c-Src controls stability of sprouting blood vessels in the developing retina independently of cell-cell adhesion through focal adhesion assembly.

Lilian Schimmel 1, Daisuke Fukuhara 2*, Mark Richards 2, Yi Jin 2, Patricia Essebier 1, Emmanuelle Frampton 1, Marie Hedlund 2, Elisabetta Dejana 2, Lena Claesson-Welsh 2, Emma Gordon 1,2*

1. Institute for Molecular Bioscience, The University of Queensland, Brisbane, Queensland 4072, Australia
2. Uppsala University, Beijer and Science for Life Laboratories, Department of Immunology, Genetics and Pathology, Rudbeck Laboratory, Uppsala 75185, Sweden.

$ Present address: Department of Paediatrics, Kyorin University School of Medicine, 6-20-2 Shinkawa, Mitaka, Tokyo 181-8611, Japan

*Correspondence: e.gordon@imb.uq.edu.au

Keywords
Angiogenesis, c-Src, adherens junctions, focal adhesions, kinase signalling
Summary Statement
Here, we show that c-Src is required for developmental angiogenic sprouting in the retina by controlling cell-matrix adhesion. This is in contrast to the previously described role for c-Src in mediating cell-cell adhesion in vascular permeability, and reveals a context dependent role for kinase signaling in endothelial cells.

Abstract
Endothelial cell adhesion is implicated in blood vessel sprout formation, yet how adhesion controls angiogenesis, and whether it occurs via rapid remodeling of adherens junctions, focal adhesion assembly, or both, remains poorly understood. Furthermore, how endothelial cell adhesion is controlled in particular tissues and under different conditions remains unexplored. Here, we identified an unexpected role for spatiotemporal c-Src activity in sprouting angiogenesis in the retina, which is in contrast to the dominant focus on c-Src’s role in maintenance of vascular integrity. Thus, mice specifically deficient in endothelial c-Src displayed significantly reduced blood vessel sprouting and loss in actin-rich filopodial protrusions at the vascular front of the developing retina. In contrast to what has been observed during vascular leakage, endothelial cell-cell adhesion was unaffected by loss of c-Src. Instead, decreased angiogenic sprouting was due to loss of focal adhesion assembly and cell-matrix adhesion, resulting in loss of sprout stability. These results demonstrate c-Src signaling at specified endothelial cell membrane compartments (adherens junctions or focal adhesions) control vascular processes in a tissue and context dependent manner.
**Introduction**

Blood vessels form complex branched networks comprised of arteries, capillaries and veins that supply oxygen and nutrients to all body tissues. Vascular outgrowth occurs through a process called sprouting angiogenesis which is essential for laying down a functional vessel network during embryonic development. Sprouting is also initiated later in life upon environmental changes such as tissue injury and growth, as well as in pathologies such as cancer and eye disease (Potente et al., 2011). A common denominator for these processes is hypoxia, which drives expression of vascular endothelial growth factors (VEGFs), acting to initiate sprouting through binding and activation of VEGF receptors (VEGFRs). Signalling downstream of these ligand/receptor complexes is essential for vascular morphogenesis, as they control processes such as endothelial cell (EC) identity, migration, proliferation and vessel permeability (Simons et al., 2016). VEGF-A/VEGFR downstream signaling converges with that of integrins, which control multiple endothelial cell processes by binding to a range of ligands, including extracellular matrix (ECM) proteins (Hynes et al., 2002). A greater understanding of the exquisite specificity of downstream signalling required for angiogenesis is now becoming appreciated, in order to precisely target specific vascular processes in disease.

During sprout formation and elongation, ECs respond to instructive cues in a collective manner whilst displaying heterogeneous gene expression, morphology and behaviour. During blood vessel formation, the importance of differential adhesion between ECs in the growing sprout has been described from studies in mouse and zebrafish (Bentley et al., 2014; Lenard et al., 2013). Differential cell-cell adhesion is modulated by the junctional localisation of the main endothelial adhesion molecule, Vascular Endothelial cadherin (VE-cadherin), whose internalisation is dependent on phosphorylation at distinct tyrosine sites within its intracellular tail (Gordon et al., 2016; Orsenigo et al., 2012; Wessel et al., 2014). In quiescent cell monolayers or *ex vivo* models, VE-cadherin is neither phosphorylated nor internalised, leading to excessively strong cell-cell adhesions, thereby inhibiting EC migration and sprout formation (Bentley et al., 2009). Conversely, in settings of high VEGF-A signaling such as in cancer, VE-cadherin phosphorylation and internalization is exaggerated, resulting in impaired adhesiveness and formation of non-functional vessels (Bentley et al., 2009). *In vivo*, VE-cadherin phosphorylation is controlled by VEGF and hemodynamic forces through a mechanosensory complex composed of VEGFR2/VEGFR3, PECAM and c-Src (Conway et al., 2013; Conway et al., 2017; Coon et al., 2015; Orsenigo et al., 2012; Tzima et al., 2005). There is still some debate as to under which conditions various signals mediate VE-cadherin dynamics, and how this controls barrier properties remains largely unknown.
VEGFR2 contains a number of tyrosine residues within its intracellular domain that are differentially phosphorylated upon VEGF-A stimulation (Koch and Claesson-Welsh, 2012). Each of these individual phosphorylation sites have specific interaction partners and the \textit{in vivo} roles of each of these are now being unravelled. Cell-cell adhesion in the blood vasculature has been identified as being downstream of the VEGFR2 Tyr949 site (Tyr951 in the human), through the VEGFR2-949/TSAd/c-Src/VE-cadherin cascade. Phosphorylation of the Tyr949 residue in mouse VEGFR2 by VEGF-A mediates binding of the T-cell specific adaptor (TSAd) protein, which is essential for activation of c-Src at cell-cell junctions (Matsumoto et al., 2005). TSAd is devoid of intrinsic kinase activity, but acts as a scaffold to recruit c-Src to junctions. Active c-Src at EC junctions can phosphorylate VE-cadherin and mediate its internalisation, thereby lowering the pool of VE-cadherin available to engage in adhesion, promoting increased leakage from the blood vessel, known as vascular permeability (Sun et al., 2012). In addition to controlling vascular permeability, VEGFR2-949/TSAd/c-Src/VE-cadherin signalling is critical for sprouting angiogenesis in certain tissues. The presence of TSAd/c-Src at cell-cell junctions, accompanied by VE-cadherin phosphorylation and internalisation, is required for sprouts to elongate in the trachea (Gordon et al., 2016). Thus, it is known that c-Src exists at junctions (Orsenigo et al., 2012), yet a second subcellular pool has also been identified at focal adhesions (FAs) (Westhoff et al., 2004). It is conceivable that the different subcellular pools of c-Src are controlled by different pathways, and depending on the instructive cues and surrounding environment, they ultimately lead to phosphorylation of distinct sets of c-Src substrates regulating cell-cell (junctions) or cell-matrix (focal adhesions) dynamics.

While we have previously identified a role for TSAd/c-Src in sprout elongation (Gordon et al., 2016), a role for c-Src in angiogenesis has remained unsettled. In the 1990’s it was reported that mice with a global deletion of either \textit{c-Src}, or related Src family kinases (SFK) \textit{Yes} and \textit{Fyn}, have normal sprouting angiogenesis, but display abnormal vessel barrier integrity (Eliceiri et al., 1999). Indeed, in mature vessels of adult mice, SFK can induce VE-cadherin phosphorylation at Tyr658 and Tyr685 in veins but not arteries, which is necessary, but not sufficient, to induce junctional breakdown and vascular leakage (Orsenigo et al., 2012). Our study (Gordon et al., 2016) hinted for the first time that c-Src does not exclusively affect vascular permeability and barrier function (Eliceiri et al., 1999; Scheppke et al., 2008; Sun et al., 2012; Weis et al., 2004) but it also plays a role in sprouting angiogenesis (Gordon et al., 2016). In agreement with our observations, when all three SFK (\textit{c-Src}, \textit{Yes} and \textit{Fyn}) are constitutively knocked out, mice die at embryonic day 9.5, a time point that is characterised by active vasculogenesis. Similarly, viral delivery of a kinase deleted c-Src results in decreased VEGF-induced angiogenesis in chicken chorioallantoic membrane (CAM) assays.
or when applied to mouse skin (Eliceiri et al., 1999). This suggests that SFK, including c-Src, may indeed be critical for early vasculogenesis and possibly angiogenesis, but a confounding factor has consistently been that the other SFK members, such as Yes and Fyn, may be able to compensate for each other.

