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LAMINAR FLOW-INDUCED ALIGNMENT OF ENDOTHELIAL CELLS REQUIRES CONTINUOUS POLARIZATION OF RAC1 ACTIVITY THROUGH THE RHO-GEF TRIO

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ABSTRACT

Endothelial cells line the lumen of the vessel wall and are constantly exposed to different types of flow patterns. Linear parts of the vessel experience laminar flow, resulting in endothelial cell alignment in the direction of flow, protecting the vessel wall from inflammation and permeability. However, in disturbed flow regions, cells fail to align, leading to vascular inflammation and permeability. Although studies have shown the effects of disturbed flow on endothelial dysfunction, little is known on the alignment process in response to long-term laminar flow. Understanding this mechanism may result in strategies that reduce vascular inflammation in disturbed flow regions. Using a novel FRET-based Rac1 biosensor with increased FRET efficiency allowed us to image over longer periods of time. We show for the first time that Rac1 remains activated and polarized during flow-induced endothelial cell alignment. The Rho-GEF Trio is crucial for flow-induced cell alignment, surprisingly not to activate Rac1 but to keep Rac1 activity at the downstream side of the cell. Moreover, Trio controlled flow-induced monolayer resistance and re-arrangement of the Golgi apparatus. In conclusion, our data show that flow-induced alignment requires the Rho-GEF Trio as a scaffold protein to control polarized Rac1 activation, endothelial permeability and Golgi re-arrangement.
INTRODUCTION

Endothelial cells lining the blood vessels are constantly exposed to shear stress. (Ballermann et al., 1998; Chien, 2007; Hahn and Schwartz, 2009) These frictional forces created by blood flow regulate important pathological and physiological responses, such as arteriogenesis (Galie et al., 2014), acute vessel tone regulation and are furthermore involved in the formation of atherosclerosis (Tzima et al., 2005; Hahn and Schwartz, 2009; Chiu and Chien, 2011). Atherosclerotic lesions mostly develop near branch points and curvatures of the arterial tree. These regions are characterized by low- and disturbed shear stress patterns, leading to failure in endothelial cell elongation and alignment (Chappell et al., 1998; Malek et al., 1999; Chiu and Chien, 2011).

Laminar shear stress however, observed in linear parts of the arteries, induces the alignment of endothelial cells in the direction of flow which is accompanied by actin cytoskeleton remodeling (Tzima et al., 2002; Tzima, 2006; Pan, 2009). This high laminar shear stress, ranging from 10 to 70 dynes/cm² in the arterial vascular network, is known to be athero-protective (Malek et al., 1999). Endothelial cells are able to sense flow by several mechanosensing mechanisms, some well-studied examples are ion channels, primary cilia expressed at the apical surface of cells, and the complex of PECAM-1, VEGFR-2 and VE-cadherin, present at endothelial cell-cell junctions (Hoger et al., 2002; Tzima et al., 2005; Van der Heiden et al., 2008). These signaling proteins play an important role in transmitting the physiological force induced by flow into intracellular signals and are therefore essential for flow-induced alignment. In order for cells to align in the direction of flow they have to remodel their actin cytoskeleton (Satcher et al., 1997). It has been well known that the family of Rho GTPases are involved in this process. The small GTPases Rac1 and RhoA, regulators of the actin cytoskeleton, are rapidly activated upon the onset of flow. Overexpression of dominant negative mutants of Rac1 or RhoA in sparse endothelial monolayers results in impaired elongation and alignment after 4 hours of flow (Wojciak-Stothard and Ridley, 2003). Notably, Rac1 is activated by flow within 30 minutes and localizes at the downstream side of the cell (Tzima et al., 2002; Goldfinger et al., 2008). Yet, until today not much is known about the spatio-temporal activation and regulation of these GTPases during long-term laminar flow conditions.

Rho-GTPases are molecular switches, cycling between a GTP-bound active state and a GDP-bound inactive state (Etienne-Manneville and Hall, 2002). Key regulators in activating GTPases are guanine nucleotide exchange factors (GEFs) (Rossman et al., 2005). Work from the group of Tzima showed the involvement of the GEFs Tiam1 and Vav2 in Rac1 activation after short-term exposure to flow (30-60 minutes) (Liu et al., 2013). Despite this, it is currently not known if these GEFs are also required for long-term flow-induced alignment of confluent monolayers and if Rac1 remains locally activated.

