Fbxw7 Controls Angiogenesis by Regulating Endothelial Notch Activity

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Abstract

Notch signaling controls fundamental aspects of angiogenic blood vessel growth including the selection of sprouting tip cells, endothelial proliferation and arterial differentiation. The E3 ubiquitin ligase Fbxw7 is part of the SCF protein complex responsible for the polyubiquitination and thereby proteosomal degradation of substrates such as Notch, c-Myc and c-Jun. Here, we show that Fbxw7 is a critical regulator of angiogenesis in the mouse retina and the zebrafish embryonic trunk, which we attribute to its role in the degradation of active Notch. Growth of retinal blood vessel was impaireed and the Notch ligand Dll4, which is also a Notch target, upregulated in inducible and endothelial cell-specific Fbxw7flox/C223EKO mutant mice. The stability of the cleaved and active Notch intracellular domain was increased after siRNA knockdown of the E3 ligase in cultured human endothelial cells. Injection of fbxw7 morpholinos interfered with the sprouting of zebrafish intersegmental vessels (ISVs). Arguing strongly that Notch and not other Fbxw7 substrates are primarily responsible for these phenotypes, the genetic inactivation of Notch pathway components reversed the impaired ISV growth in the zebrafish embryo as well as sprouting and proliferation in the mouse retina. Our findings establish that Fbxw7 is a potent positive regulator of angiogenesis that limits the activity of Notch in the endothelium of the growing vasculature.

Introduction

The Notch pathway is a versatile signaling system that controls functions as diverse as cell proliferation, differentiation and tissue patterning in a wide range of organs and animal species [1–3]. In the developing vasculature, Notch promotes arteriolar differentiation and inappropriate activation of this pathway can lead to the formation of arteriovenous (AV) shunts [4]. Notch is also an important negative regulator of endothelial sprouting and proliferation. Tip cells, the filopodia-extending endothelial cells (ECs) at the distal (leading) end of growing sprouts, express elevated levels of the Notch ligand Delta-like 4 (Dll4). This, in turn, leads to the activation of Notch receptors in adjacent stalk cells, which form the base of the sprout, and suppresses tip cell behavior in these ECs [5–7]. In line with this model, an excessive number of stalk cells, endothelial proliferation and arterial differentiation. The E3 ubiquitin ligase Fbxw7 is part of the SCF protein complex involves Fbxw7, an F-box protein and the substrate-recognizing transcription factor RBP-Jκ [1,2]. NICD lifetime is controlled by polyubiquitination and thereby proteosomal degradation, which involves Fbxw7, an F-box protein and the substrate-recognizing component of an SCF (SKP1, CUL1, F-protein)-type E3 ubiquitin ligase [15,16]. Indicating important roles of Fbxw7 in vascular development, global knockout mice were previously found to be embryonic lethal, strongly growth retarded at E10.5, and showed malformed vessels in the head and yolk sac [17,18]. Upregulated expression of several Notch target genes in a variety of different embryonic tissues argued that the mutant phenotype was indeed linked to an increased activation of the Notch pathway. However, the question whether Fbxw7 regulates angiogenesis indeed through Notch or other substrates was not investigated so far. Moreover, as the Notch pathway functions in many embryonic tissues such as the heart, neural tube and somites [17,18], it has remained unclear whether Fbxw7 activity is required in the endothelium or, possibly, in other cell types.

Here, we report the generation of EC-specific and inducible Fbxw7 mutant mice, which allowed us to investigate the function of the E3 ligase in the growing retinal vasculature, a model system and time. Among other mechanisms, the degradation of activated receptors can limit the duration of signaling and thereby control the spatiotemporal activity of pathways [13,14]. In the case of Notch receptors, ligand binding triggers the proteolytic release of the Notch intracellular domain (NICD), which is then translocated to the nucleus and binds a transcriptional complex containing the transcription factor RBP-Jκ [1,2]. NICD lifetime is controlled by polyubiquitination and thereby proteosomal degradation, which involves Fbxw7, an F-box protein and the substrate-recognizing component of an SCF (SKP1, CUL1, F-protein)-type E3 ubiquitin ligase [15,16]. Indicating important roles of Fbxw7 in vascular development, global knockout mice were previously found to be embryonic lethal, strongly growth retarded at E10.5, and showed malformed vessels in the head and yolk sac [17,18]. Upregulated expression of several Notch target genes in a variety of different embryonic tissues argued that the mutant phenotype was indeed linked to an increased activation of the Notch pathway. However, the question whether Fbxw7 regulates angiogenesis indeed through Notch or other substrates was not investigated so far. Moreover, as the Notch pathway functions in many embryonic tissues such as the heart, neural tube and somites [17,18], it has remained unclear whether Fbxw7 activity is required in the endothelium or, possibly, in other cell types.