Here, we sought to identify the role for c-Src in sprouting angiogenesis using a conditional, inducible c-Src knockout mouse model. We report that c-Src is required for sprouting angiogenesis and vessel stability in ex vivo explants, in the developing mouse trachea and retina via control of cell-matrix adhesion. In contrast, no major changes in VE-cadherin patterning or phosphorylation were observed upon loss of c-Src. Instead, we observed that central focal adhesion components Paxillin and focal adhesion kinase (FAK) were phosphorylated downstream of c-Src in endothelial cells and in the sprouting front of the mouse retina. Taken together, our study reveals a novel role for c-Src in developmental angiogenic sprouting upstream of cell-matrix adhesion but not cell-cell adhesion, providing new insights for the importance of subcellular localisation of intracellular kinases in regulating vascular adhesion and sprouting.

Results

Endothelial c-Src is required for developmental angiogenesis

Constitutive knockout of c-Src is reported to be compatible with grossly normal development (Soriano et al., 1991). However, renewed analysis of global c-Src deficient mice showed postnatal defects in developmental angiogenesis of surviving mice, with moderate decreased vascular growth and increased vessel regression (Fig S1). To specifically investigate the endothelial cell-autonomous role of c-Src, we generated mice with an inducible, endothelial specific deletion of c-Src, by crossing c-Src-floxed mice (Fig S2) with tamoxifen inducible Cdh5CreERT2 mice (Kogata et al., 2006; Wang et al., 2010) (c-Src^{fl/fl};Cdh5CreERT2). When treated with tamoxifen after birth, these mice displayed approximately 75% reduction in c-Src expression in endothelial cells isolated from lungs compared to their wildtype counterparts (Fig 1A, B). The deletion strategy (Lox sites placed between exons 7-9) could potentially result in expression of a truncated c-Src fragment, however, this was not detected (Fig S2C). Therefore, our system is an endothelial cell, c-Src loss of function model. Interestingly, expression of other SFKs, Yes and Fyn also decreased upon loss of c-Src although the effect was significant only for Fyn (Fig S3A-C). These results suggest that compensation by overexpression of other SFKs did not occur as a consequence of temporal endothelial c-Src deficiency. The possibility that Fyn was turned over at a higher rate upon loss of endothelial c-Src needs further exploration.
In agreement with our findings from mice with a deletion of c-Src partner TSAd (TSAd\textsuperscript{fl/fl};Cdh5CreERT2 (Gordon et al., 2016)), c-Src\textsuperscript{fl/fl};Cdh5CreERT2 mice displayed a decrease in capillary sprouting over the cartilage rings of the developing trachea at postnatal day (P) 5 compared to their wildtype littermates (Fig 1C, D, I). Sensitivity of tracheal vessels to loss of Src is in agreement with those of Orsenigo et al. (Orsenigo et al., 2012), who observed an arrest in permeability upon treatment with a Src inhibitor. However, in contrast to mice with deletion of upstream signalling partners in the VEGFR2-949/TSAd/c-Src/VE-cadherin pathway (TSAd\textsuperscript{fl/fl};Cdh5CreERT2 and VEGFR2\textsuperscript{Y949F/Y949F} mice (Gordon et al., 2016; Li et al., 2016)), c-Src-deficient mice displayed a significant reduction in the overall network density of the retinal vasculature (Fig 1E-H). The total vascular area (Fig 1J), outgrowth from the optic nerve (Fig 1K) and number of branch points (Fig 1L) were all reduced in c-Src\textsuperscript{fl/fl};Cdh5CreERT2 mice compared to wildtype littermates. These differences in the requirement for VEGFR2-949/TSAd suggests that sprouting in the trachea and the retina are controlled by unique mechanisms. In contrast, the number of tip cells in the retina was unaffected (Fig 1M). When deletion was induced at P1-3 and retinas analysed at P23, no changes in the vasculature in the deep or superficial plexus were observed, suggesting that a long-term deletion of c-Src is overcome possibly by compensatory mechanisms (Fig S3D-L). In contrast, we did not observe any changes in a different vascular bed of the eye, the hyaloid plexus (Fig S3M-P). These results show for the first time that c-Src is required for developmental angiogenesis in a time, and likely tissue, dependent manner.

**Endothelial c-Src is required for vascular sprouting but not tip cell identity**

To further investigate the requirement for c-Src in angiogenic sprouting, we utilised the mouse metatarsal assay to assess angiogenesis in an ex vivo setting (Song et al., 2015). In agreement with our findings in the trachea and retina, metatarsals isolated from c-Src\textsuperscript{fl/fl};Cdh5CreERT2 mice displayed a significant decrease in angiogenesis (Fig 2A, B), with a reduction in total vessel area (Fig 2C) and branch points (Fig 2D). This confirms the requirement for c-Src in angiogenic sprouting in vivo and ex vivo.

During sprouting angiogenesis, sprouts are comprised of highly migratory ‘tip’ cells with protruding filopodia, followed by their neighbouring ‘stalk’ cells, which form the stable, lumenised sprout (Gerhardt et al., 2003). To assess the cell-autonomous requirement of c-Src in tip cell identity, we crossed mice onto an mTmG reporter background (Muzumdar et al., 2007) (c-Src\textsuperscript{fl/fl};mTmG;Cdh5CreERT2) and assessed the relative contribution of GFP-positive, c-Src-deficient cells to the tip region in chimeric settings (as described previously (Aspalter et al., 2015)). In agreement with the intact number of tip cells in c-Src deficient retinas, we observed no requirement for c-Src in the formation of tip cells in metatarsals (Fig 2E, F, I) or
in the retina (Fig 2G, H, J). Thus, despite reduced sprouting in both of these assays, cells deficient in c-Src were still able reach the tip position. In agreement, c-Src activity is not as prominent in leading cells in a HUVEC scratch assay as in trailing stalk cells, indicating that the placement in the leading position is not strictly dependent on c-Src function (Fig S4). Interestingly, when mice were crossed with Lifeact-EGFP mice, to allow for high resolution visualisation of filopodia extending from tip cells and where endothelial cells display strong GFP intensity (Fraccaroli et al., 2012; Riedl et al., 2010), we observed fewer and significantly shorter filopodia in tip cells of c-Src\textsuperscript{fl/fl};Lifeact;Cd\textsuperscript{h}5CreERT2 retinas compared to wildtype Lifeact;Cd\textsuperscript{h}5CreERT2 retinas (Fig 2K-N). EGFP-positive vessels were co-stained with IsolectinB4 and VE-cadherin to confirm endothelial identity (Fig S5). This suggests that c-Src acts to promote angiogenesis independently of tip cell selection, yet is required for the proper function of tip cells by regulating filopodia formation and extension to drive the growth of sprouting vessels.

**Reduced vascular density in c-Src deficient retinas is a result of decreased vessel stability**

As we observed a reduction in total vascular area and branch points in c-Src\textsuperscript{fl/fl};Cd\textsuperscript{h}5CreERT2 retinas (Fig 1J, L), we reasoned this may be due to changes in cell proliferation, cell death or vascular regression. In c-Src\textsuperscript{fl/fl};Cd\textsuperscript{h}5CreERT2 retinas, we found no change in Ki-67-positive, proliferating cells (Fig 3A, B, E) or Cleaved Caspase-3 cells undergoing apoptosis in the vascular plexus (Fig 3C, D, F), indicating c-Src does not control endothelial cell proliferation or cell death. We also did not observe any changes in the degree of pericyte coverage of the vasculature in c-Src\textsuperscript{fl/fl};Cd\textsuperscript{h}5CreERT2 retinas (Fig 3G-I).

To study vessel regression, we performed immunostaining with type IV Collagen (CollIV), which allows for visualisation of regressed vessels by empty matrix sleeves (Franco et al., 2015). We observed a significant increase in the number of empty CollIV sleeves in the retinal vasculature of c-Src\textsuperscript{fl/fl};Cd\textsuperscript{h}5CreERT2 mice (Fig 3J-L), occurring independently of changes in apoptosis (Fig 3F). As c-Src and Fyn have been shown to be critical for the lumenisation of the vasculature (Koh et al., 2009), we performed immunostaining for intercellular adhesion molecule 2 (ICAM2) which marks the apical/luminal endothelial cell membrane (Stenzel et al., 2011). We observed ICAM2 staining was lost where empty matrix sleeves were observed (Fig 3M, N) as reported previously (Franco et al., 2015). However, the existing vasculature was able to form a lumen, suggesting c-Src is not required for the initial lumenisation of the vasculature. We also did not observe any changes in the number or association of macrophages with the vasculature or with CollIV sleeves upon loss of c-Src (Fig 3O-Q). This is pertinent, given the known role of c-Src in osteoclasts, the role of macrophages in
regression, and the recent data demonstrating Cdh5CreERT2 mice can display non-endothelial Cre activity (He et al., 2019). Taken together, these results suggest that while c-Src is not required for the formation of the vascular lumen, it is required for the stability of newly formed vessels.

