The F-BAR protein NOSTRIN participates in FGF signal transduction and vascular development

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Abstract

F-BAR proteins are multivalent adaptors that link plasma membrane and cytoskeleton and coordinate cellular processes such as membrane protrusion and migration. Yet, little is known about the function of F-BAR proteins in vivo. Here we report, that the F-BAR protein NOSTRIN is necessary for proper vascular development in zebrafish and postnatal retinal angiogenesis in mice. The loss of NOSTRIN impacts on the migration of endothelial tip cells and leads to a reduction of tip cell filopodia number and length. NOSTRIN forms a complex with the GTPase Rac1 and its exchange factor Sos1 and overexpression of NOSTRIN in cells induces Rac1 activation. Furthermore, NOSTRIN is required for fibroblast growth factor 2 dependent activation of Rac1 in primary endothelial cells and the angiogenic response to fibroblast growth factor 2 in the in vivo matrigel plug assay. We propose a novel regulatory circuit, in which NOSTRIN assembles a signalling complex containing FGFR1, Rac1 and Sos1 thereby facilitating the activation of Rac1 in endothelial cells during developmental angiogenesis.
Introduction

Developmental angiogenesis is a highly stereotypical process, leading to the establishment of organ-specific vascular branching patterns with reproducible anatomy (Larrivee et al, 2009). These are formed by angiogenic sprouts, which consist of several types of specialised endothelial cells. Tip cells are located at the leading position of the vascular sprout. They form numerous cellular protrusions, referred to as filopodia (Gerhardt et al, 2003), which constantly sense the microenvironment for guidance cues to navigate the growing vessel. Tip cells also regulate capillary branching by detecting and connecting to neighbouring sprouts. Stalk cells follow the leading tip cell; they proliferate and thereby elongate the growing branch (De Smet et al, 2009; Gerhardt et al, 2003; Larrivee et al, 2009). The assignment of these specialised functions, however, is only transient and endothelial cells dynamically shuffle their relative positioning in the angiogenic sprout (Jakobsson et al, 2010), probably due to continuous competition for the tip cell function. Finally quiescent phalanx cells build the inner lining of the new vessel after its outline has been set. (De Smet et al, 2009; Gerhardt et al, 2003; Larrivee et al, 2009).

Several attractive and repellent guidance cues and their respective receptors are known, which direct the migration of the developing blood vessel. Many of these are shared between the vascular and the neural system, such as Slits/robo receptors and Netrins/Unc5. Moreover, growth factors such as VEGF and FGF, are key regulators of vascular development, through the stimulation of directed migration and proliferation of endothelial cells (De Smet et al, 2009; Gerhardt & Betsholtz, 2005; Gerhardt et al, 2003; Horowitz & Simons, 2008; Larrivee et al, 2009). The sensing of the directional cues through filopodia as well as their translation into directed migration strongly depend on coordination of the tip cell cytoskeleton with membrane dynamics (De Smet et al, 2009; Suchting et al, 2006). Tip cells are highly polarised and the formation of lamellipodia and filopodia is one key characteristic. The activity of small GTPases is thought to be essential for tip cell function, however comparatively little is known about the factors that control cytoskeleton dynamics and small GTPase activity in the context of tip cell protrusion formation and migration (De Smet et al, 2009; Tan et al, 2008; Wang et al, 2010).

Recently F-BAR (Fes/CIP4 homology and Bin/amphiphysin/Rvs) proteins have emerged as important regulators of cell protrusion formation and migration as well as endocytosis. These processes require the concerted action of the plasma membrane and the cytoskeleton and F-BAR proteins are modular molecules that serve as multivalent adaptors that physically and functionally link both compartments. They comprise a common N-terminal F-BAR domain followed by various combinations of kinase, SH2, SH3 and GTPase interacting domains. The F-BAR domain senses and shapes membrane curvature, while a majority of F-BAR proteins uses the C-terminal domains to
interact with components and regulators of the actin cytoskeleton, e.g. actin nucleation promoting factors WASP and N-WASP, Arp2/3 and the large GTPase dynamin (Frost et al, 2009; Heath & Insall, 2008; Qualmann et al, 2011; Roberts-Galbraith & Gould, 2010; Suetsugu et al, 2010).

The F-BAR protein NOSTRIN was identified by our group as modulator of the subcellular localisation and activity of endothelial nitric oxide synthase (eNOS) and was hence termed eNOS traffic inducer (McCormick et al, 2011; Oess et al, 2006; Schilling et al, 2006; Zimmermann et al, 2002). NOSTRIN associates with membranes via its F-BAR domain (Icking et al, 2006) and binds dynamin and N-WASP through its C-terminal SH3 domain. Like other F-BAR proteins, NOSTRIN forms oligomers and hence allows simultaneous interaction with several SH3 binding partners, thereby coordinating the function of dynamin and N-WASP to facilitate the endocytosis of eNOS (Icking et al, 2005).

For many F-BAR proteins the physiological function in vivo is unclear. In this study we report an important role for the F-BAR protein NOSTRIN in developmental angiogenesis and identify the molecular mechanisms by which NOSTRIN links FGFR with the activation of the GTPase Rac1.
Results

The knockdown of NOSTRIN in developing zebrafish embryos caused vascular defects

NOSTRIN shows the highest expression in endothelial cells and highly vascularised organs (Zimmermann et al, 2002). To study the in vivo function of NOSTRIN in the vascular system, we have chosen developing zebrafish as a model, due to their ex utero development, the transparency of early embryos and the availability of transgenic strains Tg(fli1a:EGFP)Y1 and Tg(kdrl:EGFP)s843 with endothelial cell-specific expression of eGFP (Jin et al, 2005; Lawson & Weinstein, 2002). We carried out an antisense morpholino oligonucleotide (MO)-mediated knockdown (KD) of NOSTRIN in developing Tg(fli1a:EGFP)Y1 or Tg(kdrl:EGFP)s843 zebrafish, using two different MO directed against the translational start ATG (ATG MO) or the splice site inside the sequence coding for the SH3 domain (Splice MO). Zebrafish embryos injected with the NOSTRIN-targeting MO (referred to as morphants) did not show lethality or gross morphological changes during the period of inspection up to 72 hpf when compared to wildtype (WT) or Control MO-injected embryos, with the exception of axis defects in a small subset (1 %) of morphants (Fig. 1A/B).

However, the NOSTRIN morphants displayed oedema and haemorrhaging e.g. in the hindbrain and pericardial regions, indicative of a malfunctioning vascular system (Fig. 1A, 1B, 1E). Analysing the developing vasculature, an abnormal trajectory phenotype of intersegmental vessels (ISV), with improper connections formed between neighbouring ISVs in both morphants was observed. Moreover, the dorsal longitudinal anastomotic vessels (DLAV) were misshaped or interrupted and the caudal artery (CA) and caudal vein plexus (CVP) were irregular in appearance (Fig. 1C). In addition, we have found that KD of NOSTRIN leads to an impaired subintestinal vein (SIV) development (Supplementary Information, Figure S1). The specificity of the vascular defects was demonstrated by a dose-dependent rescue of the phenotype by re-introduction of NOSTRIN by sequential injection of zebrafish NOSTRIN mRNA in combination with the Splice MO (Fig. 1C, 1E, Supplementary Information, Figure S1). KD efficiency and expression of NOSTRIN after mRNA injection were verified by immunoblotting (Fig. 1D). These observations indicated an essential role of NOSTRIN for proper vascular development in zebrafish.

NOSTRIN morphants show an abnormal ISV trajectory phenotype associated with altered tip cell morphology and filopodia length

In order to analyse the development of the ISVs in more detail, we performed in vivo time-lapse microscopy of NOSTRIN morphants (Fig. 2A, images extracted from Supplementary information, Video 1, Video 2). In WT embryos, the first ISVs to originate as sprouts from the DA were visible at
20-24 hpf, grew dorsally and reached the level of the top of the neural tube at approximately 30 hpf. They bifurcated to form 2 branches extending in a T-shaped fashion along the body axis, finally joining up to form the DLAV at 32-36 hpf (Fig. 2A, left and Supplementary Information, Video 1). In contrast, in NOSTRIN morphants the ISV sprouts appeared later at approximately 24-28 hpf and failed to establish the regular ISV trajectory but formed connections with neighbouring ISVs before reaching the top of the neural tube (Fig. 2A, right and Supplementary information, Video 2). Therefore we conclude that NOSTRIN is important for the formation and/or the directed movement of ISV sprouts.

