The role of actin-binding proteins in the control of endothelial barrier integrity

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Summary
The endothelial barrier of the vasculature is of utmost importance for separating the blood stream from underlying tissues. This barrier is formed by tight and adherens junctions (TJ and AJ) that form intercellular endothelial contacts. TJ and AJ are integral membrane structures that are connected to the actin cytoskeleton via various adaptor molecules. Consequently, the actin cytoskeleton plays a crucial role in regulating the stability of endothelial cell contacts and vascular permeability. While a circumferential cortical actin ring stabilises junctions, the formation of contractile stress fibres, e.g. under inflammatory conditions, can contribute to junction destabilisation. However, the role of actin-binding proteins (ABP) in the control of vascular permeability has long been underestimated. Naturally, ABP regulate permeability via regulation of actin remodelling but some actin-binding molecules can also act independently of actin and control vascular permeability via various signalling mechanisms such as activation of small GTPases. Several studies have recently been published highlighting the importance of actin-binding molecules such as cortactin, ezrin/radixin/moesin, Arp2/3, VASP or WASP for the control of vascular permeability by various mechanisms. These proteins have been described to regulate vascular permeability under various pathophysiological conditions and are thus of clinical relevance as targets for the development of treatment strategies for disorders that are characterised by vascular hyperpermeability such as sepsis. This review highlights recent advances in determining the role of ABP in the control of endothelial cell contacts and vascular permeability.

Keywords
Actin cytoskeleton, cell-cell interactions, endothelial cells, GTPases, inflammation, vascular permeability

Introduction: Vascular permeability and the actin cytoskeleton
The endothelium lines the vasculature throughout the body and is an important barrier of the innate immune system that separates the blood stream from underlying tissues. The endothelium is comprised of endothelial cells that are connected via integral membrane structures called junctions (1). In the endothelium two major types of junctions are known, tight junctions (TJ) and adherens junctions (AJ), both of which are necessary to form a stable but selective semi-permeable endothelial barrier (2, 3). This barrier needs to be tightly controlled to allow passage of substances needed by the underlying tissue and to concomitantly prevent excessive leakage of fluids and substances that may harm underlying tissues. On the other hand, the endothelial barrier needs to be dynamic for example during acute inflammation to allow recruitment of leukocytes and resolution of inflammation (4, 5). Continuous dysfunction of the barrier as occurring during severe pathological conditions such as sepsis, ischaemia or diabetes can in some cases lead to acute organ failure (6, 7). Thus, a better understanding of the molecular mechanisms controlling vascular permeability is needed for the development of novel treatment strategies that restrict endothelial hyperpermeability under these conditions.

TJ and AJ do not only form the actual endothelial barrier by mediating selective cell-cell adhesion but they also provide mechanical strength by connecting the plasma membrane and adjacent cells to the actin cytoskeleton (8). In order for the barrier to be dynamic, this mechanical strength needs to be altered by controlled actin remodelling. Actin dynamics are crucial for the regulation of endothelial barrier stability and vascular permeability (9). The best known mediators that control actin remodelling related to endothelial barrier function are small GTPases of the Rho family that are activated in response to a plethora of different stimuli (10). However, other molecules are involved in the regulation of GTPase activation cycles, actin dynamics and junctional remodelling to control vascular permeability. Among such molecules are actin-binding proteins (ABP) that help to coordinate actin remodelling (11). ABP can directly bind actin and affect actin remodelling by several modes of action e.g. actin filament polymerisation,
branching or severing (▶Figure 1). Moreover, these molecules have recently garnered a lot of attention for their potential to act at various steps of the intracellular signalling machinery that controls endothelial barrier stability e.g. up- and downstream regulation of GTPase activation. Other actin-binding molecules 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 (▶Figure 2). Thus, actin-binding molecules have various playgrounds to exert influence on endothelial barrier stability. This review will highlight recent advances that broaden our understanding of how actin-binding molecules control signalling mechanisms and actin remodelling to regulate endothelial barrier homeostasis and vascular permeability and how these processes are altered under pathological conditions.

Classes of ABP and their impact on the actin cytoskeleton

The actin cytoskeleton is a complex and dynamic network that needs to be remodelled quickly in response to extracellular stimuli. Depending on the appropriate cellular response, actin filaments can be newly assembled, existing filaments can be elongated, branched or disassembled and actin filaments can organise into contractile arrays such as stress fibres (12). The rapid turnover of the actin cytoskeleton needs to be tightly regulated for which a plethora of actin-binding molecules exist in each cell that in turn are controlled via various signalling cascades (13). To understand the relevance of ABP for actin cytoskeletal remodelling and endothelial cell contact stability, we will first give a brief overview of the different classes of ABP. The regulation of the actin polymerisation/depolymerisation cycle by actin-binding molecules is illustrated in ▶Figure 1. Because of the complex nature of this topic, we can only provide a simplified introduction here. The interested reader is referred to other recent reviews cited in the text.

Actin nucleators

Actin nucleators are required to assemble actin filaments de novo or to produce branches on existing filaments. Production of new filament ends can be initiated by either generating free barbed ends via severing of existing filaments, by nucleating branches on existing filaments, by nucleating filaments de novo from monomers, or by uncapping capped filaments. The relative contribution of these processes to the generation of actively growing filaments is still controversial, but there is common agreement about the outstanding importance of actin nucleators for enhancing filament assembly. Nucleators are subdivided into the Arp2/3 complex that requires nucleation promoting factors to become activated, formins and tandem monomer-binding nucleators (13).

Arp2/3 complex

The actin-related protein (Arp) 2/3 complex has long been recognised as an important regulator of actin dynamics (14). Arp2/3 exhibits actin nucleating activity and promotes branching of newly polymerised actin filaments at cortical cell areas (▶Figure 1). Such branches are crucial for the formation of 3D-actin-filament networks. Filament binding and tyrosine phosphorylation of Arp2 increase nucleation activity (15). However, to become fully activated, interaction with nucleation promoting factors (NPFs) is required as described below:

Nucleation promoting factors (NPFs)

In order for the intrinsically inactive Arp2/3 complex to nucleate a new branch, it needs to be activated by NPFs. Two classes are distinguished according to their domain structure. The common feature of class I NPFs is a WH2-domain that binds G-actin (W), a connecting motif (C) and an acidic motif (A) that serves as a scaffold to unite the Arp2/3 complex, an existing filament and G-actin monomers to form a new daughter filament (16). Binding of class I NPFs to Arp2/3 causes a conformational change that triggers the nucleation activity of Arp2/3.

By contrast, class II NPFs lack WH2 domains but instead contain a repetitive sequence motif that can bind directly to F-actin. Additionally, they contain an N-terminal acidic domain that binds Arp2/3 and a C-terminal src-homology-3 domain (SH3) that can bind to other regulatory molecules, for example class I NPFs. Importantly, they stabilise newly branched filaments by preventing the dissociation of Arp2/3 (▶Figure 1). The most prominent member of class II NPFs is cortactin that has been shown to be involved in different cellular processes such as lamellipodia formation, endocytosis, organisation of cell-cell junctions and barrier stability (17). However, a recent study indicated that cortactin does not activate Arp2/3 in vivo but that it rather gets recruited to sites of established Arp2/3 activity maybe to stabilise newly branched filaments (18).

The group of class I NPFs consists of more proteins compared to class II NPFs. For example, members of the Wiskott-Aldrich syndrome protein (WASP) family (WASP in haematopoietic cells and the ubiquitous neural-WASP (N-WASP) were identified as main regulators of Arp2/3 complex activity by serving as a molecular scaffold that converts various cellular signals into actin polymerisation (19). Release of N-WASP from an autoinhibited state and activation of its nucleation promoting capacity requires binding of the active small GTPase cdc42 (20).

Another important group of class I NPFs are the WASP-family verprolin-homologous proteins (WAVE1–3). In contrast to N-WASP, WAVEs are not auto-inhibited but are instead regulated by the small GTPase Rac1 and phosphorylation since tyrosine phosphorylation of WAVE1 by src increased its affinity for the Arp2/3 complex (21).