**VE-cadherin phosphorylation is not controlled by c-Src at the sprouting front of the retina**

We hypothesised that the requirement for c-Src to stabilise the growing vascular network was due to altered VE-cadherin phosphorylation at two tyrosine residues which have been shown to be important for its internalisation, Tyr658 and Tyr685 (Orsenigo et al., 2012; Wessel et al., 2014). If VE-cadherin phosphorylation and junctional distribution were altered, we would predict the inability of sprouts to form stable connection with their neighbours, resulting in decreased vascular stability.

To characterise cell-cell junctions in the c-Src deleted retina, a number of analyses were performed. Based on immunostaining for erythrocytes, the vascular barrier in the c-Src-deficient retinas appeared unaffected as there was no apparent haemorrhage (Fig S6). Moreover, high resolution confocal imaging analyses (Bentley et al., 2014) were used to define the degree of adherens junction stability (Fig 4A-G). Regions of the retina displaying empty CollIV sleeves due to loss of c-Src expression were selected for these analyses (Fig S7). Here, the pattern of segmented VE-cadherin junctions was classified on a gradient between ‘active’ (serrated and vesicular appearance) or inactive (straighter appearance with fewer vesicles) (Fig 4E, F). Unexpectedly, no changes in VE-cadherin morphology were observed between wildtype and c-Src-deficient vessels at the sprouting front of the retina (Fig 4G), suggesting vessel stability may not be regulated by a c-Src-VE-cadherin phosphorylation cascade. Analysis of phosphorylated VE-cadherin from endothelial cells isolated from lungs also did not reveal significant changes in phosphorylation at Tyr 658 or Tyr685 (Fig 4H, I).

These results were confirmed by immunostaining for phosphorylated VE-cadherin Tyr658 (Fig 4J-N) and VE-cadherin Tyr685 (Fig 4O-S), both of which showed no significant change in intensity at VE-cadherin-positive cell junctions in the postnatal retina vasculature upon loss of c-Src. Our results suggested that the loss of vascular density and stability upon loss of c-Src in the retina is independent of VE-cadherin phosphorylation and cell-cell adhesion.

**c-Src is required for controlled, directional sprout movement**

To further investigate the dynamics of vessel growth upon loss of c-Src, we utilised the mouse metatarsal assay to visualise the sprouting vasculature in an *ex vivo* setting. We hypothesised if reduced sprouting upon loss of c-Src was independent of altered VE-cadherin junctional...
localisation and cell-cell adhesion, the dynamics of sprout elongation or sprout stability, could instead be dependent on altered cell-matrix regulation.

Using \textit{Lifeact-EGFP} metatarsals enabled us to clearly view sprouting speed, filopodial extensions and directional growth of the vasculature at high resolution. Vascular structures were shown to express CD31, confirming endothelial identity (Fig S5). Similar to what we observed in the retina, fewer filopodial extensions were visualised in sprouts deficient for c-Src (Fig 5A, B). Live imaging enabled us to visualise a highly unstable sprouting profile of vessels upon loss of c-Src. While vessels were still able to move forwards at certain time points (Fig B’ to B”’), when sprout movement over a four hour time period was quantified, we observed a significant decrease in the total distance of growth in c-Src\textsuperscript{fl/fl};\textit{Lifeact};Cdh5CreERT2 metatarsals compared to c-Src\textsuperscript{fl/fl};\textit{Lifeact} (Fig 5C). The total velocity, including both forward or backward movement of the sprout, was unchanged upon loss of c-Src (Fig 5D). However, compared to wildtype, c-Src-deficient sprouts often moved in a reverse direction (negative velocity from point to point) (Fig 5E), resulting in their net decreased forward movement (Fig 5C). When measured over 30-minute increments, c-Src-deficient sprouts moved both forwards and backwards, in contrast to wildtype sprouts which moved in a consistent forward manner (Fig 5F). This reveals that c-Src mediates controlled directional movement during angiogenesis and that c-Src-deficient sprouts are highly unstable.

**Phosphorylation of c-Src can occur independently of VEGF-A/VEGFR2 signalling**

In order to further investigate the pathways that may be up or downstream of c-Src to control vascular stability in retinal angiogenesis, we manipulated c-Src levels by knockdown using siRNA or overexpression by transducing endothelial cells using lentiviral transfection of an mKate2-tagged c-Src. So as not to interfere with c-Src protein folding or activity, the mKate2 tag was attached to the full length protein using a glycine/serine rich flexible linker peptide (as described in (Sandilands et al., 2004)). Addition of this linker/tag does not interfere with c-Src phosphorylation activity or its binding to interaction partners (Sandilands et al., 2004).

When we stimulated these c-Src manipulated endothelial cells with VEGF-A, total VEGFR2 levels decreased as a consequence of ligand-induced internalisation at similar rates across samples (Fig 6A, B) and neither VEGFR2 phosphorylation at Tyr951 (associated with TSAd binding and c-Src activity at cell junctions) or Tyr1175, were affected (Fig 6 A, C, D). These data indicate that c-Src does not alter the rate of VEGFR2 phosphorylation, internalisation or degradation, and suggest c-Src is not upstream of VEGFR2 signalling when HUVEC are grown on fibronectin. c-Src expression was reduced 3-fold when cells were treated with
siRNA, and when treated with c-Src-mKate2 lentivirus, we observed a 4.5-fold increase in c-Src protein (Fig 6A, E). Of note, in c-Src silenced cells, the remaining pool of c-Src was phosphorylated with increased stoichiometry (pSrc/total Src) both at Tyr416 and Tyr527, suggesting compensation for the reduced c-Src protein levels (Fig 6A, F, G). Moreover, in agreement with Fig 4, VE-cadherin total protein was unchanged after c-Src manipulation, suggesting c-Src is acting independently of VE-cadherin phosphorylation, internalisation and degradation/recycling (Fig 6A, H).

While total protein levels of focal adhesion components FAK and Paxillin were unchanged upon c-Src manipulations (Fig 6A, I, K), phosphorylation of FAK at one of the c-Src target sites (Jean et al., 2014; Ma et al., 2019) Tyr576 (Fig 6A, J) and Paxillin at Tyr118 were significantly increased upon c-Src-mKate2 overexpression (Fig 6A, L). These changes were not altered upon VEGF-A simulation, suggesting c-Src regulates focal adhesion component phosphorylation independently of VEGF-A activation.

**Focal adhesion activation requires c-Src during retinal angiogenesis**

To further investigate the regulation of focal adhesion components in mediating c-Src dependent vessel stability, we closely examined Paxillin phosphorylation in endothelial cells isolated from mouse lungs. We initially confirmed c-Src expression is significantly reduced in cells isolated from Src\[^{fl/fl}\];Cdh5CreERT2 mice (Fig 1A) and subsequently examined phosphorylation of Paxillin at Tyr118. We saw a trending decrease of Paxillin phosphorylation (p=0.08), in line with the hypothesis that c-Src mediates vascular stability in endothelial cells through cell-matrix adhesion assembly (Fig 7A, B). We also observed enriched phospho-c-Src immunostaining in focal adhesions in a scratch assay (Fig S4C). However, phospho-c-Src was also enriched at cell-cell junctions in a monolayer (Fig S4D), again confirming previous studies which show c-Src controls adherens junction activity (Gordon et al., 2016; Orsenigo et al., 2012), although in a context dependent manner.

To examine the effect of focal adhesion size and distribution in endothelial cells with c-Src knockdown or overexpression, we again treated cells with c-Src siRNA or c-Src-mKate2 lentivirus, grew cells on fibronectin (thought to engage integrin α5β1) (Schaffner et al., 2013) and immunostained to detect phospho-Paxillin Tyr118 (Fig 7C). A reduction of c-Src in cultured endothelial cells grown on fibronectin did not have an impact on focal adhesion number (Fig 7D), density (Fig 7E) or size (Fig 7F), despite a significant reduction in intensity of c-Src protein by immunostaining (Fig S8A). However, overexpression of c-Src resulted in an increase in number, density and size of FAs compared to cells transfected with empty-mKate2 lentivirus (Fig 7C-F). The lack of change in FA upon c-Src knockdown may be due to...
increased phosphorylation of the remaining pool of c-Src (Fig 6), or may be due to cells being grown on fibronectin matrix. In ECs, individual matrix components induce focal adhesions by clustering of their individual receptors (Dejana et al., 1988). Alteration of fibronectin fibrillogenesis is known to be mediated through Paxillin phosphorylation (Zaidel-Bar et al., 2007), however fibronectin deposition was unaffected by loss of c-Src (Fig S9). To investigate whether other matrix components may regulate how FAs are controlled by c-Src, we grew c-Src-deficient cells on vitronectin, known to preferentially engage integrin αvβ3 (Felding-Habermann and Cheresh, 1993; Horton, 1997), which resulted in decreased FA number and density (Fig 7G-K). This confirms that c-Src controls focal adhesion activity and cell-matrix connections in endothelial cells, potentially by engaging integrin αvβ3.

