RESEARCH REPORT

Dynamin 2 regulation of integrin endocytosis, but not VEGF signaling, is crucial for developmental angiogenesis

Monica Y. Lee1,2,*, Athanasia Skoura1,2,*, Eon Joo Park1,2, Shira Landskroner-Eiger1,2, Levente Jozsef1,2, Amelia K. Luciano1,2, Takahisa Murata3, Satish Pasula4, Yunzhou Dong4, Mohamed Bouaouina2, David A. Calderwood1,2,5, Shawn M. Ferguson4, Pietro De Camilli5,6 and William C. Sessa1,2,‡

ABSTRACT

Here we show that dynamin 2 (Dnm2) is essential for angiogenesis in vitro and in vivo. In cultured endothelial cells lacking Dnm2, vascular endothelial growth factor (VEGF) signaling and receptor levels are augmented whereas cell migration and morphogenesis are impaired. Mechanistically, the loss of Dnm2 increases focal adhesion size and the surface levels of multiple integrins and reduces the activation state of β1 integrin. In vivo, the constitutive or inducible loss of Dnm2 in endothelium impairs branching morphogenesis and promotes the accumulation of β1 integrin at sites of failed angiogenic sprouting. Collectively, our data show that Dnm2 uncouples VEGF signaling from function and coordinates the endocytic turnover of integrins in a manner that is crucially important for angiogenesis in vitro and in vivo.

KEY WORDS: Angiogenesis, Endocytosis, Endothelium, Mouse

INTRODUCTION

Dynamin (Dnm) is a GTPase essential for membrane fission leading to clathrin-mediated endocytosis (Ferguson and De Camilli, 2012). There are three isoforms of Dnm in mammalian cells, Dnm1, Dnm2 and Dnm3, which are encoded by distinct genes that are differentially expressed in various tissues (Ferguson et al., 2009). Mice deficient in Dnms have been generated and the loss of neuronally enriched isoforms, Dnm1 and Dnm3, results in impaired neurotransmission due to a defect in synaptic vesicle endocytosis (Ferguson et al., 2007; Raimondi et al., 2011). The loss of Dnm2 leads to early embryonic lethality and the loss of Dnm1/2 in podocytes regulates renal glomerular filtration (Soda et al., 2012). Recent evidence largely based on pharmacological inhibition of Dnm function (Gourlaouen et al., 2013; Lanahan et al., 2010; Sawamiphak et al., 2010; Wang et al., 2006; Wang et al., 2010) suggests that clathrin-mediated endocytosis is necessary for vascular endothelial growth factor (VEGF) signaling in vitro and angiogenesis in vivo. The VEGF receptor 2 (Vegfr2; Kdr – Mouse Genome Informatics) receptor complex interacts with the Eph receptor ligand ephrin B2, and this complex is internalized via clathrin to promote angiogenesis (Wang et al., 2011b). Moreover, the cytoplasmic domain of ephrin B2 interacts with a complex containing clathrin, the general clathrin adaptor complex (AP2), Dab2, Dnm2 and myosin VI (Nakayama et al., 2013). The endothelial-specific loss of Dab2 attenuates Vegfr2 internalization and angiogenesis, and loss of aPKC increases endocytosis and signaling, implying that Vegfr2 internalization is essential for signaling and angiogenesis. However, there are additional data suggesting that the cargo-specific endocytic clathrin adaptor molecules epsin 1 and epsin 2 can terminate VEGF signaling as the genetic loss of epsins impedes Vegfr2 endocytosis, increases VEGF signaling and promotes abnormal pro-angiogenic functions (Pasula et al., 2012). Indeed, the interaction of epsins with ubiquitylated Vegfr2 allows for its clathrin-mediated internalization and destruction. Clearly, the role of endocytosis in the angiogenesis response is complex and is influenced by diverse routes of endocytosis and multiple cargo-specific clathrin adaptor molecules. However, the role of the central GTPase in endocytosis, Dnm2, has not been genetically dissected in endothelium. Here, we show that Dnm2, the major isoform of Dnm expressed in endothelial cells (ECs), is essential for developmental angiogenesis. In cultured ECs depleted of Dnm2, VEGF receptor endocytosis is impaired and VEGF signaling is enhanced. Despite augmented VEGF signaling, VEGF-induced cell migration and tubular morphogenesis is impaired, implying that pathways downstream of Vegfr2 signaling are regulated by Dnm2. Mechanistically, the loss of Dnm2 or clathrin increases focal adhesion size and impairs integrin internalization thereby reducing cell motility and morphogenesis. Moreover, the loss of Dnm2 in endothelium in vivo attenuates branching angiogenesis and promotes the accumulation of β1 integrin at sites of blunted angiogenic sprouts, replicating the data in cultured ECs. Thus, Dnm2-dependent endocytosis uncouples VEGF signaling from angiogenic pathways and is crucial for the endocytic turnover of integrins and organization of EC during angiogenesis in vivo.

