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EPLIN- α and - β Isoforms Modulate Endothelial Cell Dynamics through a Spatiotemporally Differentiated Interaction with Actin

Graphical Abstract

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In Brief

Dynamics of actin bundles and branched actin filaments in ECs depend on cell activation and actin binding proteins. Taha et al. show that EPLIN- α controls branched actin filament dynamics of classical lamellipodia (cLP) and junction-associated intermittent lamellipodia (JAIL), while EPLIN- β is upregulated in hemodynamically loaded ECs to stabilize actin bundles.

Highlights

- EPLIN- α and EPLIN- β expression in ECs is stimulus-dependent *in vivo* and *in vitro*
- EPLIN- β is high in aortic and in shear stress-loaded ECs to stabilize actin bundles
- EPLIN- α is high in growing cells to control protrusion dynamics of cLP and JAIL
- Thus, EPLIN isoforms control fine-tuning of actin dynamics for cellular adaptation

EPLIN- α and - β Isoforms Modulate Endothelial Cell Dynamics through a Spatiotemporally Differentiated Interaction with Actin

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SUMMARY

Actin-binding proteins are essential for linear and branched actin filament dynamics that control shape change, cell migration, and cell junction remodeling in vascular endothelium (endothelial cells [ECs]). The epithelial protein lost in neoplasm (EPLIN) is an actin-binding protein, expressed as EPLIN- α and EPLIN- β by alternative promoters; however, the isoform-specific functions are not yet understood. Aortic compared to cava vein ECs and shear stress-exposed cultured ECs express increased EPLIN- β levels that stabilize stress fibers. In contrast, EPLIN- α expression is increased in growing and migrating ECs, is targeted to membrane protrusions, and terminates their growth via interaction with the Arp2/3 complex. The data indicate that EPLIN- α controls protrusion dynamics while EPLIN- β has an actin filament stabilizing role, which is consistent with FRAP analyses demonstrating a lower EPLIN- β turnover rate compared to EPLIN- α . Together, EPLIN isoforms differentially control actin dynamics in ECs, essential in shear stress responses, cell migration, and barrier function.

INTRODUCTION

Actin dynamics are integrated in many cellular remodeling and stabilizing processes including regulation of cell junction dynamics, cell-substrate adhesion, and cell migration. In vascular endothelial cells (ECs), actin dynamics are essential for the control of the blood-tissue barrier, angiogenesis, and wound healing. The actin filaments in ECs are components of supramo-

lecular organized cytoskeletal structures. This includes contractile actin bundles such as the junction-associated actin filaments (JAAFs) that localize at and are supposed to stabilize mature EC junctions and the cytoplasmic stress fibers, which terminate at focal contacts and adherens junctions and are supposed to help the EC to resist hemodynamic loads (Drenckhahn, 1982; Franke et al., 1984). Contractile actin bundles additionally comprise myosin II and the z-band protein α -actinin, which enables contractile force development, a feature that contributes to shear stress resistance, control of EC barrier function, and cell migration involving small Rho-GTPases (Drenckhahn and Wagner, 1986; Franke et al., 1984; Hayer et al., 2016; Millán et al., 2010; Schnittler et al., 1990; Wysolmerski and Lagunoff, 1990).

Another type of actin filaments are branched actin networks driving the extension of classical lamellipodia (cLP) during cell migration (Krause and Gautreau, 2014; Vinzenz et al., 2012), as well as the junction-associated intermittent lamellipodia (JAIL), which are small (1–5 μ m) membrane protrusions defined by their ability to directly form new vascular endothelial cadherin (VE-cadherin) adhesion sites (for review, see Cao and Schnittler, 2019). Both cLP and JAIL are driven by branched actin network formation under the control of the Arp2/3 complex downstream of Rac-1 and Wiskott-Aldrich syndrome protein (WASP)-family verprolin-homologous protein (WAVE)/WASP family proteins (Abu Taha et al., 2014; Breslin et al., 2015; Cao et al., 2017; Goley et al., 2010; Welch and Way, 2013). JAIL develops at EC junction sites with a low relative VE-cadherin concentration or where gaps appear. The actin-driven protrusions expand across the membrane of the adjacent cell and allow the formation of VE-cadherin trans-interactions by the usage of freely in the membrane diffusing VE-cadherin molecules (Abu Taha et al., 2014; Cao et al., 2017), which are called VE-cadherin plaques. Those plaque-forming, VE-cadherin molecules cluster after protrusion extension has stopped due to dissociation of branched actin

filaments. This then is followed by protrusion retraction until VE-cadherin clusters are incorporated into the junctions, and new VE-cadherin adhesions are formed. These JAIL-mediated VE-cadherin dynamics contribute to sheet migration and control junction remodeling in angiogenesis while maintaining cell junction integrity (for review, see [Cao and Schnittler, 2019](#)).

Actin-binding and -regulating proteins are fundamental in controlling actin-mediated protrusion formation, cell migration, and junction dynamics (for review, see [Krause and Gautreau, 2014](#); [Pollard, 2016](#); [Small and Resch, 2005](#); [Steffen et al., 2017](#)). The actin binding and actin bundling epithelial protein lost in neoplasm (EPLIN) was originally discovered being lost in oral tumors ([Chang et al., 1998](#)), modulating cell migration, and accompanying tumor progression and metastasis ([Jiang et al., 2008](#); [Liu et al., 2012](#); [Maul and Chang, 1999](#); [Maul et al., 2003](#)). EPLIN is further supposed to function as a tumor suppressor, since it is able to modulate epithelial-mesenchymal transition ([Zhang et al., 2011](#)) via p53 ([Ohashi et al., 2017](#)). Previous work has uncovered EPLIN as a linker protein between the E-Cadherin/ β -Catenin- α -Catenin complex and the actin filaments in the epithelium, supposed to establish and stabilize the circumferential actin filament bundles and thus impact on cell junction regulation ([Abe and Takeichi, 2008](#)), which might include mechanosensitivity ([Taguchi et al., 2011](#)). EPLIN is expressed as two isoforms from a single LIM domain and actin binding (LIMA) gene located at chromosome 12q13 under the control of alternative promoters ([Maul and Chang, 1999](#)). There is EPLIN- α , which consists of 600 amino acids and a larger EPLIN- β isoform with 759 amino acids ([Chen et al., 2000](#)). Previous work on EPLIN in ECs demonstrated that both EPLIN isoforms localize as components of the JAAF, while EPLIN- α was additionally found at JAIL ([Hofer et al., 2018](#)), which is in agreement with its impact on angiogenesis ([Chervin-Pétinot et al., 2012](#); [Sanders et al., 2010](#)).

EPLINs display a zinc finger and Lin11, Isl-1, and Mec-3 (LIM) domain ([Maul and Chang, 1999](#)) and two actin binding domains flanking the LIM domain, which allows self-dimerization and interaction with other proteins (Interactome). Of importance, EPLIN was shown to interact with the Arp2/3 complex and increases the lag phase of actin polymerization *in vitro* ([Maul et al., 2003](#)), a mechanism that might play a role in controlling membrane protrusion dynamics in endothelium. Here, we show that the EPLIN isoforms display a heterogeneous and stimulus-dependent expression. EPLIN- α controls actin dynamics including cLP and JAIL, while EPLIN- β induces and stabilizes stress fibers. We propose the different EPLIN isoforms as differentially acting tuning molecules to control and coordinate actin dynamics in endothelium.

RESULTS

Expression of EPLIN- β in ECs Depends on Hemodynamics

Since EPLIN isoform expression is under the control of two independent promoters ([Chen et al., 2000](#)), we investigated the expression of EPLIN isoforms in ECs freshly isolated from aortas and cava veins of the pig ([Schnittler et al., 1998](#)). Quantitative western blot analysis of smooth muscle free ECs displayed

EPLIN isoform-specific bands with molecular weights of about 110 kDa for EPLIN- β and 90 kDa for EPLIN- α . Impressively, EPLIN- β was about two times upregulated in pig aortic compared to pig cava vein ECs, while EPLIN- α remained the same ([Figure 1A](#)). The EPLIN isoform bands appeared as double bands, with the upper band very probably corresponding to the phosphorylated form (potentially Ser 360 and Ser 602) ([Han et al., 2007](#)). In contrast, actin expression did not show differences between aortic and cava vein and pulmonary artery ECs ([Figure S1A](#)), while expression of vimentin was much higher in aortic and left ventricular ECs as described ([Schnittler et al., 1998](#)). Alternatively, we used near-infrared detection for actin and vimentin in freshly isolated ECs, which gave the same results (H.S., unpublished data). Consistently, EPLIN- β but not EPLIN- α expression was increased upon shear stress application (18 dyn/cm²) for 30 h in confluent ($>10^5$ cells/cm²) human umbilical vein ECs (HUVECs) ([Figure 1B](#)), while again, similar to *in vivo*, the expression of actin remained unchanged (not shown). The data document a vessel type-specific and a hemodynamics-inducible expression of EPLIN- β in ECs.

EPLIN- α Expression Increases in Growing ECs

Cell migration and cell junction dynamics directly relate to formation of Arp2/3 complex-dependent plasma membrane protrusions including cLP and JAIL, respectively (for review, see [Cao and Schnittler, 2019](#); [Krause and Gautreau, 2014](#)). Since EPLIN interferes with Arp2/3 complex function ([Maul et al., 2003](#)) and that EPLIN- α localizes at JAIL and cLP ([Hofer et al., 2018](#)), we investigated whether EPLIN isoform expression correlates with cell density in HUVEC cultures. Indeed, subconfluent EC cultures ($3\text{--}4 \times 10^4$ cells/cm²) expressed about 2 times more EPLIN- α than confluent cultures ($0.9\text{--}1.2 \times 10^5$ cells/cm²), while EPLIN- β expression remained unchanged ([Figure 1C](#)). Furthermore, increasing confluence decreased EPLIN- α expression and protrusion formation, which was accompanied by an about 3 times reduction in cell migration-velocity from 0.65 $\mu\text{m}/\text{min}$ in growing cultures ($3\text{--}4 \times 10^4$ cells/cm²) to approximately 0.21 $\mu\text{m}/\text{min}$ in confluent ($0.9\text{--}1.2 \times 10^5$ cells/cm²) cultures ([Figure 1D](#); [Video S1](#)). These data suggest a relationship between EPLIN- α expression and cell density-dependent JAIL formation and cell migration rather than proliferation, since EPLIN depletion in HUVECs does not change proliferation ([Chervin-Pétinot et al., 2012](#)).

