Endothelial basement membrane limits tip cell formation by inducing Dll4/Notch signalling *in vivo*

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How individual components of the vascular basement membrane influence endothelial cell behaviour remains unclear. Here we show that laminin $\alpha 4$ (Lama4) regulates tip cell numbers and vascular density by inducing endothelial Dll4/Notch signalling *in vivo*. Lama4 deficiency leads to reduced Dll4 expression, excessive filopodia and tip cell formation in the mouse retina, phenocopying the effects of Dll4/Notch inhibition. Lama4-mediated Dll4 expression requires a combination of integrins *in vitro* and integrin $\beta 1$ *in vivo*. We conclude that appropriate laminin/integrin-induced signalling is necessary to induce physiologically functional levels of Dll4 expression and regulate branching frequency during sprouting angiogenesis *in vivo*.

Keywords: integrin; laminin; Notch signalling; sprouting angiogenesis; vascular basement membrane

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INTRODUCTION

During sprouting angiogenesis, blood vessels respond to hypoxia-induced vascular endothelial growth factor A (VEGF-A) gradients by directed filopodia extension and migration of the leading endothelial cell (EC), referred to as the tip cell (Gerhardt *et al*, 2003). The following stalk ECs proliferate and establish firm junctions to maintain and stabilize the newly formed sprout. Recent studies in mouse and zebrafish show that VEGF-A/VEGF receptor 2 (VEGFR2)-induced Dll4/Notch signalling functions to regulate tip cell formation and subsequently vessel density (Hellstrom *et al*, 2007; Leslie *et al*, 2007; Lobov *et al*, 2007; Siekmann & Lawson, 2007; Suchting *et al*, 2007). Disruption of Notch signalling by pharmacological inhibitors or by genetic deletion of the Notch receptor or one allele of Dll4 ligand results in increased tip cell formation and enhanced sprouting angiogenesis. Dll4 signalling to neighbouring ECs promotes Notch activation and thereby inhibits active tip cell formation, suggesting that endothelial tip and stalk cell specification is both spatially and temporally regulated by Dll4/Notch signalling.

Stabilization of the nascent sprout is accompanied by the recruitment of mural cells and the production of extracellular matrix (von Tell *et al*, 2006; Stratman *et al*, 2009). The abluminal basement membrane (BM) in mature blood vessels provides structural support and promotes EC adhesion, migration and survival (Hallmann *et al*, 2005; Davis & Senger, 2008). The BM mainly comprises laminin, collagen, fibronectin and proteoglycans, with various isoforms combining to form structurally and functionally distinct BMs (Timpl, 1996; Hamill *et al*, 2009). Laminins are heterotrimeric glycoproteins, composed of one α -, one β - and one γ -chain. The laminin α -chains show

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Fig 1 | Distinct expression and localization of laminin $\alpha 4$ in nascent endothelial BM at endothelial tip cells. *Lama4* (black, A and blue, B) and *Lama5* (black, C and blue, D) *in situ* hybridization (ISH) of mouse postnatal retinas (postnatal day 5). Blood vessels were stained with isolectin B4 (green, B,D) and the astrocytic network with glial fibrillary acidic protein (GFAP; red, B,D). (B,D) Three-dimensional opacity rendering of confocal images in reflection mode showing ISH BCIP/NBT reaction. *Lama4* mRNA is restricted to the migrating vascular front (A). In contrast, *Lama5* is expressed widely in the vascular plexus (C). Higher magnification of tip cells expressing *Lama4* mRNA (red arrows) in boxed area (A). *Lama4* is expressed by endothelial cells, but not astrocytes, at the sprouting vascular front (B, white arrow). Immunofluorescence staining recognizing laminin $\alpha 4$ (green, E), laminin $\alpha 5$ (green, F), co-stained with isolectin B4 (red), showed tight abluminal vascular localization of laminin $\alpha 4$. Laminin $\alpha 5$ is distributed in the astrocytic matrix ahead of the vascular plexus (asterisk, F), and less tightly associated with the endothelium (arrowheads, F). Double immunofluorescence staining on retina whole mount (G) and cryosection. (H) Laminin $\alpha 4$ (green) is present around the endothelial membrane of the sprout, but absent from endothelial protrusions and filopodia (white arrows, I-L). Counterstaining with VE-cadherin (red, I) showed localization of laminin $\alpha 4$ (green) at some endothelial junctions (yellow arrows). Other junctional profiles lack laminin $\alpha 4$ labelling (white arrows). (A–D) Transmission electron microscopy analyses, *en face* ultrathin sections. Boxes in J indicate areas of tip cell body and nascent plexus shown in higher magnification in L and M, respectively. Black arrows indicate electron-dense basement membrane (BM) fragments, whereas white arrows indicate lack of BM formation. Asterisks illustrate extracellular space. A, astrocyte; E, endothelial cell; L, lumen. Scale bars: A,C,E–H, 50 μ m; B,D,I,J, 20 μ m; K–P, 10 μ m; J, 2 μ m; K–M, 1 μ m. BCIP/NBT, 5-bromo-4-chloro-3-indolyl-phosphate/Nitro Blue tetrazolium.