To determine whether this ISV defect was associated with a change in endothelial tip cell morphology, we analysed the tip cells of growing ISVs by confocal laser scanning microscopy (CLSM) and found that in WT embryos the tip cells exhibited the characteristic elongated shape (Wang et al, 2010; Yu et al, 2010) with long filopodial extensions (15.8 % of filopodia/cell > 15 μm, 21.6 % < 5 μm). In contrast, the tip cells in morphants were stub-like and exhibited significantly shorter filopodial extensions (1.1 % > 15 μm, 42.3 % < 5 μm; Fig. 2B, Fig. 2C for full quantification). This suggests that NOSTRIN is critical for endothelial tip cell function, especially for filopodia formation, and that tip cell defects might cause the observed deviation from the stereotypical developmental pattern in the NOSTRIN morphants.

Postnatal retinal angiogenesis is impaired in NOSTRIN knockout mice

To further study the importance of NOSTRIN for tip cell function in vivo, we analysed postnatal retinal angiogenesis (Gerhardt et al, 2003) in NOSTRIN knockout (KO) mice. NOSTRIN KO mice were generated by loss-of-function genetics (Methods and Supplementary Information, Fig. S2) and will be described in detail independently of this study. In NOSTRIN KO mice the stereotypical spreading of the primary vascular plexus from the optic disc to the peripheral margin of the retina was impaired, measured as reduction of the mean vascular radius (Fig. 3A/B) and the vascularised area of the retina at postnatal day (P)2, P5 and P7 (Fig. 3A/C). In accordance, the number of branch points at the vascular front and the central region of the retina was significantly decreased in NOSTRIN KO mice at P5 (Fig. 3D). Importantly, the guiding neuronal network comprising the pre-existing astrocyte scaffold was not altered in NOSTRIN KO retinas (Supplementary Information, Fig. S3). These findings suggest an impairment of the angiogenic capacity of endothelial cells, that in general is determined i.a. by endothelial cell proliferation and migration (Larrivee et al, 2009). Indeed, proliferation was reduced in retinas of NOSTRIN KO mice, measured as the number of phospho-Histone H3- (Fig. 3G/H) or ki67-positive endothelial cell nuclei in the vascular front (Supplementary Information, Fig. S4). Detailed
analysis of the tip cells revealed, that the number of tip cells (Fig. 3E) and the number of filopodia at the leading edge of the expanding vascular plexus (Fig. 3F/J) were reduced and the average length of tip cell filopodia was greatly diminished (Fig. 3I/J). This indicates a function of NOSTRIN in the regulation of proliferation and migration as endothelial cell key characteristics and confirms the role of NOSTRIN in tip cell filopodia formation and sprouting angiogenesis in vivo.

In order to investigate whether the requirement for NOSTRIN is cell-autonomous or nonautonomous with regard to endothelial cells, we analysed key parameters of the retinal vasculature in NOSTRIN KO mice with Tie2-Cre-mediated deletion of the NOSTRIN gene (OSTRIN EC KO, Supplementary Information Fig. S5). Spreading of the vascular plexus, the vascularised area, the number of branch points, the number of tip cells and the number of tip cell filopodia were significantly reduced in NOSTRIN EC KO mice (Supplementary Information Fig. S6A-E). Moreover, we found that cell proliferation was decreased (Supplementary Information Fig. S6F/G) and observed less long filopodial extensions on NOSTRIN EC KO tip cells (Supplementary Information Fig. S6H/I). Therefore we conclude, that NOSTRIN acts cell-autonomously to facilitate endothelial cell proliferation and migration.

**NOSTRIN interacts with and activates Rac1**

Angiogenic behaviour of endothelial cells is critically controlled by the activity of small GTPases (Desmet et al, 2009; Epting et al, 2010; Tan et al, 2008; Wang et al, 2010). To understand the molecular basis for NOSTRIN function in this process, we analysed the interaction of NOSTRIN with the three prototypic Rho family GTPases, Cdc42, Rac1 and RhoA in a GST-pulldown assay. Since full size NOSTRIN is characterised by low solubility, for the following experiments we used soluble deletion mutants of NOSTRIN, which lack either the N-terminal F-BAR domain, NOSTRINΔFBAR (aa225-506), or the C-terminal SH3 domain, NOSTRINΔSH3 (aa1-440) (Fig. 4A). NOSTRIN interacted strongly and specifically with the activated form of Rac1 (GST-Rac1-GTPγS) and to lesser extent with the activated form of Cdc42 (GST-Cdc42-GTPγS), while we detected no interaction with RhoA (Fig. 4B). NOSTRIN did not interact significantly with the inactive GDP-bound forms of any of the GTPases (Fig. 4B). In order to analyse whether the interaction of NOSTRIN with Rac1 and Cdc42 was direct, we performed a GST-pulldown assay using recombinantly expressed and purified proteins. In accordance with our previous results, NOSTRIN interacted directly and specifically with the active GTPγS-bound form of Rac1 and only weakly with inactive GDP-bound and nucleotide free Rac1. We could also confirm that NOSTRIN bound weakly to active GTPγS-bound Cdc42 (Fig. 4C). We next tested whether the interaction of NOSTRIN with Rac1 depends on the presence of the HR1 motif (homology region 1, aa
304-386 in NOSTRIN; Fig. 4A), which in other F-BAR proteins mediates the interaction with small GTPases of the Rho family (Frost et al, 2009; Heath & Insall, 2008; Ho et al, 2004; Roberts-Galbraith & Gould, 2010). We used mammalian cells expressing a construct with additional deletion of the HR1 motif, NOSTRINΔFBAR/ΔHR1 (aa225-311 fused to 383-506) and found that the interaction was abolished, when the HR1 motif was deleted (Fig. 4D), indicating that the interaction between NOSTRIN and Rac1 is indeed mediated by the HR1 motif.

To test if NOSTRIN is involved in Rac1 activation, we determined Rac1 activity by PAK-CRIB pulldown experiments and found that overexpression of full size NOSTRIN led to a pronounced activation of Rac1 in comparison to the expression of GFP in control cells (Fig. 5A). To determine, if the HR1 motif was necessary for the NOSTRIN-dependent activation of Rac1, we compared Rac1 activity in cells that overexpressed NOSTRIN or the deletion mutant NOSTRINΔHR1 (aa1-311 fused to 383-506). Indeed, NOSTRINΔHR1, which is unable to bind to Rac1, did not induce Rac1 activation (Fig. 5A). This suggested that the interaction of Rac1 with NOSTRIN via its HR1 motif is necessary for Rac1 activation.

**NOSTRIN interacts with the Rac1 GEF Sos1 to activate Rac1**

A possible explanation for this NOSTRIN-induced activation of Rac1 would be the recruitment of a guanine nucleotide exchange factor (GEF). Therefore we analysed the interaction of NOSTRIN with three previously described Rac1 GEFs, Sos1, Tiam1 and Vav2 (Abe et al, 2000; Michiels et al, 1995; Sini et al, 2004). We found that GST-NOSTRIN full size (aa1-506) interacted with endogenous Sos1 in the lysates of primary endothelial cells, while we could not detect an interaction between NOSTRIN and Tiam1 or Vav2 (Fig. 5B). Since Sos1 is known to interact with SH3 domains via its proline-rich domain (Rozakis-Adcock et al, 1993), we analysed whether the SH3 domain of NOSTRIN was necessary for the NOSTRIN/Sos1 interaction in a series of experiments. For this purpose we used GST-NOSTRIN full size (aa1-506), GST-NOSTRINΔSH3 (aa1-440) and GST-NOSTRIN-SH3 (aa433-506), where the SH3 domain was fused to GST. Indeed, the interaction between NOSTRIN and Sos1 was abolished when the NOSTRIN SH3 domain was deleted (Fig. 5B, 5C) and occurred also between the isolated SH3 domain of NOSTRIN and endogenous Sos1 (Fig. 5C), indicating that the NOSTRIN SH3 domain is necessary and sufficient to mediate the interaction with Sos1. Finally, to experimentally prove that the NOSTRIN/Sos1 interaction was direct, we tested the interaction of the three GST-NOSTRIN fusion proteins with the purified recombinant His-tagged proline-rich domain of Sos1 (His-Sos1-PRD). Indeed, His-Sos1-PRD interacted with full size NOSTRIN and the isolated SH3 domain, but not with the SH3 deletion mutant, confirming our previous results (Fig. 5D).
We hypothesised that the activation of Rac1 upon NOSTRIN overexpression might be due to the recruitment of the Rac1 GEF Sos1 via the NOSTRIN SH3 domain towards the NOSTRIN binding partner Rac1. If this is correct, a NOSTRIN mutant incapable of binding Sos1, should not be able to induce Rac1 activation. Indeed, overexpression of the deletion mutant lacking the SH3 domain, NOSTRINΔSH3, which is able to bind Rac1 (Fig. 4C) but not Sos1 (Fig. 5B, 5C), did not lead to Rac1 activation in the PAK-CRIB pulldown assay (Fig. 5A). This is in accordance with the initial hypothesis that NOSTRIN recruits Sos1 to Rac1 and thereby facilitates its activation.