Using a novel Förster Resonance Energy Transfer (FRET)-based Rac1 biosensor, with increased FRET efficiency, we show for the first time that Rac1 is continuously activated and polarized during long-term flow, i.e. 12 hours. We discovered that the Rho-GEF Trio is pivotal for endothelial cell polarity through downstream localization of active Rac1 and Golgi positioning.
RESULTS

Rac1 remains activated and polarized under long-term flow conditions

It has been well established that endothelial cells transiently activate the GTPase Rac1 at the downstream side of the cells after 30 min of high laminar flow (Tzima et al., 2002). However, the activation pattern of Rac1 upon exposure to long-term flow is less well studied. To address this, we examined the spatial and temporal activation of Rac1 under long-term laminar flow for a period of 12 h at 10 dynes/cm² in real-time using FRET-based DORA Rac1 biosensor. Endothelial cells were transfected with this sensor, grown to confluence in specialized flow chambers and flow was applied. FRET-based ratiometric imaging showed that after 30 minutes of flow, Rac1 was activated at the downstream side of the endothelial cell, in accordance with previous literature (Tzima et al., 2002; Liu et al., 2013) (Figure 1A and Video 1). To our surprise, Rac1 not only remained active at the downstream side during longer periods of flow but also showed a significant increase in activation (Figure 1A). The observed Rac1 activation is crucial for flow-induced alignment, since blocking Rac1 activation with the chemical compound EHT-1864 (Shutes et al., 2007) resulted in loss of long-term induced alignment of endothelial cells (Figure 1B). Additionally, long-term flow promoted the linearization of VE-cadherin-based cell-cell junctions, together with an increase in cortical F-actin (Figure 1C). Since linearized junctions were found to promote endothelial barrier function (Seebach et al., 2007), we examined if these flow-induced linearized junctions also promoted endothelial cell monolayers. We measured the resistance of endothelial monolayers upon the onset of flow in real-time using electrical cell-substrate impedance sensing (ECIS) and found that laminar flow increased the endothelial resistance in time (Figure 1D). Interestingly, increasing the flow rate to 10 dynes/cm² additionally promoted the resistance, which is in line with improved barrier integrity (Figure 1D). These data provide evidence that long-term flow promoted continuous Rac1 activation at the downstream side of the endothelial cell. Moreover, long-term flow induced linear cell-cell junctions that improved the monolayer integrity.

The Rho-GEF Trio is required for flow-induced cell alignment

Activation of Rac1 is mediated by specific guanine-nucleotide exchange factors (GEFs) that catalyze the exchange from GDP to GTP. To address which GEF is involved in the alignment process, we reduced the expression of the Rac-GEFs Tiam1, Vav2, PREX-1 and Trio in endothelial cells using shRNA or siRNA and subjected long-term laminar flow to those cells. Interestingly, only Trio-deficient endothelial cells failed to align after long-term (i.e. 12 h) flow, whereas silencing of Tiam1, Vav2 and P-REX1 had no effect on cell alignment (Figure 2A). Quantification showed that Trio-deficient cells significantly failed to align (Figure 2B). Additionally, Trio-deficient cells that were exposed to long-term flow did not induce linear VE-cadherin-based cell-cell junctions and cortical F-actin bundles (Figure 2C).
Figure 1: The small GTPase Rac1 is and remains activated and highly polarized under long-term flow conditions. (A) HUVECs were transfected with the FRET-based DORA Rac1 biosensor, exposed to long-term flow (12 h, 10 dynes/cm²) and FRET-based ratiometric imaging showed spatio-temporal activation in time at the downstream side of the cell (indicated by arrow) (Venus/Cer3). Bar, 25 μm. The bar graph shows the activation ratio of the Rac1 biosensor in time at the downstream side of the cell. Data are means of three independent experiments ± SEM. *P<0.05. (B) Inhibition of Rac1 activity by using the chemical inhibitor EHT 1864 blocks alignment under flow, whereas the solvent control-treated HUVEC respond to flow by elongating and aligning in the direction of flow. The bar graph shows the percentage of aligned cells in under static and flow conditions for both the EHT 1864 treated and solvent control cells. Cells orientated between a 0-45° angle are quantified as being aligned. Data are means of three independent experiments ± SEM. ***P<0.001. Bar, 25 μm. (C) Long-term flow results in cell-elongation, alignment in the direction of flow and more linearized VE-cadherin-based cell-cell junctions. Bar, 25 μm. (D) Impedance measurements using Electric Cell Substrate Impedance Sensing (ECIS) under long-term flow conditions show an increase in monolayer integrity under high-laminar flow conditions.
Figure 2. Trio silencing inhibits long-term flow-induced cell elongation and alignment. (A) HUVECs were treated with Tiam1 shRNA (shTiam1), Vav2 siRNA (siVav2), P-REX1 siRNA (siP-REX1) and Trio shRNA (shTrio) and 12 h flow was applied. Only Trio-deficient cells show loss in flow-induced elongation and alignment. The efficiency of the knockdown was examined by Western blot where actin has been used as loading control. Bar, 25 μm. (B) Cells orientated between a 0-45° angle are quantified as being aligned. (C) A zoom of the junctions that long-term flow results in linearized VE-cadherin based junctions (arrow), indicative for more stable cell-cell junctions. Trio-deficient cells show more zipper-like cell junctions (arrowhead), indicative for less-stable junctions. VE-cadherin is shown in green, F-actin in red. Bar, 25 μm
These data support that Trio is required for long-term flow-induced cell alignment, linearized cell-cell junctions and formation of cortical F-actin bundles.

