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DEPARTAMENTO DE BIOMEDICINA MOLECULAR

“Papel de la inhibición del complejo Arp2/3 en la regulación de las funciones de la barrera endotelial”

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Armando Montoya García

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Director de Tesis:

Dr. Michael Schnoor

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CENTRO DE INVESTIGACIÓN Y DE ESTUDIOS AVANZADOS DEL INSTITUTO POLITÉCNICO NACIONAL

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"Rol of Arp2/3 complex inhibition in regulating the endothelial barrier functions"

THESIS

Presented by

Armando Montoya García

To obtain the degree

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DEDICATORIA

Quiero dedicar este trabajo a todas las personas que me alentaron y apoyaron para su realización, en especial mi papá y mi mamá quienes siempre han estado conmigo y gracias a sus consejos, apoyo y palabras de aliento fue posible la realización de este proyecto.

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INDEX

Abstract

The endothelium, a monolayer of endothelial cells, is a semi-permeable barrier that separates blood from underlying tissues. This barrier is mediated by cell-cell adhesion structures including the tight junctions (claudin-1, -5, and -12, occludin and JAMs), adherence junctions (VE-Cadherin), and a variety of other adhesion molecules such as PECAM-1 and nectins, all of which are connected to the actin cytoskeleton via different adaptor molecules. It is well-known that the distribution and expression of junction proteins is fundamental for the regulation of vascular permeability and leukocyte recruitment under basal and inflammatory conditions. To this end the endothelial barrier needs to be flexible; and this flexibility is controlled by the actin cytoskeleton. One element involved in this process is the heptameric Arp2/3 complex, which is responsible for the formation of branched actin filament networks. Arp2/3 action is tightly regulated: it is activated by NPFs such as WAVE, (N)-WASP, WHASH and WHAMMM; and inhibited by proteins such as PICK1, gadkin and arpin. Arpin is the most recently identified Arp2/3 inhibitor that locally antagonizes the activity of WAVE to ensure competitive Arp2/3 regulation in lamellipodia of fibroblasts. However, nothing is known about arpin expression and function in endothelial cells. Additionally, Arp2/3 can be inhibited pharmacologically by CK666, but this compound has also not been well studied in endothelial cells. Thus, in this study, I investigated the effects of arpin-depletion and CK-666 on endothelial barrier regulation. I found that endothelial cells (HUVEC) indeed expressed arpin protein; and that arpin depletion led to the formation of more actin fibers and translocation of VE-Cadherin from cell contacts to the cytosol, thus causing a strong increase in permeability. Treatment of HUVEC with the Arp2/3 inhibitor CK-666 also caused formation of more actin fibers, VE-Cadherin gaps at cell junctions and an increase in permeability under both basal and inflammatory conditions. CK-666 also reduced leukocyte transmigration under inflammatory conditions. These data demonstrate for the first time that arpin is indeed important for the regulation of endothelial barrier stability.

Resumen

El endotelio es una monocapa de células endoteliales que forma una barrera semi-permeable y separa a la sangre de los tejidos adyacentes. Ésta, es mediada por estructuras de adhesión célula-célula como las uniones estrechas (Claudina-1, -5 y -12, ocludina, y JAMs), las uniones adherentes (VE-caderina), y una variedad de otras moléculas de adhesión como PECAM-1 y nectinas, las cuales están conectadas con el citoesqueleto de actina por medio de moléculas adaptadoras. Se sabe que la distribución y expresión de estas proteínas es fundamental para la regulación de la permeabilidad vascular y el reclutamiento de leucocitos. Para tal fin, la barrera endotelial tiene conllevar un remodelamiento del citoesqueleto de actina. Un elemento que está involucrado en este proceso es el complejo heptamérico Arp2/3, el cual es responsable de la formación de ramificaciones de filamentos de actina. La actividad de Arp2/3 es regulada: es activado por los NPF como WAVE, (N)-WASP, WHASH and WHAMMM; y es inhibido por proteínas como PICK1, gadkin y arpin. Arpin es el inhibidor de Arp2/3 más recientemente identificado. Localmente antagoniza la actividad de WAVE para asegurar la regulación de Arp2/3 competitiva en lamelipodios en fibroblastos. Sin embargo, la expresión y función de arpin en células endoteliales es desconocida. Adicionalmente, el complejo Arp2/3 puede ser inhibido farmacológicamente por CK-666, pero este compuesto no ha sido bien estudiado en células endoteliales. En este estudio, se investigaron los efectos de la ausencia de arpin y del CK-666 en la regulación de la barrera endotelial. Se encontró que células HUVEC expresan arpin; y que su ausencia dirige a la formación de fibras de actina, la translocación de VE-Caderina de los contactos celulares al citoplasma y el incremento en la permeabilidad. El tratamiento con el CK-666 en HUVEC también causó la formación de más fibras de actina, la formación de brechas en los contactos de VE-Caderina y el incremento en la permeabilidad, pero redujo la transmigración de leucocitos en condiciones inflamatorias. Estos datos demuestran por primera vez que arpin es, en efecto importante para la regulación de la estabilidad de la barrera endotelial.

INTRODUCTION

THE ENDOTHELIUM

The endothelium, a monolayer of endothelial cells, forms a semi-permeable barrier that separates blood from the underlying tissue. The endothelium constitutes the inner cellular lining of the blood vessels (arteries, veins and capillaries) and the lymphatic system, and therefore is in direct contact with the blood/lymph and circulating cells. Endothelial cells are anchored to an 80-nm-thick basal lamina, and together they constitute the intimal layer of blood vessels. The basal lamina is an important component of blood vessels that works as a structural scaffold to support the endothelial layer inside of this scaffold, while the outside is covered with by smooth muscle cells or pericytes [1].

The shape of endothelial cells (ECs) varies across the vascular tree, but they are generally thin and slightly elongated, with dimensions of 50–70 μm lenght, 10–30 μm wide and 0.1–10 μm thickness[1].

ECs form a semi-permeable barrier to separate the blood stream from the underlying organs and tissues and control the transport of fluids, solutes and cells across blood vessel walls. The barrier is mediated by endothelial cell-cell adhesive structures including tight junctions (TJ), adherens junctions (AJ) and a variety of other adhesion molecules including PECAM-1 and nectins, which are connected to the actin cytoskeleton via different adaptor molecules (Figure 1)[2, 3].

AJ are formed by calcium-dependent homophilic binding bonds between extra-cellular amino-terminal domains of Vascular Endothelial-cadherins (VE-cadherin) molecules from adjacent cells. VE-cadherin also possesses two cytosolic domains, known as the juxtramembrane domain (JMD) and the carboxyl-terminal domain (CTD). The cytosolic domains of VE-cadherin are connected to the actin cytoskeleton through intracellular anchoring proteins such as p120-catenin, α-catenin, β-catenin and γ-catenin. p120-catenin binds

to the JMD, whereas β-catenin or γ-catenin bind to the CTD. β-catenin and γ-catenin also interact with α-catenin, which binds directly to F-actin or indirectly via α-actinin and vinculin. VE-cadherin together with PECAM-1 initiates and maintains endothelial cell-cell contacts, holding the ECs together to give mechanical support to the endothelium and to enable the TJ formation [2, 4-6].

Endothelial TJ constitute nearly 20% of the total endothelial junctional complexes and are involved in the regulation of macromolecular permeability [7]. TJ are formed by the homophilic interaction of cell-cell adhesion molecules such as claudins, occludin and junctional adhesion molecules (JAM) (Figure 1). Claudins are the principal barrier-forming proteins, with claudin-5 being critical for endothelial permeability. The JAM family is composed of three closely related proteins JAM-A, -B, and -C, and by the coxsakie and adenovirus receptor (CAR). JAMs mediate endothelial cell-cell interaction and regulate leukocyte transendothelial migration (TEM). Occludin, claudins and JAMs are linked to the intracellular linker proteins zona occludens (ZO)-1, ZO-2, ZO-3, and cingulin, which mediate the connection of TJ to F-actin and other scaffolding molecules such as α-catenin [2, 4, 6].

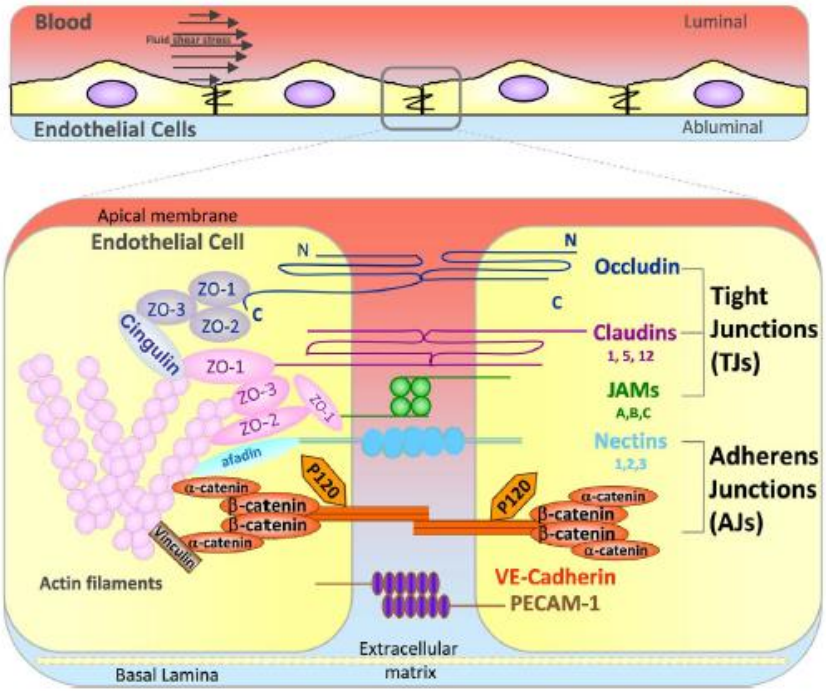


Figure 1. The main transmembrane proteins in endothelial cell-cell junctions. Endothelial cells line the blood stream and are constantly exposed to fluid shear stress (top panel). The bottom panel shows the main transmembrane proteins in endothelial cell-cell junctions (right). They are associated with TJ or AJ as indicated, except for PECAM-1, which is not associated with either type of junction. Three different claudins, JAM and nectin genes are reported to be expressed in endothelial cells (numbers/letters indicated under protein name). Intracellular scaffolding proteins link transmembrane proteins to the actin cytoskeleton. Taken from Cerutti et. al (2017) [2].

FUNCTIONS OF ENDOTHELIAL CELL-CELL JUNCTIONS DURING INFLAMMATION.

It is well accepted that strict regulation of expression, distribution and function of EC junctional proteins is essential for regulating vascular permeability to allow for selective and specific passage of blood cells and macromolecules. Opening and resealing of the junctional barrier must occur during normal physiological processes such as immune surveillance, but also during inflammatory responses[8, 9]. In response to injury or infection, controlled opening or loosening of EC junctions plays a critical role in supporting an effective inflammatory response including increased vascular permeability to

macromolecules, such as immunoregulatory and proinflammatory proteins. Hyperpermeability can be achieved via both transcellular mechanisms, possibly involving intracellular structures, and paracellular mechanisms, involving breaching of tightly connected junctions between adjacent ECs [8].

Vascular permeability is dynamically controlled by a number of extracellular stimuli or by inflammatory mediators such as vascular endothelial growth factor (VEGF), histamine, thrombin, several cytokines (TNFα, IL-1β), certain Gram-negative bacterial endotoxins (Lipopolysaccharide or LPS) and oxidized low-density lipoprotein (LDL) (Figure 2) [10].

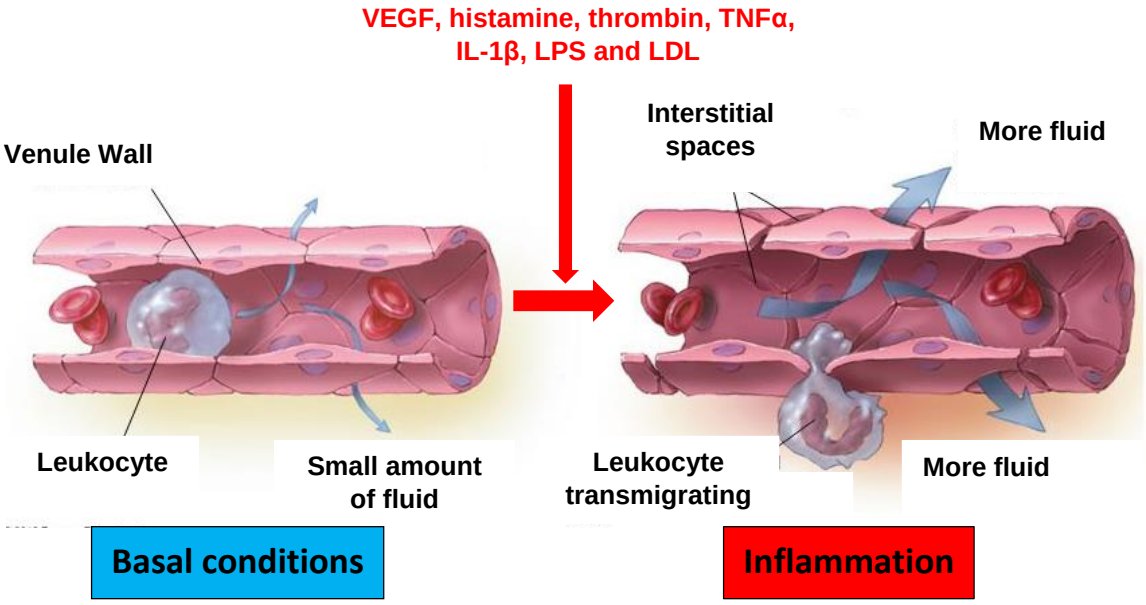


Figure 2. Dynamic regulation of vascular permeability. Endothelial permeability is dynamically controlled during inflammation by several mediators that decrease endothelial barrier function. In normal tissues or basal conditions, endothelial cells preserve basal vascular permeability at low level. When inflammation is induced by inflammatory mediators, vascular permeability increases [10].