Formins

Formins control the nucleation and polymerisation of new actin filaments or elongation of existing filaments but are not involved in actin branching (▶Figure 1). All formins are characterised by the presence of formin-homology (FH) domains FH1 and FH2
that are required for interactions with profilin-G-actin (FH1) and the barbed end of a growing actin filament (FH2) (22). The formin family is composed of seven subfamilies: mammalian diaphanous (mDia) 1–3, formin-related gene in leukocytes (FRL, or nowadays more commonly FMNL), formin homology domain proteins (FHOD), formins (FMN), disheveled-associated activators of morphogenesis (DAAM), delphilin and inverted formins (INF). In mammalian cells, formins are involved in a variety of important cellular processes such as filopodia and lamellipodia formation (23), assembly of stress fibres and actin networks for vesicular transport (24), cell adhesion (25) and stabilisation of endothelial junctions as described below.

**Actin delivery/recycling**

For efficient actin polymerisation to occur, G-actin needs to be constantly provided in sufficient amounts for rapid filament formation. Actin monomers are recycled after depolymerisation of filaments to be reused when filaments form again. The process of constant actin filament turnover driven by ATP hydrolysis is known as treadmilling (26). The delivery and recycling of actin monomers is regulated by actin-binding molecules (Figure 1). Profilin is responsible for actin delivery. It binds G-actin bound to GTP and this complex preferentially binds to barbed ends of growing filaments (27).

Proteins involved in the recycling of actin subunits are CAPs (adenylyl cyclase-associated protein) and thymosin-β4. Although these proteins have long been known, their physiologic relevance in cell biology is only beginning to be understood and their relevance for endothelial barrier stability has not yet been studied.

**Actin depolymerisation factor-homology domain (ADF-H)-containing proteins**

Five groups of proteins are characterised by an actin-depolymerising factor homology domain: ADF/Cofilins, twinfilins, drebrins, coactosins and glia maturation factors (GMFs) (28). The ADF-H domain was first discovered in the actin severing protein ADF/Cofilin, hence its name, but not all of these proteins function as actin depolymerising factors. The ADF-H domain can bind to both G- and F-actin and structural differences in the surface exposure of certain amino acid residues determine the preference for either G- or F-actin. While cofilin interacts with both G- and F-actin, twinfilin preferentially binds G-actin, drebrin and coactosin bind F-actin and GMFs do not bind actin but rather Arp2/3. Cofilin can bind to and sever existing filaments to create barbed ends for new filament growth (29) and, in general, contributes to continuous, dynamic actin turnover (Figure 1). By contrast, twinfilin, which contains two ADF-H domains, sequesters free G-actin, caps barbed filament ends and thus inhibits actin poly-
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merisation. Drebrin stabilises F-actin predominantly in dendritic spines (30) but has also been implicated in the regulation of cell-cell junctions (31).

**Actin capping**

To prevent constant growth of actin filaments, barbed ends can be blocked by capping proteins (▶Figure 1). Proteins that trigger uncapping release barbed ends for further filament growth (32). Capping plays an important role for the resulting architecture of actin networks. For example, when capping protein (CP) caps barbed ends so that existing filaments are not available for growth, Arp2/3-driven actin branching becomes preferred (33). Other prominent members of the actin capping protein group are CapZ, tropomodulin, gelsolin and Eps8. Capping can occur at both barbed and pointed ends but capping proteins seem to be specific for one side of the filament. CapZ, for example, binds to the barbed end whereas tropomodulin rather binds to the pointed end. Capping can be prevented by other actin-binding molecules such as VASP that promotes filament elongation. The versatility of capping proteins makes them important actin regulators and more physiological and pathophysiological processes in which they control actin dynamics are likely to be discovered in the future.

**Actin crosslinking**

Besides actin branching, the formation of three-dimensional actin networks can be achieved by crosslinking actin filaments into parallel bundles or orthogonal meshworks (34). Crosslinking is mediated by a group of proteins most of which contain at least two distinct actin-binding domains allowing for parallel binding of two different filaments (▶Figure 1). In the case of only one actin-binding domain, crosslinkers contain other domains that allow oligomerisation so that the oligomer can connect different actin filaments. The organisation of the domain structure within an actin-crosslinking protein determines the structure of the resulting actin meshwork. Important actin crosslinkers are α-actinin, fascin, eplin, filamins, spectrin and fimbrin. Each of these forms different actin structures. Actin crosslinking leads to strong actin networks that contribute to the stabilisation of endothelial barriers as discussed below.

**Actomyosin contraction**

Contractile actomyosin stress fibres are actin bundles that are important during various cellular processes. Stress fibres can be further crosslinked by actinins and eplin and they interact with other ABP to control their functionality such as VASP and the ERM proteins (ezrin, radixin and moesin). Stress fibres are connected to focal adhesions and also TJ/AJ in endothelial cells to transduce mechanical signals to sites of cell-matrix or cell-cell contact (▶Figure 1 and ▶Figure 2). Contractility is achieved by the interaction of actin filaments with myosin motor proteins (35). Besides muscle contraction, actomyosin contractility in non-muscle cells is mediated by the non-muscle myosins I and II. While myosin I rather connects actin to membranes to control vesicular transport, myosin II crosslinks two different actin filaments in an anti-parallel fashion leading to conformational changes within the filament that are still not completely understood (36). Myosins are build up by head, neck and tail domains and are powered by ATP hydrolysis in the head domain causing a “power stroke” that moves/contracts the myosin-head-bound actin filament. Myosins are regulated by phosphorylation via MLCK and ROCK and dephosphorylation via MLCP. These enzymes are in turn regulated by small GTPases and other ABP such as the Ca-dependent proteins caldesmon and calmodulin (as discussed below). For an overview on myosin modifications in the control of actomyosin contractility, the interested reader is referred to other excellent reviews (37, 38).

In endothelial cells, stress fibres are considered destabilising because of their appearance under inflammatory conditions when permeability increases (▶Figure 2). However, stress fibres are also necessary to support junction formation and to maintain stable junctions under shear stress generated by blood flow as discussed below. Clearly, stress fibres have ambiguous roles within different cell types that depend on the spatio-temporal generation of contractile forces. For a detailed review on how mechanical forces mediated by stress fibres are controlled at endothelial junctions see references (9, 39).

**ABP that can connect junctions to the actin cytoskeleton**

Another important class of ABP connects the cytoskeleton directly to transmembrane adhesion molecules of junctions (▶Figure 2). At TJ mainly members of the zonula occludens (ZO) family of proteins are responsible for connecting claudins, occludin and JAM-A to actin filaments. By contrast, the main component of AJ, vascular endothelial (VE)-cadherin, recruits a-catenin via β-catenin to sites of AJ assembly. Thus, a-catenin connects the VE-cadherin complex to actin filaments. Induction of mechanical tension, e.g. under inflammatory conditions, causes a conformational shift in a-catenin leading to the recruitment of other actin binding molecules such as vinculin, α-actinin and eplin to reinforce AJ (40). Another scaffold molecule is afadin that connects transmembrane adhesion molecules of both TJ and AJ to the actin cytoskeleton (41). This class of ABPs can bind to both stress fibres and the cortical actin ring making them important sensors of stabilising and destabilising mechanical forces. The mechanisms how these scaffold molecules regulate endothelial permeability are discussed below.

**ABP regulate vascular permeability by various mechanisms**

Most of the above mentioned actin-binding molecules have been also implicated in the regulation of endothelial barrier function and vascular permeability. They do so by various mechanisms involving changes in actin turnover mediated by different signalling pathways. Paracellular permeability is in principal regulated by the
stability of interendothelial TJ and AJ that strongly depend on the type of actin cytoskeleton they are connected to. On the other hand, the vascular bed determines the strength of interendothelial contacts. While the blood-brain-barrier (BBB) is characterised by strong TJ and AJ, post-capillary venules have weak TJ so that AJ becomes more important to control barrier function. The actin cytoskeleton and actin-binding molecules are of utmost importance in all vascular beds to regulate endothelial barrier function and vascular permeability. Here we describe in detail recently discovered mechanisms by which actin-binding molecules contribute to the regulation of vascular permeability (summarised in Table 1).