**NOSTRIN is required for FGF-2-dependent activation of Rac1 in primary endothelial cells**

As an F-BAR protein NOSTRIN might function as a multivalent adaptor to link Rac1 activation to an up-stream stimulus. In order to search for such novel interacting proteins, that might induce the NOSTRIN-mediated activation of Rac1, we performed a yeast-two-hybrid (Y2H) screen using the C-terminal portion of human NOSTRIN (aa362 – 506) as bait. With this approach, we identified a 255 amino acid fragment of the cytoplasmic tail of FGFR1 (aa547-801) as a novel interacting protein of NOSTRIN (Fig. 6A). We confirmed the interaction of endogenously expressed full size proteins in mammalian cells by co-immunoprecipitation of FGFR1 with NOSTRIN using a polyclonal NOSTRIN-specific antiserum (Fig. 6B).

The NOSTRIN bait fragment used for Y2H contained the SH3 domain and a sequence we refer to as intermediate domain (ID), because it lies in between the HR1 and SH3 domain (Fig. 4A, 6A). In order to determine through which domain NOSTRIN interacts with the FGFR1, we analysed the NOSTRIN ID and SH3 domain individually in the Y2H system. The bait protein consisting of the NOSTRIN ID (aa 362-434) only, was still able to interact with FGFR1, while interaction was abolished when the bait contained only the SH3 domain (aa 434-506)(Fig. 6A). Accordingly, GST-NOSTRINΔSH3 interacted with endogenous FGFR1 in a GST-pulldown assay from mammalian cell lysate, while GST-NOSTRIN-SH3 failed to interact (Fig. 6C). Finally, in a direct protein/protein interaction assay using purified proteins, GST-NOSTRIN and GST-NOSTRINΔSH3 interacted with a C-terminal fragment of FGFR1 (aa692-822), while GST-SH3 again did not interact (Fig. 6D). Taken together, this indicated that NOSTRIN and the FGFR1 interact in mammalian cells and suggests that the interaction is direct and dependent on the NOSTRIN ID and the C-terminal tail of the FGFR1.

In light of the ability of NOSTRIN to interact with FGFR1 on the one hand and to promote the activity of Rac1 on the other, the arising question was whether NOSTRIN was involved in the FGF-2-dependent activation of Rac1 in endothelial cells. To study this, we isolated primary mouse lung endothelial cells (MLECs) from NOSTRIN WT and KO mice by immuno-selection and confirmed the
expression of NOSTRIN in WT MLECs and its absence in KO cells by immunoblotting (Fig. 7A). Interaction of NOSTRIN with the FGFR1 in endothelial cells was verified by co-immunoprecipitation. NOSTRIN and FGFR1 interacted strongly after stimulation with FGF-2, while no interaction was detectable in starved cells (Fig. 7B), indicating that NOSTRIN interacted with FGFR1 in a stimulus-dependent fashion in endothelial cells. FGF-2 caused a significant activation of Rac1 in lysates of NOSTRIN WT MLECs. In contrast, FGF-2 was unable to activate Rac1 in NOSTRIN KO cells (Fig. 7C). In order to analyse, if the action of NOSTRIN is specific for the FGFR1 or might also be directed towards other pro-angiogenic growth factors, we stimulated MLECs from NOSTRIN WT and KO mice with VEGF and found that VEGF induced Rac1 activity also when NOSTRIN was absent (Supplementary Information Fig. S7). Taken together, this confirms our findings that NOSTRIN is favourable for the activation of Rac1 and demonstrates that FGF-2-dependent activation of Rac1 in primary endothelial cells depends on NOSTRIN.

**NOSTRIN is necessary for FGF-2-dependent angiogenic response in the matrigel plug assay**

Finally, we analysed the angiogenic response of WT and NOSTRIN KO mice to FGF-2 in the matrigel plug assay. For this purpose, adult male mice received two matrigel implants each, one containing vehicle and the other supplemented with FGF-2 to induce microvessel growth into the plug. After 10 days the implants were removed, processed for immunohistochemistry and the angiogenic response was measured as the area covered by PECAM-stained cells. We observed that FGF-2 induced neovascularisation of matrigel implants in WT controls, whereas the angiogenic response was impaired in NOSTRIN KO mice (Fig. 7D, 7E for quantification). In addition, in the absence of FGF-2, single, scattered PECAM-positive cells had entered the matrigel plug in WT mice and this was also reduced in the NOSTRIN KO (Fig. 7D, 7E for quantification). NOSTRIN is thus required for proper postnatal FGF-2-induced angiogenesis *in vivo*. 
Discussion

In this study we show that the F-BAR protein NOSTRIN is a novel, important factor for vascular morphogenesis. The loss of NOSTRIN causes defects in developmental angiogenesis characterised by changes in tip cell number and morphology, with a reduction of tip cell filopodia abundance and length and a decrease in endothelial cell proliferation. This is associated with the loss of the proper ISV trajectory in zebrafish and the impairment of spreading and branching of the vascular plexus of the postnatal retina in mice.

NOSTRIN KO mice are viable and we did not observe equally strong defects in vascular development during embryogenesis in mice as in zebrafish and it remains to be determined if other F-BAR proteins might at least partially compensate for the loss of NOSTRIN in the KO mouse. Phenotypic divergence between mice and zebrafish in terms of vascular development, however, is not unusual and occurs in a similar form e.g. upon the loss of NOGO A/B (Acevedo et al, 2004; Zhao et al, 2010).

During developmental angiogenesis NOSTRIN serves as a multivalent adaptor for FGFR1, Rac1 and its GEF Sos1 and the assembly of this signalling complex is necessary for the FGF-2-dependent activation of Rac1 (Fig. 7F). In the context of this current study, the function of NOSTRIN is distinct from the previously reported role of NOSTRIN in the regulation of eNOS localisation and function. This is supported by the facts that 1) eNOS-derived NO is dispensable for postnatal retinal angiogenesis, which is normal in eNOS KO mice (Al-Shabrawey et al, 2003), and 2) there is no genetic evidence for the existence of an eNOS gene in zebrafish, although immunoreactivity with a eNOS-specific antibody has been reported (North et al, 2009).

We identify NOSTRIN as a novel binding partner of FGFR1, a well-known receptor for pro-angiogenic signals in endothelial cells (De Smet et al, 2009; Horowitz & Simons, 2008; Presta et al, 2005). FGF-2 or FGFR1, respectively, have been shown to promote proliferation and migration of endothelial cells and increase vascular density and branching in various in vitro, ex vivo and in vivo models (Akimoto & Hammerman, 2003; Feraud et al, 2001; Javerzat et al, 2002; Magnusson et al, 2004; Magnusson et al, 2007; Magnusson et al, 2005; Nicoli et al, 2009; Rousseau et al, 2003; Sheikh et al, 2001; Tomanek et al, 2010; Tomanek et al, 2001; Woad et al, 2012). However, the analysis of FGFR1 function in developmental angiogenesis has been complicated by the early lethality of FGFR1 knockout mice before the onset of vascularisation (Deng et al, 1994). The defects in proliferation, migration and branching we have observed upon KD of NOSTRIN in developing zebrafish and in NOSTRIN KO mice are in accordance with the known functions of FGF-2/FGFR1 in angiogenesis.

The association of NOSTRIN with FGFR1 is direct as suggested by the Y2H interaction and verified by interaction analysis with recombinantly expressed and purified proteins. The interaction occurred in
cells cultured in the presence of serum, and upon stimulation with FGF-2, but not in starved cells, suggesting that the interaction might be regulated in an FGF-2-dependent manner. NOSTRIN interacts with the C-terminal tail of the FGFR1 (aa692-822). Interestingly, the C-terminal domain of FGFR1 (aa759-822) has been identified as crucial for mediating chemotaxis in endothelial cells (Landgren et al, 1998). This is in agreement with our model, which predicts that disassembly of the FGFR1/NOSTRIN/Rac1/Sos1 signalling complex either by the loss of NOSTRIN or truncation of the receptor would interfere with directed migration of endothelial cells.

