown and contains two Ig-like loops, a transmembrane domain, and a cytoplasmic tail with a carboxy-terminal PDZ binding domain. The star in the 6163 mutant highlights the region of mutations at amino acids 61 and 63 that forms a salt-bridge between two JAM-A molecules in cis. The DL1 mutant completely lacks the distal most Ig-like domain that mediates cis-dimerization. B, Western blots demonstrating overexpression of wild-type and mutant proteins in 293T stable transfectants. Tubulin is shown as a protein loading control. A 10 second film exposure (Fig1B1) and a 2 minute exposure (1B2) are shown to demonstrate the presence of JAM-A overexpression in the stable cell lines and to highlight the presence of endogenous JAM-A, respectively. C, Immunoflourescence labeling of 293T cells expressing mutant constructs for JAM-A protein demonstrating similar localization of endogenous JAM-A, exogenous JAM-A and mutant JAM-A to cell-cell contacts.



Supplemental Figure 2.1. Formation of JAM-A dimers in CHO cells. Plasmids encoding either full-length JAM-A or the salt-bridge JAM-A mutant 6163 were transfected into CHO cells and proteins were crosslinked with the cell impermeable cross-linker BS3. The lysates from these cells were probed with anti-JAM-A, and demonstrate the formation of a dimer at 75kD in cells expressing wild-type JAM-A, but not in those expressing 6163.

another containing the appropriate empty vector control (pIRES2-GFP). As shown in fig 1A and 1C, 293T cells normally express endogenous JAM-A which localizes to cell-cell contacts. Expression levels of vector control, wild type overexpressing, and mutant JAM-A 293T cell lines were analyzed by western blotting and are shown in Figure 1B1 after 10 seconds of exposure by enhanced chemiluminescence (ECL). Figure 1B2 highlights, by longer ECL exposure (2 min), endogenous JAM-A expression compared to that in cells stably transfected with JAM-A. Furthermore, immunofluorescence analyses indicated that in overexpressing cells, exogenous JAM-A localized to cell-cell contacts in a fashion similar to endogenous, control JAM-A (fig 1C).

Initially, we investigated the role of JAM-A dimerization on cell migration across matrix coated permeable transwell filters. As shown in Fig 2A, transwell inserts with 8.0um pores, which permit passage of 293T cells, were coated with fibronectin on the bottom of the transwell insert. Cells stably expressing the dimerization-defective JAM-A mutants or controls were added to upper chamber of the setup and incubated for three hours at 37°C. Cell migration across filters was quantified after Phallodin staining and immunofluorescence analysis by confocal microscopy. As shown in figures 2B-C, overexpression of dimerization-defective JAM-A resulted in significantly decreased transfilter migration (200-250 cells/mm^2) compared to the empty vector control as well the wild-type JAM-A overexpression control (500-600 cells/mm^2). These data suggest that the 6163 and DL1 JAM-A mutants, which prevent dimerization, have dominant-negative effects on JAM-A functions, as exemplified by the decreased levels of observed cell migration.

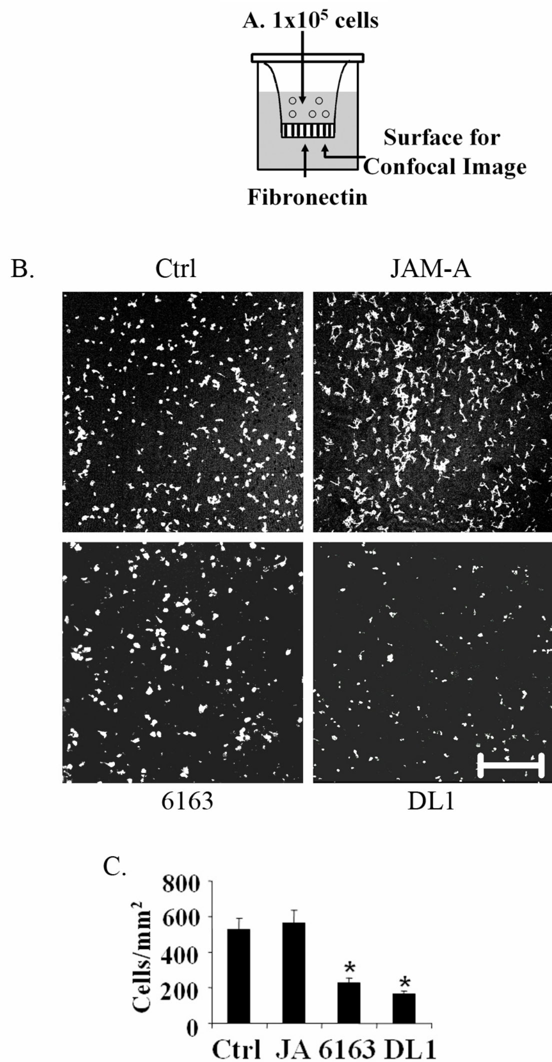


Figure 2.2. JAM-A dimerization-defective mutant effects cell migration. A, Schematic for cell migration assays. As detailed in the methods, 293T cells suspended in DMEM without serum were added to the upper chamber of transwell filters coated with fibronectin (10ug/ml) on the bottom of the filters. To assess the extent of cell migration, cells were stained with phallodin and confocal images were taken of the underside of the transwell filter. B, Cell migration assays revealed that overexpression of DL1 and 6163, but not wild-type JAM-A resulted in decreased cell migration. Scale bar is 200uM. C, The number of cells that migrated per mm^2 over three hours was determined from 3 separate filters after assessing two photomicrographs of each filter and counting the number of cells using Metamorph software. Average counts±SEM are shown. As can be seen, overexpression of 6163 and DL1 mutants significantly decreased cell migration (*, $p<0.05$).

Dimerization-defective JAM-A mutants inhibit cell spreading on fibronectin

To gain insight into mechanisms underlying inhibitory effects of the dimerization-defective JAM-A mutants on cell migration, we next analyzed mutant cells for effects on adhesion and spreading on extracellular matrix. Preliminary adhesion experiments suggested that a higher percentage of 293T cells adhered to collagen I, collagen IV, and fibronectin compared to laminin. Furthermore, adhesion was decreased after downregulation of expression of JAM-A (3). Further experiments were performed using fibronectin-coated surfaces. Serum-starved control and stably transfected cells were seeded on fibronectin-coated coverslips. After one hour, phase contrast images were taken and analyzed as detailed in the methods. As shown in figures 3A and 3B, control 293T cell lines adhered and spread on fibronectin. In contrast, cells bearing either dimerization-deficient JAM-A mutant failed to spread and remained rounded. The decrease in cell spreading in JAM-A dimerization-defective mutants versus control cells was 90%.

We hypothesized that the observed decrease in spreading would correlate with altered length of cellular extensions after prolonged growth on matrix. Phalloidin labeling was used to highlight F-actin in 293T cells actively spreading on fibronectin for two days. Indeed, confocal analysis revealed extensive elongated protrusions in control cells with an average length of 25-35 μm, whereas the length of cellular protrusions was significantly decreased in the dimerization-defective JAM-A expressing cell lines with an average length of 10-15μm (fig 4). Taken together, these data suggest that dimerization of JAM-A may be required for spreading and formation of peripheral membrane protrusions which represents an important early step in cell migration.

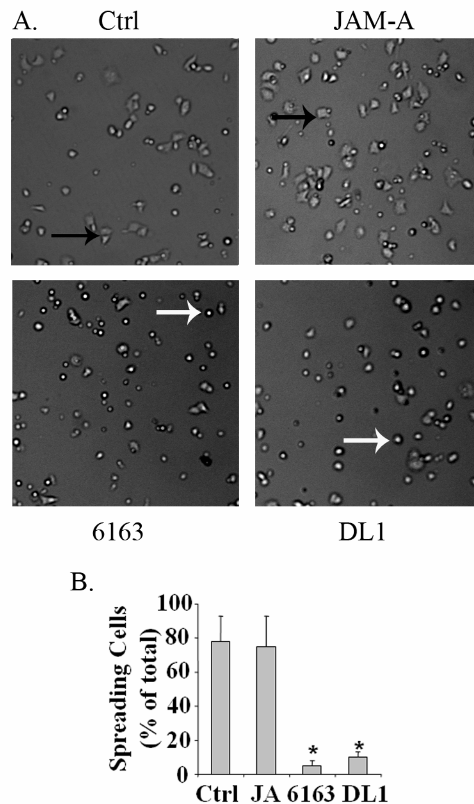


Figure 2.3. JAM-A mutants alter 293T cell spreading. A, Cell spreading assays reveal that dimerization-defective mutants 6163 and DL1 decrease cell spreading. Serum-starved 293T cells were added to fibronectin (10ug/ml) coated coverslips. After one hour at 37°C, phase contrast images were taken (2 images each for 3 coverslips per sample). Black arrows point to spreading cells and white arrows point to rounded cells. B, Spreading versus rounded cells were counted manually for each image, and average ± SEM was plotted. As can be seen, expression of dimerization-defective JAM-A significantly decreases 293T cell spreading (*, $p<0.05$).

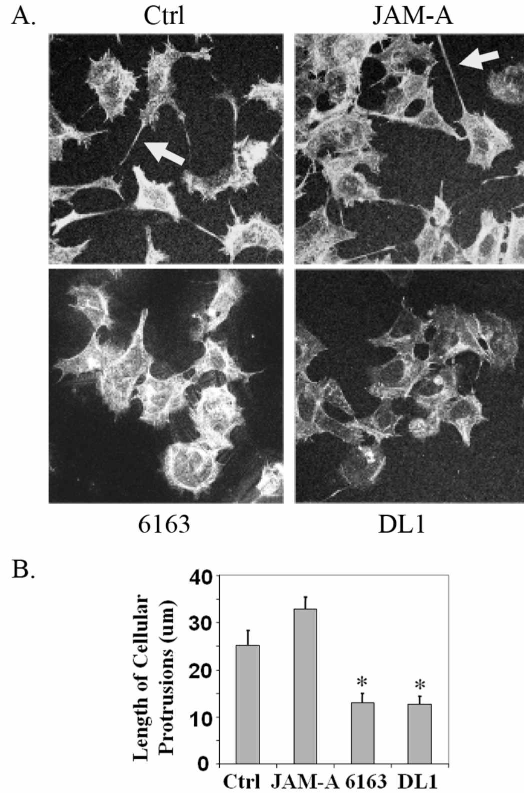


Figure 2.4. JAM-A dimerization defective-mutants decrease the length of 293T cell protrusions. A, Confocal microscopy revealed a decrease in both the length and number of cellular protrusions (white arrows) in 293T cells overexpressing the 6163 and DL1 mutants. Scale bar is 50μM. B, All cell protrusions in 8 images were measured using the program Image J. Bars are the average length in μM + SEM. As can be seen, cellular protrusions in JAM-A dimerization-defective cells are significantly shorter than those in cells expressing wild type JAM-A (*, $p<0.05$).

Dimerization defective JAM-A cell lines have a decreased density of focal concentrations of phosphorylated paxillin.

Since focal adhesion (FA) complexes are involved in cell attachment, spreading and migration, we hypothesized that expression of dimerization-defective JAM-A cell lines influences FAs. Experiments were performed to investigate if overexpression of dimerization-defective JAM-A mutants affects the assembly of FAs in 293T cells. FAs were visualized by immunofluorescence labeling of the phosphorylated forms of their major protein component, paxillin. Immunofluorescence staining revealed that PY118-paxillin was predominantly localized within discrete peripheral rod-like structures characteristic of FA in control cell lines. Interestingly, the number of these phospho-paxillin-based focal concentrations of phosphorylated paxillin per mm^2 was dramatically reduced in cells bearing either the 6163 or the DL1 JAM-A mutants (fig 5A) from 2860±680 and 2648±435 for control lines to 1020±240 and 812±203 for the 6163 and DL1 cell lines respectively (fig 5B).

JAM-A dimerization defective mutants have decreased β1 integrin protein levels.

Since integrins concentrate at focal adhesions and mediate cell attachment to matrix during cell migration, we performed experiments to analyze the effect of JAM-A on integrins. β1 integrins are abundantly expressed in epithelial cells and known to bind to extracellular matrix components including collagen I, collagen IV, and fibronectin. We previously observed decreased β1 but not β4 integrin protein levels in cells after transient knockdown of JAM-A protein (3). We thus investigated if stable overexpression of dimerization–defective JAM-A mutants results in altered expression of β1 integrins. Densitometric analyses of western blots revealed a 73% decrease in β1

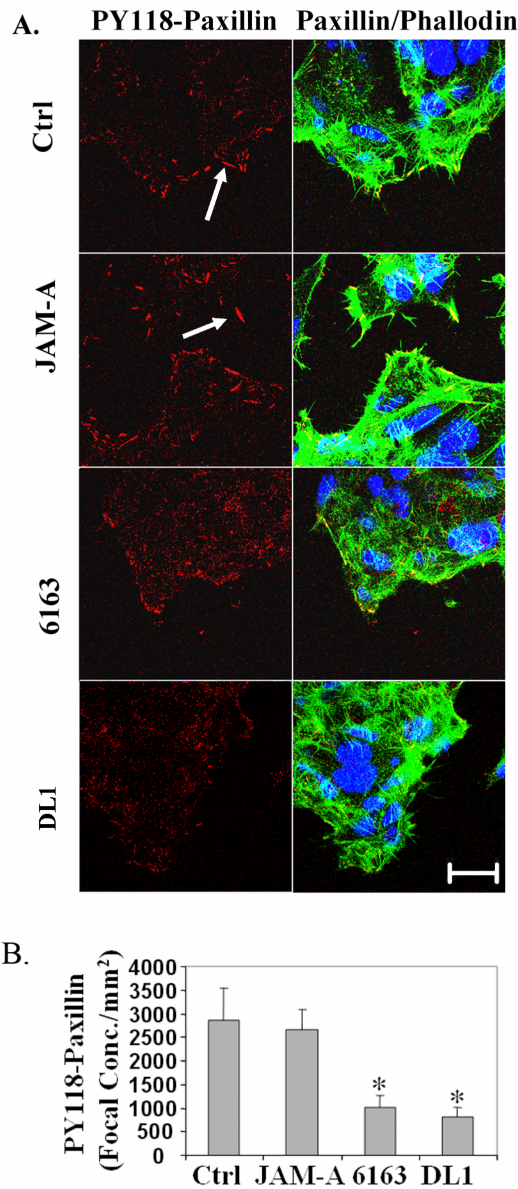


Figure 2.5. The number of PY118-Paxillin focal concentrations is decreased by overexpression of 6163 and DL1 mutants in 293T cells. A, Staining with Phallodin, anti-PY118 Paxillin, and Topro revealed that overexpression of both the 6163 and DL1 mutants significantly decreased the number and density of focal contacts as determined by PY-118 Paxillin staining. Scale bar is 20uM. B, PY118-Paxillin containing rod-shaped structures were counted with Metamorph software based on staining with anti-PY118 Paxillin. 5 slides were counted per sample and average + SEM/mm^2 was reported (*, $p<0.05$).

integrin protein levels in mutant cell lines compared to controls in subconfluent, actively spreading cells (fig 6A). Conversely, there was no effect on β4 integrin levels.

Decreased protein levels of β1 integrin in dimerization-defective JAM-A expressing cells are not due transcriptional effects.

The dimerization-defective JAM-A induced decrease in β1 integrin could be a result of either transcriptional inhibition, or altered posttranscriptional steps in β1 integrin biogenesis. To distinguish between these possibilities, we investigated if dimerization-defective JAM-A mutants have decreased β1 integrin mRNA levels. Primers that produced a single band of the appropriate size in RT-PCR were used for Quantitative real-time RT-PCR analysis (QRT-PCR). QRT-PCR demonstrated no significant change in mRNA levels in dimerization-defective JAM-A expressing 293T cells compared to the controls (fig 6B), suggesting that the expression of dimerization-defective JAM-A mutants did not result in downregulated β1 integrin gene transcription in 293T cells nor was there an affect on the stability of the mRNA. Conversely, these findings suggest that decreased β1 integrin protein levels in the mutant cell lines are the result of posttranscriptional inhibition, or accelerated degradation.

Active Rap1 is decreased in JAM-A dimerization-defective cell lines.