tissue-specific distribution patterns and contain the main cell interaction sites. According to current concepts, the formation of new sprouts requires that the activated ECs degrade the BM as laminins *in vitro* impede endothelial sprouting (Davis & Senger, 2005). A recent study illustrated that laminin 111 (LN111; $\alpha 1$, $\beta 1$ and $\gamma 1$) can promote endothelial Dll4/Notch signalling *in vitro* (Estrach et al, 2011). However, ECs *in vivo* express only laminin 411 (LN411; $\alpha 4$, $\beta 1$ and $\gamma 1$) and laminin 511 (LN511; $\alpha 5$, $\beta 1$ and $\gamma 1$), whereas laminin 111 is not considered a component of the endothelial BM (Sixt et al, 2001; Thyboll et al, 2002; Hallmann et al, 2005). Thus, the physiological substrate *in vivo* and the relevance of this concept remain unclear.

Mice lacking laminin $\alpha 4$ are born anaemic because of embryonic bleeding, suggesting blood vessel leakiness, and show enhanced blood vessel formation in pathological condition, such as tumour angiogenesis (Thyboll et al, 2002; Zhou et al, 2004). In addition, experimentally induced vessels in the cornea pocket assay show aberrant branching, dilation and fluctuation in vessel diameter, leading to microhaemorrhages (Thyboll et al, 2002), but the molecular mechanisms remain unclear.

Here we provide the first evidence for laminin $\alpha 4$ to directly regulate endothelial tip/stalk selection *in vivo* through modulation of Dll4/Notch signalling. Loss of *Lama4* caused marked hyper-sprouting and reduced Dll4/Notch signalling, whereas laminin $\alpha 4$

Fig 2 | Loss of *Lama4* results in excessive sprouting angiogenesis. Isolectin B4 immunostaining (red, A,B) showed an increased postnatal day 5 (P5) retinal vascular density in *Lama4*^{-/-} (B) compared with control (A) mice. Number of branch points per image field of P5 *Lama4*^{-/-} *Lama4*^{+/+} mice (C), $n \geq 5$. Confocal images of sprouting front of P5 control (D) and *Lama4*^{-/-} (E) retinas stained with isolectin B4 (red) showed increased number of filopodia (white dots) formation in *Lama4*^{-/-} mice (F). Control and *Lama4*^{-/-} mice, $n \geq 3$. *Pdgfrb* in situ hybridization (red) of P5 retinas were counterstained with isolectin B4 (green) showing an increased area of *Pdgfrb* expression in *Lama4*^{-/-} retinas (G,H), correlated with increased expression at *Pdgfrb* mRNA level (I) in retinas of P5 mice. Control and *Lama4*^{-/-} mice, $n \geq 7$. Anti-5-bromodeoxyuridine (BrdU) antibody (green) and isolectin B4 (red) immunostaining in P5 *Lama4*^{-/-} (K) and control (J) mice 2 h after BrdU injection. BrdU-positive endothelial cells co-labelled with isolectin were counted. Number of proliferated endothelial cells per visual image (L). Control mice, $n = 4$ and *Lama4*^{-/-}, $n = 3$. Scale bars: A,B, 200 μ m; D,E, 25 μ m; G,H,J,K, 50 μ m. *** $P < 0.0001$ and ** $P < 0.01$. Values represent mean $\pm$ s.e.m. qPCR, quantitative polymerase chain reaction.

but not $\alpha 5$ protein induces Dll4 mRNA and protein expression in an integrin-dependent manner. Our data indicate that impaired Notch signalling is the molecular mechanism underpinning the vascular defects in *Lama4* mutant mice.

