The cytoskeleton adaptor protein Sorbs1 controls the development of lymphatic and venous vessels in zebrafish

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1 **Abstract**

2

3 Lymphangiogenesis, the formation of lymphatic vessels is tightly linked to the
4 development of the venous vasculature, both at the cellular and molecular levels.
5 Here, we identify a novel role for Sorbs1, the founding member of the SoHo family of
6 cytoskeleton adaptor proteins, in vascular and lymphatic development in zebrafish.
7 We show that Sorbs1 is required for secondary sprouting and emergence of several
8 vascular structures specifically derived from the axial vein. Most notably, formation of
9 the precursor parachordal lymphatic structures is affected in *sorbs1* mutant embryos,
10 severely impacting the establishment of a proper trunk lymphatic network and leading
11 to edema development. We show that Sorbs1 is probably not part of the Vegfc
12 signaling, but instead might interacts with the BMP pathways. Mechanistically, we
13 show that Sorbs1 controls FAK/Src signaling to impact on Rac1 and RhoA GTPases-
14 regulated cytoskeleton processes. Inactivation of Sorbs1 altered cell-extracellular
15 matrix (ECM) contact rearrangement and cytoskeleton dynamics, leading to specific
16 defects in endothelial cell migratory and adhesive properties. Our data thus establish
17 Sorbs1 as an important regulator of lymphangiogenesis distinct from the Vegfc
18 signaling axis, increasing our understanding of context-specific vascular and lymphatic
19 development.

20

1 **Non-standard Abbreviations and Acronyms**

2 bp : base-pair

3 CV : Cardinal Vein

4 CVP : Caudal Vein Plexus

5 DA : Dorsal Aorta

6 DLAV : Dorsal Longitudinal Anastomotic Vessel

7 DLLV : Dorsal Longitudinal Lymphatic Vessels

8 dpf : days post-fertilization

9 EC : Endothelial Cells

10 ECM : Extracellular Matrix

11 ELV: Ectopic Longitudinal Vessel

12 EV: Ectopic Vessel

13 FA : Focal Adhesions

14 Fx : Focal Complexes

15 GAP : GTPase Activating Protein

16 GFP : Green Fluorescent Protein

17 gRNA: guide RNA

18 hpf: hours post-fertilization

19 ICV: Interconnecting Vessels

20 ISV : Intersegmental Vessel

21 Mo : Morpholino

22 NA : Nascent Adhesions

23 PCV : Posterior Cardinal Vein

24 PL: Parachordal Lymphangioblasts

25 SIV: Subintestinal Vein

26 SIVP: Subintestinal Venous Plexus

27 SoHo : Sorbs Homology Domain

28 TD: Thoracic Duct

29

1 Introduction

2

3 The adult circulatory system encompasses the blood and lymphatic vasculatures.
 4 Intimate connections link these networks at the developmental, anatomical and
 5 functional level. In the embryo, the blood vasculature develops through a sequence of
 6 events that have been extensively characterized in the past decades¹. Our
 7 understanding of lymphangiogenesis, the formation of lymphatic vessels, lags far
 8 behind that of angiogenesis, the formation of new blood vessels. Because of its critical
 9 role in tissue homeostasis and immune surveillance lymphangiogenesis has gained a
 10 lot of attention in recent years, leading to the identification of an increasing yet limited
 11 number of molecular players. Beyond these advances, recent reports also pointed to
 12 the protective and reparative therapeutic potential of enhancing lymphangiogenesis in
 13 several pathological contexts such as myocardial infarction²⁻⁴, glioblastoma⁵ and renal
 14 dysfunction⁶.

15 As for vascular development, the zebrafish model has significantly contributed
 16 to expand our understanding of lymphatic system formation and biology. More
 17 specifically, the stereotypical formation of the trunk lymphatic network has been
 18 extensively used to decipher the driving principles of lymphangiogenesis. Trunk
 19 lymphatic endothelial cell (LEC) precursors arise from transdifferentiation of venous
 20 ECs located within the posterior cardinal vein (PCV). Specification of LECs is triggered
 21 by the expression of the transcription factor Prox1a and occurs before their effective
 22 egression from the vein in a process involving asymmetric division⁷⁻⁹ and regulated by
 23 transcriptional¹⁰⁻¹⁴ and post-transcriptional programs¹⁵. At 32-34 hours post-
 24 fertilization (hpf), dorsal sprouting of these lymphatic-fated cells contributes to the
 25 transient development of a longitudinal string of parachordal lymphangioblasts (PLs)
 26 20 h later. At around 60 hpf, parachordal LECs start to migrate ventrally and dorsally
 27 to form the major trunk lymphatic network consisting of the thoracic duct (TD), the
 28 intersegmental lymphatic vessels (ISLVs) and the dorsal longitudinal lymphatic
 29 vessels (DLLV)¹⁶.

30 Illustrating the striking plasticity of endothelial cells, not all the vascular sprouts
 31 emerging from the PCV and migrating dorsally alongside the artery-derived primary
 32 intersegmental vessels (aISVs) participate in building the lymphatic vessels.
 33 Approximately half of them, almost undistinguishable except for their reduced Prox1a

1 expression, will connect and anastomose to the proximal region of aISVs to form
2 venous ISVs (vISVs)^{16–18}. Adding to the behavioral heterogeneity and specialization
3 of the venous ECs from the PCV, ventral angiogenic sprouting also occurs from the
4 posterior and anterior parts of the axial vein at different time points. ECs in the caudal
5 region sprout from the floor of the caudal vein (CV) at around 27 hpf and migrate
6 towards the ventral side of the embryo to form the caudal vascular plexus (CVP), a
7 distinctive fenestrated network of vessels¹⁹. More rostrally, formation of the
8 subintestinal venous plexus (SIVP), which will eventually provide blood supply to the
9 digestive tract starts at around 30 hpf with a process of ventral migration leading to the
10 formation at 3 days post-fertilization (dpf) of a basket-like plexus composed of vertical
11 interconnecting vessels (ICVs) that drain into a transversal subintestinal vein (SIV)^{20,21}.

12 Originating from the same parental vessel, the process of trunk
13 lymphangiogenesis is tightly intermingled with PCV-derived venous angiogenesis and
14 especially with ISV secondary sprouting that occurs concomitantly. Studies of mutants
15 isolated from forward genetic screens or associated with human diseases led to the
16 establishment of the Vegfc/Flt4 axis as the central pathway for lymphangiogenesis^{22–}
17 ²⁵. Accordingly, the currently growing list of lymphangiogenesis regulators almost
18 exclusively relates to molecules involved in Vegfc/Flt4 signaling, some of them acting
19 directly upstream such as CCBE1^{26–29} or the transcription factor HHX¹¹ or
20 downstream like the transcription factors Mafba⁷ and Yap1³⁰. Acting through multiple
21 intracellular events, including the activation of the common effector of Vegf receptors
22 Erk⁸, Vegfc signaling has been shown to control several aspects of lymphangiogenesis
23 including LEC differentiation through Prox1 expression, proliferation and migration.
24 Whereas these signaling cues seem to act indistinctly on lympho-venous sprouting,
25 with the majority of effectors impacting on both lymphatic vessel and vISV
26 formation^{22,27,31,32}, ventral angiogenesis of the caudal vascular and subintestinal
27 plexus specifically relies on BMP signaling^{19,21}.

28 While our understanding of angiogenic cues that drive formation of specific
29 vascular beds is only emerging, even less is known about the intracellular components
30 that define specific endothelial cell behavior during establishment of highly conserved
31 organ-specific vascular patterns. Sorbs1 (Cbl associated protein CAP/ponsin) belongs
32 to the SoHo family of adaptor proteins that includes two other members, Sorbs2 (Arg-
33 binding protein 2, ArgBP2) and Sorbs3 (Vinexin). Early following their discovery, SoHo
34 proteins were shown to localize to various actin-based structures, including z-discs,

stress fibers, cell-ECM and cell-cell adhesions^{33–39}. Sorbs1 interactions with several structural and signaling cytoskeletal components, such as vinculin and paxillin, strengthened the idea that it might function as an adaptor protein coordinating multiple signaling complexes regulating the actin cytoskeleton^{40,41}. In agreement with these observations, *in vitro* studies showed that Sorbs1, along with the other family members are important regulators of actin-dependent processes, such as migration, adhesion and mechano-transduction^{39,42,43}. Such cytoskeleton-based processes have been shown to be essential to support and control the morphogenic events that endothelial cells have to go through during blood and lymphatic vessel formation⁴⁴.

Little is known about the *in vivo* biological functions of SoHo proteins. Here, we report that Sorbs1 has unsuspected roles in zebrafish developmental angiogenesis and lymphangiogenesis. Using a combination of *in vivo* and *in vitro* approaches, we demonstrate that Sorbs1 controls endothelial cell adhesion signaling through modulation of specific RhoGTPases activities and consequently participates in the formation of specific venous and lymphatic structures originating from the main axial vein, both dorsally and ventrally. Surprisingly, despite its major impact on trunk lymphatic structures, Sorbs1 is not involved in Vegfc pathway but appears to participate in BMP signaling.