RESULTS AND DISCUSSION

To examine the role of endocytosis during angiogenesis, we investigated the expression of all three Dnm proteins in mouse lung ECs (MLECs) and found that Dnm2 is the major isoform expressed (supplementary material Fig. S1A). Indeed, reducing Dnm2 or clathrin heavy chain (CHC; CLTC – Human Gene Nomenclature Database) levels by 80-90% via siRNA in human umbilical vein ECs (HUVECs) significantly reduced the endocytosis of fluorescently labeled human transferrin, a well-characterized cargo molecule dependent on Dnm and clathrin (Ferguson et al., 2009) (Fig. 1A). DNM2 knockdown increased the surface accumulation of actin nucleating protein p34 subunit of the Arp2/3 complex and the clathrin adaptor adaptin 2, consistent with data in fibroblasts.
Fig. 1. Loss of Dnm2 regulates VEGF signaling but reduces angiogenesis. (A) Loss of DNM2 or CHC (50 nM of each) reduces transferrin endocytosis in HUVECs. n=4 experiments. Inset shows knockdown efficiency of corresponding siRNAs. (B) Localization of Arp2/3 complex and AP2 (adaptin 2) in control and Dnm2-deficient HUVECs. Scale bars: 7.5 μm. (C-E) Effect of VEGF stimulation (50 ng/ml) on signaling. (C) Representative blot and quantification (from four individual experiments) showing enhanced VEGF signaling via analysis of phospho-proteins. (D,E) Calcium (D) and surface Vegfr2 (E) levels in Dnm2-depleted HUVECs (from four individual experiments). (F,G) Basal, serum-driven and VEGF-driven HUVEC migration (F) and cord formation (G) was markedly impaired by Dnm2 siRNA. Data are from three independent experiments repeated in duplicates. SCR, scrambled siRNA. Error bars represent s.e.m. *P<0.05, **P<0.001.

The loss of DNM2 had no effect on cell viability or apoptosis (supplementary material Fig. S1B). As DNM2-dependent endocytosis has been implicated in VEGF signaling, HUVECs treated with scrambled or DNM2 siRNA were stimulated with VEGF (50 ng/ml) and downstream pathways examined. VEGF signaling was increased in HUVECs lacking DNM2, as demonstrated by enhanced phosphorylation of VEGFR2 (KDR – Human Gene Nomenclature Database), PLCγ, ERK and AKT (Fig. 1C) and by VEGF-induced changes in intracellular calcium (Fig. 1D). Moreover, the loss of DNM2 increased surface Vegfr2 levels as quantified by fluorescence-activated cell sorting (FACS) (Fig. 1E). Interestingly, despite enhanced VEGF signaling, serum- and VEGF-driven chemotaxis (Fig. 1F), and cord formation (Fig. 1G) are impaired whereas F-actin rich stress fibers are
increased (supplementary material Fig. S1C) in cells that lack DNM2.