Given the mentioned importance of the vascular context, we will also provide the type of endothelium/organ in which the described mechanisms have been discovered.

ABP connect endothelial junctions to the actin cytoskeleton to control vascular permeability

Tight junctions

TJ are comprised of claudins, occludins, junctional adhesion molecules (JAMs) and endothelial cell-selective molecule (ESAM) (42). These transmembrane adhesion molecules are connected to the actin cytoskeleton via members of the Zonula occludens family of proteins (ZO-1–3) that are the most important ABP that connect TJ to the actin cytoskeleton. However, they can also bind to catenins and thus connect TJ with AJ (43). It is well known that inflammatory mechanisms cause TJ disruption, internalisation of ZOs and actin remodelling whereas anti-inflammatory treatments prevent these effects (44, 45) (Figure 2). For example, monocyte-chemoattractant protein-1 (MCP-1), a pro-inflammatory chemoattractant, induced hyperpermeability and stress fibre formation via RhoA/ROCK in primary murine brain endothelial cells accompanied by redistribution of ZO-1, ZO-2, occludin and claudin-5 (46). Later, it has been discovered that MCP-1-induced redistribution of ZO-1 depended on the interaction of phosphorylated ERM proteins with ZO-1 (47). Another example is hypoxia-reoxygenation of rat brain microvascular endothelial cells (RBMEC) that also induced ZO-1 internalisation, cortical actin disruption and hyperpermeability due to production of reactive oxygen species (ROS) and activation of caspase-3. The observed effects could be ameliorated by treatment of RBMEC with the antioxidant ascorbic acid (48). Such protective effects on ZO-1, TJ integrity and thus permeability have been reported for other anti-inflammatory treatments. For example, resveratrol, an anti-inflammatory compound found e.g. in red wine, has been shown to counteract ZO-1 translocation and TJ and actin disruption in response to oxidised low-density lipoprotein in murine brain endothelial cells (49). Thus, stabilising the interaction of ZO proteins with both transmembrane adhesion molecules and the actin cytoskeleton is of vital importance for the regulation of vascular permeability.

Adherens junctions

AJ are the most prominent barrier within most interendothelial cell contacts and are formed primarily by VE-cadherin that can bind to p120-catenin via its juxtamembrane domain and to β-catenin via its C-terminal domain. Embryos lacking VE-cadherin are unable to develop a proper vascular system and die in utero after 11 days (50). β-catenin recruits a-catenin that connects the VE-cadherin complex to the actin cytoskeleton (51). α-catenin also has the ability to recruit other ABP such as vinculin and eplin thus making it a central hub for AJ homeostasis (40). Indeed, preventing dissociation of α-catenin from VE-cadherin in vivo by overexpressing a VE-cadherin/α-catenin fusion protein and thus stabilising VE-cadherin-actin interactions protected the endothelial barrier against thrombin or vascular-endothelial growth factor (VEGF)-induced hyperpermeability (52). While β-catenin binds to α-catenin to maintain the interaction between VE-cadherin and the actin cytoskeleton, p120-catenin supports catenin stability within the membrane by preventing its internalisation and supporting the connection to the actin cytoskeleton (53–55). Recently, N-WASP has been shown to directly bind to p120-catenin to link the VE-cadherin complex to cortical actin (56). N-WASP-p120-catenin interaction also triggered cortical actin formation via Arp2/3 and depletion of N-WASP led to a reassembly of cortical actin into stress fibres and increased permeability. Binding of N-WASP to p120-catenin and actin and subsequent AJ stabilisation required N-WASP phosphorylation at Y-256 by focal adhesion kinase (FAK). On the other hand, FAK has also been shown to be involved in transforming growth factor-β (TGF-β)-induced vascular permeability suggesting a destabilising role for FAK on AJ (57). In this study, rapid tyrosine phosphorylation of both FAK and src occurred after TGF-β stimulation and inhibition of both kinases ameliorated TGF-β-induced hyperpermeability. TGF-β also induced reorganisation of actin and translocation of paxillin and vinculin away from focal adhesions. However, these rearrangements were not mediated by FAK (57). More recently, pulmonary endothelial cells with a stable FAK knock-down showed increased transendothelial resistance and stronger VE-cadherin and actin stainings at cell contacts suggesting a destabilising role for FAK on AJ (58). Obviously, FAK plays an important role for the regulation of vascular permeability but its precise role still remains elusive.

