A novel pathway spatiotemporally activates Rac1 and redox signaling in response to fluid shear stress

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Introduction

Shear stress, the frictional force produced by blood flow, is a critical regulator of cardiovascular development and function. Endothelial cells lining blood vessels are equipped with numerous mechanoreceptors that function to convert mechanical force into signaling cascades that regulate diverse EC responses, such as oxidative balance, gene expression, and alignment of cytoskeletal filaments (Hahn and Schwartz, 2009). The Rho family of small GTPases are master regulators of many cellular activities (Etienne-Manneville and Hall, 2002), and are critical for the shear stress response (Tzima, 2006). The family of small GTPases cycle between inactive GDP-bound and active GTP-bound states to regulate numerous EC responses to shear stress. In particular, Rac1 signaling regulates EC alignment and polarization (Tzima et al., 2002; Wojciak-Stothard and Ridley, 2003), NF-κB-dependent gene expression (Tzima et al., 2002), and reactive oxygen species (ROS) production (Yeh et al., 1999) in response to flow. However, the mechanisms by which hemodynamic forces activate Rac1 and orchestrate such diverse cellular responses remain unknown.

Work over the past few years has identified a mechanosensory complex at cell junctions consisting of PECAM-1, VE-cadherin, and VEGFR2, that is required for the activation of a number of shear-dependent signaling pathways. Interestingly, ECs lacking PECAM-1 or VE-cadherin exhibit impaired NF-κB activation and alignment in response to shear stress (Tzima et al., 2005). As NF-κB activation and EC alignment are both Rac1-dependent processes, we hypothesized that PECAM-1 and VE-cadherin may play a role in the regulation of flow-induced Rac1 activity.

Results

PECAM-1 is required for flow-induced Rac1 activation, whereas VE-cadherin is essential for polarization of active Rac1

To gain insights into the pathway that regulates flow-induced Rac1 activation, we first tested the role of the junctional components
of the mechanosensory complex. As flow–induced cell alignment and NF-κB activation are downstream of PECAM-1 and VE-cadherin (Tzima et al., 2005), and mediated by Rac1 (Tzima et al., 2002), we hypothesized that activation of Rac1 is dependent on these adhesion receptors. Pull-down assays were performed in which Rac1 GTP loading was determined by specific binding of the active GTPase to the p21-binding domain of PAK1 (PBD). PECAM-1−/− ECs (PE-KO) failed to show activation of Rac after onset of flow, whereas null cells engineered to reexpress PECAM-1 (PE-RC) showed an increase in Rac1 GTP loading in response to flow (Fig. 1 A), similar to that seen in wild-type ECs (Tzima et al., 2002; Wojciak-Stothard and Ridley, 2003). Surprisingly, despite defects in responses downstream of Rac (Tzima et al., 2005), VE-cadherin−/− ECs (VE-KO) showed normal levels of Rac1 activation, similar to those seen in VE-cadherin–expressing ECs (VE-RC; Fig. 1 B), suggesting that VE-cadherin is not required for flow–induced GTP loading of Rac1.

Rho GTPase activation is highly spatially and temporally regulated to permit localized signaling responses. In particular, shear stress induces localized activation of Rac1, and Rac1 activity has to be spatially restricted in order for cells to align in the direction of flow (Tzima et al., 2002). To assess whether VE-cadherin is important for localized activation of Rac1 in response to flow, we used FLAIR (fluorescence activation indicator for Rho proteins; Kraynov et al., 2000). Fluorescence resonance energy transfer (FRET) images showed high Rac1 activity in both VE-RC and VE-KO ECs subjected to shear stress (Fig. 1 C). Quantitation revealed a substantial increase in the fraction of both VE-KO and VE-RC cells that scored positive for FRET (Fig. 1 D). However, when cells were scored for spatial distribution of Rac1-FRET (Fig. S1 A), we found that in the majority of VE-RC cells, Rac1 activity was strongly localized to the downstream edge, whereas Rac1 activity was randomly oriented in VE-KO ECs (Fig. 1 D). It is important to note that processing of our samples did not alter FRET observed in live ECs (Fig. S1 B). Taken together, these data demonstrate that PECAM-1 is required for global changes in Rac activation (GTP loading), whereas VE-cadherin is essential for providing the spatial information needed to polarize Rac1 activity in response to flow.