The reduction in cell motility, impaired morphogenesis and increased stress fibers triggered by the loss of DNM2 suggested that DNM2 may be regulating integrin-dependent adhesive properties. Previous work in other cell types have shown that clathrin-mediated endocytosis is essential for integrin recycling and focal adhesion disassembly and that Dnm2 can interact with clathrin adaptors or focal adhesion kinase (FAK; Ptk2 – Mouse Genome Informatics) to regulate focal adhesions (Arjonen et al., 2012; Chao et al., 2010; Chao and Kunz, 2009; Ezratty et al., 2009; Ezratty et al., 2005; Nishimura and Kaibuchi, 2007; Wang et al., 2011a). Therefore, we tested whether Dnm2 could regulate adhesion dynamics and integrin endocytosis in primary ECs. Immunofluorescence staining for paxillin and FAK (Fig. 2A) showed increased size of focal adhesions (quantified in Fig. 2B) in cells depleted of Dnm2 or CHC.

![Image](image-url)

**Fig. 2. Dnm2 regulates focal adhesion size and integrin internalization.** (A,B) Representative images of paxillin and FAK accumulation in DNM2-deficient cells (A) and quantification of these data (B; triplicate transfections of siRNA from an experiment, repeated three additional times). (C) Loss of DNM2 or CHC increases surface, but not total, levels of β1 integrin as detected by surface biotinylation (as described in Materials and Methods). Blot shown is representative of four experiments. (D,E) FACS analysis of surface αvβ5, αvβ3 and β1 integrins after DNM2 knockdown in HUVECs (D) and quantification of these data (E). n=3-4 experiments. (F) Levels of active β1 integrin (via binding FN 9-11) relative to total β1 levels (integrin activation index) in HUVECs treated with DNM2 siRNA were reduced in the absence of DNM2. Data represent results from three to four independent experiments. (G) Adhesion to fibronectin is reduced in DNM2 siRNA-treated cells. O.D., optical density. Data are from four independent experiments. SCR, scrambled siRNA. Error bars represent s.e.m. *P<0.05, **P<0.01, ***P<0.001.
(supplementary material Fig. S2). As seen in Fig. 2C, reducing levels of Dnm2 or CHC (Cltc – Mouse Genome Informatics) via siRNA-enhanced β1 integrin levels at the cell surface (surface fraction) without altering the total levels of this integrin in whole cell lysates. This was confirmed by FACS in non-permeabilized cells using β1-, αvβ3- or αvβ5 integrin selective antibodies (Fig. 2D,E). To examine if the loss of Dnm2 has a net effect on integrin activation, FACS (see supplementary material Fig. S3 for profiles and gating) was performed using a GST fusion protein composed of fibronectin type III repeats 9 through 11 (FN 9-11), which selectively binds to activated integrins (Bouaouina et al., 2012). As seen in Fig. 2F, the loss of Dnm2 reduced the ratio of active to total pools of β1 integrin, implying that despite an elevation of surface integrins by the loss of Dnm2, the net activation state was reduced. Finally, we examined whether the loss of Dnm2 affected integrin-matrix interactions by quantification of EC interactions with fibronectin, a ligand for β1 and β3 integrins. As seen in Fig. 2G, the loss of Dnm2 reduced EC adhesion to fibronectin, consistent with increased levels of inactive integrins and this effect was also seen using electric cell-substrate impedance sensing (ECIS) resistance measurements (supplementary material Fig. S4).