Localization of EPLIN in ECs *In Vivo* and in Culture

Pan-EPLIN antibody localized EPLIN at cell junctions and at stress fibers in elongated aortic and polygonal cava vein ECs *in vivo* ([Figure 2A](#)) as well as in HUVEC cultures ([Figure S1B](#)). High-resolution structured illumination microscopy (SIM) identified EPLIN as dotted-like pattern along actin filaments *in vivo* ([Figure 2A](#), cropped areas 1 and 2), in confluent EC cultures along the cell junctions, as a component of protrusions and along stress fibers as well as in the cytosol ([Figure S1B](#)). Intriguingly, EPLIN clusters appeared behind the protrusion-driving branched actin network in an approximated distance up to 1.5 μm ([Figure S1B](#)). The obtained data prompted us to investigate the particular localization and the dynamic remodeling of EPLIN isoforms by fluorescence live-cell imaging.

Figure 1. Both EPLIN Isoforms Associate with the VE-Cadherin/Catenin Complex in Vascular Endothelial Cells and Display a Differentiation-Dependent Expression In Vivo and In Vitro

(A) Representative western blots of pig aorta and cava vein endothelial cell lysates (40 μg/lane) that tested negative for α-smooth muscle actin; samples were probed with an anti-EPLIN antibody. The blots were also probed with α-tubulin as an internal loading control. Densitometry-based quantitative analysis of 42 cava veins and 8 aortas was performed. The doublets relating to EPLIN-α and EPLIN-β may be due to differentially phosphorylated forms.

(B) Representative western blots of HUVECs exposed to shear stress (18 dyn/cm² for 30 h) using a pan-EPLIN antibody. Densitometry-based quantitative analysis displays the normalized average intensity acquired from 3 independent experiments.

(C) Representative western blots of confluent and subconfluent HUVEC cultures using anti-EPLIN antibodies. Different amounts of total protein were loaded as indicated; α-tubulin served as internal control. Densitometry-based quantitative analyses show the normalized average intensity of EPLIN isoforms obtained from 4 different experiments.

(D) Migration velocities of confluent ($0.9\text{--}1.1 \times 10^5$ cells/cm²) and subconfluent ($0.31\text{--}0.35 \times 10^5$ cells/cm²) HUVEC cultures. The box-and-whisker plots show the mean values of $n = 3$ independent experiments.

(E) Pull-down of the VE-cadherin/catenin complex from HUVECs expressing either EPLIN-α-EGFP or EPLIN-β-EGFP, respectively, using GFP-Trap_A assay, indicating that both isoforms bind to the VE-cadherin/catenin complex in subconfluent HUVECs; neg ctrl, blocked agarose beads.

Error bars in (A), (B), and (C) indicate SEM. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, **** $p < 0.00005$.

Fluorescently Tagged EPLIN Isoforms Differ in Localization and Dynamics in HUVEC Cultures

Localization and dynamics of EPLIN isoforms were evaluated using EPLIN-α-mCherry, EPLIN-α-EGFP, EPLIN-β-mCherry, and EPLIN-β-EGFP (Hofer et al., 2018), which were expressed in HUVEC cultures and subsequently characterized by immunoprecipitations (IPs), by western blot analysis (Figures S2A–S2D), and by immune labeling (Figure S2E). Previous studies

showed an association of EPLIN-α with the VE-cadherin/catenin complex (Chervin-Pétinot et al., 2012), whereas both isoforms were localized at EC junctions in confluent HUVEC cultures (Hofer et al., 2018). In addition, using GFP-Trap_A assay, we demonstrate that both EPLIN-α-EGFP and EPLIN-β-EGFP co-precipitate a fraction of the VE-cadherin/catenin complex (Figure 1E), which consequently leads to a low but clear visible VE-cadherin signal. Therefore, both EPLIN isoforms are

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supposed to associate with VE-cadherin/catenin complex. Furthermore, both isoforms can associate with actin bundles and appear at EC junctions as JAAF and at stress fibers (Figures 2B and 2C). In contrast, only EPLIN- α could be regularly detected either close to or within branched actin filament networks at membrane protrusion such as JAIL and cLP (Figure 2B).

Time-lapse recordings revealed a highly dynamic and transient occurrence of EPLIN- α at cLP in single cells and JAIL at cell junctions. In agreement with previous reports (Abu Taha et al., 2014; Cao et al., 2017), JAIL size and number decreases with increasing cell density and thus EPLIN- α dynamics (Video S2). In contrast, protrusions were mostly negative for EPLIN- β -EGFP, but the latter labeled faint actin filaments in the lamella and seemed to contribute to the retraction process (Video S2). In addition, both EPLIN isoforms are part of stress fibers, which obviously exhibit lower dynamics compared to membrane protrusions (Video S2). Double expression of EPLIN- α -mCherry and EPLIN- β -EGFP in the same cell directly confirmed the differential dynamics and localization of the two EPLIN isoforms (Figure 3A; Video S3). The choice of fluorescence tags for each isoform had no detectable effect on respective, differential localization pattern, as isoform-specific differences were maintained irrespective of the tag used (Figure S2E).

The differences in the spatial and temporal occurrence of EPLIN isoforms at membrane protrusions (Figure 3A; Video S3) suggest distinct functions, which possibly derive from their differential ability to interact with actin filaments. Therefore, we used fluorescence recovery after photobleaching (FRAP) to determine the turnover rates and the immobile fraction of these isoforms. For direct comparison, FRAP analyses were performed at stress fibers, which are targeted by both EPLIN isoforms (Figure 3C). After bleaching (down to 20%–30% of the original intensities), images were acquired and recovery rates calculated. EPLIN- α recovered much more quickly ($t^{1/2} = 4.57$ s) than EPLIN- β ($t^{1/2} = 19.13$ s), which was also reflected by the difficulty to efficiently bleach the entire fluorescence pool of EPLIN- α . In contrast, the mobile fractions of both EPLIN isoforms were comparable or observed differences at least not statistically significant (Figures 3C and 3D). The data show functionally different, spatiotemporal dynamics of the two EPLIN isoforms, even within the same structure, demonstrating that EPLIN- α is more dynamic than EPLIN- β . These data further indicated that EPLIN- β might be more stable at bound actin structures than EPLIN- α . Therefore, Triton X-100 (1%) extractions were performed, showing that the bulk of EPLIN- α remained in the insoluble fraction (70%–80%) in both subconfluent and confluent cell cultures (Figures S2Fa and S2Fb), whereas the soluble fraction of EPLIN- β increased in confluent

cultures slightly (Figure S2Fc). However, the increase in EPLIN- β solubility might be related to a decreased number of stress fibers in confluent cultures. Together, the data further support the concept that EPLIN- α is involved in control of actin-mediated cell migration and cell junction remodeling, likely by controlling protrusion dynamics, while EPLIN- β might engage in stabilization of filaments in bundled structures such as stress fibers.

EPLIN- α Terminates Arp2/3-Controlled, Branched Actin Filament Protrusions

To obtain more insight into the functional role of EPLIN- α at cLP and JAIL, we expressed EPLIN- α -mCherry together with EGFP-tagged p20 (ArpC4), the 20-kDa subunit of the Arp2/3 complex mediating actin filament branching at the leading edge of cLP (Koestler et al., 2013; Lai et al., 2008; Suraneni et al., 2012; Wu et al., 2012) and of JAIL (Abu Taha et al., 2014). Spinning disc, confocal microscopy (SpDM) documents the characteristic, Arp2/3 complex-controlled extension at the leading edge of the protrusions (cLP and JAIL) (Figure 4A; Video S4). Interestingly, EPLIN- α -mCherry followed the Arp2/3 complex at a variable distance of up to 1.5 μ m, which was continuously shortened during protrusion extension until both proteins co-localized. As soon as the proteins overlapped, the forward movement of the protrusions was stopped, which coincided with the disappearance of the actin filaments, and a dissociation of both the Arp2/3 complex and EPLIN- α from actin filaments exactly at this site. This process is most likely promoted by the binding of EPLIN- α to the Arp2/3 complex (Maul et al., 2003), which presumably leads to dissociation of the Arp2/3 complex from the actin network. Subsequently, a retraction process of the protrusions takes place (Figure 4A; Video S4), as further illustrated by kymography (Figure 4B). Moreover, the mass-center of gravity of the relative fluorescence intensities of EGFP-p20 and EPLIN- α -mCherry at the protrusions was plotted as a function of time. Indeed, when EPLIN- α reached a maximum (corresponding to co-localization), the EGFP-p20 disappeared, which accompanied an inhibition of protrusion extension (Figure 4B; Video S4). To further verify the molecular interaction of EPLIN isoforms with the Arp2/3 complex, we additionally performed GFP-Trap_A pull-downs from HUVEC cultures, which expressed either EPLIN- α -EGFP or EPLIN- β -EGFP, respectively. Indeed, we found a fraction of Arp2/3 complex associated with both EPLIN isoforms (Figure 4C). All of these data thus supported the concept that EPLIN contributes to termination of protrusions, through interaction with the Arp2/3 complex in EC. Consistently, inhibition of the Arp2/3 complex by CK666 blocked both protrusion

Figure 2. Localization of EPLIN in Vascular Endothelial Cells *In Vivo* and of EPLIN Isoforms *In Vitro*

(A) Laser-scanning microscopy (LSM) of ECs in whole-mount preparations of mouse aorta (a–c) and cava vein labeled as indicated (d–f). Structured illumination microscopy (SIM) of mouse aorta (g–i) and cremaster muscle vessels (j–l) labeled as indicated. Panels 1 and 2 are cropped areas (white boxes in i and l). (B and C) Cell density-dependent localization of EPLIN- α -EGFP and EPLIN- β -EGFP in HUVEC cultures. (B and C, upper panels) LSM images of HUVECs expressing either EPLIN- α or EPLIN- β -EGFP (green) subsequently stained with phalloidin-TRITC (red) and DAPI (gray). (B, cropped areas 1–4) EPLIN- α -EGFP localization at cell junctions together with actin filaments in confluent cultures (1), at JAIL (2) demonstrated in subconfluent cultures (arrows indicate protrusion direction), and cLP (3) in sparsely plated cells behind branched actin networks and at stress fibers (4). (C, cropped areas 5–8) EPLIN- β -EGFP localization at cell junctions together with actin filaments (5). EPLIN- β is absent at JAIL (6) (arrows indicate protrusion direction), and cLP (7) as demonstrated in sparsely seeded cultures. Arrowheads point to actin-driven cLP. EPLIN- β is a component of stress fibers (8) (arrows). Scale bars as indicated.