Results

Sorbs1 genetic depletion is associated with pericardial edema formation

To investigate the function of *Sorbs1* *in vivo*, we took advantage of the zebrafish model. We performed phylogenetic analysis using results from BLAST homology searches against NCBI and Ensembl databases and identified SoHo family orthologs in zebrafish. A single *Sorbs1* ortholog (ENSDARG00000103435), two *Sorbs2* orthologs, *sorbs2a* (ENSDARG00000003046) and *sorbs2b* (ENSDARG00000061603) and a single *Sorbs3* ortholog (ENSDARG00000037476) were identified (Supplementary Figure S1A). To assess the role of *Sorbs1* in zebrafish development, we used the CRISPR/Cas9 system to generate a *sorbs1* mutant allele. We selected F1 heterozygous carriers with a 14 base pair (bp) frame-shift deletion at codon 178, in the region of the *sorbs1* gene coding for the SoHo domain. This mutation is predicted to generate a premature stop codon at codon 182 (Supplementary Figure S1B) and *sorbs1* homozygous mutants (referred to as *sorbs1*^{-/-}) from heterozygous in-crosses express no detectable *Sorbs1* protein (Supplementary Figure S1C). *Sorbs1* mutants exhibited no gross morphological abnormalities. Nevertheless, we observed that a large proportion of the *sorbs1*^{-/-} larvae exhibited large edemas around the heart and the intestinal tract, which were first detectable at 2 dpf and were clearly visible at 5 dpf (Figure 1A and 1B). We suspected that these edemas could be indicative of vascular and/or lymphatic defects, although *sorbs1* mutants displayed normal heart rates (Supplementary Figure S1D) and overall circulation. The presence of edema strongly impacted on the viability of the embryos with about 40% of edema-developing embryos dying within 10 dpf (Figure 1C). As a complementary approach, we also used a splice-blocking antisense morpholino (*sorbs1* MO) targeting the exon 3/intron 3 boundary of *sorbs1*. This morpholino efficiently prevented splicing of intron 3 and reduced *Sorbs1* protein levels when injected at 5 ng/embryo (Supplementary Figure S1E,F). Similarly to *sorbs1* mutants, the vast majority of morphant embryos exhibited edemas, a defect that was rescued by injection of RNA coding for the human *Sorbs1* ortholog (Supplementary Figure S1G). Based on these observations, we examined the expression of *Sorbs1* in endothelial cells. *Sorbs1* expression was detected *in vivo* in blood vessel endothelium by immunohistochemical analysis of various human tissues (Supplementary Figure 1H, black arrows). In addition, *Sorbs1* protein was detected in various cultured human ECs, with the highest levels being observed in venous and lymphatic ECs (Supplementary Figure 1I). In zebrafish, whole mount *in*

situ hybridization revealed a ubiquitous expression of *sorbs1* throughout development (Supplementary Figure 1J). To validate *sorbs1* expression in the zebrafish vascular endothelium, we used the *Tg(fli1a:eGFP)y1* transgenic line, in which lymphatic, arterial, and venous ECs express green fluorescent protein (GFP), and sorted ECs (*i.e.*, GFP-positive cells) and non-ECs (*i.e.*, GFP-negative cells) by flow cytometry. Quantitative PCR analysis revealed that expression of *sorbs1* was significantly higher in ECs, as compared to non-ECs (Figure 1E). The expression of *sorbs1* in ECs was maximal at around 48 hpf, when active lympho-venous sprouting is occurring (Figure 1F).

Sorbs1 is important for lymphangiogenesis in zebrafish

To explore if the presence of edema in *sorbs1*^{-/-} larvae could relate to lymphangiogenesis deficiency, we performed microscopic observation of the vasculature of *sorbs1* mutants in the *Tg(fli1a:eGFP)y1* transgenic background. Whereas, mutants showed normal morphogenesis, patterning and lumenization of the cranial and trunk primary vasculature, development of lymphangiogenic structures was severely affected in the absence of *sorbs1* (Figure 2A, Supplementary Figure S2A,B). The formation of the PLs at the horizontal trunk septum was strongly impaired: quantification analysis at 54 hpf, confirmed that the proportion of somite segments with detectable PLs was significantly reduced in *sorbs1*^{-/-} embryos, with PLs being totally absent in approximately one third of the embryos (Figure 2B). Similar defects were also detected in *sorbs1* morphants (Supplementary Figure S2C,D). At approximately 60 hpf, PLs migrate ventrally from the horizontal myoseptum to form the TD (3-6 dpf), the major lymphatic trunk vessel situated between the DA and PCV. Because *sorbs1* mutants had a lower number of PLs, we reasoned that they might also exhibit defects in TD formation. To test this, we measured the length of visible TD portions in 10 somites at 4 and 6 dpf, and expressed it as a percentage of the total length of this trunk segment (Figure 2C,D)⁴⁵. In control larvae, the observed length of the TD at 4 dpf represented approximately 49% of the trunk total length, a proportion that increased up to 60% at 6 dpf (Figure 2D). Formation of the TD was greatly impaired in *sorbs1*^{-/-} larvae, as TD length corresponded to only 19% and 26% of the 10-somite length at 4 and 6 dpf, respectively. In a large proportion of *sorbs1*^{-/-} larvae (41%, 24/58) the TD was totally absent at 4 dpf while only 14% (8/57) of control embryos had no detectable TD. As expected, the most affected *sorbs1*^{-/-} embryos (*i.e.*, embryos with

less than 20% of visible TD) had reduced life span (Figure 2E). The defects in PL and TD formation strongly suggest that *sorbs1* is important for early lymphatic development, its absence culminating in edema formation and higher embryonic mortality. Importantly, in agreement with the idea of an endothelial function for Sorbs1, PL formation defects in *sorbs1*^{-/-} mutants were rescued through endothelial specific ectopic expression of human Sorbs1 (Figure 2F,G).

Sorbs1 function in lymphangiogenesis is independent of Vegfc

Almost all currently known genetic regulators of zebrafish trunk lymphangiogenesis, act through the Vegfc signaling pathway, the major regulator of lymphangiogenesis^{46,47}. Vegfc induces expression of Prox1a in a subset of ECs in the PCV during lymphatic specification, triggering their sprouting, migration and proliferation to form the lymphatic trunk vessel network. qPCR analysis of *prox1a* expression in ECs showed no significant difference between wild-type and *sorbs1* mutants at 48 hpf (Figure 3A). Moreover, live imaging of *TgBAC(prox1a:KaITa4-4xUAS-ADV.E1b:TagRFP)*^{nim5} line confirmed the presence of Prox1a-positive endothelial cells in the PCV of *sorbs1*^{-/-} mutants (Figure 3B). However in *sorbs1*^{-/-} mutants, Prox1a-positive cells failed to sprout out of the axial vein, indicating that *sorbs1* is dispensable for lymphatic specification but seems to be required for subsequent migration of LECs (Figure 3B). To directly test a link between Sorbs1 and Vegfc signaling we analyzed the lymphatic network of double *sorbs1/vegfc* heterozygous zebrafish embryos as haploinsufficiency has been demonstrated for Vegfc in this function²⁹. We found no evidence of genetic interaction between these two genes (Figure 3C). In line with these findings, injection of a Vegfc-coding RNA in *sorbs1* mutant embryos increased formation of PLs and TD similarly to control embryos, indicating that *sorbs1*^{-/-} ECs are not affected in their potential to respond to ectopically produced Vegfc (Figure 3D, Supplementary Figure S3A).

Lack of Sorbs1 impairs secondary sprouting from the PCV

In parallel to migration of Prox1a-specified cells to form PLs, venous Prox1a-negative ECs sprout from the PCV to connect to arterial intersegmental vessels (aISVs). To evaluate the role of Sorbs1 in this process, we counted the nascent secondary sprouts emerging from the PCV at 34 hpf, *i.e.* sprouts not yet fused to a primary ISV or stabilized to form PL (arrow in Figure 4A). Compared to control, the number of sprouts

1 was significantly reduced in *sorbs1*^{-/-} Tg(*fli1a:eGFP*)y1 embryos (n=77, *P*<0.01)
 2 (Figure 4B). The vast majority of embryos (68.8%) had no visible secondary sprouts
 3 and in the remainder fraction, only 15.6% of *sorbs1*^{-/-} embryos had one secondary
 4 sprout, whereas 15.6% had 2 or 3. These defects in secondary sprouting were
 5 confirmed by looking at *sorbs1* morphants (Supplementary Figure S4A). Because
 6 approximately half of the secondary sprouts gives rise to PLs, while the other half
 7 connects to the primary ISV network and establish vISVs, the defective PCV
 8 secondary sprouting in the absence of *sorbs1* could explain the reduced number of
 9 PLs. To assess if it also affected migration of the venous sprouts that will connect to
 10 and remodel ISVs, we counted the number of vISVs, *i.e.* ISVs connected to the PCV
 11 over a 10-somite region (Figure 4C). In wild-type embryos, slightly less than half of the
 12 ISVs were connected to the PCV and thus scored as of venous identity. By contrast,
 13 the proportion of vISVs was significantly lower (35.5%) in *sorbs1* mutant embryos
 14 (Figure 4D). A similar reduction in vISVs was also observed in *sorbs1* morphants
 15 (Supplementary Figure S4B). These observations suggest that *sorbs1*
 16 knockout/knockdown affects the secondary wave of migrating ECs from the PCV,
 17 which is associated with both lymphatic and vISV network formation. In contrast,
 18 *sorbs1* is dispensable for primary sprouting from the DA.