To study the role of Dnm2 function in angiogenesis in vivo, we inactivated Dnm2 in ECs via Cre/loxP-mediated recombination of floxed Dnm2 using a Tie2 CRE mouse (supplementary material Fig. S5A,B). EC deletion of Dnm2 caused gross morphological defects at embryonic day (E) 9.5 and no live embryos remained at Fig. S5A,B). EC deletion of Dnm2 caused gross morphological defects at embryonic day (E) 9.5 and no live embryos remained at (supplementary material Fig. S5A,B). As seen in Fig. 2F, the loss of Dnm2 reduced the ratio of active to total pools of β1 integrin, implying that despite an elevation of surface integrins by the loss of Dnm2, the net activation state was reduced. Finally, we examined whether the loss of Dnm2 affected integrin-matrix interactions by quantification of EC interactions with fibronectin, a ligand for β1 and β3 integrins. As seen in Fig. 2G, the loss of Dnm2 reduced EC adhesion to fibronectin, consistent with increased levels of inactive integrins and this effect was also seen using electric cell-substrate impedance sensing (ECIS) resistance measurements (supplementary material Fig. S4).

To study the role of Dnm2 function in angiogenesis in vivo, we inactivated Dnm2 in ECs via Cre/loxP-mediated recombination of floxed Dnm2 using a Tie2 CRE mouse (supplementary material Fig. S5A,B). EC deletion of Dnm2 caused gross morphological defects at embryonic day (E) 9.5 and no live embryos remained at Fig. S5A,B). As seen in Fig. 2F, the loss of Dnm2 reduced the ratio of active to total pools of β1 integrin, implying that despite an elevation of surface integrins by the loss of Dnm2, the net activation state was reduced. Finally, we examined whether the loss of Dnm2 affected integrin-matrix interactions by quantification of EC interactions with fibronectin, a ligand for β1 and β3 integrins. As seen in Fig. 2G, the loss of Dnm2 reduced EC adhesion to fibronectin, consistent with increased levels of inactive integrins and this effect was also seen using electric cell-substrate impedance sensing (ECIS) resistance measurements (supplementary material Fig. S4).

Downregulation of Dnm2 expression was achieved by oral gavage of tamoxifen (2 mg) into pregnant females at either E9.5 or E10.5 (Benedito et al., 2009; Pitulescu et al., 2010). Two days after the initiation of gene inactivation in ECs (E11.5-E12.5), Dnm2ΔEC embryos were macroscopically indistinguishable from littermate controls; however, detailed examination revealed the formation of subcutaneous hemorrhages (Fig. 3I). Histological serial cross-sections of E12.5 embryos revealed the presence of diluted vessels filled with blood that diffused into the abluminal space (Fig. 3J, top right panel). Finally, deletion of Dnm2 at E12.5 led to significant impairments in branching in the hindlimb (Fig. 3J, middle panels) and yolk sac (Fig. 3J, bottom panels). These observations demonstrate that the absence of Dnm2 impairs developmental angiogenesis and vascular patterning in mouse embryo.

Postnatal inactivation [initiated at postnatal day (P) 1 and analyzed at P5] of Dnm2 in Dnm2ΔEC mice (P5) displayed impaired retinal vascular development demonstrated by reduced endothelial cell coverage, delayed radial growth towards the periphery (Fig. 4A,D), reduced branching (Fig. 4E,F) and impaired filopodia/tip cells at the sprouting angiogenic front of the retina, but no significant differences in the thickness of the vessels and proliferation (supplementary material Fig. S6A,B) based on phospho-histone-3 staining (Fig. 4C). Dnm2ΔEC pups (P5) were a similar weight to littermate controls (supplementary material Fig. S6C). The loss of Dnm2 had no effect on the distribution of NG2 (Cspg4 – Mouse Genome Informatics)-positive pericytes and GFAP-positive astrocytes (supplementary material Fig. S6D,E). Furthermore, Dnm2 inactivation (P5-P9) showed that remodeling of established vessels in the superficial capillary plexus at P12 is strongly altered (Fig. 4G) and this difference was not due to growth impairment (supplementary material Fig. S6F). Centrifugal outgrowth and sprouting of the nerve fiber layer (NFL) vasculature into the outer plexiform layer (OPL) was compromised in Dnm2ΔEC retinas resulting in cyst-like outgrowths (Fig. 4H, asterisks). Collectively, these data demonstrate that the postnatal lack of Dnm2 in endothelium diminishes sprouting angiogenesis.