Inflammatory mediators such as thrombin, histamine and vascular endothelial growth factor (VEGF) can regulate endothelial junctions by multiple mechanisms. They can alter the architecture of the endothelial cleft by affecting junctional protein expression, localization and stability [9, 10]. For example,

histamine markedly reduces ZO-1 expression in culture retinal ECs [11] and VEGF increases brain microvascular EC permeability by affecting occludin and ZO-1 localization at TJ and decreasing levels of occludin expression [12]. Besides, VEGF and histamine change AJ by inducing phosphorylation of the VE-cadherin cytoplasmic tail and of its associated catenins, in turn leading to the dissociation of the VE-cadherin/catenin complex and increased vascular permeability [13]. In addition, JAM-C and JAM-A are also present in intracellular compartments and are susceptible to mobilization to and from EC junctions post stimulation. In this context, ECs show a unique array of intracellular compartments such as the lateral border recycling complex [8] that is associated with junctional molecule internalization, storage and recycling.

On the other hand, ECs are critical for attracting and supporting transmigration of leukocytes [8, 14]. Specifically, at sites of inflammation, leukocytes exhibit several luminal interactions with ECs, initiating with leukocyte tethering and rolling along the endothelium, followed by slow rolling, adhesion, crawling and finally transendothelial migration or diapedesis (Figure 3). Expression of adhesion molecules on the surface of the endothelium, results from the stimulation of by several inflammatory mediators (Histamine, leukotrienes and cytokines) to initiate the neutrophil migration recruitment. E-selectin and P-selectin are upregulated within 90 minutes and maximize neutrophil recruitment leading to the tethering adhesion of free-flowing neutrophils to the surface of the endothelium [15]. Binding of neutrophils to P- and E-selectin induces signals that activate the β2 integrin LFA1 and promote slow rolling by interaction with ICAM-1. The ligation of integrins, such as talin 1, kindlin 3 and LFA1, with their ligands activates signaling pathways inside the neutrophil and promotes arrest on the endothelium. Firm adhesion of the neutrophils is followed by crawling. Neutrophils crawl endothelium through a mechanism involving MAC1 and ICAM-2 was identified as an important endothelial ligand for MAC1. Finally, neutrophils can cross the vascular endothelium and then the basement membrane via two different routes, the paracellular route between the cells or via transcellular route through the cells. This last process requires

adhesion molecules (ICAM-1, ICAM-2 and vascular cell adhesion protein 1 VCAM1) as well as junctional proteins including PECAM1, CD99 and junctional adhesion molecules (JAMs) [15, 16].

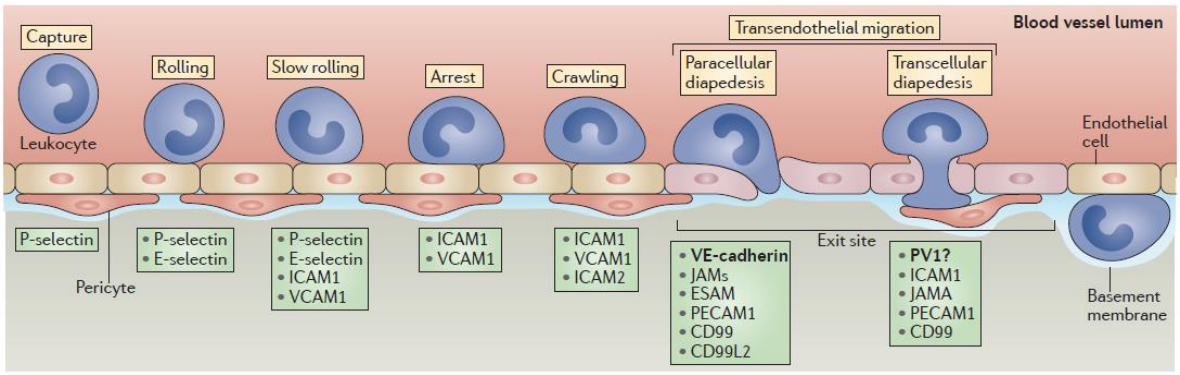


Figure 3. Leukocyte transmigration through the endothelial barrier. Expression of different receptors on endothelial cells (Green boxes) mediates the capture, rolling, arrest and crawling of leukocytes on the luminal endothelial cell surface. Leukocyte diapedesis usually occurs via the paracellular route between the cells but can also happen via the transcellular route. Some of the adhesion receptors that participate in paracellular diapedesis PECAM1 and CD99 are also relevant for transcellular diapedesis. VE-cadherin is exclusively involved in paracellular migration, functioning as a barrier to prevent transmigration. Taken from Vestweber, (2015) [13].

ACTIN DYNAMICS AT ENDOTHELIAL CELL-CELL JUNCTIONS

For the regulation of vascular permeability and leukocyte migration, the endothelial barrier needs to be flexible, and this flexibility is guaranteed by controlled actin cytoskeletal remodeling [6, 17, 18].

Actin constitutes approximately 5–15% of the total protein content in endothelial cells [1]. The actin cytoskeleton is a highly dynamic structure and undergoes polymerization and depolymerization based upon cellular demand. In cells, individual globular (G)-actin subunits alternatively polymerize in a helical fashion to form a double stranded filamentous structure known as filamentous (F)-actin [6].

Actin polymerization occurs in two sequential processes, including nucleation and elongation [19]. Nucleation occurs when three actin monomers bind

together, thus providing a site for elongation, during which ATP-bound G-actin binds and grows to form F-actin. F-actin can depolymerize due to hydrolysis of bound ATP into ADP [6, 20]. Several actin-binding proteins (ABP) have been identified that induce actin nucleation, elongation, depolymerization and cross-linking, which is the basis of actin cytoskeleton dynamics [6].

The best-known mediators that control actin dynamics related to endothelial barrier functions are small GTPases of the Rho family that are activated in response to several inflammatory mediators, such as TNFα, IL-1β, LPS, VEGF and thrombin [18, 21]. Rho, Rac and Cdc42 are the three best-characterized members of the family and are known to be regulators of the actin cytoskeleton [22]. Rho promotes formation of stress fibers, contractile actomyosin bundles and focal adhesion in fibroblast, whereas Rac and Cdc42 induces formation of lamellipodia and filopodia, respectively. Rho GTPases are implicated in several processes such as cell motility, phagocytosis, pinocytosis gene expression and organization of intercellular junctions [22]. GTPases of the Rho family contribute to endothelial barrier functions and stability via common mechanisms, such as regulation of actin dynamics, endosomal trafficking and activation of kinases or other proteins [23]. However, other molecules are involved in the regulation of GTPases activation, actin dynamics and junctional remodeling to control vascular permeability and leukocyte recruitment. Among such molecules are ABP that help to coordinate actin remodeling. ABP can directly bind actin and affect its remodeling or may affect actin remodeling by interacting with other actin regulators such as NPF. Some ABP are known to function as scaffold molecules that connect actin filaments directly to transmembrane adhesion molecules of TJ and AJ to control endothelial barrier functions. Thus, ABP have various playgrounds to exert influence on endothelial barrier stability [18].

Proteins that assemble actin filaments *de novo* or that produce branches on exiting filaments by nucleating filaments de novo from monomers are called actin nucleators [18]. There are three proteins belong to actin nucleators: spire,

formins and the actin-related protein-2/3 (Arp2/3) complex [24]. The spire proteins are conserved among metazoan species and studies with *Drosophila melanoganster* spire indicate that its four tandem G-actin binding Wiskott-Aldrich syndrome protein (WASP)-homology-2 (WH2) domains mediate association of four actin subunits and function as a scaffold for polymerizing a new actin filament [25]. Formins are conserved in most eukaryotes, biochemical and structural studies indicate that a dimer of formin-homology-2 (FH2) domains stabilizes an actin dimer or trimer to facilitate the nucleation event [26]. The Arp2/3 complex works as a template for the initiation of a new actin filament that branches off from an existing filament. Moreover, Arp2/3 requires nucleation promoting factors (NPFs) to become fully activated [24]. Recently, also inhibitory proteins have been identified that guarantee fine-tuning of Arp2/3 activity as described below.

THE ARP2/3 COMPLEX

The key nucleator for inducing branched actin networks is the seven-protein actin-related protein 2/3 (Arp2/3) complex that is conserved in almost all eukaryotes. *In vitro* studies have revealed that the Arp2/3 complex binds existing actin mother filaments and initiates the formation of daughter filaments that branch off the mother filaments at a ~70° angle [24, 27].

The Arp2/3 complex is a stable multiprotein complex of seven subunits, with a total mass of ~250 kDa. Two of these subunits are actin-related proteins, Arp2 and Arp3. The crystal structure of the complex has revealed an inactive conformation, where Arp2 and Arp3 are maintained far apart in the architecture of the complex. The other subunits of the Arp2/3 complex are p41-Arc (ArpC1), p34-Arc (ArpC2), p21-Arc (ArpC3), p20-Arc (ArpC4), and p16-Arc (ArpC5) [28] (Figure 4).

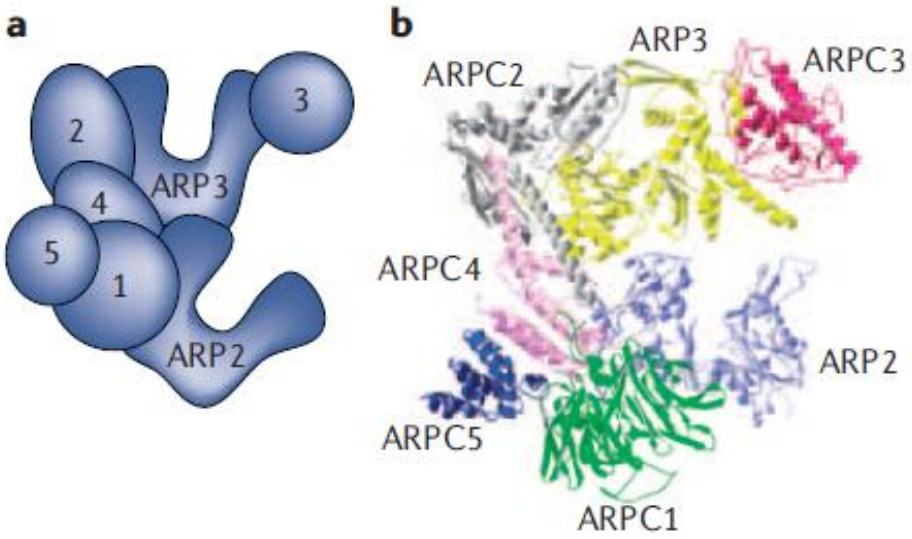


Figure 4. Structure of the Arp2/3 complex. a) Cartoon of the subunit organization in the actin related protein-2/3 (Arp2/3) complex. b) Ribbon diagram of the crystal structure of the bovine Arp2/3 complex with subunits labelled in different colors. Taken from Goley et. al (2006) [24].

The active conformation of the Arp2/3 complex brings Arp2 and Arp3 into close proximity to adopt the conformation of actin molecules within an actin filament; and this conformational change allows for the initiation of the elongation of a lateral branch [29]. However, to become fully activated, the Arp2/3 complex needs to interact with NPF (Figure 5).

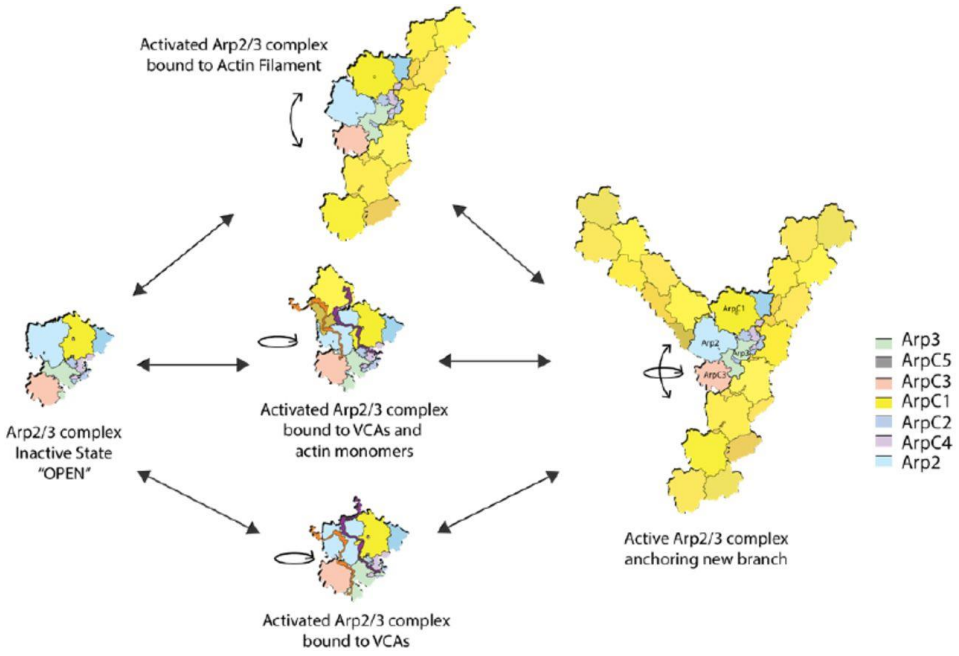


Figure 5. Model of conformational changes associated with Arp2/3 complex activation. The inactive conformation of the Arp2/3 complex maintains separated Arp2 and Arp3 (open state). Binding of VCA domain in NPFs promotes a rotational movement that brigs Arp2 and Arp3 intro proximity and favors binding to a mother filament. Upon binding to a mother filament, Arp2/3 complex experiences a further conformational change. These two movements can occur independently or in combination to fully activate Arp2/3 complex. Taken from Espinoza-Sanchez, et. al. (2017) [30]

CELLULAR FUNCTIONS OF THE ARP2/3 COMPLEX

In spite of the well-understood structure and biochemistry of the Arp2/3 complex *in vitro*, the cellular functions of Arp2/3 have mainly been inferred from its cellular location (Figure 6). For this reason, new tools have been recently identified to understand the functions of the Arp2/3 complex *in vivo*. These tools include specific and reversible small-molecule inhibitors of this complex [24, 27] such as CK-666 (Figure 7).