Recently, we demonstrated a decrease in the active form of the small GTPase Rap1 after downregulation of JAM-A expression in SK-CO15 cells (3). Furthermore, we demonstrated that downregulation of Rap1 by siRNA resulted in decreased β1 integrin levels. Thus, we examined whether Rap1 levels were altered in the JAM-A dimerization-deficient cell lines. Using standard pull-down assays to isolate active, or GTP bound Rap1, we analyzed the cell lines for activation status of Rap-1. As shown in

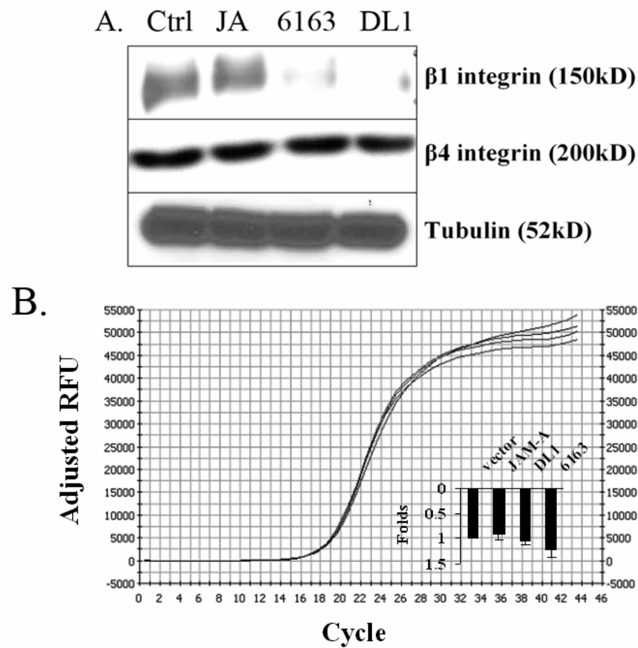
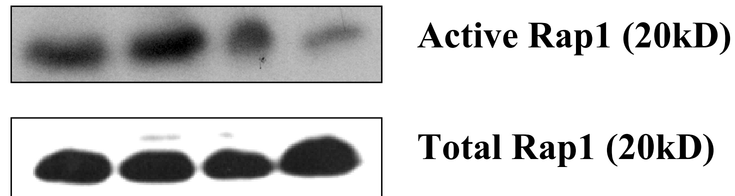
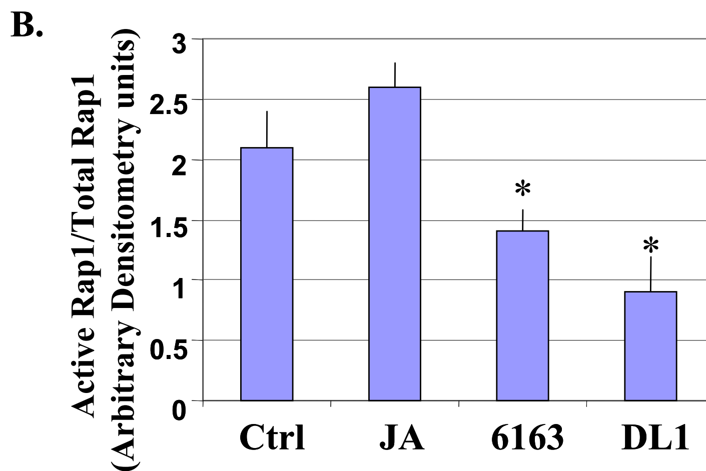


Figure 2.6. β1 Integrin protein expression, but not mRNA levels is decreased in the 293T JAM-A dimerization-defective cell lines. A, Immunoblotting revealed that 293T cells expressing 6163 or DL1 had dramatically decreased amounts of β1 integrin protein levels, with no change in β4 integrin or tubulin, which was used as a loading control. B, Real-time PCR analysis from control and transfected cell lines demonstrating no significant change in β1 Integrin mRNA for any of the overexpressing 293T cell lines.

A. Ctrl JAM-A 6163 DL1





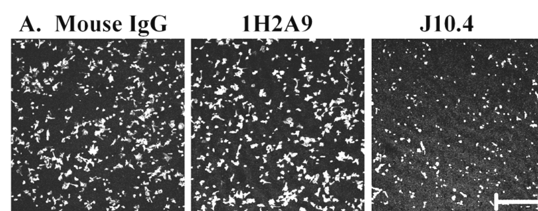
Supplemental Figure 2.2. JAM-A dimerization-defective mutants decrease Rap1 activity but not β1 Integrin mRNA levels. A, Western blot of active Rap1 obtained from control and transfected cell lines as detailed in the methods. As can be seen, there is significantly decreased levels of active Rap-1 in the dimerization defective cell lines while total levels of Rap1 remain unchanged. B, Densitometric analysis from three separate active-Rap1 pulldown assays. Data are reported in arbitrary units with each determination normalized to the total amount of Rap1 in that sample (*, $p<0.05$).

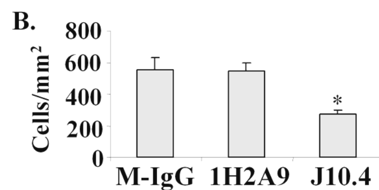
Supplemental Figure 2, the 6163 and DL1 mutant cell lines each had decreased active Rap1 levels compared to control cell lines. In concert with our previously reported findings, this observation suggests that active Rap1 may be a signaling link between JAM-A dimerization and β1 integrin protein levels.

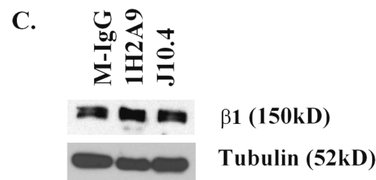
Inhibition of JAM-A dimerization with antibody reduces cell migration and β1 integrin protein levels.

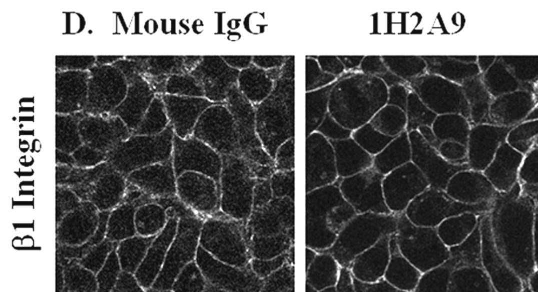
Given that expression of dimerization-defective JAM-A mutants in 293T cells inhibited cell migration and decreased β1 integrin protein level, we tested antibodies known to inhibit dimerization for effects on cell migration and β1 integrins. We were particularly interested in the time-course of effects of impaired dimerization of JAM-A since the stable mutant cell lines represent long-term (chronic) effects. We have previously shown that the JAM-A mAb J10.4 inhibits the formation of JAM-A dimers while the JAM-A mAb 1H2A9 binds to the D1 Ig loop, but does not inhibit dimerization at similar concentrations(22). 293T cells with endogenous levels of JAM-A were treated with 10 μg/ml J10.4 or 1H2A9 prior to the migration assays. As shown in figures 7A-B J10.4, but not 1H2A9, significantly inhibited 293T cell migration toward fibronectin when compared to a murine IgG control. Cells treated with murine IgG or 1H2A9 migrated at a rate of approximately 590 cells per mm^2 per 3h while migration was decreased by treatment with J10.4 to a rate of approximately 310 cells per mm^2 over three hours representing a 47% decrease. These results suggest that acute disruption of dimerization of JAM-A inhibits migration in 293T cells.

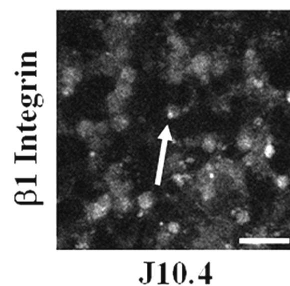
We then examined β1 integrin protein levels in cells treated with JAM-A mAbs. 293T cells were Ca^{++} depleted for 5 minutes to expose intercellular junctions followed by











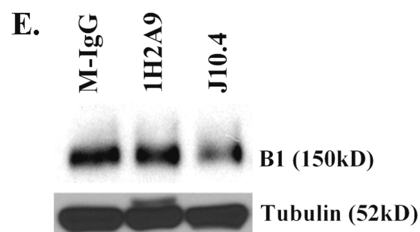


Figure 2.7. Antibodies blocking JAM-A dimerization inhibits 293T cell migration and alters β1 integrin localization. A, JAM-A antibody J10.4 (inhibits JAM-A dimerization) inhibits 293T cell migration. Serum-starved 293T cells were treated with 10 μg/ml mouse IgG, 1H2A9 (does not inhibit JAM-A dimerization) or J10.4 prior to migration assays. Confocal analyses revealed that J10.4 significantly inhibited 293T cell migration. Scale bar is 200uM. B, Blocking of JAM-A dimerization (J10.4) significantly reduced 293T cell migration compared to isotype (mouse IgG) and antigen-binding (1H2A9) antibody controls (*, $p<0.05$). C-E, 293T cells were Ca^{++} depleted for 5 minutes to expose intercellular junctions followed by treatment with J10.4 (JAM-A dimerization blocking antibody), 1H2A9 (non-dimerization blocking JAM-A antibody) or mouse IgG at 10ug/ml for 3 hours in DMEM. Western blots of cell lysates were then probed for total levels of β1 integrin (panel C) demonstrating no change in levels of β1 integrin. However, immunofluorescence photomicrographs of such cells stained for β1 integrin using a Rat anti-β1 integrin antibody (Panel D) reveal loss of lateral cell border staining after treatment with J10.4 but not 1H2A9 or mouse IgG. Note the appearance of β1 integrin labeled cytoplasmic vesicles (white arrow) in J10.4 treated cells suggesting internalization. Cells treated as in C&D with the addition of with cyclohexamide to prevent new protein synthesis show an increased rate of β1 integrin degredation after treatment with J10.4, but not with M-IgG or 1H2A9 (Panel E).

incubation with the dimerization-inhibiting mAb J10.4, the non-inhibitory JAM-A mAb 1H2A9, or mouse IgG for 3 hours (10ug/ml, 37C) followed by analysis for β1 integrin expression by western blot and immunofluorescence. As can be seen in the western blot (fig 7C), total levels of β1 integrin were not decreased after 3 hours of antibody treatment, however the immunofluorescence localization of β1 integrin in fig 7D demonstrates dramatic alterations after treatment with mAb J10.4 but not 1H2A9. Control antibody treated cells demonstrated a characteristic lateral expression pattern for β1 integrin, while J10.4 treated cells showed β1 integrin within cytoplasmic vesicles and very little staining at cell borders. These data suggests that mAb J10.4 induces internalization of β1 integrin. To examine if such stimulated endocytosis can lead to accelerated degradation of β1 integrin, new protein synthesis was inhibited with cyclohexamide and cells were incubated for four hours with mAbs J10.4, 1H2A9, or murine IgG1. As shown in Figure 7E, treatment with the dimerization inhibiting antibody J10.4 accompanied by inhibition of de novo protein synthesis resulted in enhanced degradation of β1 integrin protein (Figure 7E). These results, in concert with the mutant JAM-A data, suggest that JAM-A dimers stabilize β1 integrin at the cell surface and that disruption of JAM-A dimers is likely to trigger internalization and subsequent degradation of β1. Altered cell migration would then be an indirect consequence resulting from decreased cell surface expression of β1 integrin.

Reduced cell migration in JAM-A dimerization-defective cell lines is secondary to decreased β1 integrin protein levels.

We reasoned that decreased levels of β1 integrin observed in the dimerization-defective JAM-A expressing cell lines was linked to the observed JAM-A-mediated

decreases in cell migration. We thus downregulated β1 integrin expression using siRNA in 293T cells to determine if similar inhibitory effects would occur as observed with the JAM-A dimerization-defective cell lines. Compared to control siRNA specific for cyclophilin B, β1 integrin expression was virtually ablated in 293T cells as assessed by western blot (fig 8A). Importantly, reduction of β1 integrin expression had no effect on JAM-A protein levels (fig 8A). When such cells were tested in cell migration assays, there was significant inhibition of cell migration compared to siRNA treated controls in a fashion not significantly different from that observed with JAM-A mutants. Cells with downregulated β1 integrin protein levels migrated at a rate of 190 cells per mm^2 over the course of three hours, while cells with control siRNA treatment migrated at a rate of 470 cells per mm^2 (fig 8B-C).

To determine if the cell migration defect in the JAM-A dimerization mutant cell lines could be rescued β1 integrin was overexpressed in 293T cells stably transfected with the JAM-A dimerization-defective mutants. As shown in figure 8D, transfection resulted in increased protein levels of β1 integrin as assessed by western blotting, but did not change the levels of JAM-A expression (fig 8D). Furthermore, β1 integrin overexpression resulted in increased cell migration from 120 cells per mm^2 for the DL1 mutants and 230 cells per mm^2 for the 6163 mutant cell lines to 470 cells per mm^2 for both cell lines with β1 integrin expression restored. Thus, β1 integrin overexpression resulted in increased cell migration to a level comparable to that of the control cell lines (figs 8E-F). Taken together, these results suggest causal link between decreased β1 integrin levels and reduced migration in JAM-A dimerization defective cells.

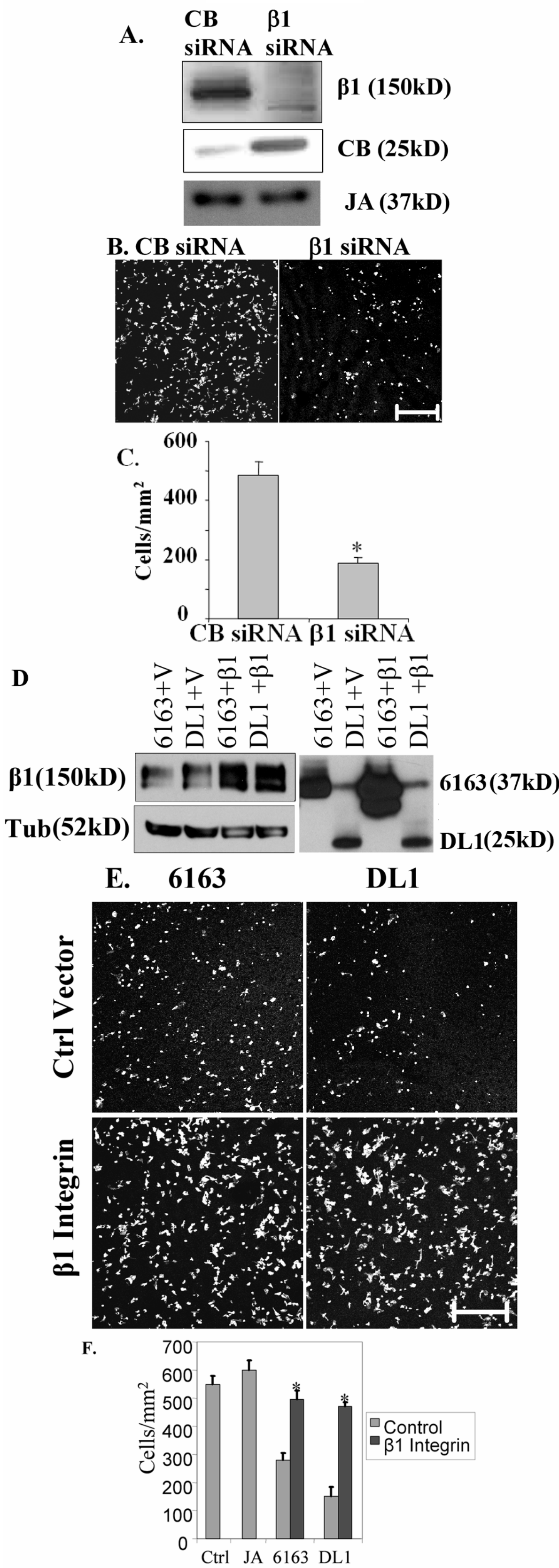


Figure 2.8. β1 Integrin is the putative effector for JAM-A – mediated regulation of 293T cell migration . A, Western blot demonstrating that transfection of 293T cells with siRNA specific for β1 integrin results in significantly decreased β1 integrin protein levels compared to transfection with siRNA targeting cyclophilin (CB). JAM-A protein levels were not changed. B-C, Transfection of 293T cells with siRNA specific for β1 integrin revealed significantly reduced 293T cell migration compared to controls treated with siRNA specific for cyclophilin B. Scale bar is 200uM. Bar graph is average number of cells per field + SEM (*, $p<0.05$). D, Western blot demonstrating increased β1 integrin protein levels in JAM-A dimerization-defective cell lines transfected with a plasmid encoding β1 integrin. JAM-A protein levels were not changed. E-F, Migration assays with mutant cell lines overexpressing β1 Integrin. As can be seen, overexpression of β1 Integrin in the 6163 and DL1 mutant cell lines restores cell migration to that of control 293T cells. Transient transfection of a control plasmid had no effect on cell migration. Scale bar is 200μM. (*, $p<0.05$ for β1 Integrin transfected cells versus control).

The PDZ domain of JAM-A is necessary for the dominant-negative effects of dimerization-defective JAM-A constructs.

To clarify mechanisms of the dominant negative effects on cell migration mediated by the dimerization-defective JAM-A mutants, we rationalized that these mutants are likely to sequester PDZ- containing JAM-A binding/signaling proteins away from native JAM-A dimers. To test this hypothesis, we created a modified DL1 mutant construct that lacks the C-terminal
PDZ binding domain termed DL1-dFLV. Furthermore, we generated a JAM-A mutant lacking only the PDZ-binding motif. Thus, if dimerization-defective JAM-A affects cell migration by sequestering PDZ-domain containing scaffolds, then DL1-dFLV should reverse the dominant negative effect of DL1 on cell migration and the dFLV-only mutant should mimic the effects of dimerization-defective JAM-A by dimerizing with wild-type JAM-A and preventing endogenous JAM-A homodimerization.