The N-terminal part of Trio is able to rescue alignment
To examine if in Trio-deficient cells, the inability to promote flow-induced linearized cell-cell junctions has functional consequences for the barrier function, we measured the monolayer resistance in Trio-deficient cells upon induction of flow. We exposed endothelial cells to long-term flow. The results showed that the flow-induced increase in endothelial cell monolayer resistance was significantly reduced in Trio-deficient endothelial cells (Figure 3A and S1A). Thus, Trio is required to maintain the barrier function under long-term laminar flow conditions.

To unravel how Trio regulates flow-induced alignment, we performed rescue experiments with different shRNA-resistant GFP-tagged mutants in Trio-deficient endothelial cells (Figure 3B). Neither the Trio-GEF1 domain only (TrioD1), known to activate Rac1 and RhoG nor the RhoA-activating GEF2 domain (TrioD2) (Blangy et al., 2000; van Rijssel et al., 2012) can rescue flow-induced alignment in Trio-deficient endothelial cells (Figure 3C and 3D). Surprisingly, only expression of the N-terminus of Trio, including the GEF1 domain, spectrin-repeats and the Sec14 domain (TrioN), significantly rescued the alignment of Trio-deficient endothelial cells (Figure 3C and 3D). Moreover, expression of TrioN also rescued the electrical resistance in Trio-deficient cells that was impaired upon exposure to flow (Figure 3E). These experiments suggest that next to the Trio-GEF1 domain other domains in the N-terminus of Trio, e.g. the spectrin-repeats or the Sec14 domain, are important for long-term flow-induced cell alignment.

Trio-GEF1 activity is not required for flow-induced alignment
To proof that GEF1 domain activity of Trio is required for cell alignment, we blocked its activity by using the Trio-GEF1 inhibitor ITX3 (Bouquier et al., 2009; van Rijssel et al., 2012). To our surprise, ITX3-treated cells aligned normally under long-term flow conditions, suggesting that GEF1 activity is not required for long-term flow-induced alignment (Figure 4A). Nevertheless, endothelial cell-cell junction organization was somewhat disturbed and there was less linearization of junctions, indicating that Trio-GEF1 activity may regulate cell-cell junction integrity independent of cell alignment.

Interestingly, both control and Trio-deficient endothelial cells showed increased Rac1 activity upon exposure to long-term flow (Figure 4B). To exclude that the activity of the Trio-GEF1 domain is involved in Trio-mediated long-term flow-induced alignment, we used a GEF catalytic-dead mutant of Trio (N1406A/D1407A). This mutant was unable to activate Rac1 and RhoG (Figure 4C). Expression of this mutant in Trio-deficient endothelial cells rescued long-term flow-induced alignment to a similar extent as TrioN-wild type (Figure 4D). These experiments indicate that the activity of the Trio-GEF1 domain is not required for Trio-mediated long-term flow-induced cell alignment.
Figure 3. The N-terminal part of Trio is able to rescue alignment and the loss in resistance in Trio-deficient cells. (A) ECIS under flow was used to measure the endothelial cell monolayer impedance in control and Trio knockdown conditions. The bar graph shows the normalized resistance after 10 h flow. Data are means of three independent experiments ± SEM. *P<0.05. (B) shRNA-insensitive GFP-tagged mutants GFP-TrioN, GFP-TrioD1 and GFP-TrioD2. Trio consists of three catalytic domains; GEF domain 1, able to activate Rac1 and RhoG; GEF domain 2, able to activate RhoA and a serine/threonine kinase domain at the C-terminus. (C) Immunofluorescent staining of shTrio HUVEC and over-expression of GFP-TrioD1, GFP-TrioD2 and GFP-TrioN and were subjected to long-term flow. GFP is shown in green, VE-cadherin in red and F-actin in white. Quantification of aligned cells after rescue of Trio expression indicates that TrioN expression is able to rescue alignment. (D) and furthermore resulted in a partial rescue of the impaired monolayer resistance under flow, as shown by ECIS. (E) Western Blot analysis confirmed the knockdown of Trio and the over-expression of GFP-TrioN. VE-cadherin expression is not affected.
Figure 4. The activity of Trio-GEF1 is not necessary for laminar long-term flow-induced alignment. (A) Trio-GEF1 activity was blocked by the chemical compound ITX3. Inhibition of GEF1 activity does not interfere with flow-induced alignment. The bar graph shows the quantification of cells that were aligned after ITX3 treatment. Data are means of three independent experiments ± SEM. ***P<0.001. Bar, 25 μm. (B) Data obtained from Rac1 G-lisa experiments show that 12 h flow increases Rac1 GTP levels in both control (shCtrl) and Trio-deficient (shTrio) cells. (C) shTrio-tagRFP cells were transduced with GFP-TrioN-N1406A/D1407A, making the GEF1 domain catalytic dead (TrioN cat. dead) and (D) cells were subjected to 12 h flow. Rescue of Trio expression with the TrioN cat. dead mutant rescued alignment.
Trio is required to localize active Rac1