**Spatial activation of Rac1 is mediated by VE-cadherin and is required for ROS production**

Rac1 is a component of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Abo et al., 1991; Knaus et al., 1991) and is widely known to act upstream of ROS production in a variety of cell types, including ECs in response to shear stress (Chiu et al., 1997; Yeh et al., 1999). Interestingly, ROS production is temporally and spatially regulated to facilitate compartmentalization of redox signaling (Usuihio-Fukai, 2009). As exposure of ECs to shear stress elicits directional cellular behaviors, including cell alignment and directional Rac activation, we hypothesized that ROS production may require both global and directional Rac1 activation in response to flow. To further test the idea that polarized Rac activity may be important for ROS production, we examined ECs transfected with a constitutively active Rac variant, V12Rac, which induces uniform high levels of Rac activity without detectable polarization toward the downstream edge (Tzima et al., 2002). Expression of V12Rac signaling events (Rajagopal et al., 2010). Vav2 mediates Rac activation in ECs in response to VEGF (Gavard and Gutkind, 2006) and is activated downstream of multiple receptor tyrosine kinases (Schiller, 2006). We therefore interrogated whether shear stress can also activate Vav2. Phosphorylation of Tyr172 revealed that shear stress induced rapid activation of Vav2 (Fig. 2 A). Interestingly, Vav2 activation was dependent on PECAM-1, as well as Src tyrosine kinase activity (Fig. 2 B; Fig. S2 C), suggesting that PECAM-1–mediated mechanotransduction and downstream Src activation (Tzima et al., 2005) are required for shear stress–induced Rac1 activation. To study the role of Vav2 in shear stress–induced Rac1 activation, we used siRNAs against Vav2 to decrease its expression in ECs. Two siRNA sequences were used to rule out the possibility of the off-target effects (Fig. S2 A). As shown in Fig. 2 C, shear stress–induced Rac1 GTP loading was compromised in Vav2-depleted ECs, whereas nonspecific siRNA had no effect, suggesting that Vav2 mediates flow–induced GTP loading of Rac. Thus, Vav2 facilitates GTP loading of Rac1 in response to shear stress.

In contrast to Vav2, silencing Tiam1 with siRNAs (Fig. S2 B) did not affect the overall GTP loading of Rac1 by flow (Fig. 2 C; Fig. S2 D), suggesting that Tiam1 is not the GEF responsible for flow–induced Rac1 activity. To assess whether Tiam1 is important for localized activation of Rac1 in response to flow, we used the FRET–based Rac1 biosensor. As observed with the affinity–based pull-down assay, when we compared the average overall Rac1 activity levels using the average Rac1-FRET values in each cell, we could not detect a difference between the Tiam1-depleted cells and control siRNA-transfected cells. However, when scored for spatial distribution, Rac activity was randomly oriented in Tiam1-depleted cells, whereas control siRNA-transfected cells exhibited an obvious directionality of activated Rac1 relative to the flow direction (Fig. 2 D). Overall, these data demonstrate distinct yet synergistic roles for two Rac GEFs in shear stress signaling: Vav2 is required for GTP-loading of Rac1, and thus functions as a canonical GEF, whereas Tiam1 is important for localized activation of Rac1.

**Vav2 mediates flow–induced Rac1 activation, whereas Tiam1 regulates spatial organization of activated Rac**

The activation of Rho proteins is mediated by specific guanine nucleotide exchange factors (GEFs) that catalyze the exchange of GDP for GTP (Hall, 2005). The mechanisms by which cells coordinate distinct sets of GEFs, GTPases, and effectors in response to a diverse array of biological stimuli remains an unanswered fundamental question (García-Mata and Burridge, 2007). We therefore sought to identify the specific GEFs that are involved in Rac1 activation in response to shear stress. We considered Vav2 and Tiam1 as candidate GEFs and examined their contribution to both GTP-loading and spatial localization of activated Rac. Tiam1 helps restrict the activity of Rac to dendritic spines via its interaction with the PDZ protein Par3 (Nishimura et al., 2005; Zhang and Macara, 2006), and Tiam1 scaffolding is also important for coupling upstream signals to downstream
Our results indicate that polarized Rac1 activation is required for flow-induced ROS production. In light of our data suggesting that Tiam1 is required for polarized Rac1 activation, we hypothesized that Tiam1 is also essential for flow-induced ROS production. Consistent with this hypothesis, ROS production was dramatically attenuated in Tiam1-depleted ECs (Fig. 4 A). Furthermore, our data indicate that Tiam1 is not required for GTP loading of Rac1, suggesting the hypothesis that Tiam1 GEF activity is not required for ROS production. We next tested the requirement for Tiam1 GEF activity in flow-induced ROS production. Rescue experiments were performed using siRNA-resistant wild-type Tiam1 and a GEF-deficient variant, 1047/1232ATiam1 (Fig. 4 B; Worthylake et al., 2000). As shown in Fig. 4 B, the wild type and a GEF-deficient Tiam1 mutant rescued flow-induced ROS production to a similar extent, suggesting that Tiam1 controls Rac1 activation and downstream redox signaling independent of its GEF activity.
the shear stress–mediated ROS production in ECs, thereby establishing that Tiam1 is an important mediator of the effect of shear stress in redox signaling.