The JAM-A mutants were transiently expressed in 293T cells (fig 9A) with a transfection efficiency of 70-90%. Transfected cells were then used in cell migration assays on permeable filters as above. In the passage of 293T cells used for this set of experiments, there was a higher rate of migration than observed in the passage of 293T cells clonally selected for the stable cell lines. In these transient transfections we observed decreased β1 integrin levels with 6163 and DL1, indicating that degradation of β1 integrin due to interference with JAM-A dimerization occurs in a relatively short time frame of less than 48 hours. In contrast, the DL1-dFLV double mutation had no effect on the levels of β1 integrin protein. Furthermore, as shown in figures 9B and 9C, deletion of the C-terminal PDZ binding motif in the DL1 mutant (DL1-dFLV) completely reversed

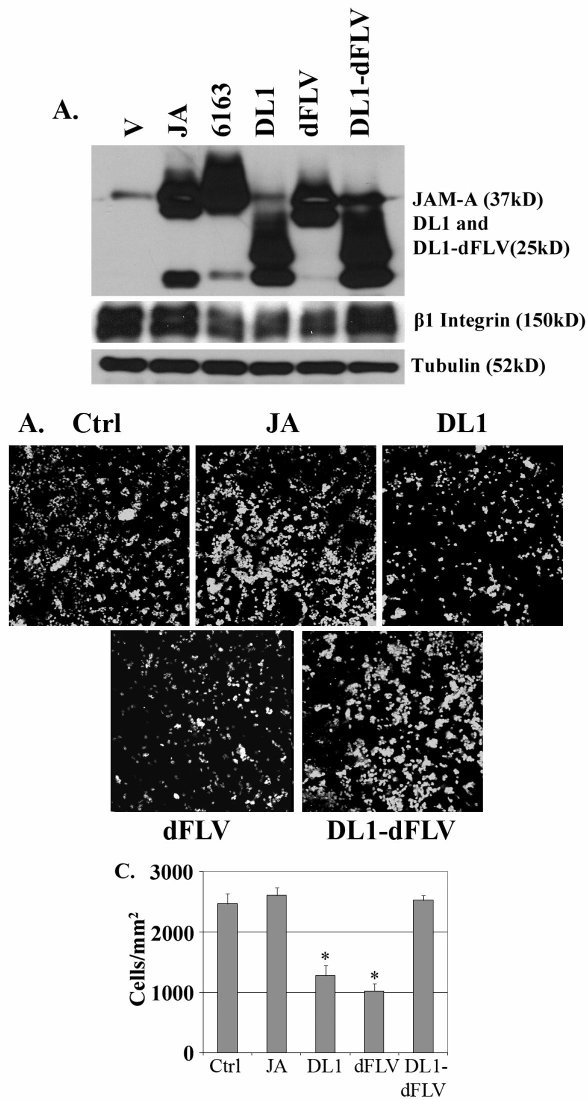


Figure 2.9. The PDZ binding domain of JAM-A is critical for dominant negative effects on cell migration and β1 integrin expression by dimerization-defective mutants. A, Expression of JAM-A mutant proteins and β1 integrin after transient transfection. As can be seen in the western blot, full-length (wild-type) and the 6163 mutant of JAM-A have an Mr of 37kD, while constructs lacking the membrane-distal loop (DL1 and DL1-dFLV) have an Mr of~ 25kD. Also shown are immunoblots for β1 integrin after transient transfection with JAM-A constructs demonstrating decreased expression with 6163, DL1 and dFLV respectively. However, transfection with the DL1-dFLV double mutant results in no change in β1 integrin protein expression. B, Transient expression of DL1 or dFLV resulted in decreased cell migration, as measured by confocal analysis of topro-3 nuclear staining, while expression of the DL1-dFLV construct had no effect. These findings suggest that the PDZ binding motif is required for regulation of β1 integrin protein levels and cell migration by the DL1 mutant. C, Average cells migrated±SEM are shown. As can be seen, overexpression of 6163 and DL1 mutants significantly decreased cell migration, while expression of the double mutant DL1-dFLV had no effect (*, $p<0.05$).

the dominant negative effects on migration observed with DL1 and restored migration to control levels. As predicted, transfection with a JAM-A mutant lacking only the PDZ-motif also decreased the levels of β1 integrin protein and cell migration. Overall, these data strongly suggest that dimerization-defective JAM-A mutants accelerated β1 integrin degradation and decreased cell migration by sequestering PDZ-containing scaffolding protein from native JAM-A dimers at the plasma membrane.

2.5 DISCUSSION

In previous studies, the N-terminal IgG loop of JAM-A has been reported as essential for homophilic dimerization and, presumably, function in intercellular junction assembly (22,23). By utilizing a DL1 mutant that lacks the N-terminal IgG loop as well as point mutations in the dimerization salt bridge (6163), we determined that loss of dimerization of the N-terminal IgG loop in JAM-A resulted in decreased cell trans-filter migration, spreading, and altered cell shape. These observations suggest that the N-terminal IgG loop, specifically the residues mediating dimerization, is important for JAM-A function in epithelial cells.

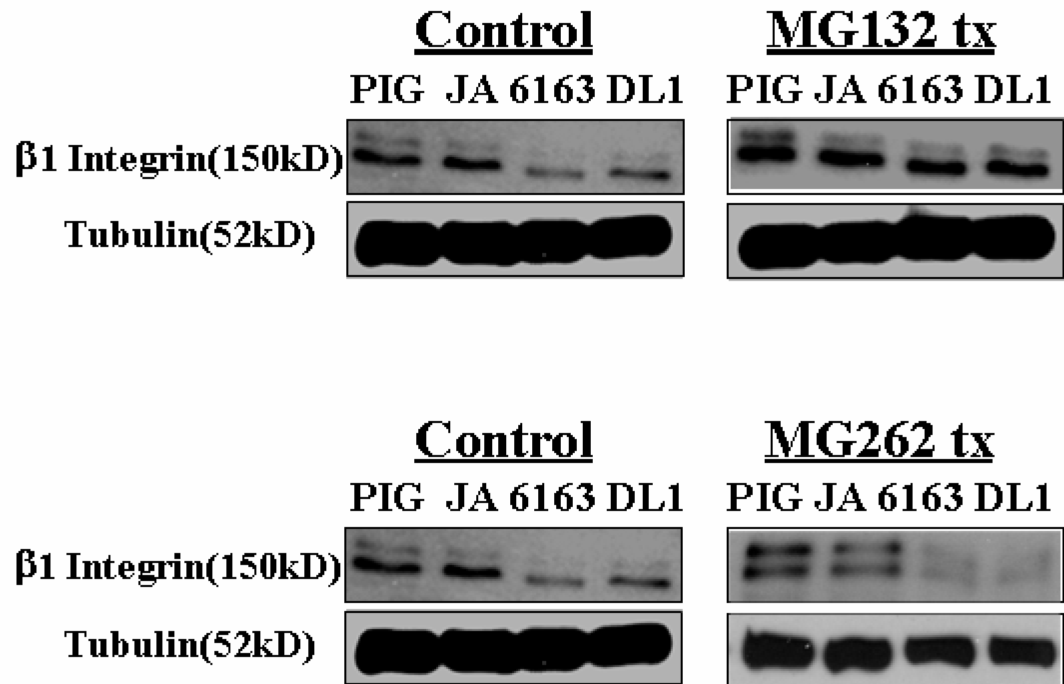
While there are no studies on the role of JAM-A migration in epithelial cells, there are a few reports examining JAM-A and migration in endothelial cells. Bazzoni *et al* reported decreased two-dimensional cell migration in JAM-A deficient endothelial cells using scratch wound assays, that was restored after transfection with full-length JAM-A but not protein lacking the PDZ binding motif (45). Furthermore, Naik *et al* reported that overexpression of JAM-A increased two-dimensional cell migration in endothelial cells through interactions with $\alpha_v\beta_3$ integrin and activation of MAPK. However, neither of these studies addressed the role of JAM-A dimerization, nor did they

provide a structural model for how JAM-A might regulate cell motility. In addition, there are no studies examining the role of JAM-A in models of cell migration in three dimensions.

We previously reported that transient downregulation of JAM-A expression in intestinal epithelial cells by siRNA resulted in altered epithelial cell morphology, decreased cell-matrix adhesion, and decreased levels of β1 integrin and Rap1 (3) however mechanistic insight(s) were lacking. This study was directed at better understanding the mechanism linking structural determinants of JAM-A to β1 integrin levels and cell migration. We determined that overexpression of JAM-A dimerization-defective mutants in 293T cells resulted in decreased cell migration across matrix-coated permeable filters, decreased spreading, and reduced length of cellular protrusions. The role of dimerization in cell migration was further confirmed through experiments demonstrating inhibition of cell migration after treatment with specific JAM-A dimer-disrupting antibodies. Furthermore, treatment with a dimerization inhibiting antibody and cyclohexamide lead to degradation of β1 integrin more quickly than treatment with cyclohexamide and IgG. Decreased cell migration in our assays correlated with decreased β1 integrin levels, alterations in β1 integrin protein localization, and decreased levels of the active form of the small GTPase Rap1, and diminished numbers of focal concentrations of phosphorylated paxillin. An effector role for β1 integrin in the observed JAM-A mediated effects was supported by experiments with siRNA specific to β1 integrin demonstrating decreased cell migration in control cell lines after downregulation of β1 integrin protein levels. Furthermore, we observed increased cell migration

comparable to that observed in control cell line levels after overexpression of β1 integrins in cells expressing JAM-A dimerization-defective mutations.

While the mechanism of decreased β1 integrin in the JAM-A dimerization mutants remains unclear, our results provide important new insights. Our data obtained using two different approaches to disrupt JAM-A dimers (JAM-A mutants and antibodies) are consistent with a scenario where disruption of JAM-A dimerization causes internalization and degradation of β1. This is consistent with the observation of no decrease in β1 integrin mRNA levels in cells expressing dimerization-defective JAM-A. We tested whether increased proteosomal degradation might account for diminished β1 integrins in JAM-A mutant cell lines. Treatment of the dimerization-defective JAM-A cell lines with the proteosome inhibitor MG262 failed to increase β1 integrins despite increasing levels of ubiquitinated proteins (Supplemental Figure 3). This finding suggests that β1 integrin degradation in our cell lines may not be mediated by the proteosome. Further studies are necessary to determine the mechanism for the degradation of β1 integrin in the presence of the JAM-A dimerization defective mutants. The mechanism(s) behind regulation of β1 integrin expression by JAM-A remains to be determined. Possibilities include direct interactions of β1 integrins with JAM-associated scaffolding proteins, activation of signaling molecules that affect β1 integrin turnover such as the small GTPase Rap1 or sequestration of negative regulators of β1 integrin stability by scaffolding complexes. In other studies, JAM-A has been reported to physically interact with αvβ3 (46) and β2 integrin (30) and regulate migration of endothelial cells (30,46), however, we have been unable to detect a direct association between JAM-A and β1 integrin in co-immunoprecipitation experiments. These



Supplemental Figure 2.3. MG132 treatment, but not MG262 treatment restored β1 integrin levels in dimerization defective stable cell lines. β1 integrin levels seen at 150kD are decreased in the presence of dimerization-defective JAM-A protein. The less specific proteosomal inhibitor MG-132 was able to rescue β1 integrin levels; however the more specific inhibitor MG-262 was unable to rescue β1 integrin levels, suggesting that a side-effect of MG-132 was responsible for the observed rescue of β1 integrin protein levels.

observations suggest that JAM-A mediates decreased β1 integrin protein levels through an indirect mechanism(s).

A signaling link between JAM-A dimerization and β1 integrin protein internalization/degradation is suggested by the correlation between decreased β1 integrin and levels of the active form of the GTPase Rap1. We previously demonstrated a decrease in active Rap1 after downregulation of JAM-A expression in SK-CO15 cells (3). Furthermore, we demonstrated that downregulation of Rap1 by siRNA resulted in decreased β1 integrin levels. Additionally, other studies have linked Rap1 activity and increased integrin protein levels and/or integrin activation (72-74). In concert with our findings in the dimerization-defective cell lines, it is thus likely that Rap1 is a signaling element between JAM-A and β1 integrin. We speculate that JAM-A dimer-dependent activation of Rap1 may be required to prevent internalization and degradation of β1 integrin.

Intriguingly, we observed that the dominant-negative effects of DL1 were abrogated after an additional mutation removed the PDZ-binding domain. Since PDZ domains are responsible for interactions with scaffolding proteins, these results suggest that the dimerization-defective mutations may affect cell migration and β1 integrin levels through sequestration of scaffolding proteins. This hypothesis is consistent with our data demonstrating that 293T cells transfected with JAM-A containing a mutation in the PDZ-binding domain (dFLV) led to decreased levels of β1 integrin protein and decreased rates of cell migration. These data suggest that the effects on β1 integrin and migration are mediated by dimerization of wild-type JAM-A with dFLV-JAM-A. Given that transfection of cells with dFLV resulted in much higher levels of expression of the

mutant than endogenous JAM-A, it is likely that a majority of the endogenous JAM-A would dimerize with the dFLV mutant. Under such conditions, functional dimers of JAM-A would not be expected to form, resulting in effects similar to those observed with dimerization-defective mutants.

From these findings, we present a hypothetical model of JAM-A function (Fig 10), In the model, cis-dimerization of JAM-A brings into close proximity two molecules of JAM-A. Each JAM-A molecule has a C-terminal PDZ-binding motif that can interact directly or indirectly with scaffolding proteins such as ZO-1 or Afadin (7,38). This scaffolding complex might interact with β1 integrin via yet unidentified partners leading to stabilization of β1 integrin at the plasma membrane. In other studies, JAM-A has been reported to physically interact with αvβ3 (46) and β2 integrin (30) and regulate migration of endothelial cells (30,46), however, we have been unable to detect a direct association between JAM-A and β1 integrin in co-immunoprecipitation experiments. Therefore it is most likely that scaffolding complexes associated with JAM-A dimers bind and regulate activity of some signaling and endocytic proteins such as Rap1 which can mediate internalization/trafficking of β1 integrin. We speculate that the dimerization-defective JAM-A expressing mutants disrupt these scaffolding complexes at endogenous JAM-A dimers by sequestering their certain components. This may activate/release yet unknown signaling cascade resulting in accelerated internalization and subsequent degradation of β1 integrin.

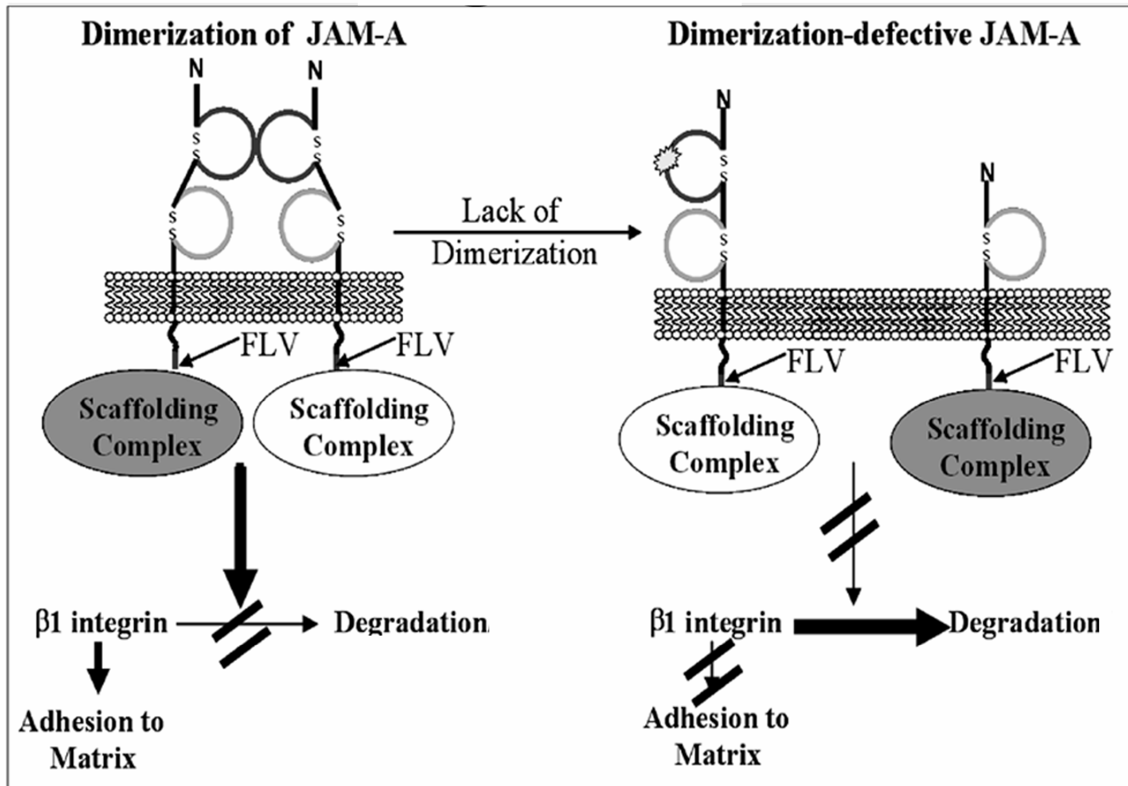


Figure 2.10. Model for effect of JAM-A dimerization on β1 integrin levels.
In this model, JAM-A dimers are required for close apposition of cytoplasmic tails bound to scaffolding complexes containing signaling elements. Under this scenario, disruption of cis-dimerization by either mutagenesis or a blocking antibody would result in loss of close apposition of these cytoplasmic tail complexes, inhibition of signaling and subsequent internalization/degradation of β1 integrin through an as of yet undetermined mechanism. Diminished cell surface expression of β1 Integrin would then result in decreased adhesion to matrix and affect cell migration.

The PDZ domain of JAM-A has been shown to bind to several scaffolding molecules, such as ZO-1, Cingulin, Occludin (38), Afadin (7) and MUPP-1 (33) amongst others. This suggests that the cytoplasmic tail of JAM-A is able to bind to different molecules and that the two tails in a JAM-A homodimer would not necessarily bind to the same scaffolding molecule, but may likely bring different molecules with signaling capacity into close proximity thus forming scaffolding complexes as discussed above.

Importantly, our model is also compatible with JAM-A dimers interacting with one another in trans, as has been observed in the mouse, but not human crystal structures (21), (5). Trans-interacting JAM-A dimers would allow for the close apposition of multiple sets of PDZ binding domains and the formation of large scaffolding/signaling complexes. Cis-dimerization may thus only be a pre-requisite for the changes described in this report to occur.

