

The non-canonical NOTCH ligand DLK1 exhibits a novel vascular role as a strong inhibitor of angiogenesis

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Aims The epidermal growth factor-like protein Delta-like 1 (DLK1) regulates multiple differentiation processes. It resembles NOTCH ligands structurally and is considered a non-canonical ligand. Given the crucial role of the NOTCH pathway in angiogenesis, we hypothesized that DLK1 could regulate angiogenesis by interfering with NOTCH. We therefore investigated the expression and function of DLK1 in the vascular endothelium and its role in the regulation of angiogenesis.

Methods and results We report DLK1 expression in the endothelium of different species, including human, cow, pig, and mouse. Angiogenesis was studied by using *in vitro* and *in vivo* models of angiotube formation in endothelial cells, retinal phenotypes in *Dlk1*-null mice, and vessel development in zebrafish. DLK1 overexpression strongly inhibited angiotube formation, whereas lung endothelial cells from *Dlk1*-null mice were highly angiogenic. *In vivo* studies demonstrated DLK1-mediated inhibition of neovessel formation and revealed an altered pattern of angiogenesis in the retinas of *Dlk1*-null mice. The expression of human DLK1 in zebrafish embryos severely altered the formation of intersegmental vessels, while knockdown of the orthologous gene was associated with ectopic and increased tumour-induced angiogenesis. NOTCH-dependent signalling as determined by gene expression reporters was inhibited by the presence of DLK1 in vascular endothelial cells. In contrast, *Dlk1*-null mice showed increased levels of NOTCH downstream targets, such as Snail and Slug.

Conclusion Our results unveil a novel inhibitory role for DLK1 in the regulation of angiogenesis, mediated by antagonism of the NOTCH pathway, and establish the basis for investigating its action in pathological settings.

Keywords Angiogenesis • Delta-like 1 • Pref-1 • Endothelium • NOTCH

1. Introduction

Delta-like 1 (DLK1), also known as Pref-1 and FA1, is a transmembrane protein pertaining to the epidermal growth factor superfamily and initially described in neuroendocrine tumours and

preadipocytes.^{1,2} It has been described that DLK1 affects several differentiation processes, including adipogenesis, muscular and neuronal differentiation, bone differentiation, and haematopoiesis.³ Several reports from our groups and others support that DLK1 may operate as a non-canonical ligand of the NOTCH pathway, as it

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lacks the DSL domain conserved in classic NOTCH ligands that mediates ligand–receptor interaction.^{4–6} This structural feature has been related to its capacity to inhibit NOTCH signalling in different cellular systems and organisms, by the interactions with specific EGF-like repeats of NOTCH.⁷ The potentially diverse effects of DLK1 would then depend on the cell context and state of NOTCH activation.⁸ To date, information on the presence of DLK1 in adult endothelia is restricted to adipose tissue vascular cells and placental blood vessels,^{9,10} and to our knowledge, there are no functional data in the vasculature.

Angiogenesis, defined as the formation of new blood vessels from pre-existing ones, is a central process in embryonic and post-natal life and the object of intense study due to its relevance for tumour progression and therapy.¹¹ Since the NOTCH signalling pathway is essential for vascular development and physiology by controlling angiogenesis in pre- and post-natal life,^{12,13} we reasoned that DLK1 could contribute to regulate this process in adult endothelial cells through the interaction with NOTCH receptors. In this study, we analysed the role of DLK1 in angiogenesis by using a series of *in vitro* and *in vivo* approaches. We find that overexpression of DLK1 inhibits migration and angioblast formation in mammalian vascular endothelial cells and disrupts normal embryonic vascularization in zebrafish. Genetic ablation of DLK1 in mice is associated with increased angiogenesis *in vitro* and with focal areas of retinal hypervascularization. Specific knockdown of the orthologous *Dlk1* of zebrafish results in ectopic angiogenesis. Moreover, in a tumour angiogenesis model in zebrafish, suppression of *Dlk1* promotes vessel migration towards the tumour cell mass. We also find that the NOTCH signalling pathway is targeted by DLK1 in the context of angiogenesis and that DLK1 antagonizes NOTCH-dependent signalling in endothelial cells, while, in contrast, this signalling is enhanced in *Dlk1*-null mice. Collectively, these results reveal a previously unknown role for DLK1 in the vasculature as a regulator of NOTCH-mediated angiogenesis.

2. Methods

2.1 Adenoviruses

Adenoviruses encoding GFP, DLK1-GFP, or DLK1-HA were obtained from Vector Biolabs. Adenoviruses were purified using the Adeno-X Maxi Purification Kit and titered using the Adeno-X Rapid Titer Kit (Clontech).

2.2 Cell culture and animal handling

Bovine aortic endothelial cells (BAEC) and porcine aortic endothelial cells (PAEC) were obtained from cow and pig aortas at a local slaughterhouse and cultured as described.¹⁴ For isolation of mouse lung endothelial cells (MLEC), mice were sacrificed by cervical dislocation and lungs were excised and collagenase-digested. The mixed population obtained was first subjected to negative selection with anti-FCsR1/II/III and then to positive selection with anti-ICAM-2 and anti-IgG-coated magnetic beads. MLEC were grown on a mixture of fibronectin, type I collagen, and 0.1% gelatine-coated plates. Isolation of human umbilical vein endothelial cells is described in Supplementary material online. They were obtained from umbilical cords of normal deliveries (after approval by the Ethics Committee of the Hospital 'Ruber Internacional') and in agreement with principles outlined in the Declaration of Helsinki. Animals were handled in agreement with the Guide for the Care and Use of Laboratory Animals contained in Directive 2010/63/EU of the European Parliament.

Approval was granted by the local ethics review board of Centro de Biología Molecular 'Severo Ochoa'.

2.3 Western blot analysis

Details are provided in Supplementary material online.

2.4 Cell proliferation assays

Endothelial cells were at a density of 3000 cells/well. XTT turnover was measured at 0, 24, 48, 72, and 96 h after 4 h incubation at 37°C to allow colour development (Cell Proliferation Kit II). BrdU incorporation at 4, 8, and 24 h was detected by anti-BrdU (1:100), followed by anti-rat (1:200; Alexa 488). Images were taken with a Nikon eclipse TE2000-U inverted-microscope coupled to a digital-sight DS-2Mv.