Figure 3. Localization and Dynamics of EPLIN- β -EGFP and EPLIN- α -mCherry at cLP in HUVEC Cultures

EPLIN- β -EGFP and EPLIN- α -mCherry were expressed in HUVEC cultures, and time-lapse recording was performed with a time difference of 1.1 s between the two images.

(A, upper panel) Overviews. Cropped areas (a1–c8) display time-dependent dynamics of EPLIN- β -EGFP (green) and EPLIN- α -mCherry (red) over about 13 min. Repeated cLP formation is documented with EPLIN- α -mCherry localizing at the forefront (arrowheads) of cLP, whereas EPLIN- β -EGFP is restricted to the lamella and behind. (A, lowest panels) A higher magnification (taken from a1–a8) of EPLIN- β -EGFP illustrates the retraction/protrusion process (dotted lines). Also compare [Video S3](#). Timescale: min.s.ms.

(B–D) FRAP analyses of EPLIN- α -EGFP and EPLIN- β -EGFP localized at stress fibers.

(B) Consecutive confocal images of a FRAP experiment in HUVEC cultures that express EPLIN- α -EGFP (upper panel) or EPLIN- β -EGFP (lower panel) pre- and post-bleaching.

(C) Plotted are the mean values of the normalized fluorescence recovery intensities $\pm$ SEM of EPLIN- α -EGFP ($n = 27$ areas) and EPLIN- β -EGFP ($n = 36$ areas) taken from 3 independent cultures.

(D) The recovery half-time (τ) of EPLIN- α -EGFP and EPLIN- β -EGFP; error bars indicate SEM. Immobile fractions (IFs) and mobile fractions (MFs) of EPLIN- α - and EPLIN- β , respectively.

** $p < 0.005$.

formation and appearance of EPLIN- α at the membrane (Figure S3A), which demonstrates the requirement of either or both Arp2/3 complex and branched actin filaments at protrusions for EPLIN- α appearance at these sites. The data acquired up to this point indicated that EPLIN- α rather than EPLIN- β inactivates the Arp2/3 complex at protrusions, and thus contributes to their termination. Furthermore, this concept could be functionally underlined by EPLIN depletion in LifeAct-EGFP-expressing HUVECs using small interfering RNA (siRNA) (siEPLIN, 20 pM) and for control non-targeting siRNA (NTRNA, 20 pM). In the same approach, we also tested whether EPLIN can contribute to the force generation of membrane protrusions (Svitkina, 2018). For this, we determined different protrusion parameters in high viscous medium ($\eta = 3.5 \text{ mPa}\cdot\text{s}$) by addition of 2% polyvinylpyrrolidone and compared it to normal medium viscosity ($\eta = 0.8 \text{ mPa}\cdot\text{s}$). This setup was previously shown to be suitable for detecting altered protrusion force development (Kage et al., 2017). SiEPLIN treatments decreased EPLIN- α expression by 79.47% and EPLIN- β by 72.62%, while expression of VE-cadherin and α -catenin remained unchanged (Figures S3B–S3D). However, the immune and phalloidin-TRITC label suggest that VE-cadherin became condensed at the cell contacts of siEPLIN treated cells, which was associated with actin recruitment as well (Figures S3E and S3F). Functionally, EPLIN depletion increased both protrusion size and protrusion duration in normal viscous medium significantly (Figures 4D and 4E). Increase of medium viscosity, however, decreased the protrusion size and the duration time in NTRNA- and siEPLIN-treated cells (Figures 4D and 4E). However, irrespective of the general decrease of both protrusion parameters upon 2% polyvinylpyrrolidone (PVP), siEPLIN-treated cells displayed increased protrusion parameters as compared to their respective controls (Figures 4D and 4E). Increased protrusion size and shorter duration time were accompanied by an increased migration velocity of siEPLIN-treated cells in normal medium, but to our surprise, no differences in migration efficiency were observed between control and siEPLIN cells in high viscous medium (Figure 4F), although both cell types migrated more slowly in 2% PVP. Thus, EPLIN knockdown could not alleviate suppression of migration in high viscous medium, as it could for protrusions (see above), indicating that effects by PVP on migration in this cell type and conditions cannot be restricted to its impact on protrusions.

EPLIN- α -EGFP Overexpression Modulates JAIL Formation and VE-Cadherin Dynamics

Since actin expression in EC is largely constant *in vivo* and in cell culture, it can be assumed that actin-binding proteins are decisive for supramolecular organization and dynamic remodeling of the different actin-containing structures. The stimulus-dependent expression of EPLIN isoforms can be regarded as a model for such a regulatory aspect. Here, we assume EPLINs as a molecular fine-tuning system for the control of actin dynamics. Thus, we used an overexpression approach to further characterize the specific functions and mechanisms of EPLIN isoforms on actin and in turn cell dynamics.

For overexpression of both EPLIN isoforms, virus titers were titrated and respective virus amounts used (Figures S4A and S4B). Overexpression of both isoforms had no influence on either VE-cadherin or α -catenin expression (Figures S4C and S4D). EPLIN- α -EGFP co-localized with actin filaments and was enriched at cell junctions, forming patch-like structures. Furthermore, the JAAFs were disintegrated, stress fibers were formed, and the VE-cadherin displayed various patterns, including invaginations and interruptions (Figure 5A). EPLIN- α -EGFP overexpression increased actin dynamics, accompanied by transient formation of EPLIN- α -EGFP patches (Figure 5A; Video S5). Higher magnification revealed that the EPLIN- α -EGFP patches appeared at JAIL-like structures, which, however, became quickly interrupted, and thus prevented a further expansion of protrusions. The aborted JAIL formation was accompanied by increased filopodia formation at the junctions (Video S5), a phenomenon also achieved by the Rac1 inhibitor EHT1864, which blocked actin-mediated JAIL formation (Cao et al., 2017). As a consequence, a vicious circle developed, which is characterized by a high frequency of aborted JAIL and formation of many filopodia (increased actin dynamics) (Video S5). Furthermore, filopodia, however, could not compensate for insufficient JAIL formation as gaps were frequently observed, which accompanied insufficient barrier function, as determined by transendothelial electrical resistance (TER) during cell growth (Figure 5B). EPLIN- α -EGFP-overexpressing cultures reached a maximum value during cell growth that was only about 68% compared to control cultures (Figure 5B). The effect was specific, since ECs overexpressing EGFP alone or moderately expressing EPLIN- α -EGFP or untreated controls all displayed the same, high TER (Figure 5B). Mechanistically, the high concentration of EPLIN- α

Figure 4. Dynamics and Association of EPLINs with the Arp2/3 Complex in Subconfluent HUVEC Cultures

(A) Dynamics of EPLIN- α -mCherry and EGFP-p20 at cLP and JAIL in subconfluent HUVEC cultures.
(a–c) Overviews, timescale (s:ms). The white boxes indicate cropped areas that were selected for documentation of spatiotemporal dynamics of cLP (cropped area 1, d–g) and JAIL (cropped area 2, h–k). EGFP-p20 (arrowheads) is followed by EPLIN- α -mCherry (red). Protrusions are terminated after colocalization of the two proteins. Timescale (mm:ss).
(B) An example of the relative appearance of EPLIN- α -mCherry and EGFP-p20 at cLP (1) analyzed by a kymograph and the determination of the center of mass from intensities using respective kymographs. Compare also Video S4. Arrows indicate interruption of protrusion.
(C) GFP-Trap_A pull-down was performed from HUVEC cultures, which express EGFP alone (control), EPLIN- α -EGFP, or EPLIN- β -EGFP, respectively. Western blot probed with ArpC/p34 antibody documents a co-precipitation of the ARP2/3 complex. neg ctrl, blocked agarose beads.
(D–F) HUVECs were transduced with LifeAct-EGFP and subsequently treated with siEPLIN or NTRNA, respectively, and subsequently placed into medium with normal viscosity ($\eta = 0.8 \text{ mPa}\cdot\text{s}$, green bars) and into medium with high viscosity ($\eta = 3.5 \text{ mPa}\cdot\text{s}$, purple bars).
(D and E) Protrusion size (D) and protrusion duration time (E) was quantitatively estimated from time-lapse videos. 45 protrusions from 3 independent experiments were manually selected.
(F) Determination of cell migration velocity in NTRNA- and siEPLIN-treated cells.
Box-and-whisker plots show the values of 3 independent experiments. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, and **** $p < 0.00005$.

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will increasingly bind to the Arp2/3 complex, reflected by the formation of the EPLIN- α -EGFP patches, which quickly terminates regular JAIL formation. In turn, we assumed that JAIL-mediated VE-cadherin dynamics is altered leading to insufficient barrier function. Therefore, we next evaluated the distribution and dynamics of VE-cadherin upon EPLIN- α -EGFP overexpression.