RESULTS AND DISCUSSION

Endothelial tip cells selectively express laminin $\alpha 4$

In situ hybridization showed restricted expression of *Lama4* at the growing vascular front of the postnatal mouse retina (Fig 1A, red arrowheads), with most abundant expression in the leading tip cells (Fig 1A,B). In comparison, expression of *Lama5* is prominent in the developing vascular plexus (Fig 1C,D). Laminin $\alpha 4$ protein is selectively distributed along the abluminal BM of all retinal vessels and appears most tightly associated with the endothelium (Fig 1E). Laminin $\alpha 5$ protein decorates the endothelium, but also retinal astrocytes ahead of and around the newly forming vessels (Fig 1F). Notably, laminin $\alpha 1$ was not detected in the retinal vasculature (supplementary Fig S1 online). The observation of strongest expression of mRNA *Lama4* in the leading tip cells indicates that tip cells not only break down BM but also actively produce nascent BM components with a specific function during sprout patterning. In addition, a recent microarray study of Dll4 heterozygous versus wild-type (WT) retinas identified high expression of several genes involved in the degradation and production of BM in endothelial tip cells (del Toro et al, 2010).

Whole-mount preparations and sections of the leading vascular front illustrate fragmented laminin $\alpha 4$ staining along the abluminal membrane of tip cells. Only the filopodia of tip cells lack laminin

$\alpha 4$ staining (Fig 1G,H). In the plexus, co-staining with VE-cadherin and isolectin B4 confirmed the tight association of laminin $\alpha 4$ with the basal and basolateral EC membrane, including areas in immediate proximity to EC junctions (Fig 1I).

En face ultrathin sections for transmission electron microscopy (TEM) analysis of retinal tip cells illustrated fragments of BM along the abluminal membrane of tip cells. Whereas the base of filopodia projections showed few and thin fragments, the proximal parts of the tip cell body and the nascent plexus showed increasingly complete BM formation (Fig 1J–M).

Loss of laminin $\alpha 4$ leads to excessive tip cell formation

To gain insight into the function of laminin $\alpha 4$, we studied the retinal vasculature of postnatal laminin $\alpha 4$ knockout mice (*Lama4*^{-/-}). *Lama4*^{-/-} retinas showed increased vascular density and excessive branching (Fig 2A–C) compared with heterozygous or wild-type (WT) littermates (in the following we consider heterozygous littermates as controls). Increased filopodia extension (Fig 2D–F) suggested excessive tip cell formation and hypersprouting as a possible cause for the increased vascular density. Indeed, *Pdgfrb*, a gene enriched in tip cells (Gerhardt et al, 2003; Hellstrom et al, 2007), was more broadly expressed in the hyperdense plexus (Fig 2G,H) and showed a twofold upregulation in *Lama4*^{-/-} retinas (Fig 2I). Aberrant filopodia extension in the proximal vascular plexus of the retinal vasculature indicated that excessive tip cell formation coincides with deficient vessel maturation and stabilization (supplementary Fig S2 online). In *Lama4*^{-/-} retinas, EC proliferation as measured by 5-bromodeoxyuridine (BrdU) incorporation (Fig 2J–L) and phospho-histone H3

Fig 3 | Reduced Dll4/Notch signalling in *Lama4*^{-/-} endothelial cells. (A) Quantitative polymerase chain reaction (qPCR) of selected Notch target genes in P5 retinas of *Lama4*^{+/+} and *Lama4*^{-/-} mice, $n \geq 3$. Confocal images of Dll4 protein labelling (green) counterstained with isolectin B4 (blue) on P5 retinas of *Lama4*^{+/+} (B,C) and *Lama4*^{-/-} (D,E) mice. The distinct vesicular labelling of Dll4 is significantly diminished in *Lama4*^{-/-} endothelial cells of the sprouting vascular front (F). Confocal images of vascular sprouts in three-dimensional embryoid bodies derived from *Lama4*^{+/+} (G,H) and *Lama4*^{-/-} (I,J) embryonic stem (ES) cells. Both vesicular cytoplasmic Dll4 protein labelling (arrowheads in G,I) and nuclear NICD labelling (arrowheads in H,J) are strongly reduced in *Lama4*^{-/-} endothelial cells. Non-endothelial NICD labelling, arrow in H. Scale bars: B,D, 50 μ m; C,E, 10 μ m; E–H, 20 μ m. * $P < 0.05$; ** $P < 0.01$ and *** $P < 0.0001$. Values represent mean $\pm$ s.e.m.