19 Along with the angiogenic dorsal sprouts, additional vascular structures are
 20 established from the PCV (Supplementary Figure S4C). Starting at 25 hpf, venous
 21 angiogenic sprouts emerge in the caudal region of the PCV and migrate ventrally
 22 through active angiogenesis to form the primordial caudal vein plexus (CVP), a
 23 complex network of vessels. During this process, ECs from the caudal vein extend
 24 protrusions towards the ventral region of the trunk to migrate and connect with each
 25 other to form the CVP at 48 hpf. We observed that while forming, the CVP from *sorbs1*
 26 mutant and knock-down embryos produced fewer ventral sprouts (Supplementary
 27 Figure S4D,E). Sprouting angiogenesis also occurs in the anterior region of the PCV
 28 leading to the formation of the SIVP. Several studies have extensively described the
 29 development of the SIVP and demonstrated that it forms from cells originating from
 30 the ventral side of the PCV, at around 30 hpf²⁰. These ECs collectively engage in a
 31 process of ventral migration and give rise at 3 dpf to a left and right basket-like plexus
 32 composed of vertical interconnecting vessels (ICVs) that drain into a transversal
 33 subintestinal vein (SIV) (Supplementary Figure S4F). Formation of the subintestinal
 34 venous plexus was affected in the absence of *sorbs1*. *Sorbs1*^{-/-} embryos showed

abnormal SIV morphology, with irregular branching and in severe cases, absence of the surrounding SIV (Supplementary Figure S4F). In sum, phenotypic characterization of *sorbs1* morphants and mutants revealed phenotypes linked to defects in the development of every major angiogenic structure that originates from the PCV. Interestingly, some of these processes rely on the Bone Morphogenetic Protein (BMP) pathway. More specifically, BMP signaling promotes ventral venous sprouting during CVP development and collective EC migration during SIV ventral expansion²¹. Its role during dorsal secondary sprouting is less clear. To examine the role of Sorbs1 in BMP-induced venous angiogenesis, we used the *Tg(hsp70l:bmp2b)* line, in which ectopic endothelial sprouting can be specifically induced from the PCV by heat-shock treatment (Figure 4E). When double transgenic *Tg(hsp70l:bmp2b; fli1a:eGFP)* embryos were heat-shocked at 39°C for 30 min at 26 hpf (*i.e.*, at the onset of PCV secondary sprouting), 40% showed ectopic vessels (EVs). Sorbs1 knockdown significantly reduced Bmp-induced sprouting from the PCV, since less than 20% of *sorbs1* morphants displayed EVs after heat-shock (Figure 4F). In these embryos, EVs were also visible in a smaller proportion of somite segments, demonstrating that *sorbs1* is implicated in the venous EC response downstream or acting in parallel to BMP.

Sorbs1 controls EC adhesion through regulation of small RhoGTPases

In order to understand the cellular and molecular mechanisms underlying Sorbs1 function during venous sprouting, we generated primary venous ECs deficient for Sorbs1 using small interfering RNA (siRNA) that efficiently and specifically suppresses the expression of Sorbs1, without affecting the viability or proliferation of ECs (Supplementary Figure S5A-D). In agreement with the observed impairment in EC migration from the PCV *in vivo*, downregulation of Sorbs1 correlated with a significant decrease in EC *in vitro* migratory capacities (Supplementary Figure S5E,F).

Members of the SoHo family are thought to function by interacting with and coordinating the activity of actin cytoskeleton regulators, including RhoGTPases^{39,48–51}. During zebrafish CVP formation, BMP has been shown to affect EC migration by promoting endothelial filopodia extension via activation of Cdc42⁷¹. We thus assessed the activity of Cdc42 by performing Rho GTPase activity assays in control and Sorbs1-depleted ECs. Levels of active Cdc42 were similar in the presence or absence of Sorbs1 (Figure 5A). In contrast, when looking at the other Rho GTPase members, we

1 found that knockdown (KD) of Sorbs1 correlated with a significant up-regulation in
 2 RhoA and a marked decrease in Rac1 activities. Reduction in Rac1 activity was
 3 associated with reduced phosphorylation of Rac1 effector kinases PAK2 and PAK4
 4 (Supplementary Figure S5G). Activation of RhoA was confirmed by looking at actin
 5 polymerization at the lamellipodia of spreading Sorbs1-KD cells. Indeed, cells depleted
 6 for Sorbs1 exhibited a denser network of actin bundles at the cell periphery and
 7 treatment with the C3 Transferase Rho inhibitor prevented appearance of peripheral
 8 F-actin in Sorbs1-KD cells, confirming the causative role of RhoA (Figure 5B,C). To
 9 get more insight into the cellular function of Sorbs1, we checked its subcellular
 10 localization in ECs and found that it localizes at cell-ECM adhesions (Supplementary
 11 Figure S5H). The formation and maturation of integrin adhesions at the leading edge
 12 of migrating cells is controlled by a precise spatio-temporal balance between the
 13 activities of Rac1 and RhoA GTPases⁵². Rac1 promotes the formation of new
 14 adhesions in regions of membrane protrusions, but also regulates adhesion turnover
 15 through downstream effectors such as PAKs and local inhibition of RhoA⁵³. In contrast,
 16 RhoA activation is associated with actomyosin-dependent stabilization and maturation
 17 of adhesions^{52,54}. We examined the possibility that Sorbs1 might control EC adhesion
 18 dynamics by modulating the activity of Rac1 and RhoA. Inactivation of Sorbs1 resulted
 19 in alterations in the pattern of EC-ECM adhesions (Figure 5D). Cell-ECM adhesions
 20 found at membrane protrusions are usually divided into two types, depending on their
 21 maturation stage. The first adhesions to appear are nascent adhesions (NA) and focal
 22 complexes (Fx), which are small dot-like structures characterized by their high content
 23 in tyrosine-phosphorylated signaling molecules, such as phospho-Paxillin⁵⁵. Few of
 24 them will elongate centripetally and mature into larger (area > 1µm²) focal adhesions
 25 (FAs), in a process relying on actin filaments⁵⁴. Compared to control siRNA-treated
 26 ECs, *Sorbs1* KD cells had a higher proportion of large FAs, which were localized more
 27 centripetally (Figure 5D,E). In contrast, the number of small phospho-Paxillin positive
 28 adhesions was reduced at the periphery of Sorbs1-deficient cells (Supplementary
 29 Figure S5I,J). Importantly, the excessive accumulation of stable FAs was correlated
 30 with a significant increase in cell adhesion onto fibronectin, providing a potential
 31 explanation for the migration defects in sorbs1-deficient cells (Figure 5F,G).

32 Expression of Sorbs1 was induced during cell adhesion onto fibronectin
 33 (Supplementary Figure S5K). This process triggers formation of the FAK-Src
 34 complex⁵⁶, which is known to induce activation of Rac1 and transient suppression of

1 RhoA, thus promoting adhesion disassembly at cell protrusions. As Sorbs1 localizes
2 to FAs and interacts with FAK, Src and several of their substrates at ligand-bound
3 integrin adhesions^{35,39,48,57} we assessed the activity of this complex upon Sorbs1
4 depletion. We found that activation of FAK, Src and their downstream target ERK was
5 decreased in Sorbs1-depleted cells (Supplementary Figure S5L).

6 Altogether these data suggest that Sorbs1 participates in the FAK-Src signaling
7 module, which controls the balance between RhoA and Rac1 activities and regulate
8 adhesion dynamics during EC migration. In that case, one should expect that
9 preventing hyperactivation of RhoA would rescue the defects associated with Sorbs1
10 deficiency. To test this hypothesis *in vivo*, we treated zebrafish embryos at 26 hpf with
11 the C3 transferase RhoA inhibitor and examined the formation of the vascular
12 structures originating from EC sprouting from the PCV. We used low doses of the C3
13 RhoA inhibitor, which had no significant impact on the vascular development of wild-
14 type embryos (Figure 5H-J). In contrast, treatment with C3 significantly improved the
15 number of sprouting ECs in the developing CVP and the proportion of vISVs in *sorbs1*
16 mutants (Figure 5H,I). Similarly, RhoA inhibitor injection improved lymphangiogenesis
17 in *sorbs1*^{-/-} embryos as PL formation was significantly increased (Figure 5J). In
18 agreement with our hypothesis, these observations altogether demonstrate that the
19 PCV sprouting defects associated with *sorbs1* deficiency are in part mediated by RhoA
20 hyperactivation.