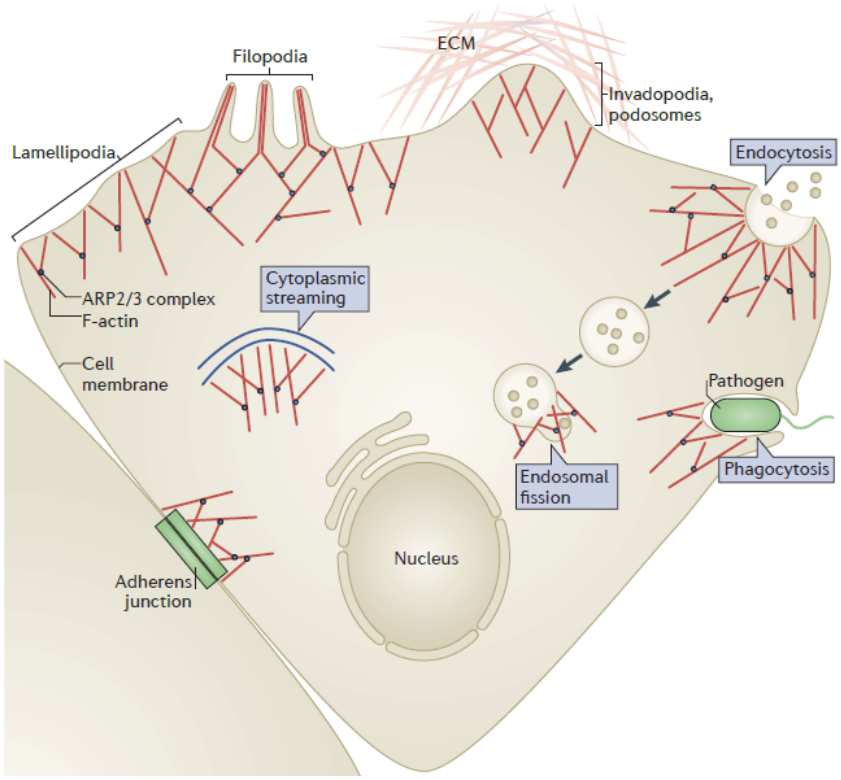


Figure 6. Cellular functions of the Arp2/3 Complex. Branched actin generated by the Arp2/3 complex localizes to the leading edge of migrating cells, AJ, phagosomes and endosomes. Polymerizing branched actin in the cytoplasm also contributes to cytoplasmic streaming (blue lines). Arp2/3-generated branched actin at the leading edge provides the protrusive force that is required to generate and extend the broad sheet-like protrusions known as lamellipodia. The leading edge of migrating cells also contains distinct thin protrusions known as filopodia, which are characterized by bundled parallel arrays of unbranched F-actin. Some of the bundled actin filaments found in filopodia are thought to be initiated from branched networks that have been generated by Arp2/3 at the leading edge. Arp2/3 also localizes to invadopodia and podosomes, which are specialized protease-rich structures implicated in cell invasion and matrix degradation. Although Arp2/3 also resides at AJ, its function at this site remains unclear. ECM, extracellular matrix. Taken from Rotty et. al (2013) [27].

CK-666 binds to the surface of the Arp2/3 complex through its fluorobenzene ring between the Arp2 and Arp3 subunits, and thus blocks the conformational rearrangement of these subunits required for activation [31] (Figure 7). CK-666 is commonly used at a concentration of 100-200 μM. When used at 100 μM, it

did not cause additional phenotypes in Arp2/3-depleted fibroblasts [32], thus highlighting the specificity of CK-666 for Arp2/3.

In endothelial cells little is known about the effects of Arp2/3 inhibition by CK-666. For example, treatment of brain endothelial cells with 80 µM CK-666 reduced methamphetamine-induced, Arp2/3-dependent actin polymerization and occludin internalization (Figure 8a). Of note, CK-666 protected against the methamphetamine-induced increase of BBB permeability *in vivo* (Figure 8b). Thus, Arp2/3 plays an important role in regulating occludin dynamics at TJ and maintaining endothelial barrier integrity in the brain [33].

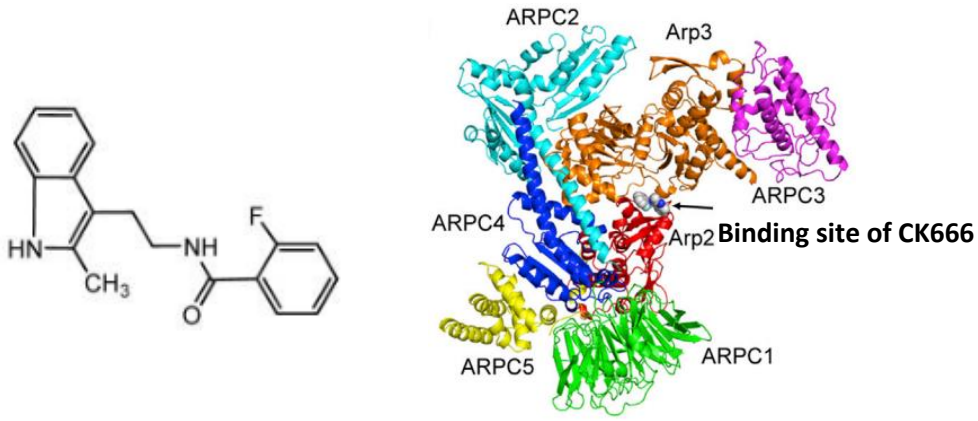


Figure 7. a) Chemical structure of CK-666. **b)** Binding site of CK-666 derived from a 2.5A° X-ray crystal structure of CK-666 bound to the Arp2/3 complex. CK666 is able to bind specifically to the Arp2/3 complex with its fluorobenzene ring between the subunits Arp2 and Arp3, thus preventing the conformational change required for activation. Taken and adapted from Nolen et. al. (2009) [34] and Hetrick et.al. (2013) [31].

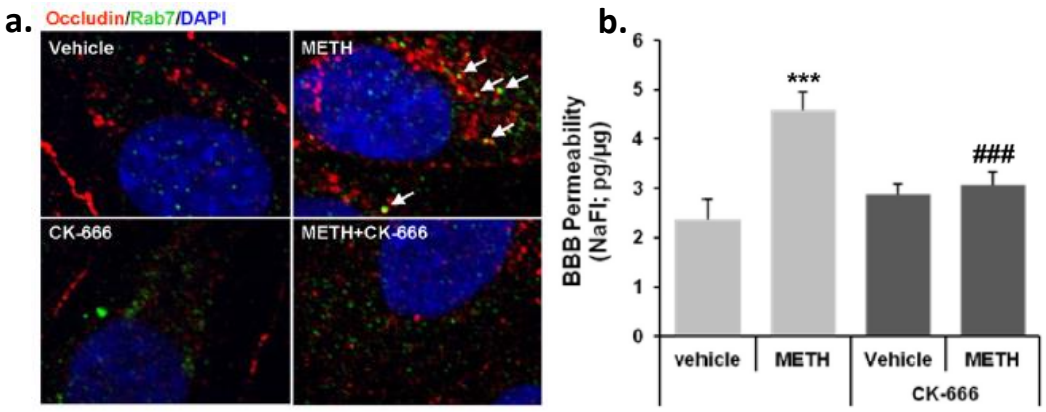


Figure 8. The Arp2/3 complex plays a role in the regulation of occludin dynamics and endothelial barrier integrity in the blood brain barrier. a) Immunostaining of brain endothelial cells with occludin (red) and Rab7 (green – small GTPases involved in the transfer of cargo from the late endosome to the lysosome). Cells were treated with CK-666 (80 µM for 1 hour), followed by exposure of methamphetamine (METH) for 3 hours. Arrows indicate co-localization of occludin and Rab7. **b)** CK-666 prevents METH-induced BBB hypermeability. Taken from Park et. al. (2013)[33]

The Arp2/3 complex also controls junction associated intermittent lamellipodia (JAIL), which are lamellipodia-like structures that appear at established endothelial junctions to stabilize cell junctions where VE-cadherin is locally and temporarily lacking. An interdependent regulation between VE-cadherin-mediated cell adhesion and Arp2/3-mediated and actin-driven JAIL formation was proposed to control VE-cadherin dynamics at endothelial AJ to guarantee cell contact stability [35, 36] (Figure 9).

Fluorescent life-cell imaging of HUVEC expressing both p20, a subunit of the Arp2/3 complex, tagged with EGFP and a VE-cadherin-mCherry fusion-protein revealed the underlying spatiotemporal mechanism. Even at established endothelial cell junctions, JAIL formation occurred and induced an overlap of plasma membranes at which VE-cadherin trans-adhesion plaques were formed over time until contacts were resealed [35, 36]. Of note, 200 µM CK-666 caused intercellular gap formation and decreased VE-cadherin-mCherry dynamics,

demonstrating that the Arp2/3 complex has indeed a critical role in VE-cadherin dynamics at AJ [36].

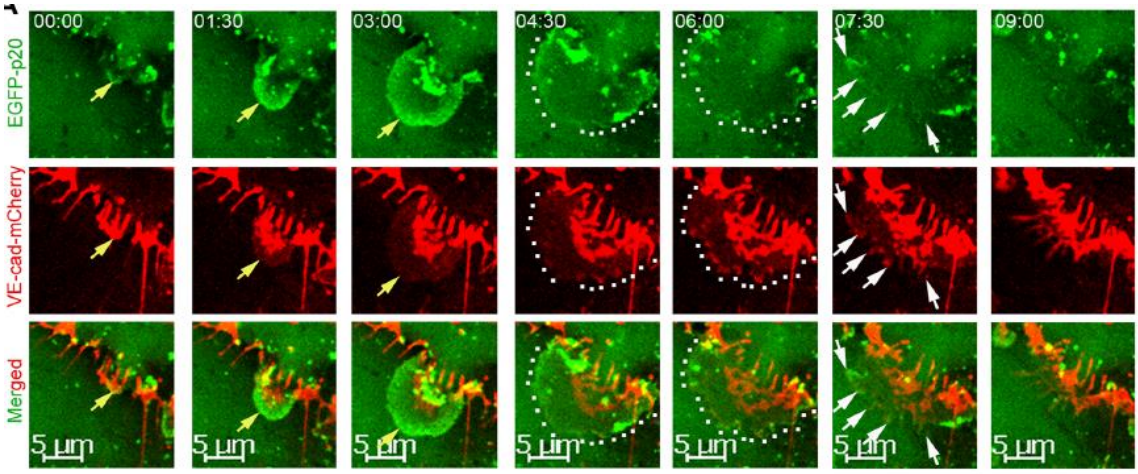


Figure 9. Time-lapse series of Arp2/3 complex-mediated JAIL formation and VE-cadherin dynamics in subconfluent HUVEC cell cultures expressing both the fusion protein EGFP-p20 (green) and VE-cadherin-mCherry (red) at high magnification. JAIL developed close to and between interruptions of VE-cadherin-m-Cherry clusters and caused new VE-cadherin adhesion plaques (middle, yellow arrows and dotted lines). VE-cadherin-mCherry plaques (middle: dotted lines) increase (white arrows) during JAIL retraction to reform AJ. Thus, JAIL formation is a mechanism that reestablishes VE-cadherin presence at AJ to stabilize the endothelial barrier. Taken from Taha et al., (2014) [36].

These data highlight the importance of Arp2/3-mediated endothelial barrier regulation. However, Arp2/3 alone is a weak activator of actin branching. Thus, several proteins exist that activate this complex depending on the sub-cellular location including endothelial junctions as described below.