Tiam1, Nox2, and Par3 polarize in response to shear stress
VE-cadherin and Tiam1 are clearly crucial for proper localization of active Rac1, which is instrumental for the subsequent activation of biological signaling events. Next, we considered whether the subcellular localization of Tiam1 itself may establish localized Rac1 activation. The onset of shear stress induced polarization of Tiam1 to the downstream edge of the cell (Fig. 5 A), similar to the pattern of Rac1 activation induced by flow (Fig. 1 C). Interestingly, we did not observe polarization of VE-cadherin in response to shear stress (Fig. 5 A), suggesting that VE-cadherin itself does not redistribute under flow, but rather provides a platform for the recruitment of other proteins, such as Tiam1 and active Rac1 to the downstream edge of the cell. In light of our evidence suggesting that polarized Rac1 activation is required for the shear stress–mediated ROS production in ECs, thereby establishing that Tiam1 is an important mediator of the effect of shear stress in redox signaling.

Our data suggest that Tiam1 controls ROS production by its ability to regulate spatial activation of Rac1. Similarly, in contrast to the in vivo situation (in which the levels of active GTPases are tightly regulated and occur in highly defined subcellular regions), when a Rho GTPase is globally activated, its effectors might aberrantly signal outside of the spatio-temporal context of their specific signaling modules (Pertz, 2010). To further evaluate the relevance of this pathway, we assessed the role of Tiam1 in ROS production in vivo. We developed a fluorescence-based assay to specifically monitor ROS production in the mouse aorta and verified that the measured fluorescence is solely due to ROS production, as it was absent in aortas treated with the ROS inhibitor diphenyleneiodonium (DPI; Fig. S3). As shown in Fig. 4 C and Fig. S4, in vivo knockdown of Tiam1 led to a marked decrease in ROS production. In this in vivo setting, we cannot distinguish between the role of Tiam1 in ECs and the contribution of other vascular cell types to ROS production. However, our in vitro data document that Tiam1 is required for
required for polarization of the microtubule-organizing center in ECs in response to flow (Tzima et al., 2003). Indeed, we observed flow-induced polarization of Par3 to the downstream edge of the cell (Fig. 5 A); additionally, expression of a dominant-negative Par6 (DN Par6) abolished shear-induced Tiam1 polarization (Fig. 5 B), whereas Tiam1 polarization in ECs expressing wild-type Par6 (WT Par6) was unaffected. These data suggest that the flow-induced polarization of Par3/6 provides a spatial cue for the recruitment of other proteins (such as Tiam1) to the downstream edge of the cell that direct proper localization of activated Rac1 and subsequent redox signaling. Next, we hypothesized that depletion of Par3 would eliminate the polarity cue for the recruitment of other proteins, and thus disrupt flow-induced ROS production. In agreement with this hypothesis, ECs depleted of Par3 using specific siRNAs displayed blunted ROS production compared with ECs transfected with a control siRNA (Fig. 5 C).