Finally, to examine whether Dnm2 regulates the localization and distribution of Vegfr2 and β1 integrin in vivo, Dnm2 was inactivated by injection of tamoxifen at P1-P3 and FACS was carried out for surface β1 integrins and Vegfr2 levels in retinal single cell isolates at P6. The loss of Dnm2 increased the surface expression of both proteins in vivo as seen in Fig. 4I,J. Moreover, β1 integrin was diffusely distributed throughout the growing vascular networks and at this time point did not show preference for expression in cells at the growing front versus mature stalk cells (Fig. 4K). However, in retinal ECs lacking Dnm2, there was a marked accumulation of β1 integrin in blunt tipped cells near the angiogenic front. Thus, Dnm2-dependent endocytosis is crucial for the proportional turnover of Vegfr2 and β1 integrin needed for sprouting angiogenesis in vivo.

Collectively, the data reveal a major role for Dnm2 in endothelial cell functions in vitro and developmental angiogenesis in vivo. Our genetic data in conjunction with results from endothelial-specific epsin-deficient mice (Pasula et al., 2012) indicates that endocytosis negatively regulates VEGF signaling. Epsins selectively bind to ubiquitylated Vegfr2, but not integrins, permitting increased Vegfr2 levels, signaling and function, whereas the loss of Dnm2 blocks endocytosis of both Vegfr2 and integrins, resulting in reduced angiogenesis. These data imply that a dominant function of Dnm2 during angiogenesis is to regulate the assembly/disassembly of cell-matrix adhesions, key molecular events that promote endothelial morphogenesis and vessel stability.

Upon first glance, our data may appear to contradict previous work showing that Vegfr2 endocytosis is necessary for signaling and angiogenesis. Previously, Lanahan et al. (Lanahan et al., 2010) documented that the loss of synectin or myosin VI did not affect the movement of Vegfr2 from the cell surface to the endosome but indeed reduced, not eliminated, intracellular VEGF signaling. Lampugnani et al. (Lampugnani et al., 2006) showed that the loss of clathrin reduced Vegfr2 endocytosis, but VEGF signaling was only suppressed in cells lacking VE-cadherin (also known as cadherin 5), not in normal ECs. In Wang et al. (Wang et al., 2010), the authors injected dynasore, a Dnm GTPase inhibitor, into the developing retina to show that Dnm was required for Vegfr2 signaling; however, a recent paper now shows this class of inhibitors block endocytosis in cells lacking all Dnms, showing that these inhibitors are non-specific (Park et al., 2013). Our work and the work on epsins clarifies that blocking endocytosis using discrete molecular tools increases VEGF signaling, consistent with a recent paper showing that Dnm2 negatively regulates epidermal growth factor receptor (EGFR) signaling (Sousa et al., 2012). However, once signaling is initiated at the cell surface, the subsequent
endocytosis of Vegfr2 can promote additional post-plasma membrane signaling.

Mechanistically, impairments in integrin internalization and inactivation are likely to explain the defective morphogenesis and remodeling observed in Dnm2\textsuperscript{I\textDelta EC} mice. Indeed, endothelial cell deletion of the β1 integrin subunit leads to early lethality (E9.5) with defects in vascular branching phenocopying the Dnm2\textsuperscript{I\textDelta EC}; Tie2Cre vascular defects (Carlson et al., 2008; Lei et al., 2008; Tanjore et al., 2008; Zovain et al., 2010). In the present paper, the loss of Dnm2 impedes endocytosis of the three integrins tested; thus, the loss of Dnm2 retards the endocytosis and recycling of integrins, resulting in the net accumulation of inactive integrins. However, Dnm2 regulation of integrins is not likely to be an exclusive mechanism because endocytosis of ephrin B2, Notch and Notch ligands or VE-cadherin may also contribute to the Dnm2 knockout phenotypes (Alghisi et al., 2009; Wang et al., 2006; Wang et al., 2011b). In summary, our results provide the first definitive molecular insights into the role of Dnm-mediated endocytosis in angiogenesis in vivo and show that VEGF signaling can be uncoupled from pro-angiogenic endothelial cell functions, suggesting that endocytosis may be an ‘Achilles heel’ to attack pathological angiogenesis in disease.
MATERIALS AND METHODS