staining (supplementary Fig S2 online) was significantly increased. However, we found no changes in the distribution or quantity of *Vegfa* expression and no upregulation of the hypoxia-regulated gene *Slc2a* in *Lama4*^{-/-} retinas (supplementary Fig S2 online), indicating that hypersprouting is not caused by increased tissue hypoxia or *Vegfa* expression. In addition, pericyte recruitment appeared unchanged in *Lama4*^{-/-} retinas (supplementary Fig S3 online).

To study the role of laminin $\alpha 4$ in a sprouting assay that does not comprise blood flow and tissue oxygenation effects, we established *Lama4*^{-/-} embryonic stem (ES) cells and prepared three-dimensional (3D) spheroid embryoid bodies (EBs; Jakobsson et al, 2007, 2010). *Lama4*^{-/-} EBs showed hypersprouting and excessive filopodia formation along the stalk and in the plexus (supplementary Fig S2 online) similar to the retina phenotype, illustrating that laminin $\alpha 4$ restricts tip cell formation independently of flow-mediated effects on vessel stabilization.

Loss of laminin $\alpha 4$ impairs Dll4/Notch signalling

The hypersprouting phenotype with increased numbers of endothelial tip cells is reminiscent of deficient Dll4/Notch signalling (Hellstrom et al, 2007; Suchting et al, 2007). In *Lama4*^{-/-} retinas, Notch target genes *Hey1*, *Hey2*, *Nrarp* and

the ligand *Dll4* were significantly downregulated (Fig 3A), and immunolabelling and quantification showed significantly reduced Dll4 protein expression (Fig 3B–F). In the sprouting EBs, strong staining of Dll4 (Fig 3G) and prominent nuclear Notch1 intracellular domain (NICD) (Fig 3H) was evident only in control ES cells. *Lama4*^{-/-} EBs showed barely detectable Dll4 and NICD staining (Fig 3I,J), confirming that the loss of laminin $\alpha 4$ affects endothelial Notch ligands/signalling. Thus, although LN111 can induce Dll4 expression in cultured ECs (Estrach et al, 2011), in the retina *in vivo* and in the EB sprouting assay other laminins that potentially remained undetected around blood vessels cannot compensate for the lack of LN111.

Laminin $\alpha 4$ directly induces Dll4

To determine how laminin $\alpha 4$ influences Dll4/Notch signalling, we cultured ECs, either human umbilical endothelial cells (HUVEC) or mouse brain ECs (bEND5), on different matrices, including LN411, LN511, collagen IV and uncoated control cell culture dishes. After 4 h, *Dll4* mRNA levels were slightly elevated in ECs cultured on LN411 (Fig 4A). In both bEND5 and HUVECs, *Dll4* mRNA was significantly upregulated on LN411 after 24 h, in contrast to cells on LN511, collagen IV (data not shown) or uncoated dishes (Fig 4A,B). Immunoblot confirmed increased Dll4