In a very recent study, the importance of Arp2/3 for continuous AJ has been highlighted. In human umbilical vein endothelial cells (HUVEC), Arp2/3-driven lamellipodia formation occurred at sites that temporarily lack VE-cadherin to facilitate the formation of new VE-cadherin containing adhesion sites. These lamellipodia formed in one cell and overlapped the VE-cadherin free portion of another cell’s membrane in order to form new VE-cadherin adhesion sites (59). These novel lamellipodial structures were termed junction-associated intermittent lamellipodia (JAIL). Arp2/3 was required for this effect since its inhibition prevented JAIL formation and caused gap formation instead. This study highlights the relevance of Arp2/3-driven-actin polymerisation to induce formation of adhesive structures that are required for the mainten-
Table 1: Overview of different actin-binding molecules that regulate vascular permeability by different modes of action.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Actin regulation</th>
<th>Barrier effect</th>
<th>Mode of endothelial barrier regulation</th>
<th>Ref.</th>
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<tbody>
<tr>
<td><strong>ABP that can connect junctions to the actin cytoskeleton</strong></td>
<td></td>
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<tr>
<td>α-catenin</td>
<td>Connects AJ to actin filaments</td>
<td>↑</td>
<td>Connects the VE-Cadherin complex to cortical actin to stabilise the endothelial barrier</td>
<td>(52)</td>
</tr>
<tr>
<td>Afadin</td>
<td>Connects both TJ and AJ to actin filaments</td>
<td>↑</td>
<td>Mediates crosstalk between TJ and AJ by interacting with ZO-1 and p120-catenin</td>
<td>(41)</td>
</tr>
<tr>
<td>EPLIN</td>
<td>Connects AJ to actin filaments</td>
<td>↑</td>
<td>Strengthens the barrier by facilitating vinculin recruitment to AJ and serves as mechano-transducer</td>
<td>(64)</td>
</tr>
<tr>
<td>Vinculin</td>
<td>Strengthens binding between F-actin and the VE-cadherin complex</td>
<td>↑</td>
<td>Vinculin is recruited by α-catenin, mediates connection to F-actin bundles and protects endothelial junctions FAK absence allows stable focal adhesions via vinculin to form a stable link between endothelia cells and ECM</td>
<td>(62)</td>
</tr>
<tr>
<td>ZO-1</td>
<td>Connects TJ to the actin cytoskeleton</td>
<td>↑</td>
<td>Provides mechanical strength for TJ by connecting it to F-actin. Internalisation of ZO-1 during inflammation or hypoxia destabilises the barrier</td>
<td>(45)</td>
</tr>
<tr>
<td><strong>Actin polymerisation/branching</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arp2/3</td>
<td>Actin branching</td>
<td>↑</td>
<td>MIM-dependent TEM resealing</td>
<td>(143)</td>
</tr>
<tr>
<td>Cortactin</td>
<td>Binds to F-actin and Arp2/3 complex, stabilises actin branches</td>
<td>↑</td>
<td>Occludin internalisation via coronin-1b-dependent endocytosis</td>
<td>(69)</td>
</tr>
<tr>
<td>Ezrin/radixin/moesin</td>
<td>Actin stress fibres formation</td>
<td>↓</td>
<td>Controls basal Rap1 activity levels</td>
<td>(91)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>↑</td>
<td>S1P strengthens cortactin-dependent cortical actin formation and promotes cortactin-MLCK interaction to stabilise TJ and AJ</td>
<td>(89, 90)</td>
</tr>
<tr>
<td>Drebrin</td>
<td>Binds F-actin and profilin</td>
<td>↑</td>
<td>Phospho-ERM, especially radixin strengthens the endothelial cytoskeleton to protect from barrier dysfunction</td>
<td>(97)</td>
</tr>
<tr>
<td>FHOD1</td>
<td>Nucleation and polymerisation of actin</td>
<td>↓</td>
<td>AGE-induced phosphorylation of ERM contributes to endothelial injury</td>
<td>(98)</td>
</tr>
<tr>
<td>mDia1</td>
<td>Nucleation of new actin filaments</td>
<td>↑</td>
<td>Thrombin-induced ERM phosphorylation and translocation of phosphorylated ERM to plasma membrane causes gap formation and endothelial barrier dysfunction</td>
<td>(96)</td>
</tr>
<tr>
<td>N-WASP</td>
<td>Actin polymerisation by activation of Arp2/3</td>
<td>↑</td>
<td>N-WASP phosphorylation at Y-256 by FAK promotes interaction with p120-catenin and Arp2 to induce cortical actin formation and stabilise AJ</td>
<td>(56)</td>
</tr>
<tr>
<td>Profilin-1</td>
<td>Addition of G-actin at barbed ends</td>
<td>↓</td>
<td>Sequestering of Src prevents phospho-dependent internalisation of VE-Cadherin induced by VEGF</td>
<td>(70)</td>
</tr>
<tr>
<td>VASP</td>
<td>Promotes actin filament elongation, anti-capping, recruits profilin/G-actin, inhibits branching</td>
<td>↑</td>
<td>VASP associates with all-spectrin to form a complex that triggers perijunctional actin filament assembly to improve cell-cell adhesion</td>
<td>(106)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>↑</td>
<td>VASP deficiency impairs integrin-mediated cell adhesion, focal adhesion formation, reduces anchorage of VE-cadherin and β1-integrin with the actin cytoskeleton</td>
<td>(107)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>↑</td>
<td>VASP deficiency increases permeability in vivo and in vitro probably by reducing actomyosin contractility</td>
<td>(103)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>↑</td>
<td>Coordinates proper actin cytoskeleton dynamics under shear stress</td>
<td>(105)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>↑</td>
<td>HIF-1α induces repression of VASP promoter during hypoxia leading to increased barrier dysfunction in vivo</td>
<td>(109)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>↑</td>
<td>VASP controls activation of Rac1 and formation of cortical actin in response to various stimuli</td>
<td>(103, 108)</td>
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The focal adhesion molecule vinculin has also been shown to be an important molecule for the regulation of vascular permeability. Vinculin binds to multiple focal adhesion and cytoskeletal proteins and permits transmission of mechanical forces between the actin cytoskeleton and the VE-cadherin complex (60, 61). Vinculin was recruited to AJ via α-catenin and reinforced the connection of the actin cytoskeleton with the VE-cadherin complex under inflammatory conditions (62). The authors suggest that junction remodelling under permeability-inducing conditions (treatment with either thrombin, TNF-α or VEGF) takes place at morphologically different, punctuated AJ termed focal AJ (FAJ) that are positive for vinculin and connected to contractile radial actin bundles. Similar AJ structures connecting adjacent endothelial cells with stress fibres under inflammatory conditions have been observed before and have been termed discontinuous AJ (63). Inhibition of the RhoA/ROCK pathway prevented FAJ formation whereas inhibition of vinculin recruitment with a truncated α-catenin protein did not (62). Nevertheless, absence of vinculin from FAJ greatly reduced their stability during thrombin treatment. Vinculin recruitment to AJ was facilitated by interaction of α-catenin with epithelial protein lost in neoplasm (eplin) that was also strongly expressed in endothelial cells (64). Eplin stabilises actin filaments by inhibiting both depolymerisation and

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<tr>
<td><strong>Protein</strong></td>
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<tr>
<td><em>α</em>-actinin</td>
</tr>
<tr>
<td>Dystrophin</td>
</tr>
<tr>
<td>Filamin A</td>
</tr>
<tr>
<td>Filamin B</td>
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<tr>
<td>HSP27</td>
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<tr>
<td>HSP90</td>
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<tr>
<td>IQGAP</td>
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### Actin severing

| **Protein** | **Actin filament severing and capping, G-actin sequestration** | **Barrier effect** | **Mode of endothelial barrier regulation** | **Ref.** |
| Cofilin-1 | Severing of actin filaments | † | Maintains actin realignment into FSS direction and stabilises AJ under FSS | (125) |
| Gelsolin | | † | Gelsolin-deficient mice show increased lung permeability. Plasma gelsolin interacts with S1P and inhibits S1P-induced cortical actin formation in endothelial cells | (132) (134) |

### Actin contraction

| **Protein** | **Actin filament contraction** | **Barrier effect** | **Mode of endothelial barrier regulation** | **Ref.** |
| Caldesmon | Regulation of actomyosin ATPase | † | Gets phosphorylated by p38-MAPK in response to thrombin or burn serum and induces actin contractility and hyperpermeability | (85, 86) |
| Myosin II | Contraction of actin filaments | † | Histamine-induced-MLC phosphorylation by MLCK contracts actin filaments at tight junctions leading to a breakdown of barrier stability. MLC gets phosphorylated by MRCK upon Rap1 activation to promote actin bundle and junction formation | (76) (79) |
branching (65). Eplin colocalised with the VE-cadherin complex in confluent HUVEC and interacted directly with α-catenin (64). This interaction served as a mechanotransmitter sensing the presence of contractile forces on AJ leading to the recruitment of vinculin to strengthen AJ bound to stress fibres. Thus, a pharmacological way to exploit protective vinculin and/or eplin functions could ameliorate pathologic conditions characterised by vascular hyperpermeability.

Another versatile ABP that connects the AJ transmembrane adhesion molecule nectin to the actin cytoskeleton and plays an important role in the recruitment of AJ and TJ molecules during endothelial cell contact formation is afadin (66). Afadin bound both p120-catenin and ZO-1 in a Rap1-dependent manner and mediated the protective effects of oxidised phospholipid products on endothelial barrier (41). Thus, afadin is a scaffold that can regulate the communication between AJ and TJ. Whether afadin is able to bind under different conditions to all three AJ, TJ and the actin cytoskeleton in parallel is an exciting question to be answered in future studies.

**Figure 2: Overview of different mechanisms involving actin-binding molecules that stabilise or destabilise the endothelial barrier.** Stabilising mechanisms are depicted in the left cell and destabilising mechanisms in the right cell. Endothelial junctions connect two adjacent cells with claudins and occludins belonging to TJ and VE-cadherin and nectins belonging to AJ. Arrows represent activation and barred lines inhibition. Stabilising mechanisms include signalling pathways involving the activation of small GTPases such as Rap1 and Rac1 leading to inhibition of stress fibre contractility and formation of cortical actin bundles that stabilise junctions (left panel). Destabilising mechanisms include signalling pathways induced by various stimuli such as thrombin or VEGF leading to internalisation of junction proteins, disassembly of focal adhesions and/or increased actomyosin contractility (right panel). All depicted pathways require single or concerted action of ABP, the mechanisms of which are explicitly explained in the text.
ABP modulate actin dynamic in response to various signalling pathways to regulate vascular permeability