Here, we report the generation of EC-specific and inducible Fbxw7 mutant mice, which allowed us to investigate the function of the E3 ligase in the growing retinal vasculature, a model system
that has been highly useful for the characterization of Notch function [8,12]. In a second approach, we interfered with zebrafish fbxw7 expression to characterize the role of the gene during development of the intersegmental vessels in the embryonic trunk, another commonly used experimental model of angiogenesis [19,20]. Our findings establish that Fbxw7 is indeed an important regulator of endothelial cell behavior in angiogenesis. Impairing Notch activity in both loss-of-function settings mentioned above was sufficient to restore angiogenic sprouting, which indicates that these phenotypes were predominantly caused by excessive Notch activation and not by other Fbxw7 substrates.

Results

Inducible Targeting Fbxw7 of in the Vascular Endothelium

For functional studies in the postnatal endothelium, mice carrying a loxP-flanked version of the Fbxw7 gene [21] were combined with Cdh5(PAC)-CreERT2 [22,23] or Pdgfb-iCre [24] transgenics. Following the postnatal administration of tamoxifen (see Material and Methods), the resulting Fbxw7iECKO mutants generated with either one of these inducible Cre lines displayed very similar phenotypes in the retinal vasculature (Figure 1A, 1C; Figure S1). Angiogenic growth was strongly impaired, which led to a smaller number of vessel branch points, fewer sprouts, a reduction in the area covered by ECs, and delayed extension of the retinal vasculature towards the periphery (Figure 1B, 1D). Both genetic mouse models consistently showed that Fbxw7 is an important positive regulator of angiogenic growth. Moreover, the EC-specific inactivation of Fbxw7 with tamoxifen-inducible Cdh5(PAC)-CreERT2 and Pdgfb-iCre transgenics demonstrated that the activity of the gene product is indeed cell-autonomously required in endothelial cells.

Previous studies on the role of Notch in the vasculature have led to a model in which DI4 presented by endothelial tips activates Notch in adjacent (stalk) ECs, which suppresses tip cell behavior in this cell population [5–7]. Notch signaling has been also implicated in the dynamic switching of ECs that compete for the tip cell position in growing sprouts [25]. While Fbxw7iECKO mutant endothelial sprouts at the distal edge of the growing retinal vascular plexus were generally short or absent (Figure 1A, 1C; Figure S1), the fraction of mutant sprouts was longer than normal and extended beyond the edge of the vascular plexus without connecting to other vessels (Figure 1A, 1C, 2A). This phenotype might indicate that the competition of ECs for the tip position or the incorporation of tip cells in new vascular connections were impaired in Fbxw7iECKO mutants. Endothelial proliferation is another important aspect of angiogenic growth that is strongly controlled by the Notch pathway [8,12]. BrdU labeling showed that the number of proliferating ECs was markedly reduced in Fbxw7iECKO mutants compared to littermate controls (Figure 2C, 2D). Likewise the siRNA mediated knockdown of Fbxw7 expression in human umbilical vein endothelial cells (HUVECs) led to a significant reduction in cell proliferation (Figure S2). Previous work has also identified the Notch pathway as a positive regulator of arterial differentiation [26,27]. Indeed, the recruitment of α-smooth muscle actin (αSMA)-positive cells, a feature that is most prominent in the arterial branch of the developing vasculature, was enhanced in Fbxw7iECKO mutants (Figure S2). Key aspects of the Fbxw7iECKO phenotype described here were produced by NICD overexpression in the postnatal endothelium (Figure S3). For this purpose, Pdgfb-iCre transgenics were bred into a heterozygous or homozygous background of Gt(ROSA)26Sor [Notch1/Fbxw7; Tg(kdrl:EGFP)s843] (Figure 3A), which enable Cre-dependent expression of the active Notch1 intracellular domain. Following postnatal administration of tamoxifen, the resulting mutants showed reduced vascular outgrowth, lower vessel density and extension of αSMA-positive vessel beds (Figure S3).

Enhanced Endothelial Notch Signaling in the Absence of Fbxw7

DI4 is not only an important activator of Notch in the vasculature, but the ligand is also a strong transcriptional target downstream of activated Notch receptors [5–7]. Thus, DI4 immunofluorescence can provide useful information about the activation of Notch in the endothelium. While anti-DI4 immunofluorescence labeled arteries and sprouting endothelial cells at the growing edge of the control retinal vasculature, staining extended from arteries into the peri-arterial capillary plexus in Fbxw7iECKO mice (Figure 3A). Moreover, many DI4-positive ECs were found in mutant capillaries behind the angiogenic front, an area that showed low DI4 immunofluorescence in control retinas (Figure 3B, 3C). qPCR on total RNA isolated from lungs, a tissue that was chosen because of its high EC content of about 20% of all cells, confirmed that DI4 expression was also elevated at the transcript level in Fbxw7iECKO mutants (Figure 3D).