Fig 4 | Non-cell autonomous laminin 411 modulates Dll4/Notch signalling. Dll4 mRNA expression levels measured by quantitative polymerase chain reaction (qPCR) in bEND5 (A) and human umbilical endothelial cells (HUVECs) (B) cultured on laminin 511 (LN511; blue bar), laminin 411 (LN411; red bar) and uncoated matrices for 4 and 24 h before analysing *Dll4* mRNA expression by qPCR, $n \geq 5$. (C) Western blot analysis of Dll4 protein levels in HUVECs cultured 24 h on either LN511 or LN411 with or without vascular endothelial growth factor (VEGF), or in uncoated dishes (0 h). (D) *Dll4* and *VEGFR2* mRNA expression levels of bEND5 endothelial cells (ECs) were significantly increased when cultured on LN411 compared with uncoated dishes, $n \geq 3$. (E) Densitometric quantification of phosphorylated vascular endothelial growth factor receptor 2 (VEGFR2) normalized to total amount of VEGFR2 levels (graph). Western blot analysis confirmed effective inhibition of VEGFR2 signalling with a VEGFR2-neutralizing antibody, DC101, in bEND5 cells cultured 24 h on LN411 or uncoated dishes, $n = 3$; dashed lines indicate rearrangement of lanes from the same blot. (F) *Dll4* expression levels of bEND5 after treatment with DC101 for 24 h. Blocking VEGFR2 signalling had a reducing effect on *Dll4* expression in uncoated and LN411-coated ECs, $n \geq 5$. (G) *Dll4* mRNA expression in bEND5 was analysed after a 24-h inhibition of Notch signalling by DAPT. *Dll4* expression was similarly decreased with or without LN411 coating, $n \geq 3$. (H–J) Confocal images of vascular sprouts in three-dimensional chimaeric embryoid bodies derived from wild-type DsRed and *Lama4*^{-/-} embryonic stem cells. Immunostaining for Dll4 (green) showed low expression in *Lama4*^{-/-} ECs of stalk cell position (asterisk, H). However, in tip cell position, *Lama4*^{-/-} cells expressed abundant Dll4 protein (arrows, I, J). Scale bars: H–J, 15 μ m. * $P < 0.05$; ** $P < 0.01$ and *** $P < 0.0001$. Values represent mean $\pm$ s.e.m. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

protein expression in VEGF-stimulated HUVECs cultured on LN411 compared with LN511 or cells without VEGF stimulation (0 h; Fig 4C).

Given that Dll4 expression is quantitatively gauged by VEGFR levels (Jakobsson et al, 2010), we asked whether LN411 affects VEGFR levels and signalling. In mouse bEND5 cells, LN411-

induced *Dll4* expression correlated with increased *Vegfr2* mRNA expression (Fig 4D). To study the relevance of VEGF–VEGFR2 signalling for LN411-mediated Dll4 induction, we used neutralizing antibodies (DC101) against mouse VEGFR2 (Fig 4E). Blocking VEGFR2 significantly reduced LN411-mediated *Dll4* expression (Fig 4F). DAPT (N-[N-(3,5-difluorophenacetyl)-L-

Fig 5 | Laminin 411-induced Dll4/Notch signalling involves integrins. (A,B) Relative contribution of laminin-binding integrins in laminin 411 (LN411) and laminin 511 (LN511) adhesion: human umbilical endothelial cells (HUVECs) were incubated with control or integrin-blocking antibodies and plated on LN411 (A) or LN511 (B)-coated surfaces. Graphs represent the percentage of adhesion inhibition, $n = 2$. (C) Densitometric analysis of western blots (D) for Dll4 expression in HUVECs cultured for 24 h on LN411 in the presence or absence of vascular endothelial growth factor (VEGF) on knockdown of integrin subunit $\alpha 2$ (*si $\alpha 2$*), $\alpha 3$ (*si $\alpha 3$*), $\alpha 6$ (*si $\alpha 6$*) by small interfering RNA treatment, $n = 3$; T0, expression prior to knockdown. (E–H) Isolectin B4 immunostaining (green) of the sprouting vascular front of control (E,F) and *Itgb1^{fllox/fllox} Pdgfb-iCre* retinas (G,H) showed aberrant filopodia formation and hypersprouting. Dll4 protein labelling (red) is reduced at the vascular front in *Itgb1^{fllox/fllox} Pdgfb-iCre* (J) compared with control retinas (I). Quantification of Dll4 immunofluorescent intensity per vascular volume (K) of postnatal day 5 (P5) control (*Itgb1^{fllox/fllox}*) and *Itgb1^{fllox/fllox} Pdgfb-iCre* retinas, $n = 2$ retinas per genotype; three visual fields were analysed for quantification. (L–Q) Visualization of P5 retinal vasculature by isolectin B4 labelling (grey) showed that none of the integrin subunits analysed, integrin $\alpha 2$ (*Itga2*, L,M), $\alpha 3$ (*Itga3*, N,O) and $\beta 3$ (*Itgb3*, P,Q), showed a significant hypersprouting phenotype. (L'–Q') Shows higher magnification images of the sprouting vascular front. Relative vascular density of *Itga2*, *Itga3* and *Itgb3^{-/-}* compared with control littermate retinas (R), $n \geq 3$; three visual fields were analysed for quantification. Scale bars: E,G, 100 μ m; F,H,I,J, 20 μ m; L–Q, 200 μ m; L'–Q', 50 μ m. * $P < 0.05$; ** $P < 0.01$ and *** $P < 0.0001$. Values represent mean $\pm$ s.e.m. NS, not significant; VEGF-A, vascular endothelial growth factor A.

alanyl]-S-phenylglycine t-butyl ester) treatment also significantly reduced Dll4 production on LN411 (Fig 4G), together illustrating that full *Dll4* induction on LN411 involves both VEGFR and Notch signalling.