A novel signaling complex that couples spatial Rac1 activation to NADPH oxidase

Par3/6 have been shown to complex with Tiam1 to provide polarity cues in numerous cell types and organisms (Chen and Macara, 2005; Mertens et al., 2005; Nishimura et al., 2005). Therefore, we investigated if these proteins also interact with each other to provide polarity cues in response to shear stress. Onset of flow induced the formation of a Par3–Tiam1 complex, which, unexpectedly, also contained VE-cadherin (Fig. 6 A). In contrast, flow did not promote increased association of Par3 with Tiam1 in the absence of VE-cadherin (Fig. 6 A). Surprisingly, shear stress also induced the association of Tiam1 and VE-cadherin with the p67phox subunit of NADPH oxidase (Fig. 6, B and C). Notably, the level of Tiam1 coimmunoprecipitation with p67phox was not increased by shear stress stimulation in the absence of VE-cadherin (Fig. 6 B), suggesting that VE-cadherin mediates ROS production by regulating Tiam1 association with p67phox. We next addressed the possibility of a direct interaction between Tiam1 and p67phox. In GST pull-down assays using recombinant proteins (Fig. 6 C), direct binding was observed between p67phox and the PHn-PHc Tiam1 variant, which encompasses the majority of the protein (Fig. 6 D). Moreover, the PHn-CC-Ex domain also associated with p67phox directly (Fig. 6 D), suggesting this region is primarily responsible for the direct interaction. Interestingly, the PDZ domain of Tiam1 did not bind to p67phox. Additionally, we performed NMR-based titration experiments where a VE-cadherin peptide comprising the last 16 amino acids was titrated into a sample containing 15N-labeled Tiam1 PDZ domain. Addition of the VE-cadherin peptide caused chemical shift perturbations in residues that are known to be important for ligand binding (Fig. S5; Shepherd et al., 2010; Shepherd and Fuentes, 2011), indicating a direct, but weak interaction. Importantly, a similar approach was used to show an interaction of the VE-cadherin C-terminal tail with the PDZ domain of Par3 (Tyler et al., 2010). Overall, these data show that Tiam1 binds to p67phox via its PHn-CC-Ex domain and that VE-cadherin binds the Tiam1 PDZ domain. Previous studies have shown that Par3 binds to the PHn-CC-Ex domain of Tiam1 through its CC domain and the C terminus of VE-cadherin through its third PDZ domain, both of which are direct (Nishimura et al., 2005; Shepherd et al., 2010; Shepherd and Fuentes, 2011).
achieved via the formation of a VE-cadherin–Par3–Tiam1 complex, which allows its coupling to the p67phox subunit of the NADPH oxidase and downstream redox signaling. In support of this model, ECs depleted of VE-cadherin, Tiam1, or Par3 show impaired ROS production in response to shear stress. Our finding that knockdown of Tiam1 in the mouse aorta also reduces ROS production clearly points to the functional relevance of this pathway in vivo.

Discussion

In this study we identify a molecular mechanism by which shear stress activates Rac1 and regulates downstream ROS production. We reveal differential roles for two components of the mechano sensory complex in this pathway: PECAM-1 is required for GTP loading of Rac; in contrast, VE-cadherin is not required for GTP loading, but is essential for polarized activation of Rac1 in response to shear stress. This polarized activation of Rac1 is achieved via the formation of a VE-cadherin–Par3–Tiam1 complex, which allows its coupling to the p67phox subunit of the NADPH oxidase and downstream redox signaling. In support of this model, ECs depleted of VE-cadherin, Tiam1, or Par3 show impaired ROS production in response to shear stress. Our finding that knockdown of Tiam1 in the mouse aorta also reduces ROS production clearly points to the functional relevance of this pathway in vivo.

The mechanisms that determine polarity, including that of Rac1, in response to shear stress is a longstanding question in the field and is an intense area of study. Recent work modeling polarized Rac1 activation in ECs predicts that an endothelial cell in a confluent monolayer would experience compression at the upstream edge of the cell and tension at the downstream edge when exposed to flow (Allen et al., 2011). Therefore, mechanosensitive
demonstrate that polarization of Rac1 signaling is a biologically meaningful event, as disruption of Rac polarity impairs flow-induced ROS production.