Arp2/3 complex

Arp2/3 is regulated by extracellular stimuli to regulate barrier function. For example, methamphetamine (METH), a well-known neurotoxic drug induced an increase of endothelial permeability at the BBB in vitro and in vivo (67, 68). Mechanistically, it has recently been observed that occludin was internalised via endocytosis upon METH treatment. METH treatment induced N-WASP activation and subsequent Arp2/3 activity (69). Interestingly, METH induced phosphorylation and inhibition of the ABP coronin-1b which is a known Arp2/3 and actin branching inhibitor. Inhibiting Arp2/3 prevented METH-induced occludin internalisation and barrier dysfunction in vivo. These data stand in contrast to the recently observed Arp2/3-driven JAILs. While Arp2/3 activation with subsequent JAIL formation triggered formation of new VE-cadherin adhesion sites to maintain barrier in HUVEC, Arp2/3-induced actin branching has obviously detrimental consequences for the BBB. It is important to keep in mind that this could be explained by the different types of endothelia involved. While BBB endothelium has well-developed TJ and forms a tight barrier, HUVEC have less well organised TJ and thus form weaker barriers. In this case, the differently developed junctions may constitute different targets for Arp2/3-dependent actin dynamics. Also the different pathophysiological circumstances have to be taken into account. While Arp2/3-driven JAIL formation may be considered as a self-restorative mechanism under basal conditions, Arp2/3-driven occludin internalisation occurs after drug treatment. Consequently, experimental results with respect to vascular permeability have to be always viewed in the context of the examined vascular bed and the kind of treatment.

mDia1

The formin mDia1 has been associated with VEGF-induced endothelial permeability (70). Activation of VEGFR2 led to VE-cadherin phosphorylation at serine 665 followed by subsequent internalisation of VE-cadherin and disruption of VE-cadherin mediated cell-cell adhesion. Importantly, treatment of the cells with angiopoietin 1 (Ang1) led to RhoA-mediated mDia1 activation and mDia1-mediated sequestering of src thus blocking src activity and inhibiting VE-cadherin internalisation (70). These data implicate mDia1 as potential target for the regulation of vascular hyper-permeability. However, mDia1 is also involved in RhoA-mediated stress fibre formation in various cell types that could have potential barrier destabilising effects in endothelia (71). This raises the possibility of mDia1 also playing a role in barrier destabilisation by increasing intracellular tension mediated by stress fibres. Understanding the precise role of mDia1 in the regulation of endothelial permeability requires further studies. The described study is also an important example of how actin-binding molecules affect signal transduction and vascular permeability without directly modulating actin dynamics.

FHOD1

In vitro studies carried out in human pulmonary artery and human aortic endothelia cells showed that phosphorylation of formin homology domain protein 1 (FHOD1) by RhoA/ROCK released FHOD1 from its autoinhibited state and induced stress fibre formation (24). FHOD1 phosphorylation occurred in a RhoA-dependent fashion after thrombin treatment and FHOD1 knockdown prevented stress-fibre formation. Thrombin is known to induce endothelial permeability via RhoA/ROCK-mediated stress fibre formation so that it seems logic that FHOD1 phosphorylation is a mechanism to increase endothelial permeability. However, in this study permeability assays have not been performed as ultimate proof. Given the observed stabilising effects of the formin mDia1 as described above and the obvious destabilising effect of the formin FHOD1, the exact roles of formins in endothelial barrier control still remain elusive.

Myosin and actomyosin contraction

Myosin light chain (MLC) phosphorylation is required to induce actomyosin contractility by triggering ATP hydrolysis necessary for the power stroke of the myosin head domain that moves the bound actin filaments in an anti-parallel fashion to contract them (Figure 1B). Both stabilising and destabilising barrier effects have been described for MLC-dependent contractile stress fibres depending on the spatio-temporal exertion of tugging forces (72). The main kinases that phosphorylate MLC and thus induce contractile forces are MLC-kinase (MLCK) and Rho kinase (ROCK) (73).

In a very recent study, Abl kinase activity was required for the induction of endothelial permeability by VEGF, thrombin and histamine in vitro and in vivo. Interestingly, loss or inhibition of Abl caused activation of the barrier-stabilising GTPases Rac1 and Rap1, stabilised VE-cadherin-dependent cell contacts and decreased actomyosin contractility due to reduced MLC phosphorylation (74). By contrast, MLCK activation by Abl tyrosine kinase has been described as important mechanism for the induction of cortical actin during S1P-induced barrier enhancement in human pulmonary endothelial cells (75). MLCK phosphorylation by Abl led to increased Arp2/3-mediated actin polymerisation and recruitment of cortactin to stabilise the rearranged actin meshwork. Abl downregulation induced a decrease in S1P-mediated actin reorganisation and reduced MLCK and cortactin tyrosine phosphorylation and barrier protection. If these discrepancies may also be explained by the different cellular context and different treatments remains to be addressed.

Histamine treatment of corneal endothelial cells also led to MLC phosphorylation and hyperpermeability via MLCK activation and MLC phosphatase inhibition (76). Additionally, phosphorylated MLC was localised at TJ together with ZO-1 suggesting that histamine-induced contractile forces pull on TJ

Actin contractility and subsequent permeability of porcine aortic endothelial cells is also induced by hypoxia-reoxygenation (77). Inhibition of MLCK protected against MLC phosphorylation and...
actin contractility in response to hypoxia-reoxygenation. Increased permeability was accompanied by a decrease of Rac1 activity and concomitant increase of RhoA signalling. Interestingly, inhibition of RhoA/ROCK signalling worsened rather than ameliorated the effects of hypoxia-reoxygenation on actin contraction and permeability. By contrast, Rac1 activation rescued the observed effects. These data show an adverse effect of RhoA and Rac signalling on MLC-dependent actin contraction and vascular permeability.

On the other hand, in bovine aortic endothelial cells, the suppression of RhoA activity by the phytoestrogen genistein has recently been shown to beneficially affect barrier function (78). Genistein suppressed thrombin-induced increases in permeability via inhibition of RhoA translocation to the membrane and MLC phosphorylation. Surprisingly, these effects were dependent on PKA-mediated RhoA activation because inhibition of PKA prevented the protective effect of genistein on thrombin-induced permeability. Additionally, RhoA inhibition led to decreased thrombin-mediated MLC phosphorylation and blocked the barrier protective effect of genistein.

Another GTPase important for barrier stabilisation by reinforcement of cortical actin in response to cAMP-producing stimuli is Rap1. Rap1-induced actin remodelling has been shown to be regulated by cdc42 activation and subsequent myotonic dystrophy kinase-related CDC42-binding kinase (MRCK)-mediated MLC phosphorylation at cell-cell contacts leading to actin bundle formation. By contrast, Rap1 counteracted general RhoA/ROCK-induced MLC phosphorylation and stress fibre formation to protect from endothelial dysfunction (79). In this respect, the Rap1 interacting protein Rasip has recently in two independent publications been shown to be important for exchange protein directly activated by cAMP (EPAC)-mediated endothelial barrier stabilisation (80, 81). Rasip-Rap1 interactions are required for inhibiting RhoA signalling leading to reduced MLC phosphorylation and actomyosin contractility and increased cortical actin formation which is in turn mediated by the Rho-GAP ArhGAP29 (80). Thus, Rap1 is important for the regulation of RhoA and MLC activation to trigger different forms of actin remodelling. These are just some very recent examples on how versatile GTPase signalling can be with respect to actin contractility. For a detailed overview of the complex effects of GTPase on MLC and actin contractility, the reader is referred to other comprehensive reviews (10, 82–84).

Another actin-binding molecule involved in the regulation of vascular permeability is caldesmon which has been shown to become phosphorylated by p38 MAPK in response to thrombin (85) or treatment with burn-injury serum (86). In both studies, caldesmon phosphorylation was paralleled by MLC phosphorylation and increased stress fibre contractility. Upon phosphorylation, caldesmon formed a complex with myosin that was prevented when cells were pretreated with a p38 inhibitor. If these events occur concomitantly to MLCK and/or ROCK activation or present a ROCK/MLCK-independent pathway to induce actin contractility and hyperpermeability remains to be tested.

**Cortactin**

Barrier-protective effects of cortactin have been recognised a decade ago when cortactin had been shown to be involved in actin remodelling under shear stress (87) and to interact with MLCK to mediate the protective effects of sphingosine-1-phosphate (SIP) on pulmonary endothelial cells (88, 89). Additionally, SIP treatment provoked cortactin translocation to the cell periphery that prevented VE-cadherin and occludin internalisation and increased permeability after platelet-activating factor or bradykinin challenge (90).