To directly investigate whether the levels of NICD were increased in Fbxw7iECKO mice, we performed Western blotting with mutant and control lung lysates (Figure 4A). This assay showed that Fbxw7 mutant samples contained more cleaved, active NICD than controls. Likewise, following knockdown of Fbxw7 expression by siRNA in cultured HUVECs, baseline NICD levels in siFbxw7 cells were substantially higher than in control siRNA-treated cells (siControl). Interestingly, addition of VEGF-A to the latter led to a transient increase in NICD at 30 minutes followed by a gradual decrease at 60 and 120 minutes after stimulation (Figure 4C, 4D). In siFbxw7 HUVECs, NICD also increased in response to VEGF-A, but levels stayed high and remained significantly above what was seen in siControl cells. Thus, reduced expression of Fbxw7 in cultured endothelial cells increased the stability of the active Notch intracellular domain.

The role of the Notch pathway in angiogenesis is strongly linked to VEGF signaling. In stalk ECs, active Notch is thought to suppress or dampen VEGF receptor expression, which contributes to the distinct behaviors of leading (tip) and trailing (stalk) cells within vascular sprouts [5–7]. In the addition to signaling by VEGF-A and VEGFR2, VEGFR3, the receptor for VEGF-C and VEGF-D, is an important regulator of angiogenesis and its expression is suppressed by active Notch [12]. In line with enhanced Notch signaling in the endothelium of Fbxw7iECKO mice, immunofluorescence showed that VEGFR3 protein expression was strongly reduced (Figure 4E, 4F).

Fbxw7 Controls Intersegmental Vessel Growth in Zebrafish

The VEGF and Notch pathways are also important regulators of angiogenesis in the zebrafish embryo, which has been shown for the growth of intersegmental vessels from the dorsal aorta in the embryonic trunk [29,30]. To investigate whether zebrafish fbxw7 might control this process, we reduced the expression of this gene with antisense morpholino oligonucleotides (MOs) that interfere with transcript splicing or translation, respectively (see Materials and Methods; Figure S4). The vasculature in these experiments was visualized by endothelial expression of green fluorescent protein in a Tg(kdrl:EGFP)ytl transgenic background [31]. Microinjection of control or either of the two fbxw7 MOs at the chosen concentration did not interfere with normal growth and general development of the embryos (Figure S4). Significant downregulation of Fbxw7
Figure 1. Inducible targeting of the \textit{Fbxw7} gene in retinal ECs. Confocal images showing the organization of the \textit{Fbxw7}\textsuperscript{iECKO} mutant and littermate control retinal vasculature at postnatal day 6 (P6) after whole-mount Isoclectin B4 staining (A and C). Postnatal, EC-specific loss-of-function mutants were either generated with the \textit{Cdh5(PAC)}-\textit{CreERT2} (A, B) or \textit{Pdgfb-iCre} (C, D) transgenics. Radial extension, branchpoint number, sprouts, and vascular area (B and D) were quantitated (control, n = 7; \textit{Fbxw7}\textsuperscript{iECKO} n = 6). Error bars indicate SEM. P value (***) < 0.00001. Scale bar is 200 μm for all microscopic images. doi:10.1371/journal.pone.0041116.g001

Figure 2. \textit{Fbxw7} promotes endothelial sprouting and proliferation. Morphology of the growing (peripheral), Isoclectin B4-stained retinal vasculature (A) and higher magnification of endothelial sprouts (B) in \textit{Fbxw7}\textsuperscript{iECKO} mutants and littermate controls, as indicated. While sprouts were reduced in number and predominantly short in the absence of \textit{Fbxw7}, isolated long protrusions were observed (arrow in A). BrdU labeling (red) showed reduced endothelial proliferation in the Isoclectin B4-stained (green, IsolB4) \textit{Fbxw7}\textsuperscript{iECKO} retinal ECs (C), which is quantitated in (D). Error bars indicate SEM (based on 8 control and 6 \textit{Fbxw7}\textsuperscript{iECKO} retinas). P value (***) is < 0.00001. Scale bars are 100 μm (A, C) and 50 μm (B). doi:10.1371/journal.pone.0041116.g002
The Role of Fbxw7 in Angiogenesis is Mainly Mediated by Notch