2.5 Cell transfection and luciferase assay

DLK1 plasmid was obtained from Origene (pCMV6 XL4, human cDNA clone, SC127962). A haemagglutinin tag was added (Vector Biolabs). The Hes-1 luciferase reporter construction, containing a 350 bp mouse promoter fragment of the Hes-1 gene inserted upstream of the luciferase gene in pGL2 Basic, has been reported elsewhere.¹⁵ Endothelial cell transfection and determination of luciferase activity are detailed in Supplementary material online.

2.6 Scratch assay

Endothelial cell monolayers were mechanically wounded and the rate of coverage of the denuded area was monitored by time-lapse microscopy for 24 h as described,¹⁶ using a Leica AF6000 LX microscopy coupled to a monochromatic Hamamatsu CCD C9100-02.

2.7 Tube-like structure formation *in vitro*

The microcapillary-like formation assay was performed by using 96-well culture plates coated with 40 µL of Matrigel (BD Bioscience) as described.¹⁷ Endothelial cells with or without vascular endothelial growth factor (VEGF; 5 ng/mL) were seeded on the polymerized Matrigel and incubated at 37°C for 5–6 h. Angiotubes were stained with MTT. Images were taken with a Nikon eclipse TE2000-U inverted microscope coupled to a digital-sight DS-2Mv. Tube formation was quantified by using the Angioquant software.

2.8 Aortic ring assay

Rings of mouse aorta were cultured in three-dimensional gels as described.¹⁸ Thoracic aortas were removed from mice sacrificed by cervical dislocation. Pieces of aortas were immediately exposed or not to recombinant adenoviruses at 1×10^9 or 5×10^9 plaque-forming units/mL. Four hours after infection, 1 mm long rings were embedded in Growth Factor Reduced Matrigel in 96-well plates. Each well contained 100 µL of MCDB131 supplemented with 2.5% mouse serum, 1% glutamine, and 1% P/S. After 6 days of culture, explants were examined by microscopy. The length of vessels was quantified by ImageJ.

2.9 Matrigel plug assay *in vivo*

Growth Factor Reduced Matrigel Phenol Red-free (500 µL), heparin (0.3 mg/mL), and VEGF (250 ng/mL), adenovirus, DAPT (10 mg/kg of body weight) or DMSO (0.01%), as appropriate, were injected subcutaneously in the mice abdominal region. Mice were anaesthetized by injecting (ip) ketamine (100 mg/kg) plus xylazine (10 mg/kg) and constantly monitored by veterinarian surveillance of reflex absence. Animals were euthanized in a CO₂ chamber after day 10 of implantation, plugs were dissected, and haemoglobin was measured using the TMB method (Sigma). Haemoglobin content was normalized to plug weight. In another set of experiments, plugs were removed for histological examination. Formaldehyde-fixed plugs were embedded in paraffin-wax and

sectioned followed by staining with haematoxylin and eosin and examination under a light microscope (Olympus DX50).

2.10 Retinal isolation and staining

P0-P14 pups were sacrificed in a CO₂ chamber and eyes enucleated and fixed overnight in 1% PFA at 4°C. Then, retinas were dissected out and whole-mount stained with biotinylated isolectin B4 (Sigma-Aldrich) followed by streptavidin, Alexa Fluor 647 conjugate (Invitrogen) as described.¹⁹ Images from 8 to 10 retinas per experimental group were acquired in a Nikon A1R confocal microscope, and quantification of tip cells, filopodia, and vascular areas was carried out with ImageJ software.

2.11 Zebrafish DLK1 gain- and loss-of-function assays

Zebrafish (*Danio rerio*) were maintained and raised under standard conditions at 28°C. Transgenic *Tg(fli1:EGFP)* and *Tg(gata1:dsRed)* embryos were used to track endothelial cell populations and blood flow. *Tg(fli1:EGFP)*; *Tg(gata1:dsRed)* were generated by standard crossing of individual lines. A plasmid encoding the human DLK1 cDNA was injected at different concentrations in one-cell stage embryos. The same plasmid without the human DLK1 cDNA was used as a control. Embryos were allowed to develop and pictures were taken at 72 hpf using a dissection microscope equipped with epifluorescence (MZ16FA, Leica) and a digital camera (DFC310FX, Leica). Sequences and experiments regarding anti-sense morpholinos (MO) are described in Supplementary material online.

2.12 Zebrafish tumour xenograft angiogenesis assay

*Tg(fli:eGFP)*²⁰ embryos were injected at one-cell stage with MO against zfDLK1 or capped hDLK1 mRNA or both, and allowed to develop at 28°C. Twenty-four hours post-fertilization fish embryos were incubated with water containing 0.2 mM 1-phenyl-2-thio-urea (Sigma) to prevent pigmentation. At 48 h post-fertilization, zebrafish embryos were dechorionated with the help of a sharp tip forceps and anaesthetized with tricaine (MS-222, Sigma). Anaesthetized embryos were transferred onto a modified agarose gel for microinjection. Before injection, tumour cells were labelled *in vitro* with 2 µg/mL of 1,1-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate (Dil, Fluka) and were further incubated for 24 h. Cells were resuspended in DMEM and 2–5 nL of tumour cell solution (25 cells/nL) was injected into the perivitelline cavity of each embryo using an Eppendorf microinjector (Femtojet 5247). After injection, the fish embryos were immediately transferred into housing-keeping water. Injected embryos were kept at 28°C and were examined 2 days after to monitor tumour-induced angiogenesis from subintestinal vessels (SIV) using a fluorescent microscope (MZ16FA, Leica) equipped with a digital camera (MZ16FA, Leica).

2.13 Zebrafish NOTCH-reporter assay

Tg(ptf1a:eGFP)^{h1} is a transgenic NOTCH-responsive zebrafish line harbouring 12 Rbp-Jκ-binding sites up-stream of the β-globin minimal promoter and the enhanced Green fluorescent protein reporter gene.²¹ *Tg(ptf1a:eGFP)*^{h1} embryos were injected at one-cell stage with MO against zfDLK1 or capped hDLK1 mRNA or were treated at 36 hpf with DAPT for 12 h and allowed to develop at 28°C. Details of the protocol are described in Supplementary material online.

2.14 RNA extraction and real-time PCR

Detailed methods and primers used are described in Supplementary material online, Table S1.

2.15 Statistical analysis

Comparisons were evaluated by Student's *t*-test for unpaired data or one-way analysis of variance when parametric tests were possible or by non-parametric tests, as appropriate.