Under control conditions (moderate expression of EPLIN- α -EGFP and EPLIN- β -EGFP, respectively), characteristic, JAIL-mediated VE-cadherin dynamics were observed, with the transient formation of VE-cadherin plaques that subsequently cluster and incorporate into cell junctions, as demonstrated in subconfluent (Figures S5A and S5B) and confluent cultures (Video S6), which is in accordance with our previous reports (Abu Taha et al., 2014; Cao et al., 2017). In contrast to the overall increased actin dynamics in EPLIN- α -overexpressing cells, VE-cadherin dynamics appeared to be slowed down (Video S6) due to abortive JAIL formation. Accordingly, VE-cadherin-mCherry irregularly distributed along the cell contacts accompanied by intercellular gaps that could obviously not be closed (Video S6). Even the many filopodia were obviously not able to compensate for the insufficient VE-cadherin dynamics, indicating that filopodia have less impact on VE-cadherin dynamics. Importantly, these data corresponded to insufficient EC migration ability, as shown in a scratch assay (Figure 5C). Together, these data strongly underline the concept that EPLIN- α terminates JAIL formation in EC, contributing to the control of VE-cadherin dynamics, and thus cell migration and barrier function.

To further evaluate the specific function of EPLIN- α in actin-mediated VE-cadherin dynamics, we used the MCF-7 carcinoma cell line, in which we ectopically expressed VE-cadherin-EGFP (MCF-VE-EGFP) (Rezaei et al., 2018). This cell line is negative for EPLIN- α , but positive for EPLIN- β (Figure 5D). MCF-VE-EGFP cells display a polygonal cell shape with an overall linear arrangement of VE-cadherin along the junctions (Figure 5D). In contrast, expression of EPLIN- α -mCherry in MCF-VE-EGFP cells causes loss of actin filaments from junctions, as reflected by EPLIN- α immune labeling, which changed the VE-cadherin pattern from linear to an overall interrupted one (Figure 5E). Time-lapse recording revealed an increased displacement

(dynamics) of VE-cadherin-EGFP in EPLIN- α -expressing cells, as demonstrated by a false color-coding of VE-cadherin-EGFP at different time points during remodeling in the same cells (Figure 5F). For a quantitative estimation of the effect, we compared the VE-cadherin pattern between EPLIN- α -negative and EPLIN- α -expressing cells. Cell boundaries with at most one interruption were counted as linear, the occurrence of multiple interruptions as interrupted (Figure 7G). These data support the concept that EPLIN- α contributes to the control of actin dynamics rather than EPLIN- β at junctions, and thus modulates VE-cadherin dynamics.

EPLIN- β -EGFP Overexpression Induces and Stabilizes Stress Fibers

Overexpression of EPLIN- β in ECs nearly doubled stress fiber formation (length and numbers), while overexpression of EPLIN- α increased them about 50% and overexpression of EGFP alone showed the same values as untreated controls (Figures 6A, 6C, and 6C1). Stress fibers, however, are contractile (Chrzanowska-Wodnicka and Burridge, 1996; Drenckhahn and Wagner, 1986; Ridley and Hall, 1992; Schnittler et al., 1990; Wysolmerski and Lagunoff, 1990), can be induced by shear stress (Franke et al., 1984), and occur in ECs *in vivo* in hemodynamically stressed vessels (Drenckhahn and Wagner, 1986; Nehls and Drenckhahn, 1991; van Geemen et al., 2014; White and Fujiwara, 1986; White et al., 1983). Thus, increased expression of EPLIN- β in arterial ECs and shear stress-exposed ECs might help to stabilize actin filaments, in particular in stress fibers. To explore this hypothesis further, we used EPLIN isoform-overexpressing, sub-confluent cells, displaying many stress fibers, and applied the Rho-associated protein kinase (ROCK) inhibitor Y27632. Physiologically, ROCK activates myosin light chain (MLC) kinase and inactivates MLC-phosphatase leading to stress fiber contraction, but also activates LIM-kinase, which inactivates cofilin-mediated actin de-polymerization through phosphorylation on serine 3 (Riento and Ridley, 2003). Accordingly, ROCK inhibitor is expected to cause stress fiber depolymerization and loss of contractility (Riento and Ridley, 2003). Indeed, 10 μ M ROCK inhibitor Y27632 depolymerized stress fibers in ECs, accompanied by loss of both EPLIN isoforms at these sites (Figures 6A–6C1),

Figure 5. Effects of EPLIN- α -EGFP Expression on the Organization and Dynamics of Cell Junctions

(A) SIM of HUVEC cultures that moderately express (1–4) or overexpress (5–8) EPLIN- α -EGFP. Cells were labeled additionally with anti-VE-cadherin antibodies and phalloidin-TRITC to stain actin filaments. The arrowheads indicate EPLIN- α -EGFP and in part actin-positive patches, which, however, are largely negative for VE-cadherin. The arrows in areas 5–8 indicate many actin and EPLIN- α -EGFP-containing filopodia close to cell junctions.

(B) Transendothelial electrical resistance (TER) was determined in growing HUVEC cultures based on impedance spectroscopy measurements. Untreated HUVECs and cultures that moderately express or overexpress EPLIN- α -EGFP, EPLIN- β -EGFP, or EGFP were examined for TER values. The ratio TER/TER (0) was plotted against time. $n = 6$ different experiments. Error bars indicate SEM.

(C) Migration rates of control HUVECs in the scratch assay. The migration rates for control cells (EGFP expression, untreated) and cells moderately expressing or overexpressing EPLIN- α -EGFP or EPLIN- β -EGFP are shown as box-and-whisker plots. The quantification was performed for 100 cells in five independent experiments.

(D) Western blot analyses of MCF-VE-EGFP cells, MDA-231 cells, and HUVECs probed with pan-EPLIN antibody, demonstrating that MCF-VE-EGFP cells are depleted of EPLIN- α .

(E) LSM of MCF-VE-EGFP cells, which are negative for EPLIN- α (upper panel) and after expression of EPLIN- α -mCherry (lower panel). Note the overall interrupted VE-cadherin-EGFP signal in EPLIN- α -mCherry-expressing cells.

(F) The superimposed image sequences of VE-cadherin-EGFP taken from time-lapse movies of EPLIN- α -negative and EPLIN- α -mCherry-expressing cells are displayed in false colors at times $t = 0'$ (green), $t = 5'$ (red), and $t = 10'$ (blue). The enhanced VE-cadherin-EGFP displacement is clearly visible in the EPLIN- α -expressing cell.

(G) Number of junction pairs that appear as linear or interrupted VE-cadherin-EGFP patterns in both cell types. About 200 cell junction pairs were analyzed in each cell type.

* $p < 0.05$, *** $p < 0.0005$, and **** $p < 0.00005$. Scale bars as indicated.

Figure 6. Effects of EPLIN Isoform Overexpression on Stress Fiber Stability after ROCK Inhibition

(A and B) Native HUVEC cultures and cultures overexpressing either EPLIN- α -EGFP, EPLIN- β -EGFP, or EGFP were left untreated (A) or treated with 10 μ M ROCK inhibitor Y27632 for 30 min (B). Cells were labeled with phalloidin-TRITC for actin filaments and DAPI for nuclear staining as indicated. (C and C1) Quantitative estimation of stress fibers length (C) and stress fiber number (C1). Data shown are from three different experiments. Error bars indicate SEM. ** $p < 0.005$.

which affected TER development in growing EC cultures as well (Figure 5B). Interestingly, overexpression recruited EPLIN- β to protrusion fronts where it co-localized with actin filaments and ArpC2 antibody (Figure S7B), a phenomenon that was never seen upon moderate expression. This presumably corresponds to a lower affinity of EPLIN- β to the Arp2/3 complex compared to EPLIN- α , and thus only becomes evident with increased expression. The aberrant EPLIN- β localization presumably also affects actin dynamics at junctions and, consequently, VE-cadherin localization and dynamics. Therefore, the reduced maximal TER of about 77.9% in growing cultures compared to controls is most likely overridden by non-physiological EPLIN- β localization at protrusions (Figure 5B). Notwithstanding this, our findings strongly indicate a stress fiber-stabilizing function of EPLIN- β , which can operate additive to physiological pathways of stress fiber formation such as ROCK activity.

EPLIN Isoforms Are Essential in Physiological Adaptation of ECs to Fluid Shear Stress

Fluid shear stress causes a quick (minutes) and transient increase in EC barrier function, which accompany

further documented by live cell imaging using EPLIN- α -mCherry and EPLIN- β -EGFP-expressing cells (Figure S6). In contrast, EPLIN- α remained unaltered under those treatments at the lamellipodia, which still displayed protrusion extension (Figures 6A–6C1 and S6). In untreated controls and in ECs expressing EGFP alone or overexpressing EPLIN- α , Y27632 decreased stress fibers to control levels, while in EPLIN- β -overexpressing cells, stress fibers disassembled by only roughly 10%–15% (Figures 6C, 6C1, and S6B). The data demonstrate that EPLIN- β rather than EPLIN- α has a stress fiber-stabilizing role, irrespective of both isoforms being capable of associating with these structures.