Fbxw7 is responsible for the polyubiquitination of multiple substrates apart from Notch [15,16]. To address whether Notch or other target proteins of Fbxw7 were responsible for the angiogenesis defects seen in zebrafish ISVs, we injected flxax7 or control morpholinos into zebrafish mutant embryos with a defective dll4 gene. These dll4 mutants display enhanced EC sprouting and proliferation [30] and thereby resemble the vascular phenotypes caused by the full or partial inhibition ofDll4 function in mice [8–10]. We found that the impaired angiogenesis seen in the ISVs of fbxax7 morphants was partially rescued in the dll4 homozygous mutant background (Figure 6). Whereas defective ISVs were seen in 97% of morphant embryos with a wild-type dll4 gene, intersegmental vessel growth was restored in 21% of heterozygous and 46% of homozygous dll4 mutants.

A similar rescue experiment was conducted in the mouse by combining Cdh5(PAC)-CreERT2 transgenic mice [23] with conditional, loxP-flanked alleles of Flk1 [21] and Rbhp [35] (the gene encoding RBP-Jκ) (Figure 7). Tamoxifen-induced targeting Fbxax7 in the postnatal endothelium in a wild-type (data not shown) or Rbhp heterozygous background strongly reduced retinal angiogenesis (Figure 7). Inactivation of the Rbhp gene in the retinal endothelium led to enhanced endothelial sprouting and proliferation reminiscent of other Notch pathway mutants. Remarkably, the combined inactivation of both Fbxax7 and Rbhp restored angiogenic growth in the resulting double mutants indicating that the Fbxax7(-/-) Rbhp(-/-) phenotype is indeed primarily a consequence of excessive Notch signaling (Figure 7). Likewise, short-term pharmacological inhibition of Notch signaling with the γ-secretase inhibitor DAPT (see Material and Methods), which blocks Notch cleavage and activation, also reversed the Fbxax7 retinal phenotype (Figure S5).

These rescue experiments demonstrate that the biological role of Fbxax7 as a positive regulator of angiogenesis is critically mediated by the degradation of Notch.

Discussion

Previous work has shown that Fbxax7, a component of an SCF-type ubiquitin ligase complex, controls several aspects of cardiovascular development in the mouse. Targeted inactivation of the gene led to embryonic lethality at midgestation and severe growth retardation, which has been attributed to defects in the mutant hematopoietic system as well as impaired cardiovascular development [17,18]. Due to the interdependent nature of morphogenetic processes in the embryonic placenta, heart, and vasculature [36,37], these findings left substantial uncertainty with regard to the exact role of Fbxax7 in the regulation of angiogenic blood vessel growth. Here we have targeted the Fbxax7 gene in the endothelium of the postnatal mouse with an inducible and cell-type-specific approach.

Our analysis of the postnatal retinal vasculature, a well-established model system of angiogenesis, demonstrated that the activity of Fbxax7 is indeed critically required in endothelial cells. EC-specific Fbxax7(-/-) mutants displayed a pronounced reduction in endothelial sprouting and proliferation indicating that the protein is an important positive regulator of both processes.

Previous work has identified active Notch as a substrate of the Fbxax7-containing ubiquitin ligase complex [15]. Notch target genes were also found to be upregulated in embryos with a full Fbxax7 gene knockout [17,18] and, accordingly, it has been proposed that the vascular phenotype of these mutants is caused by defective Notch degradation. However, in addition to active Notch, numerous other Fbxax7 substrates have been identified. Examples are the cell cycle regulator cyclin E, the proto-oncogenes c-Myc and c-Jun, or the sterol regulatory element binding protein (SREBP) [15,16], all of which might be relevant in the context of the vascular defects seen in the Fbxax7-deficient embryos. To address this important mechanistic question directly, we tested whether interfering with Notch signaling can circumvent the angiogenic growth block seen in Fbxax7 loss-of-function mutants. Indeed, inactivation of the dll4 gene was sufficient to restore angiogenesis in the trunk of a large fraction of Fbxax7-silenced zebrafish embryos. In the mouse retina, inactivation of the Rbhp gene or pharmacological inhibition of Notch signaling with DAPT were sufficient to reestablish substantial levels of angiogenic growth in the Fbxax7(-/-) mutant background. Thus, our new data directly demonstrate that excessive Notch activation is a predominant cause of compromised sprouting and proliferation in Fbxax7 loss-of-function mutants. This finding does not rule out that other Fbxax7 substrates might play minor or functionally distinct roles in the regulation of endothelial cell behavior or other aspects of vascular morphogenesis.