3. Results

3.1 DLK1 is expressed in endothelial cells where its presence correlates inversely with angiotube formation and delays wound healing

Given the scarce information on DLK1 in the vasculature, we first studied its expression in several endothelial cell types and species. We found that DLK1 was expressed in murine, human, porcine, and bovine adult endothelial cells (Figure 1A). In the description of the phenotype corresponding to the *Dlk1*-null mice, obesity and serious osteo-skeletal malformations were reported²² but no explicit allusion was made to any changes in the vasculature. For this reason, we undertook studies in MLEC derived from *Dlk1*-null mice²³ and observed increased formation of angiotubes both in the absence and presence of VEGF (Figure 1B). This phenotype was reversed by the introduction of adenoviral expression constructs bearing DLK1 (Figure 1C). In addition, BAEC overexpressing DLK1 showed delayed re-endothelization after 18 h of in-plate endothelial wounding (scratch assay) (see Supplementary material online, Figure S1A). Consistently, wound closure was significantly accelerated in MLEC from *DLK1*-null mice (see Supplementary material online, Figure S1B and C; see Supplementary material online, Videos S1 and S2). To address the contribution of proliferation and/or apoptosis to the final effect observed, we evaluated the consequences of DLK1 suppression in MLEC. We found that the absence of DLK1 significantly correlated with increased cell proliferation (see Supplementary material online, Figure S1D and E). However, DLK1 did not interfere with apoptosis or necrosis (see Supplementary material online, Figure S2). These results suggest a dual inhibitory effect of DLK1 on the migration and proliferation of these endothelial cell types.

3.2 The absence of DLK1 is associated with increased sprouting angiogenesis while DLK1 overexpression inhibits neovessel formation *in vivo*

To examine the effect of DLK1 on angiogenesis in an *ex vivo* model, we studied the outgrowth of endothelial cells from aortic explants obtained from WT or *Dlk1*-null mice. Aortic segments from null mice exhibited a significantly higher pro-angiogenic profile (Figure 2A) that was abrogated in a dose-dependent fashion when DLK1 was expressed in aorta explants of the null mutant background (Figure 2B). We then investigated the effect of DLK1 expression constructs in an *in vivo* model of ectopic angiogenesis based on the formation of vessels in Matrigel[®] plugs implanted subcutaneously in the abdominal region of healthy mice. Whereas VEGF-treated plugs showed macroscopic neovessel formation and increased haemoglobin content, functional vascular structures were drastically reduced in plugs exposed to DLK1 expression constructs compared with controls, an effect comparable in magnitude to that observed in plugs

