Role of Rac1 in Regulating the Integrity

of Endothelial Adherens Junctions

BY

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THESIS

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I dedicate this thesis to my family for their endless love and support

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LIST OF ABBREVIATIONS

AJ	Adherens Junction
ARP	actin-related protein
CaM	Calmodulin
cAMP	Cyclic Adenosine Monophosphate
CFP	Cyan Fluorescent Protein
cGMP	Cyclic Guanosine Monophosphate
COPASI	Complex Pathway Simulator
cRO	caged ROCKOUT
DN	Dominant Negative
EC	Extracellular Cadherin
E-cadherin	Epithelial-cadherin
EPAC	Exchange Proteins Activated by cAMP
EPLIN	Epithelial Protein Lost in Neoplasm
FAJ	Focal Adherens Junction
FRAP	Fluorescence Recovery After Photobleaching
FRET	Förster Resonance Energy Transfer
GAP	GTPase Activating Protein
GDI	Guanine Nucleotide Dissociation Inhibitor
GDP	Guanosine Diphosphate
GEF	Guanine Nucleotide Exchange Factor
GFP	Green Fluorescent Protein
GTP	Guanosine Triphosphate
HIF	Hypoxia-inducible Factor
HMEC	Human Microvascular Endothelial Cell
^{1}H-NMR	Proton Nuclear Magnetic Resonance
HPAEC	Human Pulmonary Arterial Endothelial Cell
IF	Immunofluorescent
IQGAP	IQRasGTPase-Activating Protein
JMD	Juxtamembrane Domain
LOV	Light Oxygen Voltage
LPS	Lipopolysaccharide
MLCII	non-muscle Myosin II Light Chain

MLCK	Myosin Light Chain Kinase
MLCP	Myosin Light Chain Phosphatase
MRCK	Myotonic dystrophy Kinase–related CDC42-binding Kinase
N-cadherin	Neural-cadherin
PA	Photo-activatable
Pak	P21 Activated Kinase
PI3K	Phosphatidylinositide 3-Kinase
PKC	Protein Kinase C
PTM	Post-translational Modification
PI	Photo-insensitive
RBD	RhoA Binding Domain
ROCK	Rho-associated coiled-coil forming protein kinase
ROS	Reactive Oxygen Species
S1P	Sphingosine 1 phosphate
SHP2	Src homology 2-domain containing tyrosine phosphatase
SP	Single Peptide
Src	Sarcoma
Tiam1	T-cell Lymphoma Invasion and Metastasis-inducing Protein 1
TJ	Tight Junction
TNF	Tumor Necrosis Factors
VE-cadherin	Vascular Endothelial-cadherin
VEGF	Vascular Endothelial Growth Factor
VE-PTP	Vascular Endothelial Protein Tyrosine Phosphatase
YFP	Yellow Fluorescent Protein

SUMMARY

The endothelium forms a semi-permeable barrier that is critical for maintaining tissue fluid homeostasis and transmigration of blood cells. Disruption of the endothelial barrier leads to accumulation of protein-rich fluids and blood cells in interestitium, leading to tissue edema. Permeability of the vessel wall is primarily regulated through inter-endothelial Adherens Junction (AJ) complex, which include Vascular Endothelial (VE)-cadherin and the associated catenin molecules. VE-cadherin, the main adhesive protein of AJs is responsible for endothelial cell-cell hemophilic interaction through formation of *trans* dimerization to bring opposing cells together and restrict vasculature permeability. Stability of VE-cadherin adhesion is regulated via linkage to the actin cytoskeleton, mediated by catenin molecules.

The members of RhoGTPase family, particularly Rac1 and RhoA, are important regulators of actin cytoskeleton. Rac1 has been shown to induce actin polymerization and mediate the barrier protective effect of mediators such as Sphingosine 1 Phosphate, while RhoA is linked to stress fiber formation and myosin II-mediated contractility. In addition, the opposing action of Rac1 and RhoA coordinate the integrity in endothelial cell barrier as they regulate formation and maturation of cell-cell contact. However the role of Rac1 in stabilizing mature endothelial AJs is not well understood.

Previous studies on Rac1 signaling have used experimental approaches that led to the global activation of Rac1 as well as activation of other signaling pathways in parallel that could mask the effect of Rac1. Here, we used a genetically encoded photo-activatable-Rac1 to restrict the spatial and temporal activity of Rac1 to study the localized and transient activity of Rac1 at the cell-cell contact. We addressed the relationship between Rac1 and stability of endothelial AJs.

SUMMARY (continued)

In the first part of the thesis, we compared the effect of Rac1 activation in sub-confluent and confluent monolayers. We observed that in the sub-confluent monolayer, Rac1 activation at the site of cell-cell contact induced lamellipodia ruffling and promoted gap sealing. Interestingly, in the mature junctions, Rac1 activation at the site of cell-cell contact resulted in the stability of VE-cadherin adhesion, independent of lamellipodia activity. Using a photo-switchable protein Dendra2 we demonstrated that Rac1 activation reduced the rate of VE-cadherin dissociation from AJs, without affecting the rate of association, leading to increased density of VE-cadherin at AJs. We coupled these findings to Rac1-induced increase in the affinity for VE-cadherin *trans* dimerization utilizing an *in-silico* modeling. Furthermore, disruption of *trans* interaction using point mutation or inhibitor peptide blocked Rac1-induced effect on VE-cadherin dynamics, suggesting that VE-cadherin *trans* dimerization is required for Rac1-induced effect.

In the second part of thesis, we investigate Rac1 downstream signaling mechanism responsible for stability of VE-cadherin adhesion. We found that Rac1 antagonized RhoA activity and reduced actomyosin-dependent tension across VE-cadherin adhesion sites. We observed that inhibiting myosin II directly using blebbistatin or through spatiotemporal photo-release of the caged Rho kinase inhibitor also reduced the rate of VE-cadherin dissociation. These finding suggest that Rac1 functioned by stabilizing VE-cadherin *trans*-dimers in mature AJs by counteracting the actomyosin tension.

Finally, in the last part of thesis, we have provided preliminary data for a possible mechanism by which Rac1 exerts its effect on the junction. We showed that IQGAP1, a scaffold protein that interacts with active form of Rac1 and Cdc42 acts downstream of Rac1 and is

SUMMARY (continued)

required for Rac1-induced gap sealing and increase in VE-cadherin density. Thus, IQGAP1 may play an important role in stability of endothelial AJs.

In conclusion, the results of this thesis project put forward a new model of VE-cadherin adhesive interaction mediated by Rac1-induced reduction of mechanical tension at AJs resulting in the stabilizing of VE-cadherin *trans*-interaction and consequently the VE-cadherin adhesion.

1. LITERATURE REVIEW

1.1. Regulation of Endothelial Barrier Integrity

The endothelium, lining the vessel wall as a single monolayer, forms a semi-permeable barrier that restricts the passage of protein-rich fluids into the underlying tissues and is critical for maintaining tissue-fluid homeostasis, transmigration of blood cells, and gas exchange [1]. In response to inflammatory mediators, the endothelial barrier is disrupted; leading to intercellular gap formation, increase in permeability of vessel wall, and accumulation of protein-rich fluid in underlying tissue [10]. In lung, this condition causes pulmonary edema [12], the hallmark of Acute Lung Injury and Acute Respiratory Distress Syndrome [13].

Endothelial permeability and the passage of protein-rich fluids and blood cells into interestium is controlled via two routes; transcellular and paracellular [14]. The transcellular route, across the endothelium, is mainly responsible for the transport of macromolecules such as albumin, the most abundant protein of blood plasma, in a receptor-mediated and caveolae-dependent fashion (i.e. transcytosis) [14]. Transport of albumin to the interestial space creates a protein concentration gradient that facilitates fluid re-absorption and thus maintains transendothelial oncotic pressure and tissue-fluid homeostasis [1, 14]. On the other hand, paracellular pathway is responsible for

controlling the passage of protein-rich solutes and fluids in interestium in a size-selective manner via inter-endothelial junctions, between the two adjacent endothelial cells [14].

The inter-endothelial junctions are composed of complexes of Adherens Junctions (AJs), Tight Junctions (TJs), and Gap Junctions [15]. While Gap Junctions form channels between adjacent cells to allow the passage of water, ions, and small molecules, AJs and TJs are responsible for bringing neighboring cells together and restricting permeability of endothelial barrier. These two junction complexes are composed of distinct molecules that display dynamic behavior with continuous remodeling at steady-state [16, 17]. Claudin and Occludin are the major adhesion proteins of the TJ complex, responsible for hemophilic and heterophilic interactions [15, 18]. These proteins interact with intracellular molecules such as Zona Occludens-1, -2, and -3, PAR-3 and -6, and Cingulin [18]. While the distribution of TJs varies in different vascular beds, they are abundant only in the microvessels of blood-brain and blood-retinal barriers, where a strict control of permeability is required [19]. Furthermore, in the semi-permeable endothelium, in contrast to the epithelium, TJ complexes are formed secondary to AJs that appear at the early-stage of cell-cell contact in cell culture as well at the early-stage of embryo development [19]. Thus, AJ complex influence formation of TJ, presumably through up-regulation of Claudin5 expression [20] mediated by Vascular Endothelial (VE)-cadherin, the primary protein of AJs and the focus of this thesis, as discussed below.

1.2. **Endothelial Adherens Junctions Complex**

1.2.1. **VE-cadherin-Mediated Adhesion**

AJ complex is the primary adhesive complex in the contiguous endothelium and is composed of transmembrane VE-cadherin and the associated cytosolic catenin molecules (p120-catenin, β-catenin, plakoglobin (also called y-catenin), and α-catenin) [21] (Figure 1). Proteins of AJ
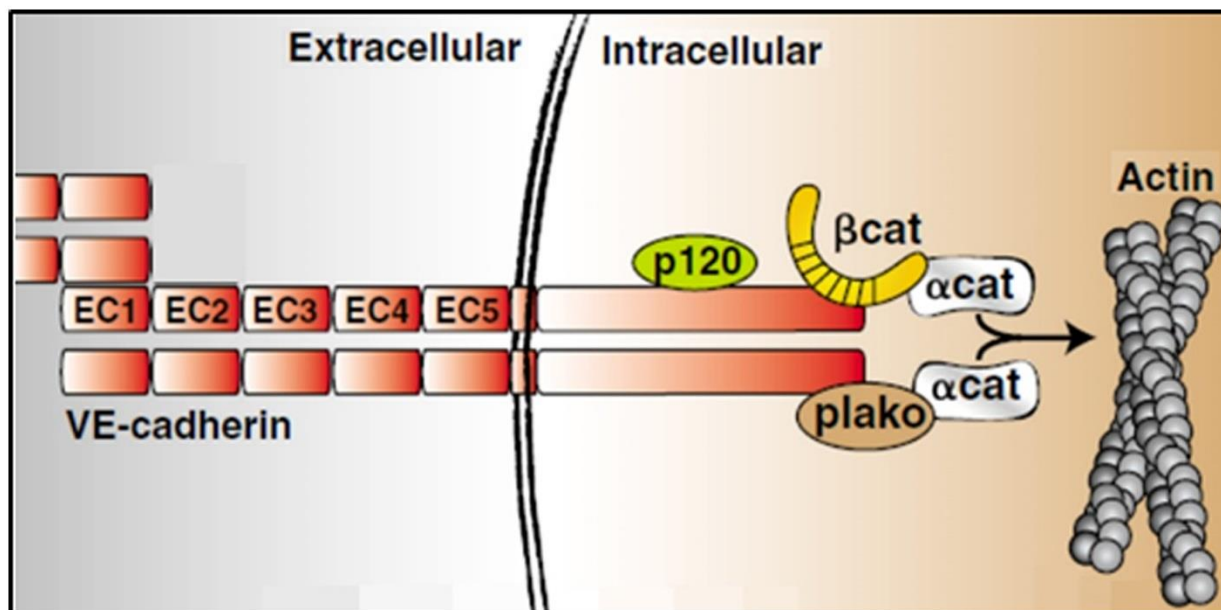
Figure 1: Endothelial Adherens Junction (AJ) Complex. VE-cadherin, the main protein of AJ, brings adjacent cells together through dimerization of its extracellular EC1 domain. From the cytoplasmic domain, VE-cadherin is linked to actin cytoskeleton via the associated catenin molecules (p120-catenin, α-catenin, β-catenin, and plakoglobin). The linkage to actin cytoskeleton is critical for stability of the AJs. Republished with modification from J Cell Sci, 2008, 121 (Pt13), 2115-22, Dejana E, Orsenigo F, Lampugnani MG. The role of adherens junctions and VE-cadherin in the control of vascular permeability. With permission from Copyright Clearance Center [3].

complex are highly dynamic as they continuously undergo remodeling to maintain barrier integrity and passage of nutrients and regulate endothelial permeability and trafficking of leukocytes during immune response [1, 22]. VE-cadherin-mediated adhesion at AJs is the primary restrictive barrier of the endothelium to plasma proteins and blood cells and the regulator of barrier integrity and plasticity that acts as a gatekeeper by bringing neighboring cells together in a calcium-dependent manner [23]. Furthermore, VE-cadherin plays a critical role during embryonic development. Depletion, targeted inactivation, or truncation of β-catenin binding domain of VE-cadherin gene, *cdh5*, leads to vascular defect and embryonic lethality in mice at E9.5 [24, 25]. This is the result of impairment in endothelial cells maturation and maintenance due to defect in respond to vascular endothelial growth factor (VEGF)-mediated survival signals [25]. Furthermore, functional blocking antibodies against the transmembrane domain of VE-cadherin impaired angiogenesis [26, 27] and increased endothelial permeability in heart and lung of adult mice [26].

VE-cadherin is a divergent member of classical cadherins that include type I (e.g. Epithelial (E)-, Neural (N)-, Placental (P)-, and C-cadherin) and type II (e.g. MN-cadherin, cadherin-8, and cadherin-11) [28]. In the endothelium, both VE-cadherin and N-cadherin are expressed, however their distribution varies with VE-cadherin localizing and clustering at the junctions to induce interaction between endothelial cells and N-cadherin exhibiting a diffused feature and being responsible for connecting the endothelial cells with the surrounding cells [29]. N-cadherin, along with E- and C-cadherins, are the most studied members of the cadherin family and the crystal structure of their ectodomain is determined [30, 31]. The cadherin structure is comprised of a large N-terminus extracellular domain, a transmembrane domain, and a shorter cytoplasmic domain that contains juxtamembrane domain (JMD) and a C-terminus domain [21]. The extracellular domain of cadherins, which drives adhesion and initiates junction assembly [17, 30], is comprised of five

~110 amino acids long tandem Extracellular Cadherin (EC) domains (EC1-EC5) that are involved in Ca^{2+}-dependent *trans-* (between cadherins on adjacent cells) and lateral *cis-* (between cadherins on the same cell) interaction [21]. On the other hand, the cytoplasmic domain (i.e. p120- and β-catenin binding sites) is not required for the recruitment of cadherin to the junction and the initial cell-cell contact [17, 30] but is responsible for stability of the interaction through association with catenin molecules and the actin cytoskeleton [32].

Based on structural and functional analysis of type I cadherins, and theoretical treatment and prediction of 2D junction formation, the low affinity strand-swap *trans*-interaction between the EC1 domains of two cadherins from opposed cells is the primary event during cell-cell adhesion [6, 30, 33]. Point mutation on residue Tryptophan (Trp)-2 that is involved in this interaction (discussed in more detail below) completely blocks adhesive capacity of the cadherin [34]. *Trans* dimerization aligns cadherins and reduces flexibility. Furthermore, the dimerization is an intrinsic moiety of the cadherin and does not require *cis*-interaction [33]. In turn, the secondary lateral *cis*-interaction, between EC3-EC4 domains of two cadherins on the same cell, is weaker in affinity and promotes lateral clustering to increase the strength of adhesion [6, 8, 30, 32] (Figure 2). Mentioned studies explain the preferential *cis*-interaction observed in the absence of calcium [35], which is due to loss of cadherins rigidity and proper orientation required to form *trans*-interaction.

Initial studies on VE-cadherin adhesion suggested that, in solution, three EC1 domains are involved in trimer formation via *cis*-interaction, followed by *trans*-interaction between the EC4 domains of two trimers on adjacent cells to form a hexamer [36, 37]. However, this model was later discarded by Brasch et al., who demonstrated, using structural analysis of full length
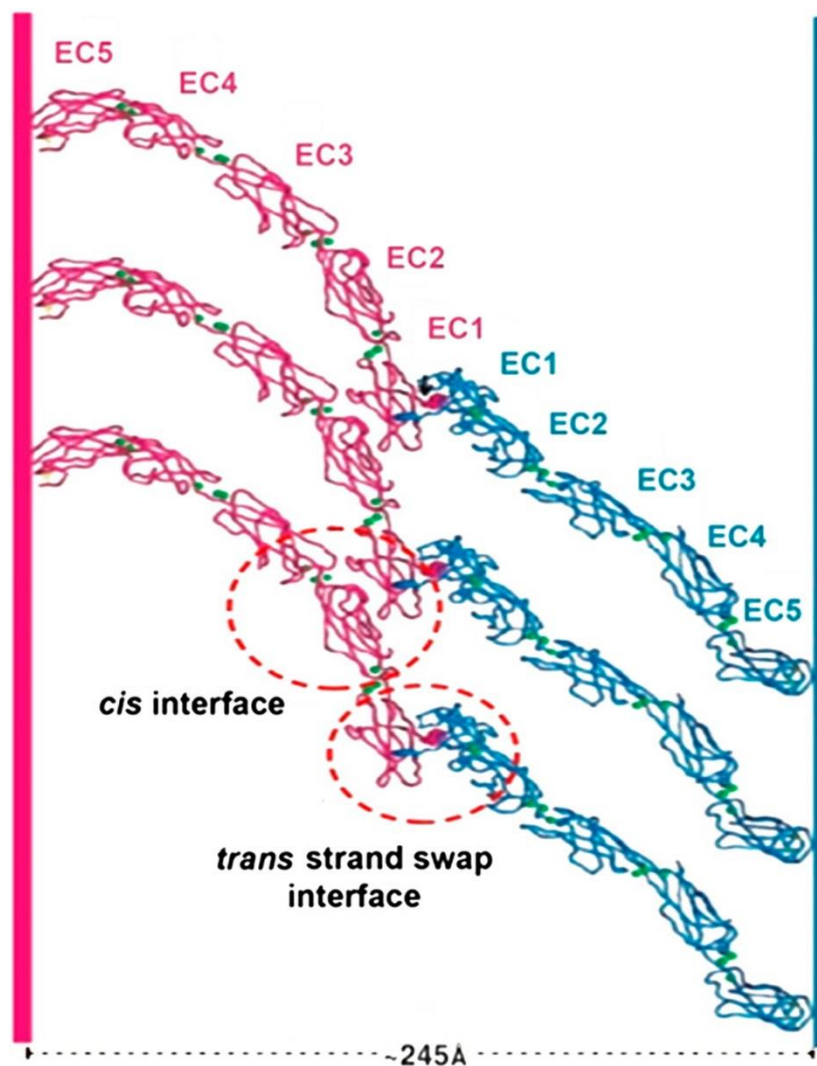
Figure 2: Classical cadherin-mediated homotypic adhesion. The cadherin extracellular domain is composed of 5 subunits (EC1-EC5) that are involved in homotypic interaction through formation of *trans* strand swap dimers on EC1 domain that align cadherins and reduce flexibility and a secondary lateral clustering through *cis* interaction that increases the strength of the adhesion. Reprinted with modification from PNAS, 2010, 107 (4), Wu Y, Jin X, Harrison O, Shapiro L, Honig BH, Ben-Shaul A., Cooperativity between trans and cis interactions in cadherin-mediated junction formation. With permission from PNAS Permissions [6].

mammalian VE-cadherin, that VE-cadherin behaves similar to other classical cadherins and forms *trans* (strand-swap) dimers between the N-terminus β-strands of two swapped EC1 domains [38]. In fact, he suggested that the higher order oligomerization observed in previous study was an artifact of bacterially expressed VE-cadherin and the lack of N-Glycosylation due to the absence of EC5 domain in the experiment setup [38]. Furthermore, Brasch et al., described VE-cadherin as a divergent classical cadherin, sharing similarities with both type I and type II cadherins [38]. In type I cadherins, dimerization occurs at conserved residue Trp2 of the EC1 domains where one Trp2 is inserted into the hydrophobic surface (assembled by Ile24, Tyr36, 78 (Ala or Ser), Ala80, and 92 (Met or Ile)) [39] of the partner EC1 domain from adjacent cell, which results in proper arrangement of the cadherin molecules. In type II cadherins, the strand-swap occurs at two conserved residues on Trp2 and Trp4, which are inserted into a larger hydrophobic surface (assembled by s Leu24, Tyr37, Ala75, Ala77, and Phe92) [39] of the partner EC1 domain. Type II cadherins also have a hydrophobic non-swapped region (Phe8, Leu10, Tyr13, Leu19, and Tyr20) that extends the hydrophobicity of the docking surface by ~ 400 $A^{\circ 2}$, from ~1600–1800 $A^{\circ 2}$ in Type I to ~2700–3300 $A^{\circ 2}$ in Type II [39]. Brasch et al., observed that similar to the characteristic of other type I cadherins, VE-cadherin lacks the large hydrophobic surface observed for type II cadherins, however VE-cadherin *trans*-interaction occurs at Trp2 and Trp4, hence its similarity to type II cadherins (Figure 3).

1.2.2. <u>Cadherin Dynamics at Adherens Junctions</u>

Cadherins are dynamic structures that undergo continuous assembly (independent of catenins and actin cytoskeleton) and ATP-dependent disassembly (i.e. endocytosis [40]) at the junction [17]. Cadherin can also undergo ATP-independent disassembly [17], however, the exact pathway(s) are still unclear. Stabilization of adhesion complex requires attachment of the
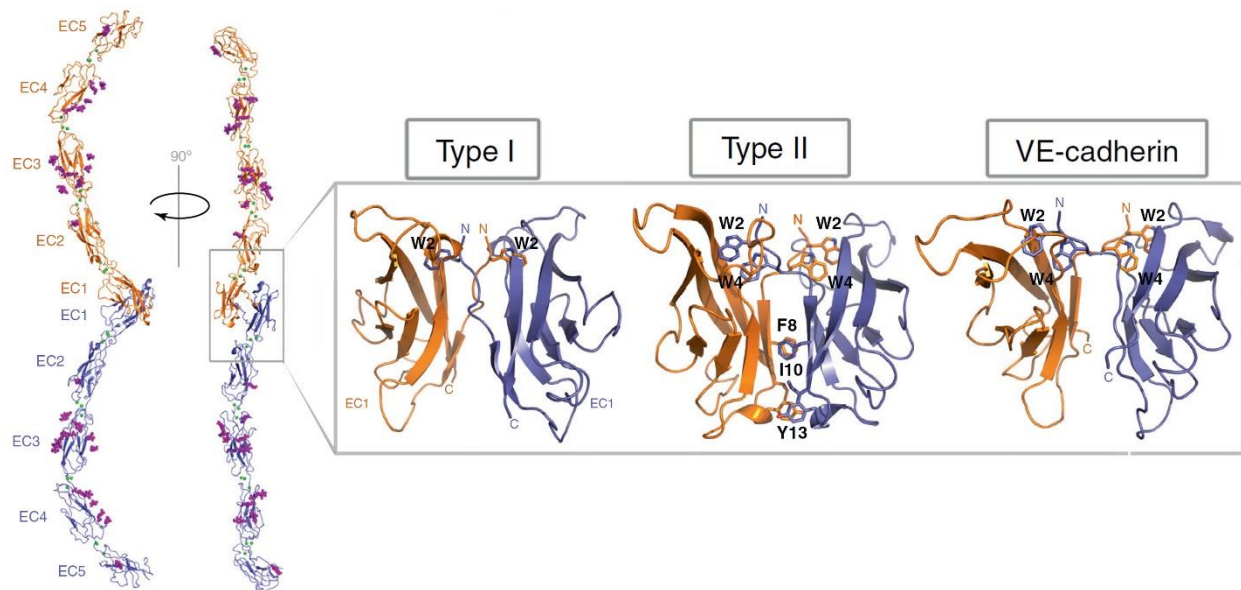
Figure 3: Structural comparison of type I-, type II-, and VE-cadherin dimerization. EC1 domains of two adjacent cadherins undergo dimerization. While in type I cadherins, dimerization occurs at conserved residue Tryptophan (W)-2 inserted in hydrophobic surface, in type II cadherin two conserved residues on W-2 and W-4, inserted into a larger hydrophobic surface, are involved in the process. VE-cadherin dimerization display similarity with both classes where, similar to type II, W-2 and W-4 are involved in dimerization, however the large hydrophobic surface is absent, similar to type I. Reprinted with modification from Trends Cell Biol, 2012, 22(6), 299-310, Brasch J, Harrison OJ, Honig B, Shapiro L., Thinking outside the cell: how cadherins drive adhesion. With permission from Elsevier [8].

cadherin intracellular domain to the actin cytoskeleton [32, 41] and is mediated by the associated catenin molecules. Disruption of the cadherin-catenin complex [42, 43], dissociation of AJ complex from the actin cytoskeleton, and disorganization of actin cytoskeleton leads to loss of barrier integrity and increased permeability [32, 44].

Post-translational modifications (PTMs) such as the phosphorylation state of VE-cadherin and the associated p120-catenin, β-catenin, and plakoglobin dictate the state of VE-cadherin remodeling. Pro-inflammatory agents such as thrombin, VEGF, platelet-activating factor (PAF), as well as tumor necrosis factors (TNF)-α-induced leukocyte diapedesis lead to phosphorylation of cadherins and catenins, dissociation of cadherin-catenin complex, and increase in endothelial permeability [1, 45-47]. Recent study on polarized VEGF signaling and endothelial permeability has shown that VEGF Receptor 2 plays a critical role in mediating the permeability response to VEGF-A through activation of p38 signaling pathway [48]. Furthermore, inhibition of p38 pathway blocks VEGF-induced increase in endothelial permeability [49], suggesting that targeting p38 pathway could be a potential therapeutic approach. Moreover, plasticity of cadherin-catenin complex plays a critical role during leukocytes transendothelial migration [50]. Blockage of leukocyte transendothelial migration in mice expressing VE-cadherin-α-catenin fusion protein that display junctional resistance to vascular leakage underscored the importance of cadherin-catenin complex stability and plasticity in maintaining barrier integrity [50]. Stability of the cadherin-catenin complex is in-part regulated by protein tyrosine phosphatases such as Src homology 2-domain containing tyrosine phosphatase (SHP2) and Vascular Endothelial Protein Tyrosine Phosphatase (VE-PTP) [42, 43]. Interaction of SHP2 with its direct substrate β-catenin prevents its phosphorylation in response to thrombin and assist with cell contact reassembly after thrombin challenge by affecting VE-cadherin mobility [43]. On the other hand, Plakoglobin plays an

important role during leukocytes transmigration, which depends on VE-cadherin phosphorylation state [42]. Plakoglobin has shown to be a direct substrate for VE-PTP, a VE-cadherin specific phosphatase and an essential regulator of cell-cell contact stability [42]. Moreover, VE-PTP is up regulated during hypoxia, which is linked to the stability of VE-cadherin adhesion [51]. Also, it is suggested that plakoglobin plays a role in localization of cadherins and reassembly of the junction by behaving as a mechano-transducer [52, 53].

Another component of the cadherin complex and a member of Armadillo family, p120-catenin, also has implication on stability of VE-cadherin adhesion through interaction with VE-cadherin JMD domain [54, 55]. p120-catenin plays a critical role on VE-cadherin turn- over and its phosphorylation state regulates VE-cadherin remodeling via clathrin-mediated endocytosis [56, 57]. Our lab has previously shown a phospho-switch mechanism by which protein kinase C (PKC)α-mediated phosphorylation of p120 at serine879 in response to pro-inflammatory mediators such as thrombin or lipopolysaccharide (LPS) leads to uncoupling of p120 from VE-cadherin and subsequent VE-cadherin internalization and AJ disassembly [57]. While the junctional p120-catenin does not directly interact with the actin cytoskeleton, the cytoplasmic pool of p120-catenin may initiate signaling pathway to induce changes in actin cytoskeleton and stability of VE-cadherin [58, 59]. Furthermore, p120-catenin's over expression is associated with increased VE-cadherin expression and decrease in leukocyte transmigration [60] by preventing VE-cadherin phosphorylation [61] and VE-cadherin internalization [60].

α-catenin is the only member of catenins that contains an actin-binding domain and can directly interact with the actin filaments. In addition, β-catenin and actin binding proteins such as α-actinin, Epithelial Protein Lost in Neoplasm (EPLIN), and vinculin, which is recruited in the presence of tension [62, 63], also bind to α-catenin. It was widely accepted that α-catenin links cadherin

complex to actin cytoskeleton by directly and simultaneously interacting with β-catenin and actin cytoskeleton. However, this notion was later disapproved in two papers by Yamada et al., and Drees et al., that used purified proteins to show that α-catenin cannot simultaneously interact with both β-catenin and actin [64, 65]. Instead, α-catenin acts as a molecular switch where the changes in its conformation, monomer versus dimmer, dictate its binding preference to β-catenin or actin-related protein 2 and 3 (Arp2/3) complex and actin filaments, respectively [64, 65]. One model for which actin cytoskeleton indirectly interacts with the AJ complex is through β-catenin or Plakoglobin interaction with the intracellular domain of cadherin that provide a bridge for α-catenin recruitment and binding. α-catenin, in turn, interacts with other players such as EPLIN via its VH3 domain [62] that links EPLIN to the actin cytoskeleton (Figure 4). α-catenin-mediated recruitment of other actin binding proteins may enhance stability of the junctions by promoting actin polymerization that works against actomyosin pulling forces exerted on the junctional complex [63], as discussed below.

1.3. **Mechanical Tension Across Adherens Junctions**

Cells experience external mechanical forces from neighboring cells and the extracellular matrix, as well as the internal force generated by actomyosin contractile machinery. Proteins of the AJ complex behave as mechano-sensors and mechano-transducers that detect and initiate a biological signaling cascade in response to mechanical force. Mechano-sensors, such as cadherins, detect changes in magnitude of the force, while mechano-transducers, such as catenins are typically a chain of proteins that respond to and alter the distribution of force [2].

α-catenin, EPLIN, and vinculin are the mehcano-transductory molecules that play an important role in regulating cadherin dynamics by mediating the myosin-generated intracellular forces (Figure 4). Dynamics of cadherin-mediated adhesion is characterized by continuous
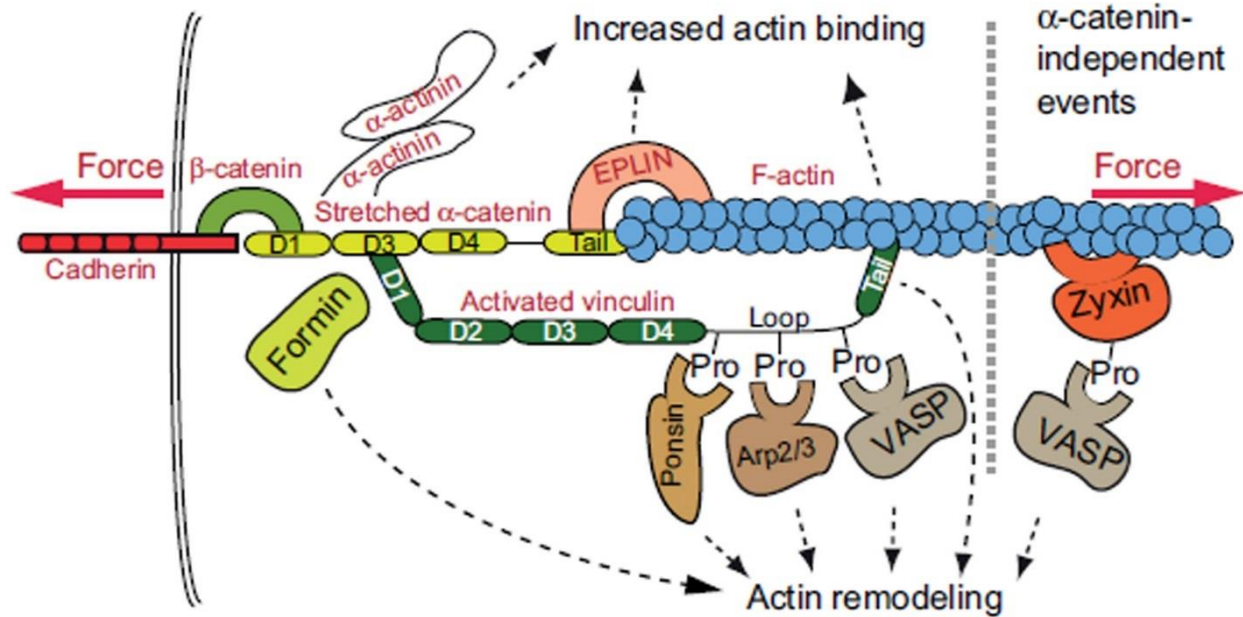
Figure 4: Linkage of cadherin cytoplasmic domain to the actin cytoskeleton. α-catenin can directly interact with actin cytoskeleton via its actin binding domain. This interaction is enhanced in the presence of actin binding proteins α-actinin and EPLIN, vinculin, leading to actin remodeling. Because α-catenin cannot simultaneously interact with actin and cadherin, β-catenin provides a bridge for α-catenin recruitment and binding. Republished from J Cell Sci, 2013, 126 (Pt2), 403-13, Huveneers S, de Rooij J., Mechanosensitive systems at the cadherin-F-actin interface. With permission from Copyright Clearance Center [2].

rearrangements of cadherin adhesive bonds within AJs [66] lateral diffusion [41], and exchange of VE-cadherin between junctional and intracellular pools [17, 40]. Interestingly, fusion of VE-cadherin with α-catenin reduced the membrane mobility of VE-cadherin [50], emphasizing on the importance of α-catenin on cadherin dynamics.