RNAi experiments and immunofluorescence
HUVECs (P2-P6) were transfected by Oligofectamine (Invitrogen) according to the manufacturer’s instructions with siRNA of control (luciferase), human DNM2 or clathrin heavy chain (CHC) designed by Qiagen. For endocytosis experiments, human Transferrin Alexa 568 (Invitrogen, T13343) was added to cells and incubated at 4°C for 15 minutes (5 mg/ml stock, 1:400). Plates were transferred back to 37°C and incubated for 30 minutes. To remove cell surface-bound transferrin, cells were washed with cold PBS, pH 2.7, containing 25 mM glycine and 3% bovine serum albumin and fixed with 4% paraformaldehyde for 15 minutes, on ice.

Fig. 4. Dnm2 is crucial for retinal angiogenesis and β1 integrin levels in vivo. (A) Representative images of reduced radial retinal EC growth in Dnm2ΔEC pups (P5). (B) Emerging tip cell front of angiogenic vessels (P5). (C) Lack of Dnm2 in EC does not influence growth, as determined by PH3 staining. (D) Quantification of data shown in A. (E,F) Loss of Dnm2 in ECs leads to reduced branching (E,F; control, n=4 and Dnm2ΔEC, n=11) and impaired filopodia. (G,H) Remodeling of established vessels in the superficial capillary plexus at P12 is strongly altered in Dnm2ΔEC pups (100 μg TMX, P5-P12). P12 Dnm2ΔEC pups with compromised centrifugal outgrowth (H). Centrifugal outgrowth of the nerve fiber layer (NFL) and compromised sprouting into the outer plexiform layer (OPL). Zooming into the NFL shows cyst-like outgrowths in Dnm2ΔEC pups. (I,J) FACS plots of retinal cells isolated from P6 mice showing elevated surface Vegfr2 (I) and β1 integrin (J) in Dnm2ΔEC pups. (K) Distribution of β1 integrin is abnormal in Dnm2ΔEC retinas. *P<0.05, **P<0.001. Scale bars: 5 mm (A); 50 μm (B); 100 μm (C); 2 mm (G,H,J); 250 μm (K).
Biotin surface labeling
HuVECs were cultured for 72 hours post-seeding, then fixed with 4% PFA/PBS, and stained with 0.1% goat anti-mouse IgG, respectively, for immunoblot analysis of β1 integrin (BD Biosciences).

Analysis of integrin activation and expression
siRNA-treated HuVECs were used for analysis of surface integrin expression and activation by measuring the binding of a fibronectin fragment (FN9-11) as described previously (Bouaouina et al., 2012). Total β1, αVβ3 and αVβ5 integrin expression levels were measured in parallel by staining, respectively, with anti-β1 (clone TS2/16, Biologend), anti-αVβ3 (clone 23C6, Biologend) and anti-αVβ5 (clone 15F11, Millipore) antibodies. Bound FN9-11 and integrin expression were detected with Allophycocyanin (APC)-conjugated streptavidin (Thermoscientific) or AlexaFluor647-conjugated donkey anti-mouse IgG, respectively, as described in supplementary material.

Generation of Dnm2 conditional deletion in the endothelium
Dnm2 conditional knockout targeting strategy was previously reported (Ferguson et al., 2009). EC Dnm2-deficient mice were generated by crossing Dnm2flox/flox females with Tie2Cre males (Jackson Laboratory). Tamoxifen (Sigma, T5648)-inducible Dnm2iE1112 (clone TS2/16, Biologend), anti-αVβ3 (clone 23C6, Biologend) and anti-αVβ5 (clone 15F11, Millipore) antibodies. Bound FN9-11 and integrin expression were detected with Allophycocyanin (APC)-conjugated streptavidin (Thermoscientific) or AlexaFluor647-conjugated donkey anti-mouse IgG, respectively, as described in supplementary material.