The existence of focal adhesion-type structures in vivo is still under considerable debate, but it is known that focal adhesion-like signalling pathways do exist in vivo in the retina (Raimondi et al., 2014). Therefore, we sought to examine differential Paxillin phosphorylation in the retina of c-Src-deficient mice. We observed that the intensity of phospho-Paxillin Tyr118 staining was brightest at the leading edge of the retina, and enriched where filopodia extend from tip cells (Fig 7L, M, P). In c-Src-deficient endothelial cells of the retina, this intensity of staining was significantly reduced (Fig 7N, O, P). This reveals that the inability of c-Src-deficient endothelial cells to form a stable cell-matrix connection in the sprouting retina causes the reduced vascular density and increased vascular regression in these mice.

Discussion
c-Src is a widely expressed non-receptor tyrosine kinase with critical functions in a multitude of cell types (Espada and Martin-Perez, 2017). Global deletion of c-Src in mice results in osteopetrosis due to impaired osteoclast function (Soriano et al., 1991), and to decreased tumour angiogenesis and vascular permeability (Eliceiri et al., 1999), however potential effects on developmental angiogenesis have not been explored. Here, we show that c-Src is important for postnatal vascular development in the retina and trachea, specifically by regulating endothelial cell-matrix adhesion, critical for the outward growth and stabilisation of new vessels during sprouting angiogenesis. In contrast, cell-cell adhesion during developmental angiogenesis appeared unaffected. We also found that the main effect of c-Src deficiency was in decreased filopodial stability and reduced postnatal outgrowth and branching within the vascular plexus of the retina, accompanied by a loss in Paxillin phosphorylation at the vascular front of the plexus.
One possible reason we did not observe changes in adherens junction activity, but reduced focal adhesion activation upon loss of c-Src in the developing retina, would be the unique properties of the endothelial barrier. In previous studies specifically deleting the adaptor molecule TSAd from endothelial cells (Gordon et al., 2016), we have previously shown that the VEGFR2-TSAd-c-Src-VE-cadherin signaling complex is required for sprouting angiogenesis in the tracheal vasculature, but not in the developing retina. While TSAd binds to the phosphosite pY949 in VEGFR2, c-Src may have more extensive, non-VEGF-dependent effects in the retina. Whether genetic loss of c-Src affects adherens junctions or FAs in settings of high VEGF exposure (such as oxygen-induced retinopathy) or across different tissue beds is the focus of ongoing work.

Phosphorylation of VE-cadherin in particular on Tyr685, is dependent on VEGFA and correlates with increased vascular permeability in vivo (Orsenigo et al., 2012; Wessel et al., 2014). Accordingly, pharmacological Src blockade or constitutive gene targeting of c-Src reduces paracellular permeability (Eliceiri et al., 1999; Paul et al., 2001). Treatment with a c-Src inhibitor results in loss of VE-cadherin-dependent permeability, providing further evidence that tracheal vessels are sensitive to c-Src-mediated VE-cadherin phosphorylation (Orsenigo et al., 2012). While we did not find changes in phospho-VE-cadherin levels or in the arrangement of adherens junctions within this study, we have not investigated whether loss of c-Src results in alterations in vascular permeability in the retina.

Potential changes in permeability may be independent of VE-cadherin phosphorylation, which would be in contrast to the well-documented pattern in non-CNS organs (Orsenigo et al., 2012). Indeed, the blood-retinal barrier is dependent on adherens junction stability, but also on regulation of endothelial transcytosis (Chow and Gu, 2017), which is mediated by caveolae. Caveolin 1 (Cav1), a known c-Src substrate (Zimnicka et al., 2016), is essential for caveolae formation (Drab et al., 2001; Razani et al., 2001). However, the exact role of Cav1 (and caveolae) in transcytosis has not been settled. While albumin transport across the endothelium requires Cav1 and caveolae, animals lacking Cav1 display increased albumin leakage in the retinal vasculature (Gu et al., 2014). Additionally, recent studies have shown no role for Cav1 and caveolae in transport across the endothelium in tumours (Sindhwani et al., 2020). Conclusively discerning the effects of c-Src deletion on endothelial transcytosis and the blood-retinal barrier cannot be done using confocal microscopy, instead, electron microscopy must be employed.

The interpretation of previous data on the role of c-Src in endothelial cell biology, whether in vitro or in vivo, is complicated by the possibility that the related EC-enriched SFKs Yes and
Fyn may have been affected as a result of compensation. While c-Src, Yes and Fyn are structurally highly related and are ubiquitously expressed, it is increasingly appreciated that they play distinct roles in the endothelium. However, their role in angiogenesis remains to be addressed using appropriate genetic models. Here, we show unexpected biology as a result of deleting c-Src in endothelial cells which are not compromised by compensatory overexpression of Yes and Fyn. In contrast, deletion of c-Src also resulted in a significant loss of Fyn expression, possibly due to that Fyn may be biologically active and turned over at a higher rate, to compensate for loss of c-Src. Fyn has been implicated in signaling downstream of VEGFR2 in endothelial cells, specifically involving Tyr1212 in VEGFR2 (Lamalice et al., 2006). These results from in vitro models were however not recapitulated when examining a VEGFR2 mutant with a Tyr to Phe exchange at the 1212 position (Testini et al., 2019). Fyn has also been implicated in lumen formation in tissue cultured endothelial cells (Kim et al., 2017). Of note, lumen formation proceeded normally in the c-Src-deficient retinal vasculature (Fig 3M, N). The in vivo role of Fyn remains to be studied using conditional, endothelial cell-specific knockout models.

While focal adhesions are known to control cell migration, precisely how they mediate endothelial cell behaviour, function and formation of a functional vasculature, is a topic of intense study. The morphology and dynamics of FAs in cultured endothelial cells are controlled by the extracellular matrix and expression pattern of integrins, but are also influenced by different stimuli such as VEGFA (Mui et al., 2016). In vivo, FAs can also be regulated by hemodynamic forces (Collins et al., 2014). c-Src is a substrate for FAK activity in FAs in vitro and is a component of the mechanosensory complex in endothelial cells (Tzima et al., 2005). However, c-Src can also act upstream of FAK, indicating the complexity of interactions between these kinases at FAs (Jean et al., 2014; Kleinschmidt and Schlaepfer, 2017). Expression of a kinase dead form of FAK results in embryonic lethality at E9.5, associated with defects in the formation of the embryonic vasculature (Lim et al., 2010) as a result of impaired FA turnover and cell motility. Interestingly, in MEFs from these mice, FAK was placed upstream of c-Src, with kinase dead FAK resulting in a loss of c-Src phosphorylation at its activating Tyr416 (Lim et al., 2010). Our data here meanwhile suggest c-Src acts upstream of FAK in ECs, as we observed increased FAK phosphorylation upon induction of c-Src expression (Fig 6). Furthermore, based on the in vivo results presented here, we can place Paxillin downstream of c-Src.

Although the exact sequence of events is not clear as detailed above, activation of integrins triggers the assembly of the multi-protein FAK/c-Src/Paxillin/talin complex linked to the cytoskeleton at nascent adhesions (Kleinschmidt and Schlaepfer, 2017). In cultured
endothelial cells, c-Src promotes FAK phosphorylation at Tyr861 and association with αvβ5 upon VEGF stimulation (Eliceiri et al., 2002). αvβ5 is also required for angiogenesis (Friedlander et al., 1995), therefore it is possible that c-Src activation downstream of this FAK-integrin complex may control FA assembly and vascular stability in the retina. β1 Integrin has also been associated with angiogenesis through correct positioning of VE-cadherin (Pulous et al., 2019; Yamamoto et al., 2015). Given the lack of detectable VE-cadherin abnormalities in c-Src-deficient retinas and the lack of changes in focal adhesions upon loss of c-Src when cells are coated on fibronectin (Fig 7), we predict our phenotype is unlikely to be downstream of β1 Integrin activation. Our results culturing c-Src-deficient cells on vitronectin are suggestive of a role for αvβ3, which has also been associated with angiogenesis. However, this effect is context dependent (Demircioglu and Hodivala-Dilke, 2016; Plow et al., 2014) and vitronectin is known to engage other integrins (Felding-Habermann and Cheresh, 1993), therefore while our results are suggestive, they are not yet conclusive. Future work aims to precisely decipher the time-dependent upstream (integrin) and downstream (FAK, talin, cytoskeleton) effectors of c-Src in sprouting angiogenesis.