The Notch pathway is a critical regulator of angiogenesis and has been linked to VEGF signaling. Expression of the ligand Dll4 is induced by VEGF-A and, conversely, DLL4-mediated activation of Notch is thought to downregulate the expression of VEGF receptors in ECs, which generates a negative feedback loop that dampens VEGF-triggered angiogenesis [5–7]. A second Notch ligand, Jagged1, opposes Dll4 and thereby promotes angiogenic growth in the skin and retina [11]. As the behavior of sprouting endothelial cells is highly dynamic, which involves positional shuffling and competition for the tip position during sprouting [25], the spatiotemporal pattern of Notch activation needs to be equally flexible. In addition to the regulation of Notch receptor and ligand
expression, modulating the lifetime of the proteolytically cleaved (active) NICD provides another level of control [38,39]. The importance of this regulatory mechanism is highlighted by our finding that Fbxw7-deficiency in ECs led to increased NICD stability and compromised endothelial sprouting in vivo. While we have not investigated the active positional switching capability of

Figure 4. Fbxw7 controls endothelial Notch signaling. Protein extracts prepared from P6 control (lanes 1 & 3 from the left) and Fbxw7ECKO lungs (lanes 2 & 4) showed increased amounts of active Notch1 (NICD-Val1744) and reduced VEGFR3 protein (A). VE-Cadherin (Cdh5) was used for normalization. Relative Fbxw7 mRNA expression in HUVECs transfected with siControl or siFbxw7 relative to siControl siRNAs (n = 3 per group) (B). Human GAPDH was used for normalization. Confocal images showing the pronounced reduction of VEGFR3 protein (green) in the endothelium (Isolectin B4; red) of the P6 Fbxw7ECKO retina (E). Scale bar is 100 μm. Quantitative analysis (with Volocity 5; n = 3 for each group) of image data confirmed reduced VEGFR3 levels (number of pixels) in mutant ECs (F). Error bars (in D and F) indicate SEM. P values are indicated as ** (p < 0.001) and * (p < 0.05).

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Figure 5. Impaired ISV growth after knockdown of zebrafish fbxw7 expression. Following injection with control morpholino (control MO) or fbxw7 transcription blocking morpholino (fbxw7 ATG MO), the fluorescent vasculature of Tg(kdrl:EGFP)transgenic embryos was examined by live microscopy. Outgrowth of ISVs from the dorsal aorta (DA) and formation of the DLAV (as indicated in the left image) were impaired in fbxw7 morphant embryos (arrowheads) at 32 hpf (A). Scale bar is 70 μm. Protein extracts from control and fbxw7 ATG MO embryos at 24 hpf and 32 hpf were analyzed by Western blotting (B). Note transient reduction of the Fbxw7 band (asterisk). Tubulin was used as loading control. Quantification of the number of ECs per ISV in control MO and fbxw7 ATG MO injected embryos at 32 hpf (based on 3 independent experiments) (C). Proliferation of endothelial cells at 32 hpf in ISVs of Tg(fli1a:nEGFP)y7 x Tg(kdrl:HsHRAS-mCherry)s896 double transgenic embryos injected with control MO or Fbxw7 ATG MO (D). Error bars indicate SEM. P value (*) in (C) is < 0.05.

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Individual Fbxw7<sup>−/−</sup> endothelial cells, our phenotypic data indicates that prolonging the lifetime of endogenously generated NICD leads to a general slowdown in angiogenic growth. This might reflect that most or all ECs - irrespective of whether they are positioned in the tip, stalk or in capillaries - are at least transiently exposed to a certain level of Notch activation, which is enhanced or prolonged in Fbxw7<sup>−/−</sup> mutants.

The overexpression of active Notch can cause vascular malformations such as the development of AV shunts [4]. As our findings show that Fbxw7 deficiency can lead to the accumulation of NICD in endothelial cells, it appears worthwhile to investigate whether mutations in the Fbxw7 gene are linked to human cardiovascular diseases. Likewise, it will be important to gain a better understanding of the modifications that mark active Notch receptors for polyubiquitination. Here, the regulated phosphorylation of Fbxw7 recognition sites (degrons) or other posttranslational modifications of the NICD are likely to be of central importance [38,39]. It also remains to be addressed whether the controlled modulation of Fbxw7 expression or activity can be utilized for therapeutic purposes in eye diseases and other settings where it is desirable to suppress angiogenic blood vessel growth.