THE NUCLEATION PROMOTING FACTORS (NPF)

NPF are activators of the Arp2/3 complex that can be divided into two subclasses. Type I NPFs are characterized by their COOH-terminal domain that contains three short peptide motifs, the verprolin-homology domain or WH2 (W or V), the cofilin-homology domain or central domain (C), and the acidic end (A) characterized by a tryptophan residue at the antepenultimate position in an acidic context. This characteristic COOH-terminus is referred to as the WCA or VCA. The CA domain binds to the Arp2/3 complex and induces

its conformational activation. The WH2 motif binds to one globular actin molecule and delivers it to rearranged Arp2/3. These two events are required to initiate an actin branch [37]. On the other hand, the NH_2-terminus varies considerably between NPF and has a regulatory role by determining how the WCA is maintained in an inactive conformation at resting conditions; and how it is exposed to allow for Arp2/3 binding and activation. Moreover, the domains present in the NH_2-terminus define the different families of type I NPF [28]. Type I NPFs include Wiskott-Aldrich syndrome protein (WASP), neural WASP (N-WASP), three WASP family verprolin-homologous proteins (WAVE1, 2 and 3), WASP and SCAR homologues (WASH) and WASP homolog associated with actin, membranes, and microtubules (WHAMM). The WASP family is composed by the ubiquitous N-WASP and the hematopoietic WASP. The main functions of (N)-WASP are in the plasma membrane after its activation by Cdc42. In the WAVE family, WAVE2 is the ubiquitous one while the other two are more tissue restricted. Basically, WAVE localizes at the edge of lamellipodia, where actin molecules incorporate into branched actin networks. The WASH family displays from 15 to 20 number of genes depending on species, because the gene is in a subtelomeric region, sensitive to recombination. WASH activates the Arp2/3 complex at the surface of endosomes. WHAMM seems to be more expressed in epithelial tissues and it is localized at the Golgi and cis-Golgi, and it is involved in anterograde transport.

On the other hand, Type II NPFs such as cortactin and its homologue HS1 have acidic domains at their amino terminus that bind the Arp2/3 complex and tandem repeat domains that bind F-actin [27], but lack complete VCA domains. They are only weak NPF. Instead, cortactin has been shown to stabilize newly generated filament branches [37].

In endothelial monolayers, the class I NPF WAVE2 is important for maintaining the endothelial barrier because promote VE-cadherin expression and correct localization at AJ, and the maturation of the cell-cell junctions [38] (Figure 10).

Moreover, the authors suggest that WAVE2 maintains the endothelial barrier stability through the activation of the Arp2/3 complex.

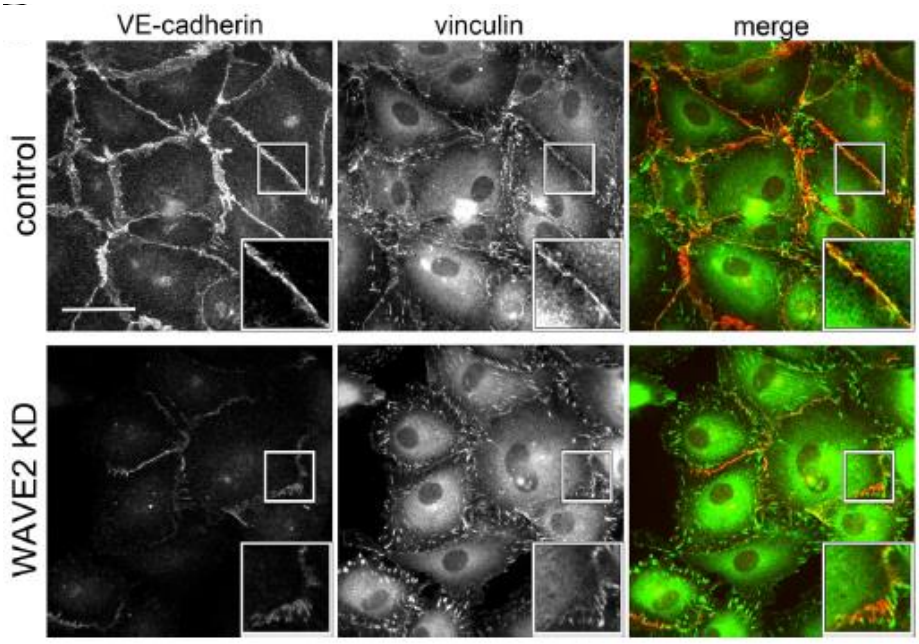


Figure 10. Functions of WAVE2 in endothelium. a) Knock-down of WAVE2 in HDMVEC (Human Dermal Microvascular Endothelial Cells) causes immature junctions based on VE-cadherin and vinculin localization; and a destabilized barrier. Taken from Mooren et. al. (2014) [38]

ARP2/3 INHIBITORY PROTEINS

The function of the Arp2/3 complex is to induce actin polymerization in response to several stimuli. Because actin filaments are both substrates and products of the branching reaction, the autocatalytic process generates an exponential increase of actin filaments [28]. For that reason, the Arp2/3 complex needs to be regulated to avoid excessive branching. The regulation of Arp2/3 complex activity is achieved by endogenous inhibitory proteins through direct binding to the Arp2/3 complex and competing with NPF. To this end, cells express certain inhibitory proteins that were recently discovered: PICK1, Gadkin and Arpin [28, 39]. The general idea is that an inhibitor might be diffuse in the cytoplasm, in order to maintain the Arp2/3 complex silent until activated. Surprisingly, these three inhibitory proteins are specifically localized at different

cellular locations, similar to the activators, suggesting that they may compete with specific NFP to control Arp2/3 activity (Figure 11) [28, 39]. In this compartmentalized theory, WAVE and arpin regulate Arp2/3 at lamellipodia, (N)-WASP and PICK1 at clathrin-coated pits, and WASH and Gakin at endosomes.

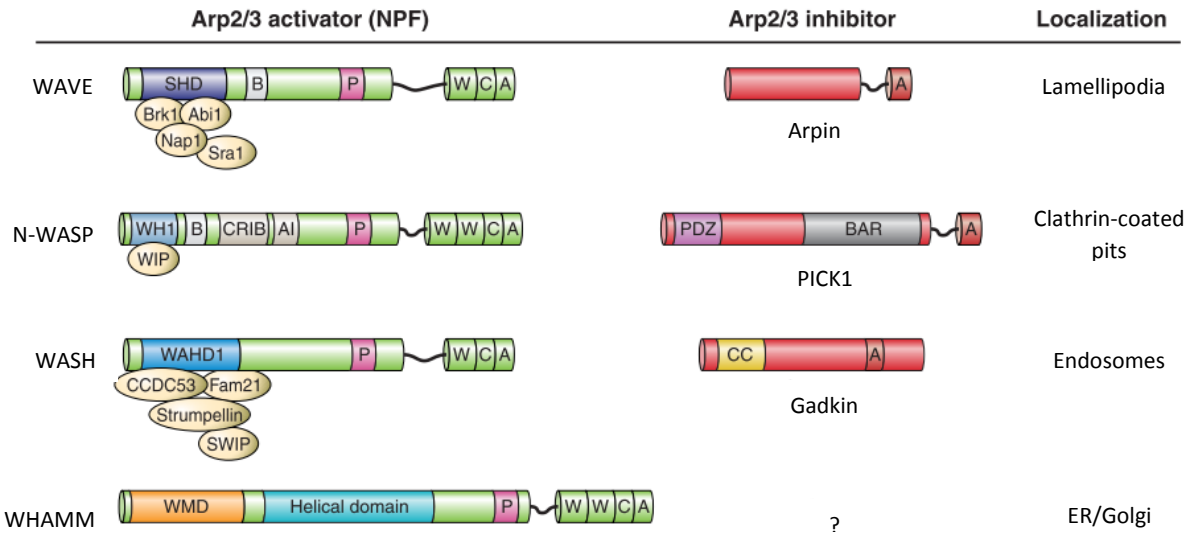


Figure 11. Organization of activating and inhibiting proteins of the Arp2/3 complex. The proteins are classified according to their sub-cellular location. All activators contain a COOH-terminal WCA domain, which binds and activates the Arp2/3 complex. All inhibitors contain an acidic motif (A), which binds also to the Arp2/3 complex to compete with NPF in the indicated sub-cellular locations. SHD, Scar/WAVE homology domain; B, basic domain; P, proline-rich region; WH1, WASP homology 1; CRIB, Cdc42 and Rac1 interactive binding region; AI, autoinhibition domain; WAHD1, WASH homology domain 1; WMD, WHAMM membrane-interacting domain; CC, coiled coiled; PDZ, PSD95-Dlg1-ZO1 domain; BAR, bin-amphiphysin-rvs domain. An inhibitory protein that would antagonize WHAMM remains to be identified. Taken from Molinie et. al (2018) [28].

Gadkin

Gadkin interacts with the Arp2/3 complex through an acidic motif and localizes under basal conditions at the surface of endosomes where it regulates the trans-Golgi network-endosomal traffic. In gadkin knockout cells, the Arp2/3 complex associated with endosomes polymerizes more F-actin suggesting that gadkin maintains the Arp2/3 complex in an inhibited conformation. It is tempting

to speculate that gadkin could antagonize the WASH complex at the surface of endosomes due to its function and localization [28], but this has not been experimentally proven yet.

PICK1

PICK1 is a protein containing a PDZ and a BAR domain. The PDZ domain connects PICK1 to numerous membrane receptors and transporters, such as AMPA receptor of the glutamate neurotransmitter. The BAR domain dimerizes and induces curved membranes through its banana-shaped membrane binding interface [40]. In the COOH-terminus, PICK1 contains an acidic motif that binds and inhibits the Arp2/3 complex. Inhibitory functions of PICK1 are regulated by the GTPase Arf1. PICK1 regulates AMPA receptor trafficking, and in particular its clathrin-dependent endocytosis. Besides, the role of PICK1 is to antagonize N-WASP at clathrin-coated pits [41, 42].

Arpin

Arpin is the most recently identified protein that can inhibit Arp2/3. It was identified as an uncharacterized protein, which contains a typical COOH-terminal acidic motif (A) with a tryptophan residue in the antepenultimate position, that binds to Arp2/3 (Figure 12a) but lacked the required WH2 and central motifs for Arp2/3 activation. *In vitro*, arpin binds to the Arp2/3 complex (Figure 12b and 12c) and acts as a competitive inhibitor of NPF [43]. Arpin binding causes the Arp2/3 complex to remain in its inactive conformation, separating the Arp2 and Arp3 subunits [44]. Arpin interacts with both the Arp2 and Arp3 subunits [44], with both possibly corresponding to the two WCA-binding sites, thus allowing for competition with NPF.

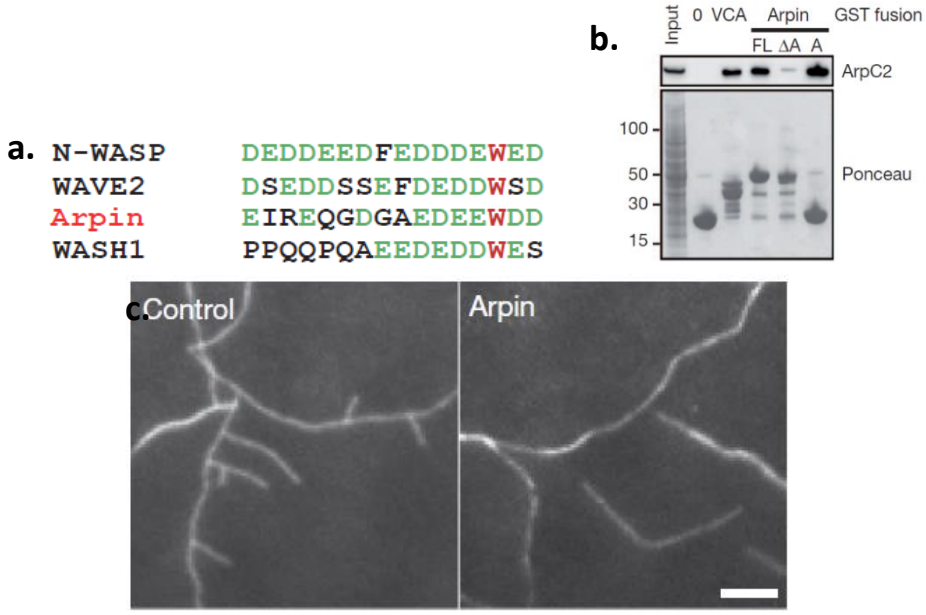


Figure 12. Arpin inhibits Arp2/3 activation *in vitro*. a) Alignment of the acidic motives of three NFP and Arpin (In red the characteristic tryptophan of the domain). **b)** Arpin binds to the Arp2/3 complex through its acidic C-terminal region. Glutathione S-transferase (GST) pulldown with full-length arpin (FL), its last 16 amino-acids (A), ArpinΔA or the VCA domain of N-WASP as a positive control. ArpC2 is a subunit of the Arp2/3 complex. c) Assembly of branched actin networks monitored by TIRF microscopy using rhodamine-labelled actin. Scale bar, 5 mm. Taken from Dang et. al (2013) [43].

Arpin mainly localizes at the lamellipodial edge in mouse embryonic fibroblasts (MEFs), where it colocalizes with Brk1, a subunit of the WAVE complex (Figure 13) [43]. This co-localization suggests that arpin can antagonize the activity of WAVE in lamellipodia. Given that WAVE2 contributes to endothelial barrier integrity, arpin may also compete with WAVE2 at endothelial junctions to regulate barrier functions.