Given the reported findings that both c-Src and FAK modulate VE-Cadherin phosphorylation, and the findings here that c-Src regulates both FAK and FAs, a key question is whether there is co-ordination between cell-cell and cell-matrix adhesions in the regulation of angiogenesis and vascular permeability, and whether this is under the control of the same signals. Close interactions between integrins and cadherins have been associated with junctional stability (Chattopadhyay et al., 2003; Pulous et al., 2019; Weber et al., 2011; Yamamoto et al., 2015), and a means of direct communication between these compartments could moreover involve direct interaction between FAK and VE-cadherin. This has been shown to be critical in the regulation of paracellular permeability in a manner independent of c-Src but dependent on FAK-mediated phosphorylation of β-catenin, independently of tension (Chen et al., 2012). Studies with a kinase dead form of FAK revealed that FAK controls VEGF-induced VE-cadherin phosphorylation at Tyr658, with c-Src implicated upstream of FAK in this process (Jean et al., 2014). Inhibition of FAK resulted in reduced c-Src at cell-cell junctions, therefore whether c-Src and FAK act in parallel or separately to regulate adherens junctions remains unsettled. To conclude, the results here indicate that cell-cell and cell-matrix adhesion in vivo can be separately controlled by c-Src, however, we do not exclude that there may be tissue- and developmental stages when coordination between the two pathways is essential.

In summary, our results are the first to tie c-Src, a known mediator of vascular permeability, to sprouting angiogenesis. While the dynamics of the assembly of the multi-protein FA
complex, and how this is regulated by c-Src remains to be fully elucidated, we observed that c-Src primarily controls cell-matrix adhesion through phosphorylation of the FA component Paxillin. As Src inhibitors are already used in clinical settings, our results open up for improved tailoring of such treatment by providing a fundamentally deeper understanding of c-Src’s role in endothelial cell signalling and how regulation of cell-cell and cell-matrix adhesion has a bearing on sprouting angiogenesis.

Materials and Methods

Antibodies

The following antibodies were used: rat anti-CD31 (BD Biosciences, 553370, RRID: AB_394816, EC isolation: 250 µg/mouse), ICAM2 (BD Biosciences, 553326, RRID: AB_394784, IHC: 1/200), rabbit anti-phospho VE-cadherin (Tyr^{656}) and (Tyr^{685}) (from E. Dejana (Orsenigo et al., 2012), WB: 1/500, IHC: 1/50), rabbit anti-phospho VE-cadherin (Tyr^{656}) (Invitrogen, 44-1144G, RRID:AB_2533583, WB:1/1000), rabbit anti-GFP (Santa Cruz, sc8334, RRID: AB_641123, IHC: 1/200), rat anti-VE-cadherin (BD Bioscience, 555289, RRID: AB_395707, IHC: 1/100), goat anti-VE-cadherin (R&D Systems, AF1002, RRID: AB_2077789, WB: 1/1000), Isolectin-B4 directly conjugated to Alexa 488, 568 and 647 (Life Technologies/Thermo Scientific, I21411, I21412 and I32450, RRID: AB_2314662, IHC: 1/500), rabbit anti-phospho Paxillin (Tyr^{118}) (Abcam, AB4833, RRID: AB_304669, IHC: 1/100), mouse anti-Src GD11 (Millipore, 05-184, RRID:AB_2302631, WB: 1/1000, ICC: 1/200), rabbit anti-phospho VEGFR2 (Tyr^{1175}) (Cell Signaling, 2478, RRID:AB_331377, WB: 1/1000), rabbit anti-phospho VEGFR2 (Tyr^{951}) (Cell Signaling, 2471, RRID:AB_331021, WB: 1/1000), goat anti-VEGFR2 (R&D, AF644, RRID:AB_355000, WB: 1/1000), rabbit anti-phospho c-Src (Tyr^{416}) (Cell Signaling, 2101, RRID:AB_331697, WB: 1/1000), rabbit anti-phospho c-Src (Tyr^{418}) (Invitrogen, 44660G, RRID:AB_2533714, ICC: 1/100), rabbit anti-Erg1/2/3 (Abcam, ab92513, RRID:AB_2630401, IHC: 1/200), mouse anti-VE-cadherin-Alexa647 (BD Bioscience, 561567, RRID:AB_10712766, ICC: 1/250), rabbit anti-phospho Paxillin (Tyr^{119}) (Invitrogen, 44-722G, RRID:AB_2533733, WB: 1/1000, ICC: 1/200), rabbit anti-phospho FAK (Tyr^{576}) (Cell Signaling, 3281, RRID:AB_331079, WB: 1/1000), mouse anti-FAK (Santa Cruz, sc-271126, RRID:AB_10614323, WB: 1/1000), rabbit anti-phospho c-Src (Tyr^{527}) (Cell Signaling, 2105, RRID:AB_331034, WB: 1/1000), rabbit anti-GAPDH (Cell Signaling, 2118, RRID:AB_561053, WB: 1/5000), mouse anti-fibronectin (BD Biosciences, 610077, RRID:AB_2105706, ICC: 1/200), rat anti-F4/80 (Biorad, MCA497R, RRID:AB_323279, IHC: 1/100), rat anti-Ter119 (Invitrogen, 14-5921-81, RRID:AB_467726, IHC: 1/200), rabbit anti-NG2 (Millipore, AB5320, RRID:AB_11213678, IHC: 1/200) rabbit anti-Cleaved Caspase 3 (Cell Signaling, 9661, RRID:AB_2341188, IHC: 1/200),...
mouse anti-Yes (BD Bioscience, 610376, RRID:AB_397759, WB: 1/1000), mouse anti-FYN (BD Bioscience, 610163, RRID:AB_397564, WB: 1/1000).

Fluorescently labelled secondary antibodies were obtained from Invitrogen (IHC: 1/250, ICC: 1/400). Horseradish peroxidase (HRP) labelled secondary antibodies were obtained from Thermo Scientific (WB: 1/5000).

**Mice**

Animals were propagated at the local animal facility under laminar airflow conditions with a 12 h light/dark cycle at a temperature of 22–25°C. All animal work was approved by the Uppsala University board of animal experimentation (permit 5.2.18-8927-16) and The University of Queensland’s Molecular Biosciences Animal Ethics Committee (permits IMB424/17 and IMB231/17/BREED). *mTmG* mice were obtained from The Jackson Laboratory (Muzumdar et al., 2007). *TdTomato* mice were obtained from Prof. Benjamin Hogan (Peter MacCallum Cancer Centre, Australia) and The Jackson Laboratory (Madisen et al., 2010). *Cdh5-CreERT2* mice were provided by Ralf Adams (MPI, Münster) (Kogata et al., 2006; Wang et al., 2010). Lifeact-EGFP mice were provided by Roland Wedlich-Söldner (University of Münster) (Riedl et al., 2010). *c-Src-/-* global knockout mice were obtained from The Jackson Laboratory (Soriano et al., 1991). *c-Src-*floxed mice were delivered from the Nice Mice, National Resource Center for Mutant Mice, Model Animal Research Center, China.

**Inducible gene deletion**

Cre activity and gene deletion were induced by intraperitoneal injections to pups (male and female) with 100 μg tamoxifen (Sigma, T5648) at P1, P2 and P3 and mice were sacrificed at P5 or P23. 30 μg tamoxifen was injected at P1 to induce mosaic deletion and mice were sacrificed at P5.

**Immunohistochemistry (IHC)**

Eyes were removed and prefixed in 4% paraformaldehyde (PFA) for 20 min at room temperature. Tracheas were fixed in 4% PFA for 15 min at room temperature. After dissection, tissues were blocked overnight at 4°C in 1% FBS (Gibco), 3% BSA (Sigma), 0.5% Triton X-100 (Sigma). Samples were incubated overnight with primary antibodies in blocking reagent, followed by washing and incubation with the appropriate secondary antibody for 2 h at room temperature, and mounted in fluorescent mounting medium (ProLong Gold Antifade, Thermo Fisher). Endothelial cells were treated with 80 ng/ml mouse VEGFA164 for 10 min before permeabilization with 3% PFA, 0.5% Triton X100 in PBS for 3 min, followed by fixation in 3% PFA in PBS for 15 min. Antibodies were added in 5% BSA, 5% donkey serum in PBS.
Images were acquired using a Zeiss LSM700 confocal microscope. For comparison purposes, different sample images of the same antigen were acquired under constant acquisition settings.

For staining with phospho-antibodies, animals were anaesthetised with ketamine:xylazine mixture (1:4). Using a peristaltic pump, cardiac perfusion was performed with 2 mL of PBS then 2 mL of fixative (1% PFA, 0.1% triethanolamine, 0.1% Triton X100, 0.1% NP-40, (pH 7.5). Eyes were removed and fixed for another 2 hr at room temperature. After dissection, retinas were blocked for 2 hr shaking at RT (0.5% Triton-X100, 0.05% Na-Deoxycholate, 1% BSA, 2% FBS, 0.02% Sodium Azide (prepared in PBS pH = 7.4). Samples were incubated overnight with primary antibodies in blocking reagent, diluted in 1 part block : 1 part 1x TBS, followed by washing 3 x 1 hour at room temperature with TBS 0.2% Triton. Secondary antibodies were added O/N at 4°C with slow agitation in 1 part Buffer B: 1 part 1x TBS, followed by washing 3 x 1 hour at room temperature with TBS 0.2% Triton and mounting mounted in fluorescent mounting medium (Fluoromount, ThermoFisher).