The physiological significance of formation of JAM-A cis-dimers is highlighted in the various functions described for JAM-A per se which include regulation of cell migration, barrier function(23), angiogenesis(32), cell adhesion(3), and determination of cell polarity(36). We recently demonstrated that loss of JAM-A leads to changes in basal intestinal permeability and increased sensitivity to DSS-induced colitis in vivo (56). In this study, loss of JAM-A was shown to result in an altered Claudin expression profile. It is tempting to hypothesize that such changes in Claudins may be due to altered JAM-A dimer-mediated signaling.

It is possible to speculate on pathophysiologic conditions that would result in dissociation of JAM-A cis-dimers. It is likely that the formation of such complexes results from low-affinity interactions that would be very sensitive to changes in levels of

JAM-A in the plasma membrane. Therefore, stimuli that decrease abundance of plasma membrane JAM-A by inhibiting protein expression or enhancing internalization would diminish cis-JAM-A dimers. Interestingly, we and others have shown that junctional proteins, including JAM-A are internalized and decrease after a variety of stimuli including exposure to inflammatory cytokines and oxidant stress (75,76). We have also observed similar internalization and diminished levels of junctional proteins and JAM-A in the mucosa from individuals with inflammatory bowel disease (77). It is thus tempting to speculate that loss of JAM-A dimers at the cell surface through inflammatory stimuli contributes to the altered permeability and pathophysiology of chronic intestinal inflammatory states. Clearly more work is needed to fully understand the physiological relevance of JAM-A dimerization.

In summary, these results suggest that dimerization of JAM-A is required for regulating several aspects of cell migration through signaling events. . Disruption of JAM-A dimerization presumably prevents the formation of scaffolding protein complexes that prevent and/or lead to signaling events that result in loss of β1 integrin and decreased cell migration. Further studies are needed to better understand the mechanisms of JAM-mediated regulation of integrin expression and cell migration as well as identification of scaffolding complexes involved in JAM-A mediated signaling events.

Chapter 3

Association of Junctional Adhesion Molecule A (JAM-A) with Afadin and PDZ-GEF2 is required for activation of Rap1A, maintenance of cellular levels of β1 integrin protein and regulation of cell migration.

Material from this chapter has been submitted to: Molecular Biology of the Cell. I am the first author and contributing authors of this article were: Winston Y. Lee, Christopher T. Capaldo, Asma Nusrat and Charles A. Parkos

3.1 Abstract

The tight junction protein JAM-A regulates several cellular processes through incompletely understood mechanisms. We previously demonstrated that JAM-A mediated regulation of cell migration is dependent upon outside-in signaling mediated by dimerization of the membrane-distal Ig loop and a PDZ binding domain on the C-terminus. A model depicting interactions of dimerized JAM-A molecules with PDZ domain-containing scaffolding proteins, was proposed that was consistent with functional data. Here we report that JAM-A is physically and functionally associated with the PDZ domain containing signaling molecules Afadin and PDZ-GEF2, but not ZO-1, in polarized intestinal epithelial cells. Both Afadin and PDZ-GEF2 were observed to colocalize and co-immunoprecipitate with JAM-A. Furthermore, association of PDZ-GEF2 with Afadin was dependent on the expression of JAM-A. Loss of JAM-A, Afadin or PDZ-GEF2, but not ZO-1 or PDZ-GEF1, similarly decreased cellular levels of activated Rap1, β1 integrin protein and the rate of epithelial cell migration. The effects observed were secondary to decreased levels of Rap1A since knockdown of Rap1A, but not Rap1B, resulted in decreased β1 integrin protein and cell migration. These and our previous findings suggest that JAM-A dimerization facilitates formation of a complex with Afadin and PDZ-GEF2 that activates Rap1A, which regulates β1 integrin levels and cell migration.

3.2 Introduction

The epithelial lining of the intestine forms a physical, protective barrier that separates lumenal contents from underlying mucosal tissue and regulates the paracellular diffusion of water, small molecules, and bacterial/viral antigens (78). Disruption of the epithelial barrier occurs during a number of inflammatory conditions, examples of which include Crohns disease and ulcerative colitis(79),(80),(81). Such damage to the epithelial barrier results in fluid/electrolyte loss as well as an influx of lumenal antigens that serve to further exacerbate the inflammatory response(82). To re-establish epithelial barrier, physical breaches in the mucosa must be repaired. Resealing of superficial breaches of the intestinal epithelial barrier is achieved through shedding of damaged cells and migration of epithelial cells to the wounded area(83),(84).

The epithelial barrier is regulated by intracellular junctions, specifically the tight junction (TJ). Proteins in the TJ play an important role in the establishment and regulation of epithelial barrier between cells(85). Junctional Adhesion Molecule A (JAM-A) is expressed in the tight junction and plays a role in the regulation of many cellular processes, including epithelial barrier function(3,56), epithelial cell invasion/migration(86), endothelial cell migration(44,45), angiogenesis(32), platelet aggregation(87) and leukocyte adhesion(10,62).

The mature JAM-A protein is composed of two extracellular immunoglobulin-like loops, a membrane-spanning segment and a cytoplasmic tail ending in a PSD-95/Dlg/ZO-1 (PDZ) binding domain. The membrane distal most extracellular Ig loop mediates homodimerization between JAM-A proteins on the same cell(21,86,88) (cis) and potentially mediates interactions between JAM-A molecules on adjacent cells(5)

(trans). Cis-dimerization has been shown to be necessary for JAM-A regulation of epithelial cell invasion(86) and epithelial barrier recovery(23). The PDZ binding domain of JAM-A has been reported to bind to the PDZ domain containing proteins Afadin and ZO-1 at endogenous levels in endothelial cells (7),(89). From these preliminary reports, Afadin and ZO-1 are good candidates as signaling molecules downstream of dimerized JAM-A. Despite these observations, whether such interactions mediate function of JAM-A is not known.

Our previous data and that from others led us to propose a model of JAM-A function based on mutations within the membrane distal extracellular Ig loop and cytoplasmic PDZ binding domain (21,86). In this model, cis-dimerization of JAM-A promotes the close apposition of JAM-A PDZ binding domains that facilitate interactions between scaffolding proteins that activate of signaling molecules. However, the scaffolding and signaling proteins downstream of the dimerized JAM-A complex that mediate function have not yet been defined.

Here we provide a model of JAM-A function through dimerization-dependent association of scaffolding proteins that activate signaling molecules to regulate cell migration. We show : (i) co-association of the guanine nucleotide exchange factor PDZ-GEF2 and Afadin with JAM-A occur in a manner dependent on the expression of JAM-A; (ii) identical effects on β1 integrin protein levels and cell migration after downregulation of expression of JAM-A, Afadin, PDZ-GEF2 or Rap1A; and (iii) that PDZ-GEF2 activates Rap1A to regulate β1 integrin and cell migration. These results suggest that dimerized JAM-A forms a scaffolding complex that promotes the association of Afadin and PDZ-GEF2 to activate Rap1A. Rap1A, in turn, regulates cell migration

through stabilization of β1 integrin levels. These findings directly implicate expression of JAM-A in the mediation of cell migration and by extension implicate JAM-A in regulation of wound healing after physical or inflammatory injury. Additionally, this is the first specific structural and mechanistic model of JAM-A function.

3.3 Results

JAM-A regulates epithelial cell migration by signaling through Afadin but not ZO-1.

We and others have previously reported that the PDZ binding domain of JAM-A is necessary for function(35,38,86), presumably due to interactions with PDZ domain-containing scaffolding proteins. We initially focused on the scaffolding proteins Afadin and ZO-1, due to published reports linking association with JAM-A (7,90). Therefore, we investigated whether these proteins may be required for JAM-A regulation of cell migration linkage to cellular function of JAM-A.

Immunofluorescence localization experiments were first performed with JAM-A, ZO-1 and Afadin. ZO-1 and JAM-A are well established TJ-associated proteins, so it was not surprising to find strong co-localization in confluent SK-CO15 cells, a transformed and polarized human intestinal epithelial cell line Afadin, on the other hand has been reported to associate with Nectin at adherens junctions (AJ)(91), but has also been observed at both the TJ and AJ by electron microscopy(89) and fractionation experiments(92). Confocal immunofluorescence analyses revealed that JAM-A, Afadin and ZO-1 all co-localize at the level of the tight junction. (Figure 1A, Supplemental Figure 1A).

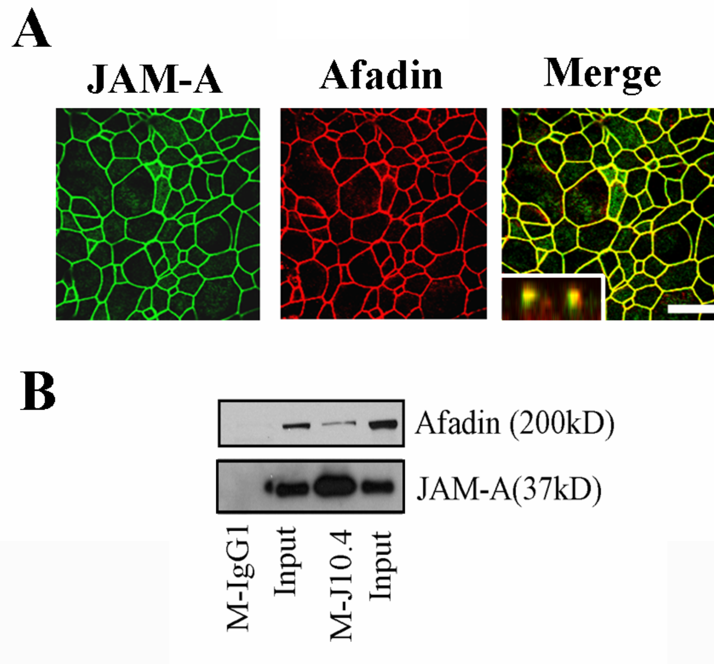
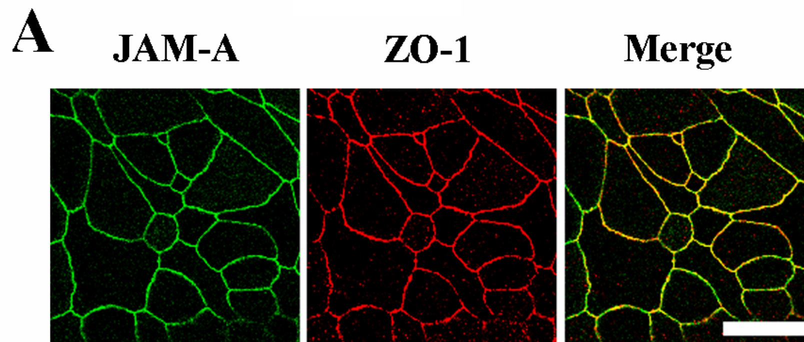
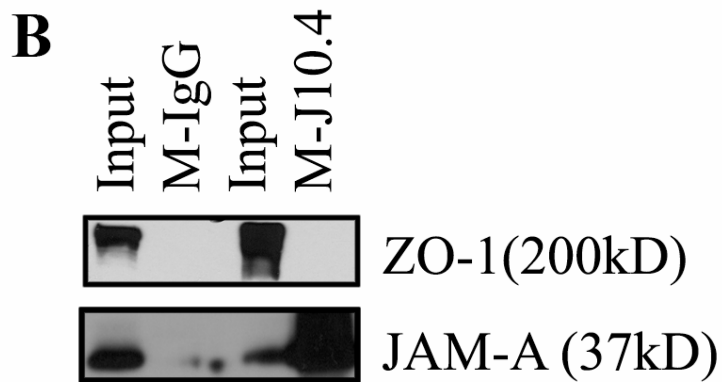
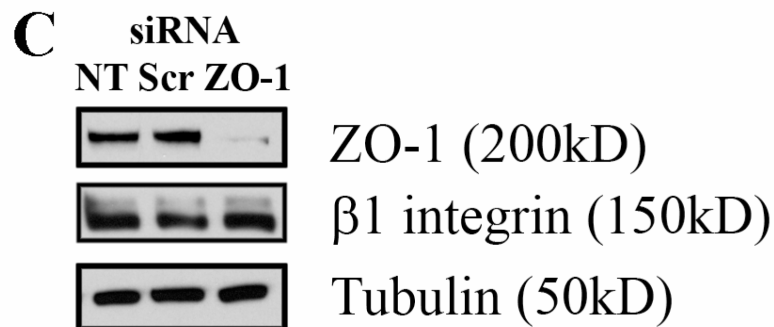


Figure 3.1A-B: *JAM-A and Afadin co-localize and co-associate*. A, By IF, JAM-A (green) and Afadin (red) colocalize as seen in the merged images in the XY and XZ (inset) planes at the level of the AJC. Scale bar is 20μm. B, Immunoprecipitations with J10.4, a mouse IgG1 anti-JAM-A antibody, detects an interaction (direct or indirect) between Afadin and JAM-A, as can be seen in the upper immunoblot, while neither JAM-A nor Afadin immunoprecipitates with the isotype control antibody.







Supplemental Figure 3.1: *ZO-1 is not in a signaling complex with JAM-A*. A, By IF, JAM-A (green) and ZO-1 (red) co-localize as seen in the merged images at the level of the AJC. Scale bar is 10µm. B, Using RIPA buffer, JAM-A and ZO-1 do not IP in SK-CO15 cells using either IgG1 isotype control or the anti-JAM-A antibody J10.4. C, ZO-1 protein expression was substantially decreased by siRNA treatment; however, loss of ZO-1 had no effect on β1 integrin levels.

To evaluate whether JAM-A forms a complex with Afadin or ZO-1, we next performed co-immunoprecipitation (IP) experiments. SK-CO15 cells were lysed in a non-ionic detergent containing solubilization (RIPA) buffer, and incubated with JAM-A mAb J10.4, an antibody that has been shown to bind to the membrane distal extracellular immunoglobulin (Ig) domain(23). Immunoprecipitated complexes were isolated using Protein-G sepharose followed by boiling in SDS-containing sample buffer and supernatants were subjected to SDS-PAGE followed by western blot. Immunoblots were then probed for JAM-A, ZO-1 and Afadin. As can be seen in Figure 1B, IPs with mAb J10.4 revealed the presence of Afadin but not ZO-1 in JAM-A-containing complexes These data suggest that, while JAM-A co-localizes with both ZO-1 and Afadin, JAM-A only forms a detergent soluble and stable complex with Afadin.

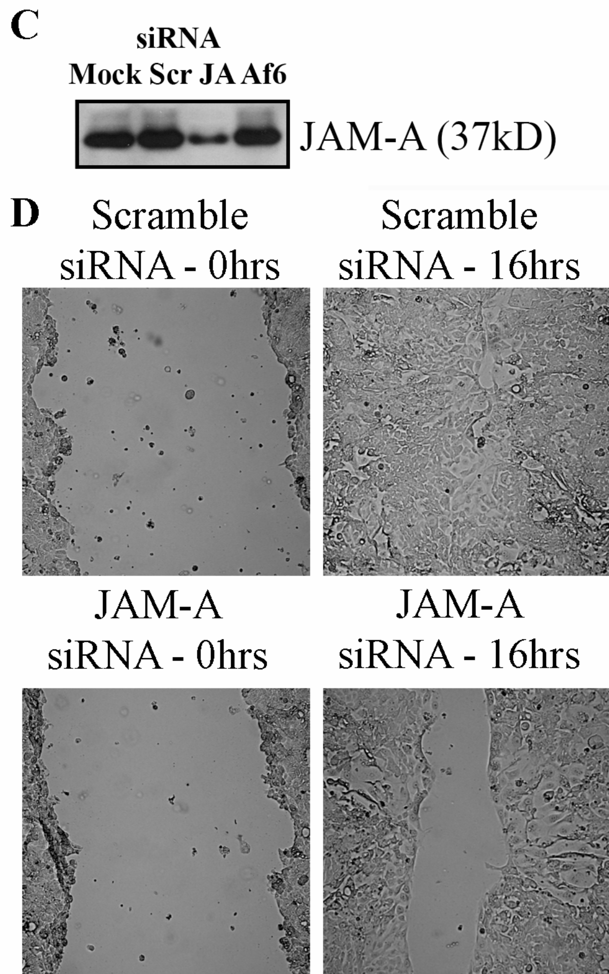
We next performed cell migration experiments to determine if Afadin and/or ZO-1 were necessary for JAM-A function. It has been previously reported that JAM-A regulates epithelial and endothelial cell migration (44,45,86). In our study and in Bazzoni et al., it was demonstrated that the JAM-A PDZ binding domain was critical for mediating JAM-A effects on cell migration. We reasoned that removal of JAM-A binding scaffold proteins, which interact with JAM-A through their PDZ domains, would similarly ablate JAM-A effects on cell migration. Thus, we determined the conditions for siRNA-mediated manipulation of JAM-A and other candidate signaling molecules in simple scratch wound migration assays using epithelial cell monolayers composed of SK-CO15 cells, which are readily transfectable. JAM-A siRNA treatment consistently resulted in greater than 80% downregulation of protein expression compared to transfection with scrambled siRNA controls. The rate of cell migration in siRNA treated

monolayers was then assessed after induction of standard scratch wounds. Cells were allowed to migrate for 16 hours and wound width was compared to that of the initial wound.. As can be seen in Figure 1C-E, SiRNA mediated downregulation of JAM-A resulted in a decrease in the rate of wound closure by 30% compared to Mock or Scramble siRNA transfected cells ($p<0.01$). These results are consistent with previous observations demonstrating that overexpression of JAM-A mutants that are defective in dimerization or lack the PDZ binding domain have decreased rates of epithelial cell migration through matrix (86). Thus, decreased expression of JAM-A reduces the rate of cell migration in epithelial monolayers.