Discussion

Although previous studies have described inactivation of SoHo family members in various animal models^{58–61} the authors did not specifically examine blood vessels and no references were made to a potential vascular phenotype. Here, using the zebrafish model, we provide the first line of *in vivo* evidence that Sorbs1 is crucial for developmental angiogenesis and lymphangiogenesis in vertebrates. We show that *sorbs1* mutant embryos exhibit specific defects in the lymphatic and venous trunk networks that correlate with edema development and impact larvae survival. The endothelial function of Sorbs1 appeared to be cell autonomous and conserved throughout vertebrates as the defects in the zebrafish vasculature could be rescued by endothelial re-expression of the human ortholog. During our investigations we found enrichment of Sorbs1 in the zebrafish endothelial compartment. However, Sorbs1 is a ubiquitous protein that interacts with multiple promiscuous cytoskeleton components and is present in actin-based structures of several cell types^{35,39}. Complementary to the endothelium, we do not exclude that additional cellular compartments might be affected in *sorbs1* knock-out embryos. Further studies would therefore be needed to better understand how these proteins might be regulated in a cell type- and/or physiological environment-specific manner.

The vertebrate vasculature is established through temporally and spatially defined angiogenic waves, during which new angiogenic structures grow out from pre-existing vessels. These new vessels do not always maintain the lymphatic, venous or arterial identity of the parental vessel. In that aspect, the process of trunk secondary sprouting from the PCV is particularly illustrative. Indeed, neighboring ECs within the PCV sprout simultaneously towards the dorsal plate. However, these sprouts rapidly diverge and develop into two differently fated structures: the intermediate pool of midline PLs that later give rise to the trunk lymphatic system and the vessel connections between the PCV and the ISVs that establish the venous intersomitic network. Induction of endothelial Prox1a expression (and therefore presumably transcriptional re-programing) in some ECs from the PCV precedes secondary sprouting and correlates with their lymphatic fate^{7,9}. Yet, recent data suggest that this specification could rather rely, at least partially, on a Notch-driven heterogeneity preexisting in the primary aISVs that are approached by the venous sprouts: aISV-forming ECs differ in their polarity and mobility before secondary sprouting, impacting

1 on ISV/PCV/DA connection outcome¹⁸. In contrast, high temporal resolution imaging
2 revealed that secondary sprouts of both fates display very similar behavior early during
3 migration, with most lymphatic sprouts connecting transiently to the aISVs before
4 assembling into PLs¹⁸. In agreement with the idea that egression of lymphatic and
5 venous sprouts from the PCV share common signaling pathways and downstream
6 intracellular mechanisms, failure in PL formation is often associated with impaired
7 arterio-venous ISV patterning^{22,27,31,32,45,62}. Our data show that lack of *Sorbs1* has no
8 obvious effect on *Prox1a* specification. However, it severely impairs secondary
9 sprouting capacities of ECs from both venous and lymphatic fates, which would
10 position *Sorbs1* as part of the cellular machineries required for the early
11 morphogenetic events underlying EC secondary sprouting from the PCV.

12 Migration of these secondary lympho-venous sprouts has been shown to rely
13 on *Vegfc* signaling^{22,27,47}. Even though this master lymphangiogenic driver also
14 controls the earlier process of *Prox1a* induction^{22,63}, some *Vegfc* downstream effectors
15 impact LECs behavior without affecting *Prox1a* specification^{30,63}, suggesting that
16 *Vegfc* can use distinct routes to instruct different lymphangiogenic steps. *Sorbs1*
17 appears to regulate lymphangiogenesis independently of *Vegfc* signaling. Indeed,
18 *sorbs1*^{-/-} mutant embryos remained highly responsive to ectopic *Vegfc* induction.
19 Whereas this result does not exclude that *Sorbs1* could be involved in endogenous
20 *Vegfc* signaling during lymphangiogenesis, genetic interaction experiments
21 demonstrated that *Sorbs1* and *Vegfc* very likely act in distinct pathways. The
22 dichotomy between *Sorbs1* and *Vegfc* signaling is rather unique as other known
23 regulators of PLs and TD formation appear to mostly function within the *Vegfc*
24 pathway.

25 Examination of other bed specific angiogenic processes gave us additional
26 insights about potential signaling pathways in which *Sorbs1* could function during
27 blood and lymphatic vessel formation. Although we did not perform systematic
28 analysis, head vascularization, which displays strong organotypic signatures including
29 during facial lymphangiogenesis^{8,64}, appeared unaffected in *sorbs1*^{-/-} mutants, with the
30 absence of periorbital edema⁶⁵. In contrast, we observed defects in the development
31 of the CVP and the SIVP networks, two structures originating from ventral migration
32 of ECs out of the axial vein. Since initiation of CVP formation¹⁹ and SIVP
33 outgrowth^{20,21}, both of which are affected in *Sorbs1* mutants/morphants, specifically
34 rely on BMP signaling, we tested and observed a clear involvement of *Sorbs1* in BMP-

1 induced ectopic sprouting from the CVP. Does that imply that the defects related to
2 sprouting events from the PCV exhibited by *sorbs1*^{-/-} embryos, including lympho-
3 venous dorsal migration, are BMP-dependent? Photo-conversion experiments have
4 revealed that the same population of progenitor cells located within the ventral side of
5 the PCV that generates lymphatic PLs also migrates rostrally to be incorporated into
6 the SIVP⁹. Although PL and SIVP formations occur in opposite direction, suggesting
7 distinct cues and signaling pathways, BMP-related transcriptional activity has been
8 described in lymphatic sprouts budding from the cardinal vein during mouse embryonic
9 development⁶⁶. Apart from a morpholino-based study suggesting a role for type II BMP
10 receptors in PL formation⁶⁷, the potential involvement of BMP signaling during the
11 early steps of lymphatic network formation has never been precisely characterized
12 beyond LEC specification^{68,69}. In the light of our findings, it would be interesting to
13 investigate this possibility and test the role of BMP signaling in the lymphatic defects
14 observed in *sorbs1* mutant embryos.

15 The context-specific phenotypes in the vasculature of *sorbs1* mutants are highly
16 remarkable and suggest that this cytoskeleton-associated protein participates in
17 establishing endothelial cell specificities required throughout PCV-derived secondary
18 venous and lymphatic beds. ECs might use different cellular and molecular
19 mechanisms to establish organ-specific vasculature. For instance, extension of
20 filopodia is crucial to the EC "sheet-like" migration during CVP morphogenesis but is
21 dispensable for the "phalanx-like" migration during ISV development^{70,71}. Lympho-
22 venous sprouting and CVP formation are particularly sensitive to microtubule
23 cytoskeleton-associated polarity⁷². Disconnection of the leading ECs from the original
24 vessel during SIVP formation is not observed during formation of ISV and CVP and
25 could suggest distinct mechanisms⁷³. Using cell culture experiments, we showed that
26 *Sorbs1*^{KD} ECs display altered adhesion dynamics and migration. Interestingly, the
27 CVP phenotype in *sorbs1* mutants is strikingly similar to that of zebrafish embryos
28 lacking various components of the ECM such as fibrillins^{74,75}. Additionally, zebrafish
29 mutant for Polydom/svep1, a large protein involved in cell adhesion to the extracellular
30 matrix, failed to form PL and TD lymphatic structures due to sprouting impairment of
31 properly specified LECs^{32,76}. This raises the intriguing possibility that venous plexus
32 and lymphatic network formation might be particularly sensitive to alterations of
33 integrin-mediated cell-ECM adhesions.