Immunohistochemistry and immunofluorescence of mouse tissues
Hematoxylin and Eosin staining was performed with Mayer’s Hematoxylin (10 minutes) and counterstained with Eosin (1 minute). Images were processed with a Nikon Eclipse 80i microscope and using the Photoshop CS software. For whole-mount staining, freshly isolated embryos were washed with a 50 mM glycine/PBS solution, pH 7.2 and subsequently harvested in protein lysis buffer. Following centrifugation, an aliquot was processed with a Nikon Eclipse 80i microscope and using the Photoshop CS software. For whole-mount staining, freshly isolated embryos and yolk sacs were processored with a Nikon Eclipse 80i microscope and using the Photoshop CS software. For whole-mount staining, freshly isolated embryos and yolk sacs were processed with a Nikon Eclipse 80i microscope and using the Photoshop CS software.

Adhesion assay
Ninety-six-well plates were coated with fibronectin (5 μg/ml, 354008, Becton Dickinson) at 4°C overnight. Plates were blocked with 10 mg/ml heat-denatured bovine serum albumin for 30 minutes. A set volume (100 μl) of HuVECs in cell suspension (5×105 cells/ml in basal medium with 0.1% BSA) were added onto plates and incubated for 30 minutes at 37°C. After incubation, non-adherent cells were removed by rinsing with PBS. Attached cells were counted in 4% PFA/PBS for 20 minutes and stained with 0.1% Triton X-100 for 1 hour. Embryos were incubated overnight at 4°C and cut into 8 μm serial cross-sections. All experimental procedures were approved by the Yale University Institutional Animal Care Use Committee.

Mouse retina vascular system analysis
For retina staining, procedures were followed as described (Pitulescu et al., 2010) with minor modifications. Pups (P1-P3) were injected with 50 μg tamoxifen and for later stages (P12) a 100 μg was used (P5-P9). Briefly, retinas were dissected out, fixed, permeabilized and stained with Alexa-594-conjugated isocyan B4 (Invitrogen, I21413; 1:200), rabbit anti-phospho-histone H3 (Cell Signaling, 9701; 1:200), rabbit anti-GFAP (Neomarkers, RB-087-A0; 1:200) and rabbit anti-NG2 (Millipore, AB5320; 1:200).

Whole retina FACS
Retinas of control or Dnm2iE1C mice were excised at P6 (n=5-7 per group) and digested in Type I Collagenase for 45 minutes at 37°C (Sigma), filtered via a 40-μm cell strainer and washed with PBS/0.5% fetal bovine serum, pH 7.4. Cells were blocked with mouse BD Fc Block (BD Pharmingen, 553142), followed by staining for either phycoerythrin (PE)-conjugated VEGFR-2 (BD Pharmingen) or PE-conjugated Integrin β1 (ebiosciences). Cells were measured by FACSaCalibur flow cytometer and analyzed using FlowJo software.

Statistical analyses
Statistical analyses were performed with Prism 5 software (GraphPad) using the two-tailed, unpaired Student’s t-test or one-way ANOVA, when appropriate. P values <0.05 were considered statistically significant.

Acknowledgements
The authors would like to acknowledge Drs Anne Eichmann, Martin Schwartz and David Cheresh for helpful comments and suggestions.

Competing interests
The authors declare no competing financial interests.

Author contributions

Funding
This work was supported by the National Institutes of Health [RO1 grants HL64793, HL61371, HL081190, HL096670 and PO1 1070205 to W.C.S.; RO1 GM068600 and GM086240 to D.A.C.]. Deposited in PMC for release after 12 months.

Supplementary material
Supplementary material available online at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.104539/-/DC1

References