VE-cadherin, as well as other junctional proteins complexes such as platelet endothelial cell adhesion molecule act a mechano-sensor that sense changes in shear rate and flow patter [67, 68] and induce changes in distribution of F-actin as well as junctional proteins and their phosphorylation state over time [67, 69-72]. Using a recently developed biosensor that measures actomyosin-mediated tension across VE-cadherin adhesion, it was shown that in response to shear stress the myosin-dependent tension across the junction was reduced as compared to static condition, concomitant with decrease in total cell-cell force [9]. Thus, it is clear that the distribution of force (as well as the magnitude) sensed and transduced by the cell must be tightly regulated to maintain cell morphology.

The magnitude and the direction of the force sensed by the cell differ depending on 1) type of the junction (i.e. Focal Adhesions, AJs, or Zonula Adhesion), which are structurally different in respect to their association with actin cytoskeletons and 2) the state of cell-cell adhesion (i.e. formation, maturation, matured), which affects organization of F-actin and localization of the associated proteins [2]. During initiation of cell-cell contact, junction re-annealing, or in response to pro-inflammatory agents, AJs display a discontinuous (or punctate-like as observed in Focal Adhesions) morphology [63]. In this conformation, stress fibers are observed and α-catenin is under tension leading to its open conformation and recruitment of vinculin (Figure 5-top) that would provide additional bonds between cadherin complex and actin and Arp2/3-mediated actin polymerization at the leading edge, thus contribute to the strengthening of the
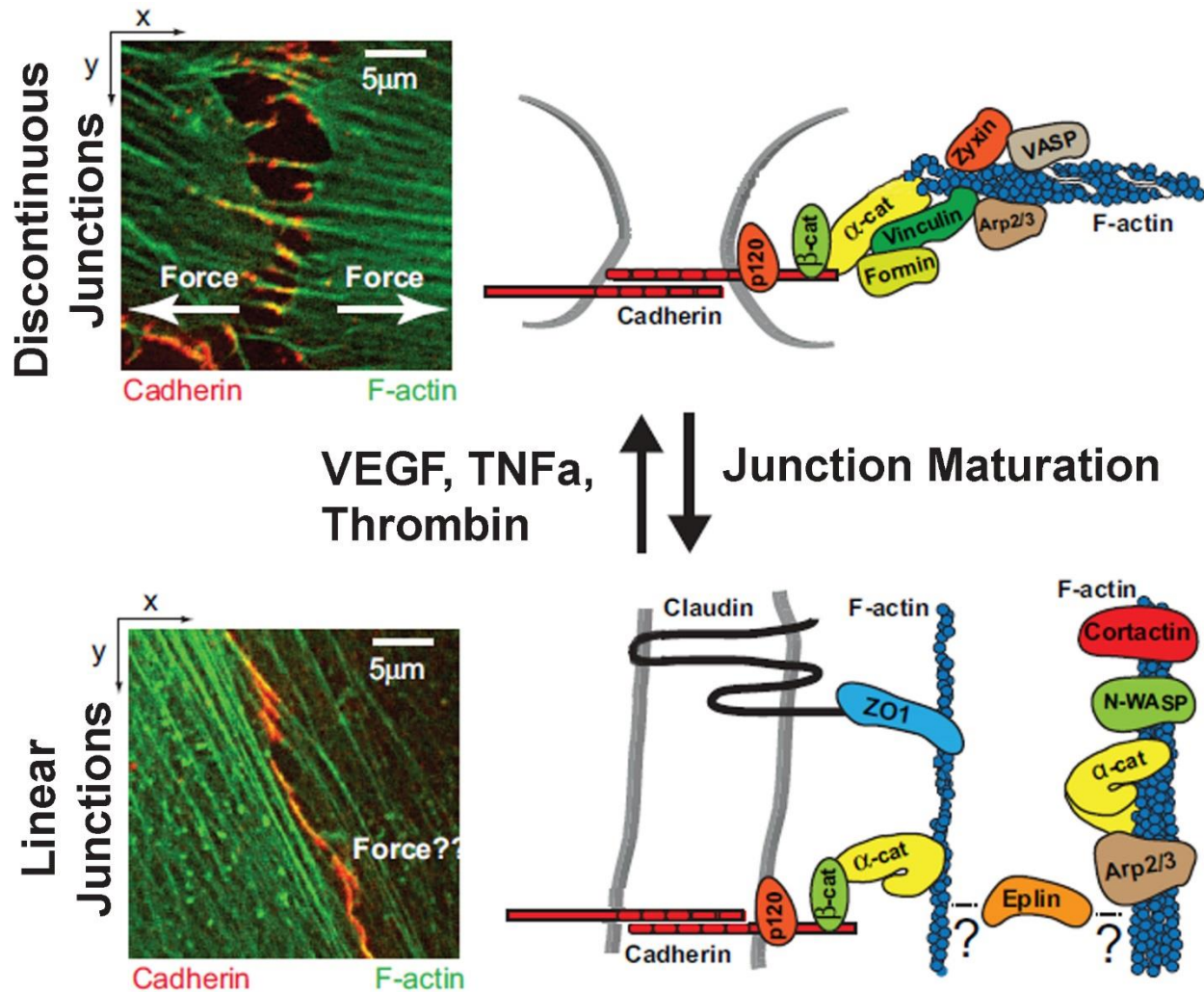
Figure 5: Cadherin' association with actin cytoskeleton dictates junction morphology. Two junctional morphologies are seen in a culture of confluent cell monolayer. Discontinuous junctions (also called punctate as seen in Focal Adhesions) are seen in response to pro-inflammatory agents or during leukocyte transmigration. In this morphology, AJ complexes are connected to actin, vertical to cell periphery, and are under actomyosin-mediated pulling forces. In matured AJs, junctions are linear with a thin actin filament around the cell periphery. Republished with modification from J Cell Sci, 2013, 126 (Pt2), 403-13, Huveneers S, de Rooij J., Mechano-sensitive systems at the cadherin-F-actin interface. With permission from Copyright Clearance Center [2].

junction [2, 63]. During maturation of cell-cell contact, there is membrane protrusion leading to increase in non-muscle Myosin II activity and formation of actomyosin bundles around the cell periphery, leading to the expansion of the junction size [73] and increased cadherin density at the junction (Figure 5-bottom). As the cell contact increases, cadherins are distributed as they move from a cluster in the middle to the edges, which is mediated by actomyosin contraction at the expanding edge [74]. In the mature conformation, the cells are under minimal tension and require α-catenin and EPLIN, however, recruitment of vinculin is not required [63].

As discussed, organization of actin cytoskeleton is critical during different stage of cell-cell contact. The member of RhoGTPases, especially Rac1 and RhoA, are critical regulators of actin cytoskeleton organization and play important roles during nascent junction formation as well as junction maturation by controlling myosin II-mediated contractile machinery and actin polymerization and are described in more detail below.

1.4. <u>Ras Superfamily of Small GTPases</u>

Ras-sarcoma (Ras)-related super-family consists of over 150 members of low molecular weight monomeric G proteins with highly conserved sequence homology among different species that are divided into 5 sub-families; Ras, Ras homologous (Rho), Ras-like nuclear protein (Ran), Ras-like proteins in brain (Rab), and ADP ribosylation factor (Arf) [75-77]. While the 36 members of Ras family, importantly H-Ras, K-Ras, and N-Ras, are well-known oncogenes, H-Ras has also show to play a role in angiogenesis and vascular permeability [78] and R-Ras, a tumor suppressor, also has barrier protective effect through interaction with Filamin A, an actin binding protein, to stabilize VE-cadherin adhesion [79]. The Rho sub-family includes 20 members and among them RhoA, Rac1, and Cdc42 are the best-studied members. These GTPases are critical regulators of actin cytoskeletons and cell-cell adhesion [80, 81] and the focus of this thesis as discussed in more

detail in section 1.5. The function of other subfamilies Rab, Arf, and Ran in terms of regulating endothelial permeability is less known and not thoroughly studied. Rab and Arf are mainly involved in vesicle transport and trafficking [82] while Ran is involved in the import and export of RNA and protein [83].

1.4.1.   <u>Regulation and Structure of GTPases</u>

GTPases cycle between active (guanosine triphosphate (GTP)-bound) and inactive (guanosine diphosphate (GDP)-bound) forms and act as binary molecular switches [84]. GTP-bound form interacts with the downstream effectors to elicit a physiological response whereas inactive form lacks this ability. Because of the high binding affinity for both GDP and GTP and slow rate of intrinsic GTP hydrolysis, the GTPase cycle is controlled by upstream regulators; GTPase Activating Protein (GAP), Guanine Nucleotide Exchange Factor (GEF), and Guanine Nucleotide Dissociation Inhibitor (GDI). GAP facilitates GTP hydrolysis and provides switch-off mechanism whereas GEF function is required for GDP to GTP exchange thus turning-on protein activity [81]. The mechanism of GEF action is a multi-step process involving formation of ternary GTPase protein-nucleotide-GEF complex and a release of nucleotide [84]. Rebinding of GTP, predominantly because of its presence at the higher concentration in the cell, results in GTPase activation. GEF merely induces GTP exchange by increasing the rate of GDP release. In contrast, GDI interacts with GDP-bound form and prevents GTP exchange. GDI binds to a prenylated C-terminus shielding the hydrophobic tail and sequesters GTPase from membrane compartment. Members of GAPs and GEF that are involved in controlling GTPases activities in endothelial cells have been extensively reviewed by van Buul et al [85].

GTPases can interact with multiple binding partners that are structurally different from one another, yet the binding motifs are very similar and overlapping among Gα protein subfamily and

GTPases [86]. All 5 GTPase sub-families contain a G domain structure at the N-terminal that consists of 5 sets of G-box binding motifs that comprise the nucleotide-binding site (also called the p-loop), the core effector domain, and the switch region (I and II), which forms the GEF binding site. The p-loop motif inside the switch I and switch II region of G-box sequence represents the site of the GTP and GDP exchange [87] and provides the interface for interaction with downstream effectors (Figure 6). The switch region undergoes conformational change upon the release of the hydrolyzed phosphate to form the open GDP state [84]. Members of Ran family have an additional sequence in the C-terminal that causes significance conformational change in the switch I and switch II region upon the release of hydrolyzed phosphate [86] (Figure 6).

1.4.2. <u>Lipid Modification of GTPases</u>

The interaction between GTPase and downstream effector requires translocation of the GTPase from cytosol to plasma membrane, which is critical for determining the downstream signaling pathway, and is controlled by lipids such as farnesylpyrophosphate and geranylgeranylpyrophosphate that induce PTMs [88]. The membrane targeting CAAX sequence (where X can be any amino acid) at C-terminus is a signature of Rho family members and also a recognition sequence for farnesylation and geranylgeranylation [76], which dictate subcellular localization and interaction. However, there are variations among Ras families; while some members of Ras family don't require PTM to localize at the membrane or don't require membrane localization to execute biological activity, others like the members of Rab family can be modified by geranylgeranyl transferase at additional C-terminal sequence motifs such as CC, CXC, CCX, CCXX, and CCXXX [76]. Arf family lacks the membrane binding sequence at the C-terminal but contain an extra sequence at the N-terminal, which undergoes myristoylation [87]
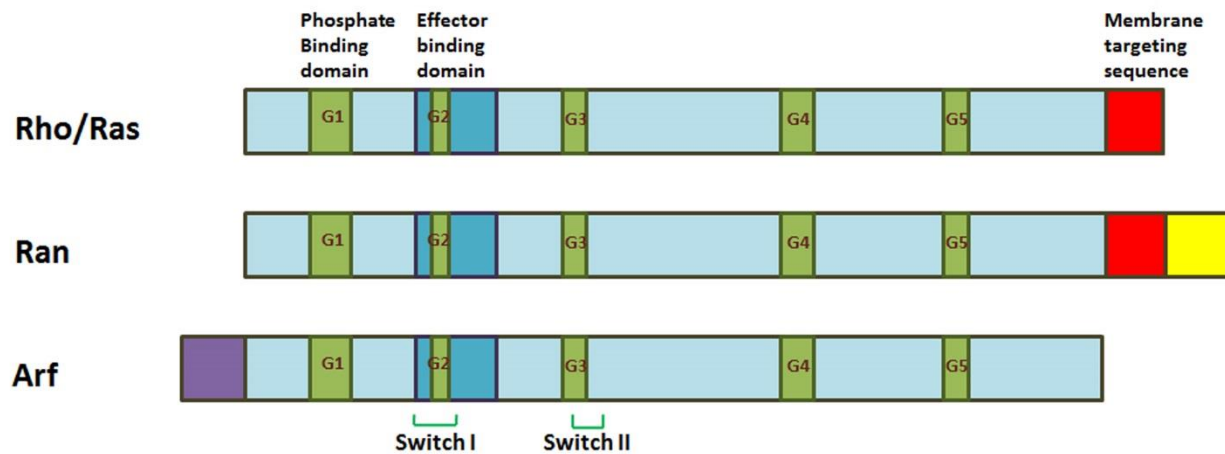
Figure 6: Schematic representation of the GTPase family binding structure. Members of Ras superfamily have highly conserved sequence homology. The 5 G-box motifs (G1-G5) (green) are nucleotide binding motifs. The switch I and Switch II are the region of GDP/GTP exchange. G domains also contain the phosphate binding domain as well as the effector binding domain (cyan). The C-terminal has the membrane targeting sequence (red), which undergoes post translational modification required for translocation to the membrane. Exceptions are members of Ran family that have an additional sequence in the C-terminal (yellow), which causes a significant conformational change in the switch I and II region, as well as some members of Arf family that have the membrane targeting sequence on the N-terminal, which undergoes myristoylation (purple).

(Figure 6). Furthermore, some members such as RhoA and RhoC are only subjected to geranylgeranylated and are localized in cytoplasm, whereas others such as RhoB can be geranylgeranylated, farnesylated, or palmitoylated and localized at plasma membrane or endosomes [76].

1.5. <u>RhoGTPases: RhoA, Rac1, Cdc42</u>

RhoA, Rac1, and Cdc42 are the best-studied members of the monomeric RhoGTPases. They were first introduced as important modulators of actin cytoskeleton dynamic and actomyosin contractile machinery by Ridley who, along with others, demonstrated that, through interaction with actin binding proteins, RhoA, Rac1, and Cdc42 induce formation of stress fiber, lamellipodia, and filopodia, respectively [89, 90]. Furthermore, their interaction with downstream activators and inhibitors initiate signaling cascade that regulate many different cellular events such as development, cell growth and division, and protein trafficking [88]. The importance of RhoGTPases in controlling endothelial junctional barrier integrity is well established [91]. While many studies have linked RhoA activation to barrier disruption, and Rac1 and Cdc42 activity to maintenance of endothelial barrier [92], these GTPases initiate many different signaling pathways in the context of barrier integrity, depending on the upstream regulators, as well as the time-frame of activity, junction morphology, etc. I will discuss the role of each of these RhoGTPases and their relationship with each other in context of endothelial barrier integrity, with the focus on the crosstalk between RhoA and Rac1 signaling and cadherin mediated adhesion.

1.5.1. <u>RhoA Regulation and Barrier Integrity</u>

RhoA is best known for stress fiber formation in response to pro-inflammatory agents such thrombin and VEGF and its association with actomyosin contractility via downstream effectors,

leading to junctional remodeling [93, 94]. The pro-inflammatory agents induce activation of RhoA-specific upstream effectors such as p115RhoGEF and GEF-H1 [95], leading to RhoA activation. Downstream of RhoA activation, Rho-associated coiled-coil forming protein kinase (ROCK) I and II inhibit myosin light chain phosphatase (MLCP), leading to phosphorylation of the light chain of non-muscle myosin II (MLCII) by myosin light chain kinase (MLCK) [96]. RhoA can also induce activation of mDia, an actin binding protein [97]. mDia can in turn localizes with its effector, profiling, at the leading edge to induce actin filament polymerization [98] (Figure 7- left side). The level of ROCK I and ROCK II are differentially regulated in endothelial cells in a time-dependent manner. While there is higher ROCK I activity under steady-state [99], treatment with pro-inflammatory agents such as TNFα or LPS results in up-regulation of both ROCKs with ROCK I activity associated with the early response to LPS [99-101] and ROCK II with LPS-mediated phosphorylation of MLC and long-term contractility and barrier disruption [99, 102]. LPS also negatively regulates the secondary messenger cyclic adenosine monophosphate (cAMP) signaling [103], which has barrier protective effect and may involve Rac1 activity. Actomyosin contraction, in turn, generates inward pulling forces on the membrane that could ultimately result in increased endothelial permeability.

1.5.2. Role of Rac1 and Cdc42 on Junctional Integrity

Rac1 activation is concentrated at the site of membrane ruffling and actin polymerization [104]. Furthermore, Rac1 has been shown to mediate the barrier protective effect of secondary messenger cAMP and bioactive lipid Sphingosine 1 Phosphate (S1P) [105, 106], which initiate
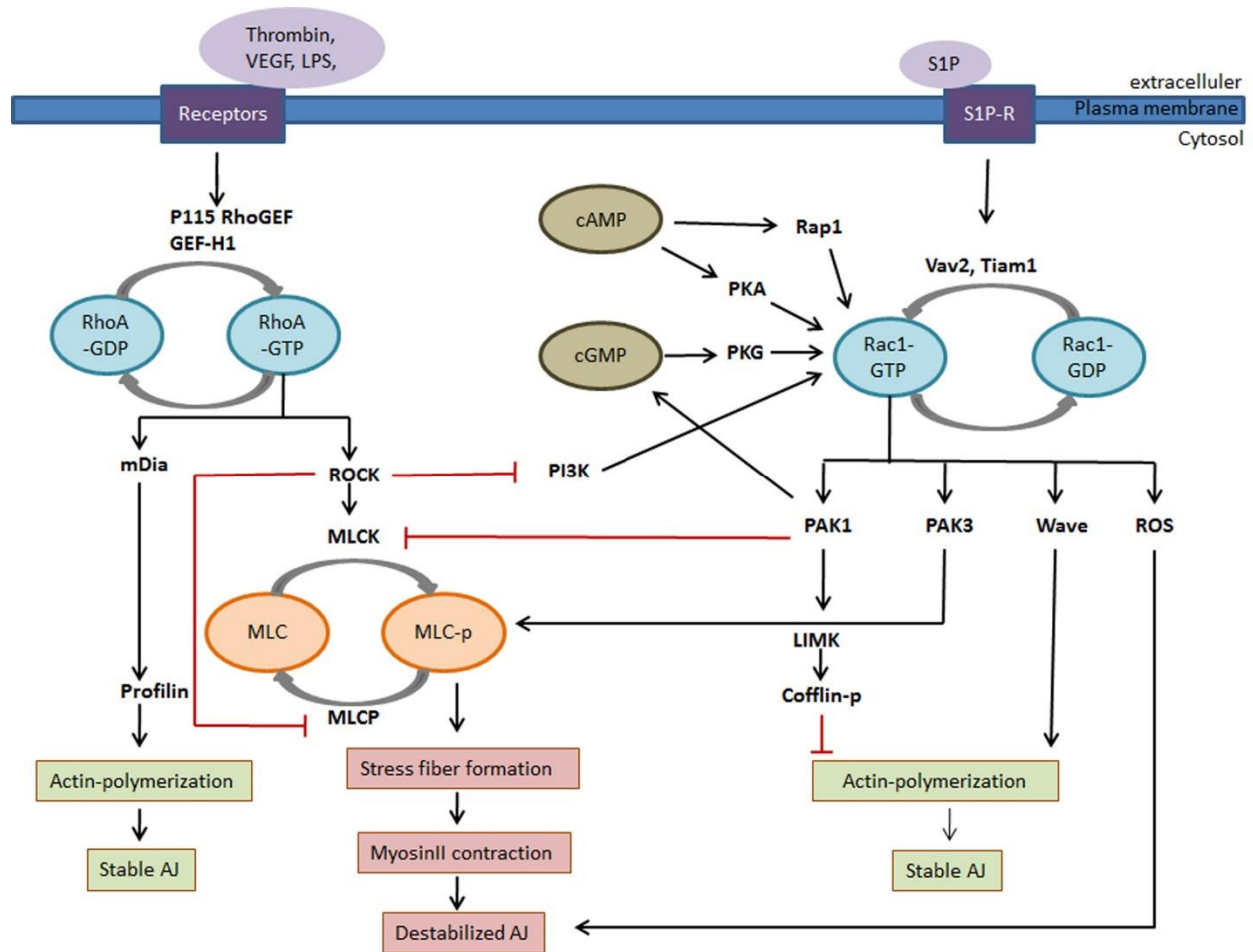
Figure 7: RhoGTPases regulate stability of AJs. Secondary messengers cAMP and cGMP as well as barrier protective mediator S1P promote Rac1 activation. Activation of Rac1 induce both actin polymerization via Wave or actin de-polymerization via Pak1. RhoA activation by pro-inflammatory agents promotes activation of downstream effector ROCK, which induces MLCII phosphorylation and actomyosin contraction. RhoA can also cooperate with Rac1 and induce actin polymerization through interaction with actin binding protein mDia.

signaling cascades leading to indirect activation of Rac1- and inhibition of RhoA- mediate pathways [105-107]. Specifically, cAMP and secondary messenger cyclic guanosine monophosphate (cGMP) activate upstream kinases such as serine/threonine Protein Kinase A and Protein Kinase G, respectively, to promote phosphorylation of actin-binding protein vasodilator-stimulated phosphoprotein, leading to Rac1 activation [108, 109]. cAMP also interacts with Rap1-specific GEF, Exchange Proteins Activated by cAMP (EPAC), and activates Rap1 GTPase [110], an upstream regulator and activator of Rac1 [111] (Figure 7- right side). Furthermore, cAMP negatively regulate RhoA activation via P190RhoGAP, a RhoA specific GAP that keeps RhoA in an inactive GDP-bound form [107] or by preventing RhoA dissociation from RhoGDI and its subsequent translocation to the membrane [112], thus playing a critical role in regulating GTPases activity and maintaining barrier integrity. Other serine/threonine protein kinases such as protein Kinase B (also known as AKT) and tyrosine protein kinases such as Sarcoma (Src) have also shown to regulate barrier integrity and mediate Rac1 signaling [113].

Upon activation, Rac1 interacts with several downstream effectors including actin binding proteins such as Wave, a member of Wiskott-Aldrich Syndrome Protein family, and Arp2/3 complex to induce actin polymerization [114], as well as members of serine/threonine P21 Activated Kinase (Pak) family. Depending on the intracellular context, Rac1 can interact with different member of Pak to induce reorganization of actin cytoskeleton. While activation of Pak1 can increase Lin1 1, Isl-1 & Mec-3 Kinase-mediated phosphorylation of actin-binding protein Cofflin and actin de-polymerization [115], Pak1 also positively regulates the cellular level of cGMP level and its activity [116]. Meanwhile, Pak3 promotes MLC phosphorylation and actomyosin contractility [117] (Figure 7). Moreover, Rac1 mediates Phosphatidylinositide 3-kinase (PI3K)-induced Hypoxia-inducible factor (HIF1α) expression, which leads to endothelial

barrier stabilization [118, 119]. On the other hand, expression of constitutively active Rac1 or VEGF-induced Rac1 activation is linked to Reactive Oxygen Species (ROS) production, and VE-cadherin phosphorylation and its subsequent internalization by β-arrestin [117, 120] and loss of endothelial barrier function. Based on the mentioned studies, it is clear that the role of Rac1 on endothelial AJ barrier integrity is far from conclusive. Furthermore these studies draw attention to the importance of the spatiotemporal control of the GTPases activities due to their involvement in many different signaling cascades.

Another important member of RhoGTPases, Cdc42, induces filopodia formation and plays a critical role in cell polarity by promoting directionality via interaction with actin binding proteins, mDia and Wave2 that induce nucleation and polymerization of actin filaments, respectively [121]. In endothelial cells, Cdc42 is essential during barrier restoration [122] and has shown to decrease junctional leakiness after LPS challenge in mice that express the constitutively active Cdc42 [123]. One mechanism by which Cdc42 may exert its effect is through interaction with downstream effector IQRasGTPase-activating protein (IQGAP). IQGAP family is presented by 3 genes in mammals, however, IQGAP1 is the only member of the family expressed in endothelial cells [124, 125]. IQGAP1 is a 190 kDa scaffold protein with sequence homology to RasGAP-related protein. However, instead of functioning as a typical GAP, IQGAP1 binds to active Cdc42, as well as Rac1 but not RhoA, and prevents GTP hydrolysis, thus prolonging the activity of GTPase bound to IQGAP1 [125, 126]. Furthermore, binding of GTPase to IQGAP1 can regulate cell adhesion by preventing IQGAP1 interaction with β-catenin [126]. In addition to its association with IQGAP1, Cdc42 also offsets Rac1 destabilizing effect on endothelial barrier through parallel activation to act as a competitive inhibitor of Rac1-induced ROS production [127].

1.6. <u>The Interplay Between Rac1 and RhoA</u>

Monomeric RhoGTPases Rac1 and RhoA have shown to express cooperative behavior, in which the cross talk between the two results in coordinated activity during various biological events, such as actin polymerization during cell migration [128] or formation of cadherin-mediated cell-cell adhesion [80]. Other studies have highlighted the antagonistic relationship of the two GTPases where activation of Rac1 promotes localization of P190RhoGAP to AJs and inhibition of RhoA activity [129, 130]. Moreover, cadherin 'outside-in' signaling orchestrated by the opposing actions of Rac1 and RhoA is responsible for remodeling of the actin cytoskeleton at the site of nascent adhesion [131-133]. In epithelial cells, spatiotemporal activity of Rac1 has shown to induce polymerization of actin filaments at the cadherin complex [134] to initiate cell-cell contact [74], whereas RhoA activity at the contact edge mediates actomyosin-dependent expansion of adhesion zone [74]. There is also cell-type (i.e. epithelium vs. endothelium) specificity in terms of the effect of Rho/ROCK-mediated actomyosin contractility. While in the epithelium, the contractile force is required for formation of mature junctions and apical and basolateral distribution, in endothelial cells where the junctions are homogenous, actomyosin contractility contributes to punctate-like (discontinuous) junctions, as described earlier.

Moreover, the level and duration of RhoA activity as well its localization within the cell affect barrier integrity. It was recently shown that while oscillation of RhoA activation at the junction is associated with membrane protrusion and gap closure, its prolonged activity leads to actomyosin contractility and barrier disruption [135]. Furthermore, this study suggested that there is micro-motion oscillation in RhoA activity. This oscillatory signal may conversely coincide with Rac1 activity where the two GTPases control each other's activity [135].

Similar to the epithelium where the increase in cadherin density is associated with increased Rac1 and Cdc42 activity and decreases RhoA activity [133], in endothelial cells, VE-cadherin-mediate adhesion has shown to provide spatial cues for both localization and GTP loading of Rac1 at the membrane through upstream effectors such as PI3K [136] and T-cell lymphoma invasion and metastasis-inducing protein 1 (Tiam1) [132] and GTP-loading through Vav2 [137], which activates Rac1 at AJs. At nascent junction Rac1 activation can promote initiation or reassembly of the adhesion zone in a lamellipodia-dependent manner [74]. However, the role of Rac1 at the mature endothelial adhesion sites is less understood. Despite numerous studies related to the function of Rac1 in regulating endothelial junctional permeability, the current models are contradictory and inconclusive. Depending on intracellular context, activation of Rac1 at the site of adhesion can lead to variety of cellular responses ranging from stabilization of AJs upon exposure of endothelial cells to shear stress or barrier protective mediator S1P and cAMP [106, 137, 138] or, in contrary, destabilization of AJs in response to vasoactive agents such as VEGF and TNFα [117, 139-141]. Furthermore, Rac1 activation in response to fluid shear stress is associated with the sites of AJs experiencing the highest tension [137, 142], suggesting that Rac1 could potentially oppose the tension across adhesion through counterbalancing RhoA activity. Moreover, Rac1 has also been implicated on tugging-force induced AJ growth [73], although it remains unclear whether Rac1 activity cooperates or suppresses these forces across cadherin adhesion. One explanation for these conflicting results is that activation of Rac1, in most cases, is accompanied by other signaling pathways that can modify Rac1-mediated effect. Another potential concern is that majority of stimuli interfere with global rather than local Rac1 activity. In this thesis project we have hypothesized that spatiotemporal activation of Rac1 at AJs stabilizes VE-cadherin

adhesion in matured junctions by regulating actomyosin-mediated pulling force on the adhesion site, thus reducing VE-cadherin dissociation from the junction.

1.6.1. <u>Statement of Aims</u>

Aim 1. To determine the effect of Rac1 activity on the steady state dynamics of VE-cadherin adhesion. Utilizing state-of-the-art photo-activatable (PA)-Rac1 to control spatiotemporal activity of Rac1 we investigated the causal relationship between localized and transient Rac1 activation at matured endothelial cell-cell junction and VE-cadherin dynamic. Furthermore, we utilized *in-silico* mathematical and computational modeling to determine the effect of Rac1 activation on VE-cadherin *trans*-interaction and stability of the adhesion.

Aim 2. To investigate Rac1's downstream signaling events responsible for stability of VE-cadherin *trans*-interaction and the potential role of Rac1 as a tension modulator. Utilizing Förster resonance energy transfer (FRET)-based biosensors and photo-inducible inhibitor of ROCK we investigated the antagonistic relationship between Rac1 and RhoA and the molecular and signaling mechanism by which transient activation of Rac1 at AJ stabilizes VE-cadherin adhesion.

2. MATERIALS AND METHODS

*Portions of the text were reproduced with permission from Daneshjou N, Sieracki N, van Nieuw Amerongen GP, Schwartz MA, Komarova YM, Malik AB. (2014) Rac1 functions as a reversible tension modulator to stabilize VE-cadherin *trans* interaction. The Journal of Cell Biology. Accepted.

2.1. <u>Plasmids and Adenovirus</u>

Wild-type human VE-cadherin adenovirus tagged to green fluorescent protein (GFP) [143] was a gift from Dr. F. Luscinskas (Brigham and Women's Hospital, Boston, MA). PA-Rac1 (PA-GFP, mCherry, and mCerulean tagged), PA-Rac1 dominant negative (DN) (mCherry and mVenus (enhanced YFP) tagged), photo-insensitive (PI)-Rac1 (mCherry tagged) [4], and RhoA-FRET biosensor [11] were gifts from Dr. K. Hahn (UNC School of Medicine, Chapel Hill, NC) and purchased from Addgene (plasmids 22022, 22027, 22030, 22029, 22017, 22028, and 12150 respectively). Rac1- and Cdc42-FRET biosensors (Raichu-Rac1/1011x and Raichu-Cdc42/1054x) were gifts from Dr. M. Matsuda (Kyoto University School of Medicine, Sakyo-ku, Kyoto, Japan) [5]. The MLCK-FRET biosensor was a gift from Dr. T L. Chew (Northwestern University Medical School, Chicago, IL) [7]. For Cyan Fluorescent Protein (CFP)-PA-Rac1DN, CFP was amplified using pECFP-C1 (BD Biosciences, San Jose, CA) as a DNA template and sub-cloned into mVenus-Rac1DN using 5'-NcoI and 3'-BamHI restriction sites. For VE-cadherin-Dendra2 construct, Dendra2 was cloned to the C-terminal of VE-cadherin by PCR-based strategy and sub-cloning into pCDNA3 vector as follow: A cDNA encoding full length human VE-cadherin and Dendra2 were amplified by PCR using the Bluescript-VE-cadherin-GFP vector [143], a gift from Dr. S K. Shaw (Women and Infants' Hospital, Brown University, Providence, RI), and pRc-CMV-

E-cadherin-Dendra2 vector [17], a gift from Dr. S. Troyanovsky (Northwestern University, Chicago, IL) as templates and inserted into pCDNA3 vector (Life Technologies, Grand Island, NY) at restriction sites 5'-KpnI and 3'-EcoRI for VE- cadherin and 5'-EcoRI and 3'-XhoI sites for Dendra2. Mutation of tryptophan residues at positions 2 and 4 to alanine (W2A/W4A) on VE-cadherin-Dendra2 was generated using site-directed mutagenesis kit (Stratagene) and confirmed by sequencing.