We hypothesized that Trio may target active Rac1 to the downstream side of the cell in response to long-term flow as Trio is required, but not directly responsible for continuous Rac1 activation under long-term flow conditions. To test this, endothelial cells were lentivirally transduced with an RFP-tagged shRNA-targeting Trio, to visualize Trio-deficient endothelial cells, and subsequently transfected with the DORA-based Rac1 biosensor. Strikingly, flow drives activation of Rac1 after 30 minutes; however, Rac1 activation was not polarized at the downstream side of the cell (Figure 5A and Video 2). This decrease in downstream localization of active Rac1 can be an important factor attributing to the loss of alignment observed in Trio-deficient cells. Quantification showed a significant reduction of Rac1 activity at the downstream side of endothelial cells after long-term flow.

Since we observed that Trio is required for long-term flow-induced linear cell-cell junctions, we focused on the distribution of Trio under these conditions. Due to the lack of proper anti-Trio antibodies for immunofluorescent staining, we expressed GFP-tagged constructs of Trio full length (FL) to study its localization. Under both static and long-term flow conditions, Trio co-localized with VE-cadherin at cell-cell junctions (Figure 5B). Interestingly, quantification of pixel overlap between VE-cadherin and GFP-TrioFL revealed that long-term flow increased VE-cadherin-Trio co-localization at cell-cell junctions. Also TrioN was recruited to cell-cell junctions and showed increased co-localization with VE-cadherin (data not shown). We next tested if the mobility of Trio that was recruited to cell-cell junctions is altered after long-term flow. Fluorescence recovery after photobleaching (FRAP) experiments revealed that long-term flow increased the immobile fraction of GFP-TrioN at cell-cell junction areas, whereas the mobility of GFP-TrioN in the cytosol was unaltered (Figure 5C). No change in mobility of VE-cadherin-GFP after exposure to flow was measured using FRAP (Figure 5C). These data show that flow recruits Trio to cell-cell junction areas.