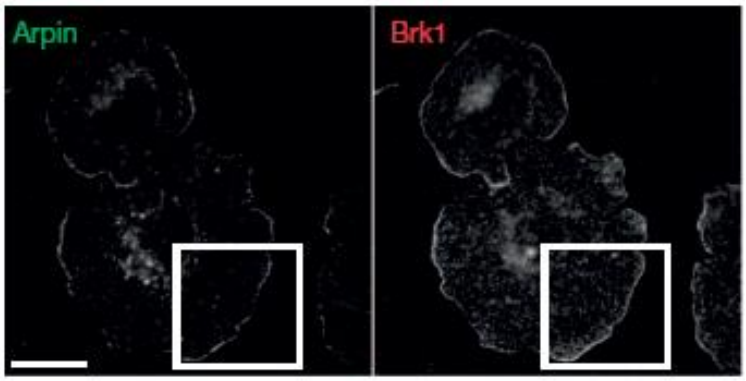


Figure 13. Arpin colocalizes with Brk1, a subunit of the WAVE complex, at the lamellipodial tip in MEFs. Scale bar, 20 um. Taken from Dang et. al (2013) [43]

JUSTIFICATION

It is well-known that under both basal and inflammatory conditions the endothelial actin cytoskeleton is a dynamic structure and changes in order to control junction architecture, vascular permeability and leukocyte transmigration. One factor that is involved in the rearrangement of the actin cytoskeleton is the Arp2/3 complex. The Arp2/3 complex is inhibited by several proteins such as the newly identified arpin, that locally antagonizes the activity of the Arp2/3 activator WAVE, and by small molecule inhibitors such as CK-666. However, the role of Arp2/3 inhibition in regulating endothelial barrier functions, such as permeability and leukocyte recruitment remains largely unknown.

HYPOTHESIS

Arpin and the Arp2/3 inhibitor CK666 regulate endothelial barrier functions under basal and inflammatory conditions.

GENERAL OBJECTIVE

Analyzing the role of Arp2/3 inhibition by arpin and CK-666 in the regulation of actin remodeling, junction architecture, permeability, and leukocyte recruitment.

PARTICULAR OBJECTIVES

- To analyze arpin expression and localization in HUVEC.
- To generate arpin knock-down HUVEC.
- To investigate actin cytoskeleton dynamics, expression and localization of junction proteins in arpin-depleted and CK666-treated HUVEC under basal and inflammatory conditions.
- To examine the effects of arpin-depletion and CK666-treatment on endothelial permeability and neutrophil transmigration.

METHODS

Table 1. Buffers and Solutions. All buffers were prepared in deionized water purified using a Mili-Q-system (Millipore).

SDS-PAGE Running Buffer, pH 8.3	20 mM Tris-HCl pH 6.8 192 mM glycine 0.1% SDS
Transfer Buffer, pH 8.3	20 mM Tris-HCl pH 6.8 192 mM glycine 0.1% SDS 20% methanol
5X SDS Loading sample buffer	250 mM Tris-HCl pH 6.8 10% SDS 30% glycerol 5% β-mercaptoethanol 0.02% bromophenol blue
TBS-T pH 8.0	150 mM NaCl 10 mM Tris-Base 0.1% Tween20
Blocking solution	5% milk powder TBS-T
PBS	138 mM NaCl 3 mM KCl 8.1 mM Na_2HPO_4 1.5 mM KH_2PO_4
RIPA lysis buffer	20 mM Tric-HCl pH 7.5 150 mM NaCl 1 mM Na_2EDTA 1 mM EGTA 1% NP-40 1% sodium deoxycholate

	cOmplete protease inhibitor and PhosSTOP® (ROCHE)
Hypotonic solution, pH = 7.4	0.2% NaCl 1% BSA 20 mM Hepes
Hypertonic solution, pH = 7.4	1.6% NaCl 1% BSA 20 mM HEPES

Table 2. Antibodies

Supplier	Antibody	Catalogue Number
Santa Cruz (California)	Anti-VE-Cadherin (C19) Anti-ICAM-1 (G-5) Goat anti-mouse IgG-HRP Goat anti-mouse IgG-HRP	sc-6458 sc-8439 sc-2005 sc-2004
Invitrogen (Carlsbad, California,)	Anti-ZO-1 Anti-Claudin-1 Anti-Occludin Anti-Tubulin Alexa Fluor-488 rabbit anti-goat IgG Alexa Fluor-568 donkey anti-goat IgG	#61-7300 #51-9000 #71-1500 #MA1-850 # A27012 # A-11057

CELL CULTURE

HUVEC (Human Umbilical Vein Endothelial Cells) were isolated from donated discarded umbilical cords. Under sterile conditions, the umbilical cords were washed with sterile water to remove excess blood. In the upper end of the cord vein, a cannula with a syringe containing PBS and Streptomycin/Penicillin was inserted into and the content was removed. Then, both cord ends were sealed with hemostatic clamps and, subsequently, 0.25% trypsin-EDTA (Sigma) was

instilled using a syringe of 25 mL until the vein was filled and then incubated at 37°C for 10 min in a water bath. Every 2 minutes, the umbilical cord was gently massaged to facilitate the digestive process. Next, the clamp of the lower end was removed, and the content recovered and centrifuged at 1500 rpm for 5 minutes. The cell pellet was resuspended in Endothelial Cell Media (ECM, ScienCell™ Research Laboratories) supplemented with 10% Fetal Bovine Serum (FBS) and plated into T25 flask (CellBind, Corning). Cells were cultivated in ECM supplemented with 10% (FBS) and kept at 37°C in a humid atmosphere containing 5% CO_2. Cells from passage 1 to 6 were used for functional experiments. For inflammatory conditions, HUVEC monolayers were treated with 15 ng/mL TNFα for 18 hours. Additionally, to analyze the effects of Arp2/3 inhibition, HUVEC monolayers were treated with 100 µM CK666 (Sigma-Aldrich, Catalogue Number: SML0006) and DMSO (vehicle) without and with the presence of 15 ng/mL TNFα for 18 hours.

HEK-293T cells were cultivated in DMEM medium supplemented with 1X L-glutamine, 1X pyruvate, 1X non-essential aminoacids, 1X sodium pyruvate and 10% FBS and kept at 37°C in a humid atmosphere containing 5% CO_2.

GENERATION OF VIRAL PARTICLES

For the generation of arpin knock-down HUVEC, shRNAs (short harpin RNAs) were designed according to the Brad Institute design rules, which are available on the web page http://www.broadinstitute.org/rnai/public/resources/rules (Genetic perturbation platform). shRNAs either target arpin or contain a scrambled sequence not targeting anything.

The resulting shRNA sequences were ordered as oligonucleotides and cloned into the expression vector pLKO.1.

Arpin-depleted HUVEC were generated using the TransLenti Viral Packaging Mix (Thermo Scientific Scientific). This kit contains the plasmids pTLA1-Pak, pTLA1-Enz, pTLA1 Env, pTLA1-Rev and pTLA1-TOFF, which encode for the proteins necessary to form the viral particles. The viral packaging plasmids and

the lentiviral vector pLKO.1 containing either arpin-specific shRNA or a scrambled control shRNA were mixed as follows:

Plasmid	µg
pTLA1-Pak	6
pTLA1-Enz	4
pTLA1-Env	6
pTLA1-Rev	4
pTLA1-TOFF	8
pLKO1-shRNA Arpin/*scr*	9

The plasmid mixture in 2.4 mL DMEM medium without serum containing 0.14 mL poliethylenimine (PEI) was incubated at room temperature for 15 minutes. Then, the mixtures was added to a culture of HEK-293T cells at 80% confluence in a 10 cm culture dish. and incubated at 37°C with 5% CO_2 for 4 h. Afterwards, the medium was exchanged with fresh DMEM medium. After 48 h, the medium was recovered and stored at 4°C. Fresh DMEM medium was added and collected again 24 h later. Recovered media at 48 h and 72 h were mixed centrifuged at 1500 rpm 4°C for 5 minutes to precipitate cell debris. The collected supernatant was then again centrifuged at 13,000 rpm, 4°C for 2 hours and the viral pellet used as described below.

GENERATING ARPIN KNOCK-DOWN HUVEC.

The viral pellet was resuspended in 3 mL HUVEC medium together with 8 µg/mL polybren. This mixture was added to monolayers of sub-confluent HUVEC in 6-well plates; and the plates were centrifuged at 3000 rpm for 1 hour to facilitate viral contact with the cells. The cells were then incubated at 37°C for 24 hours, followed by exchange with fresh medium containing 1 µg/mL puromycin to select the infected cells. A survival curve using different puromycin concentrations revealed that 1 µg/mL is the minimum concentration

killing 100% of untransfected HUVEC. The medium containing puromycin was changed every three days until infected cells grew to confluence (~4-7 days). After reaching confluency, cells were trypsinized and seeded into T25 flasks (CellBind, Corning). Knock-down efficiency was tested by Western-blot.

PROTEIN EXTRACTION AND QUANTIFICATION

HUVEC monolayers were washed once with PBS. Then, cells were lysed using RIPA lysis buffer and sonicated five times at 40% amplitude for 10 seconds. Lysates were centrifuged at 15,000 rpm at 4°C for 20 minutes to remove cell debris.

The extracted protein was quantified using the DC Protein Assay (Bio-Rad). A calibration curve was measured with a series of Bovine Gamma Globulin (BGG) dilutions ranging in concentration from 1 to 6 µg/µL. A sample of 1 µL of the lysates was mixed with 10 µL of the reagent A' (20 µL of reagent S in 1 mL of reagent A) and 80 µL of the reagent B. All samples were incubated at room temperature under gentle agitation for 15 minutes. Finally, samples were measured at 750 nm using Tecan® Infinite M200 Plate Reader. In order to determine the protein concentration, the absorbance value of the unknown samples was compared to the calibration curve using linear regression.

WESTERN-BLOT

20 µg of total protein were separated by 12% acrylamide SDS-PAGE (110 V, 120 minutes). Then the separated extracts were transferred into a nitrocellulose membrane (0.45 µm, 220 mA, 90 minutes). Subsequently, the membrane was blocked with blocking solution for 1 h. Afterwards, the primary antibody of interest (compare table 2) in blocking solution was added to the membrane and incubated overnight at 4°C. Then, the primary antibody was removed, and the membrane was washed three times with TBS-T. Next, the species-specific secondary antibody coupled to HRP was added to the membrane and incubated for 2 h at room temperature. The secondary antibody was removed, and the membrane was washed three times with TBS-T for 10

minutes. Finally, the membrane was revealed using Super West *Femto* Maximum Sensitivity Substrate (Thermo Fisher Scientific).

IMMUNOFLUORESCENCE ASSAY

HUVEC were seeded onto glass cover slips (pretreated with 1 % gelatin at 37°C for 30 minutes) in a 24-well plate. The confluent cells were fixed using 4% PFA for 10 minutes at room temperature. The fixed cells were washed with PBS three times. Next, cells were permeabilized with 0.1% Triton X-100 for 10 minutes at 4°C, then cells were washed with PBS three times. Cells were incubated with the primary antibody of interest (compare table 2) in PBS containing 1% BSA at 4°C overnight. Then, cells were washed three times with PBS for 5 min. Cells were incubated with a species-specific secondary antibody coupled to Alexa-488 or Alexa-568 in 1% BSA for 1 h at room temperature in the dark. Cells were washed three times with PBS for 5 min, in the dark. Next, the cover slips were mounted in slides using ProLong GOLD (Thermo Fisher Scientific) containing 0.5 µg/mL DAPI to stain nuclei. Cells were analyzed using Leica SPE confocal microscopy.

FITC-DEXTRAN PERMEABILITY ASSAY

HUVEC were seeded into 6.5-mm-diameter transwell® filters (CORNING, Life Science) with 0.4-µm pore size coated with 0.1% gelatin and cultivated for 48 h. Then, the media in the upper chamber was replaced with 100 µL of fresh medium containing 15 ng/mL TNFα with or without 100 µM CK-666 and the medium in the lower chamber was changed with 600 µL of fresh medium, Cells were incubated for 18 hours. Then, 0.25 mg/mL of 150 kDa FITC-dextran were added to the upper chamber. After 30 and 60 minutes, 100 µl medium were taken from the lower chamber and the fluorescence was quantified at 488 nm using a spectrofluorometer.

ISOLATION OF HUMAN NEUTROPHILS

Blood was collected from a consenting healthy donor in sodium heparin (5-10 U/mL final concentration) or acid citrate dextrose (ACD-A) blood collection

tubes. 3 mL of Histopaque 1119 and 3 ml of Histopaque 1077 (Sigma-Aldrich) were layered in a 15 mL conical centrifuge tube, and the collected blood was carefully added on the top of the Histopaque 1077 layer. The tube was centrifuged at 2750 rpm at room temperature for 30 minutes without brakes. The neutrophils were collected from the interface of the Histopaque 1119 and Histopaque 1077 layers. The neutrophil cell suspension was washed twice with 10 mL of PBS at 4°C then neutrophils were centrifugated at 1500 rpm at 4°C for 5 min. Neutrophils were resuspended in 5 mL sterile PBS at 4°C. Then 10 mL of hypotonic solution at 4°C were added to the neutrophils to lysate erythrocytes and mixed gently, one minute after 10 mL of hypertonic solution at 4°C were added to the neutrophils and mixed gently. Finally, neutrophils were centrifuged at 1500 rpm and resuspended in RPMI 1640 medium, supplemented with 10% FBS and 1% penicillin/streptomycin at 4°C. Cells were counted using a Neubauer chamber. Neutrophils were maintained at 4°C until used in transmigration assays.

TRANSENDOTHELIAL MIGRATION (TEM) ASSAY

HUVEC were seeded into 6.5-mm-diameter transwell filters with 5-µm pore size coated with 0.01% gelatin and cultivated for 48 h. Then, cells were activated with 15 µg/mL TNFα for 18 hours in the presence or absence of 100 µM CK-666.

The filters were washed once with PBS. Then, the upper reservoirs were filled with 100 µL RPMI 1640 medium containing $5x10^5$ neutrophils. The lower reservoirs were filled with 500 µL of medium with or without 50 ng/mL IL-8 as chemoattractant. After 45 min, the number of transmigrated neutrophils in the lower reservoir were counted using a Neubauer chamber.