1 Our study not only provides an important cellular and developmental *in vivo*
2 context for cytoskeleton regulation by Sorbs1, but it also discloses underlying
3 mechanistic aspects. Indeed, we demonstrate that Sorbs1 acts upstream of
4 RhoGTPases to control EC actomyosin cytoskeleton and migratory behavior. Prior to
5 this work, only few studies had alluded to potential connections between SoHo
6 proteins and RhoGTPases signaling^{50,51,77,78}. Although extension of filopodia and
7 migration of leading ECs during BMP-induced CVP morphogenesis was shown to be
8 dependent on the Cdc42 RhoGTPase⁷¹, we found that Cdc42 activity was not affected
9 in the absence of Sorbs1 in ECs. In contrast, we show that Sorbs1 controls the RhoA-
10 Rac1 balance, through the FAK-Src pathway. Integrin-mediated activation of the FAK-
11 Src complex during cell spreading and migration stimulates Rac1 activity and
12 maturation of focal complexes into stable adhesions⁷⁹. Consistent with the idea that it
13 participates in FAK-Src activation, Sorbs1 protein levels are highly and transiently
14 induced following integrin engagement onto fibronectin. FAK and Src also control
15 phosphorylation of p190RhoGAP and in addition to Rac1 activation, Sorbs1 might
16 affect focal adhesion turnover through repression of RhoA activity^{80,81}. Together with
17 the well-described antagonistic regulation of Rac1 and RhoA, these findings would be
18 consistent with the reciprocal increase in RhoA and decrease in Rac1 activities that
19 we observed in Sorbs1-depleted ECs. On this particular matter, it is noteworthy that
20 we were unable to correlate the higher RhoA activity following depletion of Sorbs1 with
21 increased stress fiber formation, cellular contractility or overall ROCK1/MLCK activity.
22 Instead, we observed a rather spatially restricted effect, with Sorbs1-depleted cells
23 exhibiting denser peripheral bundles of actin filaments. Interestingly, asynchronous
24 activation of Rac1 and RhoA activities at the cell edge is essential for membrane
25 protrusions formation and motility of non-endothelial cells⁸². Based on our
26 observations it is tempting to speculate that Sorbs1 might contribute to the spatial
27 coordination of RhoA and Rac1 activities within migrating ECs during vascular network
28 expansion. In the absence of Sorbs1, local increase in RhoA and decrease in Rac1
29 activities would be expected to reduce lamellipodia dynamics and membrane
30 protrusive activity, thus impairing EC migration. Importantly, we show that defects in
31 PCV secondary dorsal and ventral sprouting associated with Sorbs1 knockout can be
32 partially rescued by a RhoA inhibitor, indicating that RhoA activation is causal in the
33 vascular phenotype of *sorbs1*^{-/-} embryos and suggesting that Sorbs1 affects common
34 endothelial cell properties during these processes. How RhoA regulation by Sorbs1 is

1 integrated in the signaling pathways governing these processes is still an open
 2 question but it is worth noting that while Erk activation has been described to
 3 participate in LEC migration⁴⁷ and CVP formation¹⁹, adhesion-triggered
 4 phosphorylation of Erk is reduced in *Sorbs1*^{KD} ECs.

5 In summary, the results reported here indicate that the *Sorbs1* protein
 6 participates in key molecular pathways driving stage- or context-specific regulation of
 7 EC morphogenic properties during vascular development. More specifically, we
 8 identify *Sorbs1* as a novel genetic regulator of developmental lymphangiogenesis that
 9 functions independently of *Vegfc* signaling. Better understanding of these pathways
 10 and identification of novel actors provide new opportunities that can be exploited for
 11 vascular normalization strategies in various diseases.

12

1 **Materials and Methods**

2

3 *Cell Culture and transfection*

4 Human Umbilical Vein Endothelial Cells (HUVECs), Human Dermal Microvascular
5 Endothelial Cells (HDMECs), Human Mammary Epithelial Cells (HMECs), Human
6 Umbilical Artery Endothelial Cells (HUAEC), Human embryonic kidney 293 cells (HEK
7 293), Human Dermal Lymphatic Microvascular Endothelial Cells (HMVEC-dLyAd) and
8 HeLa cells were obtained from Lonza. All functional assays were performed with
9 HUVECS which were grown at 37°C in endothelial basal medium (EBM)
10 supplemented with hydrocortisone (1 µg/ml), bovine brain extract (12 µg/ml),
11 gentamicin (50 µg/ml), amphotericin B (50 ng/ml) epidermal growth factor (10 ng/ml)
12 (Lonza) and 10% Fetal Bovine serum (FBS, Perbio). Transfections of siRNA were
13 performed using the GeneTrans II (MoBiTec) reagent according to the manufacturer's
14 protocols.

15 Except for scratch-wound assays, all functional assays were performed on
16 fibronectin-plated HUVECs. Briefly, cells were harvested, left 30 min in suspension to
17 recover from trypsinisation and seeded onto fibronectin-coated dishes for 30 min.

18

19 *Migration assays*

20 Migration assays were performed as described [ref 30]. Briefly, for the scratch assay
21 a confluent HUVEC monolayer was wounded 48hrs after siRNA transfection, using a
22 sterile P200 tip to create a cell-free zone. For each wound, two different fields were
23 photographed just after injury (t = 0 h) and 16 h later. Quantification of cell migration
24 was made by measuring the percentage of area recovery using ImageJ software in 12
25 fields from 3 independent experiments.

26

27 *Adhesion assay*

28 Adhesion assays were performed essentially as described [ref 30] with slight
29 modifications. Forty-eight hours after transfection, HUVECs were seeded on
30 fibronectin precoated-wells for 30 min. After extensive washing with PBS, remaining
31 cells were stained with cristal violet. The dye was released by cell permeabilization
32 and directly proportional to the number of cells, dye concentration was measured by
33 reading absorbance at 560nm.

34

Proliferation assays

Forty-eight hours post transfection with siRNA, a colorimetric MTS assay was performed on HUVECs following the manufacturer's protocol (CellTiter 96 AQueous One Solution Cell Proliferation Assay, Promega) in 96 wells. Alternatively, semi-automatic cell counting assessment of proliferation was performed. Briefly, 24h post-transfection with siRNA, cells were seeded at 20000 cells/well in 24-well plates in triplicate. Cells were counted using the Scepter 2.0 Handheld Automated Cell Counter (Millipore) over a 2-day period and proliferation curves were generated by plotting the average cell number over time.

Immunohistochemistry

AccuMax Array (A301 VI) slides were stained with goat anti-human Sorbs1 (Abcam, ab4551) antibody. Slides were incubated overnight in optimized dilutions of primary antibodies in Antibody Diluent (Dako, S2022). Peroxidase-conjugated anti-goat Ig (Vector) was then added for 1 hour. Revelation was performed using diaminobenzidine (DAB) according to standard protocols. Images were acquired by using a FSX100 microscope (Olympus).

Immunofluorescence

For immunofluorescence experiments, HUVECs were seeded onto fibronectin coated coverslips 48 h after siRNA transfection. Cells were fixed after 30 min in 4% paraformaldehyde, permeabilized with PBS-TritonX 0.1% and incubated overnight with the appropriate primary antibody dilutions in PBS-BSA 4%. Cells were then incubated with appropriate secondary antibody dilutions for 1 h. After washing, cells were mounted with Mowiol (Sigma) and processed for immunofluorescence using a confocal Nikon A1R.

Zebrafish

Adult fish and embryos were carried on according to EU regulations on laboratory animals. All animal experiments were approved by the animal welfare committee of the University of Liège and the Université libre de Bruxelles (ULB). The zebrafish lines used in this study were: *Tg(fli1a:eGFP)^{y183}*, *Tg(hsp70l:bmp2b)⁸⁴*, *TgBAC(prox1a:KaTA4-4xUAS-ADV.E1b:TagRFP)^{nim85,86}*, *vegfcchu5055²⁹*.

1

2 *Generation of knockout lines using CRISPR/Cas9 system*

3 Cas9 mRNA and guide RNAs (gRNAs) were synthesized as described in Jao et al.⁸⁷.
 4 Briefly, the Cas9 mRNA was synthesized by *in vitro* transcription using the T3
 5 mMESSAGEMACHINE Kit (#AM1348, Ambion). The primers for the generation of
 6 DNA templates of gRNAs were designed through the CHOPCHOP software, and a T7
 7 promoter sequence was added to the 5'-upstream of the gRNA sequence. The gRNA
 8 was digested by BamHI and then submitted to *in vitro* transcription using
 9 MEGashortscriptT7 kit (#AM1354, Ambion). The size and quality of the capped mRNA
 10 and gRNA were confirmed by electrophoresis through a 2% (w/v) agarose gel. After
 11 this, 300ng/μl of Cas9 mRNA and 100 ng/μl of gRNA were co-injected into one cell-
 12 stage zebrafish embryos. Embryos were derived from the transgenic line
 13 *Tg(fli1a:eGFP)y1* cross. The injected embryos were raised to adulthood. To test
 14 mutagenesis efficiency we genotyped the zebrafish by extracting the DNA from their
 15 fin (FIN-CLIP), followed by PCR and heteroduplex melting annealing (HMA) gel. F0
 16 fish were crossed with *Tg(fli1a:eGFP)y1* fish to generate heterozygous F1 progeny,
 17 which were then genotyped by HMA gel and DNA sequencing. Heterozygous F1
 18 zebrafish were crossed with the aim to generate homozygous mutant fish.