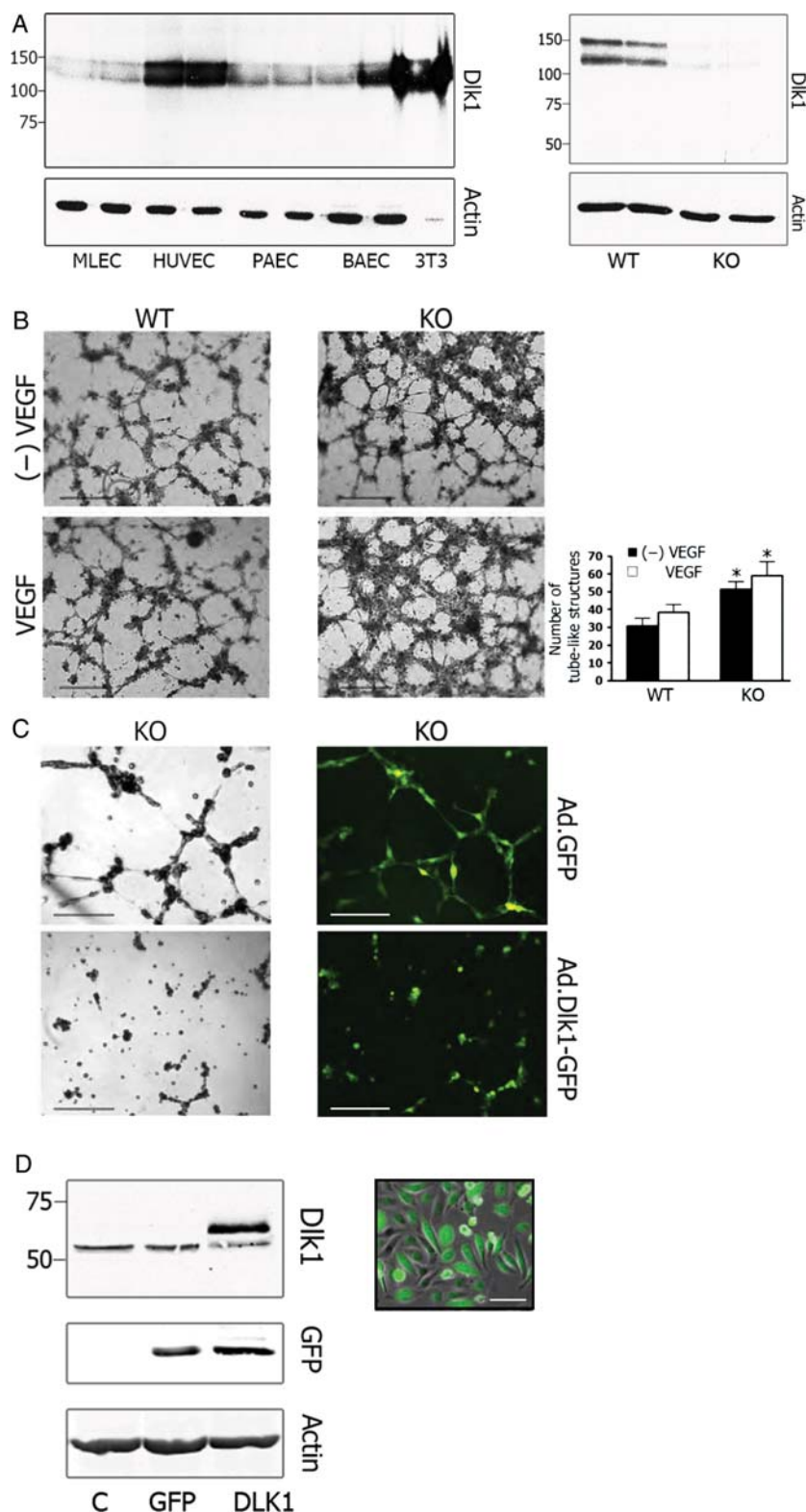


Figure 1 Expression of DLK1 in adult endothelia and reciprocal relationship between DLK1 and angiogenesis. (A) Left blot showing DLK1 dimer in non-boiled lysates (30 μ g) of endothelial cells from mouse (MLEC), human (HUVEC), pig (PAEC), and cow (BAEC) as well as 3T3 cells (1 μ g) by a specific antibody. β -Actin was used as a loading control. Right blot showing non-boiled lysates from MLEC WT and KO. Samples were assayed from two independent preparations. (B) Representative images from wild-type and DLK1-null isolated MLEC in the presence or absence of VEGF. Quantitative analysis represents the number of tube-like structures from three independent preparations per group. Values are presented as mean \pm SEM assayed per duplicate (* P < 0.05). (C) Cells from DLK1 null were infected with adenoviruses GFP or DLK1-GFP. (D) Lysates (45 μ g) from cells infected with adenoviruses GFP or DLK1-GFP were incubated with DLK1, GFP, and actin antibodies. Representative image of endothelial cells 24 h after infection at a multiplicity of infection of 500 showing an infection efficiency of \sim 90%. Scale bars: B: 500; C: 100 and D: 50 μ m.

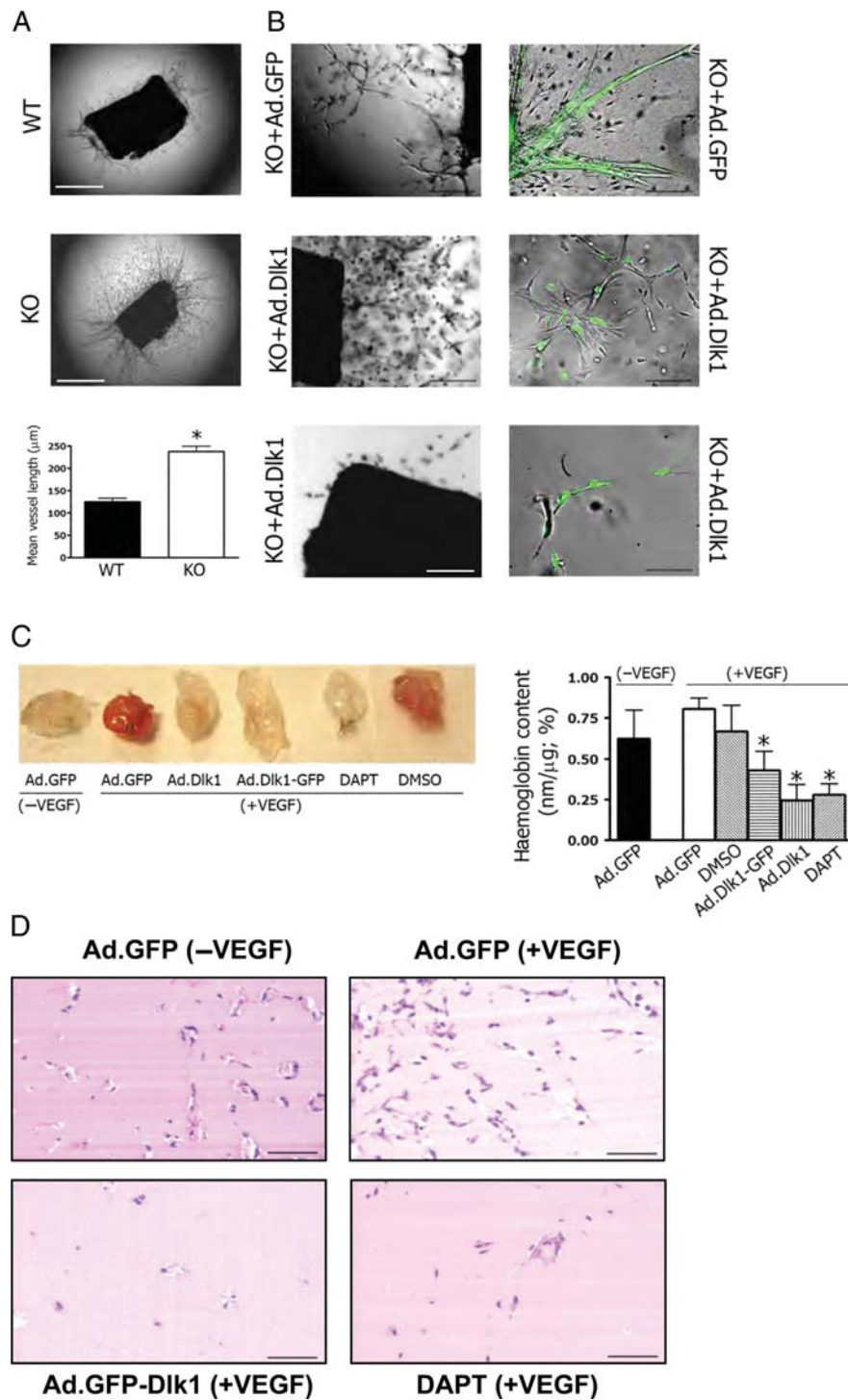


Figure 2 DLK1 regulates angiogenesis *in vivo*. (A) Representative images from wild-type and DLK1-null aorta segments. The quantification of vessel-like structures length (lower panel) shows an increase in neovessel outgrowth from explants of null mice. Values are presented as mean \pm SEM of $n = 35$ rings from six mice; $*P < 0.05$. (B) The pro-angiogenic response of the null mice is abolished in explants pre-incubated with a DLK1 adenovirus in a concentration-dependent manner, 1×10^9 (middle panel) and 5×10^9 (lower panel) plaque-forming units/mL compared with GFP adenovirus (upper panel). (C) Ectopic angiogenesis was analysed in Matrigel plugs containing adenovirus (1×10^9 plaque-forming units/mL) control, or plugs containing VEGF plus adenovirus control, Ad.DLK1, Ad.DLK1-GFP, DAPT, or vehicle (DMSO). Photograph of plugs after 10 days of implantation. The quantification of haemoglobin content (right panel) shows that the VEGF pro-angiogenic effect is reduced in plugs containing DLK1 or DAPT. Values represent mean \pm SEM from six mice ($*P < 0.05$ vs. Ad.GFP plus VEGF). (D) Haematoxylin- and eosin-stained sections of the plugs show decreased vascularization in Ad.DLK1, Ad.DLK1-GFP, and DAPT-treated plugs. Scale bars: A: 1 mm; B: 100 μm ; D: 50 μm .