As the ability of a given cell to produce Dll4 is controlled in a competitive manner by VEGFR2 levels (Jakobsson et al, 2010), we asked whether *Lama4^{-/-}* cells could compete with WT cells in mosaic vessels. Chimaeric EBs containing WT DsRed cells and *Lama4^{-/-}* cells illustrated that *Lama4*-deficient cells could form both stalk and tip cells. In stalk position, they expressed less Dll4 (Fig 4H), whereas in tip cell position they expressed abundant Dll4 protein (Fig 4I,J), indicating that neighbouring WT cells can rescue the ability of *Lama4^{-/-}* cells to produce Dll4. Given the dynamic position shuffling in the sprouts (Jakobsson et al, 2010), it is likely that *Lama4^{-/-}* cells are exposed to extracellular LN411 produced by WT cells. Curiously, *Lama4^{-/-}* tip cells were engulfed by WT neighbours such that the *Lama4^{-/-}* cells contributed minimally to the abluminal surface of the sprout (Fig 4I,J), supporting the idea that WT cells take over the role of BM formation in mosaic vessels.

Loss of laminin $\alpha 4$ phenocopies Notch inhibition

To understand the full physiological relevance of laminin- $\alpha 4$ -induced Dll4/Notch signalling, we compared EC arrangement and junctional patterning, as well as the formation of vascular lumen, in *Lama4*-deficient and DAPT-treated retinas. Co-labelling of VE-cadherin, the EC-specific transcription factor Erg and isolectin B4 illustrated increased junctional profiles with irregular trajectories, coinciding with clustering of EC nuclei in *Lama4^{-/-}* compared with control littermate retinas (supplementary Fig S4 online). Immunolabelling of the tight-junction protein ZO1 confirmed the increased amount and irregularity of EC junctions (supplementary Fig S4 online). Notch inhibition by DAPT treatment led to similar effects (supplementary Fig S5 online). Using intercellular adhesion molecule 2 (ICAM2) as a marker for luminal EC membrane, we observed that most vessels formed lumen in control and *Lama4^{-/-}* retinas (supplementary Fig S4 online), as well as in DAPT-treated retinas (supplementary Fig S5 online). However, in mutant retinas and DAPT-treated retinas, the lumen was more irregular, with wider segments, as well as local narrowing.

En face sectioning for TEM and 3D electron microscopic analysis confirmed increased numbers and length of junctional profiles, widened EC profiles, filopodia extensions and showed

a loss of abluminal space between the EC and surrounding cells in mutant compared with control vessels (supplementary Fig S5 online).

Although these results confirm a role of endothelial laminins in vessel stability, diameter control and patterning (Pollard et al, 2006; Jakobsson et al, 2008), we find that Notch inhibition and *Lama4* deficiency produce strikingly similar morphological changes, indicating that the modulation of Dll4/Notch signalling is a critical molecular mechanism underpinning the physiological role of laminin $\alpha 4$ in vessel stabilization.

Laminin- $\alpha 4$ -mediated induction of Dll4 involves integrins

To gain insight into the possible involvement of integrins in laminin-mediated induction of *Dll4*, we performed EC (HUVEC) adhesion assays using function-blocking antibodies to human integrins. Adhesion to LN411 was reduced in the presence of $\alpha 2$ and $\alpha 3$ blocking antibodies (Fig 5A), indicating that both receptors can contribute to EC adhesion to this substrate. Adhesion to LN511 involves a distinct combination of integrins, as $\alpha 6$ and $\alpha 3$ blocking antibodies are more effective in impairing adhesion to this substrate (Fig 5B). To investigate effects on Dll4 induction, we used small interfering RNA (siRNA) to knock down individual integrin subunits (supplementary Fig S6 online). LN411-induced Dll4 expression was significantly reduced on treatment with siRNA for integrin $\alpha 2$ and $\alpha 6$, but not $\alpha 3$ compared with control siRNA (Fig 5C,D).