To determine whether Afadin or ZO-1 mediates the effects of JAM-A on cell migration, analogous experiments were performed using siRNA mediated downregulation of Afadin and ZO-1 expression. As shown in Figure 2, siRNA mediated downregulation of Afadin expression inhibited wound closure at 16h by 35% compared to controls ($p<0.001$). Conversely, siRNA mediated downregulation of ZO-1 had no effect on cell migration. The similarity of observed effects on cell migration by alterations in both JAM-A and Afadin protein expression, combined with data in Figure 1 demonstrating co-association of JAM-A and Afadin suggests that JAM-A signals through Afadin but not ZO-1 to regulate cell migration.

JAM-A and Afadin effect cell migration through regulation of β1 integrin protein levels.

We previously reported that JAM-A regulates epithelial cell migration by effects on cellular levels of β1 integrin protein. Decreased epithelial invasion observed after downregulation of JAM-A expression was rescued by overexpression of β1 integrin(86).



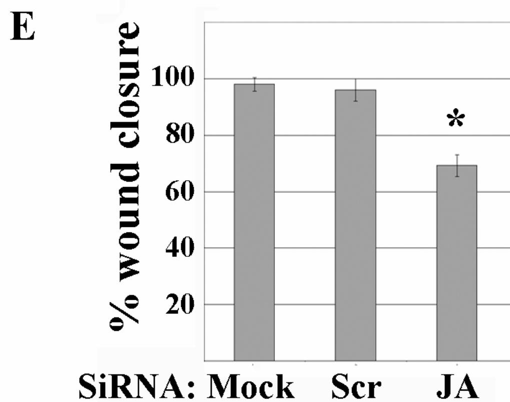


Figure 3.1C-E: *JAM-A and Afadin co-localize and co-associate*. C, Treatment with JAM-A (JA) specific siRNA decreases levels of the targeted protein compared to scramble (Scr) or mock treated controls. D-E, Scratch wound assays. Confluent monolayers of SK-CO15 cells were injured with a 10ul pipet tip using vacuum suction and the wound length was measured at 0 and 16 hours after wounding. Wound closure in Mock or Scramble treated samples was nearly 100%, while wounds with decreased JAM-A expression had only healed by 65% (*,$p<0.01$). Scale bar is 200μm.

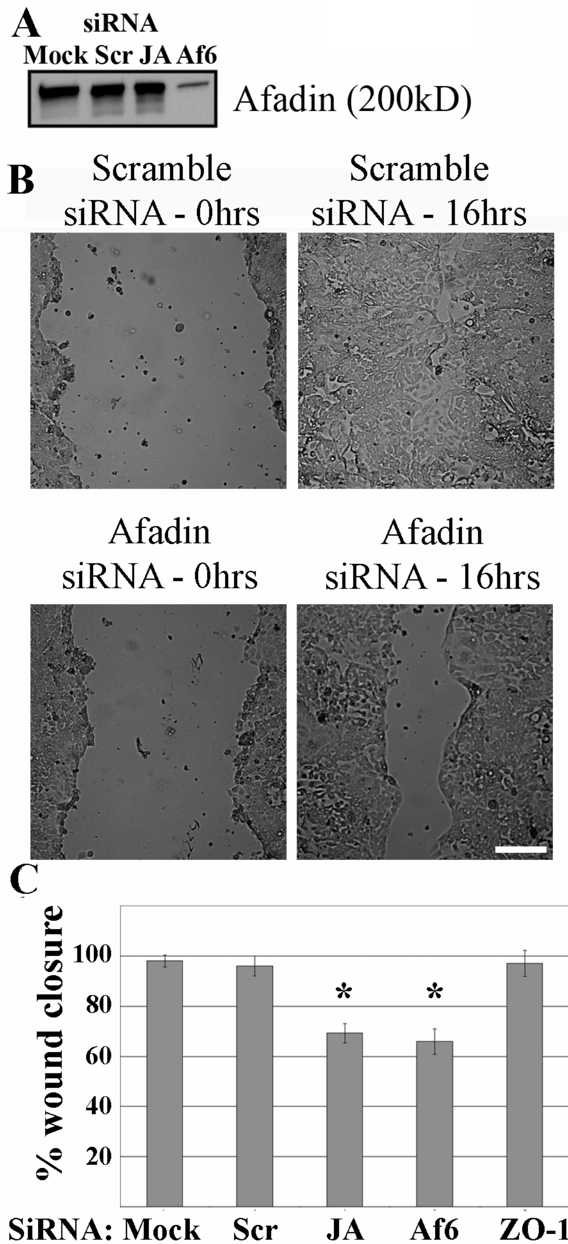


Figure 3.2: *JAM-A and Afadin siRNA treatment both decrease the rate of cell migration*. A, Treatment with Afadin (AF6) specific siRNA decreases levels of the targeted protein compared to scramble (Scr) or mock treated controls. B-C, Scratch wound assays. Confluent monolayers of SK-CO15 cells were injured with a 10ul pipet tip using vacuum suction and the wound length was measured at 0 and 16 hours after wounding. Wound closure in Mock or Scramble treated samples was nearly 100%, while wounds in JAM-A downregulated or Afadin downregulated monolayers had only healed by 65% (*,$p<0.01$) or 69% (**,$p<0.001$). Scale bar is 200µm.

We extended these observations to examine whether Afadin is necessary for JAM-A mediated signaling using the planar scratch wound assays as detailed above. Similar to that observed with loss of JAM-A, siRNA mediated downregulation of Afadin but not ZO-1 protein expression resulted in decreased levels of β1 integrin protein (Figure 3A-B and Supplemental Figure 1).

The above findings suggest that JAM-A signals through Afadin to effect cell migration by regulating cellular levels of β1 integrin protein. The Afadin-mediated effects were not an artifact of general PDZ scaffold disruption since ZO-1 did not co-immunoprecipitate with JAM-A in the polarized epithelial cell lines, and siRNA-mediated downregulation of ZO-1 expression had no effect on β1 integrin protein levels (Supplemental Figure 1). Furthermore, the effects observed after downregulation of JAM-A were not secondary to loss of Afadin since decreased expression of JAM-A did not effect expression of Afadin (Figure 3C). These data are thus consistent with an Afadin-dependent mechanism for JAM-A regulation of β1 integrin protein levels and cell migration.

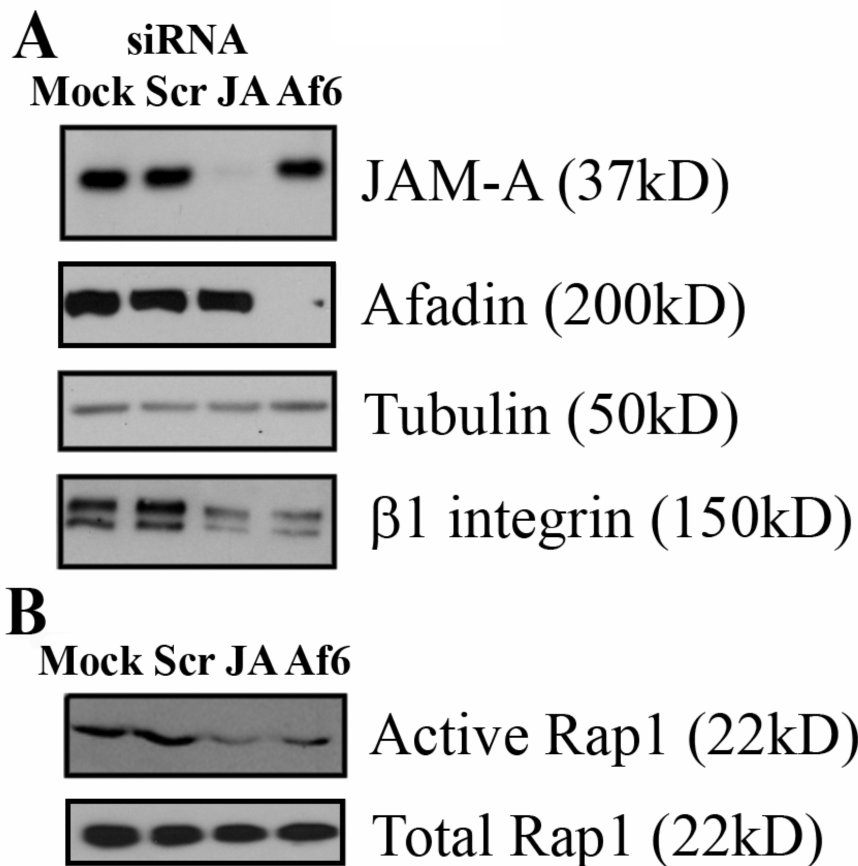
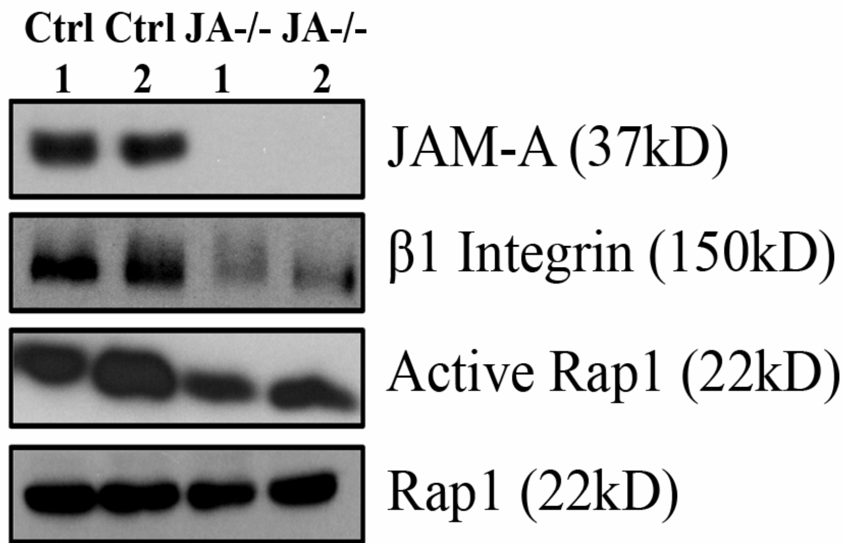


Figure 3.3: *Downregulation of Afadin or JAM-A results in decreased β1 integrin and active Rap1.* A, Treatment with JAM-A (JA) or Afadin (AF6) specific siRNA decreases levels of the targeted protein compared to scramble (Scr) or mock treated controls. Tubulin is used as a protein loading control. Downregulation of either JAM-A or Afadin decreases β1 integrin levels. B, Western blots for Rap1 for either total samples or for sample after pull down with Ral-GDS beads, which only bind to active Rap1. Active Rap1 is decreased in siRNA treated samples for either JAM-A or Afadin.

The small GTPase Rap1a mediates effects of JAM-A/Afadin on β1 integrin and cell migration.

In other studies, we observed that downregulation of JAM-A expression results in decreased cellular levels of the active form of Rap1(3,86). Interestingly, Rap1 has been reported to regulate integrin levels in epithelial cells(3) as well as integrin activation in leukocytes(72). However, these observations were made *in vitro*. To confirm that JAM-A regulates β1 integrin and Rap1 *in vivo*, we isolated colonic intestinal epithelial cells from JAM-A knockout mice and compared Rap1 and b1 integrin levels to those in wild-type controls. In JAM-A knockout mice, β1 integrin protein levels were decreased by 50% compared to wild-type controls ($p<0.05$) (Figure 4). We then assessed Rap1 activity by an assay employing Ral-GDS. Lysates of isolated colonic epithelial cells were incubated with Ral-GDS agarose beads to bind active Rap1 followed by washing and SDS-PAGE. Immunoblots were then probed for Rap1. In colonic epithelial cells from JAM-A knockout mice, total levels of Rap1 were unchanged compared to wild-type controls, however, active Rap1 levels were decreased by 49% ($p<0.05$) (Figure 4). These results suggest that JAM-A regulates Rap1 activity and β1 integrin levels both *in vitro* and *in vivo.*

Based on these data, we hypothesized that, through Afadin, JAM-A regulates activation of Rap1, which in turn, affects β1 integrin protein levels. Decreased expression of JAM-A or Afadin both lead to decreased levels of active Rap1 (Figure 3B) and similar to that observed with JAM-A and Afadin, we have also observed that siRNA mediated downregulation of Rap1 resulted in reduced β1 integrin protein levels (data not shown,(3)). However, Rap1 consists of two highly similar paralogs Rap1A and Rap1B.



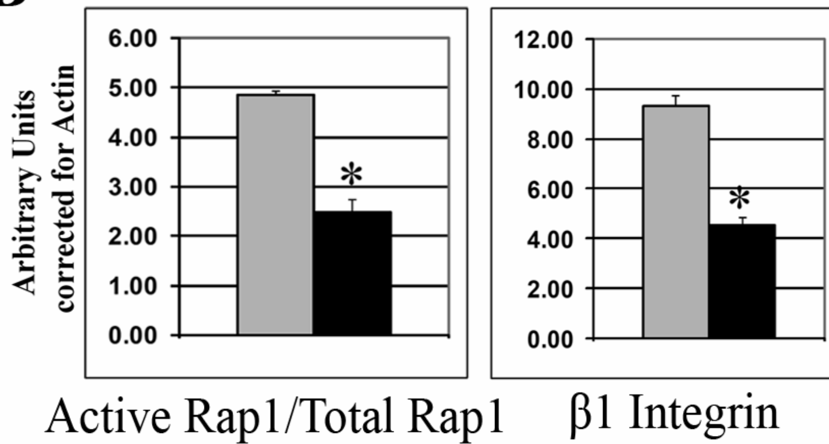


Figure 3.4: *Knockout of JAM-A in vivo results in decreased β1 integrin and active Rap1.* A-B, Immunoblots on colonic epithelial lysates from JAM-A-/- (JA-/-) C57/Bl6 mice compared to wild-type mice have decreased β1 integrin compared to controls by 50% (*, $p<0.001$) as measured by immunoblotting and corrected for actin loading control. The ratio of active to total Rap1 is decreased by 49% in JAM-A knockout mice compared to control mice (*, $p<0.001$).

We thus performed experiments examining the individual roles of Rap1A and Rap1B in JAM-A/Afadin mediated regulation of β1 integrin and cell migration. To isolate the individual effects of Rap1A and Rap1B, siRNA specific to the mRNA sequences in the 3' UTR of Rap1A and Rap1B were used since reliably specific antibody reagents are not available. As shown in Figure 5A, siRNA mediated downregulation of Rap1A and Rap1B resulted in decreased expression of Rap1 as assessed by a pan-Rap1 antibody that does not distinguish between Rap1A and Rap1B. By real-time RT-PCR, siRNA mediated downregulation of Rap1A or Rap1B reduced mRNA expression by 93 and 78 % respectively (Figure 5B). Interestingly, SiRNA mediated downregulation of Rap1A, but not Rap1B, resulted in decreased β1 integrin levels. Furthermore, decreased expression of Rap1A also resulted in a 51% decrease in cell migration compared to controls. Downregulation of Rap1B, on the other hand had no effect (Figure 5C-D). Our above findings suggest that JAM-A and Afadin activate Rap1A, which in turn stabilizes β1 integrin levels since loss of JAM-A or Afadin expression leads to concomitant decreases in β1 integrin protein consistent with loss of Rap1A, but not Rap1B.