We recently showed in vivo that cortactin-deficient mice indeed have increased basal permeability in the skin vasculature that could be attributed to reduced basal Rap1 activity levels because activation of Rap1 in cortactin-deficient endothelial cells was sufficient to revert the observed increases in permeability (91). Our data place cortactin upstream of Rap1 activation but the exact mechanism how cortactin affects Rap1 activation still awaits clarification. However, in ongoing studies, we discovered that cortactin-deficiency also causes increased ROCK-mediated MLC phosphorylation and stress fibre formation that contributes to hyperpermeability (unpublished data). Interestingly, transmigrating neutrophils cannot take advantage of loose endothelial contacts. The group of Dr. Luscinskas was the first to describe that reduced neutrophil transmigration in the absence of cortactin was due to defective ICAM-1 clustering (92). We could confirm these data in vivo and demonstrated that the defective ICAM-1 clustering resulted from decreased RhoG activity levels, a mechanism that had previously been described to be required for proper neutrophil transmigration (93). Overexpression of a constitutive active RhoG protein restored the ability of ICAM-1 to cluster and that of neutrophils to transmigrate in the absence of cortactin (91). However, leukocyte transmigration affects the endothelial barrier on multiple levels that are reviewed elsewhere (4). Of note, cortactin-dependent barrier regulation does not seem to depend on Arp2/3 or N-WASP that are important cortactin interaction partners. Thus, we have to assume that the ability of cortactin to regulate the activity of at least Rap1 and RhoG is more important for the regulation of vascular permeability than potential effects of cortactin on Arp2/3-dependent actin branching.

**Ezrin/Radixin/Moesin (ERM)**

ERM proteins are important actin-binding molecules that are targets for threonine phosphorylation in various signal pathways to induce actin remodelling and control vascular permeability. ERM was threonine-phosphorylated by PKC and p38 in response to TNF-α treatment of pulmonary endothelial cells leading to increased paracellular gap formation and permeability (94). Blocking activation of p38 or PKC or downregulating ERM expression prevented these effects induced by TNF-α. Similar responses have been observed after treatment with 2-methoxyestradiol (2ME) (95). First, a recruitment of phosphorylated ERM to the cell periphery and later after treatment with 2ME a colocalisation with F-actin branching points could be observed. Similar data have
been observed after thrombin treatment (96). Of note, this study reported differential effects of ERM after thrombin treatment. Silencing of moesin alone or all ERM inhibited thrombin-induced MLC phosphorylation, actin remodelling, gap formation and permeability whereas silencing of radixin alone aggravated these thrombin effects. Interestingly, S1P treatment of pulmonary endothelial cells has been shown to also induce ERM phosphorylation and translocation to the cell periphery where it contributed to S1P-mediated barrier reinforcement (97). In this case, ERM phosphorylation was dependent on both PKC and Rac1 activation and occurred rather on ezrin and moesin than radixin. Silencing of radixin alone or all ERM strongly reduced Rac1 activation and the barrier protective effects of S1P. Ezrin silencing alone partially attenuated these effects. By contrast, moesin silencing contributed to S1P-dependent barrier enhancement. These results reinforce the idea of differential roles of ERM proteins in the regulation of endothelial permeability with moesin having destabilising and radixin rather stabilising properties.

In agreement with the previous studies, threonine-phosphorylation of ERM occurred in response to advanced glycation end products (AGE), which are known to accumulate in different pathologies, and caused endothelial barrier dysfunction (98). In addition, AGE caused activation of the RhoA/ROCK pathway in human dermal microvascular endothelial cells to induce moesin phosphorylation, stress fibre formation and hyperpermeability (99). Inhibiting RhoA/ROCK activity or downregulating moesin decreased AGE-induced barrier dysfunction, thus confirming the role of moesin as major ERM protein promoting hyperpermeability.

Vasodilator-stimulated phosphoprotein (VASP)

VASP is a versatile ABP implicated in filament elongation, stabilization, branching inhibition and profilin/G-actin recruitment (100). VASP is also quite well studied in the regulation of endothelial barrier function. In 2002, the group of Dr. Colgan discovered VASP as a target for PKA that translocates to TJ upon S157 phosphorylation to mediate cAMP induced barrier stabilisation (101). Later, it was shown using VASP-deficient endothelioma cells that VASP is involved in cAMP-mediated and PKA-dependent Rac1 activation to promote barrier stabilisation (102, 103). By contrast, the barrier protective effects of PKG activation by cGMP could not be attributed to VASP phosphorylation even though VASP is also a PKG substrate (104). The physiologic importance of VASP in vivo was confirmed in 2007 (105). VASP-deficient animals died at the late embryonic stage due to lack of vascular structural integrity and oedema formation. In endothelial cells, VASP was required for proper responses to shear stress including regulation of actomyosin contractility highlighting the importance of VASP for endothelial homeostasis (105). VASP has been shown to interact with αI-spectrin at endothelial cell contacts to promote cortical actin formation and barrier stabilisation. Of note, increased permeability in VASP-KO endothelial cells could be restored by overexpression of wild-type (WT) VASP but not by overexpression of an αI-spectrin-binding site mutant VASP (106). Another mechanism contributing to reduced barrier function in VASP-deficient endothelioma is impaired integrin-mediated adhesion to the extracellular matrix and reduced focal adhesion formation suggesting that VASP is not only important at cell-cell junctions to control barrier functionality (107). VASP depletion in HUVEC also aggravated thrombin-induced permeability and delayed reassembly of junctions during recovery (108). Interestingly, VASP expression is regulated by hypoxia-inducible factor (HIF1α) during hypoxia (109, 110). Hypoxia caused increased permeability in microvascular endothelial cells that could be rescued by ectopic overexpression of VASP (109). VASP expression was also reduced in response to proinflammatory cytokines and absence of VASP aggravated endothelial permeability during acute lung injury (111). In another study, VASP has been reported to be phosphorylated in response to hypoxia in brain endothelial cells but its overall expression was not changed (112). The discrepancies again may be explained by the different type of endothelia or by different hypoxic conditions and once more highlight the importance of standardised endothelial cell culture conditions for given assays.

All these data confirm that VASP is a versatile actin regulating protein that exerts its effect on the actin cytoskeleton and vascular permeability at various subcellular locations by various mechanisms that can be regulated by differential phosphorylation or even on the transcriptional level.

α-actinin

α-actinin is an actin crosslinker that can also link the VE-cadherin complex by concomitantly binding to α-catenin and actin (113). Indeed, α-actinin contributed to endothelial barrier stabilisation in human pulmonary artery endothelial cells by mediating cortical actin rearrangement in response to S1P (114). Overexpression of an α-actinin mutant incapable of producing actin bundles in endothelial cells resulted in increased VE-cadherin mobility within the membrane and increased paracellular permeability for macromolecules (115). Thus, α-actinin rather affects actin connectivity to AJ.

Dystrophin

Dystrophin is a cytoplasmic ABP that is part of the dystrophin glycoprotein complex in skeletal and cardiac muscle membranes. It has been extensively studied because dystrophin protein defects lead to muscular dystrophies and cardiopathies (116). However, little is known about dystrophin in endothelial cells. Mice deficient for dystrophin showed increased BBB permeability in comparison to WT mice (117). Moreover, ZO-1, claudin-1 and actin stainings showed a strongly reduced and diffuse staining pattern in brains of dystrophin-deficient mice. Thus, dystrophin may be required for appropriate distribution of ZO-1 and proper cortical actin formation at cell contacts in brain vessels. Another recent study found that angiogenic responses of endothelial cells isolated from dystrophin-deficient mice are significantly impaired and paralleled by reduced nitric oxide (NO) production (118). How this
mechanism can be related to endothelial barrier regulation still remains a mystery.