To assess the physiological relevance *in vivo*, we studied retinal angiogenesis in mice with either global or endothelial-specific deficiency in $\alpha 2$ -, $\alpha 3$ -, $\beta 3$ - and $\beta 1$ -integrin (Fig 5E–Q). None of the mutants deficient for individual α -subunits or the $\beta 3$ subunit showed a hypersprouting phenotype reminiscent of the *Lama4* mutant (Fig 5L–Q). Endothelial deletion of $\alpha 6$ integrin, similar to $\beta 3$ integrin deletion, leads to enhanced tumour angiogenesis, but does not seem to affect developmental angiogenesis (Germain et al, 2010). Integrin $\beta 1$ combines with several α -subunits expressed in the retina (supplementary Fig S6 online), such as integrin $\alpha 2\beta 1$, $\alpha 3\beta 1$ and $\alpha 6\beta 1$ to form functional heterodimers. Using an endothelial-specific inducible approach (*Pdgfb-iCre*), we asked whether a short-term induced loss of endothelial-derived integrin $\beta 1$ results in reduced Dll4 expression and hypersprouting. Indeed, at 2–3 days post induction of Cre-recombination by tamoxifen injection, we observed a strong reduction of *itgb1* expression (supplementary Fig S6 online) coinciding with reduced Dll4 staining at the vascular front (Fig 5I–K), and a striking

peripheral hypersprouting phenotype in *Itgb1^{fllox/fllox} Pdgfb-iCre* (*Itgb1* EC^{-/-}) mice (Fig 5E–H). Taken together, these results indicate that several α -subunits in combination with the $\beta 1$ -subunit can mediate signalling events triggered by laminin $\alpha 4$ to induced Dll4 and hence regulate tip/stalk patterning.

Given that both laminin–integrin interaction and VEGF–VEGFR2 signalling induce endothelial Dll4 expression, it is interesting to note that VEGFR2 has been proposed to interact directly with integrins. For instance, integrin $\beta 3$ can form a complex with VEGFR2, promoting VEGFR2 phosphorylation (Soldi et al, 1999; Mahabeleshwar et al, 2007). In addition, interplay between integrin $\beta 1$ and VEGFR2 has been shown to regulate endothelial response towards matrix-bound VEGF-A, promoting VEGFR2 phosphorylation and integrin- $\beta 1$ -mediated cell adhesion (Chen et al, 2010). Collectively, these considerations indicate that laminin–integrin and VEGF–VEGFR2 are individually capable of inducing Dll4 expression, but fully functional levels are only reached by cooperation. Such a cooperative regulation should be of particular physiological relevance in the case of Dll4 expression, as Dll4 shows highly dose-sensitive effects *in vivo* (Duarte et al, 2004; Gale et al, 2004; Hellstrom et al, 2007; Lobov et al, 2007; Suchting et al, 2007).

On the basis of our present observations, we propose a new concept for vascular patterning, in which basal cell-matrix adhesion mechanisms (laminin–integrin) regulate critical cell-to-cell communication (Dll4–Notch) involved in cell specification, and thus modulate collective EC behaviour in branching morphogenesis.

METHODS

For full details see supplementary information online.

Animals. *Lama4^{-/-}* mice were generated previously (Thyboll et al, 2002). *Itgb1^{fllox/fllox}* mice (Raghavan et al, 2000) were crossed with *Pdgfb-iCre* mice (Claxton et al, 2008). Tamoxifen was injected twice at postnatal days 2 (P2) and 3 (P3) before eyes were collected at P6. DAPT was injected subcutaneously at P4 before collecting the eyes at P5.

Immunofluorescence. Eyes were collected at P3 to P7 and fixed in 4% paraformaldehyde for 2 h at room temperature. For whole-mount preparation, retinas were dissected and vessels were visualized using isolectin B4. For sections, eyes were passed through sucrose/PBS and embedded in TissueTec. For antibody details see supplementary information online. Retinas were analysed by confocal laser scanning microscopy.