The above findings are in contrast to our previously reported correlation between Rap1B expression and β1 integrin levels (3). In that study, we observed that siRNA mediated downregulation of Rap1B decreased β1 integrin levels. However, the previous experiments were performed using "smart-pools" of siRNA containing four separate siRNA oligonucleotides. Given that Rap1A and Rap1B are 95% conserved, we determined that the mixture of Rap1B siRNA targets previously used also resulted in partial downregulation of Rap1A, thus decreasing β1 integrin protein levels. In the

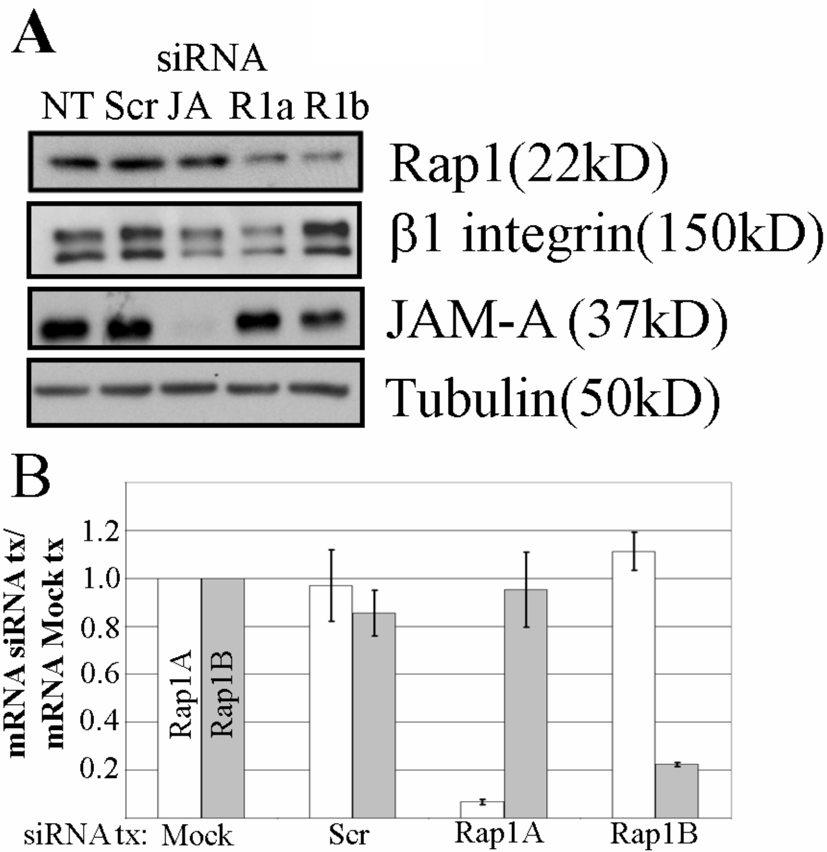
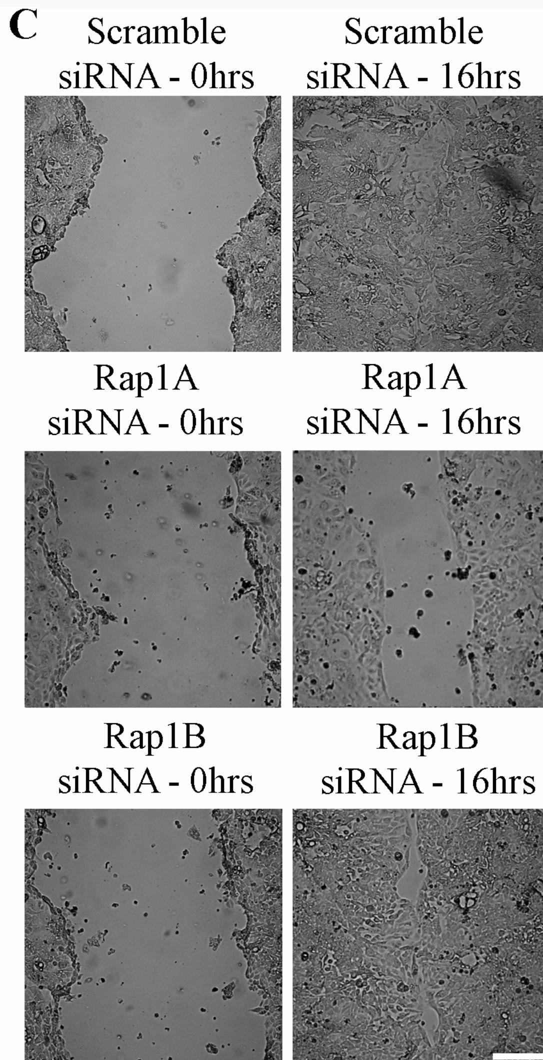


Figure 3.5A-B: *Downregulation of Rap1A causes downregulation of β1 integrin.* A, Western blots with a pan-Rap1 antibody show decreased total Rap1 levels after treatment with specific Rap1A (R1A) or Rap1B (R1B) siRNA. β1 integrin levels are decreased after JAM-A (JA) or Rap1A siRNA treatment, but not with Rap1B siRNA treatment. B, Rap1A and Rap1B siRNA treatment lead to specific downregulation of Rap1A or Rap1B mRNA as measured by real-time PCR. Values are reported as fold change compared to mock siRNA control. White bars are Rap1A message levels and gray bars are Rap1B message levels.



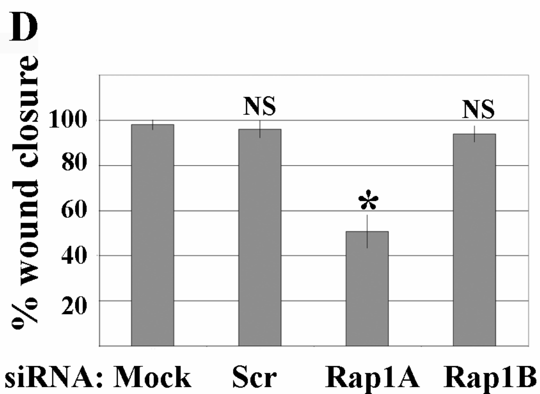


Figure 3.5C-D: *Downregulation of Rap1A or Rap1B each partially phenocopy downregulation of JAM-A.* C-D, Scratch wounds in epithelial cell monolayers with Rap1A downregulated only healed by 50% ($p<0.001$) after 16 hours, while Rap1B downregulated monolayers and control monolayers had completely healed at 16 hours. Scale bar is 200μm.

current study, we performed real-time RT-PCR results to confirm that the siRNA targets used do not cross-react between Rap1A and Rap1B. Taken together, the above results suggest that active Rap1A, not Rap1B regulate β1 integrin protein levels and the rate of cell migration.

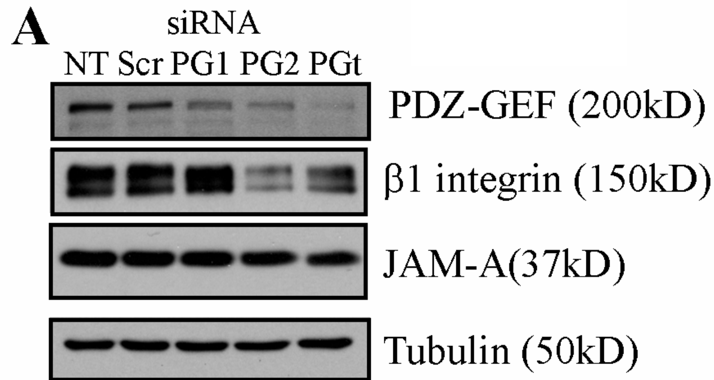
PDZ-GEF2, but not PDZ-GEF1, regulates β1 integrin protein levels and epithelial cell migration.

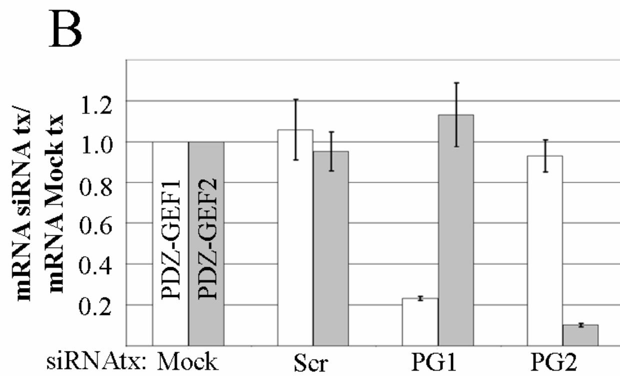
In other studies, we observed that overexpression of JAM-A mutants that are defective in dimerization or lack the PDZ binding motif similarly decrease β1 integrin levels, Rap1 and cell invasion. We thus proposed a model of JAM-A function involving dimerization of JAM-A that brings scaffolding complexes in close proximity to afford efficient downstream signaling. Our data on the co-association of JAM-A with Afadin and identical downstream effects after decreased expression of JAM-A or Afadin strongly suggest that Afadin is part of a JAM-A scaffolding complex that activates Rap1A. Interestingly, Afadin has been shown to directly associate with Rap1A(93,94). However, activation of Rap1A is dependent on Guanine nucleotide factors (GEFs). Given the dependence of JAM-A function on dimerization, we reasoned that a likely additional component of the JAM-A-associated signaling complex would be a Rap1 specific GEF. We focused on candidate GEF molecules that contain PDZ motifs given the potential for binding interactions with JAM-A. We identified two Rap1 specific GEFs: PDZ-GEF1 and PDZ-GEF2(95) that were highly expressed in SKCO15 cells as determined by PCR (not shown) and immunoblot. We obtained a rabbit polyclonal antibody raised against PDZ-GEF1 and western blots of SK-CO15 cells revealed a protein of the expected size (200kD). We next used siRNA to selectively downregulate

expression of PDZ-GEF1 and PDZ-GEF2 to determine antibody specificity. As detected by immunoblot, decreased expression of PDZ-GEF1 or PDZ-GEF2 revealed a partial reduction in total PDZ-GEF expression (Figure 6A). Specificity of siRNA mediated downregulation was confirmed by real time RT-PCR, which revealed that PDZ-GEF1 and PDZ-GEF2 mRNA were each specifically decreased by 77 and 90 % respectively (Figure 6B). To determine if either protein was involved in JAM-A mediated activation of Rap1A we performed additional siRNA experiments. As shown in Fig 6A, downregulation of expression of PDZ-GEF2, but not PDZ-GEF1, resulted in decreased β1 integrin levels and decreased cell migration. Interestingly, downregulation of either PDZ-GEF1 or PDZ-GEF2 resulted in decreased Rap1 activity. Notably, a larger decrease in total Rap1 activity was observed when PDZ-GEF1 and PDZ-GEF2 were simultaneously knocked down (Figure 6C). These data suggests that PDZ-GEF2 and not PDZ-GEF1 is activating Rap1A to stabilize β1 integrin levels and promote cell migration.

JAM-A co-associates with PDZ-GEF2.

Given that PDZ-GEF2 contains a PDZ domain that has the potential to bind the PDZ domain on JAM-A in concert with identical functional effects after decreased expression of either protein, experiments were performed to determine if JAM-A and PDZ-GEF2 associate in a signaling complex. Based on similar phenotypes seen with loss of JAM-A, Afadin and PDZ-GEF2, we hypothesized that PDZ-GEF2 may form a complex with dimerized JAM-A and Afadin that regulates activation of Rap1A. Decreased expression of any of the components of such a complex would thus reduce the levels of active Rap1A and lead to the functional and biochemical changes observed in





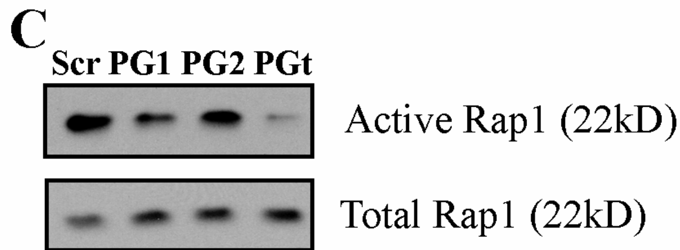


Figure 3.6: *Downregulation of PDZ-GEF2 phenocopies Rap1A downregulation and downregulation of PDZ-GEF1 phenocopies Rap1B downregulation.* A, Western blots for total PDZ-GEF show that the siRNA for PDZ-GEF1 (PG1) and PDZ-GEF2 (PG2) both decrease total PDZ-GEF (PGt) protein level, while downregulation of both more strongly decreases the level of protein. Western blots for β1 integrin indicate that loss of PDZ-GEF2, but not PDZ-GEF1, leads to decreases in β1 integrin. B, PDZ-GEF1 (PG1) and PDZ-GEF2 (PG2) siRNA treatment lead to specific downregulation of Rap1A or Rap1B mRNA as measured by real-time PCR. Values are reported as fold change compared to mock siRNA control. White bars are PG1 message levels and gray bars are PG2 message levels. C, Rap1 activity assays indicate that loss of either PDZ-GEF1 or PDZ-GEF2 leads to decreases in total Rap1, with a more pronounced loss of total Rap1 activity with downregulation of both. D-E, PDZ-GEF1 and control wounds closure was 100%, while PDZ-GEF2 siRNA treated epithelial monolayers wound closure was 64% (*, $p<0.001$). Scale bar is 200μm.

this report. To test hypothesis, co-localization and co-immunoprecipitation studies with PDZ-GEF2 were performed.

As shown in Figure 7A, JAM-A and PDZ-GEF localize at cell-cell junctions as observed by immunofluorescence in SKCO-15 cells. In experiments using JAM-A mAb J10.4, co-immunoprecipitation with endogenous PDZ-GEF was also observed (Figure 7B). Furthermore, to determine if Afadin was also associated in a complex with PDZ-GEF2, immunoprecipitation experiments with an anti-Afadin antibody were performed in SKCO-15 cells transfected with Flag-tagged PDZ-GEF2. Since the proposed model would require the presence of JAM-A for an association of PDZ-GEF with Afadin, as each scaffolding protein would independently interact with JAM-A, we treated cells with control siRNA or with siRNA targeting JAM-A expression. In this way, we could directly test if JAM-A was required for an association of PDZ-GEF with Afadin. As shown in Figure 7, Afadin co-immunoprecipitated with JAM-A and Flag-PDZ-GEF2 in lysates from control cells that express JAM-A.. However, in cells with decreased JAM-A expression, Flag-PDZ-GEF2 did not co-immunoprecipitate with Afadin. These and our other findings suggest that JAM-A dimerization forms the nucleus of a complex containing Afadin and PDZ-GEF2, which in turn activates Rap1A to stabilize β1 integrin levels and promote cell migration.

3.4 Discussion

In this study we report that intestinal epithelial JAM-A associates with Afadin and PDZ-GEF2 and to activate Rap1A. Decreased expression of any of these components results in reduced protein levels of β1 integrin. Reduced β1 integrin protein levels result in reduction of the rate of cell migration.

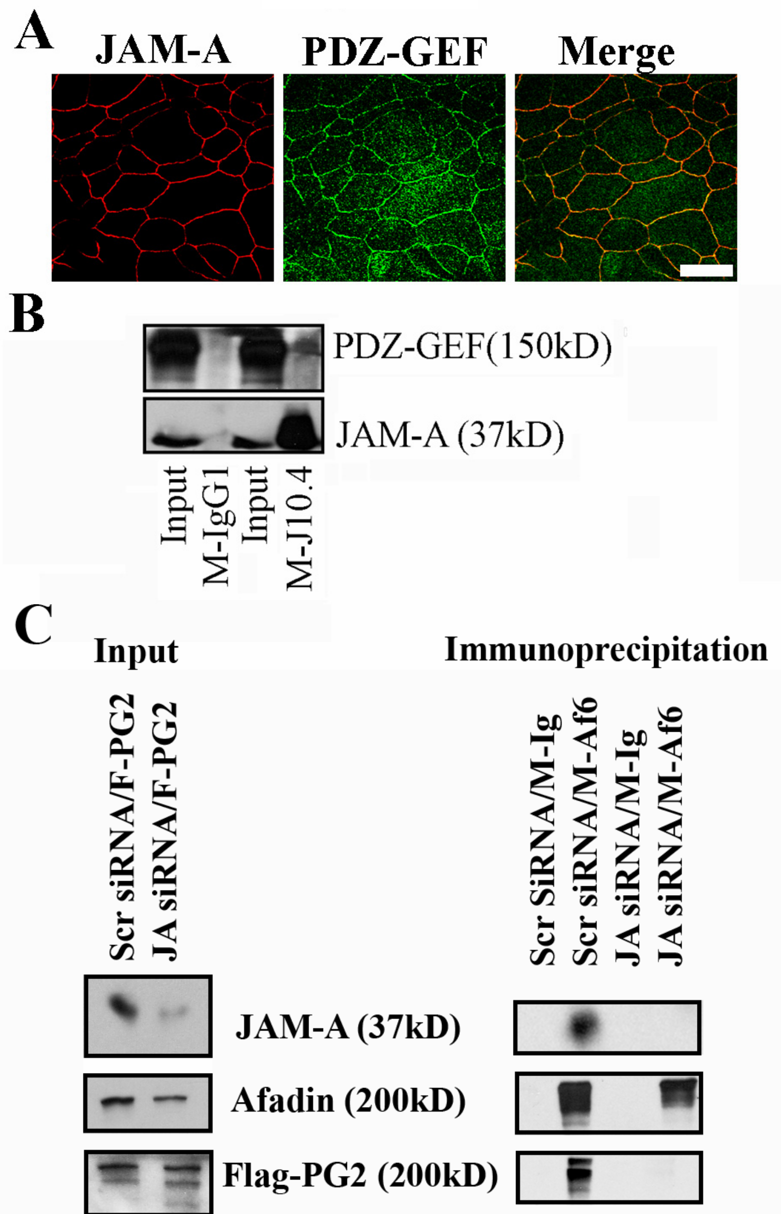


Figure 3.7: *JAM-A and PDZ-GEF co-localize and co-associate*. A, By IF, JAM-A (red) and total PDZ-GEF (green) co-localize as seen in the merged image. Scale bar is 20μm. B, immunoprecipitations with J10.4, a mouse IgG1 anti-JAM-A antibody, pulls down PDZ-GEF, as can be seen in the upper immunoblot, while neither JAM-A nor PDZ-GEF is pulled down by the isotype control antibody. C, Overexpression of Flag-PDZ-GEF2 allows for IP of the Flag tagged PDZ-GEF2 with Mouse anti-Afadin antibodies in the presence of JAM-A. If JAM-A expression is removed via siRNA, PDZ-GEF2 and Afadin appear to lose their ability to associate.

Epithelial cell migration is necessary for maintenance of epithelial barrier function and the repair of superficial injuries in the intestinal mucosa. Decreased wound healing *in vivo* would be expected to result in increased severity and delayed recovery of a variety of conditions associated with mucosal injury. Indeed these results are consistent with recent reports increased severity of disease in a chemically induced mouse model of colitis (56),(96). These reports demonstrated increased levels of colonic mucosla injury and inflammation in JAM-A deficient animals compared to wild type control mice. This *in vivo* data is consistent with our in-vitro observations implicating JAM-A as an important regulator of epithelial wound healing.