2.1.1. Genetically Encoded Photo-activatable Rac1 Construct

Localized and transient activation of Rac1 was accomplished by utilizing a genetically encoded PA probe that consists of a photo-sensitive Light Oxygen Voltage (LOV) domain from *Avena sativa* Phototropin1 bearing a constitutively active (GTP-bound) $Rac1^{V12}$ mutant (PA-Rac1) [4] (Figure 8). The LOV domain poses a closed conformation in dark, which prevents the release of $Rac1^{V12}$. Upon irradiation with 458nm laser beam, the change in conformation of LOV domain leads to a transient release and interaction of $Rac1^{V12}$ with downstream effector [4]. For control, a photo-insensitive $Rac1^{V12}$ (PI-Rac1) probe bearing a mutation on Cysteine 39 to Alanine (C39A) in a flavin-binding LOV2 domain [144] that prevents LOV conformational change as well as a photo-activatable probe bearing a dominant negative $Rac1^{T17N}$ mutant (PA-Rac1DN) were used.

2.1.2. VE-cadherin-Dendra2 Construct

For studying VE-cadherin kinetics, a photo-switchable fluorescent protein, Dendra2, was cloned to the C-terminal of VE-cadherin. Dendra2, a ~600 base pair protein, contains a functional group that exhibits a shift in emission spectrum from 488 nm (green) to 543 nm (red) maximum wavelength after photo-conversion with 405 nm laser beam [145] (Figure 9).
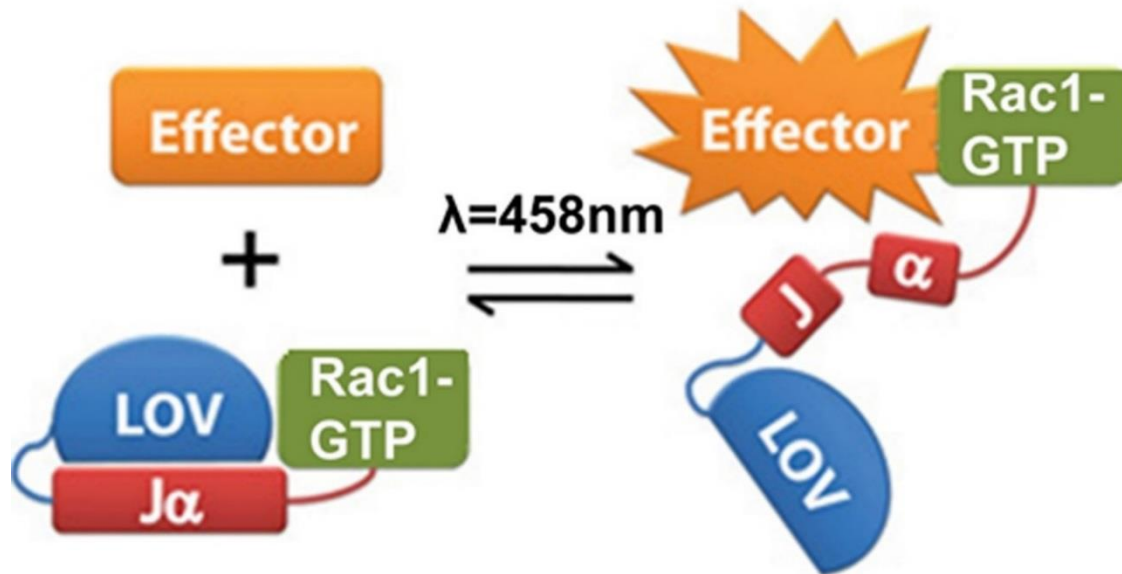
Figure 8: Genetically encoded photo-activatable (PA)-Rac1. The probe consists of a constitutively active Rac1 bound to a light sensitive domain (LOV) that blocks Rac1 activity. LOC undergoes conformational change upon irradiation with 458nm laser beam leading to a transient and localized release of active Rac1. Republished with modification from Nature, 2009, 461 (7260), Wu YI, Frey D, Lungu OI, Jaehrig, A, Schlichting I, Kuhlman B, Hahn KM., A genetically encoded photoactivatable Rac controls the mobility of living cells. With permission from Nature Publishing Group [4].
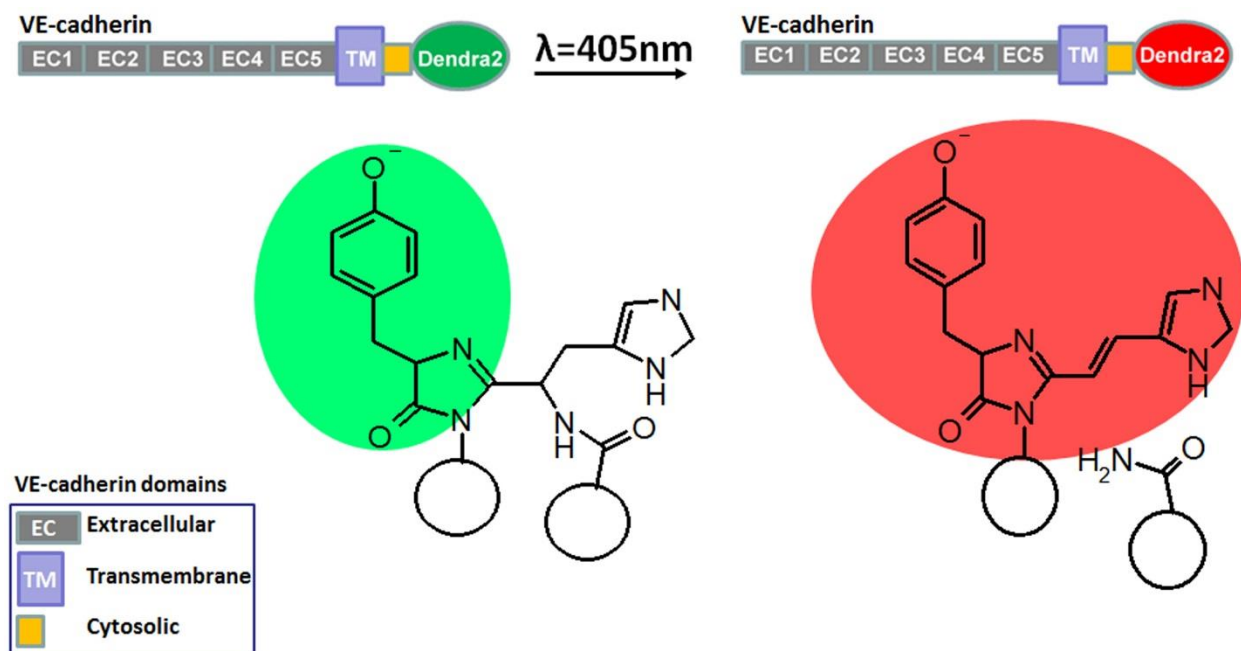
Figure 9: Photo-switchable Dendra2 as a tool to study VE-cadherin kinetics. Fluorescent protein Dendra2 is cloned to the C-terminus of VE-cadherin.Dendra2 contains a photo-switchable functional group, which exhibits an irreversible shift in emission spectrum from green to red after photo-conversion with 405nm laser beam, enabling direct monitoring of VE-cadherin dynamics.

2.1.3. FRET-based Biosensors to Measure RhoGTPases Activity

To measure RhoGTPases activity, single chain biosensors with intra-molecular FRET were used (Figure 10). Rac1 and Cdc42 biosensors [5] are comprised of Yellow Fluorescent Protein (YFP) and CFP fluorophores bound to the specific GTPase (Rac1 or Cdc42) and their shared downstream effector, Pak1. When the GTPase is in inactive form (GDP-bound) the CFP emission spectrum is at 475nm. Once the GTPase become active (GTP-bound), it binds to Pak1, leading to the close proximity of YFP and CFP and the change in CFP emission spectrum to 530nm (Figure 10A).

The RhoA biosensor is similar, however, the YFP and CFP fluorophores are inserted between RhoA and RhoA binding domain (RBD) of rhotekin, a RhoA-specific effector that interacts with active RhoA, and joined by an unstructured linker [11]. The C-terminus of RhoA is free and can interact with a RhoA-GDI to regulate its localization at the membrane. Activation of RhoA, leads to binding to RBD, which brings YFP and CFP into close proximity to each other causing the change in emission spectrum, as described above (Figure 10B).

2.1.4. Myosin Light Chain Kinase FRET Biosensor

The biosensor is used to measure both the localization and the activation state of MLCK. The biosensor take advantage of the fact that MLCK activity is dependent on the availability of Ca^{2+}/calmodulin (CaM), and is designed to detect CaM binding to MLCK (which is dependent on Ca^{2+} level) [7]. The YFP and CFP modules are inserted in the C-terminal of MLCK and are linked by a MLCK-derived Ca^{2+}/CaM binding domain. In the absence of Ca^{2+}/CaM (inactive state) the fluorophores can interact and produce FRET. In the presence of Ca^{2+}/CaM (active
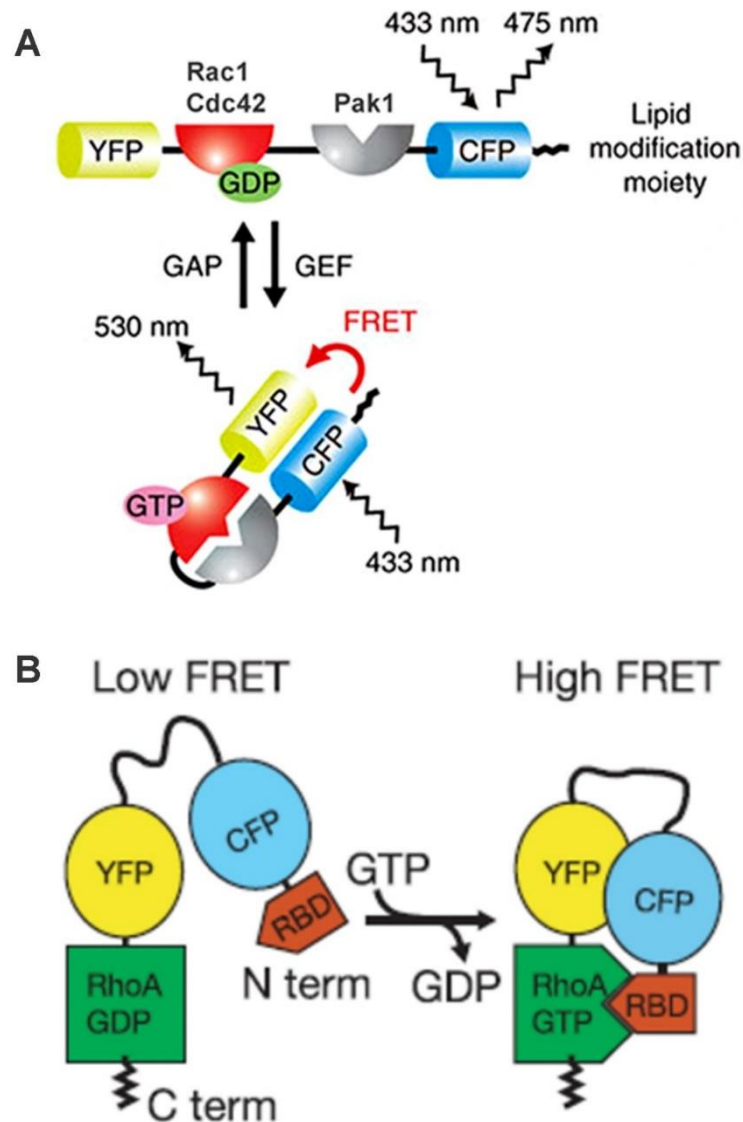
Figure 10: Design of RhoGTPase FRET-based biosensors. A. YFP and CFP are bound to the GTPase (Rac1 or Cdc42) and Pak1. Pak1 interacts with the active GTPase, leading to the close proximity of YFP and CFP and change in CFP emission spectrum. Republished from MCB, 2002, 22 (18), Itoh RE, Kurokawa K, Ohba Y, Yoshizaki H, Mochizuki N, Matsuda M., Activation of rac and cdc42 video imaged by fluorescent resonance energy transfer-based single-molecule probes in the membrane of living cells. With permission from American Society for Microbiology [5]. **B.** YFP and CFP are separated by an unstructured linker, between RhoA and a RhoA binding domain (RBD) that interacts with GTP-bound RhoA. The Interaction leads to change in CFP emission spectrum, which produces FRET signal. Republished from Nature, 2006, 440 (7087), Pertz O, Hodgson L, Klemke RL, Hahn KM., Spatiotemporal dynamics of RhoA activity in migrating cells. With permission from Nature Publishing Group [11].

state), CaM interacts with its binding domain in the linker and MLCK and prevents FRET signal (Figure 11). Note that the FRET signal is inversely correlated with MLCK activity.

2.1.5. <u>VE-cadherin Tension FRET-based Biosensor</u>

FRET-based VE-cadherin biosensor (VE-t) [9] (Figure 12A) consists of mTFP1 (enhanced CFP) and Venus fluorophores inserted between the p120- and β-catenin binding domains and separated by an elastic linker. The sensor measures the actomyosin-mediated tension across VE-cadherin adhesion. Increase in contractile force leads to increase in distance between the two sensor modules and drop in FRET signal. Conversely, the tailless control (Δtail) biosensor (Figure 12B) lacks the β-catenin binding domains and does not experience the contractile force, thus exhibiting the highest FRET signal. Note that high FRET signal correlates with low tension.

2.2. <u>Synthesis of Caged ROCK Inhibitor</u>

Photo-activatable caged ROCK inhibitor, Rockout (cRO) was synthesized according to [146] with some modification (Figure 13). Briefly, starting material 6-nitropiperonyloxymethyl-chloride (NPOM-Cl) was first prepared according to [147]. A mixture of 10.0 mg ROCK inhibitor III (ROCKOUT; emd Millipore) and 1 mL dimethylformamide was stirred on ice for 5 minutes. 1.4 mg sodium hydride (0.058 mmol) was added to the mixture, stirred on ice for additional hour, and frozen solid using a dry ice/acetone bath. Next, 0.3 mL dimethylformamide containing NPOM-Cl (20.5 mg, 0.079 mmol) was added to the frozen mixture and stir at room temperature for 12 hours before quenching with 1 mL saturated sodium bicarbonate. The solution was extracted with ethyl acetate (10 mL) and dried over sodium sulfate and concentration *in vacuo*. The crude product was purified via silica column chromatography. A mobile phase of 7:3 hexanes:ethyl acetate (v/v) removed byproducts and unreacted material and
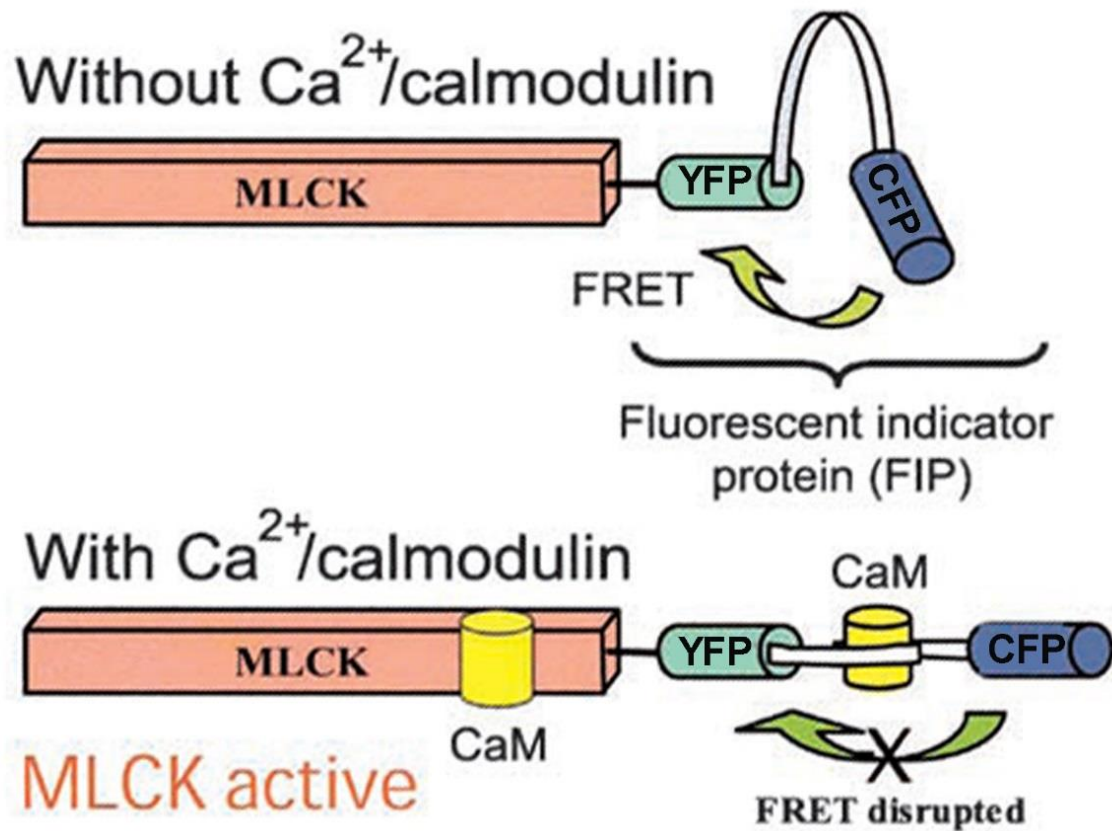
Figure 11: Design of MLCK FRET biosensor. The biosensor activity depends on the level of Ca^{2+}/CaM. YFP and CFP are inserted at C-terminal of MLCK and are separated by a Ca^{2+}/CaM binding domain. In the presence of Ca^{2+}/CaM, CaM interacts with its binding sites and disrupts FRET signal. Republished with modification from J Cell Biol, 2002, 156 (3), Chew TL, Wolf WA, Gallagher PJ, Matsumura F, Chisholm RL., A fluorescent resonant energy transfer-based biosensor reveals transient and regional myosin light chain kinase activation in lamella and cleavage furrows. With permission from Rockefeller University Press [7].
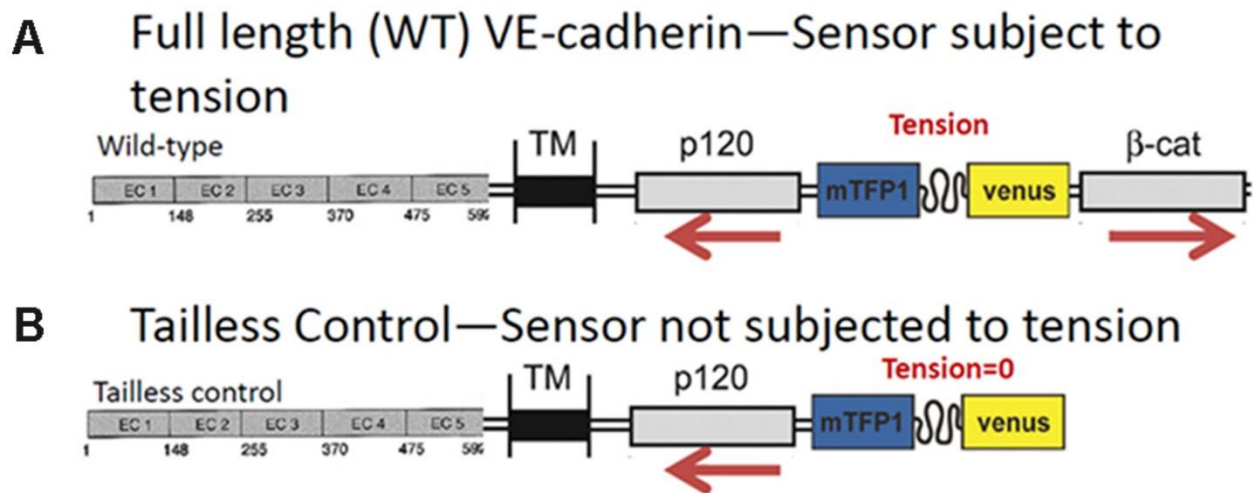
Figure 12: Design of VE-cadherin FRET-based tension biosensor. A. The sensor modules (mTFP and Venus) are inserted between the p120- and β-catenin binding domains and separated via an elastic linker. Increase in tension from actomyosin results in a distance increase between the two fluorophores and decrease in the FRET signal. **B.** The tailless biosensor is used as a positive control. It lacks β-catenin and is not subjected to tension, thus showing the highest FRET signal. Republished from Curr Biol, 2013, 23 (11), Conway DE, Breckenridge MT, Hinde E, Gratton E, Chen CS, Schwartz MA., Fluid shear stress in endothelial cells modulates mechanical tension across VE-cadherin and PECAM-1. With permission from Elsevier [9].
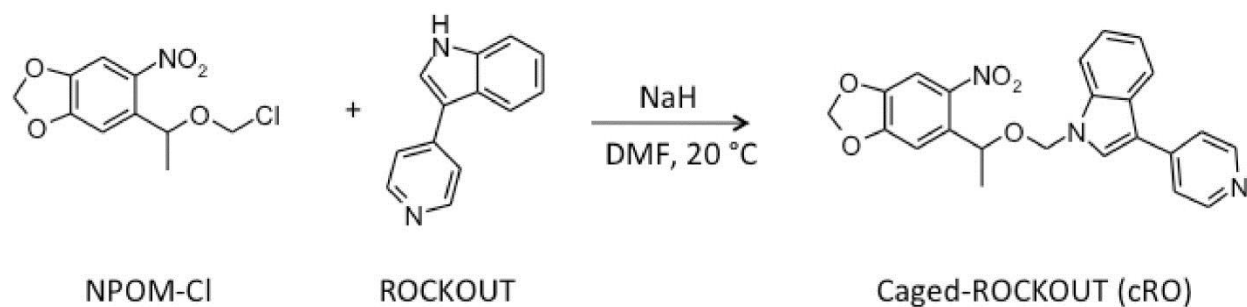
Figure 13: Schematic of caged ROCK inhibitor synthesis. Addition of 6-nitropiperonyloxymethyl-chloride (NPOM-Cl) to ROCK III inhibitor, ROCKOUT (RO) in the presence of sodium hydride (NaH) and dimethylformamide (DMF) at 20°C leads to a 24% yield caged RO (cRO).

the product was eluded with a mobile phase of 100% ethyl acetate to yield 5.2 mg (24% yield) of pure cRO as yellow flakes. Purity was assessed via Proton Nuclear Magnetic Resonance (^{1}H-NMR) spectroscopy (Figure 14A). ^{1}H-NMR spectra were acquired on a Bruker Avance 360 MHz NMR spectrometer and were obtained in CDCl$_3$ using 0.01% TMS as an internal standard. Electrospray mass spectra (positive ion mode) was obtained on a Shimadzu IT-TOF mass spectrometer (Figure 14B).

2.3. Cell Culture, Transfection, and Treatment

Human dermal microvascular endothelial cells (HMECs) [148] were grown in MCDB 131 medium (GIBCO) supplemented with 10% FBS, h-EGF (0.003 mg/ml), hydrocortisone (0.001 mg/ml), and L-glutamine. Primary human pulmonary arterial endothelial cells (HPAECs) were grown in EBM-2 culture medium (Lonza) supplemented with 15% FBS and EGM-2 bullet kit (Lonza) and were used at passages 2-6. All cell lines were maintained at 37°C and 5% CO$_2$. For the hypoxic condition, HPAECs were incubated at 37°C with 1% O$_2$ and 5% CO$_2$ for 16 hours.

Endothelial cells were plated on glass-bottom cover slips coated with 0.2% gelatin and transfected at 70-80% confluency using X-tremeGENE HP DNA transfection reagent according to manufacture protocol (Roche). For adenoviral infection, endothelial cells were exposed to the adenoviral particles overnight and were used for live-cell imaging at 24 to 72 hours post infection. siRNA treatment were performed 48 hours prior to imaging using GeneSilencer siRNA transfection reagent kit and according to manufacture protocol (Genlantis). Procedure was handled according to NIH guidelines and safety for materials containing BSL-2 organisms.

A

B
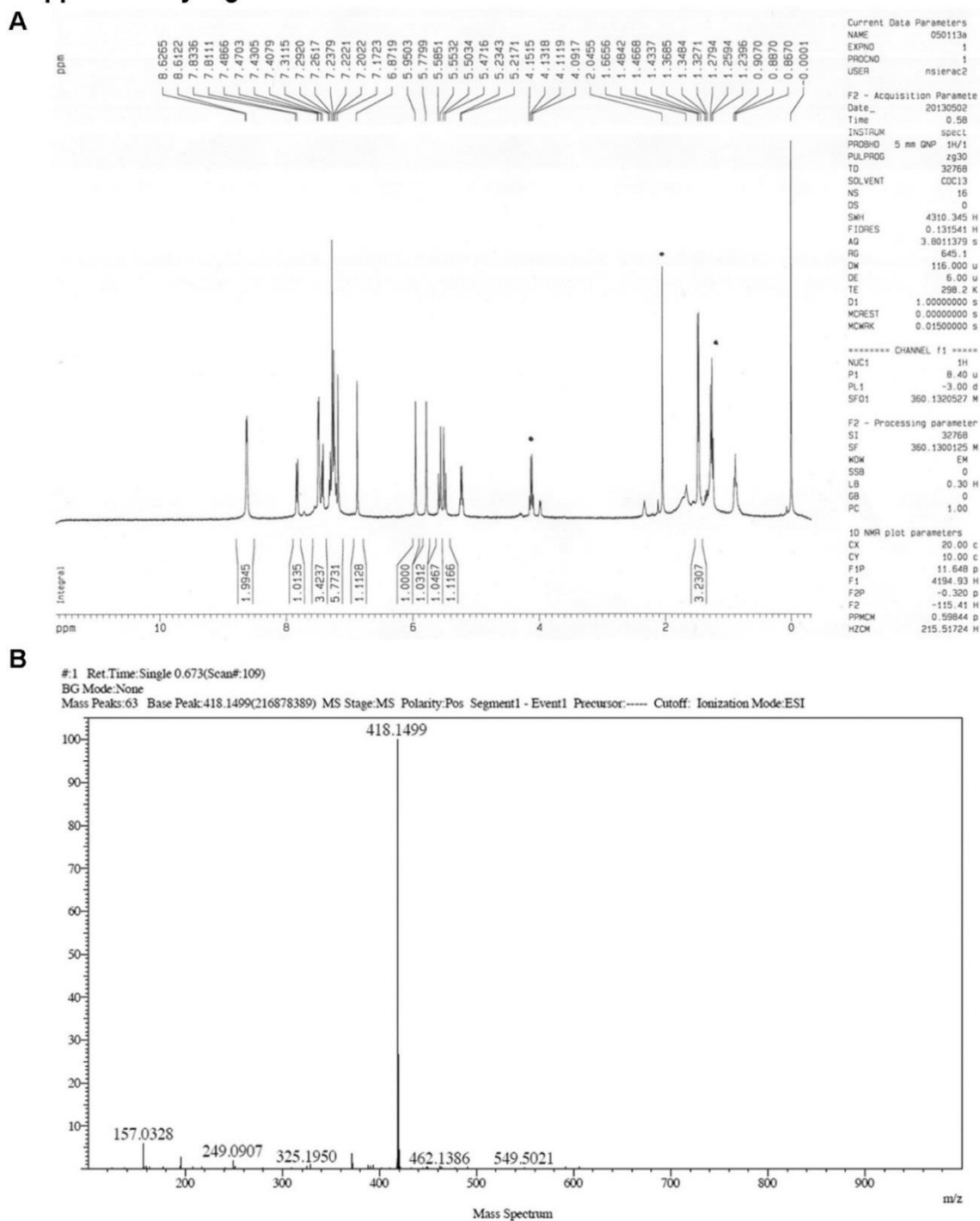
Figure 14: Characterization of caged ROCK inhibitor. A. ^{1}H-NMR spectrum (360 MHz) of caged-ROCKOUT (cRO) obtained in CDCl$_3$. Asterisks indicate ethyl acetate solvent impurity. **B.** Electrospray mass spectrum (positive ion mode) of cRO (C$_{23}$H$_{18}$N$_3$O$_5$). Expected mass (M+1H$^+$) of 418.1397 m/z correlates with obtained mass of 418.1499 m/z.

Cells were treated with 20μM cRO or 10μM blebbistatin (Toronto Research Chemicals) for 10 minutes prior to experiments and 200μM Single Peptide (SP) [149] for 2 hours prior to experiments.

2.4. <u>Immunofluorescence (IF) Staining and Analysis of MLCII Phosphorylation.</u>

Cells expressing mCerulean-PA-Rac1 were kept in dark or exposed to white light for 10 min. Cells were fixed with 4% Paraformaldehyde (Electron Microscopy Sciences) and permeabilized with 0.2% Triton X-100. Non-specific sites were blocked with 4% Bovine Serum Albumin. Samples were stained for phospho-MLCII using rabbit polyclonal phospho-Thr18/Ser19-specific antibody (Cell Signaling; 3674) and TRITC-conjugated donkey anti-rabbit antibody (Jackson ImmunoResearch Laboratories), and mounted in ProLong Gold reagent (Molecular Probes). Z-stack images were obtained using confocal microscope (LSM 510 Meta; Carl Zeiss) equipped with a 63x, 1.2 NA water immersion objective lenses and Ar ion and dual HeNe lasers. Image handling and analyses were performed using MetaMorph software (Molecular Devices, Sunnyville, CA). Relative phosphorylation of MLCII was expressed as average pixel intensity above background noise. Z-stack images were projected onto a single image, threshold to remove background noise and the average pixel intensities of the threshold images were measured for each cell.

2.5. <u>IF Staining for VE-cadherin Expression.</u>

HMECs and HPAECs were fixed and blocked as described above but not permeabilized. Samples were stained for VE-cadherin using mouse monoclonal antibody BV9 against VE-cadherin extracellular domain (Abcam; ab7047) and FITC-conjugated anti-mouse antibody (Jackson ImmunoResearch Laboratories), and mounted in ProLong Gold reagent (Molecular Probes). Z-stack images and analysis were performed as described above.

2.6. <u>Transendothelial Resistance (TER) Measurements</u>

Cells were plated onto gelatin-coated 8W1E gold electrodes (Applied Biophysics). Different concentrations of SP were added 30 minutes prior to the measurement of changes in electrical resistance using an Electric Cell Substrate Impedance Sensing system (Applied Biophysics Inc.).

2.7. <u>Live Cell Imaging</u>

Endothelial cells were imaged in phenol red free media (Lonza) supplemented with 5% FBS at 37°C using the stage heater (Tempcontrol-37; Carl Zeiss, Thornwood, NJ). Time-lapse images were acquired using confocal microscope (LSM 510 and LSM 710; Carl Zeiss) equipped with 63x water, 100x oil, and 63x oil immersion objective lenses and Ar ion and dual HeNe lasers.

To determine the effect of Rac1 on gap sealing or VE-cadherin density at AJs, cells co-expressing VE-cadherin-GFP or GFP-actin and mCherry tagged PA-Rac1 were imaged with λ=488nm and λ=543nm laser, respectively. Photo-activation was achieved with λ=458nm laser beam at 70% power for ~10 seconds at a selected region and images in green channel were acquired every 5 seconds. To determine the diffusion of PA-Rac1, cells co-expressing mCherry-actin and PA-Rac1-PA-GFP were imaged with λ=543nm and λ=488nm laser, respectively. PA-Rac1 and PA-GFP were simultaneously photo-activated with λ=458nm at 70% power and λ=405nm at ~12% power laser beams, respectively. For studying VE-cadherin kinetics, cells co-expressing VE-cadherin-Dendra2 and CFP tagged PA-Rac1 were imaged with λ=488nm and λ=543nm for green and red states of Dendra2, respectively, and λ=458nm for CFP. Dendra2 and cRO were photo-converted or photo-uncaged with λ=405 nm laser at 8-12% power. Images in green and red channels were acquired every 5 seconds, separately or simultaneously. For FRET emission ratio imaging, 12-bit CFP (λ = 458 nm; BP500/20), FRET (λ = 458 nm; LP530), and YFP (λ = 514 nm; LP530) were acquired.