19

20 *Morpholino injection*

21 One-cell stage *Tg(fli1a:eGFP)y1* embryos were injected with 5 ng of Sorbs1 splice-
 22 blocking (5'-TCCCCAAATGCTCTTCTTACCAGTA-3') and control morpholino (5'-
 23 CCTCTTACCTCAGTTACAATTTATA-3'). We performed rescue experiments by
 24 injecting RNA molecules (60ng/μl) from *in vitro* transcription reactions using linearized
 25 PCS2+ vector coding for human Sorbs1.

26

27 *RNA extraction and PCR amplification*

28 RNA was extracted from zebrafish embryos using Trizol reagent (Invitrogen) according
 29 to the manufacturer's protocol. RNA from HUVECs was prepared using the nucleospin
 30 RNA kit (Macherey Nagel). RNA integrity and concentration were assessed by
 31 spectrophotometry analysis (Nanodrop, Thermo Scientific). Reverse transcription
 32 reactions were done using the RevertAid H Minus First Strand cDNA Synthesis Kit
 33 (Fermentas) with random hexamer primers. The cDNA was then submitted to
 34 quantitative real time PCR using Sybrgreen technology (Eurogentec) on a Stepone

apparatus (Applied Biosystems) or to end-point PCR amplification followed by gel electrophoresis analysis.

Primers used for end-point PCR are: Zebrafish *sorbs1*: ATCATCGATGTGCACTAACGTG (Forward) and CTCCAGCAGAGGGCACAG (Reverse). Primers used for quantitative real-time PCR are: Zebrafish *sorbs1*: GCCAGGAAAGTCTTCAGTGC (Forward) and TCTGCTTCACCGTCACTCAC (Reverse); Zebrafish *prox1a*: TGTCATTTGCGCTCGCGCTG (Forward) and ACCGCAACCCGAAGACAGTG (Reverse). Zebrafish *elfa*: CTTCTCAGGCTGACTGTGC (Forward) and CCGCTAGCATTACCCTCC (Reverse). Primers used for mutagenesis efficiency analysis by PCR are: TGAGACTCCAGCAGACATGG (Forward) and ACAATTACAGCTGGAGAACTACA (Reverse).

Whole mount in situ hybridization

An antisense RNA DIG-probe was generated by transcription from linearized pCS1 vector containing *Sorbs1* coding sequence using SP6 RNA polymerase kit where UTPs were labelled with digoxigenin (DIG) (Roche, 11175025910).

Whole mount in situ hybridization was performed in 12, 24 and 48 hpf embryos and in 3dpf larvae. Every time point was fixed with paraformaldehyde 4% overnight at 40C and then dehydrated and rehydrated through methanol and PBS 1x (Gibco)-Tween 5% washes. Embryos were permeabilized with 10µg/mL of proteinase K and then re-fixed with paraformaldehyde 4%. Antisense probe hybridization was performed using 100 ng of *sorbs1*-DIG-probes hybridization buffer containing 5% dextran sulfate at 65 °C overnight. The use of a DIG alkaline phosphatase-conjugated antibody (Roche, 11093274910, dilution 1/3000), and its substrates BCIP and NBT, enabled the colorimetric detection of *sorbs1* transcript. Pictures were taken with an Olympus SZx10 stereomicroscope.

Phenotyping

Embryos were anesthetized with Tricaine 0,4% in order to perform phenotypical analysis. Analysis and pictures of overall zebrafish morphology and edemas were performed under a stereomicroscope. Analyses of zebrafish vasculature were performed under a fluorescent stereomicroscope, whereas confocal pictures were taken on live embryos embedded in low melting point agarose (0.8%) on a confocal

Nikon A1R. 3D color projections were done using the volume view-slices mode and the volume view-z depth blending functions of NIS-Element A1R1 Software. Lightsheet Zeiss Z1 was used in order to perform time-lapse video of the emerging secondary sprouts at 36 hpf from the trunk vasculature of zebrafish embryos from crossing *TgBAC(prox1a:Ka/TA4-4xUAS-ADV.E1b:TagRFP)^{nim5}* and *sorbs1^{-/-}* homozygous lines which were embedded in low melting point agarose (0.8%) with Tricaine 0.4%.

For rescue experiments with RhoA inhibitor, control, *sorbs1* mutants or morphants embryos were incubated with C3 Transferase RhoA inhibitor (#CT04-A, Cytoskeleton, Inc.) (1µg/mL) at 26 hpf before analyses of CVP structures at 28 hpf and of the proportion of aISV/vISV at 48 hpf. For PL development rescue, RhoA inhibitor (1 µM) was injected in the circulation of wild type and *sorbs1^{-/-}* at 28 hpf, and PLs were quantified with a fluorescent stereomicroscope at 54hpf.

For testing the interaction with *vegfc*, embryos from *sorbs1^{+/-}* and *vegfc^{-/-}* crosses were submitted to phenotyping before being genotyped by BsaI digestion (*vegfc*) or HRM (*sorbs1*). WT and *sorbs1^{-/-}* embryos were injected at the one-cell stage with 200 pg/embryo of human VEGFC plasmid (kindly provided by Dr. Schulte-Merker laboratory) before PL and TD quantification.

Antibodies and RNA interference (RNAi)

Anti-Sorbs1 was obtained from Abcam (#Ab4551). Anti-PAK4 (#3242), PAK2 (#2608), Src (#2123), ERK1/2 (#9102) and phosphorylated Src (#2101S), paxillin (#2541S) and ERK1/2 (#9101) were purchased from Cell signaling. Anti-paxillin (610051), FAK (#ab72140) and its phosphorylated form (# 44-624G) were from Biosciences, Abcam and Invitrogen, respectively. Non-targeting control siRNA and siRNA duplexes targeting Sorbs1 (5'-UUAAGUCCUGAGUGCUCUUC-3') were synthesized and purchased from Eurogentec.

Rho GTPase pull down activity assay

SiRNA-treated HUVECs were cultivated for 30 minutes on fibronectin. After harvesting, total cellular active RhoA levels were measured using the Rho Activity Assay (Cytoskeleton Inc., BK036) following the manufacturer's guidelines. In short, cell lysate (approximately 500µg of total protein) was incubated for 1 hour at 4 °C with GST-Rhotekin beads. Bound activated RhoA was eluted from the beads and analyzed

by western blotting using a RhoA antibody. To measure the levels of active Rac1 and Cdc42 were measured using the Rac1 and CDC42 Activity Assay (Cytoskeleton Inc., BK035 and BK034, respectively), cells were lysed in a buffer containing 5 mM DTT, 50 mM Tris pH 7.2, 1% tritonx-100 (10%), 0,5% deoxycholate (20%), 0,1% SDS (20%) and 500 mM NaCl 5M. Extracts were then incubated 1h at 4°C with GST-PAK beads. Bound activated Rac1 and Cdc42 were eluted from the beads and analyzed by western blotting using dedicated antibodies.

Statistical Analysis

Unless stated otherwise, experiments were performed at least three times independently and graphs represent means +/- standard deviation. Normality tests were performed and when the data were considered normal, statistical analysis were performed by two tailed Student's t-test or a Pearson's chi-squared test. Mann-Whitney U and Wilcoxon rank-sum test were used otherwise.

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- 19

Figure Legends

Figure 1: *Sorbs1* expression is enriched in the endothelium and its knockout in zebrafish results in cardiac edemas

(A-B) Transmitted light images of live wild-type (WT) and *sorbs1* mutants (*sorbs1*^{-/-}) zebrafish embryos at 5 days post-fertilization (dpf) (A) and quantification of the percentage of embryos displaying edemas (B). (n=number of embryos; *** $P < 0.001$; Fischer exact test). The arrow indicates an example of edema observed in *sorbs1*^{-/-} embryo. Scale bar represents 250 μ m.

(C) Quantification of the percentage of survival for *sorbs1*^{-/-} embryos presenting or not edemas from 4 to 10 dpf. (n=24 and n=23, respectively).

(D-E) RT-qPCR analysis of *sorbs1* expression relative to Elfa at 48hpf in endothelial cells (ECs, GFP+) vs non-endothelial cells (non-ECs, GFP-) sorted from WT *Tg(fli1a:eGFP)y1* embryos by FACS (Fluorescence Activated Cell Sorting) technology (D). Relative endothelial expression of *Sorbs1* was quantified at different time points of embryonic development (E). (* $P < 0.05$, Unpaired t-test).

Figure 2: *Sorbs1* is necessary for trunk lymphangiogenesis *in vivo*

(A-B) Confocal microscopy analysis of the trunk vasculature in WT and *sorbs1*^{-/-} *Tg(fli1a:eGFP)y1* embryos at 54 hpf (A) used to quantify the number of paracordal lymphangioblasts (PLs) (white arrows in A) over 10 somite segments (B). Dorsal aorta (DA), post cardinal vein (PCV), intersegmental vessels (ISV) or dorsal longitudinal anastomotic vessels (DLAV). (n=number of embryos; * $P < 0.05$; Mann-Whitney *U*-test). Scale bars represent 50 μ m.