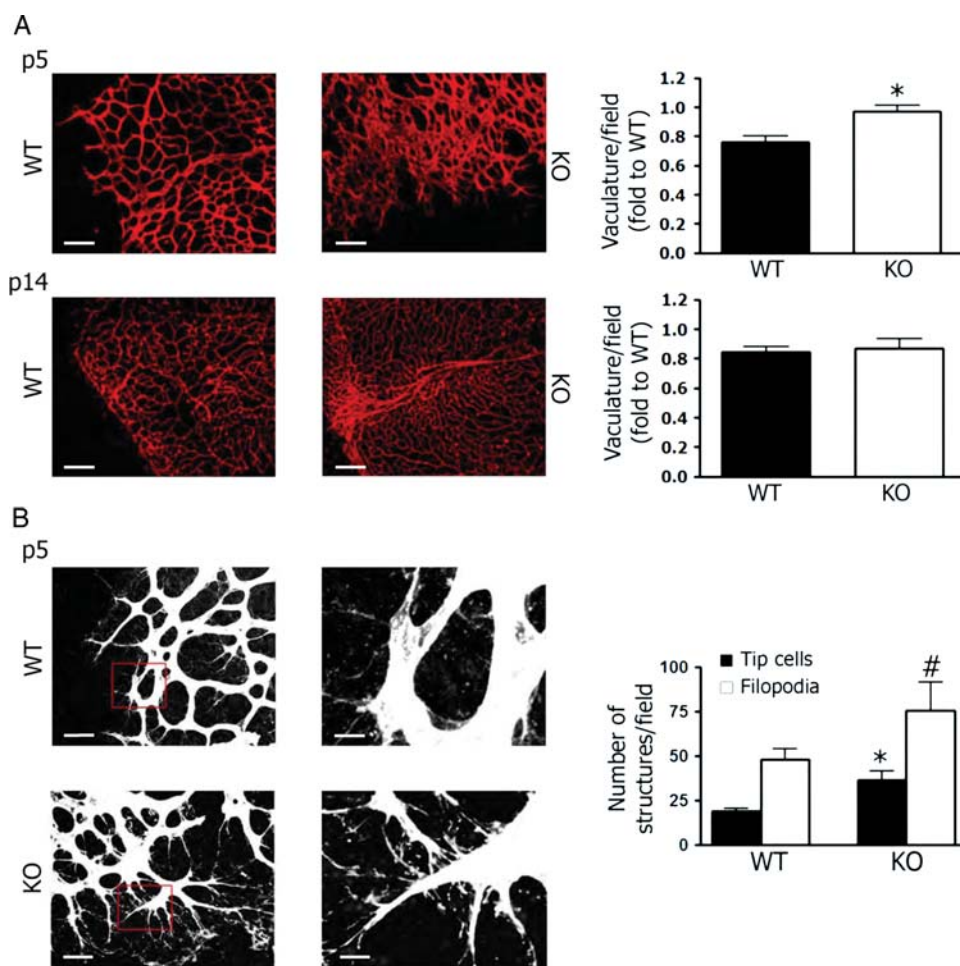


Figure 3 Altered retinal angiogenesis in DLK1-null mice. (A) Confocal images of retinas from wild-type or DLK1-null p5 and p14 pups stained for isolectin B4. Retinas of null mice p5 show a hyperfused plexus compared with wild-type. Right panel depicts the quantification of retinal areas covered by vessels. Bars represent mean \pm SEM from 10 mice ($*P < 0.05$). (B) Isolectin B4 immunofluorescence showing increased tip cell and filopodia in the DLK1-null p5 angiogenic front. Squared areas are blown-up in the right panels. Bar graph depicts the quantitative analysis of tip cells and filopodia ($*P < 0.05$). Scale bars: A: 200; B: 50 (left side) and 20 (right side) μm .

treated with the γ -secretase inhibitor DAPT which is a established inhibitor of the NOTCH pathway²⁴ (Figure 2C and D).

3.3 The absence of DLK1 correlates with focal retinal hyperangiogenesis

To confirm the antiangiogenic role of DLK1 *in vivo*, we studied the potential differences in retinal vessel formation in *Dlk1*-null mice compared with their wild-type counterparts. Retinas from null mice showed focal hypervascularization at p5 and p14 (Figure 3A; see Supplementary material online, Figure S3). This hypervascularization was enhanced at p5, indicating a more prominent role of DLK1 in the regulation of vascular leading edge progression (p5) than in remodeling (p14). In the developing murine retina, sprouting angiogenesis correlates well with the formation of endothelial tip cells and filopodia.¹² A detailed study of the affected regions in the retinas of *Dlk1*-null mice demonstrated an increased number of filopodia and tip cells (Figure 3B), consistent with a dysregulation of angiogenesis. Collectively, our results indicate that DLK1 acts as a brake for

proliferation, migration, and angiogenesis in the vascular endothelium of post-natal mammals.

3.4 DLK1 regulates vascular development and tumour angiogenesis in zebrafish

To investigate whether the role of DLK1 in angiogenesis could be extended to other vertebrate models, we performed experiments in embryos from *Danio Rerio* (zebrafish), in which the human version of DLK1 was microinjected and overexpressed in one-cell stage embryo. Three days after the injection, embryos expressing DLK1 showed an abnormal pattern of dorsal vascularization that was DLK1 dose-dependent, manifested in aberrant intersegmental vessel branching and a lack of an established dorsal longitudinal anastomotic vessel (Figure 4A–F; see Supplementary material online, Figure S4). To decipher whether DLK1 played a role by itself in the development of zebrafish vasculature, the endogenous orthologous mRNA was targeted with an MO. Upon MO injection, 6 days old larvae showed ectopic subintestinal angiogenesis (Figure 4G–J). This phenotype was MO dose-dependent and was rescued by the concomitant