2.8. **Image Processing**

All images in green channel were corrected for photo-bleaching using a modified version of a Matlab software [150]. A photo-bleaching coefficient for each image was determined from decay kinetics of fluorescent intensity outside of irradiation zone. Each image of time-lapse sequence was corrected for photo-bleaching, exported to 16-bit format using LSM program, and analyzed using MetaMorph software (Molecular Devices, Sunnyville, CA).

To determine the length of lamellipodia protrusion, a line scan along the protrusive edge was created that provided a representation of average intensity (y-axis) over distance in pixel (x-axis). The change in distance under the curve was determined and values were converted to microns (1μm=7.8pixel). The area of gap between two adjacent cells were measure at each time point by drawing a region around the gap and the change in gap area was determined between the first and last time points. The rate of gap sealing was determined from the slope of linear regression.

To determine the rate of PA-Rac1 diffusion, the integrated fluorescent intensity of GFP after photo-activation within the irradiation zone was measured, plotted over time, and fitted to a single exponential decay curve; $N = N_0 e^{-kt}$. The half-life of diffusion was determined from the following equation: $t_{1/2} = \ln(2)/k$, where ln is natural log and k is the rate constant.

VE-cadherin-GFP density at AJs was expressed as average fluorescent intensity of GFP at AJs (above background noise) within the photo-activation zone. The increase in VE-cadherin fluorescence after activation of PA-Rac1 or uncaging of cRO above the initial basal values was expressed as relative fluorescent amplitude. The rate constants were calculated by fitting data to a single exponential rise curve.

VE-cadherin-GFP adhesion area was determined by selecting pixels of high intensity values (i.e., VE-cadherin adhesion) using a threshold mapping function in MetaMorph software. The percent threshold area within the photo-activation zone was measured before and at 5 minute of PA-Rac1 photo-activation.

VE-cadherin kinetics at AJs were assessed with VE-cadherin-Dendra2. The changes in average fluorescent intensities at 488nm and 543 nm maximum emission spectra were measured inside the photo-conversion zone. The rate constants for VE-cadherin dissociation (at 543 nm) and association (at 488 nm) were calculated from decay and recovery kinetics, respectively, after Dendra2 photo-conversion. The rate of VE-cadherin-Dendra2 lateral movement within the cell-cell junction was determined from the rate of fluorescence lateral movement at $\lambda=543$ nm outside of the photo-conversion region using a kymograph analyses.

For FRET analysis, the correction for YFP and CFP photo-bleaching was performed as described above. Photo-bleaching coefficient obtained for YFP was used to correct both YFP and FRET images. CFP image was corrected using CFP photo-bleaching coefficient. YFP image was used to create a binary mask with a value of zero outside the cell and a value of one inside the cell. To generate a ratio image, the FRET image was first multiplied by a binary mask image and then divided by the CFP image. For analysis of MLCK activity, because FRET efficiency was inversely related to the activity, CFP was multiplied by a binary mask image and then divided by the FRET image. The ratio images were rescaled to the lower value and a linear pseudo-color table was applied to generate the color-coded image map. The relative activity of RhoGTPases and tension were expressed as mean pixel intensity of FRET/CFP ratio (or CFP/FRET ratio for MLCK) within the irradiation zone at AJs. The changes in activity or tension across VE-cadherin adhesion were determined before and after PA-Rac1 activation or cRO photo-uncaging.

2.9. <u>**Prediction of Peptide Binding**</u>

PepSite program (http://pepsite2.russelllab.org/) [151] was used to predict the site of SP binding to VE-cadherin EC1 domain. The PDB code and chain identifier for VE-cadherin and the peptide sequence were given to the program. PepSite created matrices based on the preferred environment for every residue in the SP when bound. These matrices were then used to score the surface of VE-cadherin EC1 domain to find the best binding sites for SP as well as a preferred orientation for binding.

2.10. <u>**Computational Modeling and Parameter Estimation**</u>

An *in-silico* model was used to describe VE-cadherin adhesion (Equations 1.1-1.2) as an equilibrium between VE-cadherin monomers (reaction *a*) and *trans*-dimers (reaction *d*). After assuming symmetry in VE-cadherin distribution in mature cell-cell adhesions, as illustrated in Figure 33, the equilibrium between monomer and dimer was described by the mass balance equations [**1.1.- 1.2.**].

$$\frac{da}{dt} = j - k_a a - 2k_d^+ a^2 + 2k_d^- d \qquad \text{[1.1.]}$$

$$\frac{dd}{dt} = k_d^+ a^2 - k_d^- d \qquad \text{[1.2.]}$$

Where;

j and $k_a a$ are the rates of VE-cadherin monomer-junction association and dissociation, respectively;

k_d^+ and k_d^- are the "on" and "off" rate constants between monomer and *trans*-dimer;

Complex Pathway Simulator (COPASI) software was used to solve the mass balance equations by fitting them to the experimental data and perform parameter estimation. We assumed that all VE-cadherin-Dendra2 molecules emitted green fluorescence prior to photo-conversion and obeyed rules established by equations 1.1-1.2. The equilibrium relations between different states of VE-cadherin species were determined from [**1.1-1.2**] as following:

$$a = \frac{j}{k_a} \qquad \textbf{[2.1.]}$$

$$d = \frac{a^2}{K_d} \qquad \textbf{[2.2.]}$$

Where;

$K_d = k_d^- / k_d^+$ describes the affinity between two opposing VE-cadherin molecules forming *trans*-dimer.

The total concentration equation is then:

$$s = a + 2a \qquad \textbf{[3.1.]}$$

However, only a fraction of these molecules (at equal probability) emitted a red fluorescence after photo-conversion. It was experimentally estimated that 70% of VE-cadherin-Dendra2 molecules underwent a photo-conversion. These photo-converted molecules were distinguished from the others using an asterisk ($*$) symbol. We assumed that VE-cadherin molecules existing either as a monomer or as a part of the dimer had an equal probability for photo-conversion, and were assigned with number of asterisks equal to the number of photo-converted molecules within the complex. For example, monomer had only one asterisk and dimer could have either one or two asterisk. Therefore, the governing reactions [1-5] shown below (based on Figure 33) can be described as: VE-cadherin monomer-junction association and dissociation with '$*$' depicting the

number of photo-converted molecules (ranging from 0 to 1) [reactions 1 and 2] and reversible dimerization of two monomers with '∗' depicting the number of photo-converted molecules (ranging from 0 to 2) [reactions 3-5].

1
$$\xrightarrow{j} a \xrightarrow{k_a} \emptyset$$

2
$$a_* \xrightarrow{k_a} \emptyset$$

3
$$2a \underset{k_d^-}{\overset{k_d^+}{\rightleftharpoons}} d$$

4
$$a_* + a \underset{k_d^-}{\overset{k_d^+}{\rightleftharpoons}} d_*$$

5
$$2a_* \underset{k_d^-}{\overset{k_d^+}{\rightleftharpoons}} d_{**}$$
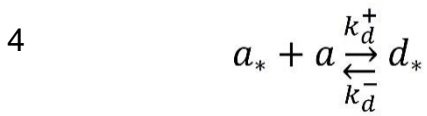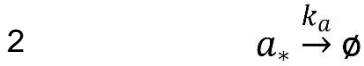
Subsequent to photo-bleaching the governing mass balance equations are:

$$\frac{da}{dt} = j - k_a a - 2k_d^+ a^2 + 2k_d^- d - k_d^+ a a_* + k_d^- d_* \qquad \text{[4.1.]}$$

$$\frac{da_*}{dt} = -k_a a_* - k_d^+ a a_* + k_d^- d_* - 2k_d^+ a_*^2 + 2k_d^- d_{**} \qquad \text{[4.2.]}$$

$$\frac{dd}{dt} = k_d^+ a^2 - k_d^- d \qquad \text{[4.3.]}$$

$$\frac{dd_*}{dt} = k_d^+ a a_* - k_d^- d_* \qquad \text{[4.4.]}$$

$$\frac{dd_{**}}{dt} = k_d^+ a_*^2 - k_d^- d_{**} \qquad \text{[4.5.]}$$

The equilibrium relations presented in 2.1-2.2 are shown with γ, the fraction of photo-converted molecules (ranging from 0 to 1) at time zero in 5.1- 5.5. a_t is the total amount of a (which is 1). Introduction of γ allows us to derive the initial equilibrium relations for the model that includes photo-converted and non-photo-converted VE-cadherin species, under the assumption that the monomers are photo-converted independently of one another even if they form a *trans* dimer.

$$a = (1 - \gamma)a_t \qquad \text{[5.1.]}$$

$$a_* = \gamma a_t \qquad \text{[5.2.]}$$

$$d = \frac{a^2}{K_d} = \frac{(1 - \gamma)^2 a_t}{K_d} \qquad \text{[5.3.]}$$

$$d_* = 2(\frac{a\, a_*}{K_d}) = \frac{2\gamma(1 - \gamma)a_t}{K_d} \qquad \text{[5.4.]}$$

$$d_{**} = \frac{a_*^2}{K_d} = \frac{\gamma^2 a_t}{K_d} \qquad \text{[5.5.]}$$

These reactions and the steady state values were given to COPASI. Values for j and k_a were based on experimental measurements (Figures 29 and 30). The value for j was fixed at 0.01 sec^{-1} and the value for k_a was fixed at 0.003 sec^{-1} for basal condition and 0.0016 sec^{-1} after PA-Rac1 photo-activation (obtained from VE-cadherin-Dendra2 experiment). For k_d^- a lower bound of 1 sec^{-1} and higher bound of 2 sec^{-1} and for k_d^+ a lower bound of 1 mM^{-1} sec^{-1} and higher bound of 100 mM^{-1} sec^{-1} were given to COPASI based on previous publication [152, 153]. A constraint was added for the binding affinity (K_d) with lower bound of 0.01 mM and upper bound of 1 mM,

also based on previous publications [152-154]. A set of values for basal k_d^+ and k_d^- parameters was estimated by fitting the experimental data (Figure 29) to the total concentration equation:

$$s_* = a_* + d_* + 2d_{**} \qquad\qquad \textbf{[6.1.]}$$

The equation establishes the relation between the photo-bleach signal (s_*) and the forms of photo-bleached *trans* dimers. This process was repeated to fit experimental data (Figure 29) and to estimate parameters after PA-Rac1 photo-activation. In this scenario, the constraint for k_d^- was removed to assess the effect of Rac1.

3. ROLE OF RAC1 ON STABILITY OF ENDOTHELIAL AJS

*Portions of the text, figures 22-33 and table 1 were reproduced with permission from Daneshjou N, Sieracki N, van Nieuw Amerongen GP, Schwartz MA, Komarova YM, Malik AB. (2014) Rac1 functions as a reversible tension modulator to stabilize VE-cadherin *trans* interaction. The Journal of Cell Biology. Accepted.

3.1. <u>Distribution of RhoGTPases Under Normal and Hypoxic Conditions</u>

Distribution of the RhoGTPases is dependent on the cell type and the state of junction maturation. In the migrating fibroblast, RhoA is at the leading edge of the migratory cell and localizes at the site of protrusion and is followed by a delayed activation of Rac1 [11]. During initiation of cell-cell contact, antagonistic relationship between Rac1 and RhoA derive the adhesion formation [74]. Disruption of the junctions in response to thrombin is linked to increase RhoA activity, whereas Rac1 and Cdc42 are up regulated during re-annealing of the junction [155]. The mature endothelial cell monolayer is characterized by the balanced between Rac1 and RhoA activities, with a non-linear shift towards Rac1 and Cdc42 activity [156], however, the functional activity of these GTPases is not clear. Therefore, our first study was to determine the spatial distribution of these GTPases activities in our system using FRET-based biosensors [5, 11], Materials and Methods; Figure 10). We observed that the activity of Rac1 and Cdc42, and not RhoA was concentrated at mature junctions of HMECs (Figure 15). Furthermore, exposure of cells to hypoxic condition for 16 hours, which stabilizes VE-cadherin adhesion, led to an increase in both Rac1 and Cdc42 junctional activity (Figure 16), consistent with the increase in HIF2α expression and the up-regulation of VE-PTP [51], which interacts with and stabilizes VE-cadherin [42]. These data suggested an important contribution of Rac1 and Cdc42 junctional
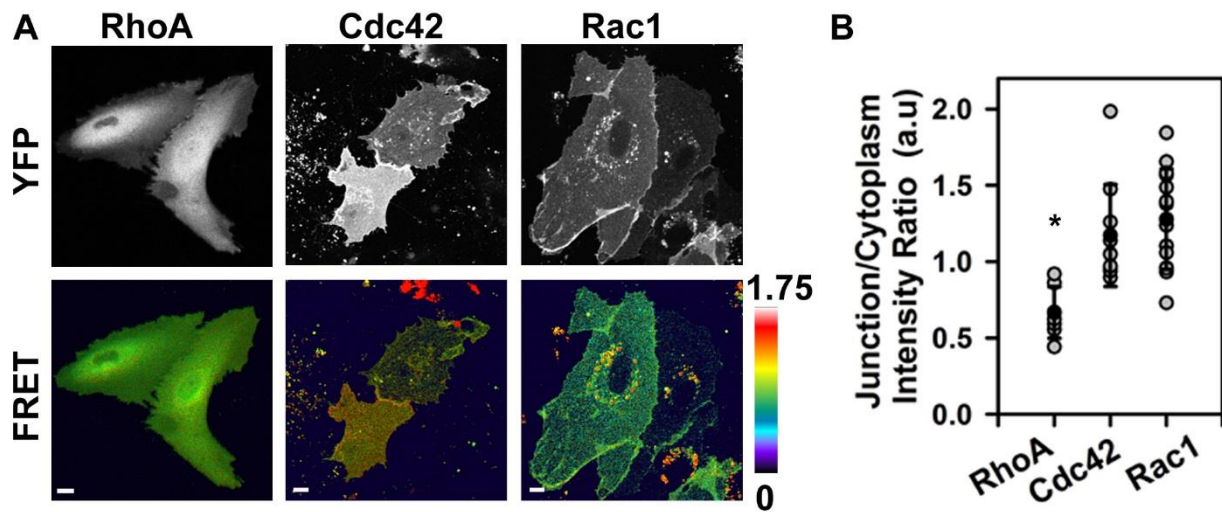
Figure 15: Distribution of RhoGTPases. A. HMECs expressing RhoA, Cdc42, or Rac1 biosensors (Top panel) and the FRET/CFP ratio (bottom panel). Ratio images were scaled from 0 to 1.75 and color-coded as indicated on the right. Scale bar, 10μm. **B.** Junctional activity relative to cytoplasm for RhoA (0.67±0.17), Cdc42 (1.17±0.33), and Rac1 (1.28±0.33); mean±SD. n=7-14; *p*<0.05.
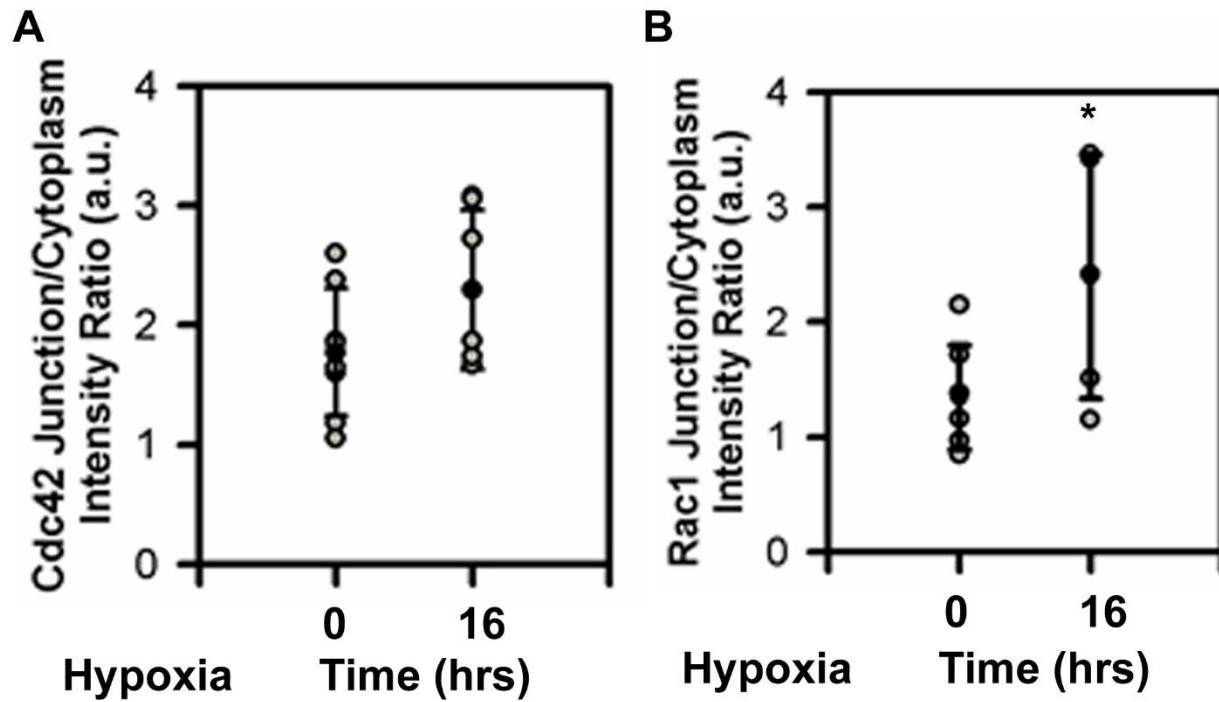
Figure 16: Effect of hypoxia on RhoGTPases activities. A-B. HMECs expressing Cdc42 (**A**) or Rac1 (**B**) biosensors were subjected to 16 hours of hypoxic condition. Junctional activity relative to cytoplasm were increased for both Cdc42 from (1.74±0.75) to (2.18±0.67) (**A**) and Rac1 from (1.37±0.49) to (2.39±1.06) (**B**); mean±SD. n=5-6; *p*<0.05.

activities in response to hypoxia and up-regulation of VE-cadherin, which may initiate an "outside-in" signaling cascade that regulate GTPases activities and barrier integrity. Due to the observed increase in Rac1 activity and the importance of Rac1 during initiation and strengthening of the cell-cell contact [134] and mediating the barrier protective effect of mediators such as S1P [106, 138], we focused our studies on the effect of localized Rac1 activation on stability of endothelial AJs.

3.2. <u>PA-Rac1 as a Tool to Control the Spatiotemporal Activity of Rac1</u>

We utilized PA-Rac1 probe bearing a constitutively active Rac1 bound to a light-sensitive domain (Materials and Methods; Figure 8) to study the effect of spatiotemporal activation of Rac1 at AJ on barrier integrity. Photo-activation of PA-Rac1 with 458nm laser beam resulted in the transient photo-release of constitutively active Rac1 mutant that occurred with a half-life ($t_{1/2}$) of ~25 seconds, which mimics the transient 'switch-on' property of endogenous Rac1 induced by mediators such as the AJ stabilizing lipid S1P [74, 106, 138]. In comparison with the global activation of Rac1 with other experimental tools, which have linked Rac1 activity to a range of responses from stabilization of endothelial AJs on exposure to shear stress or S1P [106, 137, 138] to disassembly of AJs in response to VEGF and TNFα [117, 139-141], the spatiotemporal activation of Rac1 with PA-Rac1 probe allowed us to study the effect of localized and transient Rac1 activation at AJs while preventing activation of other parallel signaling pathways that may influence the outcome or mask Rac1 effect [14, 92].

Due to the cytosolic nature of the probe, PA-Rac1 is subjected to diffusion. Its short reported half-life led us to investigate the rate at which PA-Rac1 diffused and whether its transient effect being studies at our hands was masked by its diffusive property. To determine the diffusion rate of PA-Rac1, we utilized a photo-activatable GFP protein (PA-GFP) tagged to PA-Rac1 (PA-Rac1-

PA-GFP), which emits green fluorescence after photo-activation with 405nm laser beam. We observed that PA-Rac1 diffused with a half-life of 72±16 seconds (Figure 17). Our finding confirmed that the diffusion of PA-Rac1 with cytosolic compartments was secondary to its transient activity and thus the effect of Rac1 activation in our studies would be merely due to Rac1's transient activation.

3.3. <u>Rac1 Induces Lamellipodia Protrusion in Sub-confluent Monolayer</u>

Rac1 is a known mediator of actin cytoskeleton and its role on lamellipodia protrusion at the nascent cell-cell contact site to induce junction annealing or to promote gap closure in response to injury is well studied [74, 134, 157]. To determine the effect of Rac1 activation in sub-confluent monolayer on actin organization, under steady-state, we photo-activated PA-Rac1 in endothelial cells expressing GFP-actin. We observed an immediate establishment of ruffling at the site of photo-activation followed by rapid relocation of the ruffle to other regions (Figure 18), consistent with the transient effect of Rac1. Our data suggests that the brief photo-activation of the probe is sufficient for Rac1 to interact with downstream effectors and induce a signaling event, in this case lamellipodia ruffling. Consistent with this data, photo-activation of PA-Rac1, but not the light insensitive control probe (PI-Rac1), at the site of cell-cell contact in sub-confluent monolayer, where small gaps were observed, induced lamellipodia protrusion and gap sealing with the rate constant of ~8 nm^2/min (Figure 19). These findings are consistent with the known function of Rac1 at nascent junctions and during junction re-annealing and gap sealing by promoting lamellipodia ruffling and membrane protrusion [157].

mCherry-actin   **PA-GFP-PA-Rac1**
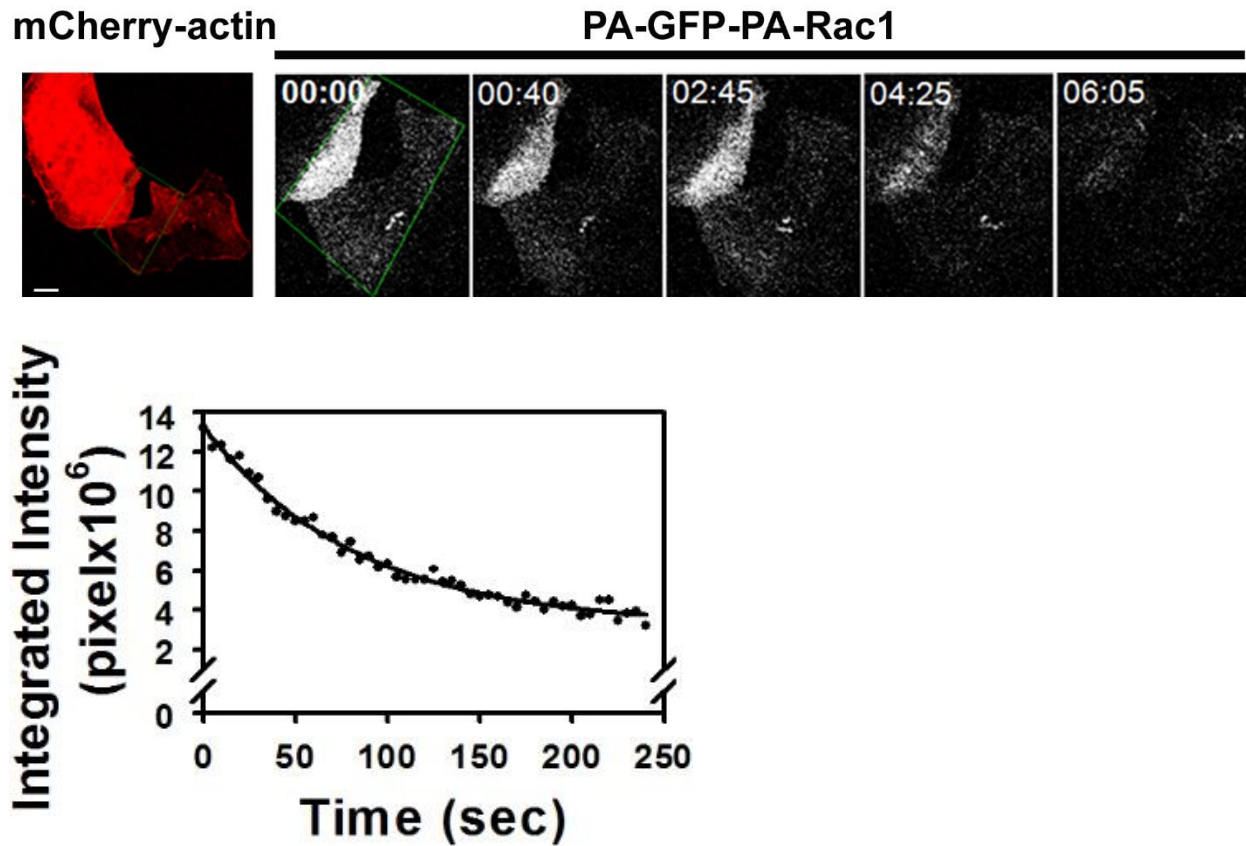
Figure 17: PA-Rac1 diffusion at the sites of AJs. HMECs expressing mCherry-actin (red) and PA-GFP-PA-Rac1 (shown enlarged; white) after simultaneous photo-activation of PA-GFP and PA-Rac1 within the indicated region at time 00:00. Time in minute and second. Scale bar, 10μM. The graph represents integrated intensity within photo-activation zone as a function of time and fitted to exponential decay to determine the diffusion coefficient (0.01±0.002) and half-life (72±16 sec); mean±SD. n=4.
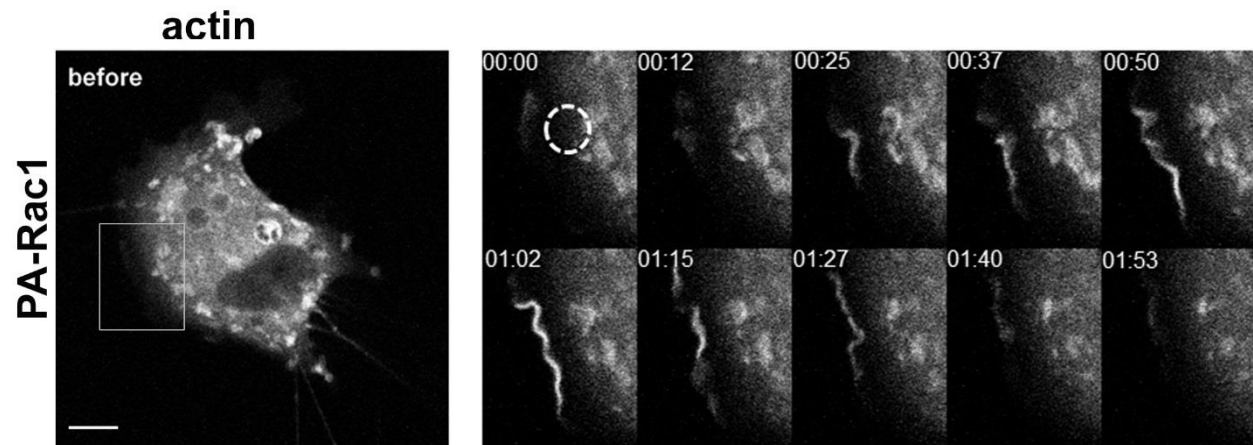
Figure 18: PA-Rac1 photo-activation induces lamellipodia ruffling in sub-confluent monolayers. Time-lapse imaging of HMECs expressing GFP-actin (white) and mCherry-PA-Rac1 (co-transfection not shown) within the rectangular region after photo-activation of PA-Rac1 within the circle at time 00:00. Time in minute and second. Scale bar, 10μM.
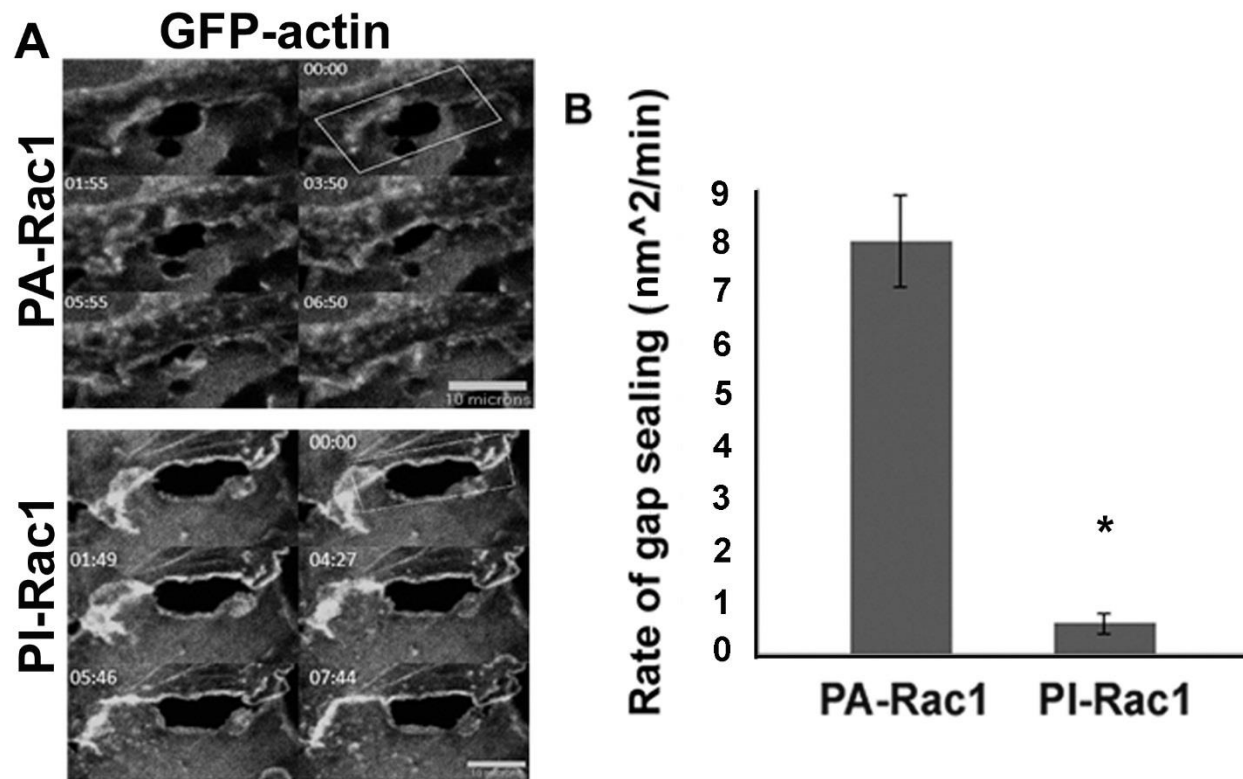
Figure 19: PA-Rac1 photo-activation induces gap sealing. A. Time-lapse imaging of HMECs expressing GFP-actin (white) and mCherry tagged PA-Rac1 or PI-Rac1 (co-transfection not shown) before and after photo-activation within the indicated region at time 00:00. Time in minute and second. Scale bar, 10μM. **B.** Rate of gap sealing is measured after PA-Rac1 or PI-Rac1 photo-activation. mean±SD. n=6. *p*<0.05.

3.4. <u>Rac1 Activation at Mature AJs Increases VE-cadherin Density</u>

The role of Rac1 at mature endothelial cell-cell adhesions is not well understood. Interestingly, Rac1-induced actin ruffling observed in sub-confluent monolayers was absent at the mature junctions of confluent monolayer of endothelial cells. While continuous ruffling was observed at these junctions, photo-activation of PA-Rac1 or the control probes (PI-Rac1 or a probe bearing a dominant negative Rac1; PA-Rac1DN) did not have any significant effect on the size of lamellipodia protrusion (Figure 20).