Trio regulates flow-induced cell polarity

Interestingly, McCue and colleagues showed that flow induced planar cell polarity (PCP) in endothelial cells by phosphorylation of GSK3β and translocation of the microtubule organizing center (MTOC) which in turn determines the localization of the Golgi apparatus (McCue et al., 2006); (Yadav and Linstedt, 2011). As expected, long-term flow significantly increased the translocation of the Golgi to the downstream side of the cell (Figure 6A and 6B). In contrast, this polarized localization was perturbed in Trio-deficient endothelial cells (Figure 6A and 6B), suggesting that Trio is involved in flow-induced re-arrangement of the Golgi apparatus. Yet, flow-induced GSK3β phosphorylation is not affected in Trio-deficient endothelial cells (Figure 6C). These data show that Trio acts downstream from GSK3β phosphorylation, but upstream from Golgi rearrangements and regulates polarized Rac1 activation required for long-term flow-induced endothelial cell alignment.
Figure 5. Trio expression is a prerequisite for targeting active Rac1 at the downstream side of the cell and localization of Trio at cell-cell junctions is promoted by exposure to long-term laminar flow. (A) HUVECs were first transduced with shRNA targeting Trio, containing a RFP tag and subsequently transfected with the FRET-based DORA Rac1 biosensor. Ratiometric imaging showed reduced Rac1 activation at the downstream side of the cell in time (indicated by arrows) (Venus/Cer3). The bar graph shows the activation ratio of the Rac1 biosensor in time at the downstream side of the cell. Data are means of three independent experiments ± SEM. **P<0.005. Bar, 25 μm. (B) GFP-tagged full length Trio (GFP-TrioFL) was expressed in HUVECs. Upon induction of flow, Trio localization at cell-cell junctions increases, as shown by the pixel overlap between Trio and VE-cadherin. Bar, 25 mm. (C) Flow induced an increase in the immobile fraction of GFP-TrioN at the cell membrane shown by FRAP, whereas mobility of TrioN in the cytosol or VE-cadherin-GFP at junctions was not affected. Data are means of three independent experiments ± SEM. ***P<0.001.
Figure 6. Flow-induced cell polarity is regulated by Trio. (A) shCtrl and shTrio HUVEC under static and flow conditions were immunostained with Hoechst to visualize nuclei (blue) and Golgin to visualize the Golgi apparatus (green). Bar, 25 mm. In control conditions long-term flow induces Golgi localization at the downstream side of the cell in (B) 60% of the cases which is affected in Trio-deficient cells. Bar, 25 μm. Data are means of three independent experiments ± SEM. *P<0.05. (C) Western blot analysis showed 12 h of flow (10 dynes/cm²) induced phosphorylation of GSK3β, independently of Trio.
DISCUSSION

In this study we show that Trio controls continuous polarized localization of Rac1 GTP in order to mediate endothelial cell alignment in response to exposure to long-term flow. We additionally found that Trio serves as a scaffolding protein rather than a Rac-GEF upon exposure to long-term flow.

For short-term exposure to high laminar flow, it has been shown that the Rho-GEF Vav2 is of importance in Rac1 GTP loading, whereas the Rac-GEF Tiam1 is responsible for local and polarized distribution of active Rac1 (Liu et al., 2013). We confirmed that subjecting endothelial cells to short periods of flow induced polarized activation of Rac1. Our data indicate that the Rho-GEF Trio is responsible for long-term induced continuous polarization of active Rac1 and subsequent cell alignment. However, we found that Vav2 and Tiam1 were not required for long-term flow-induced cell alignment. This discrepancy of the role of different GEFs in signaling between long-term versus short-term flow may be explained by the fact that there are differences between long- and short-term flow. Short-term flow, like disturbed flow, leads to low-level inflammation by activating the transcription factor NF-κB (Cicha et al., 2008; Hahn and Schwartz, 2009; Petzold et al., 2009; Chiu and Chien, 2011; Wang et al., 2013). Vav2/Tiam1 may account for the phenotype of endothelial cells after short-term flow. However, if Vav2 and/or Tiam1 are also directly involved in short-term flow-induced NF-κB activation and inflammation is not known.

When exposure to laminar flow was extended to several hours and can be considered as long-term flow, this inflammatory phenotype declines to basal levels (Orr et al., 2006, 2008). In fact, exposure of endothelial cells to long-term laminar flow induced an anti-inflammatory phenotype that involved multiple different signaling pathways required for the alignment process (Traub and Berk, 1998; Civelekoglu-Scholey et al., 2005; Yamawaki et al., 2005; Tzima, 2006; Chien, 2007; Mowbray et al., 2008; Liu et al., 2013). Thus, we hypothesize that Trio is controlling signals that are induced by long-term flow whereas other GEFs such as Vav2 and Tiam-1 are involved in short-term or disturbed flow-induced signals.

We show here that endothelial cell alignment requires continuous Rac1 activation at the downstream side of the cell during exposure to long-term flow. Because we additionally show that the Rac-GEF Trio is required for proper cell alignment, we were surprised to find that the GEF activity of Trio is not involved in this process. Instead, the adjacent spectrin-repeats and the Sec14 domain appear to be crucial, since a TrioN-catalytic dead mutant partially rescued cell alignment in Trio-deficient cells. In particular spectrin-repeats, three-helix bundle structures, which can be found in many proteins, are able to unfold after application of mechanical stress and therefore have the ability to interact with other proteins (Djinovic-Carugo et al., 2002; Law et al., 2003). This makes Trio, besides a GEF, also a potential scaffolding protein that is involved in recruiting protein complexes to specific locations in the cell, for instance under long-term flow conditions.
To underscore this element, we show that Trio is involved in stabilizing VE-cadherin-based cell-cell junctions by controlling flow-induced linearization of VE-cadherin-based junctions and promoting the barrier function. VE-cadherin has been recognized as one of the main mechanotransducers in endothelial cells to translate changes in flow conditions (Tzima et al., 2005). Also, we find that long-term flow promotes the localization and stability of Trio at VE-cadherin-based cell-cell junctions and increases the immobile fraction of Trio at these specific sites. Moreover, VE-cadherin and Trio strongly co-localize at these sites. Therefore, we hypothesized that Trio and VE-cadherin interact with each other upon exposure to long-term flow. It is therefore tempting to speculate that Trio collaborates with the VE-cadherin/PECAM/VEGFR2 complex at cell-cell junctions to regulate alignment by acting as a scaffold protein by recruiting a yet undefined Rac-activating protein complex.