Profilin-1
Profilin-1 has been well studied in the context of actin turnover in various cells but little is known about the role of profilin-1 in the regulation of endothelial barrier function. In one study, siRNA-mediated silencing of profilin-1 in HUVECs was associated with overall reduced F-actin staining and reduced assembly of focal adhesions (119). Interestingly, in VEGF-stimulated and profilin-1-depleted cells, VE-cadherin and ZO-1 translocalisation away from cell contacts was inhibited. This study suggests that profilin-1 may be required for proper T-junctions and focal adhesion maintenance. By contrast, a more recent study showed that profilin-1 was associated with endothelial cell injury induced by AGE (120). In this study, AGE-treated HUVEC12 cells showed increased expression of profilin-1 and formation of actin stress fibres that was accompanied by increased levels of ROS and ICAM-1 but decreased NO production. Profilin-1 silencing significantly ameliorated AGE-induced endothelial dysfunction. The authors proposed that AGE-induced production of ROS and activation of PKC to induce nuclear factor kB-mediated profilin-1 expression is required for AGE-driven endothelial cell injury. The discrepancies between these studies may again be due to different stimuli and cell types. More studies are required to unravel the exact role of profilin-1 in the endothelium.

Filamins
Filamins are actin crosslinkers that can act as a molecular scaffold for different molecules and are involved in several important cellular processes (121). Filamin A was reported to interact with R-Ras and this interaction was required for maintaining endothelial barrier function in human coronary artery endothelial cells (122). siRNA-mediated knockdown of R-Ras or filamin A increased phosphorylation of VE-cadherin at Y731 via src and reduced transendothelial electrical resistance (TER) and paracellular flux of FITC-dextran. Filamin A is also capable of anchoring different plasma membrane proteins to filamentous actin and could therefore be a key molecule for barrier integrity. In fact, filamin A knockdown caused changes in ZO-1, VE-cadherin and stress fibre formation in LPS-treated human lung microvascular endothelial cells and rendered both quiescent and LPS-treated cells more susceptible to endothelial dysfunction (123). However, and in contrast to VASP, filamin A was not required for PKA-dependent alleviation of LPS-induced endothelial permeability. Filamin B was identified as a histone deacetylase 7 (HDAC7) interacting partner and silencing of filamin B repressed VEGF-mediated cytoplasmic accumulation of HDAC7 and permeability in human primary endothelial cells (124). This process was dependent on PKC and ubiquitination of filamin B since PKC inhibition prevented VEGF-induced ubiquitination of filamin B and its interaction with HDAC7 while silencing ubiquitin blocked HDAC7-mediated gene expression and endothelial permeability. Thus, filamins have versatile functions in the control of vascular permeability and more studies are warranted to improve our understanding of these functions.

Cofilin-1
The actin severing and depolymerising protein cofilin is ubiquitously expressed and is required for actin reorganisation and its activity is regulated by phosphorylation at serine 3 (29). However, cofilin-1 has not been well studied in endothelial cells. A recent study investigated cofilin-1 function in endothelial barrier control under shear stress (125). Confluent monolayers of bovine aortic endothelial cells (BAECs) under flow shear-stress (FSS) exhibited a strong accumulation of phosphorylated cofilin-1 and LIMK, a kinase that phosphorylates cofilin-1, within nuclei. However, the relevance of this location has not been further explored. Overexpression of phospho-defective or phospho-mimetic cofilin-1 mutants (S3A or S3D) disturbed correct actin alignment in the direction of the applied FSS and barrier integrity as determined by gaps in VE-cadherin staining. These findings highlight the importance of cofilin-1 phosphorylation dynamics for barrier homeostasis under FSS. Inhibition of the stress kinases INK and p38 mimicked the observed effects suggesting a role for these kinases in dynamic cofilin-1 phosphorylation.

Drebrin
Drebrin is an actin binding protein that stabilises actin filaments and has been implicated in the regulation of neuronal dendritic spines and stabilisation of cell adhesion (126–128). In endothelial and epithelial cells, drebrin accumulation at AJ has been observed (128). Recently, drebrin-depleted HUVEC showed reduced TER and a higher tendency for dissociation under FSS. This was accompanied by reduced expression of the AJ molecule nectin at cell contacts while other adhesion molecules such as VE-cadherin, occludin and PECAM-1 where not affected. Thus, drebrin has a specific role in the regulation of nectin-dependent endothelial cell-cell adhesion (129). Moreover, drebrin was associated to afadin to stabilise nectin-dependent junctions by serving as a hinge between the nectin/afadin complex and the F-actin network. Obviously, drebrin is important for the regulation of endothelial barrier homeostasis under FSS. If drebrin also plays a role in stabilising AJ under various pathological conditions remains to be tested.

Gelsolin
Gelsolin is another actin-binding molecule that has severing, capping and actin-monomer sequestering abilities but has so far been little studied in endothelial cells (130). Gelsolin-deficient mice were observed to easily develop ischaemic brain injury (131) and to exhibit increased pulmonary vascular permeability compared to WT controls (132). Interestingly, gelsolin can also be secreted into the blood stream to clear circulating actin and to interact with lipidic bioactive substances such as lysophosphatidic acid or lipopolysaccharide (LPS) (133). For example, plasma hypogelsoline-
mia occurs during systemic inflammation such as sepsis. Of note, plasma gelsolin could bind to and sequester the barrier-protective molecule S1P and repressed the ability of S1P to induce cortical actin formation (134). Thus, the correct balance between plasma gelsolin and permeability-inducing (LPS) and inhibiting (S1P) substances may be a key feature for the control of vascular permeability during acute and chronic inflammations. This is an intriguing example of differential functions of an actin-binding molecule depending on its intra- or extra-cellular location.

Heat-shock proteins (HSP) HSP27-HSP90

Members of the heat shock protein (HSP) family are actin binding molecules of which HSP27 and HSP90 have been directly associated with endothelial permeability regulation. These proteins act as multichaperone complexes with a wide range of clients, many of which are involved in inflammatory responses. Inhibition of HSP90 locks HSP90 dimers in the open configuration, interferes with client interaction and can suppress certain proinflammatory mediators (135). In a murine sepsis model, HSP90 inhibition reduced inflammation and prolonged survival (136). HSP90 inhibition prevented TER decreases in bovine pulmonary arterial endothelial cells elicited by either TGF-β1, thrombin, VEGF or LPS by inhibiting MLC phosphorylation and actomyosin contractility (137, 138). HSP90 inhibition during induced permeability was also associated with reduced src activity, less VE-cadherin and β-catenin phosphorylation and overall increased barrier function. Additionally, HSP90 inhibition decreased phosphorylation of HSP27 and HSP90/HSP27 interaction in response to TGF-β1. TGF-β1 exerts its function via the receptor activin receptor-like kinase 5 (ALK5) and expression of a constitutively active ALK5 mutant resembled the above described permeability-inducing effect of TGF-β1 (139). Importantly, HSP90 inhibition protected the endothelial barrier also under these conditions. Thus, HSP90 inhibition exerts a general barrier protective effect.

Hypoxia-induced permeability has been shown to be dependent on the activation of p38 MAPK leading to increased intercellular gap formation and RhoA/ROCK-dependent stress fibre formation. However, hypoxia also causes phosphorylation of HSP27 that has a rather protective effect during hypoxia (140). Overexpression of a phospho-mimetic HSP27 also increased stress fibre formation but surprisingly protected endothelial cells from hypoxia- or TGF-β-induced loss of barrier function. Thus, even though hypoxia is associated with endothelial dysfunction it may in parallel also induce a HSP27-dependent protective mechanism that protects from complete loss of barrier functionality.