In confluent cultures, EPLIN- β -EGFP overexpression also rearranged actin filaments and stress fibers were formed (Figure S7A),

increased protrusion dynamics at the junctions in a Rac-dependent manner (DePaola et al., 2001; Hofer et al., 2018; Seebach et al., 2007). Therefore, we investigated this effect in relation to EPLIN localization and dynamics. Under control conditions, the application of fluid shear stress (18 dyn/cm²) recruited EPLIN to cell junctions together with actin bundling and VE-cadherin cluster formation (Figure 7A). Results were confirmed by live-cell imaging of EPLIN- α -EGFP- and EPLIN- β -EGFP-expressing ECs. Shear stress of 18 dyn/cm² increased both EPLIN- α -EGFP fluorescence at junctions within minutes, whereas an increase of EPLIN- β -EGFP fluorescence was delayed (Figure 7B). The time course of EPLIN- α -EGFP dynamics can be assigned to increased JAIL

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formation, which is in agreement with previously described VE-cadherin-EGFP dynamics and actin recruitment to the junctions under shear stress (Fraccaroli et al., 2015; Hofer et al., 2018; Seebach et al., 2015). We simultaneously determined the TER from impedance spectroscopy measurements and found the characteristic transient increase in NTRNA-treated control cells (Figures 7C and 7D). In contrast, siEPLIN treatment abolished the transient TER increase completely and resulted in a progressive TER downregulation within 120 min (Figures 7C and 7D). Together with the increased shear stress-induced JAIL formation, these data support the concept that EPLINs function as mechano-responsive molecules involved in rapid, shear stress-induced tightening of EC junctions.

In addition, long-time exposure of ECs to shear stress leads to EC elongation and alignment, which is accompanied by cell re-orientation, individual cell migration leading to cell elongation along the shear stress axis in a process maintaining cell integrity (Dieterich et al., 2000; Seebach et al., 2007). Such a complex process requires a highly balanced, subcellular cell junction regulation, as can be achieved by JAIL dynamics, which we propose is EPLIN dependent. To explore this further, HUVECs were treated with siEPLIN and NTRNA as control, and exposed to fluid shear stress of 18 dyn/cm² for 8 h. IF labeling of siEPLIN-treated cells displayed a mosaic culture, consisting of EPLIN-depleted and EPLIN-expressing cells (Figure S7C). Quantitative analyses confirmed a reduction in mean EPLIN fluorescence intensity (Figure 7E). siEPLIN cells further displayed a significant inhibition of shear stress-induced alignment, as documented by determination of the aspect ratio in all cells, while NTRNA-treated controls aligned regularly (Figures 7E, 7F, and S7C). These data demonstrate that EPLINs significantly contribute to EC junction regulation under hemodynamic load of fluid shear stress, and both through immediate and long-term effects. Depletion of both isoforms in HUVEC as well as expression of EPLIN- α -mCherry in MCF-VE-EGFP cells supported the concept that EPLIN- α is the major determinant of this effect, since EPLIN- α controls cLP and JAIL, and thus influences VE-cadherin dynamics at the EC junctions. This mechanism also contributes to cell adhesion and maintenance of monolayer integrity during cell re-arrangements and remodeling.

DISCUSSION

The vascular endothelium has many control and regulating functions in the vascular system, including inflammation, wound healing, and hypoxia. Dynamic EC remodeling includes shape change, polarized cell migration, modulation of endothelial barrier function, and changes in gene expression (Dejana et al., 2017), parameters that are modulated by fluid shear stress (Davies et al., 2013) under epigenetic control (Bondareva et al., 2019). EC remodeling depends on the dynamics of actin, which is a major component of stress fibers and JAAF as well as branched networks driving cLP and JAIL in ECs. In these processes, actin binding and actin regulating proteins are key elements modulating actin assembly and disassembly via nucleation, elongation, capping, and severing, but also bundling (Pollard, 2016; Small and Resch, 2005; Steffen et al., 2017). Apart from principal, continuous generation and turnover of actin structures mediated for instance by major assembly factors such as Arp2/3 complex, it is reasonable to assume that a fine-tuning of actin dynamics is also necessary. We propose that the EPLIN isoforms have such a function in vascular endothelium, as a number of effects could only be detected after challenging cells derived from this tissue.

In this work, we aimed to understand the physiological function of actin binding by EPLIN isoforms in primary isolates of cultured vascular endothelium. This cell type exhibits a high plasticity requiring quick and well-adjusted actin dynamics for many adaptive processes. Here, we demonstrate that EPLIN- β induces and stabilizes stress fibers, while EPLIN- α is more dynamic and fine-tunes the subcellular, actin-driven membrane protrusions, specifically cLP and JAIL.

The conclusion of EPLIN- α to operate as a fine-tuning molecule for control of protrusion dynamics is supported by several observations. First, physiologically, EPLIN- α but not EPLIN- β localized at cLP and JAIL, both of which control EC migration and barrier function in wound healing and angiogenesis (for review, see Cao and Schnittler, 2019). Thus, the upregulation of EPLIN- α in growing cells with high migration activity seems to coincide with increased protrusion dynamics, which is also consistent with the notion that EPLIN- α expression can be stimulated by serum due to a serum responsive element upstream of the EPLIN- α promoter (Chen et al., 2000). Second, EPLIN- α balances protrusion dynamics, since time-lapse recordings

Figure 7. Distribution of EPLIN in HUVEC Cultures after Application of Shear Stress

Confluent HUVEC cultures were exposed to unidirectional shear stress of either 0.5 and 18 dyn/cm², respectively, for 15 min as well as for 6–8 h, as indicated. Cells were labeled with anti-pan-EPLIN and phalloidin-TRITC that mark actin filaments, as indicated.

(A) Overview (a–f). The white boxes indicate cropped areas (g–l). The arrows point to cell junctions.

(B) HUVECs moderately expressing either EPLIN- α -EGFP or EPLIN- β -EGFP were exposed to low shear stress (0.055 dyn/cm²) for 20 min followed by upregulation to 18 dyn/cm² for 1 h. Time-lapse recordings were performed and normalized average intensities of EPLIN- α -EGFP or EPLIN- β -EGFP appearing at the junctions plotted as a function of time using the Cell Border Tracker (Seebach et al., 2015). n = 3 independent experiments.

(C) Transendothelial electrical resistance (TER) was determined by impedance spectroscopy under shear stress conditions, as indicated. Representative curves of TER developments were plotted relative to baseline TER values (TER/TER₀) in HUVEC cultures treated with siEPLIN or NTRNA, as indicated.

(D) Bar plot demonstrating the increase of TER within 10–15 min after the onset of shear stress. Data shown arose from n = 5 independent experiments.

(E and F) siEPLIN- (n = 472 cells) and NTRNA-treated (n = 358 cells) HUVEC (8–9 × 10⁴ cells/cm²) were exposed to 18 dyn/cm² shear stress for 8 h. IF labeling of VE-cadherin marks cell junctions, while EPLIN antibodies identify EPLIN-depleted and EPLIN-expressing cells (also compare Figure S7C).

(F) Frequency distribution of fluorescence intensities in siEPLIN- and NTRNA-treated HUVECs. (Inset) Mean fluorescence intensity of all cells.

(F) Aspect ratio of siEPLIN- and NTRNA-treated HUVEC cultures after application of shear stress, as indicated.

The data shown are from 3 independent experiments. Error bars indicate SEM. **p < 0.005, ***p < 0.0005, and ****p < 0.00005.

document that EPLIN- α follows Arp2/3 complex-controlled branched actin filaments, continuously shortening the distance to the front while protrusions expand. Complete co-localization of EPLIN- α with the Arp2/3 complex immediately blocked further protrusion, which is in line with the observation that EPLIN- α extends the delay phase of actin assembly induced by Arp2/3 complex *in vitro* without altering rates of spontaneous actin polymerization (Maul et al., 2003). This inhibitory effect seems to play a fine-tuning role in controlling protrusion extension, since ablation of both EPLIN isoforms increased protrusion-size and -duration time in our experiments, as well as migration velocity. This conclusion is supported by the Arp2/3 complex being co-precipitated with both EPLIN isoforms. Our data are also consistent with previous investigations in MDA-7 cells, human mammary epithelial cells, and HeLa cells where actin-stabilizing roles of EPLIN (not specified) at the expense of plasma membrane protrusions via inhibition of Arp2/3 complex and Rac1 were observed (Maul et al., 2003). The impact of EPLIN- α in actin-mediated VE-cadherin dynamics was further supported in EPLIN- α -negative MCF-VE-EGFP cells (Rezaei et al., 2018), which displayed an interrupted VE-cadherin pattern and an increased VE-cadherin dynamics after EPLIN- α expression. These data further support the idea that EPLIN- α is more responsible for actin dynamics at the junctions than EPLIN- β . Third, in contrast to moderate EPLIN- α expression, its overexpression increased actin dynamics and at the same time reduced VE-cadherin dynamics. Both effects can be traced back to the abortive interruption of JAIL, which also underlines its role in control of junction actin dynamics. In this setting, the disorganized VE-cadherin pattern and the reduced VE-cadherin dynamics can easily be explained, since proper JAIL formation drives and remodels VE-cadherin at the junctions (Abu Taha et al., 2014; Cao et al., 2017). These results are thus in agreement with expression of EPLIN- α in EPLIN- α -negative MCF-VE-EGFP, which changed VE-cadherin patterns from linear to interrupted and increased dynamics. Furthermore, blocking regular JAIL formation results in increased filopodia, which are not able to drive VE-cadherin dynamics and thus remodeling. Both effects contribute to insufficient migration and an insufficient development of endothelial barrier function. Fourth, siEPLIN blocked an initial TER increase upon shear stress application. Under physiological conditions, onset of fluid shear stress quickly increased membrane protrusion that directly correlated with increased barrier function (DePaola et al., 2001; Hofer et al., 2018; Seebach et al., 2007), and as shown in this study, accompanied both EPLIN- α and at least delayed EPLIN- β recruitment to junctions. Although EPLIN- α terminates cLP and JAIL formation, ablation of both isoforms by siEPLIN blocked the initial shear stress-induced barrier function increase, and accompanied a progressive barrier function decrease. This insufficient EC junction regulation also compromised the ability of long-term adaptation of EC to shear stress, in particular involving collective cell migration and subsequent alignment. Since collective cell migration has been shown in angiogenesis and wound healing to require polarized (front/rear) JAIL formation, which at the same time maintains junction integrity (Cao et al., 2017), we propose EPLINs as critical molecules in controlling actin dynamics during shear stress-induced alignment. The data are in further agreement with impaired

angiogenesis after EPLIN depletion, as previously described (Chervin-Pétinot et al., 2012). In contrast to these and our data, a previous work indicated that miR-95-5p negatively controls EPLIN (not specified) expression, which in turn was proposed to promote angiogenesis, via forces migration. However, the physiological process of angiogenesis requires coordinated EC junction dynamics while maintaining integrity (Cao et al., 2017) even under flow conditions. More work is required to clarify what other genes are further controlled by miR-93-5p and if both EPLIN isoforms are targeted in addition to other genes. Together, the described EPLIN- α functions underline the requirement for a well-balanced cLP and JAIL dynamics, controlled by agonistic and antagonistic molecules to accomplish physiological reactions.