(C) Z-maximum projections of confocal images of the trunk vasculature from 4 dpf *Tg(fli1a:eGFP)y1* WT or *sorbs1* knock-out embryos. Schematic representations of arterial (red), venous (light blue) and lymphatic (green) vessels are showed below the confocal pictures. Dorsal aorta (DA), Posterior Cardinal Vein (PCV), Thoracic duct (TD). Scale bars represent 50 μ m.

(D) Quantification of the thoracic duct (TD) extent over 10 segments at 4 and 6 dpf in WT and *sorbs1*^{-/-} embryos. (n=number of embryos; *** $P < 0.001$; Mann-Whitney *U*-test).

(E) Analysis of *sorbs1*^{-/-} embryo survival over time in relation to their TD defects.

(F-G) Z-maximum projections of confocal images of the trunk vasculature of 54 hpf *Tg(fli1a:eGFP)y1 sorbs1* knock-out embryos expressing transgenic endothelial constructs coding for human Sorbs1 or not (F) and quantification of the number of PLs in the indicated condition (G). BFP is used as transgenesis marker. (n=number of embryos, ns=non-significant; ** $P<0.01$; Mann–Whitney *U*-test). Scale bars represent 50 μ m.

Figure 3: Sorbs1 functions independently of Vegfc signaling during *in vivo* lymphangiogenesis.

(A) RT-qPCR analysis of *prox1a* relative expression at 48hpf in endothelial cells (ECs) sorted from WT and *sorbs1*^{-/-} *Tg(fli1a:eGFP)y1* embryos by FACS (Fluorescence Activated Cell Sorting) technology. (ns= non-significant, Unpaired t-test). Results are means from 5 experiments.

(B) Frames (Z-maximum projections) from time-lapse lightsheet imaging of *prox1* expressing ECs sprouting from the PCV in WT and *sorbs1*^{-/-} *TgBAC(prox1a:KalTA4-4xUAS-ADV.E1b:TagRFP)*^{nim5} embryos. White arrows point to Prox1-positive ECs that sprouted to form PLs in WT but failed to migrate dorsally in *sorbs1*^{-/-}. Scale bars represent 25 μ m.

(C) Quantification of PL extent within the trunk region of 54 hpf *Tg(fli1a:eGFP)y1* embryos from the indicated genotype resulting from the incross of *sorbs1*^{+/-} with *vegfc*^{+/-} embryos (n= number of embryos; ns=non-significant; Mann–Whitney *U*-test).

(D) Quantification of the trunk PL (54 hpf) in WT or *sorbs1*^{-/-} *Tg(fli1a:eGFP)y1* embryos injected or not with human Vegfc. (n= number of embryos; ** $P<0.01$; Mann–Whitney *U*-test).

Figure 4: Sorbs1 depletion results in defects in secondary sprouting from the PCV.

(A) Confocal image (top) and schematic drawing (bottom) of secondary sprouts (arrow) emerging from the PCV in *Tg(fli1a:eGFP)y1* embryos at 34 hpf . The DA and the primary ISVs are in red and the PCV is in blue. Scale bars represent 25 μ m.

(B) Quantification of secondary sprouts visible at 34 hpf in WT and *sorbs1* mutants (*sorbs1*^{-/-}). (n=number of embryos, ** $P<0.01$; Mann–Whitney *U*-test).

1 (C-D) Color-coded Z-maximum projections of confocal images of the trunk regions of
 2 *Tg(fli1a:eGFP)y1* WT embryos and its schematic representation (C). The depth-
 3 associated color scale (warm colors = deep, cold colors = surface) allowed distinction
 4 between vISVs and aISVs to quantify their proportion at 48 hpf in a 10 somites trunk
 5 region of WT or *sorbs1*^{-/-} embryos. (n= number of embryos, *** $P < 0.001$; χ^2 with Yates
 6 correction). Dorsal aorta (DA), light post cardinal vein (PCV), arterial Intersegmental
 7 vessel (aISV) venous intersegmental vessel (vISV). Scale bars represent 50 μ m.
 8 (E) Confocal image representation as described in C of 54hpf *Tg(hsp70l:bmp2b*;
 9 *fli1a:eGFP)* heat-shocked embryos that were heat-shocked at 26 hpf used to illustrate
 10 formation of ectopic vessels (EVs, indicated with dotted lines) (A). Scale bars
 11 represent 50 μ m.
 12 (F) Quantification of ectopic vessel (EV) growing from the PCV at 28 hpf in
 13 *Tg(hsp70l:bmp2b; fli1a:eGFP)* embryos injected with Ctl or *sorbs1* Mo before (-) or
 14 after (+) a heat-shock treatment at 26hpf. (n=number of embryos, ** $P < 0.01$, ns=non-
 15 significant, χ^2 pairwise proportion test with Holm correction).

16
 17 **Figure 5: Sorbs1 controls EC adhesive properties via RhoGTPases *in vitro* and**
 18 ***in vivo***

19 (A) Cdc42, Rac1 and RhoA activity in HUVECs transfected with control (Ctl) or *Sorbs1*
 20 siRNA. Histogram is from Western blot densitometric analysis of three independent
 21 experiments and represent the ratio between bound active- and total amount of each
 22 RhoGTPase in the lysate, relative to control cells. (** $P < 0.01$, * $P < 0.05$, ns=non-
 23 significant, Student's t test).
 24 (B) Confocal pictures of peripheral F-Actin (phalloidin staining) in Ctl or *Sorbs1* siRNA
 25 transfected HUVECS treated (+) or not (-) with the C3 RhoA inhibitor. Images are
 26 shown using an intensity-based color look-up table (bottom, from blue = low to red =
 27 high). Scale bars represent 25 μ m.
 28 (C) Quantification of the relative peripheral F-actin signal calculated based on images
 29 acquired as in (B) (n=48, 51 and 31 cells; *** $P < 0.001$, Student's t test).
 30 (D-E) Adhesion complexes were analyzed by confocal microscopy (D) after
 31 immunostaining of paxillin and phosphor-paxillin in HUVECs transfected with control
 32 or *Sorbs1* siRNA. Scale bars are 10 μ m. Nascent adhesions (NA) and focal complexes
 33 (Fx) are identified (solid arrows) by their small size, peripheral location and high p-
 34 Paxillin/Paxillin ratio content. Larger and more mature focal adhesion (FA, dashed

1 arrows) were defined as bigger than $1\mu\text{m}^2$ and their proportion in each condition was
2 quantified (E). (n=21; * $P < 0.05$, Student's t test).
3 (F-G) Representative micrographs (F) and quantification (G) of adhesion assays
4 performed with HUVECs transfected with control siRNA or with siRNA against *Sorbs1*
5 as described in the method section. Scale bars represent 100 μm . (n=3 independent
6 experiments; ** $P < 0.01$, Student's t test).
7 (H,I) WT and *sorbs1*^{-/-} embryos were treated (+) or not (-) with RhoA inhibitor at 26 (H)
8 or 28 (I) hpf and the number of sprouting cells at the edge of developing CVP at 28
9 hpf (H) or the percentage of aISV/vISV at 48 hpf (I) were quantified (n=number of
10 embryos; *** $P < 0.001$, * $P < 0.05$; ns=non-significant; Mann–Whitney U-test (H) ; χ^2
11 with Yates correction(I)).
12 (J) Quantification of the number of PLs in 10 somites at 54hpf in WT and *sorbs1*^{-/-}
13 embryos injected with RhoA inhibitor or left untreated (n=number of embryos;
14 *** $P < 0.001$, * $P < 0.05$; ns=non-significant; Mann–Whitney U-test).
15

Supplemental Figure Legends

Supplemental Figure 1: Sorbs1 expression in endothelial cells *in vitro* and *in vivo* and verification of its depletion in the zebrafish model.

(A) Phylogenetic tree was constructed from multiple human, mouse and potential zebrafish mRNA of *Sorbs1*, *Sorbs2* and *Sorbs3* with ClustalW software. One zebrafish ortholog was identified for *sorbs1* (z*Sorbs1*) and *sorbs3* (z*Sorbs3*) and two for *sorbs2* (z*Sorbs2a* and z*Sorbs2b*).

(B) DNA and amino-acid sequences of the wild-type (WT) and 14-bp deletion (-14) in *sorbs1* alleles following CRISPR/ Cas9-based editing.

(C) Western blotting analysis of protein extracts from wild-type (WT) and *Sorbs1* mutant (*sorbs1*^{-/-}) embryos, using anti-Sorbs1 antibody. GAPDH was used as loading control.

(D) Quantification of heart beats per minute of wild-type (WT) and *sorbs1*^{-/-} homozygote embryos at 2dpf. Depletion of *Sorbs1* has no effect on the heartbeat compared to wild-type embryos (n= number of embryos, ns= non-significant, Mann–Whitney U-test).