The establishment and maintenance of cell polarity is crucial for many biological functions and is regulated by conserved protein complexes. Interactions between Par complex proteins and Rho GTPases are essential for the proper regulation of cell and cytoskeletal polarity in a wide variety of polarized cell types. For example, Par3 functions to restrict Rac-GTP formation to dendritic spines by binding to Tiam1 (Nishimura et al., 2005; Zhang and Macara, 2006). Similarly, the Par3/6 polarity complex, in conjunction with Tiam1-mediated Rac1 signaling, controls...
VE-cadherin is required for flow-induced association of Tiam1 with p67phox, suggesting that VE-cadherin mediates ROS production by regulating the association of these proteins. During the preparation of this manuscript, another group reported that VE-cadherin and Tiam1 mediate shear-induced Rac1 GTP loading and barrier enhancement in brain microvascular ECs (Walsh et al., 2011). Our data show that VE-cadherin and Tiam1 are not required for GTP loading of Rac1 but are important for polarized activation of Rac1. A possible reason for this discrepancy could be the origin of ECs used in that study and/or the approaches used to inhibit VE-cadherin and Tiam1. Here, we identify Vav2 as the critical GEF for shear-induced GTP-loading of Rac1. Our data indicate impaired Rac1 activation in Vav2-depleted ECs. Notably, Vav2 localization is not polarized under shear (unpublished data).

apical–basal cell polarity in contacting epithelial cells (Mertens et al., 2005). A recent study reported that Tiam1 interactions with different scaffolding proteins couple distinct upstream signals to localized Rac1 activation and specific downstream pathways (Rajagopal et al., 2010). Our results now identify a novel role for Tiam1 as an adaptor in a VE-cadherin–p67phox–Par3 polarity complex that provides the spatial cues required for ROS production. We show that shear stress induces polarization of Par3/6, which mediates the polarization of Tiam1; Tiam1 binds to p67phox via its PHn-CC-Ex domain and to VE-cadherin via its PDZ domain. Surprisingly, VE-cadherin does not polarize under shear forces. These data suggest that VE-cadherin provides the membrane anchor that stabilizes other polarized proteins, such as the Par complex, to the downstream edge of the cell. Interestingly, VE-cadherin is required for flow-induced association of Tiam1 with p67phox, suggesting that VE-cadherin mediates ROS production by regulating the association of these proteins. During the preparation of this manuscript, another group reported that VE-cadherin and Tiam1 mediate shear-induced Rac1 GTP loading and barrier enhancement in brain microvascular ECs (Walsh et al., 2011). Our data show that VE-cadherin and Tiam1 are not required for GTP loading of Rac1 but are important for polarized activation of Rac1. A possible reason for this discrepancy could be the origin of ECs used in that study and/or the approaches used to inhibit VE-cadherin and Tiam1. Here, we identify Vav2 as the critical GEF for shear-induced GTP-loading of Rac1. Our data indicate impaired Rac1 activation in Vav2-depleted ECs. Notably, Vav2 localization is not polarized under shear (unpublished data).

Figure 6. A novel signaling complex that couples spatial Rac1 activation to the NADPH oxidase. (A) VE-RC or VE-KO ECs were transiently transfected with HA-tagged Tiam1 expression construct. 24 h after transfection, cells were sheared for indicated times or kept as static control. Cell lysates were immunoprecipitated with an anti-HA antibody followed by immunoblotting with anti-Par3, anti–VE-cadherin, or anti-HA antibodies. Three experiments were performed for each cell type and numbers below the gel panels represent the fold of band intensity relative to t = 0 min. (B) VE-RC or VE-KO ECs were transiently transfected with HA-tagged Tiam1 and Flag-tagged p67phox. 24 h after transfection, cells were sheared for indicated times or kept as static control and cell lysates immunoprecipitated with anti-Flag M2 agarose. The immune complexes were subjected to SDS-PAGE followed by immunoblotting with anti-HA and anti-Flag antibodies. Three experiments were performed for each cell type and numbers below the gel panels represent the fold of band intensity relative to t = 0. (C) Tiam1 variants used in the in vitro direct binding assays. (D) Equal amounts of GST or GST-p67 fusions were incubated with indicated His-tagged Tiam1 variants in vitro. Bound proteins were detected by immunoblotting with an anti-6xHis monoclonal antibody. The white line indicates where the Western blot was cut to remove irrelevant lanes. (E) Shear stress regulates Rac1 activation through two distinct GEFs, Vav2 and Tiam1. Vav2 controls the global Rac1 GTP loading while Tiam1 links Rac1 to the flow-dependent polarity complex composed of Par3 and VE-cadherin. Tiam1 also couples the polarity complex to the NADPH oxidase for ROS production in a flow-dependent manner.
This result agrees with our results indicating Tiam1 as the GEF required for Rac1 polarization. ECs depleted of Tiam1 (with Vav2 expression intact) globally activate Rac1 in response to shear, but lack downstream Rac1 polarity (Fig. 2D). These results highlight the differential roles of the two GEFs in this system: Vav2 is required for global GTP loading and activation of Rac1, whereas Tiam1 is required for recruitment of activated Rac1 to the downstream edge of the cell.