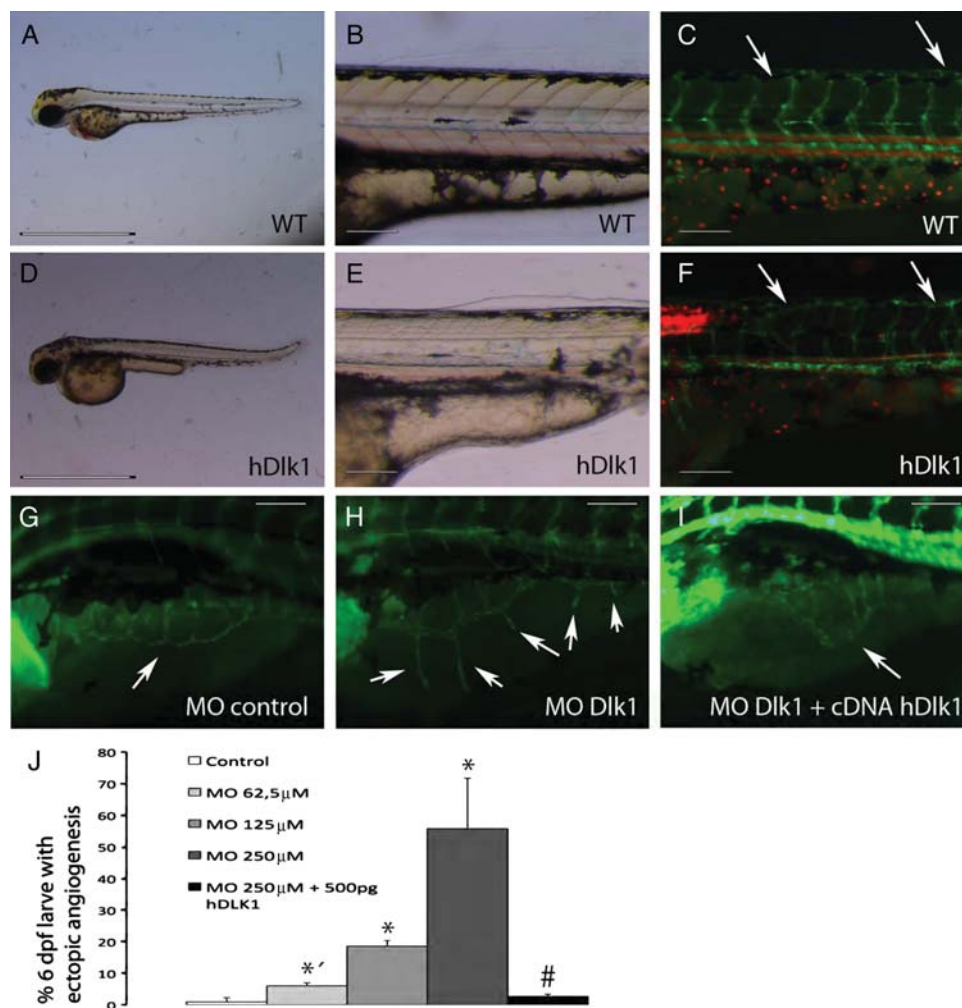


Figure 4 DLK1 affects angiogenesis in WT zebrafish embryos. (A–F) Tg(Fli:eGFP); Tg(gata1:dsRed) embryos at 72 h post-fertilization. (G–I) Tg(fli:eGFP) at day 6 post-fertilization. (A, B, D, and E) Bright-field images. (C and F) Fluorescent microscope images. General morphology of wild-type embryos (A) and intersegmental artery sprouts and GATA-dsRED-labelled erythrocytes (B and C) from a wild-type embryo injected with 500 pg of control cDNA plasmid. (D–F) Embryos injected with 500 pg of DLK1 cDNA. In (C) and (F), arrows highlight intersegmental arteries. (G) Embryo injected with 250 μM control MO oligonucleotide (MO control). (H) Ectopic sprouts of the SIV in a DLK1-morphant embryo. (I) Phenotype after co-injection of DLK1 MO (MO Dlk1) and DLK1 cDNA. (J) Quantification of ectopic angiogenesis in day 6 pf larvae injected with control MO, DLK1 MO alone, or co-injected with human DLK1 plasmid (hDLK1). Arrows identify SIV. Each experiment, which included 100–300 embryos, was repeated at least twice. Bars represent mean \pm SEM (* P < 0.05 vs. control; * P < 0.025 vs. control; # P < 0.025 vs. MO 250 μM). Scale bars: A and D: 2 mm; B, C and E–I: 200 μm.

expression of human DLK1 (Figure 4j). In a model of zebrafish xenograft tumour formation,²⁵ suppression of DLK1 resulted in a clearly visible migration of vessels towards the tumour cell mass, an effect reversed by co-injection with human DLK1 mRNA (Figure 5A–C). Our results suggest that DLK1 plays a role in the developing vasculature of zebrafish, hence supporting that this protein contributes to setting the general angiogenic programme.

3.5 DLK1 inhibits angiogenesis by antagonizing the NOTCH pathway

To study the potential interaction of DLK1 with the NOTCH signalling pathway in the context of angiogenesis, we used several approaches. First, we found that expression of the NOTCH-dependent luciferase reporter Hes-1 was reduced in a dose-dependent manner, in endothelial

cells expressing DLK1 (Figure 6A). This occurred at levels similar to the effect observed after treatment with the γ -secretase inhibitor DAPT (Figure 6B). We then studied the abundance of NOTCH signalling pathway-related proteins in MLEC from *Dlk1*-null mice and observed that the levels of the Notch1 intracellular domain (NICD) indicative of Notch1 activation and the downstream transcriptional effector Hey1 were markedly augmented (Figure 6C), thus suggesting that the absence of *Dlk1* might contribute to derepress the Notch pathway. As Notch receptors have been shown to regulate Snail family of proteins in specific types of endothelial cells,^{26,27} we examined whether their expression was altered in endothelial cells from *Dlk1*-null mice. We found increased levels of Snail mRNA and Slug protein in MLEC of *Dlk1*-null mice, compared with MLEC from wild-type mice (Figure 6D), suggesting that DLK1 is inhibiting the expression of Snail and Slug.