The main functional role of EPLIN- β , however, appears to be the control of stress fiber formation and stability. This conclusion derives from various observations, including that EPLIN- β displays reduced turnover rates and is upregulated in hemodynamically loaded ECs (arteries *in vivo* and after fluid shear stress in cell culture), and last but not least, that its overexpression induces stress fibers. All of these data are in agreement with reports describing that EPLIN- β expression increases actin filament formation nearly twice as much as EPLIN- α does, a phenomenon most likely dependent on a serum-responsive element placed upstream of the EPLIN- α promoter (Maul et al., 2003). Furthermore, EPLIN-enriched stress fibers are less sensitive to their disassembly upon application of the ROCK inhibitor, indicating its stabilizing function. The EPLIN- β function on stress fiber induction and stabilization might be of relevance in certain physiological adaptations and diseases that accompany stress fiber formation, such as disturbed or high shear stress levels, acute and chronic wound healing, as well as inflammation (Moores et al., 2014; Prasain and Stevens, 2009; Schnittler et al., 2014). In addition, EPLIN- β might also have a function on cell junctions where it could stabilize JAAF, whereas EPLIN- α provides a means for the control of dynamic remodeling such as in JAIL.

Finally, EPLIN has been proposed to bind to the VE-cadherin/ α -catenin complex (Chervin-Pétinot et al., 2012), in analogy to the E-cadherin/E- α -catenin complex in epithelium (Abe and Takeichi, 2008). However, it needs to be analyzed if both or only one of the EPLIN isoforms, which are localized at the EC junctions, binds to α -catenin. Based on the data presented here, and given that EPLIN binding to α -catenin stabilizes the cortical actin filament bundles in epithelium (Abe and Takeichi, 2008), EPLIN- β might be a suitable candidate for such a function. The localization of EPLIN- α , however, is most likely coincident with small JAIL developing even in confluent cultures, although it also localizes at the JAAF. At these sites, EPLIN- α might help to induce their dissociation, a process that plays an essential role in activation of EC junctions under wound healing conditions and in inflammation. Overexpression of each of the isoforms caused abnormal dynamics of actin and VE-cadherin distribution and compromised TER development and cell migration. While these effects observed after EPLIN- α overexpression obviously relate to the abortive JAIL formation, the consequences of EPLIN- β overexpression are not so easy to explain, since EPLIN- β -mediated effects on disturbed cell junctions might be

overridden by non-physiological EPLIN- β localization at protrusions under these conditions.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.celrep.2019.09.043>.

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The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
rabbit polyclonal anti-EPLIN	Bethyl-Biomol	Cat#A300-225A; RRID:AB_2249975
goat polyclonal anti-VE-cadherin	Santa Cruz	Cat#Sc-6458200; RRID:AB_2077955
mouse monoclonal anti- α -catenin	BD Transduction Laboratories	Cat#610194; RRID:AB_397593
mouse anti- α -tubulin	Sigma-Aldrich	Cat#T5168; RRID:AB_477579
mouse anti-EGFP	Clontech	Cat#632569
mouse anti-GAPDH	Chemikon	Cat#MAB374; RRID:AB_2107445
rabbit anti-mCherry	Dirk Lindemann (TU-Dresden)	N/A
rabbit anti ArpC/P34	Millipore	Cat#07-227; RRID:AB_310447
mouse anti- α -smooth muscle actin	Sigma-Aldrich	Cat#A5228; RRID:AB_262054
phalloidin-TRITC	Sigma-Aldrich	Cat#P1951; RRID:AB_2315148
donkey-anti-rabbit IgG Secondary Antibody, Alexa Fluor 488	Life/Invitrogen	Cat#A21206; RRID:AB_141708
donkey-anti-goat IgG Secondary Antibody, Alexa Fluor 488	Life/Invitrogen	Cat#A11055; RRID:AB_2534102
donkey-anti-goat IgG Secondary Antibody, Alexa Fluor 568	Life/Invitrogen	Cat#A11057; RRID:AB_142581
donkey-anti-rabbit IgG Secondary Antibody, Alexa Fluor 568	Life/Invitrogen	Cat#A10042; RRID:AB_2534017
donkey-anti-goat IgG Secondary Antibody, Cy5	Jackson	Cat#705-175-147; RRID:AB_2340415
donkey-anti-mouse IgG, IRDye 800CW	LI-COR Biotechnology	Cat#926-32212; RRID:AB_621847
donkey-anti-rabbit IgG, IRDye 800CW	LI-COR Biotechnology	Cat#926-32213; RRID:AB_621848
donkey-anti-goat IgG, IRDye 800CW	LI-COR Biotechnology	Cat#926-32214; RRID:AB_621846
donkey-anti-mouse IgG, IRDye 680CW	LI-COR Biotechnology	Cat#926-32222; RRID:AB_621844
donkey-anti-rabbit IgG, IRDye 680CW	LI-COR Biotechnology	Cat#926-32223; RRID:AB_621845
donkey-anti-goat IgG, IRDye 680CW	LI-COR Biotechnology	Cat#926-32224; RRID:AB_1575081
Bacterial and Virus Strains		
EGFP, LentiVirus	Hofer et al., 2018	N/A
EGFP-p20, LentiVirus	Abu Taha et al., 2014	N/A
EPLIN- α -mCherry, LentiVirus	Hofer et al., 2018	N/A
EPLIN- α -EGFP, LentiVirus	Hofer et al., 2018	N/A
EPLIN- β -EGFP, LentiVirus	Hofer et al., 2018	N/A
VE-cad-mCherry, LentiVirus	Abu Taha et al., 2014	N/A
Chemicals, Peptides, and Recombinant Proteins		
DAPI	Roche	Cat#10236276001
CK-666	Calbiochem	Cat#182515
CK-689	Calbiochem	Cat#182517
Experimental Models: Cell Lines		
Human: umbilical cord vein endothelial cells (HUVEC)	This paper	N/A
MCF-7-VE-EGFP	Rezaei et al., 2018	N/A
MDA-231	ATCC	Cat#ATCC® HTB26; RRID:CVCL_0062
Experimental Models: Organisms/Strains		
Mouse: C57BL/6	Zentrale Tierexperimentelle Einheit, UK Münster	N/A

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Oligonucleotides		
ON-TARGET plus NON-targeting siRNA#1 sequence: (UGGUUUACAUGUCGACUAA)	Dharmacon	Cat#D-001810-01-20
ON-TARGET plus SMART pool siRNA J-010663-09, LIMA1 sequence: (GCUUAAACAUUACGACUGA)	Dharmacon	Cat#L-010663-02-0005
ON-TARGET plus SMART pool siRNA J-010663-17, LIMA1 sequence: (UGUUAGUGUUAGCGAGCCA)	Dharmacon	Cat#L-010663-02-0005
ON-TARGET plus SMART pool siRNA J-010663-18, LIMA1 sequence: (GAAGAAGCUAAGACGAUCU)	Dharmacon	Cat#L-010663-02-0005
ON-TARGET plus SMART pool siRNA J-010663-19, LIMA1 sequence: (AGGUUAAGAGUGAGGUUCA)	Dharmacon	Cat#L-010663-02-0005
Software and Algorithms		
Cell border tracker	Seebach et al., 2015	N/A
Fiji	N/A	https://fiji.sc/
Graphpad Quickcalcs	Graphpad	https://www.graphpad.com/quickcalcs/
MATLAB	Mathworks	N/A
ZEN	Carl Zeiss Jena	N/A
Odyssey	LICOR	N/A

LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Hans Schnittler (Hans.Schnittler@uni-muenster.de). All unique/stable reagents generated in this study are available from the Lead Contact with a completed Materials Transfer Agreement.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell culture

Human umbilical vein endothelial cells (HUVEC) were isolated and cultured in endothelial cell growth medium (Promocell, Heidelberg, Germany) as described elsewhere (Wahl-Jensen et al., 2005). The use of HUVEC was according to the principles outlined in the Declaration of Helsinki and was approved by the ethics boards of the WW-University of Muenster (2009-537-f-S). HUVEC from passage 1 were used for experiments and seeded on crosslinked gelatin-coated culture dishes (Wahl-Jensen et al., 2005): briefly, culture dishes were coated with 0.5% gelatin/phosphate-buffered saline (PBS) (Sigma) for 30 min at 37 °C, subsequently crosslinked with 2% glutaraldehyde (Sigma-Aldrich) for 10-15 min, and incubated with 70% ethanol/water for 30-60 min at room temperature (RT). After washing 5 times with PBS, dishes were exposed to 2 mM glycine/PBS overnight at RT. This coating allows the cells to deposit their own extracellular matrix proteins, which leads to a more physiological cell behavior (Seebach et al., 2007). For cell density-dependent studies HUVEC were seeded at densities of either 1×10^5 cells/cm² or 2.5×10^4 cells/cm² to achieve confluent or subconfluent culture conditions, respectively, within 24 h. To investigate the effect of cell density on the morphological distribution of cell contact proteins in the same cultures, a drop of a cell suspension (100 μ l) was added to a glass-bottomed chamber, which led to a convex bubble due to surface tension. This procedure leads to cell accumulation in the center, while the cell density decreases with increasing distance from the culture center. As a final result, a defined cell density gradient is obtained. MCF-VE-cadherin carcinoma cells were generated by expression of VE-cadherin-EGFP in MCF-7 cells and maintained in RPMI medium supplemented with 10% cultured in as described elsewhere (Rezaei et al., 2018).