(E) RT-PCR analysis on total RNA from embryos injected with control morpholino (Ctl Mo) or with a splice-blocking morpholino against *sorbs1* (*sorbs1* Mo).

(F) Western blotting analysis of total protein extracts from 48hpf embryos injected with control (Ctl) and *sorbs1* ATG-blocking Morpholino, using an anti-Sorbs1 antibody. Actin was used as loading control.

(G) Quantification of the percentage of edemas-bearing embryos observed at 2 dpf in embryos injected with control or *sorbs1* morpholino, together or not with human *Sorbs1* mRNA. (n= number of embryos, *** $P < 0.001$, ns= non-significant; Fisher exact test).

(H) Expression of *Sorbs1* in various human tissues assessed by immunohistochemistry. Typical *Sorbs1* staining in endothelial cells is illustrated for the indicated tissues. Boxes correspond to the enlarged area showing expression of *Sorbs1* in blood vessels (arrows). Scale bars represent 30 μ m and 100 μ m respectively in large and zoomed picture.

(I) Western blotting analysis of *Sorbs1* expression in various human endothelial cells: HDMECs (Human Dermal Microvascular Endothelial Cells), HMECs (Human

1 Mammary Epithelial Cells), HUAECs (Human Umbilical Artery Endothelial Cells),
 2 HUVECs (Human Umbilical Endothelial Cells), HMVEC-dLyAd (Human Dermal
 3 Lymphatic Microvascular Endothelial Cells), HEK293 (Human Embryonic Kidney
 4 293) and Hela cells. HSP90 was used as a loading control.

5 (J) Whole mount in situ hybridization using a digoxigenin-labeled antisense *sorbs1*
 6 probe at different time points: 12 somites, 24, 48 and 72 hpf. Scale bars represent
 7 100 μ m.

8

9 **Supplemental Figure 2: Lack of *Sorbs1* expression does not affect cranial**
 10 **vasculature pattern**

11

12 (A) Maximal intensity projection of a confocal z-stack of the cranial vasculature of
 13 *Tg(fli1a:eGFP)y1* wild-type (WT) and *sorbs1* mutant (*sorbs1*^{-/-}) embryos at 60 hpf in
 14 dorsal views (anterior to the left) and wire diagram of the brain vasculature in dorso-
 15 lateral view. Red vessels in the 3D renderings represent the intra-cerebral central
 16 arteries (CtAs) and gray vessels represent the perineural vessels (primordial hindbrain
 17 channels: PHBC and basilar artery: BA).

18 (B) Quantification of the corresponding hindbrain CtAs in 8 wild-type (WT), 24 *sorbs1*
 19 heterozygous (*sorbs1*^{+/-}) and 7 *sorbs1* homozygous (*sorbs1*^{-/-}) embryos at 60 hpf.
 20 Error bars represent median $\pm$ interquartile range; (n= number of embryos; ns= non-
 21 significant, Kruskal–Wallis test).

22 (C) Confocal pictures of the trunk vasculature show no gross vascular defects at 48 hpf in
 23 *sorbs1* morphant and Ctl embryos. Defects in PL formation were detected in *sorbs1*
 24 morphants (bottom) compared to Ctl embryos (top, white arrowheads). DA: Dorsal
 25 Aorta; PCV: Posterior Cardinal Vein; DLAV: Dorsal Longitudinal Anastomic Vessels;
 26 ISV: Intersegmental Vessels. Scale bars represent 50 μ m.

27 (D) Quantification of PLs was performed at 48 hpf between 10 somites in Ctl and *sorbs1*
 28 morphant embryos as imaged in (C) (n=number of embryos; **P*<0.05; two-tailed
 29 Mann–Whitney *U*-test).

30

31 **Supplemental Figure 3: *Sorbs1* depletion does not affect *pro1xa* expression in**
 32 **endothelial cells.**

(A) Quantification of the trunk TD extent (4dpf) in WT or *sorbs1*^{-/-} *Tg(fli1a:eGFP)y1* embryos injected or not with human Vegfc. (n= number of embryos; ***P*<0.01; Mann–Whitney *U*-test).

Supplemental Figure 4: *Sorbs1* depletion leads to defects in secondary sprouting from the PCV.

(A) Quantification of secondary sprouts in control and *sorbs1*-morphant embryos at 36 hpf was established using the five indicated categories. (n = number of embryos ; **P*<0.05; two-tailed Mann–Whitney *U*-test).

(B) Percentages of vISVs and aISVs were quantified at 48 hpf in a 10 somite region in the trunk of embryos injected with control or *sorbs1* morpholino. (n= number of embryos; **P*< 0.01; χ^2 without Yates correction).

(C) Schematic representation of arterial (red) and venous (blue) circulation in the zebrafish embryo. Blue arrows indicate the direction of endothelial cell migration during the formation of PCV-derived angiogenic structures. vISVs: venous Intersegmental vessels; CVP: caudal vein plexus, SIVP: subintestinal venous plexus

(D) Confocal imaging of CVP tip cells (white arrows) from 28 hpf wild-type and *sorbs1*^{-/-} embryos. Scale bars represent 40 μ m.

(E) Quantification of tip cell numbers were performed at 28hpf in control and mutant *sorbs1* embryos, as well as in embryos injected with control or *sorbs1* Mo (n= number of embryos; * *P*< 0.01; ****P*<0.001; two-tailed Mann–Whitney *U*-test).

(F) Confocal pictures of subintestinal plexus of three different phenotypes encountered in WT (first picture) and *sorbs1*^{-/-} (second and third picture) *Tg(fli1a:eGFP)y1* embryos taken at 80 hpf. Scale bars represent 40 μ m.

(G) Quantification of SIV phenotypes as illustrated in F (n=number of embryos, * *P*<0.05; two-tailed Mann–Whitney *U*-test).

Supplemental Figure 5: *Sorbs1* deletion affects migratory and adhesive properties of ECs

- 1(A) HUVECs were transfected with siRNA targeting *Sorbs1* or with a control siRNA.
2 Efficiency of RNA silencing was assessed by qRT-PCR 48h after transfection. GAPDH
3 was used as internal control.
- 4(B) *Sorbs1* protein levels were examined in cells described in (A) by Western blotting
5 analysis using *Sorbs1* specific antibody. Actin was used as loading control.
- 6(C) HUVECs were transfected as in (A). Cell viability relative to control was assessed
7 using an MTS assay, as described in the method section. Results are mean $\pm$ SD of
8 3 independent experiments, each performed in triplicate (ns=non-significant, Student's
9 t test).
- 10(D) HUVECs were transfected as in (A), harvested 24 h after transfection and plated in
11 triplicate at a defined density. Cell number was then assessed by semi-automatic
12 counting (see the method section) at 48 h and 72 h after transfection. Results are
13 presented as the average $\pm$ SD increase in cell number, from 3 independent
14 experiments (ns= non-significant, Student's t test).
- 15(E) Micrographs representing HUVECs transfected with control siRNA or with siRNA
16 against *Sorbs1* submitted to a scratch-wound assay.
- 17(F) Quantification of the scratch-wound assay as described in E. Histogram represent
18 mean $\pm$ sd of 3 independent experiments (*: $P < 0.05$, Student's t test).
- 19(G) Rac1 activity in HUVECs transfected with control (Ctl) or *Sorbs1* siRNA. Rac1
20 activation was assessed by Western blot analysis of PAK2 and PAK4 phosphorylation
21 using phospho-specific antibodies. Total amounts of PAK2 and PAK4 were used as
22 loading controls.
- 23(H) Co-localization of *Sorbs1* and adhesion complexes was analyzed in HUVECs by
24 confocal microscopy using antibodies specific to paxillin and *sorbs1*.
- 25(I) The size distribution of adhesions was established from HUVECs (n=21) visualized as
26 in Figure 5D. Adhesions were classified into two categories based on their size: [0.2-
27 0.4 μm^2], [0.4-1 μm^2] (NAs + FX). (*: $P < 0.05$, ns= non-significant, Student's t test).
- 28(J) Phosphorylation of Paxillin was assessed in Ctl and *Sorbs1*-depleted cells using a
29 phospho-specific antibody. Total amount of Paxillin was used as loading control.
- 30(K) HUVECs were seeded onto fibronectin for the indicated times and the expression of
31 *Sorbs1* was analyzed by Western blotting with a specific antibody. Actin levels were
32 used as loading control.

(L) FAK-Src-ERK signaling was assessed in Ctl and *Sorbs1*-depleted cells. Activated FAK
and ERK and inactivated Src were detected using phospho-specific antibodies. Total
amounts of the corresponding proteins were used as loading controls.

4

Figure 1

Figure 2

A

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B

C

D

Figure 3

A

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B **C** **D** **E** **F**

Figure 4

Figure 5

A **B** **C** **D** **E** **F** **G** **H** **J** **I**

A

Supplementary Figure 4

Supplementary Figure 5