Several members of the F-BAR protein family have been implicated in the trafficking of transmembrane receptors such as the EGF- and the PDGF-receptor. However, no direct interaction of the receptor and the F-BAR protein has been reported in either case (Hu et al, 2009; Toguchi et al, 2010). We have shown previously that NOSTRIN facilitates the internalisation of eNOS through its binding to dynamin and N-WASP (Icking et al, 2005), therefore it is conceivable that NOSTRIN might also influence FGFR1 endocytosis. In respect to the specificity of NOSTRIN towards FGFR1, we so far do not have indications that NOSTRIN might bind to other trans-membrane receptors. In our Y2H interaction screen we did not find other growth factor receptors to interact with NOSTRIN and the VEGF-dependent activation of Rac1 in endothelial cells was unaffected when NOSTRIN was absent, indicating that NOSTRIN does not bind to a broad variety of receptors or unspecifically modulates signal transduction.

In addition to the direct interaction with FGFR1, in this study we have identified the small GTPase Rac1 and its GEF Sos1 as novel direct binding partners of NOSTRIN. The interaction of NOSTRIN with Rac1 is mediated by the HR1 motif, an interaction motif shared between several F-BAR proteins (Frost et al, 2009; Heath & Insall, 2008). The interaction with Sos1 involves NOSTRIN’s SH3 domain and the PRD of Sos1. Moreover NOSTRIN induces Rac1 activation dependent on both the HR1 and the SH3 domain, suggesting that NOSTRIN might serve as an adaptor to facilitate the interaction of Rac1 and its GEF in order to promote Rac1 activation. NOSTRIN preferentially interacts with active Rac1 and this would be consistent with the idea that NOSTRIN participates in an activation loop, amplifying Rac1 activation. Endothelial Rac1 is important for cell migration and vascular development in mice (D’Amico et al, 2009; Tan et al, 2008) and for developmental angiogenesis in zebrafish (De Smet et al, 2009; Epting et al, 2010; Wang et al, 2010), however, the precise role of Rac1 in endothelial tip cell function is not fully understood. Correct spatial positioning and activation of Rac1 are important for the highly polarized character of endothelial cells (De Smet et al, 2009; Tzima, 2006) and it might be possible that NOSTRIN serves to coordinate FGFR1 stimulation with spatial activation of Rac1. The dual effect of FGF2/FGFR1 on directed migration and proliferation is also observed in the case of VEGF-A/VEGFR2, where the VEGF-A gradient serves as tip cell guidance
cue and the VEGF-A concentration regulates proliferation in stalk cells (Gerhardt et al, 2003). Therefore the NOSTRIN/Rac1/Sos1 signalling complex might serve to detect the FGF-2 signal both in tip and stalk cells; while it is translated into directional migration in tip cells, it stimulates proliferation in stalk cells. However, we cannot rule out that other FGFR1-dependent signal cascades might be involved e.g. MAP kinase signalling.

The pro-angiogenic effect of FGF-2 is diminished in vivo in the matrigel plug assay, if NOSTRIN is absent. In accordance with our findings, it has been shown that the FGF-2-induced angiogenesis into matrigel plugs depends on Rac1 (Dormond et al, 2001), highlighting the importance of Rac1 to mediate the angiogenic effect of FGF-2. In addition, we have employed the zebrafish yolk sac angiogenesis assay (Nicoli et al, 2009) to test for FGF-2 induced angiogenesis from the SIV basket in vivo. However, the impaired SIV development in NOSTRIN morphants prevented a conclusive interpretation. Taken together, our data strongly suggest that NOSTRIN is indeed involved in FGFR1-dependent signal transduction in vitro and in vivo. Our model is different from the previously proposed mechanism, where FGF-2 has been shown to activate Rac1 independently of FGFR1 through the low affinity heparan sulfate proteoglycan syndecan-4, involving the action of the RhoG/ELMO1/Dock180 GEF complex (Elfenbein et al, 2009). A potential crosstalk between the different pathways controlling the spatial activation of Rac1 remains to be determined.

F-BAR proteins are typically involved in the generation of positive membrane curvature and plasma membrane invaginations, e.g. during endocytosis (Frost et al, 2009; Heath & Insall, 2008; Qualmann et al, 2011). In contrast, I-BAR (inverse BAR) proteins generate negative membrane curvature and induce plasma membrane protrusions, such as lamellipodia and filopodia (Ahmed et al, 2010; Zhao et al, 2011). In this study we have observed, that the loss of NOSTRIN affects filopodia formation, but we did not analyse if this is a direct effect of loss of the F-BAR domain or a consequence of altered Rac1 activity or cytoskeletal processes. Both scenarios seem possible, since - deviating from the classification described above - the F-BAR domain of srGAP2 (Slit/robo Rho GTPase activating protein-2) generates negative membrane curvature and promotes the formation of plasma membrane protrusions, suggesting that F-BAR proteins can have a more versatile function in membrane dynamics (Guerrier et al, 2009; Zhao et al, 2011).

With this study we demonstrate that NOSTRIN is necessary for proliferation, directed migration and filopodia formation in endothelial cells. NOSTRIN is the first F-BAR protein for which a function in developmental angiogenesis is demonstrated and illustrates an evolving common theme in F-BAR protein biology (Hu et al, 2009; Koduru et al, 2010; Toguchi et al, 2010): in addition to co-ordinating events involving the plasma membrane and the cytoskeleton, such as endocytosis, F-BAR proteins
serve to integrate extracellular signals and are essential for complex biological processes such as neuronal guidance and vascular development.
Material and methods

Zebrafish MO injection and analysis

Control MO CCTCTACCTCAGTTACAATTTAT, translation blocking ATG MO GCTGCTCAGGGGTCTTTCATCTTC and splice blocking MO TCAACACGTCTCCTGGCCAGATC were diluted in ultrapure water with 0.05 % phenol red. 3.3 ng of Control MO, 3.3 ng of ATG MO or 5 ng of Splice MO were injected into 2-8 cell stage Tg(fli1a:EGFP)y1 (Lawson & Weinstein, 2002) or Tg(kdrl:EGFP)s843 (Jin et al., 2005) zebrafish embryos. For rescue experiments full size zebrafish NOSTRIN cDNA clone: IRBOp991F08104D (NCBI Reference Sequence: NM_001039724.3) was purchased from Imagenes and cloned into the vector pcDNA3.1 (Invitrogen). mRNA was prepared using the mMessage mMachine kit (Ambion) and 15 pg co-injected with the splice MO if not stated otherwise. After all injections embryos were kept at 28° C in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, 0.33 mM MgSO4). At indicated time points embryos were dechorionated, anaesthetized with tricaine (Sigma) and mounted in 1.2 % low-melting-point agarose (Roth) in E3 medium. Bright field images were acquired using a Leica MZ165 dissecting microscope (Fig. 1A-C). Fluorescent images were acquired using a Leica TCS SP5 confocal microscope (Fig. 1D). Time-lapse microscopy images were captured every 10 min for 24 hours with Leica Live Cell Imaging microscope (Fig. 2A, Supplementary Video 1 and 2). Length of tip cell filopodia was determined from high resolution images captured with Zeiss LSM510 confocal microscope using ImageJ software (Fig. 2B).

Zebrafish protein lysates were prepared in lysis buffer (1 % NP-40, 150 mM NaCl, 2 mM EDTA, 50 mM Tris pH 7.4, protease inhibitor cocktail (Roche)) and analysed by immunoblotting.

Generation of NOSTRIN knockout mouse

NOSTRIN targeting vector construction and knockout mouse generation were carried out by genOway (www.genoway.com; Lyon, France). The targeting vector contained two loxP sites, one in the long homology region (6.0 kb, homologous to a sequence containing exons 3, 4 and 5) and a second one in the short homology region (2.0 kb, homologous to exon 6) of the NOSTRIN gene (GenBank accession number NM_181547), and a neomycin resistance gene cassette for positive selection flanked by Frt sites. Homologous recombination after introduction of the linearised vector in 129sv/Pas embryonic stem cells and neomycin selection resulted in generation of ES cell clones with a recombined NOSTRIN locus containing one loxP site between exon 3 and 4 and the Frt-flanked neomycin resistance gene cassette followed by the second loxP-site between exons 5 and 6. ES cell clones containing the recombined NOSTRIN allele were used for injection into C57BL/6J blastocysts and generation of chimera. Male offspring of chimera with recombined NOSTRIN allele was further mated with C57BL/6J Cre deleter females to induce excision of the floxed sequence (exons 4 and 5).
to generate the NOSTRIN knockout allele. The presence of the NOSTRIN knockout allele in the offspring was confirmed by Southern blotting and PCR. Heterozygous NOSTRIN knockout mice were backcrossed into C57BL/6J mice for 6 generations.