The Nox family of proteins is essential in normal physiology (Brown and Griendling, 2009). Nox proteins and ROS have been shown to regulate many fundamental physiological processes, including cell growth, differentiation, apoptosis, angiogenesis, and cytoskeletal remodeling. In the vasculature, ROS are required for endothelial cell proliferation and migration during angiogenesis (Ushio-Fukai, 2007), as well as flow-mediated vascular remodeling via activation of MMPs (Castier et al., 2005). Within the endothelium, the membrane-bound Noxa2 subunit of the NADPH oxidase has been shown to be a major source to EC ROS production (Brown and Griendling, 2009). Our data suggest that Noxa2 may also play a role in flow-mediated ROS production. In addition, NADPH oxidases are now recognized to have specific subcellular localizations, and compartmentalization of redox signaling is thought to involve the interaction of NADPH oxidase subunits with distinct signaling platforms associated with different subcellular sites (Ushio-Fukai, 2009). Thus, recruitment of p67phox to a VE-cadherin–Tiam1 scaffold in response to flow may reflect a general strategy of targeting oxidant production to particular subcellular sites as a means to gain signal specificity (Ushio-Fukai, 2009). Overall, these results indicate that VE-cadherin is required for the flow-induced Par3–Tiam1 complex formation and that these proteins might be components of a pathway that links spatial cues with redox signaling (Fig. 6E). Importantly, E-cadherin was similarly required for establishing polarity during cell migration (Desai et al., 2009), indicating that this pathway may represent a general role for cell–cell adhesion in polarization.

The work described here identifies a novel signaling module that links cell–cell junctions, polarity cues, and redox signaling in response to blood flow. This signaling pathway constitutes a novel paradigm that demonstrates distinct yet synergistic roles for two Rac GEFs: one GEF regulates global Rac1 activation, while the second determines spatial activation of Rac1. Compartmentalization of Rac1 activity is achieved via formation of a VE-cadherin–Par3–Tiam1 complex and is required for coupling to the p67phox subunit of the NADPH oxidase and downstream redox signaling. The identification of a molecular cascade required for spatial mechanosignaling and NADPH oxidase activation provides new insights into mechanisms of ROS signaling in cellular responses related to signal transduction, inflammatory diseases, and atherosclerosis.

Materials and methods

Cell culture and shear stress

VE-cadherin null (VE-KO) and reconstituted (VE-RC) cells were prepared as described previously (Carmeliet et al., 1999). In brief, ECs were derived from undifferentiated mouse embryonic stem cells from VE-cadherin–null embryos and were transduced with a retrovirus expressing the Polyoma middle-T oncogene (VE-KO). VE-RC cells were obtained generated by reintroducing human VE-cadherin. VERC and VEKO cells were grown in Dulbecco’s Modified Eagle’s Medium (DMEM; Invitrogen) containing 10% FBS (Invitrogen), 5 μg/ml endothelial cell growth supplement (ECGS; Sigma-Aldrich), and 100 μg/ml heparin and penicillin/streptomycin (Invitrogen). PECAM-1 knock-out (PE-KO) and reconstituted (PE-RC) cells were cultured in DMEM containing 10% FBS, 10 μM 2-mercaptoethanol (Invitrogen), 1x nonessential amino acids (NEAA; Invitrogen), and penicillin/streptomycin. Human umbilical vein endothelial cells (HUVECs) were cultured in EGM medium (Lonza) according to the manufacturer’s suggested procedure. Bovine aortic endothelial cells (BAECs) were cultured in DMEM supplemented with 10% FBS and penicillin/streptomycin. For shear stress experiments, endothelial cells were plated on 10 μg/ml fibronectin and allowed to grow for 5 h in medium containing 10% FBS. Cells were then starved overnight in medium containing 0.5% FBS. Slides were loaded onto a parallel plate flow chamber in 0.5% FBS and 12 dynes/cm² of shear stress was applied for indicated times. The starvation time was shortened to 1 h if HUVECs were used in shear stress.