Using our tools in hand, we investigated the role of spatiotemporal Rac1 activity in regulating VE-cadherin dynamics at mature AJs of confluent endothelial monolayers. We first confirmed that there is similar level of endogenous VE-cadherin expression in both primary endothelial cells, HAPECs, and the immortalized endothelial cell line, HMECs used in our studies (Figure 21). We observed that photo-activation of PA-Rac1 in different endothelial cell types expressing VE-cadherin-GFP induced VE-cadherin accumulation within the photo-activation zone at similar rate constants of 0.17 ± 0.16 min^{-1} for HPAECs and 0.14 ± 0.13 min^{-1} for HMECs while photo-activation of PI-Rac1 or PA-Rac1DN had no significant effect (Figure 22 and 23). Importantly, the Rac1-induced increase in VE-cadherin density was not accompanied by expansion of adhesion zone (Figure 24), suggesting that any effect of lamellipodia activity was negligible. These results showed that spatiotemporal activation of Rac1 at mature AJs functioned by increasing VE-cadherin density of the junctions, independent of lamellipodia activity (Figure 25).
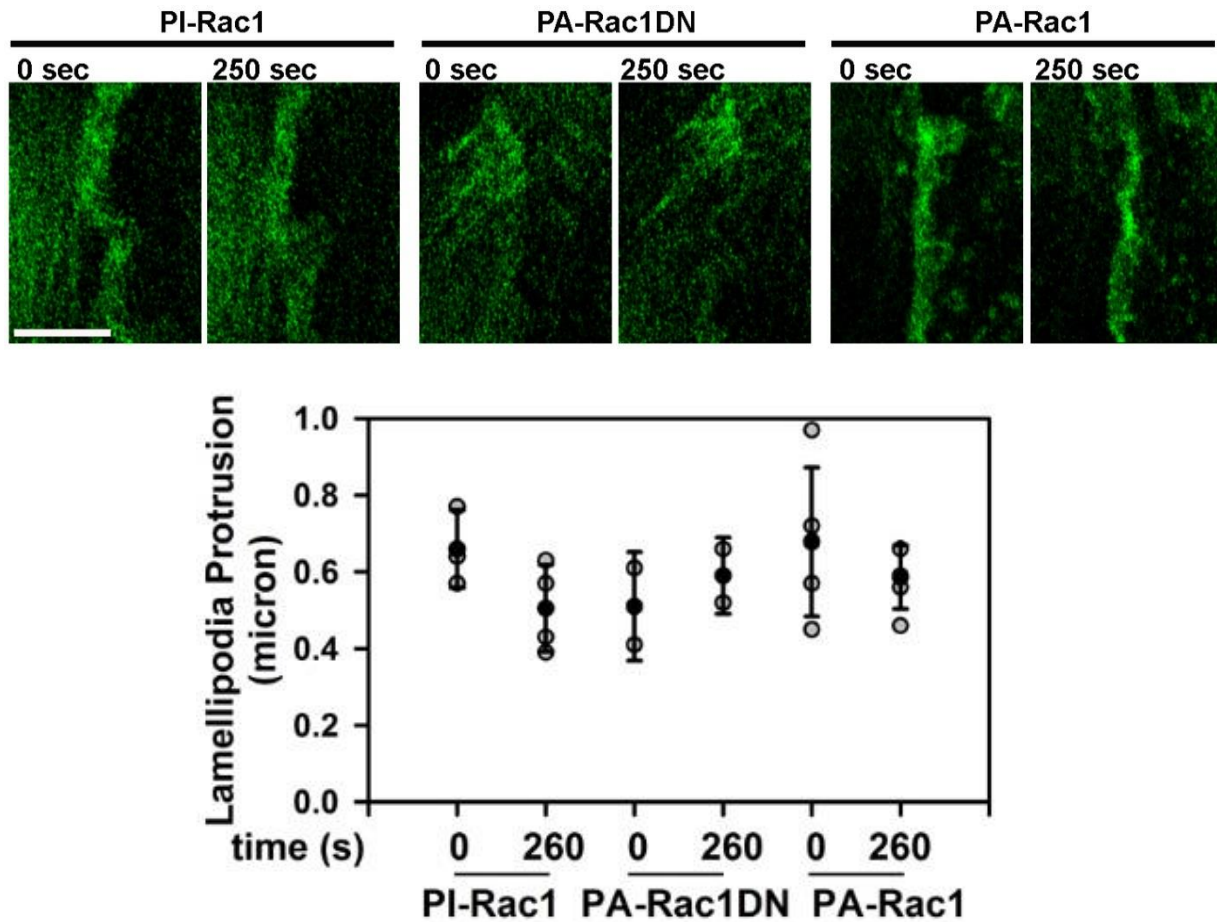
Figure 20: PA-Rac1 photo-activation does not affect lamellipodia protrusion at mature AJs. Junctions expressing GFP-actin at time 0sec and 250 sec after photo-activation of PI-Rac1, PA-Rac1DN, or PA-Rac1. Scale bar, 10μM. Expansion of adhesion zone was compared at time 0 sec (before photo-activation) and 260 sec. No significant changes were observed. mean±SD. n= 2-5 cells.
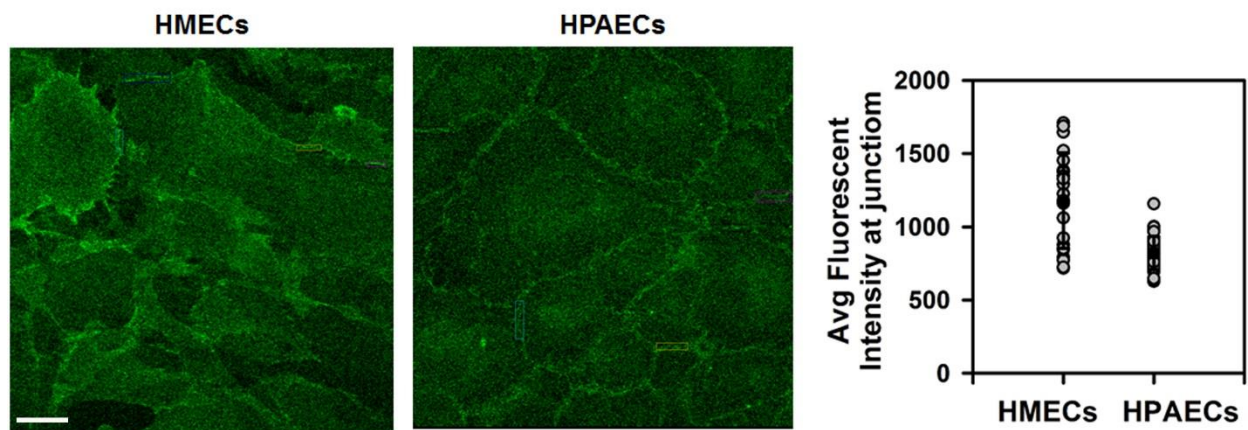
Figure 21: Expression level of VE-cadherin in different endothelial cell monolayers. HMECs and HPAECs are stained for VE-cadherin. Scale bar, 20µM Expression level at junctions is presented as average fluorescent intensity. mean±SD. n=22-36 junctions.

A **VE-cad-GFP**

B

C

D

E

F
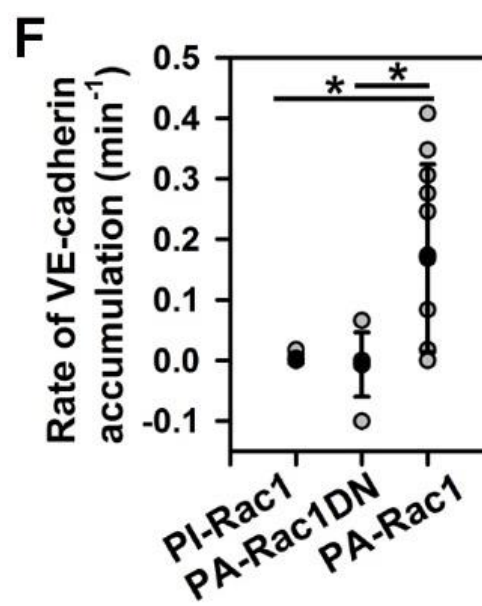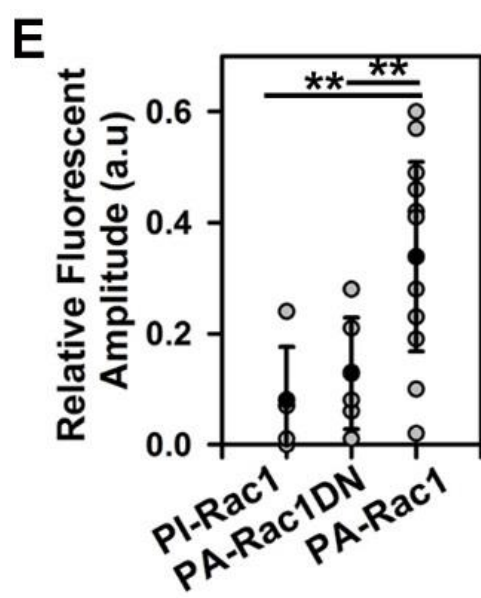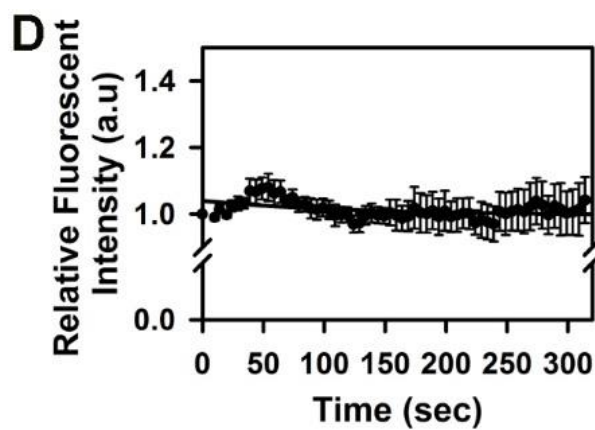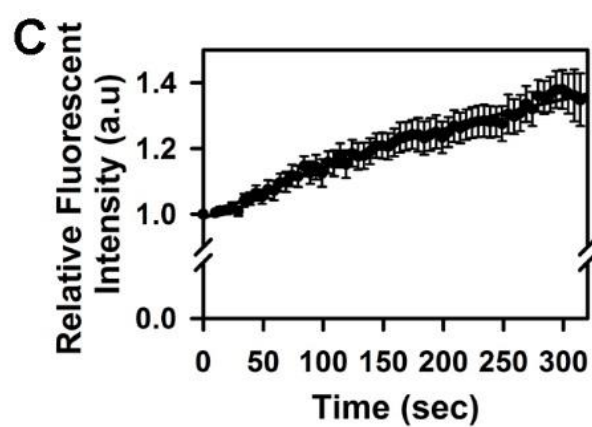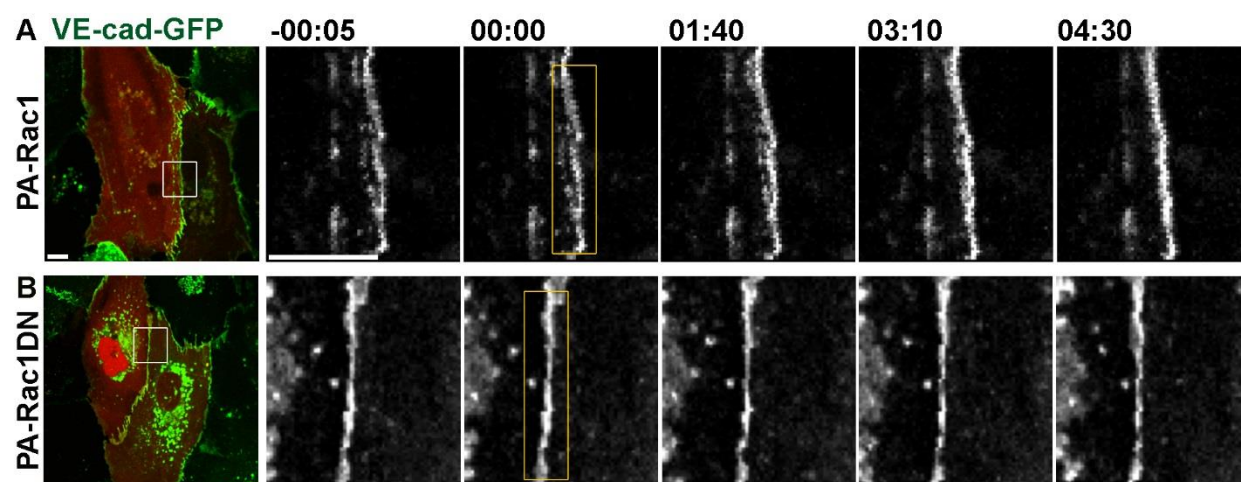
Figure 22: Photo-activation of Rac1 at AJs increases VE-cadherin density in HPAECs. A-B. Time-lapse images of VE-cadherin-GFP (green) in cells expressing either mCherry (red) tagged PA-Rac1 **(A)** or PA-Rac1DN **(B)** before and after photo-activation inside the yellow rectangular region at time 00:00. Time in minutes and seconds is shown. Scale bar, 10µm, and 5µm for inserts. **C-D**. Relative changes in VE-cadherin-GFP fluorescence intensity at AJs within photo-activation zone for PA-Rac1 **(C)** or PA-Rac1DN **(D)** as shown in **A** and **B**; mean±SEM. n= 6-14. **E.** Amplitude of VE-cadherin-GFP accumulation after photo-activation of PI-Rac1 (0.08±0.1), PA-Rac1DN (0.13±0.1), or PA-Rac1 (0.34±0.17); mean±SD. n= 5-14; $p<0.005$. **F.** Rate constant for VE-cadherin-GFP accumulation after photo-activation of PA-Rac1 was $0.17±0.16$ min^{-1} whereas no significant change was observed with PI-Rac1 or PA-Rac1DN; mean±SD. n=5-11; $p<0.05$.

A VE-cad-GFP   -00:18   00:00   01:43   03:10   04:30

PA-Rac1

B

PA-Rac1DN

C

D

E

F
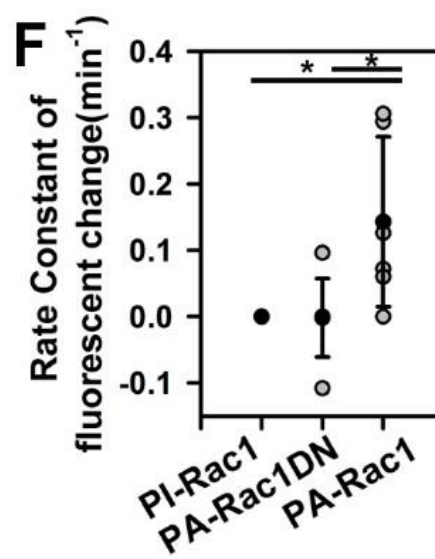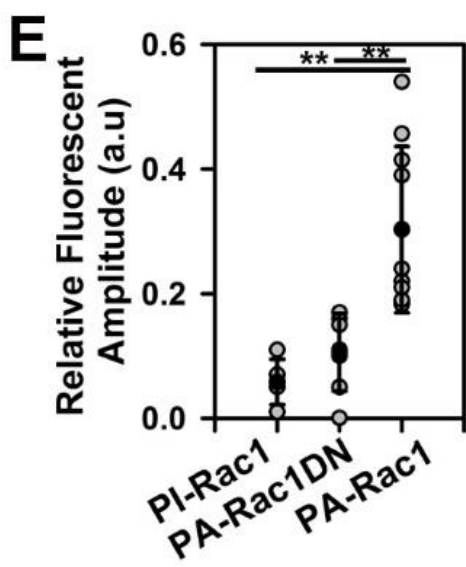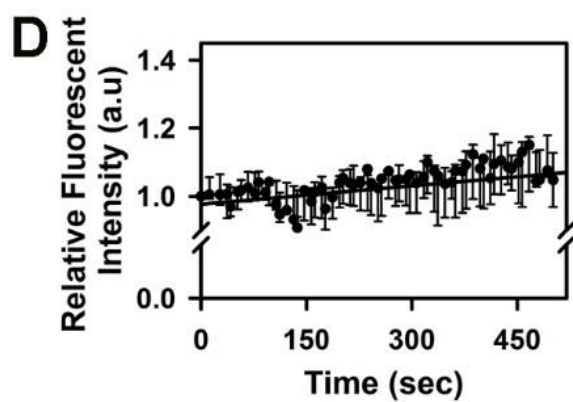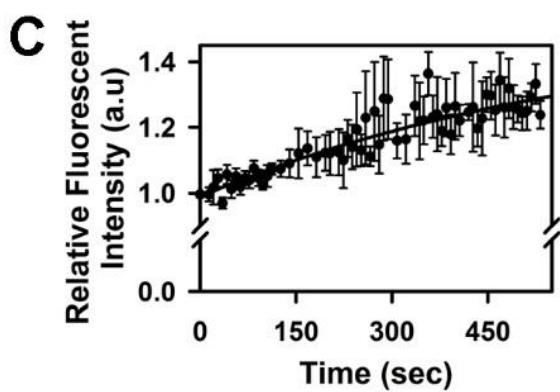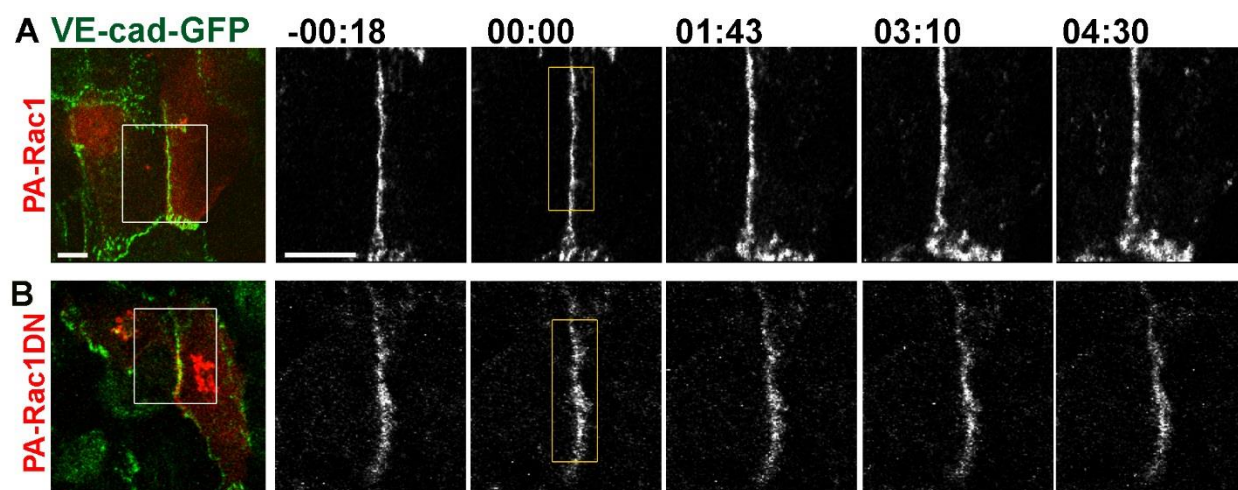
Figure 23: Photo-activation of Rac1 at AJs increases VE-cadherin density in HMECs. A-B. Time-lapse images of VE-cadherin-GFP (green) in cells expressing either mCherry (red) tagged PA-Rac1 **(A)** or PA-Rac1DN **(B)** before and after photo-activation inside the yellow rectangular region at time 00:00. Time in minutes and seconds is shown. Scale bar, 10µm, and 5µm for inserts. **C-D**. Relative changes in VE-cadherin-GFP fluorescent intensity after photo-activation of PA-Rac1 **(A)** or PA-Rac1DN **(B)**. mean±SEM. n= 8-10. **E.** Amplitude of VE-cadherin-GFP accumulation after photo-activation of PI-Rac1 (0.06±0.04), PA-Rac1DN (0.11±0.06), or PA-Rac1 (0.30±0.13); mean±SD. n= 5-10; $p<0.005$. **F.** Rate constant for VE-cadherin-GFP accumulation after photo-activation of PA-Rac1 was 0.14±0.13 min^{-1} whereas no significant changes were observed after activation of PI-Rac1 or PA-Rac1DN; mean±SD. n=5-8; $p<0.05$.
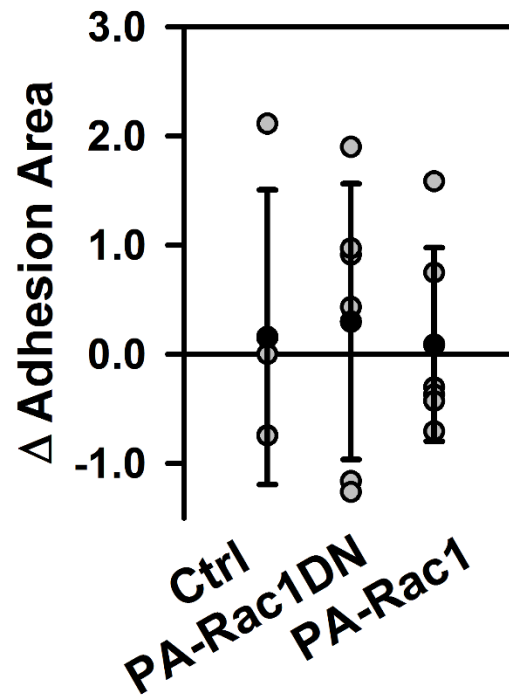
Figure 24: PA-Rac1 photo-activation does not affect VE-cadherin adhesion area. Changes in VE-cadherin adhesion area within irradiation zone after photo-activation of PA-Rac1 or PA-Rac1DN, and in adjacent cells (ctrl). mean±SD. n= 4-6.
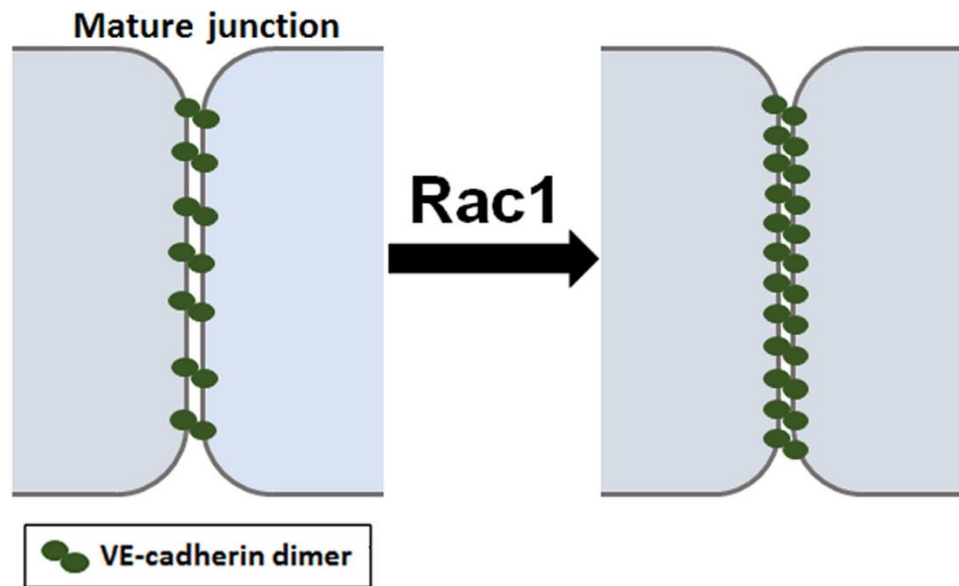
Figure 25: Schematic model of Rac1-induced VE-cadherin accumulation. Model demonstrating the relationship between Rac1 activity and VE-cadherin density at mature AJs.

3.5. <u>Activation of Rac1 at AJs Reduces VE-cadherin Dissociation Rate.</u>

Cadherin-mediated adhesion is a dynamic event characterized by continuous rearrangements of the cadherin adhesive bonds [66], lateral movement of VE-cadherin molecules within the AJs [41], and exchange of cadherin between the junctional and intracellular pools [17, 40]. Recruitment of cadherin is a spontaneous process followed by active ATP-dependent and independent release of cadherin from AJs [17]. To determine how Rac1 regulated VE-cadherin dynamics at AJs, we tagged VE-cadherin to the photo-switchable fluorescent protein Dendra2.

VE-cadherin-Dendra2 allowed us to directly monitor VE-cadherin dynamic at AJs and to overcome several limitations related to the use of a more conventional method fluorescence recovery after photobleaching (FRAP). Using FRAP approach, one does not account for diffusion; FRAP measurement assumes that all binding sites are saturated and that all molecules are bleached at the same rate. Another assumption made by FRAP is spontaneous association. Therefore, FRAP experiments do not provide information regarding whether the photo-bleaching recovery at AJ is due to recruitment of new cadherin molecules to AJ or formation of new AJs at the region of photo-bleaching. Using VE-cadherin-Dendra2 construct, we can determine VE-cadherin behavior at AJs from the fluorescent intensity kinetics at 488nm and 543 nm after Dendra2 photo-conversion (Figure 26A). The kinetics of fluorescent recovery at 488nm described the flux of new VE-cadherin molecules into AJs, namely the "association", whereas the decay kinetics at 543 nm represented the net effect of VE-cadherin lateral movement and removal of VE-cadherin from AJs, namely "dissociation" (Figure 26A). The representation of expected results from VE-cadherin-Dendra2 experiment is shown for better appreciation of the tool (Figure 26B). Importantly, irradiation of VE-cadherin-Dendra2 with 458 nm laser beam
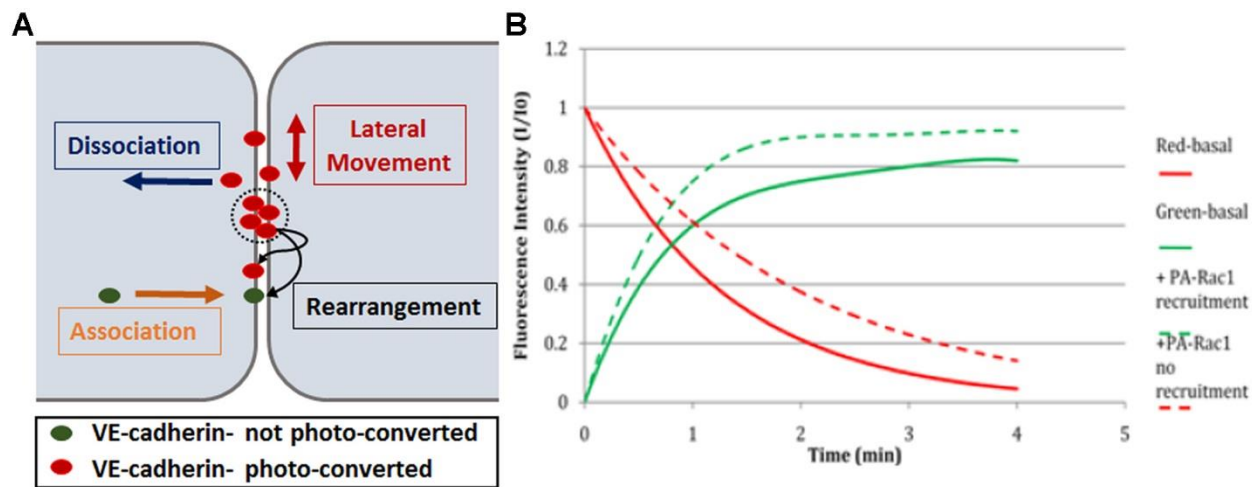
Figure 26: Schematic model of VE-cadherin dynamic. A. Model representing VE-cadherin-Dendra2 kinetics at AJs. Photo-converted VE-cadherin molecules (red) within the irradiation zone (circle) undergo lateral movement and dissociation from AJs resulting in fluorescence intensity decay. They also undergo re-arrangement of adhesive bonds with other photo-converted or newly recruited (green) molecules. **B.** Dendra2 is used to determine the rate of VE-cadherin association (solid green) and dissociation (solid red) from AJs. If Rac1 activation promotes recruitment of new cadherin molecules to AJs, we expect an increase in association rate (dotted green) with no change in the dissociation rate. However, if another mechanism is involved, we expect a decrease in dissociation rate (dotted red) with no change in association rate.

required for PA-Rac1 activation did not interfere with photo-conversion of Dendra2 (Figure 27), thus Dendra2 enabled assessment of VE-cadherin dynamics.

We observed that VE-cadherin lateral movement contributed little to the decay kinetics and PA-Rac1 photo-activation had no significant effect on the rate (Figure 28), indicating that lateral movement negligibly contributed to the kinetics decay. However, VE-cadherin underwent rapid exchange between junctional and intracellular pools (Figure 29 and 30). Photo-activation of PA-Rac1, in contrast to PA-Rac1DN, significantly decreased the rate constant of VE-cadherin dissociation (Figure 29) without an alteration in the association rate (Figure 30). This shift in VE-cadherin kinetics following Rac1 activation was the result of spatiotemporal activity of PA-Rac1 since no changes in VE-cadherin kinetics were observed in the absence of photo-activation of PA-Rac1 (Figure 31), indicating that the shift in VE-cadherin kinetics was secondary to the spatiotemporal activation of Rac1. Together, these results show that Rac1 stabilized VE-cadherin adhesions at mature AJs primarily by decreasing the rate of VE-cadherin dissociation but without affecting the rate of association.

3.6. Relationship Between Stability of Adhesive Bonds and VE-cadherin Kinetics

We next investigated the relationship between VE-cadherin kinetics at AJs as modulated by Rac1 activity and stability of *trans*-dimers. Structural analysis of extracellular domain of VE-cadherin has shown that the strand-swap (*trans*) dimerization is the result of exchange between N-terminal β-strands of opposing ectodomains [30, 38] and is the primary event during cell-cell adhesion responsible for VE-cadherin homotypic interaction [33, 158].