**Analysis of mouse postnatal retinal angiogenesis**

For flat mount retina staining, the intact eye was fixed in 4 % paraformaldehyde (PFA). The retina was dissected, rinsed with PBS, and permeabilized in blocking buffer (1 % BSA and 0.5 % Triton-X-100 in PBS). After three washes with Pblec buffer (0.5 % Triton-X-100, 1 mM CaCl$_2$, 1 mM MgCl$_2$, 0.1 mM MnCl$_2$ in PBS, pH 6.8), the retina was incubated in Pblec containing FITC-conjugated isoelectin B4 (1:100, Sigma). For proliferation analysis additional staining with a phospho-Histone H3-specific antibody (anti-p-H3-Ser-10 mitosis marker, 1:100, MerckMillipore) in combination with an Alexa546-conjugated anti-rabbit antibody (1:200, Invitrogen) was carried out. Stained retinas were flat mounted and viewed with a Zeiss LSM 510 META confocal microscope. High-resolution CLSM Images were analysed using Axiovision software.

**Matrigel plug assay**

Growth factor reduced matrigel (BD Bioscience) was thawed overnight at 4°C and supplemented with 0.0025 U/ml heparin. Optional, 150 ng/ml FGF-2 was added to the matrigel. Eight week old male wild type C57BL/6J and NOSTRIN knockout mice were anaesthetized by subcutaneous injection of 4 % chloralhydrate solution (three animals per genotype). 500 µl matrigel supplemented with 150 ng/ml FGF-2 were injected subcutaneously, dorsolaterally on the right side of the animal and as a negative control matrigel without growth factor was injected on the left side. After ten days mice were sacrifised and matrigel plugs dissected from the tissue, embedded in Tissue-Tek (Sakura) and frozen at -80°C. Frozen matrigel plugs were cut on a microtome at 16 µm slice thickness. Sections were fixed in acetone and stained with PECAM-1 antibody in combination with secondary biotin goat anti-rat antibody, Streptavidin-Horseradish Peroxidase Pre-dilute (BD Pharmingen) and DAB substrate kit (BD Pharmingen). Sections were counterstained with Mayer’s hematoxylin solution. Images (10 per matrigel plug) were acquired using a Leica MZ165 dissecting microscope and PECAM-1 staining positive area determined with Adobe Photoshop CS4 software.

**Isolation of mouse lung endothelial cells**

Isolation of MLECs was carried out as described (Sawamiphak et al, 2010). In summary, lungs from 3-6 P4-P7 old pups were minced and digested with collagenase. Endothelial cells were sorted using rat
anti-mouse PECAM-1 antibody (Pharmingen) coupled to anti-rat IgG coupled magnetic beads (Invitrogen). Cells were used for analysis in passage 2 and 3.

**Yeast two hybrid (Y2H) screening**

Y2H screening was performed according to the Yeast Protocol Handbook (Clontech). In brief, NOSTRINaa362-506 was cloned into pGBKT7 vector (coding for the DNA binding domain of Gal4, Gal4BD) and used as bait. Human kidney cDNA library in pACT2 vector (coding for the activator domain of Gal4, Gal4AD) was co-transformed together with the bait into yeast strain AH109 and growth on synthetic drop-out media (-W/-L/-H) was assessed to select for NOSTRIN interaction partners. Among others one clone was identified as aa547-801 of fibroblast growth factor receptor 1. For “one-on-one” Y2H analysis NOSTRIN deletion mutants were cloned into pBD vector and cotransformed with pACT2-FGFR1aa547-801 into yeast (Fig. 6A). Selection was done as above.

**Purification of GST- and His-tag fusion proteins**

GST-fusion proteins of Rac1, the CRIB domain of PAK (PAK-CRIB), C-terminal part of FGFR1, NOSTRIN and NOSTRIN deletion constructs were purified on glutathione (GSH)-Sepharose (GE Healthcare) as previously described (Icking et al, 2006). For the small GTPases and PAK-CRIB, lysis and wash buffer contained 5 mM MgCl$_2$. (His)$_6$-tagged fusion proteins of the Sos1 proline rich region (His-Sos-PRD) were purified from E. coli BL21 as previously described for (His)$_6$-tagged NOSTRIN (Icking et al, 2006).

**SFV-mediated NOSTRIN expression and Rho GTPase interaction assay**

Infection of CHO cells with Semliki Forest Virus was performed as described before (Zimmermann et al, 2002). Cells expressing SFV-NOSTRIN-ΔF-BAR or SFV-NOSTRIN-ΔF-BAR/ΔHR1 were lysed in GTPase lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM MgCl$_2$, 0.5 % Triton-X-100, 10 μM Aprotinin, 1 mM Benzamidin, 1mM PMSF). GDP or GTP$_\gamma$S (Sigma) was added to the cell lysates (final concentration 0.1 mM). GST-Rac1 coupled to GSH sepharose was loaded with either GDP or GTP$_\gamma$S in GTPase loading buffer (20 mM Tris-HCl pH 7.4, 25 mM NaCl, 5 mM EDTA, 2 mM of GDP or GTP$_\gamma$S), finally MgCl$_2$ was added (final concentration 10 mM). Nucleotide loaded GSH sepharose bound GTPases were added to pre-cleared lysates and incubated at 4°C rotating for 4 h. The beads were washed six times with wash buffer 1 (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 2.5 mM MgCl$_2$, 0.2 % Triton-X-100) and three times with wash buffer 2 (20 mM Tris-HCl pH 7.4, 2.5 mM MgCl$_2$). Bound proteins were eluted with sample buffer (63 mM Tris-HCl, pH 6.8, 2.5 % SDS, 5 % glycerol, 5 % β-mercaptoethanol, 0.005 % bromophenol blue) and binding of NOSTRIN to the small GTPases was analysed by immunoblotting.
**Rac GTPase activation assay**

Lysates of SFV-NOSTRIN, SFV-NOSTRIN-ΔSH3 or SFV-NOSTRIN-ΔHR1 expressing cells were prepared in PAK-CRIB lysis buffer (50 mM Tris-HCl pH 7.5, 200 mM NaCl, 10 mM MgCl$_2$, 5 % Glycerol, 1 % Triton-X-100, 1 mM PMSF, 10 µM Aprotinin, 1 mM Benzamidin). EDTA was added to the lysates (final concentration 24 µM). Lysates were incubated with GST-PAK-CRIB (20 µg) coupled to GSH sepharose for 40 min rotating at 4 °C. Beads were washed three times with PAK wash buffer (25 mM Tris-HCl pH 7.5, 40 mM NaCl, 30 mM MgCl$_2$, 1 mM DTT, 1 % NP-40) and two times with PAK wash buffer without detergent. Bound proteins were eluted with sample buffer. Precipitation of endogenous, active Rac1 was monitored by immunoblotting. Alternatively, Rac1 activity in MLECs was measured after 6 h of starvation in serum free medium and stimulation with 25 ng/ml FGF-2 (PeproTech) using the Active Rac1 pulldown and detection kit (Pierce Biotechnology) according to the manufacturer’s instructions.