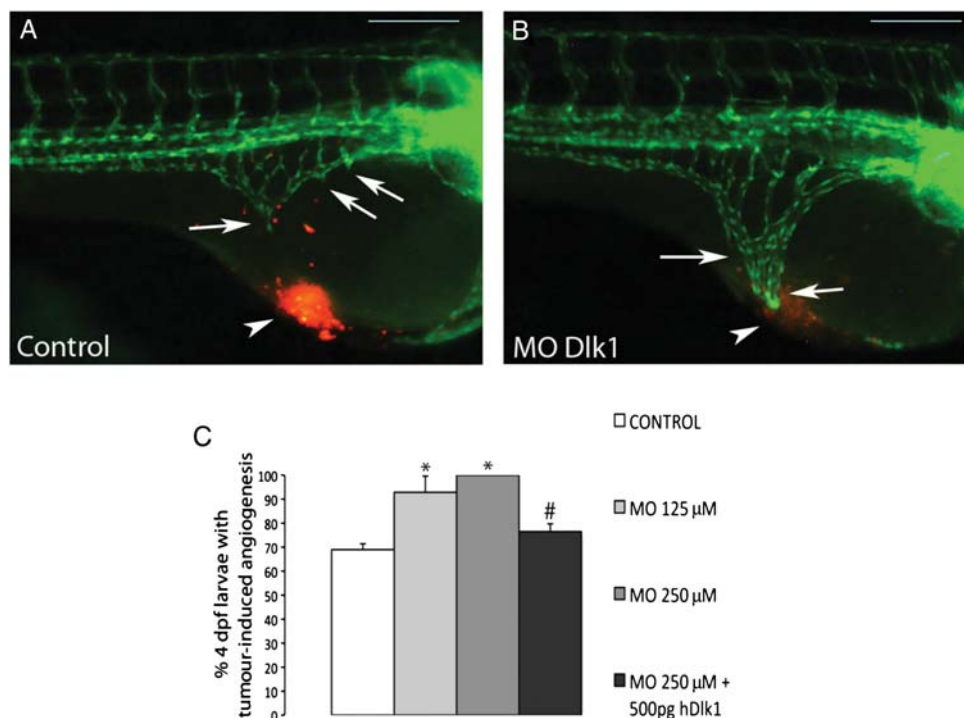


Figure 5 Knockdown of DLK1 promotes, and DLK1 inhibits, tumour-induced angiogenesis in zebrafish xenograft assay. Fluorescent microscopic images of (A) 4 dpf control and (B) MO-injected larvae xenografted with MDA-MB-435 tumour cells at 2 dpf. Arrows highlight SIV and arrowheads identify tumour cells. (C) Quantification of embryos showing tumour-induced angiogenesis when injected with MO against DLK1 at different concentrations or co-injected with human Dlk1 capped mRNA. Each condition, which included 25–50 embryos, was repeated at least twice. Bars represent mean \pm SEM (* $P < 0.025$ vs. control; # $P < 0.025$ vs. MO 250 μ M). Scale bars: 200 μ m.

3.6 DLK1 inhibits angiogenesis upstream of NOTCH intracellular signalling

To evaluate the step at which DLK1 was interfering with the NOTCH signalling pathway, we performed studies of angiotube formation in PAEC in resting conditions and in PAEC overexpressing NICD, which are engineered to express active NOTCH-dependent signals in a ligand–receptor independent manner. In keeping with previous reports,²⁷ PAEC stably expressing NICD showed high levels of Hes-1-dependent luciferase expression (data not shown). Expression of DLK1 resulted in the expected inhibition of angiotube formation, both in the presence and absence of VEGF (see Supplementary material online, Figure S5, upper panels). In contrast, angiotube formation in cells with constitutive NICD expression was not reduced by DLK1 (see Supplementary material online, Figure S5, lower panels), thus suggesting that DLK1 acts at the level of NOTCH receptors to regulate angiogenesis. To confirm that DLK1 was also inhibiting NOTCH signalling in another model, we performed a series of studies in zebrafish embryos expressing the NOTCH-dependent fluorescent effector RbpJk. As shown in Supplementary material online, Figure S6A and B, DAPT drastically reduced fluorescence associated with NOTCH signalling, while antagonism of DLK1 with an MO moderately enhanced it (see Supplementary material online, Figure S6C). In contrast, injection of embryos with the human Dlk1 mRNA promoted a marked abrogation of NOTCH-dependent fluorescence (see Supplementary material online, Figure S6D). Taken together, these results support the notion that DLK1 is negatively regulating

NOTCH signalling, likely by interfering with canonical ligand–receptor interaction.

4. Discussion

Since the initial description of DLK1 as an inhibitor of adipocyte and neuroendocrine differentiation, a growing body of evidence suggests that it may act as a general inhibitor of differentiation, including cell types of non-neuroendocrine lineage such as osteoblasts,²⁸ myocytes,²⁹ haematopoietic cells,³⁰ and chondrocytes.³¹ Work by one of our groups has established that DLK1 may antagonize the NOTCH pathway in several settings and that this antagonism relies on its capacity to act as a non-canonical ligand.⁴ Precisely, because an essential role of the NOTCH pathway is to regulate angiogenesis, we asked what role, if any, could be played by DLK1 in the context of this fundamental biological process. This work shows for the first time to our knowledge that DLK1 is: (i) expressed in adult endothelial cells and (ii) it acts as a powerful antiangiogenic factor in several models and organisms. Furthermore, we have been able to provide evidence supporting that this inhibition of angiogenesis is mediated by interference with the NOTCH pathway and plausibly with canonical ligand–receptor interaction.

Several reports lend a basis for the interaction between DLK1 and the NOTCH 1 receptor, suggesting that the former exerts an antagonistic role due to its capacity to bind to those NOTCH EGF-like repeats also involved in the interaction with NOTCH ligands.^{4,7,8}