The relationship between Rac1 activity and stability of VE-cadherin trans-dimers at mature AJs was initially assessed using an *in-silico* model. This model describes VE-cadherin adhesion
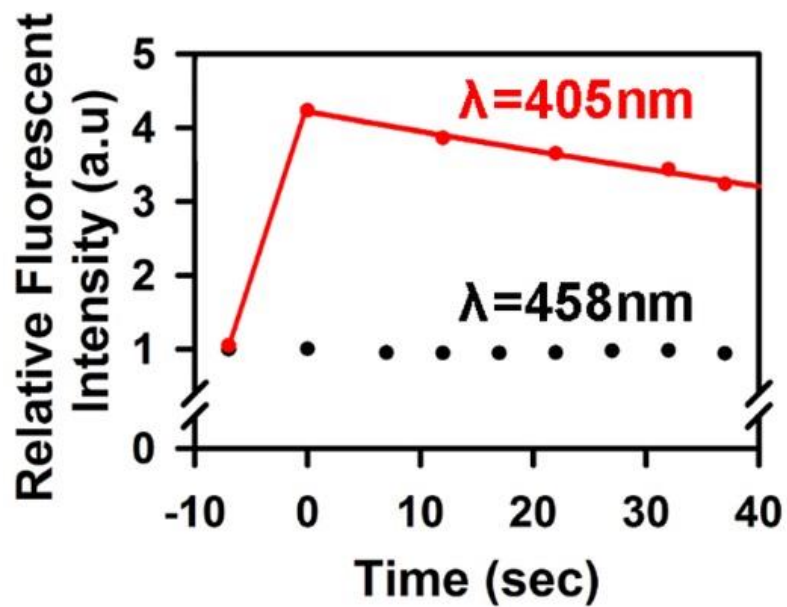
Figure 27: Dendra2 is not photo-converted with 458nm laser beam. Changes in fluorescent emission at 543nm after irradiation of VE-cadherin-Dendra2 with λ=405nm and λ=458nm laser beams at time zero
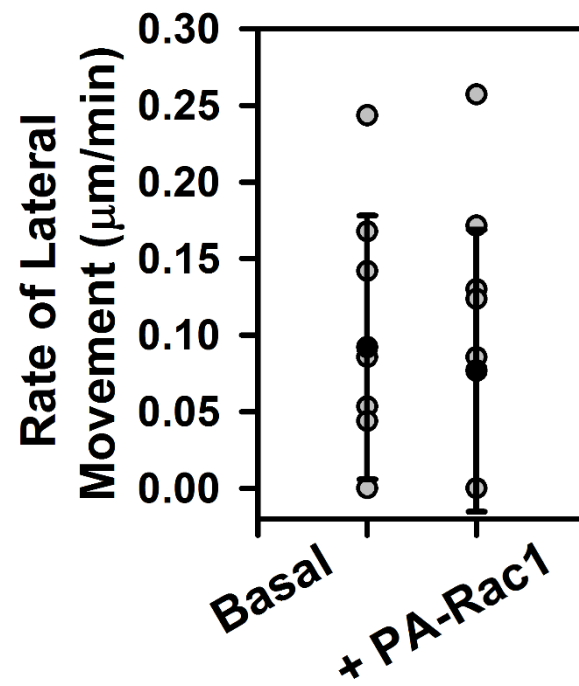
Figure 28: PA-Rac1 photo-activation does not affect the rate of VE-cadherin lateral movement. Rate of lateral movement of VE-cadherin-Dendra2 for basal condition (0.09±0.09 μm/min) and after PA-Rac1 photo-activation (0.08±0.09 μm/min); mean±SD; n=8-10.
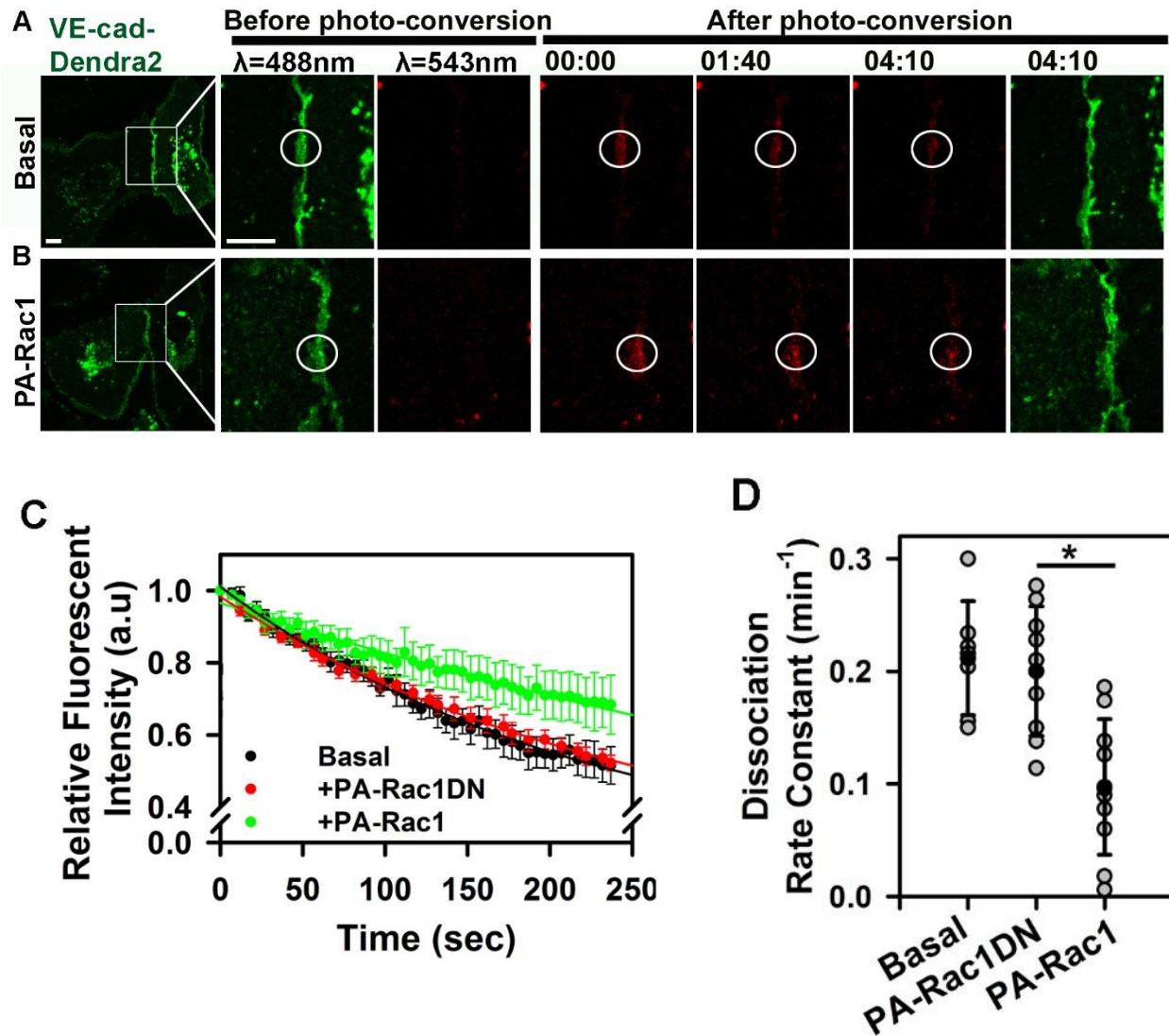
Figure 29: PA-Rac1 Photo-activation at AJs decreases rate of VE-cadherin dissociation. **A-B**. Time-lapse images of VE-cadherin-Dendra2 before (green) and after photo-conversion (red, circle) at time 00:00 for basal **(A)** and PA-Rac1 photo-activation **(B)**. Scale bar, 10 μm. **C**. VE-cadherin dissociation rate is calculated from decay kinetics of Dendra2 after photo-conversion; mean±SEM, n= 7-10. Photo-activation of PA-Rac1, and not PA-Rac1DN, reduced the rate of VE-cadherin dissociation. **D**. Rate constants of VE-cadherin dissociation calculated from **C** for basal (0.21 ± 0.05 min^{-1}), PA-Rac1DN (0.2 ± 0.06 min^{-1}), and PA-Rac1 (0.1 ± 0.06 min^{-1}); mean±SD, n=7-10; $p<0.05$.
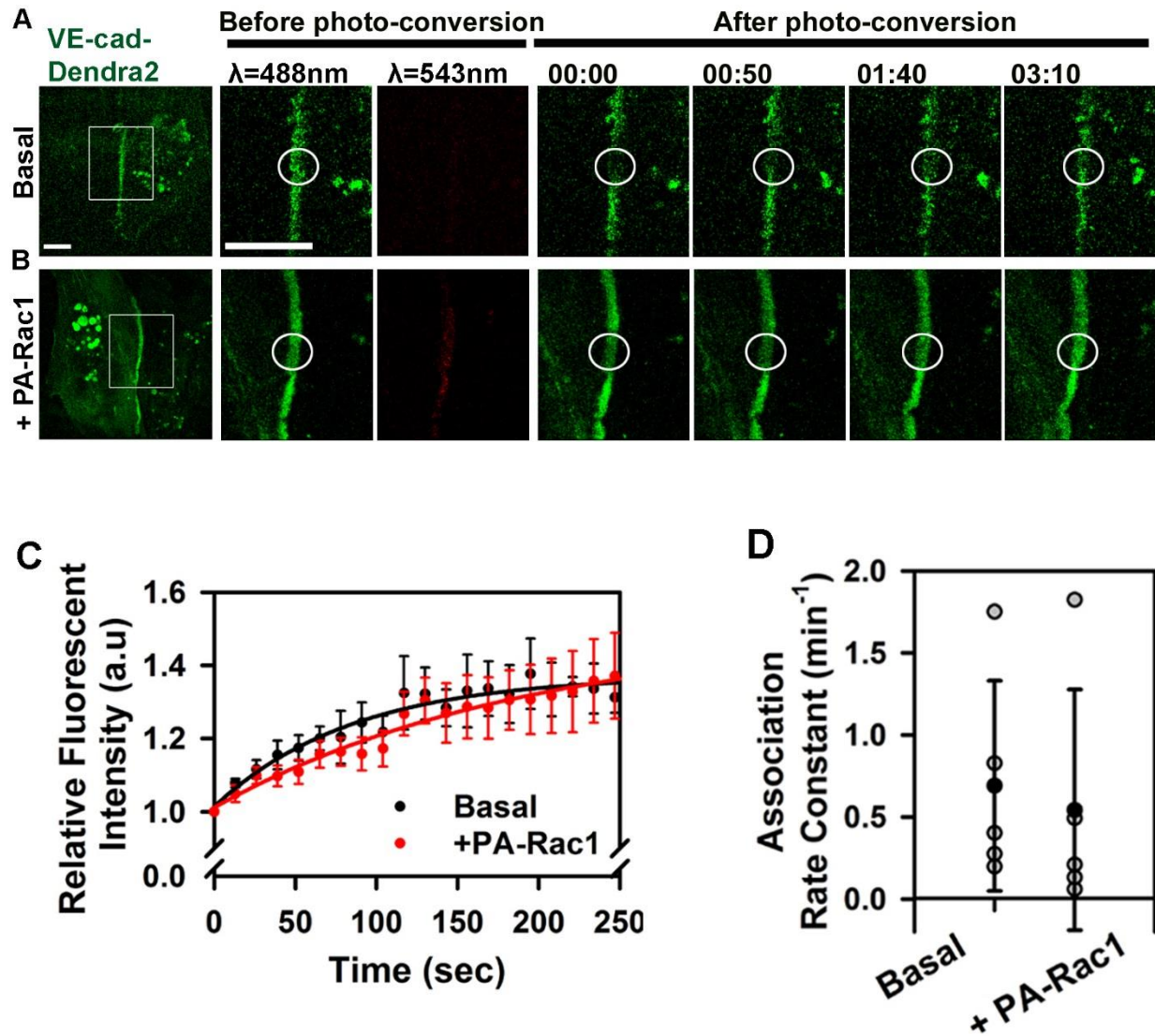
Figure 30: PA-Rac1 Photo-activation at AJs does not affect VE-cadherin recruitment. A-B. Time-lapse images of VE-cadherin-Dendra2 emission at 488 nm before and after photo-conversion within the circular region under basal condition (**A**) and after activation of PA-Rac1 (**B**). The recovery in fluorescent intensity at 488nm is due to recruitment of VE-cadherin to AJs. Scale bar, 10 μm. **C**. VE-cadherin association rate is determined from recovery kinetics of Dendra2 after photo-conversion; mean±SEM, n=7-9. **D**. Rate constants of VE-cadherin association calculated from **C** for basal (0.69±0.64 min^{-1}) and PA-Rac1 (0.54±0.73 min^{-1}); mean±SD, n=5.
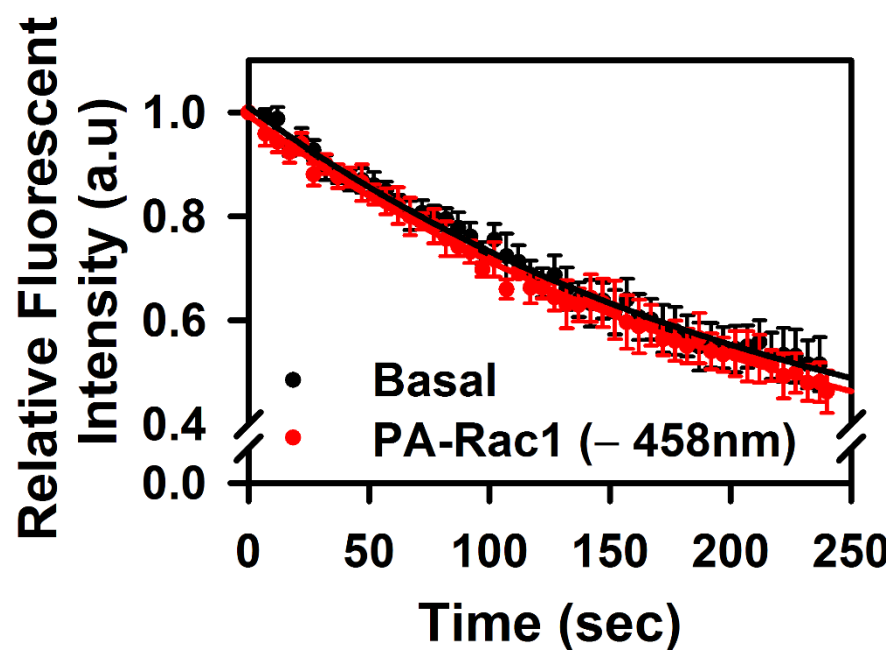
Figure 31: Photo-activation of PA-Rac1 is required for the shift in VE-cadherin kinetics. Cells were transfected with VE-cadherin-Dendra2 alone or with PA-Rac1. Changes in relative fluorescent intensity of VE-cadherin-Dendra2 were monitored after Dendra2 photo-conversion; no PA-Rac1 photo-activation was performed; mean±SEM. n= 3 and 7.

(Materials and Methods) as equilibrium between VE-cadherin monomers and *trans*-dimers (Figure 32), which are defined by mass balance equations and solved using COPASI software (Materials and Methods). COPASI produced an optimal fit to our experimental data (i.e., monomer-junction association (j) and dissociation (k_a) rate constants (Figures 29 and 30) yielding a set of values for *in-silico* "on" rate for *trans*-interaction (Table 1). Further, the reduction in dissociation rate constant as observed after Rac1 activation in the Dendra2 experiments was accompanied by approximately 2-fold decrease in the "off" rate constant for *trans*-interaction (k_a^-) (Table 1). The model based on the above assumptions predicted that Rac1 increases the affinity between *trans*-dimers and stabilizes VE-cadherin adhesion.

To validate experimentally the *in-silico* results, we determined the monomer-junction dissociation rate constant for VE-cadherin-Dendra2 bearing a substitution of two highly conserved tryptophan (W) residues at positions 2 and 4, the swapping elements required for *trans*-interaction [38], to alanine (*A*). VE-cadherin W2A/W4A mutant displayed an approximately 3-fold increase in the dissociation rate as compared to the wild type (wt) VE-cadherin (Figure 33 A-C). These data showed an inverse relationship between assembly of VE-cadherin adhesive bonds and dissociation rate, and they fully supported the *in-silico* simulation above. Importantly, transient activation of PA-Rac1 did not restore the dynamics of the VE-cadherin mutant (Figure 33 C), indicating the essential role of *trans*-dimerization in mediating Rac1-induced stabilization of VE-cadherin adhesions.

As a complimentary approach, we used an inhibitory single peptide, (SP; RVDAE) that was reported to target VE-cadherin adhesive interface and prevents formation of new *trans* interaction [149]. To validate published data, we used a recently available crystal structure of chicken VE-cadherin and threaded human VE-cadherin sequence into chicken crystal structure.
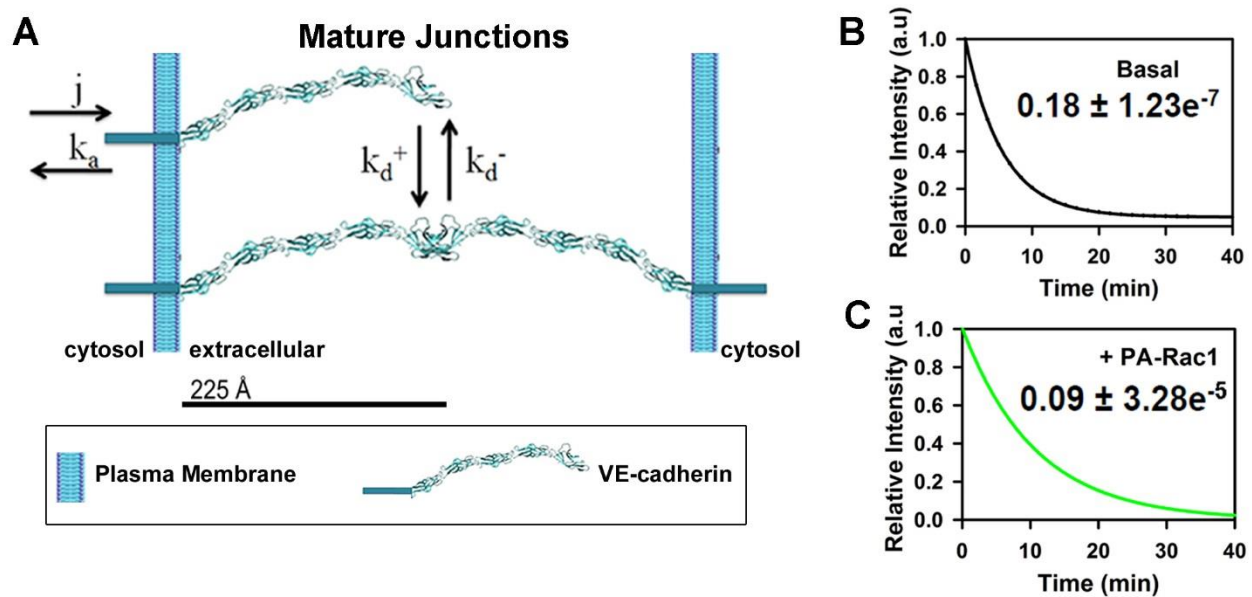
Figure 32: Relationship between VE-cadherin kinetics and stability of adhesive bonds. A. Mathematical model describing VE-cadherin monomer-junction association and dissociation rate constants (j and k_a), followed by reversible *trans* dimerization characterized by k_d^+ and k_d^-. **B-C**. COPASI-fitted experimental data from Fig. 2D and F for basal **(B)** and after PA-Rac1 photo-activation **(C);** dissociation rate constants in min^{-1} ± SEM.

	k_d^+ $(mM^{-1}.s^{-1})$	k_d^- (s^{-1})	K_d (k_d^-/k_d^+) (mM)
Basal	19.15	1.96	0.1
+PA-Rac1	23.97	0.87	0.04

Table 1: COPASI-estimated values for VE-cadherin dimerization. Estimated values for k_d^+, k_d^-, and affinity of *trans* interaction (K_d) described in Figure 33.
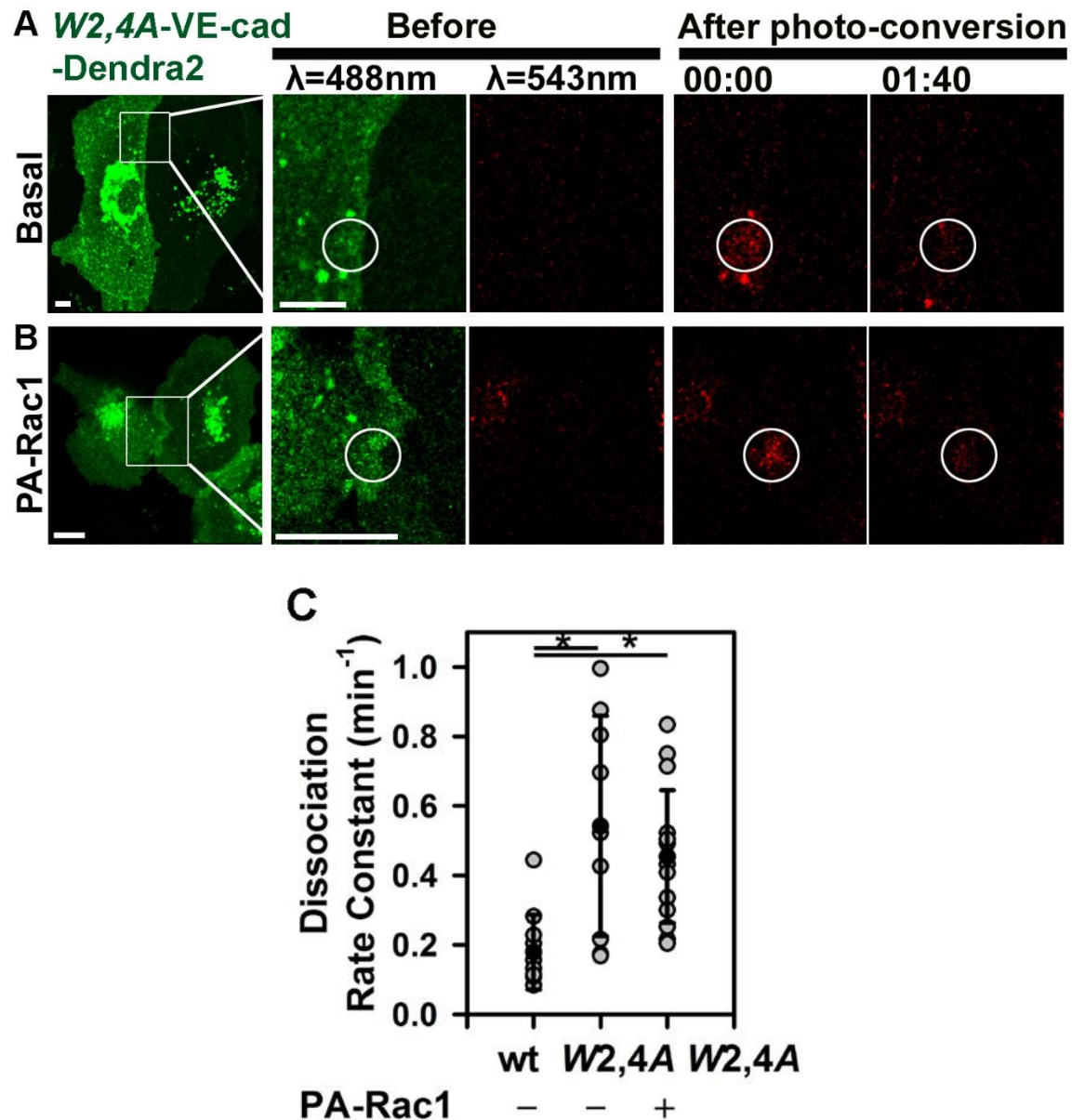
Figure 33: VE-cadherin *trans* interaction is required for Rac1-mediated stability of the adhesion. A-B. Time lapse images of VE-cadherin-Dendra2 *W2,4A* before and after photo-conversion for basal **(A)** and PA-Rac1 activation **(B)**. Scale bar, 10 μm. **C.** Dissociation rate constant for wild type (wt) (0.18 ± 0.11 min^{-1}), *W2,4A* mutant without (0.54 ± 0.32 min^{-1}) and with Rac1 photo-activation (0.45 ± 0.19 min^{-1}); mean$\pm$SD. n=9-15; $p<0.05$.

We showed that the strand swap is in the same conformation (Figure 34 A). Using PepSite 2.0 modeling approach we predicted peptide RVDAE binding site. The model prediction suggested tight binding of RVDAE to the monomer, binding directly to Trp 2/Trp 4 (Figure 34 B), thus suggesting that the peptide prevents the formation of new *trans* dimers.

We tested the effect of SP treatment (i.e. blocking formation of *trans* interaction) on VE-cadherin accumulation. We observed that pre-treatment of endothelial cells with 200μM SP decreased junction integrity as measured by the reduction in endothelial electrical resistance (Figure 35A). Thus, we used this concentration of SP to determine its effect on VE-cadherin density. We observed that pretreatment of endothelial cells with SP hindered Rac1-induced increase in VE-cadherin accumulation at AJs (Figure 35 B-C).

In conclusion, our *in-silico* modeling along with our experimental approaches suggest that Rac1 stabilizes VE-cadherin at the mature AJs by increasing the affinity for *trans*-dimerization to reduce its dissociation from the junction and importantly the *trans*-dimerization is indispensable for Rac1-mediated effect.

A
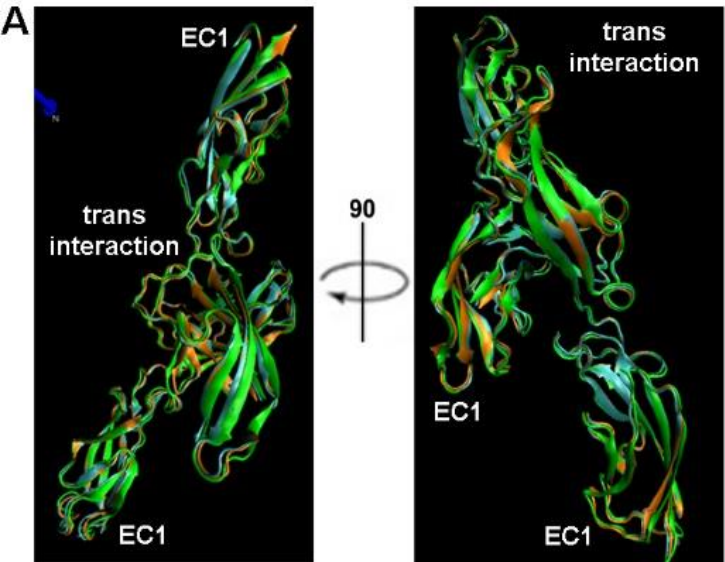
Chicken VE-cadherin crystal – CYAN
Human VE-cadherin-Preliminary Model – ORANGE
Human VE-cadherin – Energy Mimimized - GREEN

B
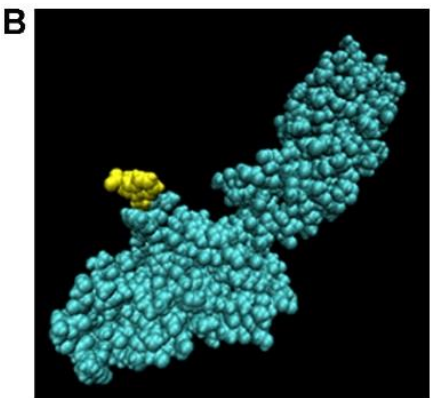
rank	p-value	N					
1	0.1049	4	arg-1	val-2	asp-3	ala-4	-----
2	0.107	4	arg-1	val-2	asp-3	ala-4	-----
3	0.1143	4	arg-1	val-2	asp-3	ala-4	-----
4	0.1163	4	arg-1	val-2	asp-3	ala-4	-----
5	0.1293	4	arg-1	val-2	asp-3	ala-4	-----
6	0.1485	4	arg-1	val-2	asp-3	ala-4	-----
7	0.1656	4	arg-1	-----	asp-3	ala-4	glu-5
8	0.1741	4	arg-1	val-2	asp-3	ala-4	-----
9	0.1847	4	arg-1	val-2	asp-3	ala-4	-----
10	0.1847	4	arg-1	val-2	asp-3	ala-4	-----

Each row from the table above is colored according to statistical significance (p-value) following the color scale below (red = highly significant; yellow = moderately significant; white = not significant).

0.00   0.25

Figure 34: Model for SP interaction. A. Threaded human VE-cadherin sequence into chicken VE-cadherin crystal structure, showing that the strand swap is in the same conformation. **B,** In human VE-cadherin, sequence 'RVDAE' (yellow) binds tightly to Trp/Trp loop.
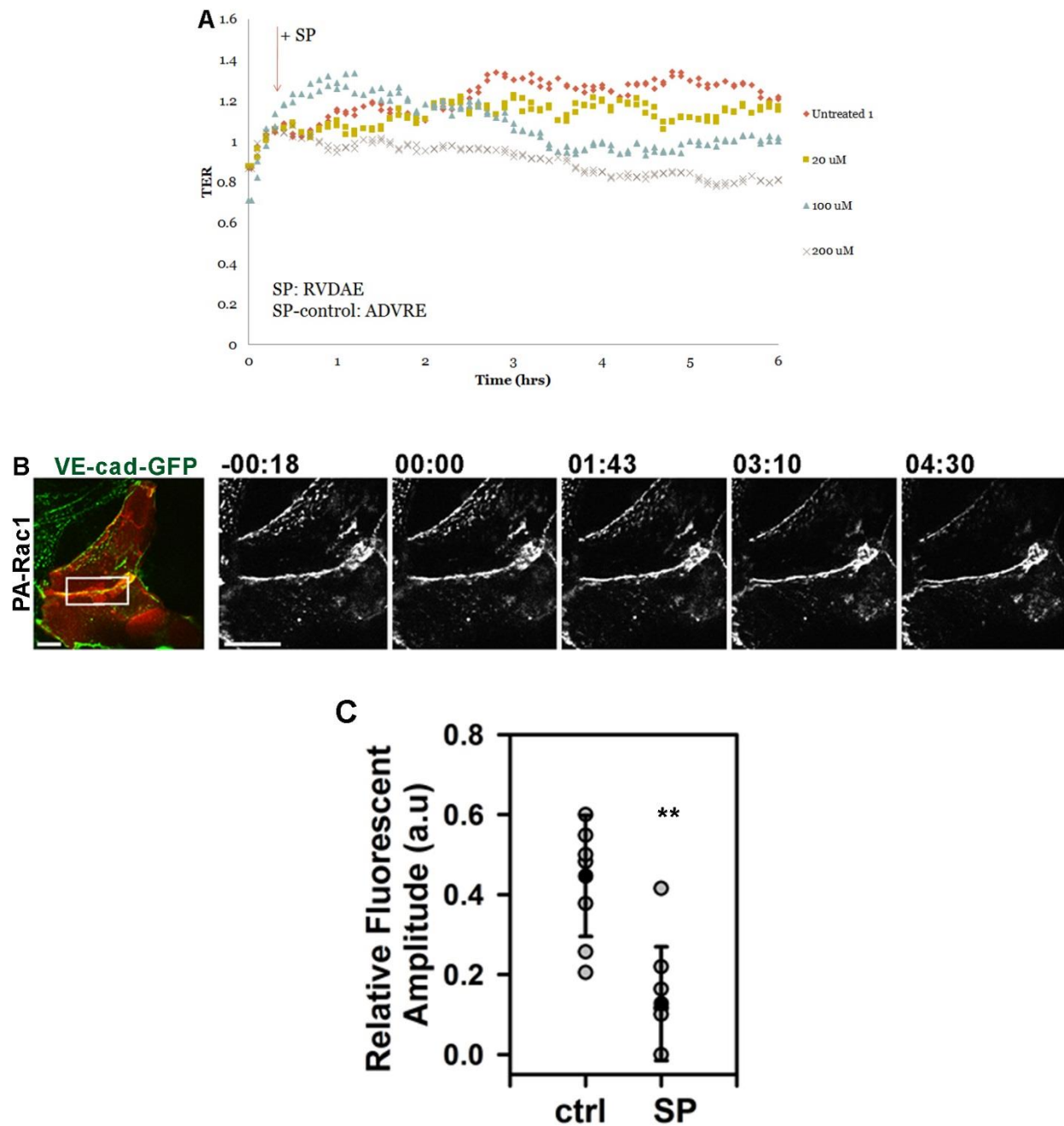
Figure 35: Inhibition of VE-cadherin *trans* interaction formation prevents Rac1-induced effect. A. Real-time TER measurement in ~90% confluent cells treated with different concentration of SP; mean values over time are shown; n =5. **B.** Time-lapse images of VE-cadherin-GFP (green) in cells expressing mCherry (red) tagged PA-Rac1 and incubated with 200 μM of SP for 3 hours before and after photo-activation inside the yellow rectangular region at time 00:00. Time in minutes and seconds is shown. Scale bar, 10μm. **C.** Amplitude of VE-cadherin-GFP accumulation after photo-activation of SP control (0.45±0.15) or SP (0.13±0.14) peptides; mean±SD. n= 8; *p*<0.005.

4. RAC1 AS A REVERSIBLE TENSION MODULATOR

*Portions of the text and figures 36-38, 40-44 were reproduced with permission from Daneshjou N, Sieracki N, van Nieuw Amerongen GP, Schwartz MA, Komarova YM, Malik AB. (2014) Rac1 functions as a reversible tension modulator to stabilize VE-cadherin *trans* interaction. The Journal of Cell Biology. Accepted.

4.1. <u>Rac1 Modulates Actomyosin Tension Across VE-cadherin Adhesion</u>

We next investigated the mechanism of the Rac1 molecular "switch" responsible for stabilizing VE-cadherin *trans*-interaction. In the first section of the thesis we showed that Rac1-mediated stability of VE-cadherin adhesion was independent of the known function of Rac1 on lamellipodia protrusion. Based on studies demonstrating that activation of Rac1 occurs at the sites of AJs experiencing the highest tension in response to fluid shear stress [137, 142], we surmised that Rac1 could potentially function by opposing this tension across VE-cadherin adhesion through counterbalancing RhoA activity (Figure 36). To test these concepts, we first used a FRET-based biosensor to measure RhoA activity at AJs [11]. Consistent with the known function of Rac1 in counterbalancing RhoA activity [74], we observed that activation of PA-Rac1, but not PI-Rac1, at AJs induced spatial reduction of RhoA activity (Figure 37).