**NOSTRIN-FGFR1 interaction analysis**

For co-immunoprecipitation HeLa cell lysates were prepared in lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 2 mM EDTA, 1 % NP-40, 25 mM NaF, 1mM Na$_3$VO, supplemented with protease inhibitor cocktail (Roche)) and immunoprecipitation was performed using rabbit anti mouse NOSTRIN antiserum in combination with protein A/G-agarose (Santa Cruz). Precipitated proteins were analysed by immunoblotting. Co-immunoprecipitation from MLEC lysates was performed accordingly using rabbit anti-FGFR1 antibody (Santa Cruz, sc-121). For pulldown experiments NIH-3T3 cell lysates were prepared in lysis buffer (as above) and incubated with GSH-Sepharose beads (GE Healthcare) coupled with equal amounts of GST-tagged full size NOSTRIN or NOSTRIN deletion constructs. Subsequently beads were washed three times with the lysis buffer, boiled in sample buffer and binding of FGFR1 to NOSTRIN was analysed by immunoblotting. For direct interaction studies the C-terminal part of human FGFR1, encompassing aa692-822 was subcloned from RZPD full length cDNA clone no. IRAKp961IJ0214Q2 into pGEX4T1 vector, expressed as GST-fusion protein and released by thrombin cleavage. Equal amounts of the C-terminal part of FGFR1 were incubated with GST-NOSTRIN or GST-NOSTRIN deletion constructs coupled to GSH-Sepharose beads in incubation buffer (20 mM Tris pH 8.4, 150 mM NaCl, 2.5 mM CaCl$_2$, 10 % Glycerol, 1 % Triton-X-100, 1 mM DTT supplemented with protease inhibitor cocktail (Roche)). Finally Sepharose beads were washed three times with the incubation buffer and two times with PBS. Bound proteins were analysed by immunoblotting.
**Immunoblotting and Immunohistochemistry**

In this study 2 different NOSTRIN antibodies were used. (1) A monoclonal antibody raised in mouse against human NOSTRIN and described previously (Mookerjee et al, 2007). This was used for immunoblotting in Fig 4B-D, Fig. 5A/B, 6B (2) A polyclonal antiserum against mouse NOSTRIN was produced using recombinant GST-tagged NOSTRINaa337-506 purified from E. coli BL21 as antigen. Standard procedures were used for immunization and bleeding of rabbits. This was used for immunoblotting in Fig. 1D, Fig. 7A/B. Additional antibodies were directed against GAPDH (Abcam ab8245) Fig. 7A; FGFR1 (polyclonal FGFR1-specific antiserum (Mohammadi et al, 1991) Fig. 6B/C; FGFR1 (Santa Cruz sc-121) Fig. 6D; GST (Amersham 27-4577-01) Fig. 4B; His-Tag (Bethyl A190-114A) Fig 5E; Rac1 (BD Pharningen 610650) Fig. 5A/B, 7C; Sos1 (Santa Cruz sc-256) Fig. 5C/D; Tiam1 (Santa Cruz sc-872); Vav2 (Acris AP220506); vinculin (Sigma v9131) Fig. 1D. For immunohistochemistry an antibody directed against PECAM-1 was used (BD Pharningen 553370) in combination with biotinylated goat anti-rat secondary antibody (BD Pharningen).

**Statistics**

Data are expressed as the mean ± s.e.m., and statistical evaluation was performed by unpaired two-tailed t-test or two-way ANOVA with Bonferroni post tests by GraphPad Prism. Values of p<0.05 were considered statistically significant.

**Ethical review**

Zebrafish were maintained under standard conditions at the MPI for Heart and Lung Research, Bad Nauheim. Mice were maintained at the animal facility of the Goethe University Frankfurt, Medical School. All animal experiments were performed in compliance with the relevant laws and institutional guidelines and were approved by local animal ethics committees.
Acknowledgements

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Author Contribution


Conflict of Interest

The authors state that there are no competing commercial interests in relation to the work described herein.
References