Antibodies and inhibitors

Src inhibitors SU6656 and PP2 were purchased from EMD Millipore. Vav2, phospho-Vav2, and Tiam1 were purchased from Santa Cruz Biotechnology, Inc. The Vav2 antibody used for immunoprecipitation, anti-myc, and anti-HA antibodies were from Cell Signaling Technology. The Rac1 antibody was obtained from BD and the VE-cadherin antibody was from Enzo Life Sciences. The anti-FLAG and actin antibodies were purchased from Sigma-Aldrich and the anti-Par3 antibody was purchased from Cell Signaling Technology.

RNA interference and plasmid transfections

The siRNA sequences used in the study were as follows: siControl, 5′-UAA-GGCUCUAGAAGAGAUC-3′; human siTiam1 #1, 5′-GGCGAGGAAAG-GGUUAGUCU-3′; human siTiam1 #2, 5′-GAAACCCUUAGAGGUAGAG-3′; human siVav2 #1, 5′-GGACACCGAGCUGUUGUAAU-3′; human siVav2 #2, 5′-AGUCCCGUGCUAGUCUAC-3′; mouse siTiam1 #1, 5′-UCACAGCUUGA-AACAGGAU-3′; mouse siTiam1 #2, 5′-GCGAAGGAGCGGUUU-CUU-3′; mouse siPar3, 5′-CGUAAAUCUCAUAACUAAU-3′. The siRNAs were introduced into the cells as described previously using a calcium phosphate method (Liu et al., 2008). Plasmid constructs were transfected into VERC or VEKO cells using Lipofectamine LT (Invitrogen). To transfect HUVECs with plasmids or siRNA, the Amoeba Nucleofector kit and Nucleofector program A-034 were used. The wild-type pcDNA3-Tiam1 and the GEF-deficient mutant pcDNA3-Tiam1-1047/1232A were generous gifts from Dr. Channing J. Der (UNC-Chapel Hill, Chapel Hill, NC). The siRNA-resistant Tiam1 rescue plasmids were generated by changing the target sequence of human siTiam1 #2 to 5′-GCCAGUCCUCGGUUUAGAC-3′ (mutated nucleotides underlined) in the context of wild-type pcDNA3-Tiam1 or 1047A/1232A-Tiam1. Plasmid constructs (GFP-Tiam1-WT, m3GFP-Par3, and WTPar6, and DNPar6) were transfected into BAECs using the Effectene Transfection Reagent (QIAGEN) according to the manufacturer’s protocol. Confluent monolayers were sheared 48 h after transfection.

Rac pull-down assays

Cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed in Rac pull-down buffer containing 50 mM Tris, pH 7.6, 150 mM NaCl, 0.5 mM MgCl₂, 1% Triton X-100 supplemented with 1 mM phenylmethylsulfonyl fluoride (PMF), 1 μg/ml aprotinin, and 1 μg/ml leupeptin. The cell lysates were spun at 15,000 g for 5 min and the cleared supernatant incubated with 20 μg of purified recombinant GST-PBD for 30 min at 4°C. The beads were subsequently washed with Rac pull-down buffer and bound proteins eluted with SDS sample buffer and analyzed by Western blotting using a monoclonal anti-Rac antibody (BD). Whole-cell lysates were also analyzed for the normalization of protein loading.

FRET assays

EGFP-Rac and mCherry-PBD were validated as an appropriated fluorophore pair for FRET analysis. EGFP-tagged Rac and pcDNA3.1(+)mCherry-PBD were cotransfected into endothelial cells using Lipofectamine LTX or Amoeba Nucleofector and subjected to shear stress for 0 or 30 min. Images of fixed cells were acquired using a laser scanning confocal microscope (710 LSCM; Carl Zeiss). Calculations to account for bleedthrough and background were performed as described previously (Kraynov et al., 2000; Tzima et al., 2002). In brief, after subtracting the background from all images, the EGFP-Rac images were thresholded and used to generate a binary image with values within the cell = 1 and outside the cell = 0. The FRET and mCherry-PBD images were multiplied by the binary image and a fraction of the EGFP-Rac and mCherry-PBD images was subtracted from the FRET image. The corrected 8-bit FRET images were displayed using pseudocolor, where blue is closest to 0 and red closest to 100.
ROS assays
Endothelial cells were preincubated with 10 µM of 2,7-dichlorodihydrofluorescein diacetate (H₂DCFDA) at 37°C for 30 min before the onset of flow. shear stress was applied to the cells in continued presence of the dye for indicated times. Cells were rinsed with PBS and lysed in PBS containing 0.2% Triton X-100 and 1 mM Na-acycteylcyteine. Fluorescence was measured using the 485-nm excitation/535-nm emission filter in a Wallac Victor 1420 multi-label plate reader (PerkinElmer) and normalized to total protein concentrations in the lysates (Bradford assay; Thermo Fisher Scientific).