RhoA is a known mediator of actomyosin contractility through interaction with its downstream kinase ROCK to maintain MLCII phosphorylation state [96]. To test whether the Rac1-induced stability of VE-cadherin adhesion is mediated by antagonizing RhoA-mediated actomyosin contractility (Figure 37), we investigated the effect of PA-Rac1 activation on MLCII phosphorylation. Consistent with our RhoA activity results, exposure of endothelial cells
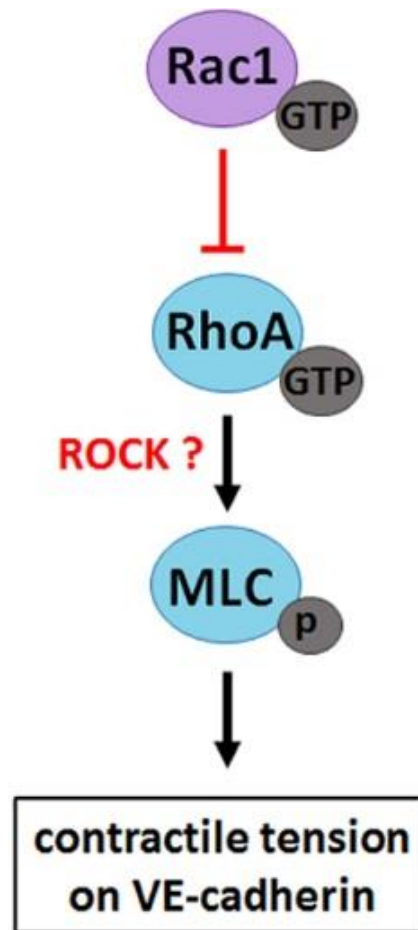
Figure 36: Working Model for Rac1-mediated signaling at AJs. Model of Rac1-mediated inhibition of RhoA/ROCK-signaling and mechanical force across VE-cadherin adhesion.
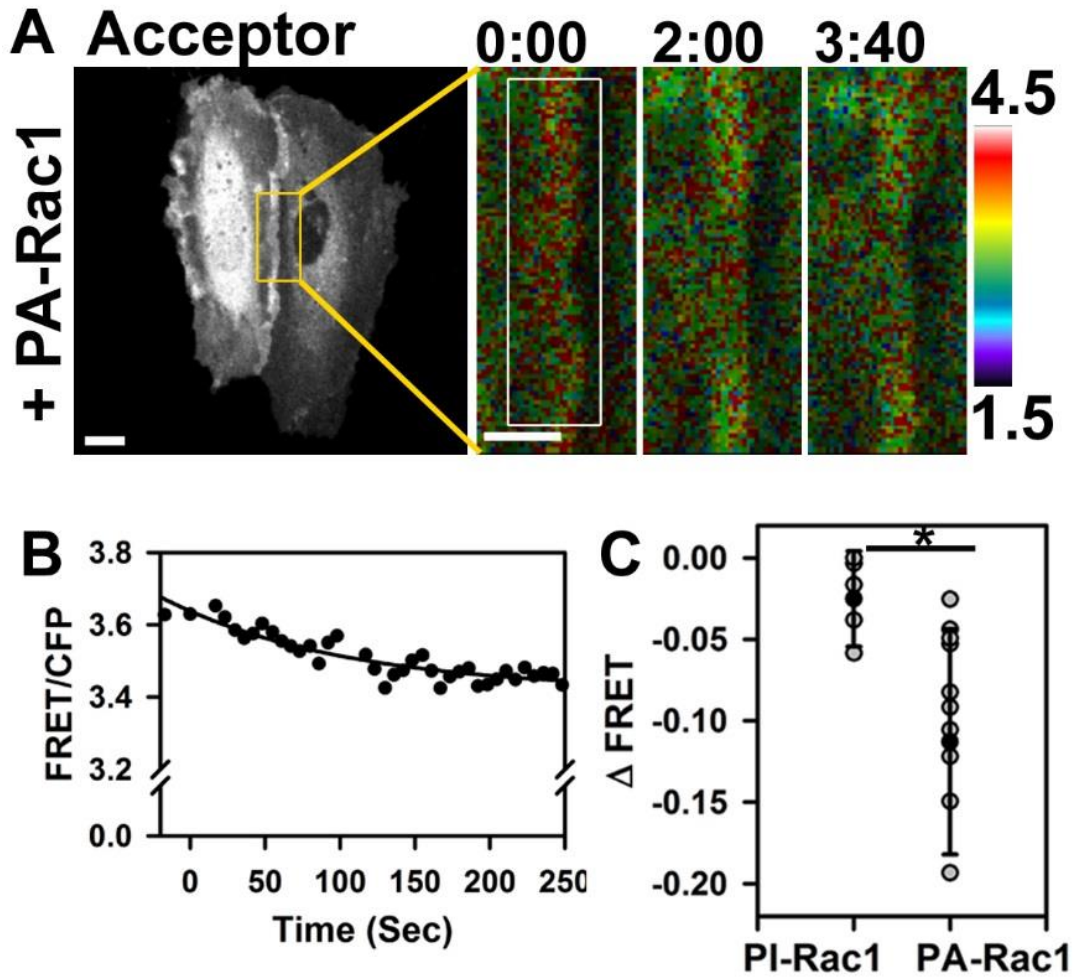
Figure 37: Rac1 counterbalances RhoA activity. A-B. Cells expressing RhoA biosensor and PA-Rac1. Changes in FRET/CFP ratio after PA-Rac1 photo-activation. Ratio images were scaled from 1.5 to 4.5 and color-coded as indicated on the right. Scale bar, 10µm, and 5µm for inserts. **C.** Changes in RhoA activity after photo-activation of PI-Rac1 (-0.03±0.03) or PA-Rac1 (-0.11±0.07); mean±SD. n=5-10; *p*<0.05.

expressing PA-Rac1 to white light resulted in significant reduction of MLCII phosphorylation as compared to non-transfected cells or cells that were kept in dark (Figure 38).

Previous study by Sanders et al., showed that the constitutively active Pak1, a downstream effector of Rac1, reduced MLCK activity and subsequently the phosphorylation level of MLC [159]. Consistently, the constitutively active Rac1 also resulted in the decrease in MLC phosphorylation, which they proposed could partially be reversed by co-expression of Pak1 auto-inhibitory domain [159], suggesting that Rac1 may control MLCK activity. MLCK is a Ca^{2+}/CaM regulated kinase downstream of RhoA, responsible for MLC phosphorylation on serine 19 and actomyosin contractility. There are two MLCK isoforms; a ~210KD long MLCK, primarily expressed in the non-muscle cells and a ~108-125 KD short MLCK, expressed in both smooth and non-muscle cells [160]. To this end, we used a MLCK FRET biosensor [7] to measure the level of MLCK activity in response to PA-Rac1 photo-activation. Similar to previous findings, we observed a significant reduction in the level of MLCK activity after photo-activation of PA-Rac1 but not PI-Rac1 (Figure 39). Taken together, these findings suggested that Rac1 might regulate VE-cadherin dynamics or stability of VE-cadherin adhesion at AJs by counterbalancing RhoA downstream signaling and could potentially modulate RhoA-dependent tugging forces across VE-cadherin adhesion.

To directly measure actomyosin-mediated tension across VE-cadherin adhesion, we next used a FRET-based tension biosensor for VE-cadherin (VE-t) [9]. Endothelial monolayer basally showed low FRET/CFP ratio at the cell-cell adhesion (Figure 40, Insert 1) indicative of constitutive intracellular forces across VE-cadherin adhesion in the confluent endothelium. At the AJ zone subjected to Rac1 activation, we observed that basal tension of ~ 2.4 nN/molecule [9] was reduced to the level of the tailless control (Δtail), a probe that experiences no tension [9]
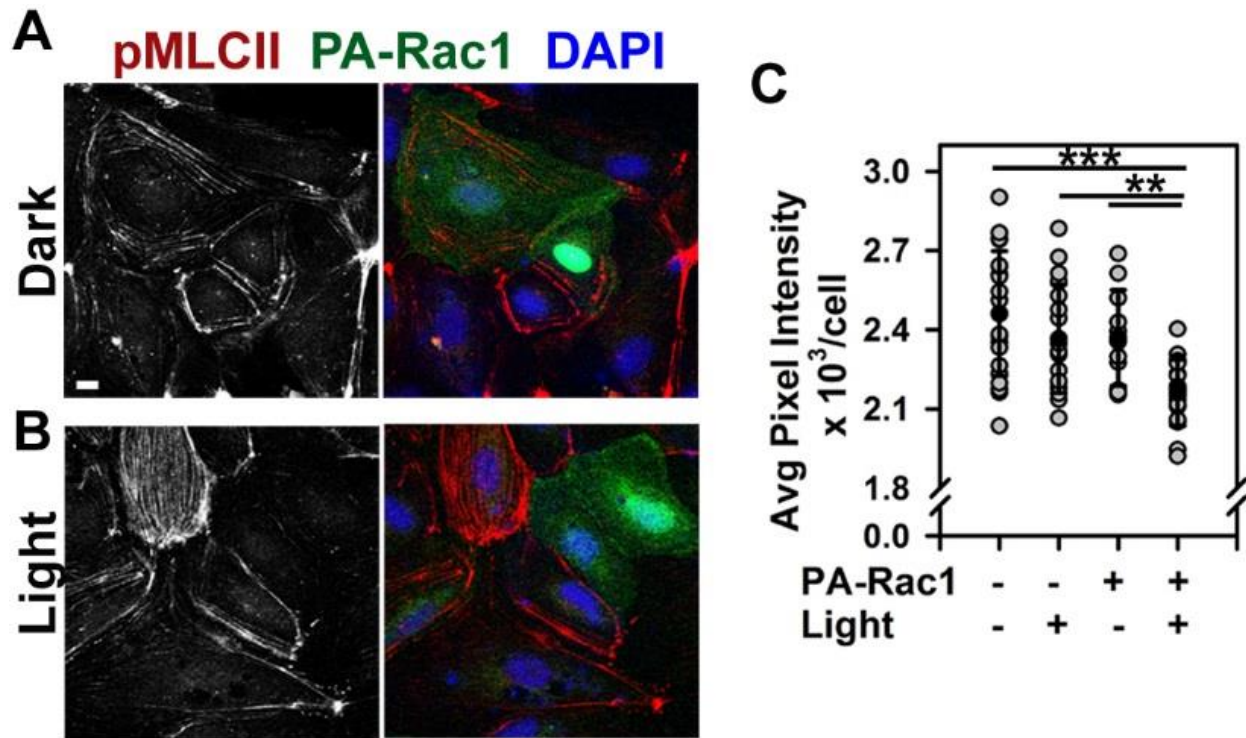
Figure 38: PA-Rac1 photo-activation reduces MLCII activity. A-B. Immunofluorescence staining for myosin light chain phsophorylation (pMLCII) (red) without (dark; **A**) and with (light; **B**) photo-activation of Rac1. Scale bar, 10μm. **C**. Quantification of p-MLCII in E-F; mean±SD, n=11-21; **, $p<0.005$ and ***, $p<0.0005$.
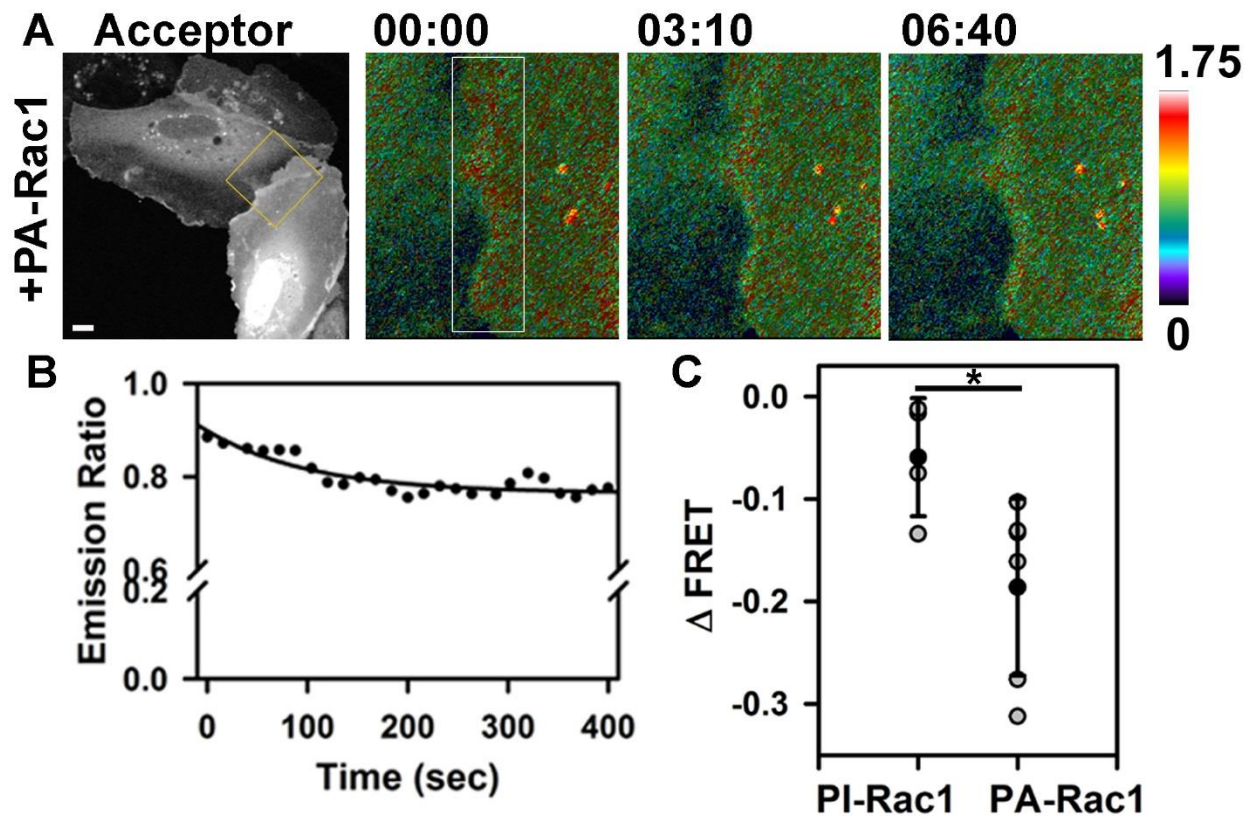
Figure 39: PA-Rac1 photo-activation reduces MLCK activity. A-B. Cells expressing MLCK biosensor and PA-Rac1. Changes in CFP/FRET ratio after PA-Rac1 photo-activation. Ratio images were scaled from 0 to 1.75 and color-coded as indicated on the right. Scale bar, 10μm. **C.** Changes in MLCK activity after photo-activation of PI-Rac1 (-0.06±0.06) or PA-Rac1 (-0.19±0.09); mean±SD. n=4-6; *p*<0.05.
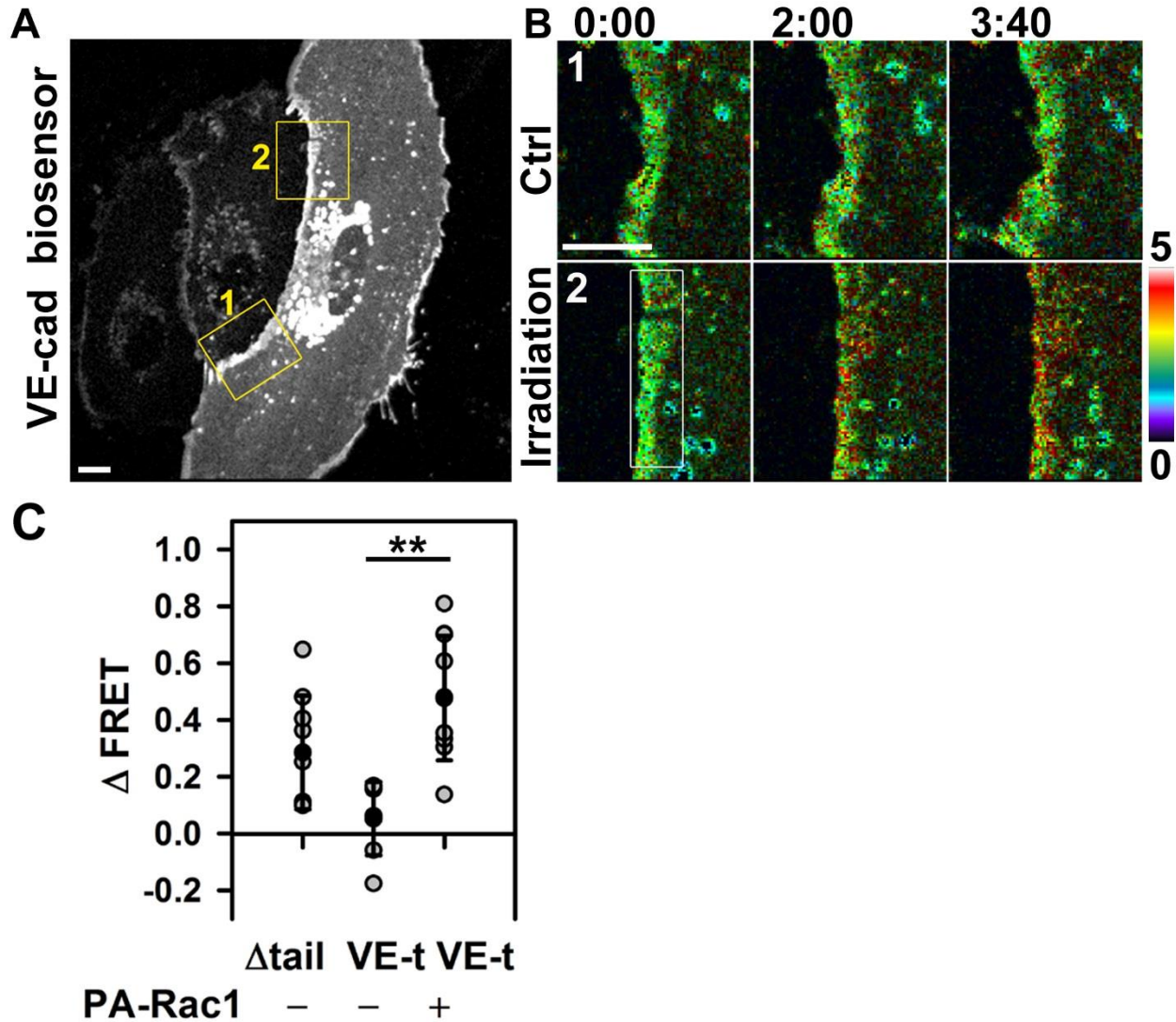
Figure 40: Rac1 modulates actomyosin tension across VE-cadherin adhesion. A. Cells expressing VE-cadherin tension biosensor (VE-t) and PA-Rac1. Control (ctrl; 1) and PA-Rac1 photo-activation (irradiation; 2) zones are shown enlarged in **B**. **B.** Spatial increase in FRET/CFP ratio was observed at the site of PA-Rac1 photo-activation inside the rectangular region (2) but not within the adjacent zone (1). Ratio images were scaled from 0 to 5. Scale bar, 10µm. **C.** Changes in FRET ratio for the tailless (tail) control and VE-t without or with PA-Rac1 activation; mean±SD. n= 7-10; $p<0.005$.

(Figure 40, insert 2). Thus, transient activation of PA-Rac1 at AJs caused spatial relaxation and reduced in tension across VE-cadherin adhesions similar to the effect seen on applying fluid shear stress to confluent endothelial monolayers [9]. Thus, at AJs Rac1 functioned by counteracting RhoA activated signaling to reduce the tension across VE-cadherin adhesion and stabilize VE-cadherin *trans*-interaction.

4.2. <u>Inhibition of Actomyosin Tension at AJs Stabilizes VE-cadherin Adhesion</u>

Rac1 is known to play a critical role in the mechanism of tugging-force-induced AJ growth [73], although it remains unclear whether Rac1 cooperates with mechanical forces or opposes the effects of these forces on VE-cadherin adhesion. Therefore, we further tested the assumption of the model that transient inhibition of RhoA signaling downstream of Rac1 activation and the subsequent reduction in tension at AJs should itself be sufficient to stabilize VE-cadherin *trans*-interaction. ROCK is a primary downstream effector of RhoA that inhibits MLCP in phosphorylation-dependent manner and phosphorylates MLCII leading to actomyosin contractility [161]. To establish causal relationship between RhoA/ROCK signaling and stability of VE-cadherin adhesion, we locally photo released the caged ROCK inhibitor, Rockout (cRO) [146] synthesized as described in Materials and Methods. Similar to our data with photo-activation of PA-Rac1, Photo-release of the cRO at AJs significantly decreased the tension across VE-cadherin adhesion (Figure 41) and VE-cadherin dissociation rate from AJs (Figure 42; +cRO-UV). cRO release thus also promoted VE-cadherin accumulation at AJs within the irradiation zone (Figure 43). Importantly, direct inhibition of MLCII ATPase activity with small molecule inhibitor blebbistatin (Blb) [162, 163] similarly reduced the VE-cadherin dissociation rate (Figure 42; +Blb).
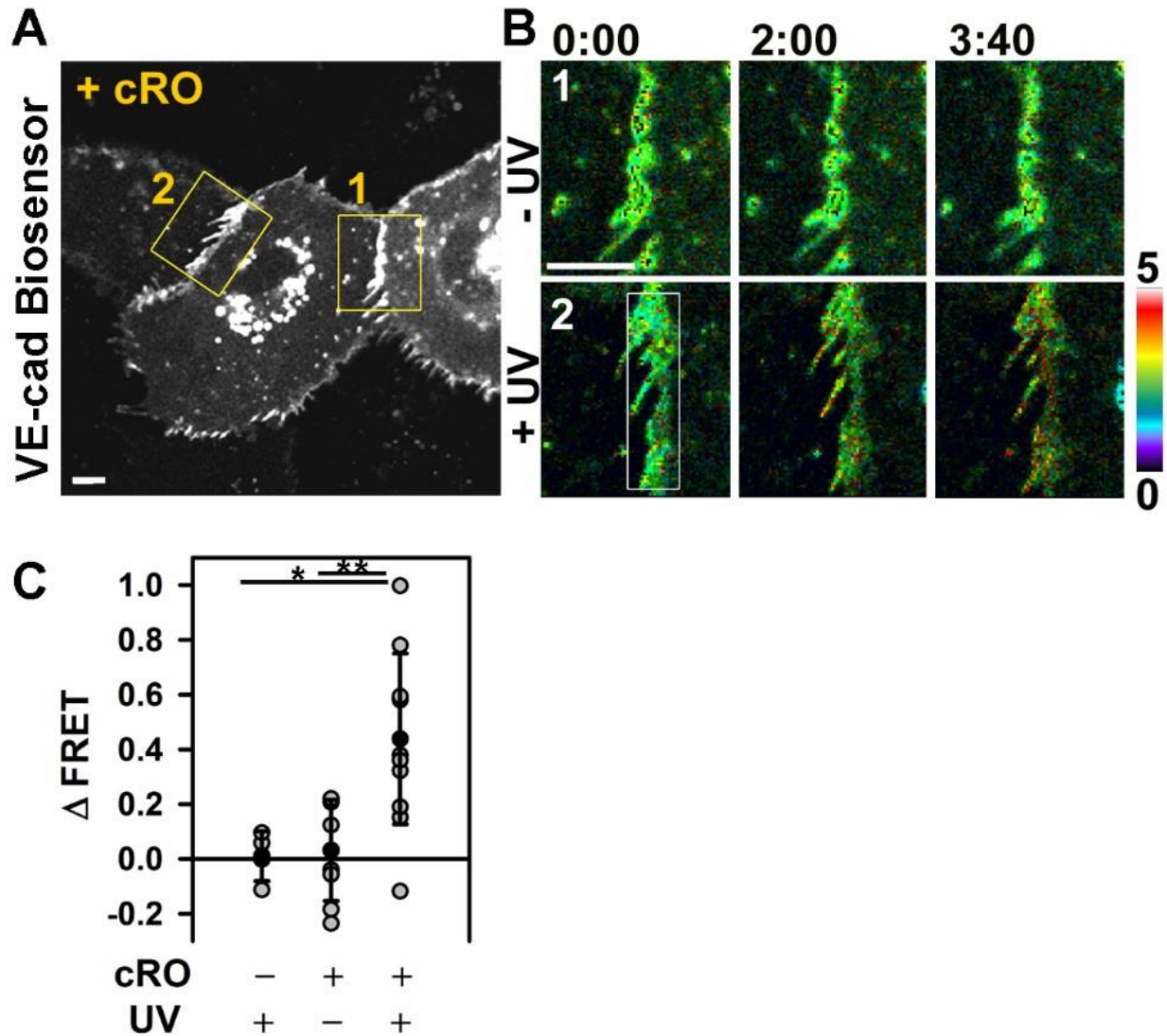
Figure 41: Spatial inhibition of ROCK reduces actomyosin tension across VE-cadherin adhesion. A. Cells expressing VE-t and treated with cRO. FRE/CFP ratio was determined within the control (-UV; 1) and cRO photo-uncaging (+UV; 2) zones; shown enlarged in **B**. **B.** Spatial increase in FRET/CFP ratio after cRO photo-uncaging within irradiation zone (2) and not inside adjacent zone (1). Ratio images were scaled from 0 to 5. Scale bar, 10μm. **C.** Changes in FRET ratio for VE-t with and without cRO photo-uncaging; mean±SD. n= 8-11; $p<0.05$ and $p<0.005$.
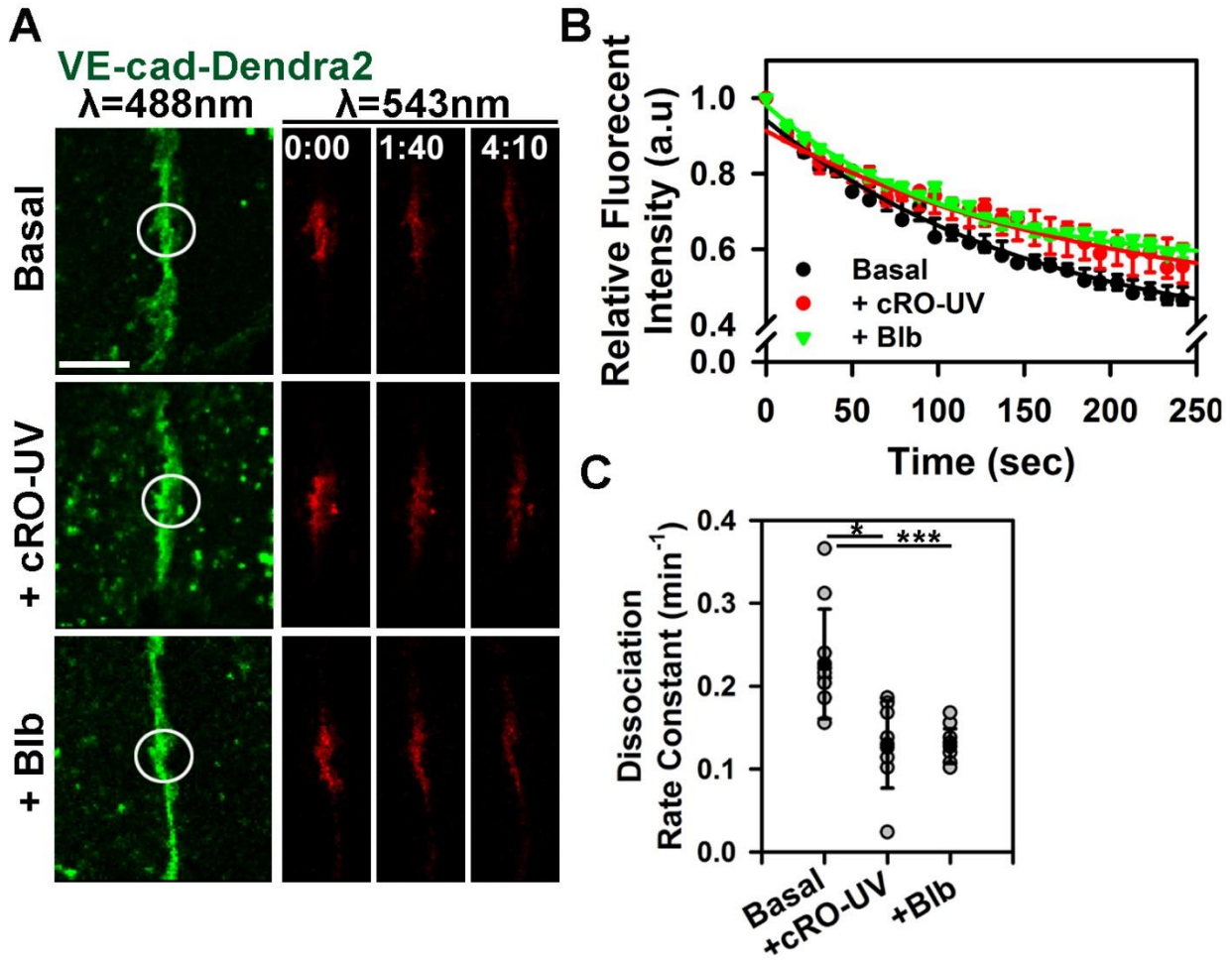
Figure 42: Inhibition of actomyosin tension at AJs reduces the rate of VE-cadherin dissociation. A. Time-lapse images of VE-cadherin-Dendra2 before and after photo-conversion in cells untreated (top panel; basal), or treated with cRO (middle panel) following photo-uncaging or blebbistatin (bottom panel; +Blb). Scale bar, 10 μm. **B**. VE-cadherin dissociation rates; mean±SEM. n= 6-12. **C**. Rate constant of VE-cadherin dissociation calculated from **B** for basal ($0.23 \pm 0.07 min^{-1}$), cRO photo-uncaging (0.13 ± 0.05 min^{-1}), and blebbistatin (0.13 ± 0.02 min^{-1}); mean±SD. n=8-11 cells; *, $p < 0.05$ and ***, $p < 0.0005$.
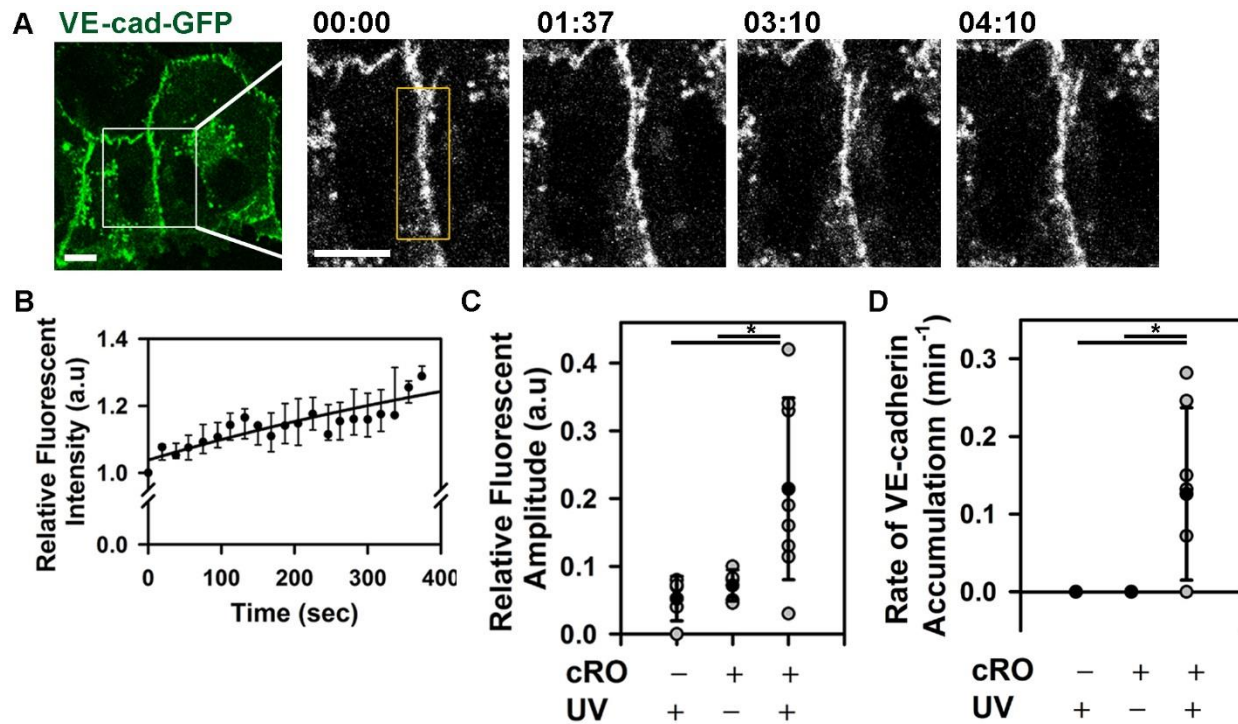
Figure 43: Spatial inhibition of ROCK at AJs increases VE-cadherin density. A. Time-lapse images of VE-cadherin-GFP before and after cRO photo-uncaging within the rectangular region at time 00:00. Scale bar, 10 μm. **B**. Relative changes in VE-cadherin-GFP fluorescent intensity at AJs after cRO photo-uncaging; mean±SEM. n=8. **C**. Amplitude of VE-cadherin-GFP accumulation at AJs in cells irradiated with 405nm (0.05±0.03) or treated with cRO without (0.07±0.02) and with photo-uncaging (0.21±0.13); mean±SD. n=5-8; $p<0.05$. **D**. Rate constant for VE-cadherin-GFP accumulation after cRO photo-uncaging of was 0.13±0.11 min^{-1} whereas no significant changes were observed in cells irradiated with 405nm laser beam or cells treated with cRO but without photo-uncaging; n=5-7; $p<0.05$.

Together, out data demonstrated that localized and transient Rac1 activation at AJs counteracted the RhoA/ROCK pathway to reduce the actomyosin-mediated tension across VE-cadherin adhesion that thereby stabilized VE-cadherin adhesive bonds and reduced VE-cadherin dissociation from the junction (Figure 44).
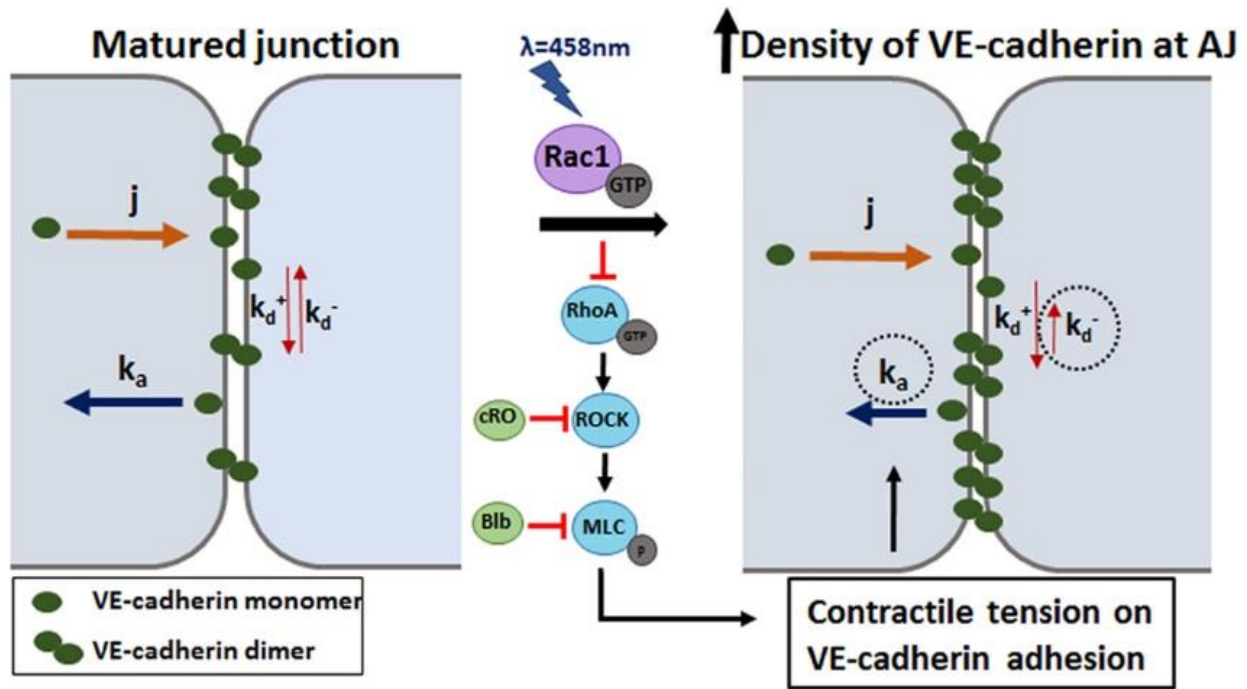
Figure 44: Working model. Working model of Rac1-mediated stabilization of VE-cadherin adhesion. Rac1 functions by inhibiting RhoA/ROCK mediated signaling and relieving actomyosin tension across the adhesion, leading to increased affinity for VE-cadherin *trans*-interaction and decreased VE-cadherin dissociation from AJs.