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Figure legends

Figure 1 MO-mediated KD of NOSTRIN in developing zebrafish embryos cause vascular defects. *Tg(fli1a:EGFP)*<sup>y1</sup> zebrafish embryos were injected with 3.3 ng Control MO, 3.3 ng ATG MO, 5 ng Splice MO or 15 pg zebrafish NOSTRIN mRNA, if not indicated otherwise. All zebrafish images are lateral views with the anterior to the left and the dorsal side up. (A) Development of NOSTRIN morphants. Embryos injected with the Splice MO showed an overall regular development at 48 hpf when compared to WT embryos or embryos injected with Control MO (left). Higher magnification of embryo heads, black arrowhead pointing to hindbrain oedema in embryo injected with the Splice MO (right). (B) Cranial haemorrhaging (left and middle) and pericardial oedema and haemorrhaging (right) in embryos injected with the Splice MO analysed at 72 hpf, black arrowheads indicating red blood cells. (C) CLSM images of trunk regions taken at 48 hpf, with the vascular structures visualised by eGFP fluorescence and labelled ISV (intersegmental vessel), CA (caudal artery), DLAV (dorsal longitudinal anastomotic vessel) and CVP (caudal vein plexus) showed regular development in the WT and embryo injected with Control MO. In embryos injected with the ATG or Splice MO false connections between neighbouring ISVs were formed (indicated by white arrow heads), the DLAV was interrupted (indicated by asterisks) and the caudal vein plexus was irregular in appearance in both morphants. Co-injection of NOSTRIN mRNA with the Splice MO almost completely re-established the ISV trajectory, DLAV integrity and CVP regularity. (D) The efficient KD of NOSTRIN after injection of ATG MO or Splice MO and the expression of NOSTRIN after mRNA co-injection were verified by immunoblotting of whole fish lysates prepared at 48 hpf using a NOSTRIN-specific antiserum, an immunoblot of vinculin demonstrated loading of equal amounts. (E) Quantitative analysis of live embryos at 48 hpf treated as indicated; vascular phenotype included abnormal ISV trajectory, DLAV discontinuity, oedema, haemorrhaging.
Figure 2 NOSTRIN morphants show an abnormal ISV trajectory phenotype associated with altered tip cell morphology and filopodia length. (A) Time-lapse microscopy of developing ISVs. Images are lateral views with the anterior to the left and the dorsal side up and were extracted from Supplementary Video 1 (WT) and 2 (ATG MO). Images show developing ISVs and DLAVs in the trunk region of WT Tg(fli1a:EGFP)y1 embryos (left) in comparison to embryos injected with ATG MO (right) visualised by eGFP fluorescence and analysed at the indicated time points between 20 and 44 hpf. The white arrowhead indicates an ISV tip cell in the ATG MO-injected embryo, which did not migrate more dorsally than the level of the horizontal myoseptum after 32 hpf, but extended a prominent protrusion along the longitudinal body axis at approx. 36 hpf. It formed an irregular connection with the neighbouring ISV at the level of the horizontal myoseptum at approx. 42 hpf. (B) Reduced filopodia length of ISV tip cells. CLSM images of leading tip cells of ISV sprouts in the trunk region of WT Tg(kdr1:eGFP)s843 (top) and ATG-MO injected embryos (bottom) visualised by eGFP fluorescence and analysed at 24 hpf. The scale bar represents 15 \( \mu \text{m} \). (C) Distribution of tip cell filopodia length. Analysis of filopodia length in ISV tip cells equivalent to those shown in (B) revealed a reduction in the percentage of long filopodia in the ATG MO-injected embryos in comparison to WT. For analysis of filopodia length images of 6-8 tip cells from 2 WT and 2 ATG MO injected embryos were used.
Figure 3 Postnatal retinal angiogenesis is impaired in NOSTRIN knockout mice. (A) Side-by-side comparison of the retinal vasculature of NOSTRIN wild type (WT) and knockout (KO) mice. Retina flat mounts of postnatal days (P)2, P5 and P7, vasculature visualised by isolectin B4-FITC staining and analysed by CLSM. Dashed circles denote the vascular front in the KO, circles indicate the retinal margin in both genotypes. Scale bars represent 500 µm. (B) Quantification of retinal vasculature spreading. The mean vascular radius (distance between vascular front and centre of the optic disk) of 4-6 retinas for each genotype and time point was calculated and expressed relative to the mean retinal radius. (C) Comparison of vascularised area. The vascularised area of 4-6 retinas for each genotype and time point was determined using AxioVision software (Rel 4.8.2 Zeiss). (D) Comparison of number of branch points. Branch points were counted in 5-8 fields in the front (peripheral to two-thirds of the vascular radius) and the central part of the vascularised area (between one-third and two-thirds of the vascular radius), 4 retinas for each genotype at P5. (E) Quantification of tip cell number. Tip cells (defined as blind-end endothelial protrusions with associated filopodial bursts at the angiogenic front) were counted in 15 fields per retina, 5-6 retinas for each genotype. (F) Quantification of filopodia number. Filopodia of tip cells at the vascular front were counted in 15 fields per retina, 5-6 retinas for each genotype and expressed as filopodia number per 100 µm vessel length. (G) Vascular front of retina flat mounts at P5 with mitotic nuclei stained using a p-H3-specific antibody in combination with isolectin B4-FITC as described above. Scale bars represent 70 µm. (H) Quantification of phospho-Histone H3 (p-H3)-positive endothelial cells. p-H3-positive cells were counted in 8 fields per retina (vascular front) and 6 retinas per genotype. (I) Distribution of tip cell filopodia length. Measurement of filopodia length was done on 6 fields per retina, 5 retinas for each genotype. (J) Comparison of retinal tip cells at the vascular front. Representative high magnification CLSM images of the vascular front of P5 retina flat mounts as depicted in (A) show that tip cells in the KO have less and shorter filopodial protrusions than tip cells in WT retinas. Scale bars represent 20 µm. Data shown in (B), (C), (D), (E), (F) and (H) are means ± s.e.m.. Data were analysed by unpaired two-tailed t-test and p-values are shown.
Figure 4 NOSTRIN interacts with Rac1 via the HR1 motif. (A) Domain structure of NOSTRIN. Amino acid numbers indicate the N- and C-terminal borders of the F-BAR, HR1, ID (intermediate domain) and SH3 domains. (B) NOSTRIN interacts with Rac1. GDP- or GTPγS-loaded GST-Cdc42, GST-Rac1 and GST-RhoA were used for GST-pulldown experiments using cell lysates expressing NOSTRIN. NOSTRIN (NOSTRINΔF-BAR) interacted specifically with GTPγS-loaded GST-Rac1 and to a lesser extent with GTPγS-loaded GST-Cdc42, while it did not interact with RhoA, independent of the nucleotide bound. (C) NOSTRIN interacts directly with active Rac1. Nucleotide free (NF), GDP- or GTPγS-loaded GST-Rac1 or GST-Cdc42 were used for GST-pulldown experiments in combination with purified recombinant NOSTRINΔSH3. 5% of the amount of purified NOSTRINΔSH3 used for GST-pulldown is shown for comparison of protein levels (5% input). NOSTRIN (NOSTRINΔSH3) interacted strongly and directly with GTPγS-loaded GST-Rac1. (D) NOSTRIN interacts with Rac1 via the HR1 motif. GDP- or GTPγS-loaded GST-Rac1 was used for GST-pulldown experiments using cell lysates expressing NOSTRINΔF-BAR and NOSTRINΔF-BAR/ΔHR1. Immunoblot from cell lysates shows expression of equal amounts of NOSTRINΔF-BAR and NOSTRINΔF-BAR/ΔHR1 (left). In the GST-Rac1 pulldown GTPγS-loaded GST-Rac1 interacted specifically with NOSTRINΔF-BAR, but not with NOSTRINΔF-BAR/ΔHR1 (right). (B-D) NOSTRIN was detected by immunoblotting with NOSTRIN-specific antibody (Mookerjee et al, 2007). Equal amounts of GST-Rac1, GST-Cdc42 or GST-RhoA, respectively, were used, detected by immunoblotting with a GST-specific antibody or Ponceau staining, as indicated.
Figure 5 NOSTRIN interacts with the Rac1 GEF Sos1 and induces Rac1 activation. (A) NOSTRIN overexpression induces Rac1 activation depending on the presence of the HR1 and the SH3 motif. NOSTRIN, NOSTRINΔHR1, NOSTRINΔSH3 or GFP were expressed using the SFV-system and the activity of Rac1 measured as amount of Rac1-GTP precipitated with the CRIB domain of PAK (PAK-CRIB assay). Equal amounts of Rac1 (input Rac1) and NOSTRIN, NOSTRINΔHR1 and NOSTRINΔSH3 were applied. Overexpression of NOSTRIN induced strong Rac1 activation in comparison to GFP, the deletion mutants NOSTRINΔSH3 and NOSTRINΔHR1 did not induce Rac1 activation. (B) NOSTRIN interacts with Sos1. GST-pulldown from primary mouse lung endothelial cells using GST-NOSTRIN, GST-NOSTRINΔSH3 or GST alone indicated specific interaction of full size NOSTRIN with endogenous Sos1, but not with Tiam1 or Vav2. Deletion of the SH3 domain in NOSTRINΔSH3 resulted in loss of the NOSTRIN/Sos1 interaction. (C) NOSTRIN SH3 domain is sufficient for interaction with Sos1. GST-pulldown from cell lysate using GST-NOSTRIN, GST-NOSTRINΔSH3, GST-SH3 or GST alone indicated specific interaction of endogenous Sos1 with GST-NOSTRIN and the isolated SH3 domain GST-SH3. (D) NOSTRIN SH3 domain binds the proline-rich domain of Sos1. GST-pulldown with recombinantly expressed and purified proline-rich domain of Sos1 (His-Sos1-PRD) confirmed specific and direct interaction of Sos1 with GST-NOSTRIN and GST-SH3.
Figure 6 NOSTRIN interacts with FGFR1 (A) Y2H interaction analysis between FGFR1 and NOSTRIN. Co-expression of a fusion protein of the Gal4 activator domain (Gal4AD) with the cytoplasmic tail of the FGFR1 (Gal4AD-FGFR1aa547-801) with 3 separate fusion proteins between the Gal4 DNA binding domain (Gal4BD) with distinct C-terminal NOSTRIN fragments (Gal4BD-NOSTRINaa362-506, Gal4BD-NOSTRINaa362-434 and Gal4BD-NOSTRINaa434-506). Gal4BD co-transformed with Gal4AD-FGFR1aa547-801 served as control. Each co-transformed yeast clone was spotted onto growth medium devoid of tryptophan and leucin (−WL) and growth medium devoid of tryptophan, leucine and histidine (−WLH) in 2 different dilutions. Growth of co-transformed yeast colonies on −WL indicates lack of toxicity, growth on −WLH of yeast co-transformants with Gal4AD-FGFR1aa547-801 in combination with either Gal4BD-NOSTRINaa362-506 or Gal4BD-NOSTRINaa362-434 indicates interaction, while no growth of yeast co-transformants with Gal4AD-FGFR1aa547-801 in combination with Gal4BD-NOSTRINaa434-506 indicates a lack of interaction. (B) NOSTRIN interacts with FGFR1 in mammalian cells. Co-immunoprecipitation of endogenous FGFR1 with endogenous NOSTRIN from cell lysates using a polyclonal NOSTRIN-specific antiserum for immunoprecipitation (IP). Lack of co-immunoprecipitation with pre-immune serum (PIS) served as specificity control. 5 % of the volume of the cell lysate used for IP is shown for comparison of protein levels (5 % input). Proteins are detected by immunoblotting with FGFR1-specific antiserum and a NOSTRIN-specific antibody (Mookerjee et al, 2007). (C) NOSTRIN interacts with FGFR1 independently of the SH3 domain. GST-pulldown experiment using GST-NOSTRIN, GST-NOSTRINΔSH3, GST-SH3 or GST alone to pulldown endogenous FGFR1 from cell lysates. Lack of interaction with GST indicates specificity. Proteins are detected by immunoblotting with polyclonal FGFR1-specific antiserum. (D) Direct protein/protein interaction analysis. Recombinantly expressed and purified GST-NOSTRIN full size, GST-NOSTRINΔSH3, GST-SH3 or GST used in combination with a recombinantly expressed and purified C-terminal fragment of FGFR1 comprising aa 692-822, confirming interaction of FGFR1 with GST-NOSTRIN and GST-NOSTRINΔSH3 (FGFR1 aa692-822 was chosen because it could be purified as a soluble protein in sufficient amounts). Proteins were detected by immunoblotting with an FGFR1-specific antibody.
Figure 7 NOSTRIN is required for the FGF-2-dependent activation of Rac1 in primary endothelial cells and for the angiogenic response to FGF-2 in the matrigel plug assay. (A) NOSTRIN expression in mouse lung endothelial cells (MLECs). Confirmation by immunoblotting with a NOSTRIN-specific antiserum of the expression of NOSTRIN in MLECs isolated from WT mice and its absence in MLECs isolated from NOSTRIN KO mice; an immunoblot of GAPDH is shown as loading control. (B) NOSTRIN interacts with FGFR1 in MLECs. Specific co-immunoprecipitation of NOSTRIN with FGFR1 from lysates prepared from MLECs pretreated with FGF-2 (25 ng/ml, 5 min) or vehicle. An FGFR1-specific antibody was used for precipitation. NOSTRIN was detected by immunoblotting with a NOSTRIN-specific antiserum. (C) Comparison of FGF-2-dependent activation of Rac1 in MLECs isolated from WT or NOSTRIN KO mice. PAK-CRIB assays performed using equal amounts of Rac1 (input Rac1) and GST-PAK-CRIB. FGF treatment (25 ng/ml) induced Rac1 activity in WT, but not in KO MLECs. (D) Comparison of representative PECAM-stained cryosections of matrigel plugs implanted in WT and NOSTRIN KO mice in the absence (control) or the presence of FGF-2. FGF-2 (150 ng/ml) induced a strong angiogenic response after 10 d in WT, but not in NOSTRIN KO mice. (E) Quantification of PECAM-stained area and statistical analysis of 10 sections per matrigel plug, from 3 matrigel plugs per treatment and genotype. Data shown are means ± s.e.m. analysed by two-way ANOVA with Bonferroni post-test; p<0.01 (F) Model of the FGFR1/NOSTRIN/Rac1/Sos1 complex, discussed in the main text (TK – tyrosine kinase domain).
Supplementary Legends

Video 1 Development of ISVs in WT zebrafish embryos between 20 and 44 hpf. Time-lapse microscopy images of the developing vasculature of the trunk region of WT Tg(fli1a:EGFP)$^{y1}$ embryos visualised by eGFP fluorescence. Frames were taken at 10 min intervals between 20 and 44 hpf. Shown are lateral views with the anterior to the left and the dorsal side up.