RESULTS

As nothing is known about arpin in endothelial cells, first, I analyzed arpin expression in human umbilical vein endothelial cells (HUVEC) under basal and inflammatory conditions. Arpin is expressed under basal conditions, but was not changed after treatment with TNF-α (Figure 14). Blots for ICAM-1 were performed to ensure that the inflammatory stimulation worked. As expected, total VE-cadherin expression did not change.

To unravel the localization of arpin in HUVEC under basal and inflammatory conditions, I performed immunofluorescence stainings and found that arpin is localized throughout the cytoplasm; but also in vicinity to actin fibers, which makes sense given that arpin is a regulator of Arp2/3, and thus actin dynamics. Of note, arpin also appeared near the junctions under both conditions (Figure 15). Nevertheless, to confirm junction localization, it will be necessary to perform co-immunofluorescence stainings with arpin and antibodies against junction proteins such as VE-Cadherin or PECAM-1. Interestingly, only under inflammatory conditions, arpin seemed to be present in the nucleus (Figure 15). This agrees with bioinformatics analysis demonstrating that arpin has a non-canonic nuclear localization signal. However, to confirm this finding it will be necessary to perform Western blots of nuclear extracts and immunofluorescence stainings of arpin together with a nucleus marker.

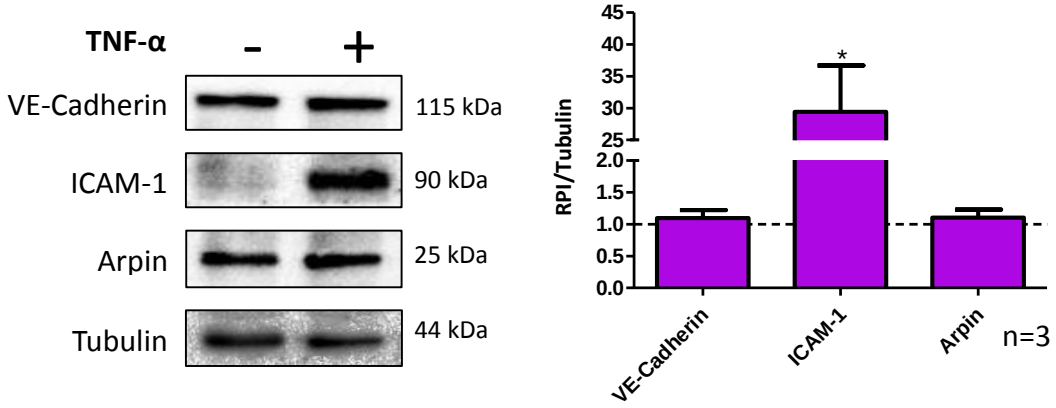


Figure 14. HUVEC express equal amounts of arpin under both basal and inflammatory conditions. Representative blot of three independent experiments, with tubulin as loading control. The graph on the right shows relative pixel intensities of each protein normalized with tubulin. Data are represented as mean plus SEM *p<0.05.

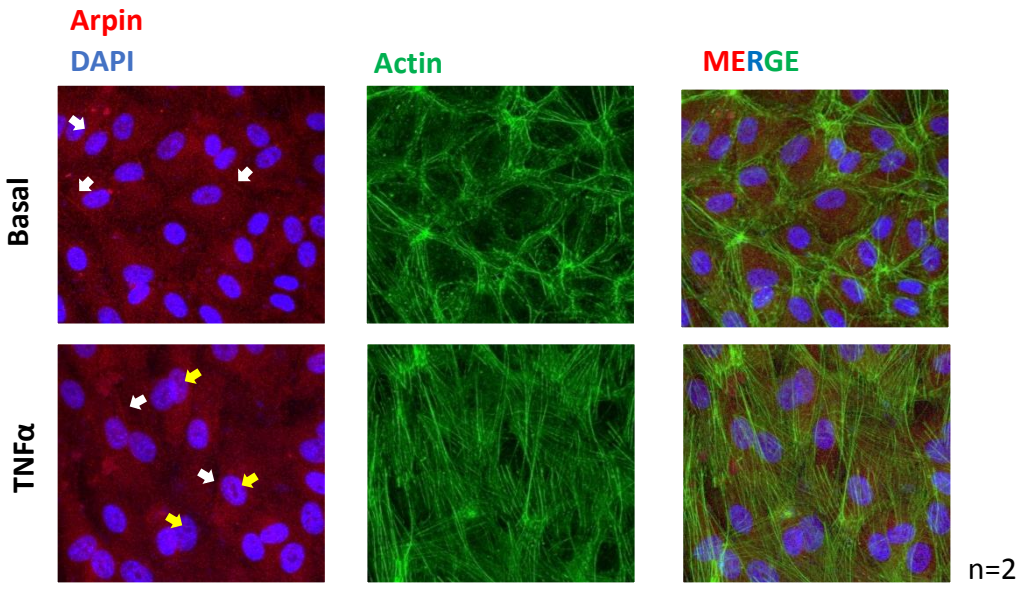


Figure 15. Arpin is localized throughout the cytoplasm, and partially co-localizes with F-Actin and at cell -cell contacts. HUVEC stained with arpin in red and F-actin using Alexa 488-Phalloidin. Basal conditions (Top) and treatment with TNFα (Bottom). White arrows indicate that arpin is localized at cell-cell contacts and yellow arrows indicate that under the stimulus of TNFα seems to be presence of arpin in nucleus. Bar=20 µm

Generation of arpin-depleted HUVEC

In order to analyze the role of endothelial arpin, it was generated stable arpin-depleted HUVEC using lentiviral particles that express two different arpin-specific shRNA sequences (shArpin1 and shArpin2) or a scrambled (Scr) control; which all also provide puromycin resistance. Arpin protein levels were down-regulated by more than 90% in these cells (Figure 16).

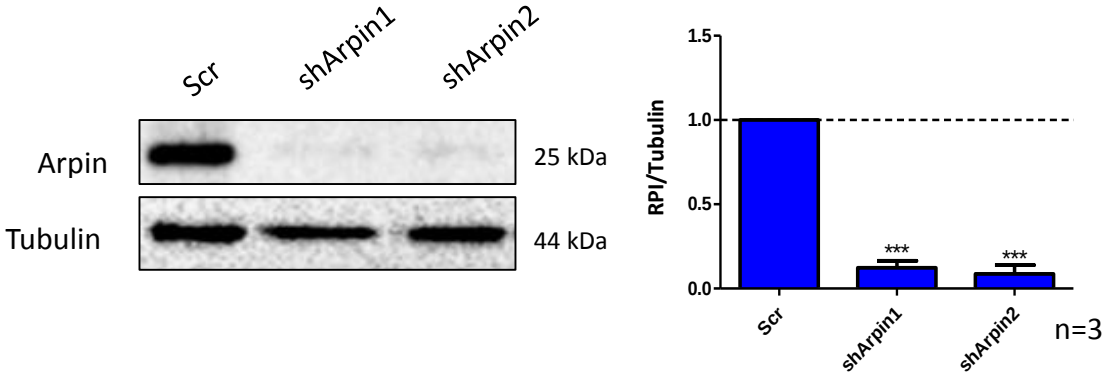


Figure 16. Stable arpin depletion in HUVEC. Representative blot for three independent experiments is shown, with tubulin as loading control. The graph to the right shows relative pixel intensities of arpin normalized with tubulin. Data represented as mean plus SEM; ***p<0.001.

Arpin depletion does not alter total protein expression, but the localization of different junction proteins

Next, I analyzed the expression of the junctional proteins VE-Cadherin, occludin, claudin-1 and ZO-1 and the adhesion molecule ICAM-1 by Western-blot in arpin-depleted HUVEC (or Arpin KD) as these proteins are essential for the control of endothelial barrier integrity under both basal and inflammatory conditions. However, I did not see any changes in the total protein expression of these proteins in the absence of arpin neither under basal nor under inflammatory conditions (Figure 17). ICAM-1 was barely expressed under basal conditions, but was strongly induced by TNF treatment, confirming that the inflammatory stimulus worked. However, although the expression of these

molecules did not change, their localization may very well be altered in the absence of arpin, as it is well known that junction proteins are internalized during inflammation in an actin-dependent fashion.

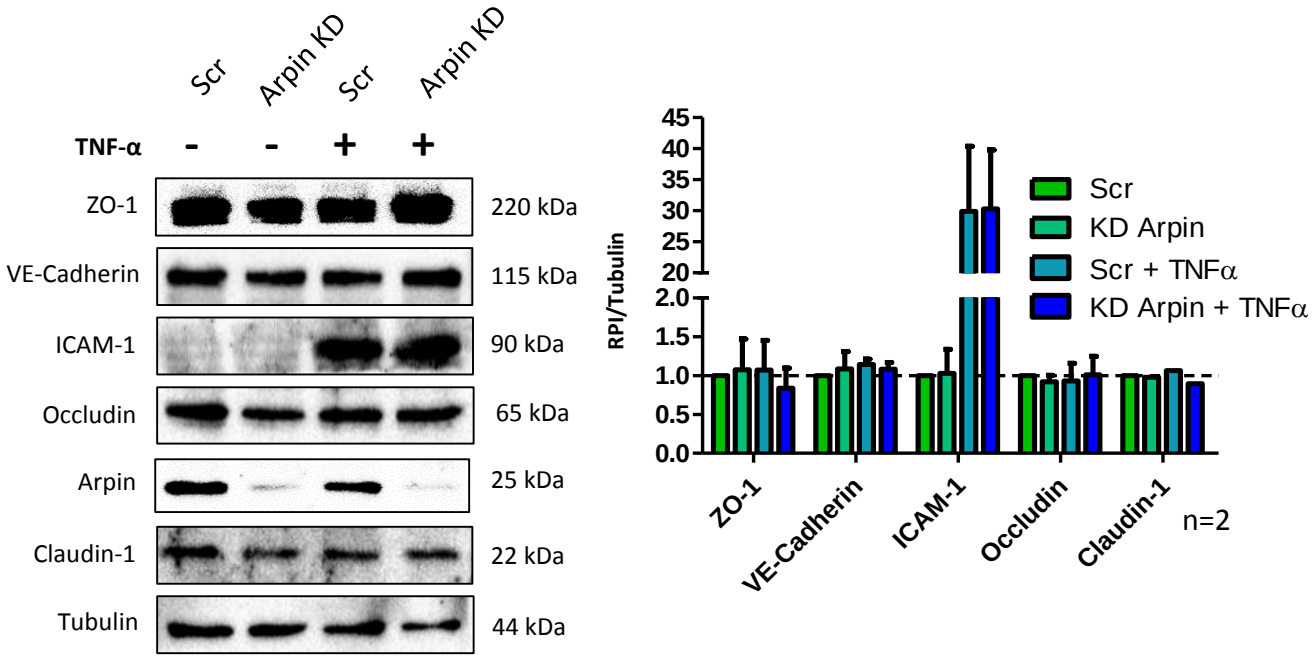


Figure 17. Arpin-depletion (Arpin KD) does not change the expression of the indicated junction proteins or the apical adhesion molecule ICAM-1. Representative blots for junction proteins in arpin-depleted HUVEC with tubulin as loading control. The graph on the right shows relative pixel intensities of each protein normalized with tubulin.

To analyze the cellular location of these molecules, it was performed immunofluorescence stainings in order to observe VE-cadherin distribution and indeed it was observed significant changes in the distribution of VE-cadherin (Figure 18). Even under basal conditions, VE-cadherin gaps formed at some cellular junctions in the absence of arpin (Figure 18, white arrows), most likely due to internalization as some VE-cadherin could be observed in the cytoplasm (Figure 18, yellow arrows). Moreover, arpin knock-down cells showed formation of more actin fibers under basal conditions (Figure 18, blue arrows) that could contribute to junction destabilization by contractile tension. On the other hand, arpin-depleted HUVEC treated with TNFα showed even more

internalization of VE-cadherin into the cytoplasm with the loss of the VE-cadherin continuity and even more actin fibers than under basal conditions (Figure 18, yellow and blue arrows, respectively).