Materials and Methods

Inducible Mutant Mice

Mice carrying a loxP-flanked (floxed) Fbxw7 gene [21] were combined with Cdh5(PAC)-CreERT2 [22,23] or Pdgfb-iCre [24] transgenic lines, respectively. All Fbxw7<sup>−/−</sup> mutants shown were obtained with the Cdh5(PAC)-CreERT2 strain unless indicated otherwise. Tamoxifen-injected Cre-negative littermates were used as controls. For the generation of double mutants, floxed alleles of the Rbhp gene [35], floxed Fbxw7 and the Cdh5(PAC)-CreERT2 transgene were combined by crossing over. To overexpress NICD in the postnatal endothelium, Pdgfb-iCre heterozygotes were bred into a heterozygous or homozygous background of Gt(ROSA)26Sor<sub>tm1(Notch1.DA45F)</sub> transgenics [20]. Detailed protocols for the administration of tamoxifen were published previously [11,22]. Mutant phenotypes were analyzed at postnatal day 6 (P6) unless other information is provided.

DAPT treatment was performed according to Benedito et al. [11] with minor modifications. Following the tamoxifen injection, pups were twice intraperitoneally injected (at P4 and P5) with 0.1 mg/g γ-secretase inhibitor IX (DAPT; Calbiochem, 565770) andretinas were collected 48 hrs later at P6.

All animal experiments were performed in strict accordance with the relevant laws and institutional guidelines at Cancer Research UK and the Max Planck Institute for Molecular Biomedicine. All protocols were approved by animal ethics committees of Cancer Research UK (UK) and the state of North Rhine-Westphalia (Germany), respectively, and all efforts were made to minimize suffering.

Whole-mount Immunohistochemistry of the Retinal Vasculature

P6 mouse eyes were fixed in 4% PFA for 2 hrs at room temperature (RT) or on ice. Retinas were blocked in blocking buffer (1% BSA, 0.3% TritonX-100 in PBS) followed by incubation with primary antibodies in Phc e (1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 0.1 mM MnCl<sub>2</sub>, 1% TritonX-100 in PBS) for RT-fixed retinas or in 0.5 Phc e (Phc e +0.5% TritonX-100) for samples fixed on ice. Endothelial cells were visualized with biotinylated Griffonia Simplicifolia lectin I (Isol lectin B4) (Vector Labs, B1205). The following primary antibodies were used: goat anti-mouse Dll4 (R&D Systems, AF1389), rat anti-mouse VEGFR3/Fit4 (Bioscience, 14-5988), and mouse C3-conjugated anti-alpha-smooth muscle actin (Sigma-Aldrich, C6196). The corresponding secondary antibodies were coupled with AlexaFluor 488, 546, or 647 (Invitrogen).

BrdU Staining of Proliferating Cells

For the detection of proliferating cells, 300 μg of 5-bromo-2’-deoxyuridine (BrdU) (Invitrogen, B23151) was injected intraperitoneally 3 hrs before the animals were humanely killed. Eyes were fixed in 4% PFA for 2 hrs at RT. After Isol lectin B4 staining, retinas were re-fixed in 4% PFA for 30 min at room temperature, washed 3 times with PBS, incubated in 50% Formaldehyde/1× SSC (0.15 M NaCl and 0.015 M Na-citrate) [PH 7.0] for 1 hr at 65°C, incubated in 2 N HCl for 30 min in a 37°C water bath, neutralized with 0.1 M Tris-HCl [pH 8.0], blocked in blocking buffer for 2 hrs at room temperature, and incubated overnight with a mouse anti-BrdU antibody (BD Biosciences, 347580). Secondary detection was performed with an Alexa Fluor 546-coupled secondary antibody (Invitrogen, A11003).

Quantitative Analysis and Imaging

Radial extension, length of vascular growth from the optic nerve, branchpoints, number of sprouts, and vascular area of P6 retinas were quantified as described previously [11]. Student’s t-test was used for statistical analysis.

Whole-mount images of the retinal vasculature and zebrafish embryos were taken with a Leica TCPS5 confocal microscope using the following objectives: 10×/0.30 NA (Figures 1 and 7), 20×/0.70 NA (Figures 2, 3, and 4), and 20×/0.50 NA (Figure 5).
Low magnification images were acquired with a Leica MZ16F stereomicroscope. Images were converted to greyscale in Volocity 5 (Improvision) and managed in Photoshop CS3 (Adobe Systems Inc.).

Endothelial Cell Culture and siRNA Knockdown Experiments

Human umbilical vein endothelial cells (HUVECs) were purchased from Invitrogen and were maintained in Medium200 (M200) supplemented with low serum growth supplement (LSGS) (Invitrogen). HUVECs at passage 3 to 6 were used for experiments.