Video 2 Development of ISVs in NOSTRIN KD zebrafish embryos between 20 and 44 hpf. Time-lapse microscopy images of the developing vasculature of the trunk region of NOSTRIN KD Tg(fli1a:EGFP)$^{y1}$ embryos injected with ATG MO. The vasculature was visualised by eGFP fluorescence. Frames were taken at 10 min intervals between 20 and 44 hpf. Shown are lateral views with the anterior to the left and the dorsal side up.
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Video 2 Development of ISVs in NOSTRIN KD zebrafish embryos between 20 and 44 hpf. Time-lapse microscopy images of the developing vasculature of the trunk region of NOSTRIN KD Tg(fli1a:EGFP)y1 embryos injected with ATG MO. The vasculature was visualised by eGFP fluorescence. Frames were taken at 10 min intervals between 20 and 44 hpf. Shown are lateral views with the anterior to the left and the dorsal side up.
Supplementary Figure 1. Knockdown of NOSTRIN in zebrafish interferes with the development of the subintestinal vein (SIV) basket. Tg(fli1a:EGFP)y1 zebrafish embryos were injected with 3.3 ng Control MO or 5 ng Splice MO or left uninjected (WT). For the rescue 15 pg of zebrafish NOSTRIN mRNA was coinjected with Splice MO. SIV baskets were imaged at 48 hpf using CLSM. Images are lateral views with the anterior to the left and the dorsal side up.
Supplementary Figure 2. (A) NOSTRIN knockout in mice is achieved by Cre-mediated excision of exons 4 and 5 of the Nostrin gene. Schematic presentation of the endogenous Nostrin locus (WT) with the binding sites for genotyping primers indicated by arrows (top). Two loxP sites and a neomycin resistance gene cassette were introduced by homologous recombination to generate the recombined Nostrin locus (middle). Cre-mediated excision of the loxP-flanked sequence results in deletion of the exons 4 and 5 in the Nostrin KO locus (bottom). (B) Genotyping of NOSTRIN KO mice by PCR. Mice were genotyped by PCR using the primers mNSTfwd (5'-CCTAGAGCTGACTCCTGCTGTGAGAGG-3') and mNSTrev (5'-CTCATACTGGTAAGCA GAAAAGCATCGTTT-3') indicated in (A). Amplified DNA sequences had the expected sizes of 2170 bp indicating the WT allele or 570 bp indicating the KO allele, respectively. (C) NOSTRIN is highly expressed in lung and liver and expression is lost in the NOSTRIN KO. Comparison of the level of NOSTRIN protein in various tissues of WT and NOSTRIN KO mice, analysed by immunoblotting with a NOSTRIN-specific polyclonal antiserum. Immunoblot against GAPDH (Abcam) is shown as loading control.
Supplementary Figure 3. Analysis of mouse retina astrocyte network. The vascular radius is greater in the retinas of WT than in the retinas of KO mice, but the radius of the astrocyte network is unchanged in the NOSTRIN KO compared to WT. Specimen were treated as described for analysis of mouse postnatal retinal angiogenesis and in addition stained with GFAP (glial fibrillary acidic protein)-specific antibody (1:1000, Dako) in combination with an Alexa 546-conjugated anti-rabbit antibody (1:200, Invitrogen). Scale bars represent 300 μm.
Supplementary Figure 4. Analysis of endothelial cell proliferation in mouse retina. Proliferation of endothelial cells in retinas isolated from NOSTRIN KO is decreased when compared to WT mice. Retinas were isolated from P5 WT and NOSTRIN KO mice and stained with isolectin B4-FITC and Ki67 (1:100, Millipore) in combination with an Alexa 546-conjugated anti-rabbit antibody (1:200, Invitrogen). Scale bars represent 70 μm.
Supplementary Information Figure S5 Kovacevic

(A) NOSTRIN endothelial specific knockout in mice is achieved by Cre-mediated excision of exons 4 and 5 of the Nostrin gene in endothelium. Schematic presentation of the endogenous Nostrin locus (WT) with the binding sites for genotyping primers indicated by arrows (top). Two loxP sites and a neomycin resistance gene cassette were introduced by homologous recombination to generate the recombined Nostrin locus (middle). Neomycin cassette was excised by crossing with Flp-recombinase-expressing deleter mice to generate Nostrin<sup>flp/flp</sup> control mice (CTL). CTL mice were further bred with Tie2-Cre mice in order to achieve the excision of the loxP-flanked sequence leading to deletion of the exons 4 and 5 in the Nostrin KO locus in endothelium (EC KO, bottom). (B) Genotyping of EC KO mice by PCR. EC KO mice were genotyped by PCR using the following primers: flpfw (5'-AGGTTAGGAGAGCCAGGGACCTAGC-3'), flprev (5'-CTCATACTGGTAAGCAGAAAAGCATTTT-3'), Crefw (5'-GCGGTCTGGCAGTAAAAACTATC-3'), Crerev (5'-GTGAAACAGCATTGCTGTGACTT-3'), controlfw (5'-CTAGGGCCACAGAAAATGCTAGTC-3') and controlrev (5'-GTAGGGGAAATCTTGCATCATCC-3'). Amplified DNA sequences had the expected sizes of 722 bp indicating the Nostrin<sup>flp/flp</sup> allele, the 554 bp indicating the Nostrin WT allele, 324 bp indicating internal positive control and 100-bp indicating Cre-recombinase. (C) The expression of NOSTRIN in lung tissue is almost completely lost in the EC KO mouse. Comparison of the level of NOSTRIN protein expression in lung tissue of WT, CTL and EC KO mice, analysed by immunoblotting with a NOSTRIN-specific polyclonal antiserum. Immunoblot against eNOS (Transduction laboratories) is shown as loading control.
Supplementary Figure 6. Analysis of postnatal retina in NOSTRIN endothelial cell-specific KO mice (EC KO). The analysis was performed on retinas isolated from littermates Nostrin^ flox/flox (CTL) and Nostrin^ flox/floxTie2-Cre-^ (EC KO) at P4 as described in Materials and Methods and in Figure 3 legend. Mean vascular radius (A) and vascularised area (B) are significantly reduced in EC KO mice. The number of branch points at the vascular front (C, left) and in the central (C, right) region of the retina are reduced when compared to retinas isolated from CTL mice. In addition, the tip cell number (D) and filopodia number (E) are significantly decreased in EC KO. Furthermore, we found less proliferating cells (shown by phospho-Histone H3 staining) in EC KO retinas (overview F, quantification G). Scale bars represent 50 μm. Finally, the tip cells in retinas of EC KO mice exhibit less of long filopodia when compared to CTL mice (overview I, quantification H). Scale bars represent 20 μm. In general, the effect of endothelial cell-specific NOSTRIN KO on postnatal retinal angiogenesis in EC KO mice reproduces the effect of loss of NOSTRIN in the global NOSTRIN KO mice (Figure 3).
Supplementary Figure 7. Comparison of VEGF dependent activation of Rac1 in MLECs isolated from WT or NOSTRIN KO mice measured with PAK-CRIB assay. Equal amounts of Rac1 (input Rac1) and GST-PAK-CRIB were applied. The cells were starved for 6 hr and stimulated with 100 ng/ml VEGF-C (ReliaTech) as described (Wang et al. 2010). VEGF stimulation induced Rac1 activity in both WT and KO MLECs.