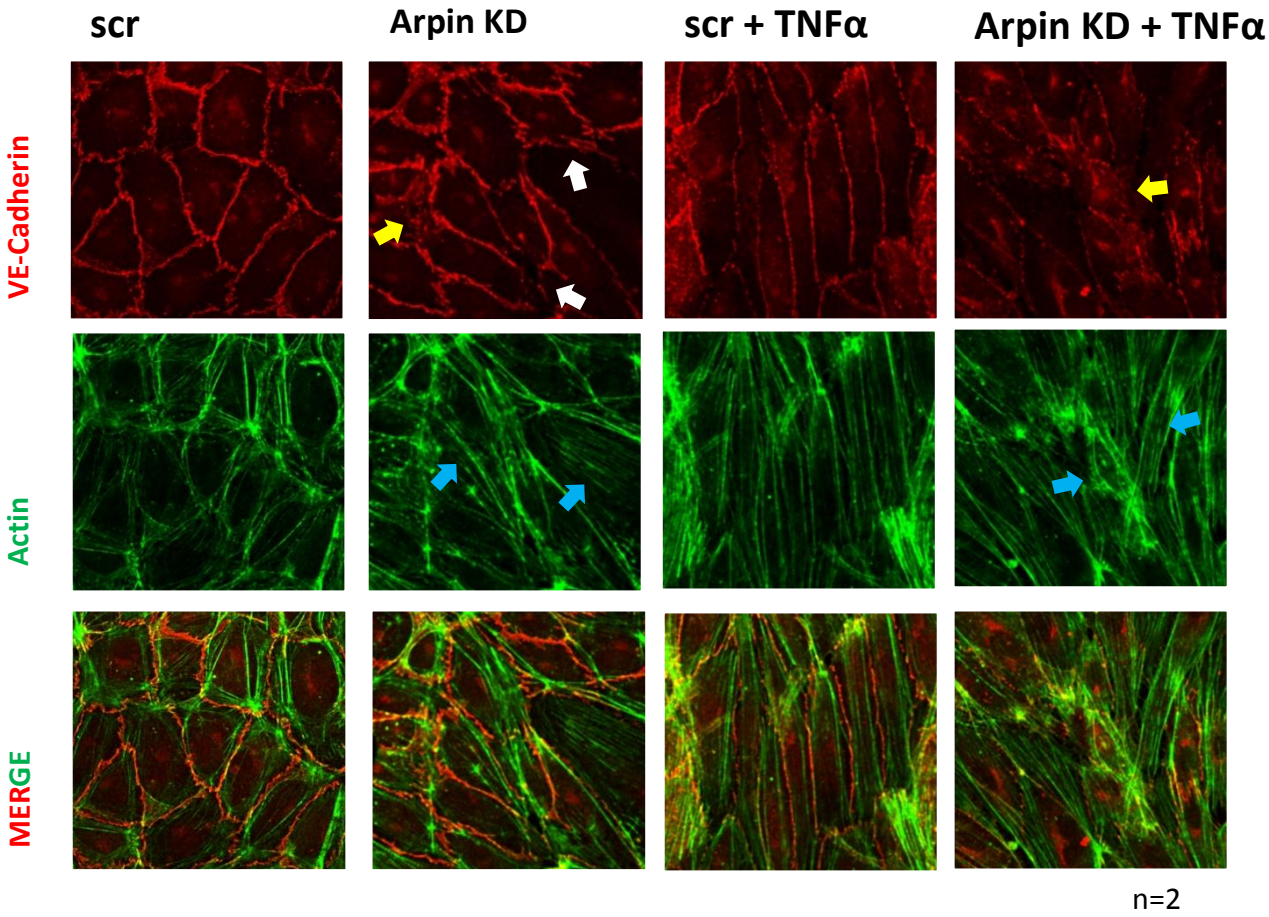


Figure 18. Arpin-depletion in HUVEC alters localization of VE-cadherin and generates actin fibers. HUVEC stained for VE-Cadherin in red and F-actin using Alexa 488-Phalloidin in green. Under basal conditions arpin-depletion causes some VE-cadherin gaps (Top panel, white arrows) and presence of VE-cadherin in the cytoplasm (Top panel, yellow arrow). Moreover, arpin-depletion causes more actin fibers crossing the cell body (Middle panel, blue arrows). After TNFα treatment, VE-cadherin seems to be more internalized (Top panel, yellow arrow) and even more actin fibers form than under basal conditions (Middle panel, blue arrows). Bar= 20 μm

Arpin-depleted HUVEC have increased permeability

Given these changes in the distribution of VE-cadherin and the remodeling of the actin cytoskeleton in the arpin-depleted HUVEC, I was next investigated

whether arpin depletion would affect permeability. *In vitro* filter-based permeability assays showed that arpin-depleted HUVEC are more permeable compared to control cells under basal conditions (Figure 19). Of note, no additional increase in permeability was observed after treatment of arpin-depleted HUVEC with TNFα suggesting that the basal increase in permeability is already maximal. All these results suggest that arpin participates in the correct localization of VE-Cadherin and thus in the regulation of vascular permeability. To unravel whether these effects might be due to arpin-dependent effects on Arp2/3, we decided to analyze junction regulation using a pharmacological Arp2/3 inhibitor and to compare the effects.

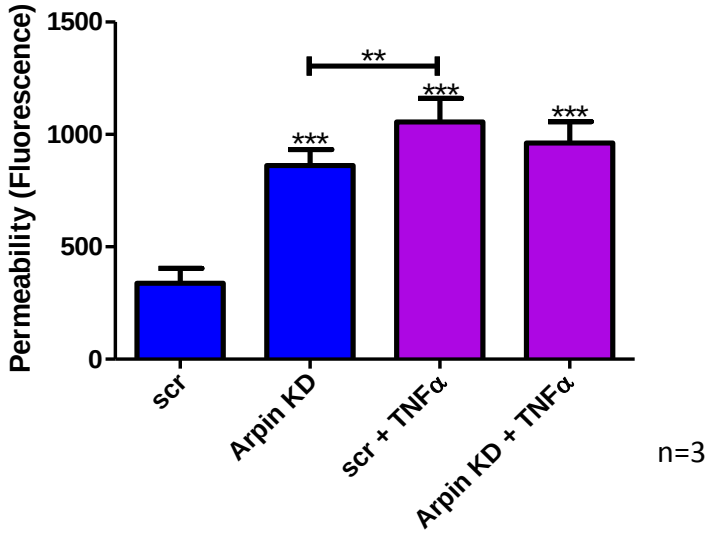


Figure 19. Depletion of arpin increases permeability. Under basal conditions arpin-depletion in HUVEC increases permeability, but no additional increase in permeability is observed under inflammatory conditions. Data represented as mean plus SEM **p<0.01, ***p<0.001.

Inhibition of the Arp2/3 complex by CK-666 increases endothelial permeability.

As a second approach to study Arp2/3 inhibition, it was performed *in vitro* filter-based permeability assays with HUVEC treated with the Arp2/3 complex inhibitor, CK-666, or its vehicle DMSO under basal and inflammatory conditions

(Figure 20). Surprisingly, the inhibitor caused a significant increase in permeability under basal conditions. Of note, with the administration of TNFα and CK-666, there was an additional increase in permeability.

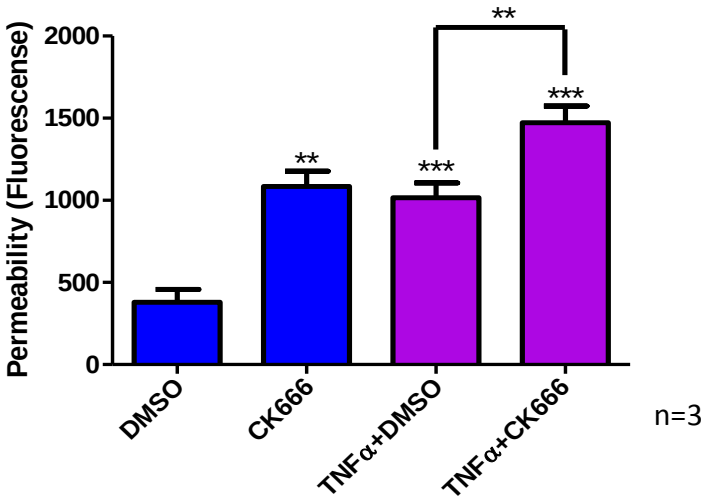


Figure 20. Inhibition of the Arp2/3 complex by CK-666 increases permeability under basal and inflammatory conditions. Under basal conditions, CK-666 increases permeability. Moreover, under inflammatory conditions, TNFα+CK-666, causes an even more pronounced increase in permeability. Data are represented as mean plus SEM **p<0.01, ***p<0.001.

CK-666 does not alter junction protein expression

In order to analyze the mechanisms causing this increase in permeability with CK-666, I next examined the expression of different junction proteins. Western-blots for VE-Cadherin, ZO-1, occludin, claudin-1 and the adhesion molecule ICAM-1 revealed no differences in total protein amounts after treatment with CK-666 neither under basal nor inflammatory conditions (Figure 21).

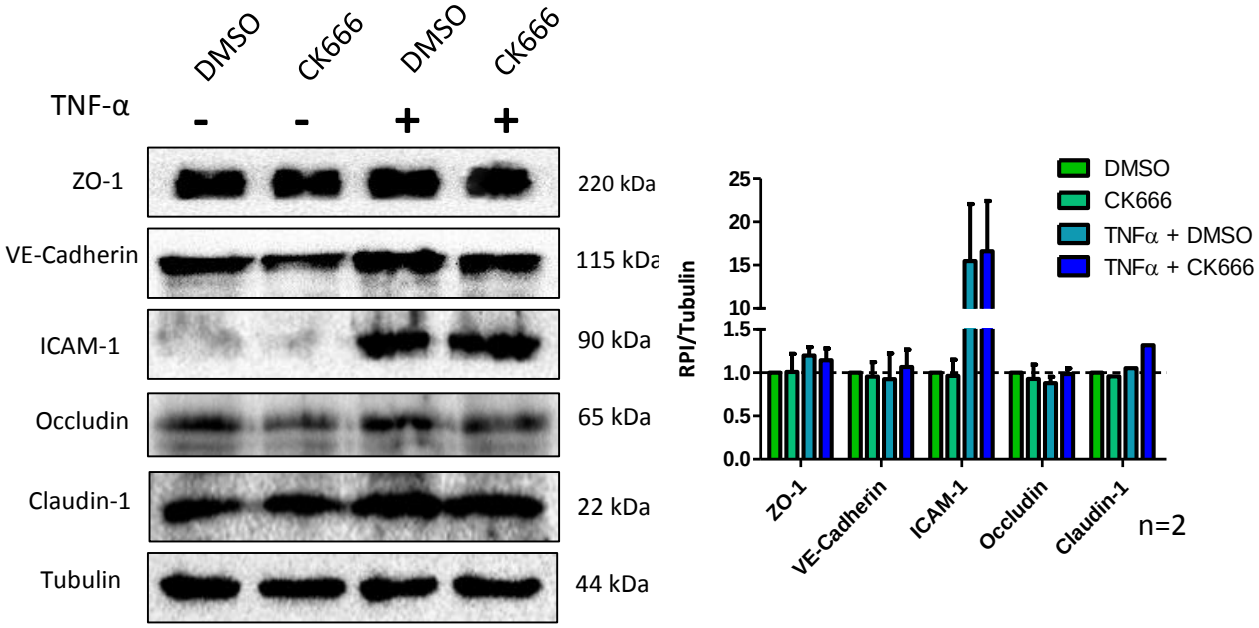
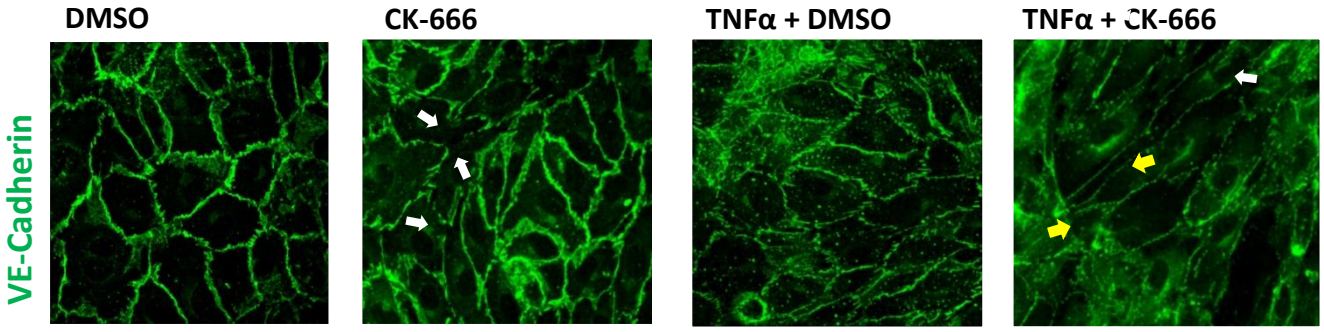


Figure 21. CK-666 administration does not change the expression of junction proteins or the expression of the adhesion molecule ICAM-1. Representative blots for junction proteins in HUVEC treated with CK-666; with tubulin as loading control. The graph on the right shows relative pixel intensity of each protein normalized with tubulin.

CK-666 causes VE-cadherin gaps

Next, I performed immunofluorescence stainings for VE-cadherin and actin. HUVEC monolayers treated with CK-666 showed formation of VE-cadherin gaps at cellular junctions even under basal conditions (Figure 22, white arrows) and some cells have the presence of VE-cadherin in the cytoplasm. Moreover, in some parts VE-cadherin staining seemed even thicker. On the other hand, in HUVEC monolayers treated with both CK-666 and TNFα, it was observed a stronger destruction of VE-cadherin contacts including more VE-cadherin gaps and more presence of VE-cadherin in the cytoplasm compared to HUVEC treated with vehicle and TNFα. Thus, it is likely that these changes in junction architecture are causing the increased permeability observed after CK-666 treatment.