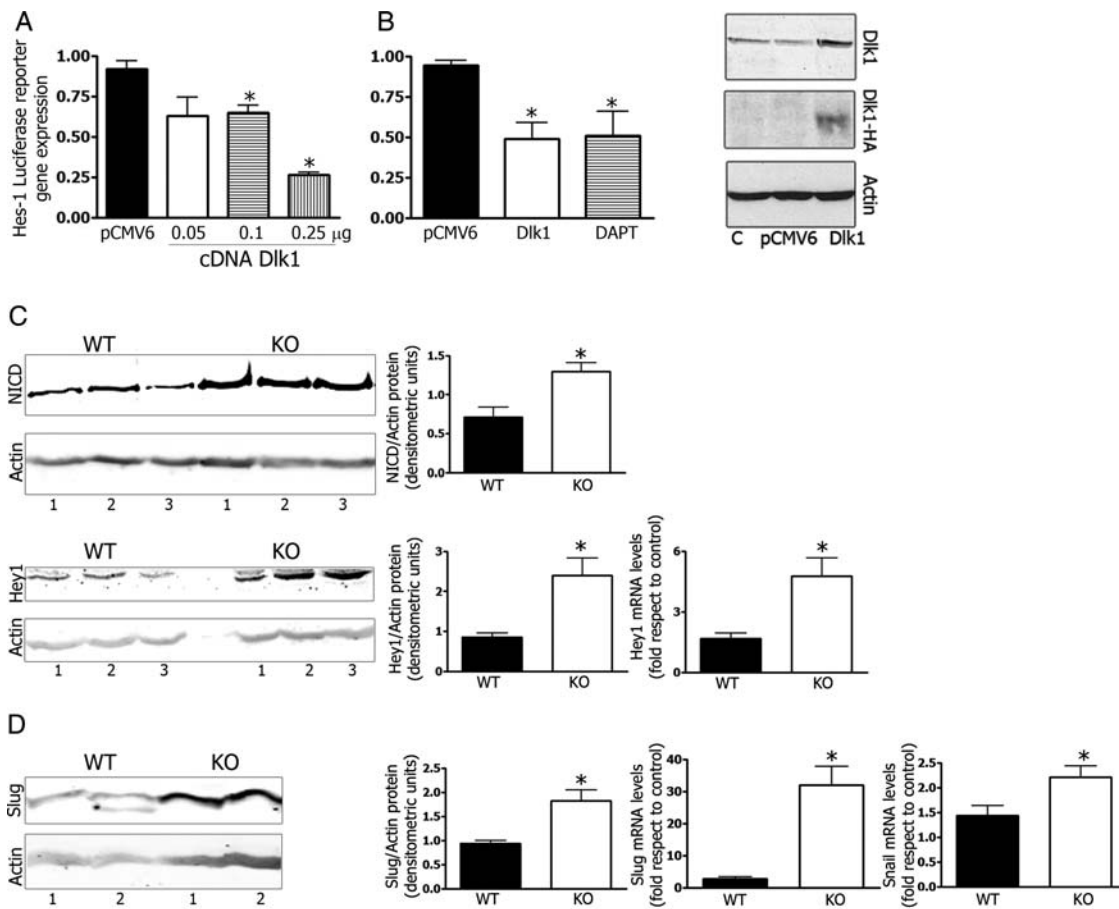


Figure 6 DLK1 inhibits angiogenesis by antagonizing the NOTCH signalling pathway. (A) Quantification of NOTCH signalling in bovine endothelial cells measured by Hes-1 activity. (B) cDNA DLK1 (0.25 μg) decreases Hes-1 activity to a similar extent than 0.5 μM of the γ-secretase inhibitor, DAPT. Bars represent mean ± SEM of three independent experiments assayed per triplicate (**P* < 0.05). Lysates from control cells or cells transfected with a control vector or with DLK1-HA (50 μg) were incubated with DLK1, HA, and actin antibodies. Densitometric analysis yielded a 2.5-fold increased DLK1 protein with respect to controls. (C) Representative western blots (*n* = 3) from Notch intracellular domain (NICD, upper panel) and Hey1 (lower panel) showing protein up-regulation in lung endothelial cells from KO mice. Quantification of four different mouse preparations per group by densitometry and qRT-PCR (mean ± SEM; **P* < 0.05). (D) Representative western blot of Slug protein (*n* = 3). Quantitative qRT-PCR and densitometric analysis showing Slug and Snail are enhanced in DLK1 KO mice (*n* = 4, mean ± SEM; **P* < 0.05).

However, other authors have proposed alternative modes of action for DLK1 invoking direct interaction with fibronectin³² and other molecules, such as IGFBP1,⁶ although these interactions do not involve the DLK1 EGF-like region. It has been established that three of the five canonical NOTCH ligands (Jagged 1, Dll1, and Dll4) are expressed in endothelial cells and play critical roles in vascular angiogenesis.¹² For example, maintenance of arterial identity relies on the Notch1–Dll interaction.^{33,34} Heterozygous deletion of Dll4 in mice or pharmacological inhibition of NOTCH signalling was associated with increased numbers of filopodia-extending endothelial tip cells and increased expression of tip cell marker genes compared with controls,³⁵ thus suggesting that Dll4 is a negative regulator of vascular sprouting. Dll4 and Jagged1 have opposing effects on sprouting angiogenesis mediated by VEGF signalling, by controlling the equilibrium between tip and stalk cells through a mechanism regulated by the glycosyltransferase Fringe, which post-translationally modifies the NOTCH receptors.³³ In zebrafish, the absence of NOTCH promotes a shift towards the tip cell phenotype, favouring vessel sprouting, and

loss of Dll4 was associated with an increased endothelial cell number.³⁶ In addition, several studies have found that the NOTCH pathway is generally related to angiogenesis inhibition, at least when the canonical ligand Dll4 is involved (see Phng and Gerhardt³⁷ for review). The interaction between canonical and non-canonical NOTCH ligands is a complex one³⁸ and further studies are needed to establish the precise interplay of DLK1 with the canonical NOTCH ligands in the vasculature. The phenotype found in the retinas of *Dlk1*-null mice (increased number of vessels, tip cells, and filopodia in selective areas) is consistent with the antiangiogenic action of DLK1 observed in cultured cells and in the zebrafish model. Our data clearly show that Dlk1 interferes with Notch signalling but do not define the precise level of interaction or the Notch canonical ligand potentially affected. Thus, a straightforward interpretation of discrepancies between the vascular phenotype observed in this study in mice and zebrafish and those previously reported in models bearing genetic modifications of NOTCH canonical ligands is still premature.

Antagonism of the NOTCH pathway by the use of decoys has proven to be effective in animal models of tumour progression³⁹ and the use of endogenous inhibitors of the NOTCH pathway has been claimed to represent a potential therapeutic avenue.⁴⁰ The regulation of angiogenesis in tumours is the object of intense study in the context of providing effective means of deferring or suppressing human tumoral growth. However, tumours seem to find mechanisms of escape from anti-VEGF-directed therapies,⁴¹ and hence, it is essential to search for new candidates and targets mediating an antiangiogenic effect. In this pathological setting, and supported by our observations in zebrafish tumour-induced angiogenesis, it will be worth determining the potential of DLK1 to abrogate tumour-dependent angiogenesis in mammals.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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Conflict of interest: A provisional patent based on this work was filed in October 2010 (ES1641.793) by Consejo Superior de Investigaciones Científicas (CSIC).

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