This study provides further details highlighting a pathway downstream of dimerized JAM-A that regulates cell migration, We recently reported that in epithelial cells, JAM-A dimerization and the JAM-A PDZ binding motif are necessary for stabilization of β1 integrin and regulation of 3D migration (86). These findings are consistent with those observed in endothelial cells where decreased cell migration in JAM-A–deficient endothelial cells that was restored after transfection with full-length JAM-A but not protein lacking the PDZ binding motif(45). Furthermore, Naik *et al.* reported that overexpression of JAM-A increased cell migration in endothelial cells through $\alpha_v\beta_3$ integrin and activation of MAP kinase(44).In the current study, our scratch wound assays revealed that, similar to epithelial cell migration through matrix, decreased levels of β1 integrin protein resulted in decreased rates of cell migration and rescued levels of β1 integrin led to a restoration of normal rates of cell migration. Collectively, these observations indicate that JAM-A regulates both two and three dimensional cell migration through maintenance of cellular β1 integrin protein levels.

The mechanism(s) that lie behind intracellular JAM-A signaling are poorly understood. Based on our previous data, we hypothesized that JAM-A regulation of 3D epithelial cell migration was dependent on cis-homodimerization that brings into close apposition of at least two JAM-A PDZ binding domains. It was proposed that such closely apposed PDZ binding domains would allow for efficient binding to a scaffolding protein complex . The interactions of such scaffolding proteins might then result in signaling that regulates β1 integrin protein levels(86) and cell migration.

To further explore the above hypothesis, we tested whether JAM-A associates with candidate PDZ domain signaling molecules in polarized intestinal epithelial cells using standard biochemical and immunolocalization techniques. We then tested for functional linkage to JAM-A by comparing the effects of loss of these associated proteins with those observed after loss of JAM-A. For each candidate protein tested, multiple siRNA targets for each gene were used independently of each other to test for protein knockdown. The two most promising targets were then *separately* tested for effects on β1 integrin levels and cell migration. We used two separate oligonucleotides for each candidate molecule tested to diminish the possibility of siRNA-mediated interferon and off-target effects and to increase the likelihood of specificity of findings (97). In all experiments, a maximum concentration of siRNA used was 50nM, which is well below the concentrations reported to induce activation of toll-like receptor 3 (~330nM)(98). For each gene targeted, the two siRNA oligomers that resulted in the greatest decrease in protein expression were then combined and used for the experiments presented in this report.

Using the above approach, we observed that decreased expression of JAM-A, Afadin, PDZ-GEF2 or Rap1A, but not ZO-1, PDZ-GEF1 nor Rap1B, resulted in reduced levels of β1 integrin protein as well as decreased rates of cell migration. Additionally, decreased expression JAM-A, Afadin and PDZ-GEF2 all decreased levels of active Rap1. Similar biochemical and functional effects after decreased expression of these proteins indicated that they are either in the same signaling pathway or separately activate the same effectors. However, since JAM-A, Afadin, and PDZ-GEF2 are part of a complex dependent on JAM-A expression, it is most likely that these proteins participate in a single signaling pathway.

While decreased expression of PDZ-GEF1 had no effect on β1 integrin levels or cell migration, there was a decrease in total Rap1 activity. Furthermore, simultaneous knockdown of PDZ-GEF1 and PDZ-GEF2 resulted in larger decreases in Rap1 activity. These observations would be explained if PDZ-GEF1 specifically regulates Rap1B., Further studies are necessary to elucidate the functional consequences of decreased Rap1B activity.

Together, the data from this and previous studies support the hypothesis that a complex of dimerized JAM-A, Afadin and PDZ-GEF2 regulate β1 integrin at the protein level by controlling Rap1A activation. Such fine-tuning the levels of β1 integrin could serve as a regulator of cell migration under a variety of circumstances. As highlighted in figure 8, our observations support a model of JAM-A –mediated regulation of cell migration through the formation of a PDZ-dependent signaling complex. In this model, JAM-A dimerization leads to the close apposition PDZ-GEF2 and Afadin. Rap1A brought into close proximity to PDZ-GEF2 by Afadin is maintained in a active state to

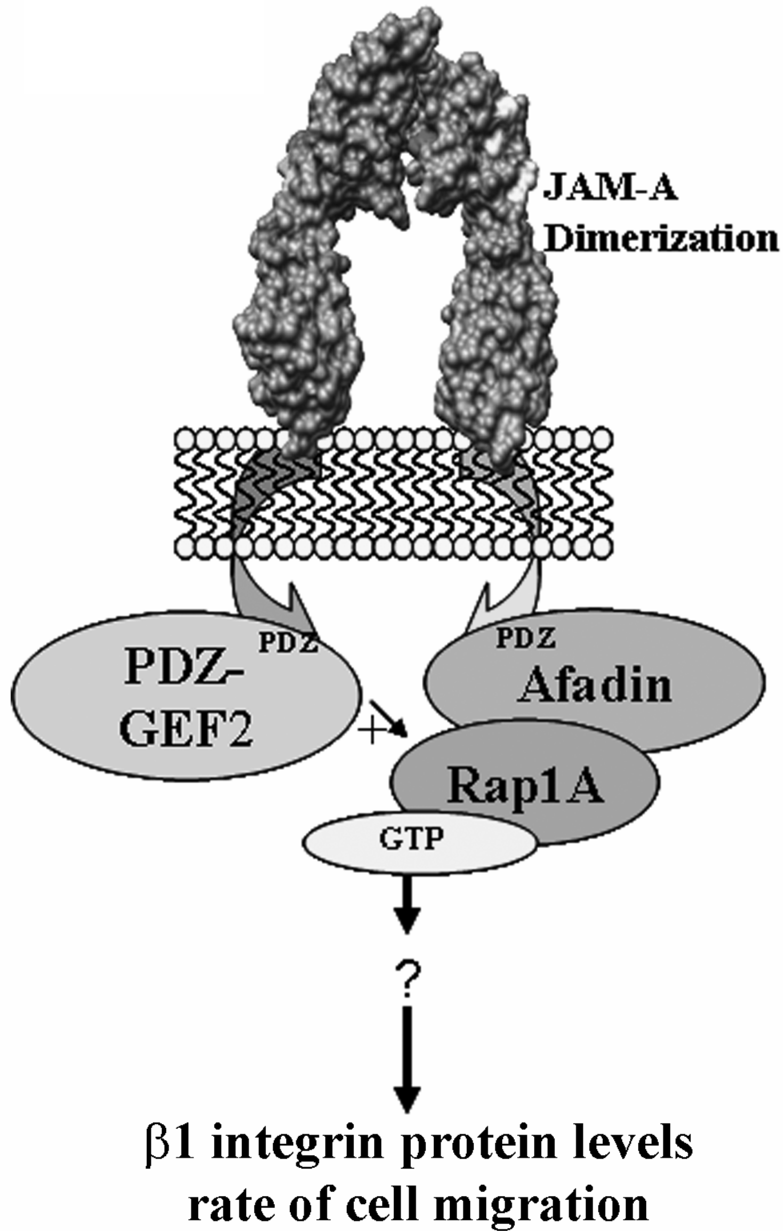


Figure 3.8: *Model for the molecular basis of JAM-A signaling.* Dimerized JAM-A can interact with Afadin and PDZ-GEF2, possibly directly (as shown) or indirectly. Rap1A then interacts with Afadin and can be activated by the spatially close PDZ-GEF2.

promote cell migration by stabilizing β1 integrin levels through, as of yet, unclear mechanisms. The protein interactions depicted in Figure 8 may also occur via indirect mechanisms as additional, as of yet unidentified, scaffolding proteins may be part of the complex.

It is important to note that the murine crystal structure of JAM-A predicts the formation of dimers on the same cell (cis) that interact between cells in trans (5). Indeed, the signaling events outlined in figure 8 would be enhanced through trans interactions of JAM-A cis-dimers between cells. Trans-interactions as well as cis interactions would result in each JAM-A dimer having two trans binding sites, allowing for localized high density polymerization of JAM-A between cells. Such localized high concentrations of JAM-A between cell-cell contacts would result in large signaling complexes serving to amplify JAM-A mediated signals. The nature of such predicted trans-interactions between cis-dimers of JAM-A remain to be defined through rigorous biochemical studies.

In summary, our findings support a model of JAM-A dimerization-mediated signaling through interactions with Afadin and PDZ-GEF2 resulting in activation of Rap1A, which stabilizes β1 integrin levels and regulates cell migration. Additional mechanistic studies are necessary to determine how activated Rap1A stabilizes β1 integrin levels in epithelial cells. We hypothesize that other reported JAM-A functions may use similar mechanisms for intracellular signaling; however, there are likely to be cell-type specific signaling intermediates and effector proteins. Further studies are needed to determine if the general mechanism described in this report is applicable to JAM-A –mediated regulation of other cellular processes.

3.5 Methods

Cell culture: All experiments used SKCO-15 human colonic epithelial cells, which were grown in Dulbecco's Modification of Eagle's Medium (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 (Cellgro). The cells were subcultured and harvested with 0.05% trypsin with EDTA in Hanks' balanced salt solution (Sigma). For immunoblotting and DIC imaging, cells were plated in T25 flasks (Corning) at a number such that their density was 25% the day of transfections. For filter based studies, cells were plated at a density of $1x10^5$ cells/0.33 cm^2.

Western blots: Monolayers of epithelial cells that were between 60 and 90% confluent (subconfluent) were homogenized in RIPA lysis buffer containing 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. Lysis buffer was supplemented with protease and phosphatase inhibitor cocktails from Sigma (1:100 dilution). Protein concentrations in lysates were quantified by BCA assay. Lysates were cleared by centrifugation and immediately boiled in reducing SDS sample buffer. Additionally, isolated murine epithelial sheets of JAM-A (-/-) and control mice were analyzed for β1 integrin and Rap1 by western blot analysis. Distal colonic epithelial cell lysates were prepared after the serosa and external longitudinal layer of the muscularis propria were stripped away. Isolated epithelial sheets were subsequently lysed in RIPA buffer. Mice were genotyped and raised as reported previously reported (56). SDS-PAGE and western blots were performed by standard methods. Tubulin was used as a protein loading control. Each western blot shown is representative of at least three independent experiments.

Immunoprecipitations(IP): Cells were grown to between 60 and 90% confluence (subconfluent) in T25 flasks (Corning) and then lysed with 1ml RIPA buffer and douncing. Samples were then centrifuged to remove cell debris and 100ul of supernatant was saved for input sample. The remaining supernatant was used for IPs. Sample was precleared for 45 minutes at 4C with sepharose beads followed by incubation for 1 hour at 4C with Protein G-coupled to sepharose (GE). Beads were washed 3x with sample buffer. Beads were boiled for 15 minutes with SDS sample buffer and the entire sample was loaded for analysis by western blot. Each IP was repeated at least 3 times.

Immunofluorescence Microscopy(IF): Cells were grown on 0.3um pore transwell filters (CoStar), fixed in 100% Ethanol at -20C for 20 minutes and blocked in 1% BSA in HBSS+ for 1 hour. Primary antibodies were diluted in blocking buffer and incubated with cells for one hour at 25°C. The cells were washed in HBSS+ and then incubated in fluorescently labeled secondary antibodies for 45 minutes at room temperature. Labeled cells were then washed and mounted in Prolong Antifade Agent (Molecular Probes). A Zeiss laser scanning microscope was used to capture confocal fluorescence images.

Differential interference contrast microscopy (DIC): Pictures were obtained using an Axiovert 35 light microscope at 5x power using the DIC .4 filter and saved using Axiomatic Imaging Software (Zeiss). Images were then imported directly into Adobe Photoshop and saved as TIFF files for figures.

Antibodies: The murine monoclonal anti-JAM-A antibody J10.4 was previously described*(23)* and the rabbit polyclonal anti-PDZ-GEF was a kind gift of Dr. Mochizuki(99). Other antibodies were commercially purchased: polyclonal rabbit anti-JAM-A (Zymed), monoclonal mouse anti-Afadin (BD transduction), monoclonal mouse

anti-tubulin (Sigma), polyclonal rabbit anti-actin (Sigma), rabbit polyclonal Rap1 (Upstate) , monoclonal rabbit anti-β1 integrin (Novus), monoclonal rabbit anti-JAM-A (Novus), polyclonal rabbit ZO-1 (Zymed), polyclonal Rabbit anti-Flag (Sigma), goat anti-rabbit-HRP (Jackson Immunolabs), and goat anti-mouse-HRP (Jackson Immunolabs).

SiRNA experiments and DNA transfection: For siRNA protein targets, four to ten oligonucleotides were designed for each protein target and protein downregulation was verified by western blot. SiRNA oligonucleotides were obtained from Dharmacon or Qiagen. Controls included a scramble control from Qiagen (All-stars Negative Control) as well as mock transfected controls. All targets used are listed in Table 1. Every transfection was done at a total concentration of 50nM siRNA. Transfections were performed using HiPerFect (Qiagen) according to the manufacturer's instructions. Assays were performed 72 hours after transfection.

DNA transfections in SKCO-15 cells were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol when the cells were 50% confluent and similar to previously published protocols(86). Cells were used in assays 48 hours post-transfection.

Rap1 Activity Assay: A Rap1 activity assay was performed according to the manufacturer's instructions (Upstate). Briefly, cells were lysed in a tris and triton-X based lysis buffer at 4C. Cell debris was removed by centrifugation and 100uls of sample was saved as input to determine total Rap1 levels. 50ug of protein for each sample was then incubated at 4C for 45 minutes with Ral-GDS agarose beads to bind active Rap1. Beads were washed three times with the lysis buffer followed by boiling in SDS sample

buffer. The entire sample was analyzed by western blot with detection using a polyclonal Rap1 antibody from Upstate. Each activity assay was repeated at least 3 times.

Scratch wound Assay: Cells were seeded in 24 well plates at a density resulting in 50-70% confluence 24 hours later. Cells were treated with siRNA 24 hours after seeding. Wounds were made in confluent monolayers 48 hours after siRNA treatment using a pipet tip under vacuum suction. A straight line was drawn across the bottom of each well, and then pictures were taken with the line at the bottom of the viewing field. Wounds were measured from the exact vertical middle of each picture such that the initial measurement and the measurement 16 hours later were taken from the same vertical spot in each well. Representative images are shown for each figure and results are reported as % of wound closed. % wound closure is equal to (initial width – width at 16 hrs)/initial width. These measurements were taken in pixels, which linearly correlates to distance. Data are from a representative experiment of 3 independent experiments with 4 sample replicates per experiment.

Constructs and cloning: A PDZ-GEF2 construct was cloned from SK-CO15 cDNA. mRNA from SK-CO15 cells, which were 75% confluent, was isolated using trizol (Invitrogen). cDNA was then produced using the first-strand cDNA synthesis kit (Invitrogen). PDZ-GEF2 was amplified by PCR with Phusion (NEB) polymerase and an N-terminal Flag tag and BamHI and XhoI restriction sites were added in the primers. The forward primer containing the flag tag was: 5’aataaagcttgccaccATGGATTACAAGGACGACGATGACAAGATGAACTCACCCGTGGACCCT-3’. The reverse primer was 5’- TTCAAGAAATGTCCTGTAAGTT-3’.

The PCR product was inserted into pCDNA3.0 using the engineered restriction sites and sequence was verified.

RT-PCR: RT-PCR was performed using the Superscript III one-step PCR kit from Invitrogen according to the manufacturer's instructions. The annealing Tm used for all primers was 57C. The PDZ-GEF1 and 2 primers used have been previously described and verified(100).

Real-time PCR- Quantitative real-time PCR was performed to determine mRNA levels. PCR amplification was performed using the GeneAmp 5700 Sequence Detection System (PE Applied Biosystems). PCR was run using the following protocol: initial activation at 94°C for 3 min, 40 cycles of 94°C for 15 s, 52°C for 30s, and 72°C for 30s. Each sample was performed in triplicate. Direct detection of PCR product was monitored in real-time by measuring the increase in fluorescence caused by the binding of SYBR Green I Dye (Invitrogen) to dsDNA, using the ABI Gene Amp 5700 sequence detection system (Applied Biosystems). Results of the real-time PCR data were represented as fold change, which was determined by applying the formula $2^{\Delta\Delta Ct}$, where ΔCt = Ct of target gene - Ct of endogenous control gene (signal regulatory peptide), and $\Delta\Delta Ct$ = ΔCt of samples for target gene (siRNA treated) - ΔCt of the control for the target gene (Mock siRNA treated). The primers used were: Rap1A.F: 5' - TGGATACTGCAGGGACAGAGCAAT - 3'; Rap1A.R: 5' - ACATCTTCCGTGTCCTTAACCCGT - 3'; Rap1B.F: 5' - AGGCGTTGGAAAGTCTGCTTTGAC - 3'; Rap1B.R: 5' - ATTGCTCCGTTCCTGCAGTATCCA - 3'; PDZ-GEF1.F: 5' - AAATTCGTCACGTTGGCCGAATGG - 3'; PDZ-GEF1.R: 5' -

ACTCCGCCATTTCTTCTTCCGAGT - 3’; PDZ-GEF2.F: 5’ - TGTTGACTCCATGTCTGCAGCTCT - 3’; PDZ-GEF2.R: 5’ - ACCCAGGGCCATGTTGACTATGAT - 3’.

Statistics: For comparisons samples in experiments with only two groups, student’s t-test was used. One way Anova was used for comparisons in experiments with greater than two groups with post-hoc analysis performed by Graphpad (Graphpad software) to determine p values for sample groups compared to controls. $p<0.05$ was considered significant in either case.

Chapter 4

Conclusions

Some of the material in this Chapter will appear in the Annals of the New York Academy of Sciences: Mechanisms of outside-in signaling at the Tight Junction by Junctional Adhesion Molecule A. Dr. Charles A. Parkos is a co-author of this review.