Hyaloid vasculature analysis

Hyaloid vessels were dissected from P5 eyes. Eyeballs were removed and fixed 20 min 4% PFA at room temperature. Using an insulin syringe, eyeballs were injected with 5% gelatin (Sigma) in PBS around the cornea into the eyeball space, 4 injections of approximately 50-100 µl total. Eyeballs were left on ice for 30-45 min to allow gelatin to set, before removal of the remaining eye tissue to isolate the gelatin plug (containing the hyaloid plexus). The hyaloid plexus plug was transferred to a glass slide, before addition of antibodies for 20 min at room temperature, rinsed with PBS and then mounted in fluorescent mounting medium (ProLong Gold Antifade with DAPI, Thermo Fisher).

Immunocytochemistry (ICC)

Immunofluorescent staining was in general performed on HUVECs cultured on 12 mm glass coverslips coated with 5 µg/ml FN (Sigma) or 10 µg/ml Vitronectin (Sigma) until confluent, washed with PBS++ (supplemented with 1 mM CaCl2 and 0.5 mM MgCl2), fixed in 4% PFA (Sigma), blocked for 30 min with 3% BSA, 0.3% Triton-X-100 (Sigma). Primary antibodies were incubated in 1.5% BSA for 60 min at room temperature, followed by washing and incubation with the appropriate secondary antibody for 45 min at room temperature and mounted in ProlongGold + Dapi solution (Cell Signaling Technologies). Z-stack image acquisition was performed on a confocal laser scanning microscope (Zeiss LSM880) using a 40x NA 1.3 or 63x NA 1.4 oil immersion objective.
**VE-cadherin patching image analysis**

VE-cadherin junctional patterning was assessed using a blinded image analysis approach. 3D retinal image stacks were processed for VE-cadherin morphology, into ‘patches’ of 16 x 16 μm. Images were acquired with Zeiss LSM700 confocal microscope, x 63, numerical aperture 1.4 objectives. The VE-cadherin morphology in each patch was hand-classified on a scale from 1: ‘active’ (irregular/serrated morphology with diffuse/vesicular regions, colour labeled red in output images) to 6: ‘inactive’ (straighter morphology with less vesicular staining, colour labeled blue in heatmap images). Heatmap images were generated using specialised Matlab software described previously (Bentley et al., 2014).

**Image analysis**

Phospho-VE-cadherin analysis was performed using ImageJ. First, total VE-cadherin was thresholded and area measured and saved as a mask. Then, phospho-VE-cadherin was thresholded, masked with the total VE-cadherin, and total area was measured.

Focal adhesion analysis (Figure 7) was performed using ImageJ. p-Paxillin staining was thresholded and VE-cadherin was used to draw 2-3 cells per image and added as ROI. Subsequent particle analysis of each ROI was used to measure particle number, size and density per cell.

Phospho(p)-Paxillin retinal analysis was performed using Imaris (Bitplane). Vascular area was defined using Isolectin-B4 staining to define a 3D mask. Within the vascular mask, the Sum Intensity of p-Paxillin was recorded, and normalised to the total area of the vascular mask. To account for variations in staining intensity between samples, 2 non-vascular areas per image were analysed for p-Paxillin and the SumInt/area of the vascular mask was normalised to the average of the non-vascular SumInt/area. 3-4 images were quantified per retina and averaged to determine the p-Paxillin intensity per retina (n is one retina).

Quantification of endothelial cell proliferation, Collagen IV empty sleeves, VE-cadherin phosphorylation and phospho-Paxillin were all performed at the plexus region at the vascular front of the retina. When imaging, arterial and venous regions were carefully avoided. All images shown are representative of images that were quantified.

**Isolation of lung endothelial cells**

Endothelial cells were isolated as described previously (Li et al., 2016). Mouse lungs were collected at P10, minced and digested in 10 ml Dulbecco’s PBS medium containing 2 mg/ml 1 collagenase type I (Sigma), for 1 h at 37°C with shaking, followed by filtration through a 70-
μm disposable cell strainer (BD Falcon). Cells were centrifuged at 400g for 8 min at 4°C, suspended in cold PBS with 0.1% bovine serum albumin (BSA) and incubated with anti-rat immunoglobulin G-coated magnetic beads (Dynabeads sheep anti-Rat IgG, Invitrogen) pre-coupled with rat anti-mouse CD31 for 10 min, with gentle agitation. Beads were separated using a magnetic particle concentrator (Dynal MPC-S, Invitrogen). The beads were washed with PBS, and endothelial cells were suspended in EGM-2 plus medium, supplemented with singlequots (Lonza CC-5035). To induce Cre activity, cells were treated with 1 μM of 4-hydroxytamoxifen (Sigma).

Metatarsal Assay
Metatarsals were isolated from E16.5 mice using a protocol adapted from (Song et al., 2015). After dissection, one metatarsal per well was placed in a μ-Plate 24 well ibiTreat plate, #1.5 polymer coverslip (Ibidi) and left in 170 μl of MEM-alpha (Gibco) with 10% FCS and 1% penicillin/streptomycin (Sigma). After 3 days, media was replaced with 300 μl per well and media changed every subsequent day. To induce Cre activity, cells were treated with 1 μM of 4-hydroxytamoxifen (Sigma) after 5 days. After 14 days, metatarsals were imaged every minute over 4 hours with a Zeiss LSM700 confocal microscope, x 63, numerical aperture 1.4 objectives. Alternatively, metatarsals were fixed with 4% PFA in PBS for 20 min and Antibodies were added 3% Trixon X-100, 1% Tween, 0.5% BSA in PBS.

Cell culture and treatments
Human umbilical vein endothelial cells (HUVECs) purchased from Lonza (Cat # CC-2935), were cultured until passage 5 in EGM-2 plus medium, supplemented with singlequots (Lonza CC-5035). Overnight starvation with EBM-2 plus basal medium, followed by stimulation with recombinant human VEGF-A, 100 ng/ml (R&D systems) for indicated duration. Human Embryonic Kidney (HEK)-293T cells were maintained in DMEM with L-glutamine and sodium pyruvate (Invitrogen), containing 10% (v/v) heat-inactivated fetal bovine serum (GE healthcare Australia), 100 U/ml penicillin and streptomycin (Life Technologies Australia). All cells were cultured at 37°C and 5% CO2.

c-Src-GFP was kindly provided by Dr. Margaret Frame, the University of Edinburgh, UK. The c-Src fragment was obtained by digesting the c-Src-GFP plasmid with XhoI and BamHI and inserted into pmKate2-N plasmid (Evrogen). The c-SRC-mKate2 was then sub-cloned into the pLenti-MP2 plasmid (Addgene #36097) and mKate2 only was sub-cloned into pLenti-MP2 as control for lentiviral construction. Lentiviral constructs were packaged into lentivirus in HEK-293T cells by means of third generation lentiviral packaging plasmids (Dull et al., 1998).
Lentivirus containing supernatant was harvested on day 2 and 3 after transfection. Lentivirus was concentrated by Lenti-X concentrator (Clontech, Cat# 631232). HEK cells were transfected with the expression vectors according to manufacturer’s protocol with PEI 2500 (BioScientific) and lentiviral transduced target HUVECs were used for assays after 48-72h.

siRNA targeting c-Src (VPDSIRNA2D, SASI_Hs01_00112907) and scrambled non-silencing control siRNA (SIC001) both used at working concentration of 20 nM were purchased from Sigma. siRNA transfections were performed according to manufacturer’s protocol using Polyplus INTERFERin (In Vitro Technologies) and cells were used for assays after 48h.

**Western blotting**

After washing once with PBS+/+ (1mM CaCl and 0.5 mM MgCl) cells were lysed in 95°C SDS-sample buffer containing 4% β-mecapto-ethanol. Samples were boiled at 95°C for 5-10 minutes to denature proteins. Proteins were separated on 4-15% mini protean TGX precast gel (Biorad) in running buffer (200 mM Glycine, 25 mM Tris, 0.1% SDS (pH8.6)), transferred to nitrocellulose membrane (BioRad Cat#1620112) in blot buffer (48 mM Tris, 39 mM Glycine, 0.04% SDS, 20% MeOH) and subsequently blocked with 5% (w/v) BSA (Sigma) in Tris-buffered saline with Tween 20 (TBST) for 30 minutes. The immunoblots were analysed using primary antibodies incubated overnight at 4°C and secondary antibodies linked to horseradish peroxidase (HRP) (Thermo Fischer Scientific) incubated for 1h at room temperature, after each step immunoblots were washed 4x with TBST. HRP signals were visualized by enhanced chemiluminescence (ECL) (BioRad) and imaged with Chemidoc.