To proof that Trio interacts with VE-cadherin under flow-conditions, biochemical immunoprecipitation studies would be required. However, due to the limited number of cells present in the flow chambers, it was technically not possible to perform these experiments.

Another hallmark of adaptations under long-term flow conditions is induction of cell polarity. Recent studies showed a role of glycogen synthase kinase-3β (GSK3β) in regulating cell polarization during cell migration to wound edges (Etienne-Manneville and Hall, 2003; Kim and Kimmel, 2006). In line with this, McCue and co-workers found that shear stress phosphorylates GSK3β and that this is crucial for flow-induced alignment (McCue et al., 2006). We underscored these data and additionally provide evidence that long-term flow redistributes the Golgi to the downstream side of endothelial cells. Interestingly, our data demonstrate that Trio did control redistribution of the Golgi but did not change flow-induced GSK3β phosphorylation, raising the possibility that Trio exerts its function on long-term flow-induced polarity downstream from GSK3β and upstream from Golgi re-orientation. Whether Golgi re-orientation requires Rac1 activity under these conditions is not known.

In conclusion, Trio is required in long-term flow-induced continuous polarization of Rac1 activity and Golgi re-orientation, acting as a scaffolding protein, rather than a Rac-GEF. Trio may potentially scaffold other Rac-GEFs to locally promote GTP exchange on Rac1. Our findings may help to find novel therapies to target Trio distribution in order to promote endothelial cell alignment at sites of disturbed flow and thereby prevent vascular inflammation.
MATERIALS AND METHODS

Antibodies and reagents

Trio (clone D-20), Vav2 (clone H-200), and VE-cadherin (F8) antibodies were from Tebu-Bio (Heerhugowaard, Netherlands). Actin (clone AC-40) mAb (clone PM6/317) antibody was purchased from Sigma-Aldrich (Zwijndrecht, Netherlands). Mouse polyclonal Trio antibody was from Abnova (Heidelberg, Germany). Secondary horseradish peroxidase (HRP)-conjugated goat-anti-mouse, goat-anti-rabbit and rabbit-anti-goat antibodies were purchased from Dako (Heverlee, Belgium). PECAM (CD31; clone HEK170) was purchased from Sanquin (Amsterdam, the Netherlands). Directly labeled VE-cadherin was purchased from BD (clone 55-7H1). To visualize F-actin filaments, differently labeled was phalloidin used (Invitrogen, Bleiswijk, the Netherlands), as well as Hoechst 33258 to visualize the nucleus. Secondary Infrared labeled anti-mouse, anti-rabbit and anti-goat antibodies, used for visualization of proteins by means of Odyssey, were from Westburg (Leusden, the Netherlands).

Cell culture and transfection

Primary Human Umbilical Vein Endothelial Cells (HUVEC) and Human Arterial Endothelial Cells (HAEC) were purchased from Lonza (Baltimore, MD) and were maintained on fibronectin (30 mg/ml) coated tissue culture treated culture flasks (TPP, Switzerland) or glass slides in EGM2-containing SingleQuots (Lonza). Endothelial cells were cultured up to passage four. HUVECs and HAECs were subjected to shear stress for periods as indicated. To inhibit Trio-GEFD1 activity, and thus Rac1 and RhoG activity, cells were pre-treated for 2 h with ITX3 (75 μM), purchased from ChemBridge (San Diego, USA) (Bouquier et al., 2009) and then subjected to shear stress for different periods of time. To specifically inhibit Rac1 activity cells were pre-treated with 12.5 μM EHT 1864 for 2 h, before applying shear stress. HUVECs and HAECs were transfected with GFP-Trio full length (GFP-TrioFL) via electroporation using the Neon transfection system (1 pulse, 1350V, 30 msec) according to manufacturer's protocol (Life Technologies, Bleiswijk, the Netherlands). shRNA constructs targeting Trio (shTrio#1; TRC864 and shTrio#2; TRC10561), a non-specific control shRNA (shCtrl; shc002) and Tiam1 shRNA were used to produce lentivirus in HEK 293T cells by using the third generation packaging plasmids (Hope et al., 1990; Dull et al., 1998). The lentivirus-containing supernatant was harvested after two and three days after transfection, filtered and concentrated by using Lenti-X Concentrator (Clontech, Saint-Germain-en-Laye, France) according to manufacturer's protocol. Lentivirally transduced cells were used three days after transduction for further processing. Adenovirally transduced cells were used two days after transduction for imaging studies.