For the VEGF-A stimulation assay, HUVECs were transfected with Stealth RNA interference oligonucleotides (Invitrogen) against either control (300 nM) [Negative Medium GC:12935-300, Invitrogen] or human FBXW7 (mixture of #1, HSS124318 and #3, HSS124320, Invitrogen; 150 nM each) using Oligofectamine (Invitrogen) according to manufacturer’s instructions. At 72 hrs post-transfection, confluent HUVECs were serum-starved in M200 supplemented with 0.5% fetal bovine serum (FCS; Biochrom AG) for 4 hrs. After stimulation with VEGF-A165 (50 ng/ml) [ReliaTech GmbH, 300076L] in M200 supplemented with 0.5% FCS, cells were incubated and collected with Laemmli sample buffer at the indicated time points.

For the extraction of RNA, the RNeasy Mini kit (Qiagen) was used to purify total RNA from HUVECs at 72 hrs post-transfection. 500 ng RNA/reaction were used to generate cDNA using the iScript cDNA Synthesis Kit (BioRad).

Figure 7. Rbpj inactivation reverses the impaired vascular growth of FBXW7 mutant mice. Whole-mount Isolectin B4 staining of the retinal vasculature in P6 control, FBXW7iECKO/Rbpj+/iECKO (FBXW7 homozygous & Rbpj heterozygous EC-specific KO), FBXW7iECKO/Rbpj+iECKO (FBXW7 & Rbpj EC-specific double KO), and FBXW7+/iECKO/Rbpj+iECKO (FBXW7 heterozygous & Rbpj homozygous EC-specific KO), mice, as indicated. Inactivation of both Rbpj alleles enhanced EC proliferation and sprouting, and restored retinal angiogenesis in the FBXW7 mutant background. Scale bar represents 200 µm. Three independent experiments were performed with similar results.

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To assess the growth for siControl and siFbxw7 HUVECs, cells were detached, diluted and counted with a Neubauer chamber at 72 hrs post-transfection. Six independent transfections were used for quantitation.

Processing of Mouse Lung Lysates

Lungs were isolated from P6 mice. Left lobes were used for RNA analysis, whereas right lobes were used for protein analysis in individual pups. The RNeasy Mini kit (Qiagen) was used to purify total RNA. 500 ng RNA/reaction were used to generate cDNA using the iScript cDNA Synthesis Kit (BioRad).

For the extraction of protein, lung samples were lysed in RIPA buffer (50 mM Tris/HCl [pH 8.0], 150 mM NaCl, 1% NP-40, 0.1% SDS, and 0.5% sodium deoxycholate) supplemented with protease and phosphatase inhibitor cocktails (Thermo Scientific). Samples were sonicated 4× for 10 sec followed by incubation for 30 min at 4°C. Lysates were then centrifuged for 30 min at 16,000 x g at 4°C. Supernatants were collected and combined with Laemmli sample buffer.

Western Blot Analysis

Western blots of lung or HUVEC samples were probed with the following antibodies: rat anti-mouse VEGER3/Fht4 (eBioscience, 14-5988), rabbit anti-cleaved Notch1 (NIDC-Val174) (Cell Signaling Technology, 2421), rabbit anti-activated Notch1 (NIDC) (Abcam, ab8925), rabbit anti-alpha-tubulin (Sigma-Aldrich, T5168), and rabbit anti-Cdc4/Fbxw7 (Millipore, ab10620).

Quantitative PCR Analysis

Real-time quantitative PCR reactions were performed in duplicate per reaction using the Taqman Gene Expression Assay system (Applied Biosystems). The following Taqman primers were used: mCdh5 (Mm00441691_m1), mFbxw7 (Mm00504445_m1), mDll4 (Mm00444169_m1), hGAPDH (FAM Dye/MGB Probe, 4352934), and hFbxw7 (Hs01015617_m1).

Zebrafish Strains

Zebrafish were maintained in a recirculating aquaculture system under standard laboratory conditions at 27°C. Embryos were grown at 28.5°C and staged in hours post fertilization according to Kimmel et al. [40]. The transgenic lines used were: Tg(fbxw7:EGFP); Tg(fbxw7:EGFP)3465, and Tg(fbxw7:EGFP)3581, which express mCherry under the control of FBXW7. Zebrafish were genotyped by PCR and subsequently staged in hours post fertilization (hpf).

Fbxw7 Controls Endothelial Notch

To determine changes in Fbxw7 cell number following Fbxw7 knockdown, control MO and Fbxw7 ATG MO-injected embryos were examined live or fixed for 2 hrs with 4% PFA at room temperature and analyzed by confocal microscopy (Leica TCS SP5). Confocal stacks and movies were assembled using Imaris Software (Bitplane). From each embryo, the number of cells in four ISVs, located between the 12–16th somite were counted. Endothelial cell nuclei were visualized by transgenic GFP expression using the Tg(fbxw7:EGFP)1076. Five to six embryos were analyzed per injection sample.