Preparation of mouse tissue

The C57 mice are bred in the ZTE (Zentrale Tierexperimentelle Einrichtung) of the Medical Faculty of the University of Münster. For each examination, four male and four female animals were studied, with the Cremaster muscle naturally delivered by male animals. Aortas, distal cava veins, and cremaster muscles were prepared from mice euthanized according to the German legislation for the protection of animals; the use of the animals was approved by the Landesamt fuer Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen (AZ 84-02.05.50.17.011). The tissue was washed with PBS containing 1 mM CaCl₂ and subsequently fixed in 4% PFA in PBS for 1 h at 4 °C.

Isolation of pig cava vein and aortic endothelial cells

Pig cava veins and aortas were obtained from the local slaughterhouse. Endothelial cells were isolated as described elsewhere (Schnittler et al., 1998); briefly, vessels were opened and washed with PBS at 4°C. Endothelial cells were gently scraped off with a soft rubber policeman in PBS supplemented with 1 mM phenylmethylsulfonyl fluoride, 25 µg/ml leupeptin, 25 µg/ml aprotinin, and 25 µg/ml pepstatin, and centrifuged at 200 g for 10 min. Contaminating erythrocytes were lysed at RT in 153 mM NH₄Cl buffered with 16 mM TRIS HCl, pH 7.6, and subsequently centrifuged. This procedure was repeated up to 3 times until all erythrocytes were lysed. Isolated cells were dissolved in 6% SDS sample buffer and tested by western blotting for smooth muscle cell contamination using an antibody against smooth muscle actin. Only preparations free of smooth muscle actin were used for further analyses.

METHOD DETAILS

Immune Staining of HUVECs

For immune labeling HUVEC were fixed with freshly prepared 2% paraformaldehyde dissolved in PBS for 10 min at RT. The cells are then washed three times for 5 minutes each, using PBS with 1% BSA (PBS/BSA). Cell membranes were made permeable with 0.1% Triton X-100 in PBS for 10 min at 4°C, washed again three times PBS/BSA, followed by incubation with the respective antibody as indicated for 1 h at RT or overnight at 4°C, washed again and exposed to appropriate secondary antibodies as indicated. Cultures were mounted in Dakofluorescence mounting medium and evaluated by either LSM or SIM using LSM 780 supplemented with ELYRA super resolution module (Carl Zeiss, Göttingen, Germany).

Immune staining of mouse tissue

Immune staining was performed on free-floating tissue samples after permeabilization with 1% Triton X-100 in PBS/1% BSA for 1 h at RT, washing with PBS, and incubation with the first antibody in PBS/2% BSA at 4°C overnight. After 3 washing steps with PBS/0.1% Triton X-100 for 15 min the tissue was incubated with secondary antibodies and phalloidin-TRITC in PBS/2% BSA for 2 h at RT. After washing steps with PBS/0.1% Triton X-100, 1 µg/ml DAPI/PBS was added for 30 min. The tissue was mounted with DAKO mounting medium.

Cellular fractionation and immunoprecipitation

Cell fractionation of HUVEC cultures was performed at 4°C on ice. All buffers used contained protease inhibitors as follows; 1 mM phenylmethylsulfonyl fluoride, 25 µg/ml leupeptin, 25 µg/ml aprotinin, and 25 µg/ml pepstatin. HUVEC cultures were rinsed with PBS and extracted using lysis buffer (20 mM Tris/HCl, pH 8.0, 150 mM NaCl, 2 mM CaCl₂, 1% Triton X-100, 0.04% sodium acid and protease inhibitor) for 10 min with continuous shaking at 4°C on ice. Cells were scraped and centrifuged for 5 min at 13,200 rpm at 4°C. Triton X-100-soluble and -insoluble samples were heated at 95°C for 5 min, aliquoted, and stored at –20°C until further processing.

Immunoprecipitation using GFP-Trap_A (Chromo Tek, GmbH, Planegg-Martinsried) was performed from subconfluent HUVEC cultures expressing, EGFP, EPLIN-α-EGFP and EPLIN-β-EGFP respectively, according to the manufacturer's protocol.

Western blotting

For western blot analyses, HUVEC cultures were lysed in 6% sample buffer as described elsewhere (Schnittler et al., 1998). Prior to gel loading, the total protein concentration of SDS-solubilized or Triton-extracted samples was determined by the amido black method (Dieckmann-Schuppert and Schnittler, 1997); this was followed by western blotting using standard protocols as described elsewhere using exactly same amounts of protein loaded on each lane (Dieckmann-Schuppert and Schnittler, 1997). After protein transfer (via semidry blotting) membranes were incubated with appropriate antibodies and subsequently detected by secondary near-infrared-labeled antibodies using the Li-Cor system (Li-Cor Odyssey Infrared Reading System, Homburg, Germany). Quantitative analyses were performed using the odyssey software package (Li-Cor). Quantitative determination of total actin in cultured and freshly isolated endothelial cells was performed by SDS-polyacrylamide gel electrophoresis (PAGE). Western blot analysis was performed using the monoclonal mouse pan actin antibody (Amersham, Braunschweig, Germany) followed by near-infrared-labeled secondary antibody. Alternatively bound immunoglobulins were detected by [¹²⁵I] protein A (NEN Du Pont, Berkely, USA) using 0.5 µCi/lane and autoradiography. The labeled bands were excised and their radioactivity counted in a Hewlett-Packard γ-counter. Background label was determined using unlabeled pieces from the same nitrocellulose sheets.

Plasmid construction, lentivirus production, and gene transduction

Generation of fluorescence-tagged EPLIN isoforms (EPLIN-α-mCherry, EPLIN-α-EGFP, EPLIN-β-mCherry, and EPLIN-β-EGFP and its cloning into lentiral pFUGW vector were recently described in detail (Hofer et al., 2018). Prior to gene transduction into endothelial cells the respective virus titers were determined by bioassay using HUVEC cultures. After transduction with different volumes of virus-containing solutions in Promocell –/– (antibiotic and serum free), cells were incubated for 1 h and subsequently suspended in full medium. Medium exchange was performed after 24 h and cells were cultured further until use (usually 2–3 days). Western blotting and fluorescence microscopy were used to analyze expression levels. For overexpression, the virus titers were determined (see above) and the respective virus load was applied to the HUVEC cultures (see Figure S5A).

Downregulation of EPLIN by siRNA

Downregulation of EPLIN in HUVEC cultures was performed by the siRNA (siEPLIN) approach using the Magnet-Assisted Transfection protocol (MATra from Promokine, Heidelberg, Germany). Briefly, cultures were washed with antibiotic- and serum-free Promocell medium. ON-TARGET plus NON-targeting siRNA#1 (sequence: UGGUUUACAUGUCGACUAA) or ON-TARGET plus SMART pool siRNA, LIMA1 (sequences: GCUUAAACAUUACGACUGA, UGUUAGUGUUAGCGAGCCA, GAAGAAGCUAAGACGAUCU, AGGUUAAGAGUGAGGUUCA) (Dharmacon, Lafayette, USA) were diluted in serum-free medium together with the MATra siRNA-reagent (Promokine) and incubated for 20 min. Solutions were added to the cells and exposed to the magnetic field for 15 min at 37°C with 5% CO₂ and further cultured for 4–6 h. After medium exchange, the cells were cultured, usually for 72 h, until the protein was downregulated.

Phase contrast live-cell imaging, scratch assays and determination of cell migration

Phase contrast-based live-cell imaging was performed on an automated Observer (Carl Zeiss, Göttingen, Germany) microscope. A heating insert (PeCon, Erbach, Germany) and a motorized microscope dish (Carl Zeiss, Göttingen, Germany) allows repeated image acquisitions of up to 12 samples (different areas in each sample) at 37°C with 5% CO₂.

For the classical scratch assay, a linear wound was set in confluent HUVEC cultures using a soft needle. Cell migration was determined by phase contrast-based time-lapse microscopy. Alternatively to normal medium with a viscosity of $\eta = 0,8$ mPas, we used high viscous medium with $\eta = 3,5$ mPas at 37° and 5% CO₂. Cell migration was analyzed manually using ImageJ and the chemotaxis and migration tool software (IBIDI, Munich, Germany).

Fluorescence live-cell imaging

Fluorescence live-cell imaging of HUVEC cultures and MCF-VE-EGFP cells expressing fusion proteins was performed by SpDM, (Carl Zeiss, Göttingen, Germany) at 37°C with 5% CO₂ using a culture dish perfusion system consisting of glass-bottomed dish of 1 cm in diameter glued into a 35-mm Petri dishes. A cask cover with an inlet and outlet connected to silicon tubes allows application of drugs. Image acquisition was performed using either Plan Apo 1.3 oil 40X or alpha Plan Apo 63X 1.46 oil objective lenses (Carl Zeiss, Göttingen, Germany). EGFP was excited by a 488-nm laser line (Argon laser) and emission was recorded using a 38 HE Green filter. mCherry was excited by a 543-nm laser line and detected through a 43 HE DsRed filter. The ZEN software (Carl Zeiss, Göttingen, Germany) was used for image acquisition and analyses, as indicated.

Kymographs and correlative analyses of EGFP-p20 and EPLIN- α -mCherry

Kymographs were generated using ImageJ. Intensity profile (y axis) was plotted as a function of time (x axis). For correlative analyses of EGFP-p20 and EPLIN- α -mCherry, a fixed region from time lapse recordings of 1,6 μ m x 9 μ m length for cLP was selected, showing segments of the moving front. After subtracting the background intensity, the center of mass of the EPLIN- α -mCherry and the EGFP-p20 fluorescent signal within the region was computed along the protrusion direction, identifying the main localization of both molecules. This localization is not faithful if the region only shows background intensity (as happens during frames 1–180 in Figure 4E (1)), which is why the overall intensity within the region is also shown.