FRET-based biosensor analysis

The Dimerization-Optimized Reporter for Activation (DORA) Rac1 biosensor was a kind gift from Y. Wu (University of Connecticut Health Center, Farmington, USA). Development of the DORA single-chain Rac1 biosensor is detailed elsewhere (Y. Wu, unpublished data). Briefly, dimeric Cerulean3 coupled to the Rac1 effector p21-activated protein kinase (PAK) is linked via ribosomal protein-based linker (L9H) with circular-permutated Venus coupled to Rac1. Prior to the experiment, cells were electroporated using the Neon transfection system (Life Technologies). In short, HUVEC were transfected with the Rac1-biosensor via electroporation (1 pulse, 1300V, 30 msec) and were used 24 h post-transfection. A Zeiss Observer Z1 microscope equipped with a 40x NA 1.3 oil immersion objective, HXP 120 V excitation light source, a Chroma 510 DCSP dichroic splitter and two Hamamatsu ORCA-R2 digital CCD cameras were used for simultaneous monitoring of Cer3 and Venus emission. Zeiss Zen 2012 microscope software was used to control the system. Offline ratio analyses between Cer3 and Venus images were processed utilizing the MBF imageJ collection (Tony Collings). Image stacks were background corrected, subsequently stacks were aligned and a smooth filter to both image stacks were applied to improve image quality by noise reduction. An image threshold was applied exclusively to the Venus image stack, converting background pixels to ‘not a number’ (NaN), eliminating artefacts in ratio image stemming from the background noise. Finally, Venus/Cer3 ratio was calculated were high activation is shown in red/white and low activity is displayed in blue/black.
**Knockdown with siRNA**

Vav2 and PREX-1 siRNA SMARTpools were purchased from Dharmacon (Lafayette, CO). Cells were transfected with 1 nM siRNA by means of interferin transfection reagent (Polyplus/Westburg, Leusden, Netherlands) according to the manufacturer's instructions. After 72 h, cells were assayed as described below. Note that the oligos efficiently reduced protein expression in HUVECs as well in HAECs.

**Adenovirus production**

GFP-TrioD1, GFP-TrioD2, GFP-TrioFL and GFP-TrioN, were obtained as previously described. (van Rijssel et al., 2012) Adenovirus expressing GFP-TrioD1, GFP-TrioD2, GFP-TrioFL and GFP-TrioN was produced by transfecting PacI-digested (Westburg, Leiden) constructs into HEK293T cells.

**Western Blotting**

Cells in IBIDI-slides (IBIDI, Planegg, Germany) were washed three times with ice-cold phosphate-buffered saline (PBS) containing 1 mM CaCl₂ and 0.5 mM MgCl₂ and boiled in SDS-sample buffer containing 4% β-mercaptoethanol. Samples were analyzed by SDS-PAGE or 3-8% Tris-Acetate gradient gels (Invitrogen) and subsequently transferred to a 0.2 μm nitrocellulose membrane (Whatman, Dassel, Germany) and blocked with blocking buffer containing 5% (wt/vol) non-fat dry milk in Tris-buffered saline with Tween-20 (TBST). The nitrocellulose membrane was incubated with specific primary antibodies for 1 h at room temperature, followed by incubation with secondary HRP-conjugated antibodies for 1 h at room temperature. Between all the incubation steps, the blots were washed at least three times with TBST for 10 min. Staining was either visualized with a enhanced chemiluminescence detection system (Pierce, Rockford, IL) or by infrared imaging by using the Oddyssey (LI-COR Biosciences, Lincoln, USA).