4.1. Summary of Results

4.1.1 Introduction

Junctional Adhesion Molecule A (JAM-A) is expressed at tight junctions of endothelial and epithelial cells as well as on a variety of hematopoietic cells. The functional significance of JAM-A is only just being realized, with many studies published in recent years detailing connections between JAM-A and inflammation,(24,56) angiogenesis,(32,101) hypertension(102,103) ischemia/reperfusion(50) and atherosclerosis.(104,105) At a cellular level, JAM-A function has been linked with regulation of cell migration/invasion,(86) platelet adhesion,(87) cell polarization,(35) cell proliferation(56) and epithelial or endothelial barrier function.(3,22,23,56) However, the mechanisms by which JAM-A mediates these processes had not been well elucidated prior to the work presented here.

4.1.2 JAM-A and outside-in signaling: evidence for dimerization-mediated regulation of cell function.

Several reviews have detailed responses and phenotypes in different systems after manipulating JAM-A expression(106-109) and are not the focus of these chapters. Here we focus on data derived from interference with JAM-A dimerization and/or downregulation of JAM-A expression in order to provide a current overview of the structure-function relationships for various domains of JAM-A. These structure-function relationships, primarily investigated in Chapter 2, were used to construct a hypothetical model of JAM-A-mediated signaling, the testing of which is described below and in Chapter 3.

According to both the mouse(5) and human(21) crystal structures, JAM-A forms a homodimer in cis. Cis-dimerization is mediated by an R-EWK motif found in JAM-A at amino acids 59 through 63 and is conserved in a number of closely related family members, including JAM-B, JAM-C and Coxsackie-Adenovirus Receptor (CAR). JAM-A dimerization is readily assessed biochemically since it occurs naturally in solution between exogenously expressed JAM-A molecules. Single substitutions for any of the amino acids involved in the ionic interactions important for dimerization ablates the ability for JAM-A to dimerize in solution.(68) Homodimerization can also be blocked by treatment with function-blocking antibodies such as J10.4(22,23) or BV11.(4) These antibodies prevent dimerization of JAM-A through steric hindrance and are useful tools for looking at the relationship between JAM-A function and JAM-A dimerization.

293T cells are a human embryonic kidney epithelial cell line that are readily transfectable and endogenously express JAM-A at a moderately low level,(86) making them a good model in which to study JAM-A structure/function relationships, as was done in Chapter 2. Intriguingly, treatment of 293T cells with the JAM-A dimerization-inhibiting antibody J10.4 significantly reduces the rate of cell migration. This effect is likely mediated by reduced stability of β1 integrin protein levels, as treatment with J10.4 also causes internalization and eventual degradation of β1 integrin protein but has no effect on levels of β1 integrin mRNA levels.(86)

The above findings have been confirmed using 293T cells stably transfected with wild-type JAM-A constructs or dimerization-defective JAM-A constructs. Indeed, dimerization-defective JAM-A mutants seem to behave in a dominant negative fashion, causing decreased β1 integrin protein levels as well as decreased cell invasion similar to

that observed after treatment with J10.4. The role of β1 integrin as the effector for cell invasion in these *in vitro* assays has also been confirmed. Overexpression of β1 integrin in a background of JAM-A dimerization-defective mutants restores the rate of 293T cell migration to wild-type levels.(86)

From these observations, we have proposed a hypothetical model of JAM-A function (**Fig 4.1**), which was then tested in the study described in Chapter 3. In this model, cis-dimerization of JAM-A appears to brings into close proximity at least two molecules of JAM-A. Each JAM-A molecule has a PDZ-binding motif that mediates interactions with PDZ containing scaffolding proteins such as Afadin, as functionally demonstrated in Chapter 3 and biochemically reported in previous studies.(4,7) The close apposition of these scaffolding molecules likely then mediates signals that, in turn, increase β1 integrin stability.

4.1.3 The role of JAM-A extracellular dimerization and scaffolding complex formation in intracellular signaling.

In Chapter 2, evidence was presented implicating JAM-A in the regulation of cellular levels of β1 integrin and cell migration. Furthermore, it was reported that dimerization and a PDZ binding motif are required for these JAM-A mediated effects. To gain further insight into the JAM-A mediated signaling pathway that mediates these effects, we reduced the expression of candidate TJ scaffolding proteins using siRNA in a model intestinal epithelial cell line SK-C015. In Chapter 3, we report that in a fashion identical to that observed with loss of JAM-A expression, downregulation of the TJ scaffolding protein Afadin resulted in decreased activation of the small GTPase Rap1, reduced β1 integrin protein levels and a slower rate of cell

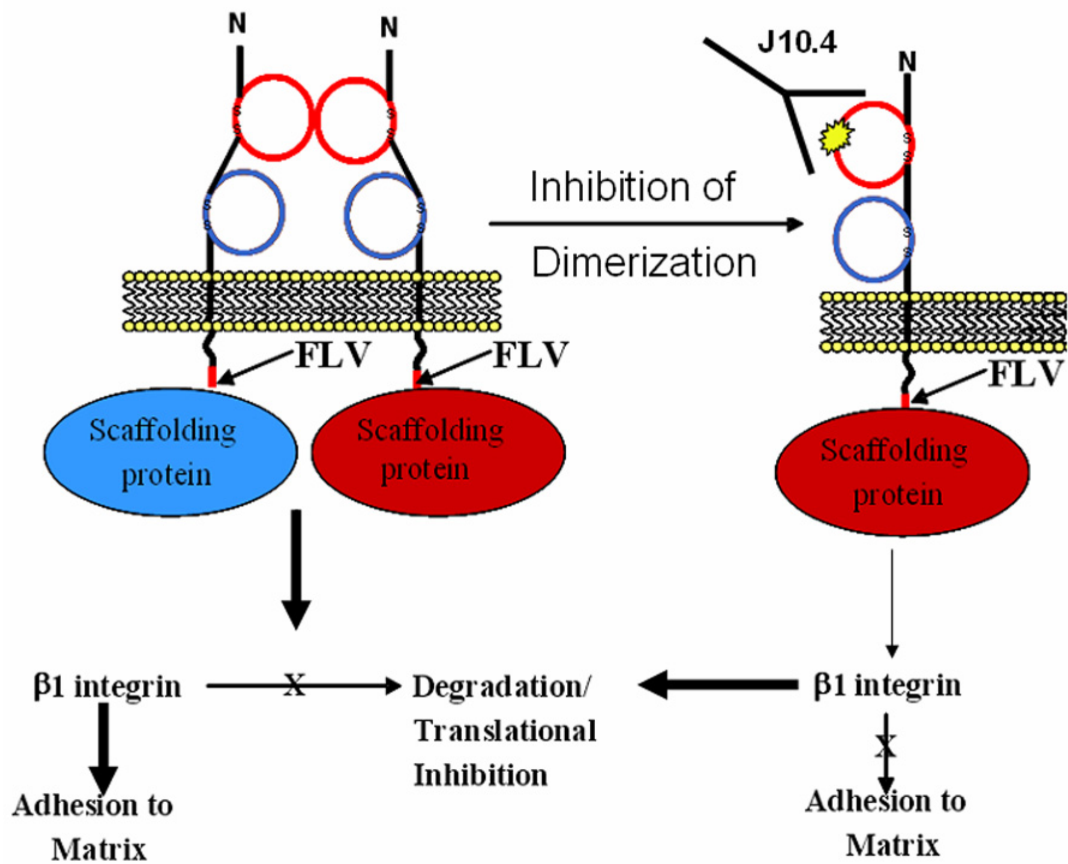


Figure 4.1: *Model for JAM-A outside-in signaling.* JAM-A dimerization leads to the close apposition of scaffolding proteins. Scaffolding proteins activate signaling molecules to stabilize β1 integrin at the cell surface through unknown mechanisms. Interference with JAM-A dimerization prevents close apposition of scaffolding proteins and prevents subsequent signaling.

migration. Immunofluorescence (IF) and immunoprecipitation (IP) studies revealed co-association of Afadin with JAM-A. Interestingly, the small GTPase Rap1 is known to interact with Afadin and regulate integrin function. Selective downregulation of Rap1A, but not Rap1B, resulted in decreased β1 integrin protein levels and a reduced rate of cell migration. Since Rap1 activation is dependent on guanine nucleotide exchange factors (GEFs), we assessed expression of GEFs *in vitro* and identified PDZ-GEF1 and PDZ-GEF2 as major transcripts in colonic epithelia. IF and IP studies revealed co-association of the PDZ-GEF2 with Afadin that was dependent upon the expression of JAM-A. Loss of both PDZ-GEF1 and PDZ-GEF2 expression led to diminished Rap1 activity, however, only PDZ-GEF2 downregulation resulted in decreased β1 integrin levels and a decreased rate of cell migration. These results suggest that dimerized JAM-A signals through a complex containing both Afadin and PDZ-GEF2 which, in turn, activates Rap1A to regulate β1 integrin levels and cell migration. Thus, the data from chapters 2 and 3 present a novel mechanism for how JAM-A dimerization activates Rap1A. Future studies will focus on how increased Rap1A activity functions to stabilize β1 integrin levels.

4.2. Different functions of JAM-A may be mediated through activation of distinct scaffolding and signaling molecules.

We have presented evidence suggesting that dimerization of JAM-A and the binding of the PDZ binding domains of JAM-A to scaffolding proteins leads to the formation of signaling complexes. Different complexes containing distinct components would thus be likely to produce different JAM-A functional responses. For example, as reported in chapter 3 dimerized JAM-A bound to Afadin and other GTPase exchange

factors/activating proteins stabilize β1 integrin levels and regulate cell migration.(86) However, while Afadin reduces trans-epithelial resistance (TER) we have not observed that loss of Rap1 has an effect on TER across confluent epithelial monolayers. By contrast, downregulation of JAM-A expression significantly diminishes TER and alters expression of various claudin proteins.(3,56) As another example, loss of JAM-A in vivo results in enhanced proliferation, which suggests that additional nuclear signaling events occur, the nature of which remain to be determined. The role of other PDZ containing scaffold proteins in regulating these processes remains to be determined. These observations suggest that JAM-A expression and dimerization is able to activate various signaling molecules through a variety of distinct mechanisms. Signaling through diverse scaffolding and signaling molecules would account for the variety of phenotypes observed after loss of JAM-A expression and/or interference with JAM-A dimerization.

4.3. <u>JAM-A, PDZ-GEF1, Rap1B and cell morphology.</u>

An example of another JAM-A signaling pathway emerged during the course of the research that led to the publication detailed in Chapter 3. In cell culture, using an intestinal epithelial cell line (SK-CO15), cells with decreased expression of JAM-A had altered cell morphology when subconfluent (**Figure 4.2**) (22). These data provides insight into another JAM-A signaling cascade. During the course of our investigations into the β1 integrin signaling cascade, we observed that decreased expression of JAM-A, Afadin, PDZ-GEF1 and Rap1B, but not PDZ-GEF2 or Rap1A resulted in similar altered cell morphology as shown in Figure 5.2. Combined with the association and localization data presented in Chapter 3, this indicates that PDZ-GEF1 activates Rap1B in the

presence of JAM-A to regulate cell morphology. The mechanism of control of cell morphology by

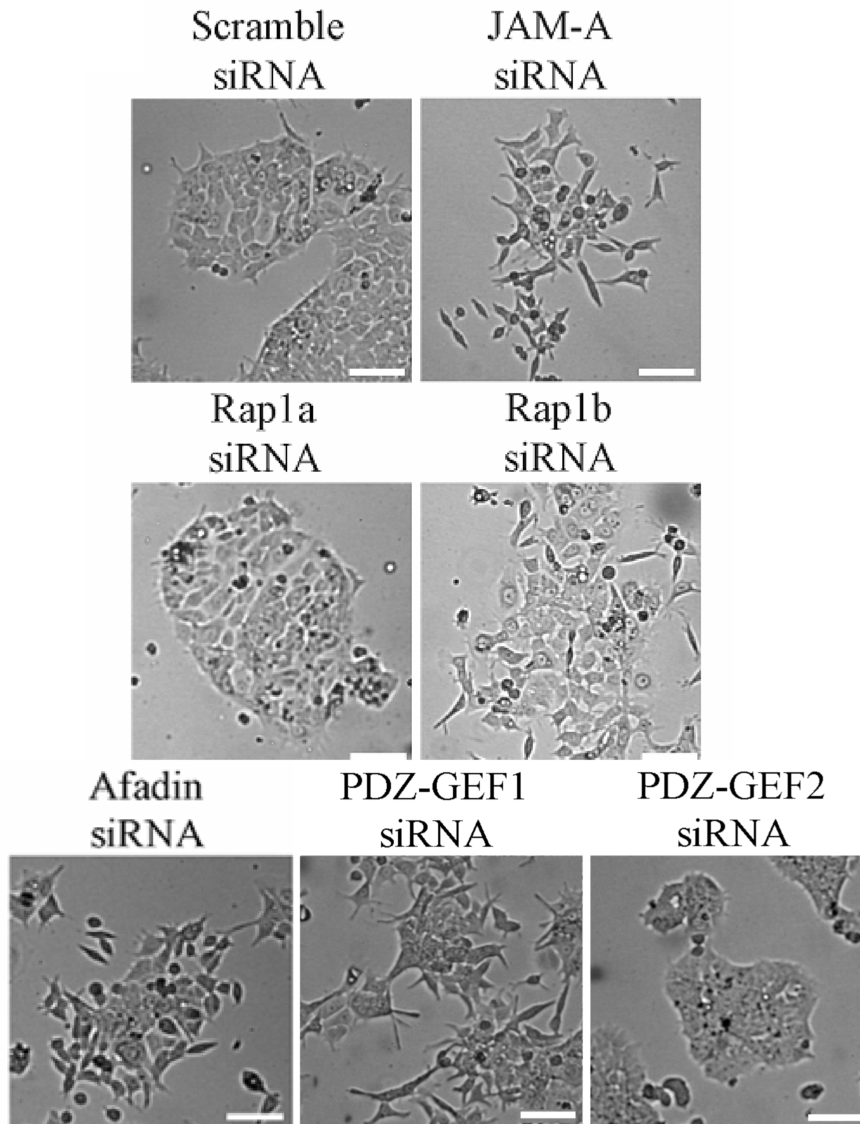


Figure 5.2: *Alterations in cell shape after siRNA treatment in Subconfluent SK-CO15 cells.* Treatment with siRNA for JAM-A, Afadin, PDZ-GEF1, or Rap1B causes a change in cell shape compared to control treatments. The cells change from forming round epithelial clusters to forming individualized cells with prominent protrusions.

activated Rap1B remains unknown. However, these data does indicate a second signaling cascade that is regulated by JAM-A and increases the likelihood that JAM-A functions in a similar matter to control other cellular functions.

4.4 Other potential interacting domains on JAM-A

These chapters have focused extensively on systems where expression of JAM-A has been downregulated or on JAM-A mutants with defects in cis-dimerization or PDZ binding domains. Respectively, these domains consist of either a subsection of the distal most Ig-like loop or the last three amino acids of the cytoplasmic tail. Thus, a functional role of other regions on the protein has not been excluded.

Indeed, there is evidence for other functional domains on JAM-A. One example consists of the domain comprising the epitope for F11, an antibody for which JAM-A/F11R was originally named(64). Peptides mimicking part of the distal Ig domain (D1) block both F11 binding to JAM-A and platelet adhesion to endothelial cells.(87) However, these peptides map to a different area on D1 that is distinct from the cis-dimerization region,(21) indicating a potentially separate functional domain. Additionally, the proximal Ig-like domain (D2) may also have functional significance. There are reports indicating that D2 binds to the leukocyte integrin LFA-1 and mediates leukocyte transmigration,(110) thus it is also possible that D2 is responsible for some JAM-A mediated functions through, as of yet, unknown interactions.

4.5. Therapeutic potential for targeting of JAM-A homodimerization.

JAM-A has been linked to pathophysiology of several diseases. In the introduction, it was discussed how JAM-A cellular adhesion and angiogenesis, thus their expression may be key in the growth of primary tumors, tumor metastasis and survival of

cancer cells. Dimerization of JAM-A may contribute to increased invasion, particularly in light of the data presented in chapter 2 and chapter 3. Additionally, given the roles of the JAM-A in cell permeability, cell polarization and heterophilic ligand binding, JAM-A likely plays a key role in a number of inflammatory conditions such as ischemia/reperfusion injury (49). Expression of JAM-A has also been implicated in atherosclerosis and blood pressure as wild-type endothelial cells lacking ApoE have more severe atherosclerosis compared to endothelial cells deficient for JAM-A and rats with spontaneously increased JAM-A have higher blood pressure(58,59). Inhibition of JAM-A by targeting the homo-dimerization domain either through a small molecule or antibody approach could help with any or all of these conditions by disrupting JAM-A function. Further studies examining other functional regions of JAM-A would possibly lead to other therapeutic targets beyond the homodimerization domain.

4.6 Conclusions

This thesis outlines a novel outside-in signaling mechanism for JAM-A function. The data presented here suggests that JAM-A dimerization recruits scaffolding proteins, specifically Afadin and PDZ-GEF2 in our example, to activate signaling molecules, such as Rap1A. The activated signaling molecules then serve to control cellular phenotypes such as the rate of cell migration. This is the first description of a specific, detailed mechanism for the function of a JAM family member and may permit generalization for the mechanism of action of JAM-A in other context and mechanisms of action for other JAM family proteins. Hopefully, the data from this thesis will lead to work exploring similar mechanisms for other JAM-related phenotypes and lead to the development of novel therapeutics that target the easily accessible extracellular JAM-A homo-

dimerization interface for the control of conditions such as cancer metastasis, inflammation, atherosclerosis and hypertension.

Chapter 5

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