Fluorescence recovery after photobleaching (FRAP)

HUVEC cultures expressing EPLIN- α -EGFP or EPLIN- β -EGFP were used for FRAP analyses, as described previously (Laugsch et al., 2013) with a few modifications. HUVEC were seeded with a density of 2×10^4 cells/cm², cultured over night and subsequently transduced with EPLIN- α -EGFP or EPLIN- β -EGFP (titrated lentivirus) after another day. Analyses were performed using a Zeiss Laser Scanning Microscope 780 Elyra PS.1 equipped with a 25 mW Argon (458, 488, and 514 nm laser lines), a 20 mW DPSS (561 nm), and a 30 mW diode (405 nm) laser. Pre- and post-bleach images were taken after excitation with the 488 nm laser (2% power) and 493–556 nm for emission using a Plan Aplanachromat 63x objective/1.4 NA oil DIC immersion lens. Prior to bleaching, 20 images were acquired. Circular regions of interest (ROI) with a radius of 3 μ m were selected and bleached with 100% power of the 405-, 458-, 488- and 561-nm laser lines. Fluorescence recovery was continuously recorded for 100 s. The Image series was imported into Fiji to quantify the fluorescence intensity in the ROI. The resulting time course of the fluorescence intensity was further analyzed in MATLAB. In particular, after background subtraction, the fluorescence intensity in the ROI was normalized to the fluorescence intensity before bleaching. The resulting curve of the recovery phase was fitted with a Levenberg-Marquardt algorithm to the following exponential function with the fitting parameters M_f , b , τ using MATLAB:

$$I(t) = M_f * (1 - \exp(-t/\tau)) + b$$

where τ is the time constant, b the fluorescence immediately after photobleaching, and M_f the mobile fraction. The differences between tau and the mobile fraction (MF) were tested for significance for EPLIN- α -EGFP and EPLIN- β -EGFP using the t test.

Impedance spectroscopy under static and shear stress conditions

Impedance spectroscopy is a biophysical method to precisely determine the transcellular electrical resistance (TER) of monolayer forming cells such as epithelium and endothelium (Giaever and Keese, 1984; Wegener and Seebach, 2014). Therefore, 400 nm thick titanium electrodes (one large area reference electrode and one to three small area measuring electrodes) were evaporated on glass

glass-slides, which were subsequently coated with cross-linked gelatin and seeded with HUVEC at a density of 2.5×10^4 cells/cm² and further cultivated until cells became confluent ($0.8\text{--}1 \times 10^5$ cells/cm²). Titanium electrodes are very stable and reusable, and no further adhesion promoters such as cell-toxic chromium are required. The impedance $|Z|$ corresponds to the ratio between the amplitude of the alternating voltage (U_0) and the amplitude of the corresponding alternating current (I_0). The impedance of the cell cultures were analyzed in the range between 10 Hz and 1 MHz using an self assembled impedance generator and analyzer with components obtained from (National instruments, Austin, Texas, USA) supplemented with a measuring software (MOS Technologies, Telgte, Germany) written in LabView (National Instruments, Austin Texas, USA). Measurements and analyzes for a complete spectrum takes about 50 s (Giaever and Keese, 1984; Wegener and Seebach, 2014). A computer controlled relay allows switching between different electrodes to analyze sequentially the time course of the TER for several cell cultures (Kronstein et al., 2012; Seebach et al., 2000, 2016).

For impedance spectroscopy (ISP) under static conditions reusable 8-well stainless steel culture supports were used equipped with impedance spectroscopy glass plates on which one titanium measuring electrode was evaporated for each well (MOS Technologies, Telgte, Germany). The stainless steel block served as reference electrode. ISP under shear stress conditions (see below) was performed on HUVEC, which are cultured on round perfectly parallel glass slides of 60 mm in diameter, evaporated with three titanium measuring electrodes and one reference electrodes (Seebach et al., 2000, 2007). From impedance spectroscopy data under static and shear stress conditions, the TER was determined using TER analytical software (MOS Technologies, Telgte, Germany) and was calculated as $TER(t)/TER(t = 0)$, which is the TER value at a given time point (t) divided by the TER at the time point zero (Kronstein et al., 2012; Seebach et al., 2000, 2016).

Shear stress experiments

Fluid shear stress experiments were performed using the BioTechFlow system (BTF system) (MOS Technologies, Telgte, Germany). The BTF-system was designed to generate well-defined unidirectional or oscillatory stationary flow conditions using the cone and plate principle. In particular, above a perfect plan-parallel fixed glass culture support carrying the EC, which is mounted in a stainless steel chamber, a transparent rotating cone (2,5°) is placed above with the cone tip exactly reaching the EC layer. For a sufficiently low Reynolds number, rotation of the cone generates a stable three-dimensional laminar flow, which ensures a continuous medium exchange above the EC cultures. Analytical and numerical analyzes revealed a steady, laminar and three-dimensional flow of a Newtonian fluid (culture medium) at low Reynolds numbers, which also ensures a medium exchange without disturbing the laminar flow profile. The cone rotation causes a frictional force on the EC layer, which occurs with a time delay of 10^{-3} seconds thus allowing the investigation of very quick effects and a quick change in flow direction where required. The entire cone-plate system is transparent for microscopic evaluation and time-lapse recordings during shear stress exposure (Buschmann et al., 2005; Dieterich et al., 2000; Schnittler et al., 1993). Two different BTF-arrangements were used in this study. For biochemical and alignment studies we used our fully automated BTF system consists of 9 arranged BTF-units that are placed on a revolver plate. In each of the BTF-units, a freely configurable shear stress type (oscillating, unidirectional pulsating or stationary) and shear stress level can be generated, which is set via the cone speed. Furthermore, each of the BTF-unit can be placed automatically over a modified inverse Zeiss Axiovert 20 microscope equipped with an autofocus developed in-house that is based on fast Fourier transformation. The setup allows time-lapse recordings of different cell culture areas in all BTF chambers even during ISP recordings in one run. For shear stress application HUVEC were cultured on cross-linked gelatin coated glass plates until the desired cell density was reached. Shear stress was applied in Promocell medium supplemented with 3% PVP (Sigma-Aldrich, Deisenhofen, Germany), and in the presence of 5% CO₂. Experiments and respective controls were performed in parallel.

Quantification of shear stress-induced EC alignment

Shear stress induced alignment of HUVEC upon siEPLIN and NTEPLIN treatment was quantified after immune labeling with VE-cadherin and EPLIN antibodies using the CellBorderTracker (CBT). Based on the VE-cadherin staining, Cell segmentation was performed using the CellBorderTracker (CBT) (Seebach et al., 2015). The segmentation was imported into Fiji, converted to an 8-bit binary image and analyzed with the “Analyze Particle”-function to determine the aspect ratio of all cells in the image. This parameter represents the ratio between the major and minor axis of an ellipse, which is fitted to the outline of each segmented cell (Seebach et al., 2015). EPLIN intensity was measured in the segmented cells and the aspect ratio was calculated using Fiji.

Fluorescence live-cell imaging under shear stress

Fluorescence live-cell imaging under shear stress conditions was performed using a single BTF-unit (BTF-Fluor), which was mounted on a SpDM (Carl Zeiss, Göttingen, Germany) in 5% CO₂ at 37°C. HUVEC cultures expressing either EPLIN- α -EGFP or EPLIN- β -EGFP respectively were cultured on 0.17 mm-thick, 60 mm in diameter round glass coverslips mounted in specially designed BTF-Fluor culture mounting supports. The mounting supports consist of a metal chamber, which is stabilized by a metal cross avoiding bending of the thin glass slides. For time-lapse recordings either a Plan Apo 1.3 oil 40X or an alpha Plan Apochromat 63x objective/1.4 NA oil DIC immersion lens was used. Fluorescence time-lapse recording was applied on HUVEC cultures exposed to shear stress using SpDM as described above in detail.

Determination of protrusion size and duration time

Protrusion size and duration time were performed from time lapse recordings (2 s interval) of HUVEC cultures expressing LifeAct-EGFP in normal viscous medium ($\eta = 0,8$ mPas) and high viscous medium ($\eta = 3,5$ mPas) at 37° and 5% CO_2 . For the determination of the protrusion size, the time lapse movies were screened for protrusion development in the NTRNA-treated and siEPLIN treated HUVEC, respectively. Manual segmentation of 45 lamellipodia from 3 different cultures was performed using Fiji (Schindelin et al., 2012) to determine protrusion size and duration time of lamellipodia at time point when extensions were maximal.

Determination of stress fibers number and length

EPLIN- α and EPLIN- β or EGFP were overexpressed in HUVEC cultures. Subsequently, cells were fixed by 2% formaldehyde/PBS, pH 7.3 and stained with phalloidin TRITC and DAPI respectively under standardized conditions. Images were acquired using LSM 780 (Zeiss, Oberkochen, Germany) and subsequently analyzed by Fiji (Schindelin et al., 2012). Briefly, a self-written macro detects all ridges within the image (Steger, 1998) and determines the number, length and intensity of all detected structures with a directionality ratio (euclidan distance between start and end-point / total length of the ridge) above a certain threshold (0.98). The method is illustrated in Figure S6.

QUANTIFICATION AND STATISTICAL ANALYSIS

Information of replicates is indicated in figure legends. For TER data analysis a LabView software was implemented in our working group, which calculates the TER values from impedance spectroscopy measurements as well as mean values and the standard deviation. Significances were tested using GraphPad Prism statistical software (San Diego, <https://www.graphpad.com/quickcalcs/ttest1>), which was also used for all other datasets to test for significance, standard deviation ($\pm$ SD) and standard error of the mean (SEM) respectively. Differences were considered significant when $p < 0.05$.

DATA AND CODE AVAILABILITY

This study did not generate any unique datasets or code.