**Acknowledgements**

We thank Holger Gerhardt (MDC Berlin) and Roland Wedlich-Söldner (University of Münster) for providing Lifeact-EGFP mice. We thank Margaret Frame (University of Edinburgh) for providing the c-Src-GFP plasmid. We are grateful to Pernilla Martinsson for expert technical assistance, and to UQBR and UU animal house husbandry staff for assistance with mouse colony maintenance. We thank Benjamin Hogan, Robert Parton and Nicholas Condon for helpful discussions. We thank Matt Sweet for providing the F4/80 antibody.

**Author contributions**

L.S. and E.G. designed and performed experiments, analysed data and wrote the manuscript. D.F. designed and performed experiments and analysed data. M.R. and Y.J. performed experiments, analysed data and wrote the manuscript. P.E. performed experiments and analysed data. E.F. and M.H. performed experiments. E.D. supplied essential reagents. L.C.W designed experiments, analysed data and wrote the manuscript.
Competing interests
The authors declare no competing interests.

Funding
E.G. was supported by a Wenner-Gren Postdoctoral Fellowship, an ARC DECRA (DE170100167) and NHMRC Project Grant (APP1158002). D.F. was supported by the Gustav Adolf Johansson Foundation, Uppsala University. M.R was supported by EMBO (ALTF 923-2016) and the Swedish Society for Medical Research (SSMF; 201912). L.C.W. was supported by the Swedish Cancer foundation (CAN2016/578), the Knut and Alice Wallenberg foundation (KAW 2015.0030) and a Fondation Leducq Transatlantic Network of Excellence Grant in Neurovascular Disease (17 CVD 03). KAW also supported LCW with a Wallenberg Scholar grant (2015.0275).
References

Aspalter, I. M., Gordon, E., Dubrac, A., Ragab, A., Narloch, J., Vizan, P., Geudens, I.,
Collins, R. T., Franco, C. A., Abrahams, C. L., et al. (2015). Alk1 and Alk5 inhibition
by Nrp1 controls vascular sprouting downstream of Notch. Nature communications 6,
7264.

Bentley, K., Franco, C. A., Philippides, A., Blanco, R., Dierkes, M., Gebala, V., Stanchi,
F., Jones, M., Aspalter, I. M., Cagna, G., et al. (2014). The role of differential VE-
cadherin dynamics in cell rearrangement during angiogenesis. Nature cell biology 16,
309-321.

Bentley, K., Mariggi, G., Gerhardt, H. and Bates, P. A. (2009). Tipping the balance:
robustness of tip cell selection, migration and fusion in angiogenesis. PLoS Comput
Biol 5, e1000549.

Chattopadhyay, N., Wang, Z., Ashman, L. K., Brady-Kalnay, S. M. and Kreidberg, J. A.
(2003). alpha3beta1 integrin-CD151, a component of the cadherin-catenin complex,
regulates PTPmu expression and cell-cell adhesion. The Journal of cell biology 163,
1351-1362.

Chen, X. L., Nam, J. O., Jean, C., Lawson, C., Walsh, C. T., Goka, E., Lim, S. T., Tomar,
A., Tancioni, I., Uryu, S., et al. (2012). VEGF-induced vascular permeability is
mediated by FAK. Developmental cell 22, 146-157.

Chow, B. W. and Gu, C. (2017). Gradual Suppression of Transcytosis Governs Functional
Blood-Retinal Barrier Formation. Neuron 93, 1325-1333 e1323.

Collins, C., Osborne, L. D., Guilluy, C., Chen, Z., O’Brien, E. T., 3rd, Reader, J. S.,
Burrage, K., Superfine, R. and Tzima, E. (2014). Haemodynamic and extracellular
matrix cues regulate the mechanical phenotype and stiffness of aortic endothelial cells.
Nature communications 5, 3984.

Conway, D. E., Breckenridge, M. T., Hinde, E., Gratton, E., Chen, C. S. and Schwartz, M.
A. (2013). Fluid shear stress on endothelial cells modulates mechanical tension across
VE-cadherin and PECAM-1. Curr Biol 23, 1024-1030.

Conway, D. E., Coon, B. G., Budatha, M., Arsenovic, P. T., Orsenigo, F., Wessel, F.,
Zhang, J., Zhuang, Z., Dejana, E., Vestweber, D., et al. (2017). VE-Cadherin
Phosphorylation Regulates Endothelial Fluid Shear Stress Responses through the
Polarity Protein LGN. Curr Biol 27, 2727.

Coon, B. G., Baeyens, N., Han, J., Budatha, M., Ross, T. D., Fang, J. S., Yun, S., Thomas,
J. L. and Schwartz, M. A. (2015). Intramembrane binding of VE-cadherin to VEGFR2
and VEGFR3 assembles the endothelial mechanoensory complex. The Journal of
cell biology 208, 975-986.
Dejana, E., Colella, S., Conforti, G., Abbadini, M., Gaboli, M. and Marchisio, P. C. (1988). Fibronectin and vitronectin regulate the organization of their respective Arg-Gly-Asp adhesion receptors in cultured human endothelial cells. *The Journal of cell biology* **107**, 1215-1223.

Demircioğlu, F. and Hodivala-Dilke, K. (2016). alphavbeta3 Integrin and tumour blood vessels-learning from the past to shape the future. *Curr Opin Cell Biol* **42**, 121-127.

Drab, M., Verkade, P., Elger, M., Kasper, M., Lohn, M., Lauterbach, B., Menne, J., Lindschau, C., Mende, F., Luft, F. C., et al. (2001). Loss of caveolae, vascular dysfunction, and pulmonary defects in caveolin-1 gene-disrupted mice. *Science* **293**, 2449-2452.

Dull, T., Zufferey, R., Kelly, M., Mandel, R. J., Nguyen, M., Trono, D. and Naldini, L. (1998). A third-generation lentivirus vector with a conditional packaging system. *J Virol* **72**, 8463-8471.

Eliceiri, B. P., Paul, R., Schwartzberg, P. L., Hood, J. D., Leng, J. and Cheresh, D. A. (1999). Selective requirement for Src kinases during VEGF-induced angiogenesis and vascular permeability. *Mol Cell* **4**, 915-924.

Eliceiri, B. P., Puente, X. S., Hood, J. D., Stupack, D. G., Schlaepfer, D. D., Huang, X. Z., Sheppard, D. and Cheresh, D. A. (2002). Src-mediated coupling of focal adhesion kinase to integrin alpha(v)beta5 in vascular endothelial growth factor signaling. *The Journal of cell biology* **157**, 149-160.

Espada, J. and Martin-Perez, J. (2017). An Update on Src Family of Nonreceptor Tyrosine Kinases Biology. *Int Rev Cell Mol Biol* **331**, 83-122.

Felding-Habermann, B. and Cheresh, D. A. (1993). Vitronectin and its receptors. *Curr Opin Cell Biol* **5**, 864-868.

Fraccaroli, A., Franco, C. A., Rognoni, E., Neto, F., Rehberg, M., Aszodi, A., Wedlich-Soldner, R., Pohl, U., Gerhardt, H. and Montanez, E. (2012). Visualization of endothelial actin cytoskeleton in the mouse retina. *PloS one* **7**, e47488.

Franco, C. A., Jones, M. L., Bernabeu, M. O., Geudens, I., Mathivet, T., Rosa, A., Lopes, F. M., Lima, A. P., Ragab, A., Collins, R. T., et al. (2015). Dynamic endothelial cell rearrangements drive developmental vessel regression. *PLoS Biol* **13**, e1002125.

Friedlander, M., Brooks, P. C., Shaffer, R. W., Kincaid, C. M., Varner, J. A. and Cheresh, D. A. (1995). Definition of two angiogenic pathways by distinct alpha v integrins. *Science* **270**, 1500-1502.

Gerhardt, H., Golding, M., Fruttiger, M., Ruhrberg, C., Lundkvist, A., Abramsson, A., Jeltsch, M., Mitchell, C., Alitalo, K., Shima, D., et al. (2003). VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. *The Journal of cell biology* **161**, 1163-1177.
Gordon, E. J., Fukuhara, D., Westrom, S., Padhan, N., Sjostrom, E. O., van Meeteren, L., He, L., Orsenigo, F., Dejana, E., Bentley, K., et al. (2016). The endothelial adaptor molecule TSAd is required for VEGF-induced angiogenic sprouting through junctional c-Src activation. *Sci Signal* **9**, ra72.

Gu, X., Fliesler, S. J., Zhao, Y. Y., Stallcup, W. B., Cohen, A. W. and Elliott, M. H. (2014). Loss of caveolin-1 causes blood-retinal barrier breakdown, venous enlargement, and mural cell alteration. *Am J Pathol* **184**, 541-555.