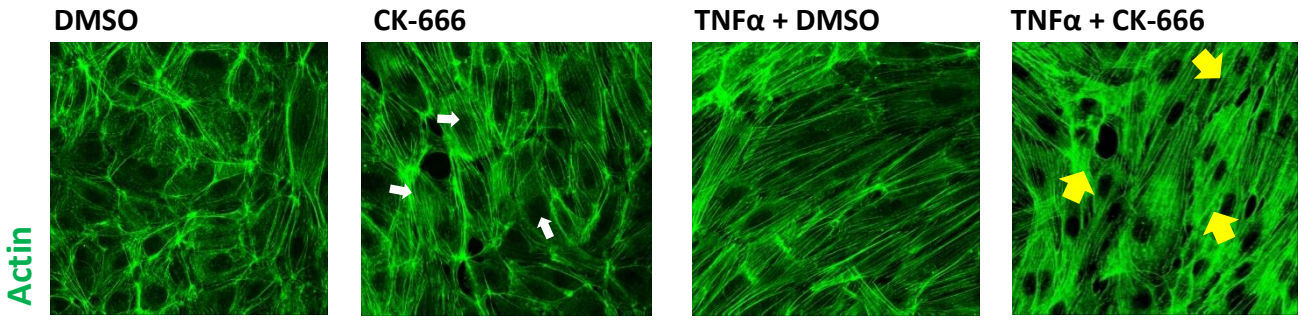


n=3

Figure 22. Treatment of HUVEC with CK-666 causes junction gaps, and VE-cadherin internalization. CK-666, under basal conditions, causes VE-cadherin gaps (white arrows). Under inflammatory conditions, CK-666 causes more VE-cadherin gaps (white arrows) and more VE-Cadherin internalization into the cytoplasm (yellow arrows) compared to HUVEC treated with CK-666 or TNFα+Vehicle (DMSO). HUVEC stained for VE-Cadherin in green. Bar= 20 µm

CK-666 induces formation of actin fibers

To analyze Arp2/3 inhibition effects on endothelial actin remodeling, it was performed actin staining using phalloidin conjugated to Alexa-488. Apparently, CK-666 treatment caused increased formation of actin fibers crossing the cell body (Figure 23, white arrows). On the other hand, TNFα treatment causes the expected cell elongation and stress fiber formation. Of note, HUVEC treated with both TNFα and CK-666 showed even more actin fibers (Figure 23, yellow arrows) and a loss of actin in the adhesion zones or intersection actin zones, suggesting an important role of the Arp2/3 complex in maintaining the endothelial barrier junctions.



n=1

Figure 23. Treatment of HUVEC with CK666 causes increased actin fiber formation. Under basal conditions, CK-666 causes some actin fibers formation (white arrows), but under inflammatory conditions, CK-666 causes more actin fibers crossing the cell body (white arrows) compared to HUVEC treated with TNFα+vehicle. HUVEC stained for F-actin using Alexa 488-Phalloidin. Bar=20 μm

CK-666 reduces neutrophil transendothelial migration

Finally, CK666 was used to examine the role of endothelial Arp2/3 during neutrophil recruitment. *In vitro* filter-based neutrophil transmigration assays using monolayers of endothelial cells treated with TNFα and CK-666 showed a significant decrease in the numbers of transmigrated neutrophils (Figure 24). Given that the number of transmigrated neutrophils is very low without an inflammatory stimulus, CK-666 did not affect neutrophil transmigration under basal conditions.

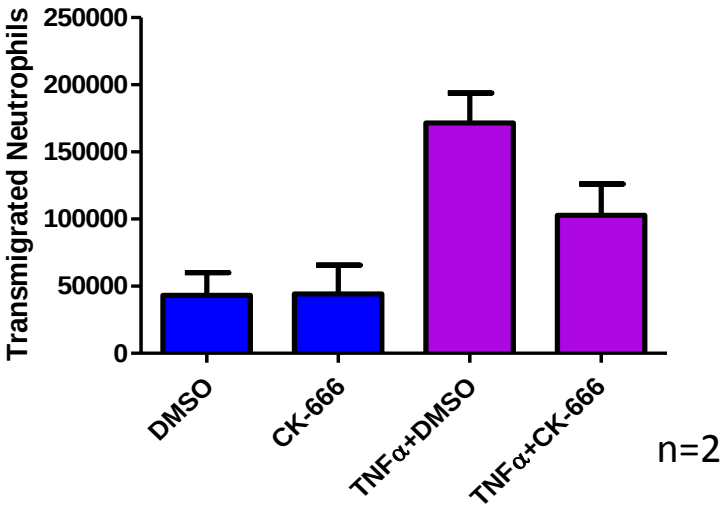


Figure 24. CK-666 reduces neutrophil transmigration during inflammation. As a preliminary result, CK-666 does not affect neutrophil transmigration under basal conditions, but under inflammatory conditions CK-666 reduces neutrophil transmigration.

DISCUSSION

In this project, I studied the effects of Arp2/3 inhibition on endothelial barrier functions using two different approaches: First, we studied the role of arpin, the most recently discovered Arp2/3 inhibitor; and second, we used the small molecule inhibitor of the Arp2/3 complex, CK-666. We chose to analyze arpin as it can antagonize the activity of the NPF WAVE2 at lamellipodial tips [43], and previous data demonstrated the important role of WAVE2 for the regulation of endothelial junction architecture and barrier integrity [38]. We hypothesized that arpin could be closely related with the regulation of both junction architecture and endothelial permeability. On the other hand, we chose to work with CK-666 because it is a specific inhibitor of the Arp2/3 complex and it has become a valuable tool to study Arp2/3 complex functions because the deficiency of any of the Arp2/3 complex subunits is lethal [45]. Moreover, we decided to pursue both approaches because it has not yet been ruled out that arpin may function independently of its ability to inhibit Arp2/3. Arpin depletion can theoretically be considered as an activation or gain-of-function of the Arp2/3 complex; whereas CK-666 is considered as inhibition or loss-of-function" of Arp2/3. Surprisingly, with both approaches we found similar results, i.e. VE-cadherin gap formation and internalization, increased formation of actin fibers and increased permeability. The data seem counterintuitive, but may point to novel and interesting Arp2/3-independent functions of arpin in endothelial cells as discussed below.

Here, we showed for the first time that arpin-depleted HUVEC generated more actin fibers, which resembled the structure of stress fibers, which are cross-linked actin filament bundles, that span a length of 1-2 micrometers [46]. Even, there are four types of actin fibers, dorsal stress fibers, ventral stress fibers, transverse arcs, and the perinuclear actin cap [47], it is just observed several ventral stress fibers with a few dorsal stress fibers in arpin-depleted HUVEC. A similar result was seen in HUVEC treated with CK-666, in which we observed actin fibers crossing the cell body or ventral stress fibers. This finding using

CK-666 makes sense as Arp2/3 inhibition would prevent formation of branched actin filaments leaving more G-actin available for spire, formins and other actin nucleators to form linear fibers and/or stress fibers [24]. In order to prove that the observed filaments are indeed contractile stress fibers that contribute to endothelial barrier dysfunction due to the pulling tension forces they generate, we have to measure the levels of ROCK1, MLCK and phosphorylated myosin light chain (MLC) [48]. In the case of arpin-depleted HUVEC, such explanation would not apply, given that arpin depletion should theoretically be accompanied by more active Arp2/3 given its known role as Arp2/3 inhibitor. Thus, in HUVEC, it seems more likely that arpin is only a weak inhibitor of Arp2/3 in endothelial cells and may affect actin remodeling differently. To unravel how arpin depletion triggers actin fiber formation in endothelial cells will be a challenging task for the future.

Besides the actin cytoskeleton, junction architecture controls endothelial barrier functions. Neither arpin-depletion nor CK-666 treatment modified the total protein expression of the junction proteins VE-Cadherin, ZO-1, claudin-1 and occludin. Of note, using both approaches, immunofluorescence stainings of VE-Cadherin revealed significant VE-Cadherin gaps and internalization. Interestingly, arpin depletion caused more VE-cadherin internalization under both basal and inflammatory conditions than CK-666. It is well known that VE-cadherin is essential for junction formation and endothelial barrier maintenance [4, 49]. Several studies on VE-cadherin and actin have contributed to the concepts for the regulation of cell permeability. There are four main routes involving post-translational modifications of VE-cadherin and actin cytoskeleton remodeling that modulate VE-cadherin internalization and vascular permeability under inflammatory conditions: (1) phosphorylation by PAK (p21-activated kinase), Scr and FAK (focal adhesion kinase) in the C-terminal domain leads to dissociation of the VE-cadherin/catenin complex and loss of connection to the actin cytoskeleton; (2) p120-catenin and VE-cadherin phosphorylation by several signaling pathways such as PI3K, Src and PAK signaling, leads to internalization via clathrin-coated vesicles; (3) mechanical

forces exerted by RhoA-induced actin stress fibers that weaken VE-cadherin-mediated cell-cell adhesion; and (4) intracellular cleavage of VE-cadherin by the metalloproteinase ADAM-10 [50, 51]. The Arp2/3 complex is known to be involved in clathrin-coated vesicle formation and actin remodeling at junctions to regulate cell adhesion [49, 52]. Thus, Arp2/3 complex and VE-cadherin are both closely involved in endothelial barrier maintenance and vascular permeability regulation suggesting that Arp2/3 inhibition by CK-666 alters barrier functions by controlling VE-cadherin adhesions, maybe by controlling mechanical forces exerted by the actin cytoskeleton at AJ. On the other hand, arpin-depletion also causes VE-cadherin gaps and internalization. These results suggest that arpin could influence VE-cadherin adhesion strength at AJ by mechanisms independent of Arp2/3 inhibition to maintain the endothelial barrier. One possible mechanism is direct binding to VE-cadherin in order to interfere with the sites of phosphorylation or ubiquitination that could prevent VE-cadherin/catenin dissociation and VE-cadherin internalization or degradation. Bioinformatics analyses revealed a possible direct interaction of arpin with E-cadherin. Given that E-cadherin and VE-cadherin have high structural similarity in its C-terminal domain, it is possible arpin could maintain endothelial stability by directly binding the intracellular VE-cadherin domain and stabilize its localization within junctions.

Increased actin fiber formation together with increased VE-cadherin internalization suggests that endothelial permeability would be increased. In permeability assays, indeed both arpin-depletion and CK-666 administration increased permeability, which is line with the above discussed findings. Previous studies reported that lung endothelial cells treated with CK-666 had decreased electrical resistance and increased permeability to 70kDa FITC-avidin, which is in agreement with our finding [53]. Arpin-depletion also caused an increase in permeability, which is not surprising given that it also induces VE-cadherin gaps, however, this finding suggests again that arpin may serve roles other than Arp2/3 inhibition in endothelial cells. On the other hand, under inflammatory conditions, arpin-depletion did not further increase permeability

compared with basal conditions suggesting that the basal increase in permeability is already maximal due to the VE-cadherin gaps caused by arpin deficiency alone; i.e. TNF-induced VE-cadherin gaps do not add significantly to the existing gaps in arpin-depleted HUVEC. However, HUVEC treated with CK-666 and TNFα increased the permeability even more compared to HUVEC administrated with TNFα and the vehicle suggesting that the gaps caused by CK-666-mediated Arp2/3 inhibition are not sufficient to induce maximal permeability as seen after an inflammatory stimulus. Similar results were found in human lung microvascular endothelial cells (HLMVECs), in which co-treatment with CK-666 and thrombin increased intercellular gap formation and increased permeability even more that just treatment with CK-666 or thrombin alone [53]. These data together clearly demonstrate that Arp2/3 activity is essential for maintenance of endothelial barrier stability and vascular permeability under both basal and inflammatory conditions. However, our data also support the idea that arpin may act through mechanisms independent of Arp2/3 inhibition to regulate endothelial barrier stability. We think that arpin could regulate endothelial barrier stability under basal conditions by binding to VE-cadherin in its C-terminal domain to regulate post-translational modifications. To test this idea, we are planning to perform experiments including immunocytochemistry and advanced microscopy techniques such high resolution 3D-SIM and FRET, and co-immunoprecipitations of arpin and VE-cadherin. Besides, recording live-cell images and videos using confocal microscopy after different stimulations will be helpful for unraveling in detail arpin functions in endothelial cells.

We also analyzed leukocyte transmigration, which is another inflammatory process actively supported by endothelial cells. *In vitro* assays surprisingly revealed that CK-666 reduced neutrophil transmigration across HUVEC. This finding is in the first instance also counterintuitive because HUVEC treated with TNFα and CK-666 had increased permeability, but neutrophils were not able to exploit the open cell contacts. Similar results have been previously observed in cortactin-depleted endothelial cells [54], in which cortactin deficiency caused

an increase in permeability but impaired neutrophil transmigration. In this study, cortactin-depleted endothelial cells were unable to cluster ICAM-1 and to correctly support neutrophil adhesion on their apical surface so that neutrophils were not able to reach the open contacts. Thus, it will be important to analyze the formation of ICAM-1 clusters CK-666 treated HUVEC because similar mechanisms may occur after Arp2/3 inhibition as ICAM-1 clustering is actin-dependent. Total expression of ICAM-1 in TNF and CK666-treated was not changed, but localization and clustering might be affected so that we need to perform cytochemistry in HUVEC-neutrophils co-cultures. Such experiments will then also be important to perform with arpin-depleted HUVEC to unravel whether arpin affects neutrophil transmigration, and whether this occurs in a similar fashion as after Arp2/3 inhibition. Moreover, intravital microscopy of inflamed and CK-666-treated cremaster muscles will be performed to analyze which step of the extravasation cascade is regulated by the Arp2/3 complex (i.e tethering, rolling, adhesion, or diapedesis).

CONCLUSIONS

In summary, our data support that the correct regulation of the Arp2/3 complex is important for the maintenance of the endothelial barrier functions. Our data also suggest that arpin has a potential role of sustaining endothelial barrier stability by a mechanism independent of Arp2/3 inhibition.

PERSPECTIVES

- To analyze leukocyte transmigration using arpin-depleted HUVEC
- To investigate the mechanisms of VE-Cadherin internalization without arpin and with CK-666
- To examine other tight junction proteins and PECAM-1 in arpin-depleted HUVEC and after CK-666 treatment
- To analyze by intravital microscopy recruitment of neutrophils using CK666
- To unravel mechanisms of arpin action independent of Arp2/3 inhibition

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