5. THE INTERPLAY BETWEEN RAC1 AND IQGAP1

5.1. IQGAP1 may be Required for Rac1-induced Signaling at AJs

The scaffold protein IQGAP1 binds to active Rac1 and Cdc42 and prolong their activity [125, 126]. Furthermore, IQGAP1 has shown to be necessary for VE-cadherin-mediated adhesion [164] and down regulation of Rac1 by siRNA prevents translocation of IQGAP1 to the membrane [165], suggesting that IQGAP1 may act downstream of Rac1. To address this possibility, we first determined the effect of localized Rac1 activation on IQGAP1 accumulation at the AJs. Our findings suggested that photo-activation of PA-Rac1 promotes junctional accumulation of IQGAP1 at the site of photo-activation and with similar rate as that observed for VE-cadherin accumulation (Figure 45), suggesting that the increased VE-cadherin density at AJs and IQGAP1 accumulation in response to Rac1 activation may occur in concert.

To further investigate the role of IAGQP1 on Rac1-induced effect, we depleted IQGAP1 using siRNA treatment, which decrease IQGAP1 level by ~70% (Figure 46). We observed that depletion of IQGAP1 prevented Rac1-mediated signaling as observed by lack of PA-Rac1 ability in sub-confluent monolayer of cells to induce gap sealing after photo-activation (Figure 47) as well as the failure of PA-Rac1 photo-activation to induce VE-cadherin accumulation at the junction (Figure 48). While much more investigation needs to be done, these data suggest that IQGAP1 may play an important role in mediating Rac1-induced VE-cadherin stability at AJs and is one of the focuses for future direction (See Future Directions).
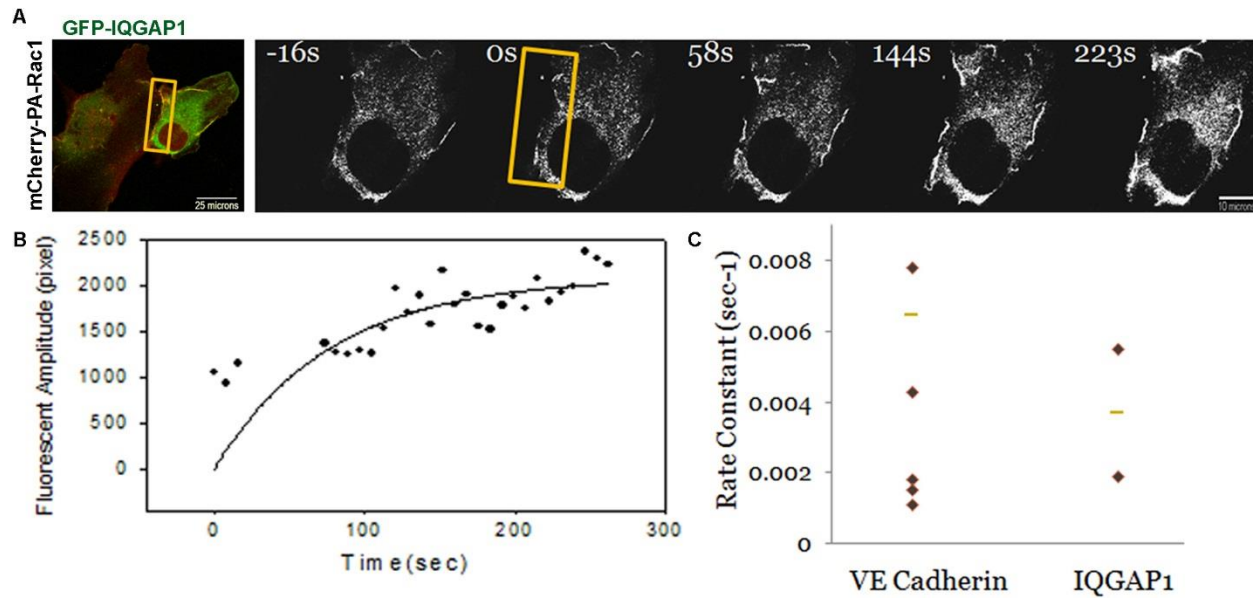
Figure 45: PA-Rac1 photo-activation promotes IQGAP1 accumulation at AJs. A. Time-lapse images of GFP-IQGAP1 before and after PA-Rac1 photo-activation within the rectangular region at time 0s. Time in seconds shown. Scale bar, 25 µm and 10 µm. **B**. Relative changes in VE-cadherin-GFP fluorescent intensity at AJs after PA-Rac1 photo-activation. **C**. Amplitude of IQGAP1 and VE-cadherin accumulation at AJs after PA-Rac1 photo-activation. n=2-6.

Figure 46: Depletion of IQGAP1 using siRNA. Cells treated with IQGAP1siRNA for 72 hours. 200nM siRNA reduced IQGAP1 expression by ~70%.
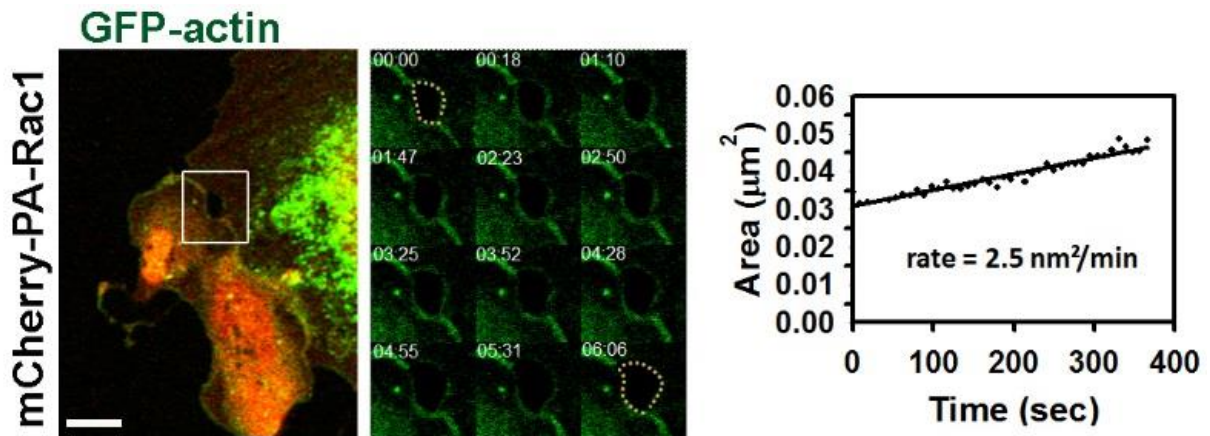
Figure 47: Depletion of IQGAP1 blocks Rac1-induced gap sealing. Cells expressing GFP actin and mCherry-PA-Rac1. Time-lapse images of GFP-actin inside the rectangular region after PA-Rac1 photo-activation at time 00:00. Time in seconds and minutes shown. Scale bar, 10 μm. Rate of gap sealing is measured after PA-Rac1 photo-activation. Depletion of IQGAP1 not only prevents gap sealing, but also slightly increases the size of the gap.
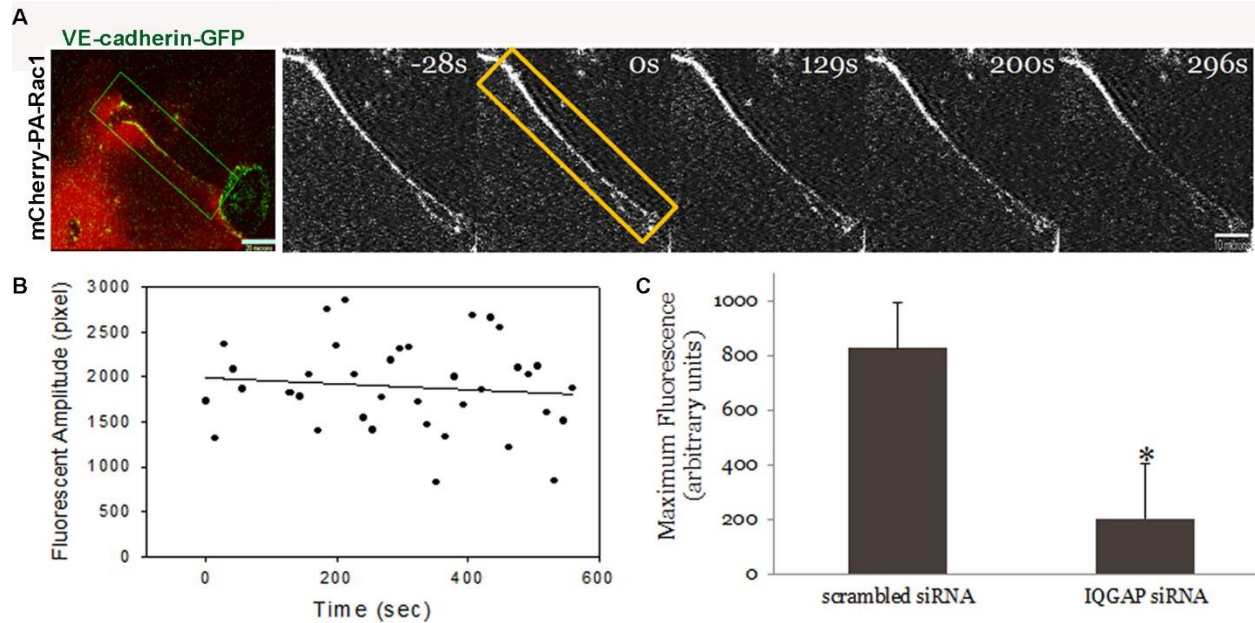
Figure 48: Depletion of IQGAP1 blocks Rac1-induced VE-cadherin accumulation at AJs.
A. Time-lapse images of VE-cadherin-GFP before and after PA-Rac1 photo-activation within the rectangular region at time 0s. Time in seconds shown. Scale bar, 20 μm and 10 μm. **B.** Relative changes in VE-cadherin-GFP fluorescent intensity at AJs after PA-Rac1 photo-activation. **C.** Amplitude of VE-cadherin accumulation at AJs after PA-Rac1 photo-activation in cells treated with scrambled or IQGAP1 siRNA. mean±SD. n=6; $p<0.05$.

6. CONCLUSIONS AND DISCUSSION

*Portions of the text were reproduced with permission from Daneshjou N, Sieracki N, van Nieuw Amerongen GP, Schwartz MA, Komarova YM, Malik AB. (2014) Rac1 functions as a reversible tension modulator to stabilize VE-cadherin *trans* interaction. The Journal of Cell Biology. Accepted.

VE-cadherin-mediated adhesion is dynamic in nature. *In-vivo* studies suggest that many regulatory pathways as well as the microenvironment (e.g. vascular bed and shear flow) dictate the dynamic state of VE-cadherin [70, 166], which is critical for regulating barrier function under physiological and pathophysiological condition. Even in confluent monolayer of cultured endothelial cells, as described in this thesis, VE-cadherin exhibits a dynamic behavior that dictates the junction morphology. Both the concentration of cadherin at the junction (i.e. density) and the state of the junction maturation, which subsequently affects cadherin distribution, dictate the dynamic behavior of the junction [63, 167].

Endothelial cells adapt to environmental cues via mechano-sensors and mechano-transducers that translate the extracellular mechanical stimuli and initiate intracellular signaling cascades to induce reorganization of actin cytoskeleton [69]. This adaptive behavior is critical during stages of vascular development and angiogenesis [168, 169] as well as during immune response where an increase in endothelial permeability, mediated by actomyosin contractile machinery, is required for leukocyte transmigration to the site of inflammation [50]. Disturbance in the response to these stimuli can have detrimental outcomes and lead to vascular diseases such as atherosclerosis [170].

The junctional proteins and the actin cytoskeleton are the two main elements that regulate barrier integrity. Thus, understanding the crosstalk between the two is the critical first step towards

future therapeutic development. Importantly, organization of the F-actin affects the state of cell-cell adhesion [167, 171]. In the endothelium, three major state of F-actin is observed. These include the cortical actin, which constitutes the thin and thick actin bundles distributed along the cell periphery and are associated with stable junctions [172], the non-contractile filopodia-like filaments [173], which are developed from the pre-existing lamellipodia during the initiation of cell-cell contact or in response to pro-inflammatory mediators and are matured into the contractile stress fibers that promote formation of discontinuous junctions [172].

At the nascent cell-cell contact, Rac1-mediated and Arp2/3-driven lamellipodia protrusion promotes the initial cell-cell contact as well as closure of inter-endothelial gaps after injury [157, 174]. Consistent with the known function of Rac1 in inducing lamellipodia protrusion, our initial studies also showed lamellipodia ruffling in response to Rac1 activation. Interestingly, localized and transient activation of Rac1 was sufficient to induce gap sealing under steady-state in sub-confluent monolayer of endothelial cells at the site of photo-activation. This is consistent with previous study on wound healing in response to leukocyte trans-migration that showed generation of Rac1-mediate ventral lamellipodia to initiate gap closure [174].

As junctions mature, VE-cadherin density increases, leading to linearization of cadherin complex and subsequent decrease in junction size and lamellipodia activity [167]. Here, the cortical actin filaments, along the cell periphery replace lamellipodia to stabilize VE-cadherin adhesion [172]. During junction disassembly, myosin II-mediated stress fibers induce contractile force and promote cadherin remodeling [63, 172]. Thus, regulating Myosin II-mediated contractile machinery is critical for stability of the endothelial AJs.

While the increase in the actomyosin contractility and stress fiber formation is associated with discontinuous junctions, some studies linked the increase in tension to the positive regulation of

the junction size [73] or the junctional remodeling in response to thrombin [63]. Along the same line, Bell's model in 1978 proposed that the off rate of a bond is proportional to the applied force [175]. Thus, as the applied force is increased, the off rate should exponentially rises. While at first look these statements seem contradictory, it may well be that the morphology of the junction (nascent, matured, and remodeled) and subsequently the direction of mechanical force applied to the junction (cortical actin filaments along the cell periphery vs. stress fibers perpendicular to the membrane) dictate the outcome response. While many studies have focused on the changes in actin cytoskeleton organization and actomyosin-mediated tension during nascent adhesion or during contact remodeling [63, 74, 172], the underling mechanisms that maintain the stability of the mature VE-cadherin adhesion need to be elucidated. In our studies, we mainly focused on mature cell-cell contact that contains the continuous and linear VE-cadherin and provided a correlative relationship between actomyosin-mediated mechanical tension and stability of VE-cadherin adhesion as mediated by spatiotemporal activity of Rac1.

While Rac1-mediated E-cadherin accumulation has been described previously [176, 177], the effect on VE-cadherin adhesion and its physiological mechanism on barrier integrity is far from confirmatory, since the effect of Rac1 is linked to both barrier protection and barrier destabilization in endothelium. Activation of Rac1 in response to circulating vasoactive VEGF promotes VE-cadherin internalization and junctional destabilization [120], resulting in vascular hyper-permeability (vascular leakage and edema) and is permissive for the formation of new blood vessels (angiogenesis) [139, 140]. TNF-α-induced barrier disruption is also mediated by a Rac1-specific GEF, phosphatidylinositol (3,4,5)-trisphosphate–dependent Rac exchanger 1 (P-Rex1) and Rac1-induced ROS production [141], which leads to VE-cadherin tyrosine phosphorylation, decrease in adhesion, and increase in endothelial permeability [117]. Rac1 and its upstream

effector Tiam1 are also linked to mediating PAF, a biologically active substance, that induces endothelial barrier disruption by bringing changes to actin organization [178].

In contrast, shear stress and barrier protective mediator S1P enhance endothelial barrier through activation of Rac1 [106, 137, 138]. In addition, cadherin-mediated adhesion provides spatial cues necessary for the recruitment of Rac1 at cell-cell contacts via upstream effectors such as PI3K [136], Tiam1 [132] and Vav2 [137]. These conflicting findings indicate multiple possible outcome of Rac1 activation on cadherin mediated cell-cell junction and emphasizes on the importance of controlling and restricting the spatial and temporal activity of Rac1 to prevent its global activation that can induce activation of multiple pathways, which may mask the effect of Rac1.

Meanwhile other studies have suggested that VE-cadherin localization at the endothelial junctions, in contrast to E-cadherin localization, is independent of RhoA/Rac1 activity [179]. This notion is underscored by the less prominent accumulation of Rac1 in endothelial vs. epithelial junctions; in the endothelium Rac1 is hardly detectable in the junctions [105], whereas 80% of epithelial junctions stain positive for Rac1 [157]. Thus, the regulation of endothelial junctions by Rac1 differs remarkably from epithelial junctions and little is known about the molecular basis of Rac1's action at the level of VE-cadherin dynamics.

There are evidence for an antagonistic relationship between Rac1 and RhoA and their localized and cooperative activity during nascent epithelial cell-cell adhesion [74]. Whether in endothelial cells the balance of spatiotemporal Rac1 and RhoA activities determine the contractility and tension applied to the junctions has not been established. Previous study on the spatiotemporal relationship of RhoA activity and junctional disassembly found no evidence of junctional RhoA activation co-localizing with junctional disassembly [135], suggesting that the mechanical forces

can work at distance and the cortical tension does not need to be regulated locally at the particular AJ in respond to altered RhoGTPase activity. Moreover, the study by Liu et al., proposed a cooperative, rather than an antagonistic, model where Rac1 and myosin-mediated tugging force cooperated to determine the ultimate size of the junctions [73]. However, the conclusions on these studies were based on microinjection of active RhoA and global inhibition of ROCK by pharmacological inhibitors that did not allow targeting spatiotemporal distinct activities. These approaches are not sensitive enough to elucidate the spatiotemporal events and can be argued that they likely masked the spatiotemporal changes.

In our studies, we provide the first direct evidence that local and transient Rac1 activation regulate VE-cadherin dynamic and is sufficient for stabilization of VE-cadherin adhesion at AJs. In our proposed model, localized activation of Rac1 at VE-cadherin adhesion sites modulated actomyosin-mediated tension across VE-cadherin adhesion and stabilized VE-cadherin adhesive bonds. Interestingly, Rac1-mediated effect occurred independently of formation of lamellipodia protrusions, a well-known effect of Rac1. Instead, Rac1 antagonized RhoA/ROCK-mediated actomyosin contractile force and the mechano-tension applied to VE-cadherin adhesion sites. This model is based on results showing that spatiotemporal activation of Rac1 decreased VE-cadherin dissociation rate from AJs by enhancing the affinity for VE-cadherin *trans* dimerization and thus increased local VE-cadherin density. The present findings help to explain effects of mediators such as S1P which enhance endothelial AJ integrity through the activation of Rac1 [106, 138].

Previous study by Hong et al., described cadherin dissociation (also called disassembly) as an ATP-dependent mechanism, suggesting that the dissociation kinetics may be due to cadherin endocytosis [17]. However, inactivation of the endocytic pathway by point mutation on the dilucine motif and lysine 738 did not affect the dynamic behavior of the cadherin [17], suggesting

that other mechanism(s) may be involved in the disassembly process. Our preliminary data (not shown in this thesis) using Dynasore, a non-competitive inhibitor of dynamin 1 and 2 [180], the GTPases that mediate VE-cadherin endocytosis by pinching-off the clathrin-coated vesicles, also led to similar observation, emphasizing on the existence of an ATP-independent mechanism by which VE-cadherins dissociate from the junction. In our studies, we ruled out lateral displacement as a possible contributor to VE-cadherin dissociation. We speculate that VE-cadherin diffusion along the cell periphery may be a potential mechanism, however this hypothesis is yet to be validated.

VE-cadherin adhesion is mediated through interaction of the extracellular ectodomains of the adjacent cadherins. Several models have been proposed to describe cadherin homophilic interaction. Initially, studies by Taveau et al., proposed a head-head interaction mediated by *cis* trimers in one cadherin to form a hexamer through *trans* interaction with the neighboring cadherin [36]. On the other hand, Tomschy at al., proposed that two cadherins in the same cell first form a dimmer through lateral *cis* interaction, which is followed by *trans* interaction between cadherins in neighboring cells [181]. However, the VE-cadherin hexamer complex was found to be an artifact of bacterially expressed VE-cadherin that lacked N-glycolysation [38] and it was suggested that VE-cadherin behave similar to other classical cadherins where the two partner EC1 domains are swapped to from *trans* dimerization. Several subsequent studies show that monomeric (His-tagged) cadherins are functional, in direct contrast to the Tomschy's proposal. The postulated *cis*-interactions interpreted based on the electron microscopy data have not been substantiated by any other studies, and differ from the *cis*-interaction model proposed on the basis of more recent crystallographic data. In addition, there is substantial solution binding data indicating that *trans* dimers form in solution [154, 158], but *cis* interactions are not detected (and have only been

suggested through *in-silico* modeling [6, 182])-in direct contradiction to the postulate that *cis* dimers are required for adhesive function.

Our own studies, using the micropipette aspiration technique (data not shown in this thesis), to determine intrinsic 2D binding affinity and off-rate of VE-cadherin *trans*-dimerization during the initial phase of interaction showed a kinetic pattern consistent with type1 classical cadherin ectodomains and defined VE-cadherin *trans*-interaction as the primary adhesion event. Thus, we had good reasons to confine our *in-silico* model to the *trans*-interaction and not include *cis*-interaction aspect in our model. Consistently, kinetic measurements of cadherin-mediated cell adhesion showed that cadherin monomers first form *trans* bonds- the initial binding is not between dimers [33, 183]. Interestingly, E-cadherin bearing a D1A point mutation that stabilized *trans*-swapped dimers also demonstrated increased retention time of the adhesive clusters [32]. In present study, the decrease in VE-cadherin dissociation rate mediated by Rac1 was proportional to the increase in affinity of VE-cadherin *trans*-interaction. Destabilization of this interaction with double W2A/W4A point mutations [38] increased VE-cadherin dissociation rate and mitigated the effect of Rac1 activation on VE-cadherin dynamics. These date together demonstrate the fundamental role of Rac1 activity at AJs in stabilizing VE-cadherin *trans*-interaction.

It is noteworthy to mention that the PA-Rac1DN construct had no net effect on VE-cadherin dynamic. This was interesting to us since over-expression of the dominant negative Rac1 is shown to compete for binding and sequestering upstream GEFs and to subsequently alter endogenous Rac1 activity [184]. Thus we expected that PA-Rac1DN would have a negative effect on VE-cadherin dynamics (i.e. decrease in VE-cadherin accumulation and increase in the dissociation rate), which we did not observe. We reasoned that this might be due to the transient and reversible

nature of the construct; therefore the short half-life of the dominant negative mutant will not provide the sufficient time for competing for GEF binding.

Activation of Rac1 functioned by reducing myosin-dependent tension across VE-cadherin adhesion. The data demonstrated that similar to Rac1 activation, inhibition of downstream RhoA/ROCK signaling increased VE-cadherin density. In newly formed junctions in doublets of cell, inhibition of ROCK reduced E-cadherin concentration at the junction [171], in contrast to our observation with the caged ROCK inhibitor, emphasizing on the morphological differences between newly formed versus matured junctions. Our studies suggest that in mature endothelial contacts, Rac1-mediated counterbalancing of RhoA and reduction of the tension at VE-cadherin junctions plays a critical role in stabilizing *trans*-dimers. Our finding contrasts previous study using RhoA biosensor, where no spatial relationship with enhanced RhoA activity and inter-endothelial gap formation was observed [135].

A study by Ando et al., showed that myosin II promotes two independent pathways resulting in different junctional morphology depending on the associated upstream effector. While Rho/ROCK mediated contractile forces were associated with stress fiber formation and a punctate morphology as seen during junctional remodeling, Cdc42 and its downstream effector myotonic dystrophy kinase–related CDC42-binding kinase (MRCK) dependent pathway resulted in MLCII-dependent cortical bundle formation to stabilize the linear junctions [185]. Furthermore, they showed that Rap1, a member of GTPase family, is the key mediator of junctional stability by spatially controlling the non-muscle MLCII activity through localization of active CDC42 and MRCK while suppressing Rho/ROCK pathway [185]. These data is not only in agreement with our finding in terms of role of Rac1 in regulating actomyosin mediated tension applied to VE-cadherin adhesion but also suggest that activation of Rac1 at AJs may also activate Cdc42 parallel

to or upstream of RhoA signaling to enhance the junctions. Our data describing a possible role of IQGAP1 in Rac1-mediated signaling also points in this direction. Since IQGAP1 interacts with both active Rac1 and Cdc42 and based on the results from our lab suggesting that Cdc42 may act downstream of IQGAP1 (data not published), one can hypothesize that the interplay between Rac1, IQGAP1, and Cdc42 may play an important role in stability of VE-cadherin adhesion.

One question raised from these findings is how Rac1 inhibits RhoA activity. One possible explanation could be through activation of a RhoA-specific GAP, P190RhoGAP. Our lab has previously shown that p190RhoGAP-A inhibits RhoA activity and its GAP activity is controlled by tyrosine nitration at Y^{1105} [186]. In epithelial cells, it appears that P190RhoGAP-B is the isoform responsible for controlling RhoA activity [187], suggesting that there may be cell-type specificity. However, both isoforms require interaction with p120-catenin to inhibit RhoA activity and protect against vascular permeability [59, 187], possibly through SHP2 activity that regulate VE-cadherin and β-catenin phosphorylation state and their interaction with p120-catenin [188]. Our lab has previously shown that the binding affinity between p120 and VE-cadherin is critical for maintaining vascular permeability and is dependent on the phosphorylation state of p120, mediated by PKCα [57]. The lung of mice depleted of PKCα showed reduction in vascular permeability in response to inflammation [57]. Interestingly, it has been suggested that Rac1 and its upstream effectors Tiam1 and Vav2 are required for localization of P190RhoGAP to the junction [129, 189]. Previous studies have highlighted the cross talk between the RhoGTPases and the secondary messengers, such as cAMP and cGMP [103, 107-110, 116]. In response to inflammation, Rac1 may initiate a signaling pathway to block the negative effect of the secondary messenger PKCα on p120-catenin to stabilize the binding affinity between p120-catenin and VE-cadherin. This interaction could then recruit p190RhoGAP to the junction to inhibit RhoA activity.

This mechanism could explain how Rac1 inhibits RhoA mediated signaling to stabilize VE-cadherin adhesion. Thus, it would be interesting to determine the effect of Rac1 activity at the level of cell-cell junction on localization, activation, and tyrosine nitration of P190RhoGAP.

Our studies provide the first kinetic data based on the use of cutting-edge technologies and probes to explain the molecular mechanisms by which Rac1 regulates AJs assembly. Our findings have allowed us to develop the model describing the regulation of endothelial junctions by Rac1 as well as its implications for regulating vascular integrity. Moreover, our data directly indicate that transient decrease in myosin-II activity is sufficient to stabilize VE-cadherin at AJs. Our results based on inhibiting myosin-II activity both downstream of Rac1 activation and directly with blebbistatin best fit the model that the reduction in tension is coupled to reduction of actomyosin force at AJs and is responsible for the VE-cadherin accumulation and increased VE-cadherin binding affinity at AJs. These studies provide strong causal link between Rac1's localized oppression of Rho/ROCK/MLCII pathway and VE-cadherin density at AJs.

Previous study on the importance of the plasticity of cadherin-catenin complex showed that the stronger attachment of catenin to actin cytoskeleton stabilized the complex and reduced VE-cadherin turn-over, with larger immobile fraction [50]. This was concomitant with reduction in leukocyte transendothelial migration [50]. In line with these findings, our data suggest that activation of Rac1 at the junction, through inhibition of RhoA/ROCK signaling, reduces actomyosin tension across VE-cadherin adhesion, thus stabilizes cadherin-catenin attachment to cortical actin.

Leukocytes transmigration requires opening of endothelial cell-cell junction, possibly through disruption of VE-cadherin adhesive bonds [50]. Our data on Rac1-mediated stability of VE-cadherin adhesion could explain how Rac1 activation affects leukocytes transmigration. Rac1 and

RhoA are molecular switches that are transiently activated. One possible mechanism that regulate AJ dynamic during transendothelial migration could be the antagonistic and oscillatory relationship between the two GTPases. RhoA activation at the cell-cell contact could help open the endothelial junctions through increase in actomyosin contractility to provide the route for transmigration, which is in turn followed by Rac1 activation to enhance the affinity of VE-cadherin *trans* interaction and re-seal the junctions.

In conclusion, we have described a novel mechanism by which Rac1 relieves myosin-dependent tension across VE-cadherin adhesion and stabilizes *trans*-interaction. The "tug of war" between Rac1 and RhoA ensures stability of AJs and ensures prompt reversibility of loss of cell-cell adhesion occurring with inflammatory mediators that disrupt the AJ barrier, and thus regulates tissue fluid balance.

7. FUTURE DIRECTIONS

With a global perspective, one remaining question is how activation of Rac1 at cell-cell junction reduces RhoA activity. In another word, to conclusively and directly show that the increase in VE-cadherin accumulation at the AJ is driven by Rac1-mediated inhibition of the RhoA/ROCK pathway. To show this properly it would be interest to knock-down the putative intermediary between Rac1 and RhoA activity. As discussed earlier and based on published data and our own studies, a possible intermediate is p190RhoGAP. Therefore, it would be interesting to deplete the GAP function using siRNA technique and show that the shift in VE-cadherin association with AJs upon photo-activation of Rac1 no longer occur.

Other important question is whether Rac1 and RhoA antagonistically fluctuate. Although micro-fluctuations of RhoA were previously observed in endothelial monolayer [135], a continuous reciprocal on and off signaling of Rac1 and RhoA has not been demonstrated. Due to limitations in our techniques we could not explore this aspect, however, with future advancement in the FRET-based technologies, it would be interesting to perform simultaneous analysis of the RhoA and Rac1 spatiotemporal activities.

Based on our preliminary experiments suggesting that Rac1 regulates barrier integrity in an IQGAP1-dependent manner, it would be interesting to address the potentially important role of IQGAP1 as an essential effector of Rac1 regulating AJs stability. Knowing that Cdc42 is also another major effector of IQGAP1 and since Cdc42 activity is also linked to barrier stability, it is of importance to understand the inter-relationship between Rac1, IQGAP1, and Cdc42. The availability of the photo-activatable Cdc42 probe in our lab would offer exciting avenues to explore and to define the effect of spatiotemporal Cdc42 activity on junctional integrity.

Based on our VE-cadherin kinetics studies we concluded that Rac1 reduces VE-cadherin dissociation, however we did not go into detail as what accounts for the rate of dissociation. Previous study by Hong et al., suggested that cadherin disassembly is an ATP dependent event [17]. Possible mechanisms that account for this active event are cadherin internalization through clathrin-mediated pathway, lateral movement along the junctions, or actomyosin-dependent removal of cadherins from the junction. However, Hong's data showed that depletion of ATP did not completely abrogate cadherin disassembly [17], suggesting that other non-active mechanisms such as passive diffusion along the cell periphery could contribute to this pathway. In our system, we determined that VE-cadherin lateral movement along the junction did not significantly contribute to the dissociation rate. Other possible mechanism is VE-cadherin internalization. However, our preliminary data using dynamin inhibitor along with Hong's study [17] suggest that the internalization mechanism could not alone explain cadherin dissociation, signifying the involvement of other mechanism(s). Thus, it would be of great interest to resolve the underlying mechanism(s) that contribute to the dissociation of VE-cadherin from the AJs.

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ABSTRACTS

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