ABP regulate micro-wound healing to prevent vascular hyperpermeability

Wound healing is a fundamental process that governs maintenance of endothelial barrier function after injury. Macroscopic or, more common, microscopic wounds of the vasculature need to be closed rapidly to avoid blood loss or leakage of blood components into underlying tissues. Micro-wounds or paracellular gaps disrupting endothelial barrier function can occur for example during excessive diapedesis (141). Wound healing is generally accompanied by abundant actin remodelling controlled by concerted action of various actin-binding molecules. For example, Martinelli et al. studied endothelial junction remodelling during the formation of transcellular pores and paracellular gaps induced by diapedesis (142). Using in vitro models of inflammatory leukocyte recruitment the authors showed that endothelial cells produce new structures termed ventral lamellipodia (VL) in response to tension loss that are generated by Arp2/3-dependent actin branching to rapidly cover and close micro-wounds behind a transmigrated neutrophil. Such VL were also formed in response to mechanical microwounding. VL formation depended on the presence of active Rac1 and the actin-binding molecules IQGAP and cortactin and led to rapid re-assembly of punctual VE-cadherin adhesion sites into functional adherens junctions. Overexpression of either dominant-negative Rac1 or cortactin significantly inhibited VL protrusion and microwound closure (142). It is most likely that cortactin is required at sites of VL formation to stabilise Arp2/3-mediated actin branching and VL formation in response to Rac1 activation. However, experimental evidence for this assumption is still missing. Thus, VL-driven microwound closure depends on synchronised activity of actin-binding molecules to restore endothelial barrier integrity after micro-wounding. This study also nicely demonstrates that in addition to cellular signals inducing endothelial dysfunction per se, compromising endothelial self-restoring capabilities via targeting actin-binding molecules may be equally important events for producing vascular hyperpermeability.

In another recent study, formation of transendothelial cell macropartures (TEM) induced by the oedema toxin of Bacillus anthracis caused endothelial barrier dysfunction in vivo and in vitro (143). If not closed rapidly, TEM can lead to hyperpermeability and bacterial dissemination. Induction of TEMs required cAMP-dependent activation of both PKA and Epac and the cellular response to close TEM involved the ABP Missing-in-Metastasis (MIM), MIM was rapidly recruited to sites of TEM-induced membrane curves via its I-BAR-domain that sensed the curved membrane morphology, MIM, which contains domains for interaction with both Arp2/3 and actin, in turn recruited Arp2/3 to these sites and triggered the formation of Arp2/3-dependent actin waves that closed the TEM to restore the endothelial barrier. By contrast, siRNA-mediated downregulation of MIM led to a reduced formation of actin waves and an accumulation of TEM highlighting the importance of MIM for TEM closure. The data of these studies highlight the importance of self-repair mechanisms that are controlled by actin-binding molecules to maintain the endothelial barrier and prevent vascular hyperpermeability under inflammatory conditions or during bacterial infection.

The roundabout family of neuronal guidance genes is a group of transmembrane receptors implicated in numerous cellular functions including myogenesis, leukocyte chemotaxis, neuronal migration and angiogenesis. The endothelial-specific Robo4 has recently been implicated in the regulation of vascular permeability (144, 145). These receptors are not known to bind actin directly but have been shown to bind to and signal via N-WASP and WIP
to induce actin branching and endothelial motility (146). It is tempting to speculate that this induction of actin remodelling contributes to the observed endothelial barrier stabilisation after Robo4 activation (144). Providing experimental evidence for this notion will be an exciting future task since Robo receptors may serve as targets for the treatment of pathologic conditions characterised by hyperpermeability.

Conclusions

Clearly, the concerted action of actin-binding molecules is crucial for proper regulation of endothelial barrier functions and vascular permeability. Mechanisms of how actin-binding molecules affect the actin cytoskeleton itself have been extensively studied in various model organisms and cell types. However, data describing consequences for vascular permeability are not quite as abundant and in part contradictory depending on the vascular bed or permeability-inducing stimuli. The abundance of ABP and their regulators renders this a challenging but rewarding field of investigation. Several open questions need to be addressed in future studies to shed new light on the function of actin-binding molecules in endothelia and to assess the usefulness of ABP as targets for treatment of pathologies characterised by vascular hyperpermeability. First, how are various signalling mechanisms leading to remodelling of actin and endothelial junctions integrated in space and time by actin-binding molecules? Second, actin-binding molecules are characterised by domain structures allowing parallel interaction with various other molecules including other actin-binding molecules. Thus, what mechanisms regulate the recruitment and concerted action of multiple actin-binding molecules to control both actin and junction dynamics? Third, a lot of known mechanisms of junction regulation by actin-binding molecules have been shown in epithelia. If they also apply in endothelia still awaits confirmation in most cases. Fourth, although several knock-out mouse models exist that have confirmed a physiologic relevance for actin-binding molecules even in the vasculature, other molecules still await that confirmation. Finally, little is known from clinical studies. In this respect, tissue biopsies of patients suffering from diseases with vascular hyperpermeability need to be examined for variations in ABP expression and location.

The many studies that discovered regulatory functions of various ABP on endothelial barrier integrity render ABP attractive candidates for novel strategies to treat pathologic inflammatory conditions involving life-threatening vascular hyperpermeability. However, we have to keep in mind that targeting these proteins for therapeutic purposes requires drugs to be either delivered into the cell or to target extracellular receptors that specifically regulate ABP. In this respect, it will be crucial to unravel the importance and specificity of different signalling pathways that control ABP-mediated barrier regulation in health and disease. Given the complexity of vascular permeability regulation and the numerous ABP, we are most likely only at the beginning of understanding the exact interplay between the actin cytoskeleton and the endothelial barrier. Thus, the development of tailored ABP-specific drugs poses a challenging task. Improving (microscopic) technologies will facilitate future investigation on the interaction of actin-binding molecules with junctional complexes and actin filaments and will most likely reveal more mechanisms that contribute to endothelial barrier regulation.

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Conflicts of interest

None declared.

Abbreviations

AI, adherens junctions; ABP, actin-binding proteins; ADF-H, actin de-polymerising factor-homology domain; AGE, advanced glycan end product; ALK5, activin receptor-like kinase 5; ANG1, angiopoietin 1; ARP, actin-related proteins; BBB, blood brain barrier; CAMP, cyclic adenosine monophosphate; CAP, adenylly cyclase-associated protein; Cdc42, cell division control portal 42; CP, capping protein; DAAM, dishevelled-associated activators of morphogenesis; ESAM, endothelial cell selective adhesion molecule; EPLIN, epithelial protein lost in neoplasms; ERM, ezrin, radixin, moesin; FAJ, focal AJ; FAK, focal adhesion kinase; FH1,2, formin homology 1, 2; FHOD, formin homology domain proteins; FMNL, formin-related gene in leukocytes; FSS, fluid shear stress; HDAC7, histone deacetylase 7; HUVEC, human umbilical vein endothelial cells; HSP, heat-shock protein; ICAM-1, intercellular adhesion molecule 1; JAIL, junction-associated intermittent lamellipodia; JAM, junctional adhesion molecule; LPS, lipopolysaccharide; MAPK, mitogen-activated kinase; MCP-1, monocyte chemotactic protein-1; mDia1, mammalian diaphanous-1; 2ME, 2-methoxyestradiol; METH, methamphetamine; MIM, missing-in-metastasis; ML, myosin light chain; MLCK, myosin light chain kinase; MLCP, myosin light chain phosphatase; MRCK, myotonic dystrophy kinase-related cdc42-binding kinase; NPF, nucleation-promoting factor; PECAM-1, platelet endothelial cell adhesion molecule 1; PKA, protein kinase A; Rap1, Ras-related protein 1; RBMEC, rat brain microvascular endothelial cells; ROCK, Rhos-associated protein kinase; ROS, reactive oxygen species; S1P, sphingosine-1-phosphate; TER, transendothelial electrical resistance; TEM, transendothelial cell macroapertures; TGF-β, transforming growth factor-β; TJ, tight junctions; TNF-α, tumour necrosis factor-α; VASP, vasodilator-stimulated phosphoprotein; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; VL, ventral lamellipodia; WASP, Wiskott-Aldrich syndrome protein; WAVE, Wasp-family verprolin-homologous proteins; ZO, zonula occludens.

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