For time-lapse microscopy, embryos were dechorionated manually and mounted in agarose embryo arrays as described previously [42]. To prevent movement of embryos, medium was supplemented with 19.2 mg/l (0.0192%) tricaine (ethyl-3 amino benzoox methane sulfonated salt). Pigmentation was inhibited by adding phenyliourea to a final concentration of 0.003%.

Rescue of the fbxw7 Phenotype in Zebrafish

Embryos generated by intercrossing dll4f/e6 cells were cultured with DAPT, a Notch inhibitor, at 72 hours after Fbxw7 knockdown (A). For control, E14.5 embryos were injected at the one cell stage with Fbxw7 ATG MO morphtm. The injected embryos were at 32 hpf for 2 hrs with 4% PFA at room temperature, analyzed using a Leica M165FC fluorescence microscope, and genotyped individually by PCR and subsequent restriction analysis. Primers used for genotyping were: dll4f/e6 FWD 5’-AGTTTTAGATTGTGCTGG AACATCTTGTATA-3’ and dll4f/e6 REV 5’-GACATATTTTCAATCAAATCCAGT-3’.

Supporting Information

Figure S1 Defective retinal angiogenesis in Fbxw7 loss-of-function mutants. Confocal images of whole-mount Isolectin B4-stained retinas from P6 Fbxw7<sup>−/−</sup> (left) and control (right) embryos. Note increased SMA signal in the mutant vasculature. Scale bar is 500 μm. (TIF)

Figure S2 Fbxw7 controls HUVEC proliferation and smooth muscle cell recruitment. Quantiation of cell numbers in cultured siFbxw7 (Fbxw7 silenced) or siControl HUVECs, as indicated, at 72 hours after siRNA transfection (A). Error bars indicate SEM. P value is <0.001. Confocal image of P9 whole-mount Fbxw7<sup>−/−</sup> (right) and control (left) retinas after staining with anti-smooth muscle actin (SMA) antibody (B). Note increased SMA signal in the mutant vasculature. Scale bar represents 500 μm. (TIF)

Figure S3 Endothelial overexpression of NIDC phenocopies Fbxw7 defects. Confocal images of P6 control and endothelial cell-specific NIDC gain-of-function retinas (A). Pdgfb-iCre transgenics were combined with Gt(ROSA)26Sor(J1)tm1(Notch1)Dam/J mice. Confocal images of P9 whole-mount Fbxw7<sup>−/−</sup> (middle column) or homozygous mice (right column). Pdgfb-iCre transgenics were combined with Gt(ROSA)26Sor(J1)tm1(Notch1)Dam/J (middle column) or homozygous mice (right column). ECs were visualized by Isolectin B4 (green), smooth muscle cells by αSMA immunofluorescence. Bottom panels show higher magnification of insets in the middle row. Arrows indicate extension of αSMA staining into peri-arterial capillary beds. Radial extension of the vascular plexus towards the periphery (B) was reduced in NIDC/+ heterozygotes and NIDC/NIDC homozygotes.
Splice MO in two independent experiments. Beta-actin PCR products were used as loading control.

32 hpf injected with control (genesis was not increased in vehicle (DMSO)-injected Tg(kdrl:EGFP)s843 fluorescence (B, D) of fbxw7 transcripts in zebrafish embryos injected with Splice MO in two independent experiments. Beta-actin PCR products were used as loading control.

Figure S5 Notch inhibition restores vascular growth in Fbxw7 mutants. Confocal images of whole-mount Isolatec B4-stained retinas. Notch inhibition was achieved by administration of the γ-secretase inhibitor DAPT, which interferes with Notch cleavage and signaling, for 48 hrs prior to the isolation of the retinas at P6. DAPT partially restored enhanced sprouting and proliferation in the Fbxw7iECKO vasculature. In contrast, angiogenesis was not increased in vehicle (DMSO)-injected Fbxw7iECKO mutants. The phenotype of DMSO or DAPT-treated control retinas is shown in the upper row. Scale bar is 200 μm.

Figure S4 Vascular defects caused by the knockdown of zebrafish fbxw7. Bright-field images (A, C) and endothelial fluorescence (B, D) of Tg(kdrl:EGFP)Δ230–250 fbxw7 embryos at 32 hpf injected with control (control MO), fbxw7 translation-blocking (ATG MO) or fbxw7 splicing-blocking (Splice MO) morpholinos, as indicated. The knockdown of fbxw7 impaired ISV outgrowth and prevented the formation of the DLAV, while the size and general growth of the morphant embryos were unaffected. Scale bar is 200 μm. PCR analysis (E) showing the reduction of fbxw7 transcripts in zebrafish embryos injected with Splice MO in two independent experiments. Beta-actin PCR products were used as loading control.

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