Immunoprecipitations and Western blotting
Cells were harvested in lysis buffer (20 mM Hepes, pH 7.4, 150 mM NaCl, 50 mM KCl, 1% Triton X-100, 1% NP-40, protease and phosphatase inhibitors, 10 µg/ml leupeptin, 1 mM PMSF, 1 mM Na-acycteylcyteine, and 1 mM β-glycero-phosphate. Lysates were precleared with 50 µl protein A/G plus Sepharose beads (Santa Cruz Biotechnology, Inc.) for 1 h at 4°C. Supernatants were then incubated with 30 µl protein A/G plus Sepharose previously coupled to the primary antibodies for 2 h at 4°C with continuous agitation. The beads were washed three times with lysis buffer supplemented with protease and phosphatase inhibitors and the immune complexes were eluted in 2× SDS sample buffer. Associated proteins were subjected to SDS-PAGE and Western blotting using the appropriate primary antibodies and IRDye-conjugated anti-mouse or anti-rabbit antibodies (Rockland). Images of Western blotting were obtained with an Odyssey infrared scanner system (LI-COR Biosciences).

In vivo RNAi and en face staining of mouse aorta
50 µg of mouse Tiam1 or control siRNAs were mixed with jetPEI in vivo delivery reagent (Polyplus) and injected into C57BL/6 mice (>30 wk) through the retro-orbital veins for two or three consecutive days (Sheel et al., 2008). 72–96 h after the initial injection, aorta arches were isolated and processed for en face staining as described previously (Chen and Tzima, 2009). In brief, aortas were perfusion-fixed and dissected out under dissection microscope. The aortic arches were cut longitudinally and pinned flat with endothelium facing up onto a Superfrost/Plus glass slide (Thermo Fisher Scientific). Confocal images were obtained using the 60× 1.40 NA oil objective and a digital camera (ORCA-ER; Hamamatsu Photonics) and were analyzed using Image software (National Institutes of Health).

Quantification and statistical analysis
The intensities of immunoblotting or immunofluorescence staining were quantified using ImageJ. Data were analyzed by performing two-tailed Student’s t test (between two groups) and one-way ANOVA (among multiple groups) as appropriate. Statistical significance was defined as P < 0.05.

Online supplemental material
Fig. S1 provides additional information on FRET analysis. Fig. S2 provides additional siRNA and inhibitors studies that demonstrate shear-induced activation of Vav2 is required for Rac1 GTP-loading. Fig. S3 demonstrates specific detection of ROS production in vivo. Fig. S4 shows that successful knockdown of Tiam1 in vivo blunts ROS production in the mouse aorta. Fig. S5 demonstrates that Tiam1 binds to VE-cadherin. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201207115/DC1.

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Author contributions: E. Tzima initiated and supervised the project; designed experiments and analyzed data. Y. Liu and C. Collins performed experiments and analyzed data. W.B. Kiosses performed FRET experiments and analyzed data. A.M. Murray, M. Joshi, T.R. Shepherd, and E. Fuentes performed experiments and analyzed data. Y. Liu, C. Collins, and E. Tzima wrote the manuscript.

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References
Endothelial cells were preincubated with 10 µM of 2,7-dichlorodihydrofluorescein diacetate (H₂DCFDA) at 37°C for 30 min before the onset of flow. shear stress was applied to the cells in continued presence of the dye for indicated times. Cells were rinsed with PBS and lysed in PBS containing 0.2% Triton X-100 and 1 mM Na-acycteylcyteine. Fluorescence was measured using the 485-nm excitation/535-nm emission filter in a Wallac Victor 1420 multi-label plate reader (PerkinElmer) and normalized to total protein concentrations in the lysates (Bradford assay; Thermo Fisher Scientific).