**Immunofluorescence**

Immunofluoroscently stained endothelial cells were grown in fibronectin coated IBIDI-slides (IBIDI). After treatment, cells were washed twice with room temperature PBS (phosphate-buffered saline, 1 mM CaCl₂, 0.5 mM MgCl₂) and subsequently fixed in 3.7% (vol/vol) formaldehyde in PBS (+1 mM CaCl₂, 0.5 mM MgCl₂) for 10 min. After fixation, cells were permeabilized in PBS-T (PBS + 0.1 % Triton-X100) for 10 min. Next, cells were incubated with primary and secondary antibodies and ,between each incubation, washed three times with PBS (+1 mM CaCl₂, 0.5 mM MgCl₂). Finally cells were kept in PBS (+1 mM CaCl₂, 0.5 mM MgCl₂) until imaging with a confocal laser-scanning microscope (LSM510 META, Carl Zeiss MicroImaging, Jena, Germany). FRAP experiments were performed using 50 iterations with 488-nm laser illumination at maximum power (25 mW). Fluorescence recovery was measured by time-lapse imaging. Prism 6 (GraphPad Software, La Jolla, CA) was used for statistical analysis and nonlinear regression. A single-exponential association was used for curve fitting: \( Y = Y^{\text{max}}(1 - \exp(-KX)) \), which starts at zero and ascends to \( Y^{\text{max}} \) with a rate constant \( K \), where \( Y^{\text{max}} \) represents the mobile fraction and \( K \) represents the time characteristic of the curve.

**Laminar pulsatile flow system**

All cell cultures were kept in a humidified atmosphere of 95% air and 5% CO₂ at 37 °C. Prior to the experiment, cells were seeded at semi-confluence in IBIDI VI-microslides (IBIDI). Four hours after seeding, the slide was connected to a peristaltic pump, equipped with eight roller heads to decrease the pulse and a bubble trap (Technical University of Denmark, Kgs. Lyngby, Denmark) to filter out air bubbles in the closed flow system. A surface area of 0.8 cm² was exposed to fluid shear stress generated by perfusing culture medium over the cells. The physiological shear stress in arteries, 10 dynes/cm², was used for all experiments, unless stated otherwise.

**Electric cell-substrate impedance sensing under laminar flow**

Endothelial monolayer integrity was determined by measuring the electrical resistance using Electric Cell-Substrate Impedance Sensing (ECIS). Flow chamber electrode arrays (8F10E; Applied
Biophysics, Troy, NY) were pretreated with 10mM L-cysteine (Sigma-Aldrich) for 15 min at 37 °C, subsequently washed twice with 0.9% NaCl and coated with fibronectin (Sanquin) in 0.9% NaCl for 1 h at 37 °C. Cells were seeded at 300,000 cells per slide (2.5cm²) and grown to confluence. Continuous resistance measurements were performed at 37 °C at 5% CO₂ with the ECIS Z (Theta) system controller (Applied Biophysics). After forming a stable monolayer, the cells were subjected to flow (2.5-10 dynes/cm²) for several hours.

Statistical analysis
For statistical analysis between experimental groups, the Student's t-test was used. A two-sided p value of ≤0.05 was considered to be significant. Unless stated otherwise, a representative experiment out of at least three independent experiments is shown.

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DISCLOSURES
None
REFERENCES


Online Figure 1. Trio is required for the flow-induced increase in monolayer integrity. ECIS under flow was used to measure endothelial monolayer integrity in control and Trio knock-down conditions. An increase in monolayer integrity in control conditions was observed after applying laminar flow to the cells. This flow-induced increase in monolayer integrity was largely reduced in Trio-deficient cells.

LEGENDS FOR VIDEO FILES

Video 1. FRET-based Rac1 biosensor exposed to long-term laminar flow shows downstream Rac1 activity. HUVECs were transfected with the FRET-based biosensor, exposed to long-term flow (12 h, 10 dynes/cm²). Movie shows spatio-temporal activation of Rac1 (Venus/Cer3) in time at the downstream side of the cell. Black/dark colors represent low Rac1 activity, whereas white/red colors represent high Rac1 activity. Arrow indicates the flow direction.

Video 2. Trio-deficient cells show reduced polarization of downstream Rac1 activity upon exposure to long-term flow. HUVECs were first transduced with shRNA targeting Trio, containing an RFP-tag and are subsequently transfected with the FRET-based Rac1 biosensor. Next, cells were exposed to long-term flow (12 h, 10 dynes/cm²). Movie shows spatio-temporal activation of Rac1 (Venus/Cer3), albeit reduced at the downstream side. Black/dark colors represent low Rac1 activity, whereas white/red colors represent high Rac1 activity. Arrow indicates the flow direction. shTrio-tagRFP shows visualizes knock-down cells.