In situ hybridization. Mouse *Vegfa*, *Pdgfb* and *Lama4* complementary DNA probes were digoxigenin labelled and used for whole-mount retina *in situ* hybridization as previously described (Fruttiger, 2002).

BrdU labelling. BrdU was injected 2 h before eyes were isolated, fixed and dehydrated. After dissection, retinas were treated with proteinase K and DNaseI as described (Abramsson et al, 2007). ECs were visualized by isolectin B4 and BrdU was detected using mouse anti-BrdU Alexa 488 (Molecular Probes).

Quantifications. Branchpoints were counted per 400 μm^2 visual field. Relative vascular density was determined by measuring isolectin-B4-positive surface area in relation to the total vascularized area using ImageJ. Filopodia were counted and normalized to leading vascular membrane length. Statistical significance was verified using *t*-test.

Dll4 labelling quantification was performed using Imaris 'surface' function on 16-bit $\times$ 40 images of sprouting region of *Lama4^{+/-}* and *Lama4^{-/-}* retinas.

Quantitative PCR. Eyes were collected in RNeasy (Qiagen). RNA was isolated from tissues and cells, and quantity and quality of RNA was determined. RNA was converted using SuperScriptIII (Invitrogen), followed by quantitative real-time PCR. Gene expression assays specific for either mice or humans were from Applied Biosystems.

Cell culture. HUVECs and mouse brain ECs (bEND5) were seeded on plates and coated with 10 $\mu\text{g}/\text{ml}$ CollagenIV, LN411, LN511 or with PBS at 4 °C. Matrix substrates were aspirated and cells were cultured for up to 72 h.

For details and Notch and VEGFR2 inhibition, see supplementary information online.

SDS gel electrophoresis and western blotting. Cell lysates were obtained in RIPA (RadiolImmunoPrecipitation Assay) buffer. Equal amounts of protein samples were run on 4–12% Bis-Tris gradient gel and blotted according to NuPage System manufacturer's instruction. For antibodies used, see supplementary information online.

ES cell generation and embryoid body assay. Two *Lama4^{-/-}* cell lines were established and used for generation of 3D EBs as described (Jakobsson et al, 2007). DsRed-MST ES cell line used as control was a kind gift from Dr Andras Nagy.

Embryoid bodies in collagen I gels were processed for whole-mount immunohistochemical staining as described previously (Jakobsson et al, 2007). Antibodies are listed in supplementary information online.

Adhesion assays with integrin-blocking antibodies. HUVECs were used in adhesion assays in the presence of integrin-blocking antibodies against $\alpha 2$ (AK7), $\alpha 3$ (ASC-1), $\alpha 6$ (GoH3) or anti-major histocompatibility complex antibody (W6.32) as control.

Knockdown of integrin subunits by siRNA. siRNA transfection was carried out using Magnetofection technology (polyMag; OZ Biosciences). ON-TARGETplus SMARTpool L-004571 and L-007214 (Dharmacon) directed against $\alpha 3$ and $\alpha 6$, respectively, as well as siRNA targeting $\alpha 2$ integrin and luciferase as a control (si Ctrl), were used at a final concentration of 100 nM.

Protein extraction. Protein was extracted 24 h after plating on different extracellular matrices using ice-cold RIPA buffer. Lysates were analysed by immunoblotting.

FACS analysis of surface-protein expression. Cells were detached and incubated with antibodies against $\alpha 2$ (AK-7), $\alpha 3$ (ASC-1) or $\alpha 6$ (GoH3) integrins, followed by secondary fluorescein isothiocyanate-conjugated antibody. Cells were fixed in 1% formaldehyde and mean fluorescence intensities were determined.

For TEM and focused ion beam scanning electron microscopy (FIB/SEM), see supplementary information online.

Supplementary information is available at EMBO reports online (<http://www.emboreports.org>).

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Author contributions: D.S., C.A.F. and H.G. designed and performed experiments and analysed data. S.E. and A.M. performed integrin adhesion experiments and analysed data. A.S. generated FIB/SEM data sets, H.A., L.C. and H.G. segmented and analysed FIB/SEM data, I.R. generated *Lama4*^{-/-} ES cells. A.D., S.R. and K.T. generated the LN411 and LN511 protein. L.S. provided *Lama4*^{-/-} mice and laminin-specific antibodies. D.S., C.A.F. and H.G. wrote the manuscript.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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