He, Q., Li, X., Singh, K., Luo, Z., Meija-Cordova, M., Jamalpour, M., Lindahl, B., Kriz, V., Vuolteenaho, R., Ulvmar, M., et al. (2019). The Cdh5-CreERT2 transgene causes conditional Shb gene deletion in hematopoietic cells with consequences for immune cell responses to tumors. *Sci Rep* **9**, 7548.

Horton, M. A. (1997). The alpha v beta 3 integrin "vitronectin receptor". *Int J Biochem Cell Biol* **29**, 721-725.

Hynes, R. O., Lively, J. C., McCarty, J. H., Taverna, D., Francis, S. E., Hodivala-Dilke, K. and Xiao, Q. (2002). The diverse roles of integrins and their ligands in angiogenesis. *Cold Spring Harb Symp Quant Biol* **67**, 143-153.

Jean, C., Chen, X. L., Nam, J. O., Tancioni, I., Uryu, S., Lawson, C., Ward, K. K., Walsh, C. T., Miller, N. L., Ghassemian, M., et al. (2014). Inhibition of endothelial FAK activity prevents tumor metastasis by enhancing barrier function. *The Journal of cell biology* **204**, 247-263.

Kim, D. J., Norden, P. R., Salvador, J., Barry, D. M., Bowers, S. L. K., Cleaver, O. and Davis, G. E. (2017). Src- and Fyn-dependent apical membrane trafficking events control endothelial lumen formation during vascular tube morphogenesis. *PloS one* **12**, e0184461.

Kleinschmidt, E. G. and Schlaepfer, D. D. (2017). Focal adhesion kinase signaling in unexpected places. *Curr Opin Cell Biol* **45**, 24-30.

Koch, S. and Claesson-Welsh, L. (2012). Signal transduction by vascular endothelial growth factor receptors. *Cold Spring Harb Perspect Med* **2**, a006502.

Kogata, N., Arai, Y., Pearson, J. T., Hashimoto, K., Hidaka, K., Koyama, T., Somekawa, S., Nakaoka, Y., Ogawa, M., Adams, R. H., et al. (2006). Cardiac ischemia activates vascular endothelial cadherin promoter in both preexisting vascular cells and bone marrow cells involved in neovascularization. *Circulation research* **98**, 897-904.

Koh, W., Sachidanandam, K., Stratman, A. N., Sacharidou, A., Mayo, A. M., Murphy, E. A., Cheresh, D. A. and Davis, G. E. (2009). Formation of endothelial lumens requires a coordinated PKCε-spilian-, Src-, Pak- and Raf-kinase-dependent signaling cascade downstream of Cdc42 activation. *J Cell Sci* **122**, 1812-1822.
Lamalice, L., Houle, F. and Huot, J. (2006). Phosphorylation of Tyr1214 within VEGFR-2 triggers the recruitment of Nck and activation of Fyn leading to SAPK2/p38 activation and endothelial cell migration in response to VEGF. *The Journal of biological chemistry* **281**, 34009-34020.

Lenard, A., Ellertsdottir, E., Herwig, L., Krudewig, A., Sauteur, L., Belting, H. G. and Affolter, M. (2013). In vivo analysis reveals a highly stereotypic morphogenetic pathway of vascular anastomosis. *Developmental cell* **25**, 492-506.

Li, X., Padhan, N., Sjostrom, E. O., Roche, F. P., Testini, C., Honkura, N., Sainz-Jaspeado, M., Gordon, E., Bentley, K., Philippides, A., et al. (2016). VEGFR2 pY949 signalling regulates adherens junction integrity and metastatic spread. *Nature communications* **7**, 11017.

Lim, S. T., Chen, X. L., Tomar, A., Miller, N. L., Yoo, J. and Schlaeffer, D. D. (2010). Knock-in mutation reveals an essential role for focal adhesion kinase activity in blood vessel morphogenesis and cell motility-polarity but not cell proliferation. *The Journal of biological chemistry* **285**, 21526-21536.

Ma, X., Zhang, L., Song, J., Nguyen, E., Lee, R. S., Rodgers, S. J., Li, F., Huang, C., Schittenhelm, R. B., Chan, H., et al. (2019). Characterization of the Src-regulated kinome identifies SGK1 as a key mediator of Src-induced transformation. *Nature communications* **10**, 296.

Madisen, L., Zwingman, T. A., Sunkin, S. M., Oh, S. W., Zariwala, H. A., Gu, H., Ng, L. L., Palmiter, R. D., Hawrylycz, M. J., Jones, A. R., et al. (2010). A robust and high-throughput Cre reporting and characterization system for the whole mouse brain. *Nat Neurosci* **13**, 133-140.

Matsumoto, T., Bohman, S., Dixelius, J., Berge, T., Dimberg, A., Magnusson, P., Wang, L., Wikner, C., Qi, J. H., Wernstedt, C., et al. (2005). VEGF receptor-2 Y951 signaling and a role for the adapter molecule TSAd in tumor angiogenesis. *The EMBO journal* **24**, 2342-2353.

Mui, K. L., Chen, C. S. and Assoian, R. K. (2016). The mechanical regulation of integrin-cadherin crosstalk organizes cells, signaling and forces. *J Cell Sci* **129**, 1093-1100.

Muzumdar, M. D., Tasic, B., Miyamichi, K., Li, L. and Luo, L. (2007). A global double-fluorescent Cre reporter mouse. *Genesis* **45**, 593-605.

Orsenigo, F., Giampietro, C., Ferrari, A., Corada, M., Galaup, A., Sigismund, S., Ristagno, G., Maddaluno, L., Koh, G. Y., Franco, D., et al. (2012). Phosphorylation of VE-cadherin is modulated by haemodynamic forces and contributes to the regulation of vascular permeability in vivo. *Nature communications* **3**, 1208.
Paul, R., Zhang, Z. G., Eliceiri, B. P., Jiang, Q., Boccia, A. D., Zhang, R. L., Chopp, M. and Cheresh, D. A. (2001). Src deficiency or blockade of Src activity in mice provides cerebral protection following stroke. *Nature medicine* 7, 222-227.

Plow, E. F., Meller, J. and Byzova, T. V. (2014). Integrin function in vascular biology: a view from 2013. *Curr Opin Hematol* 21, 241-247.

Potente, M., Gerhardt, H. and Carmeliet, P. (2011). Basic and therapeutic aspects of angiogenesis. *Cell* 146, 873-887.

Pulous, F. E., Grimsley-Myers, C. M., Kansal, S., Kowalczyk, A. P. and Petrich, B. G. (2019). Talin-Dependent Integrin Activation Regulates VE-Cadherin Localization and Endothelial Cell Barrier Function. *Circulation research* 124, 891-903.

Raimondi, C., Fantin, A., Lampropoulou, A., Denti, L., Chikh, A. and Ruhrberg, C. (2014). Imatinib inhibits VEGF-independent angiogenesis by targeting neuropilin 1-dependent ABL1 activation in endothelial cells. *The Journal of experimental medicine* 211, 1167-1183.

Razani, B., Engelman, J. A., Wang, X. B., Schubert, W., Zhang, X. L., Marks, C. B., Macaluso, F., Russell, R. G., Li, M., Pestell, R. G., et al. (2001). Caveolin-1 null mice are viable but show evidence of hyperproliferative and vascular abnormalities. *The Journal of biological chemistry* 276, 38121-38138.

Riedl, J., Flynn, K. C., Raducanu, A., Gartner, F., Beck, G., Bosl, M., Bradke, F., Massberg, S., Aszodi, A., Sixt, M., et al. (2010). Lifeact mice for studying F-actin dynamics. *Nat Methods* 7, 168-169.

Sandilands, E., Cans, C., Fincham, V. J., Brunton, V. G., Mellor, H., Prendergast, G. C., Norman, J. C., Superti-Furga, G. and Frame, M. C. (2004). RhoB and actin polymerization coordinate Src activation with endosome-mediated delivery to the membrane. *Developmental cell* 7, 855-869.

Schaffner, F., Ray, A. M. and Don tenwill, M. (2013). Integrin alpha5beta1, the Fibronectin Receptor, as a Pertinent Therapeutic Target in Solid Tumors. *Cancers (Basel)* 5, 27-47.

Scheppke, L., Aguilar, E., Gariano, R. F., Jacobson, R., Hood, J., Doukas, J., Cao, J., Noronha, G., Yee, S., Weis, S., et al. (2008). Retinal vascular permeability suppression by topical application of a novel VEGFR2/Src kinase inhibitor in mice and rabbits. *The Journal of clinical investigation* 118, 2337-2346.

Simons, M., Gordon, E. and Claesson-Welsh, L. (2016). Mechanisms and regulation of endothelial VEGF receptor signalling. *Nat Rev Mol Cell Biol* 17, 611-625.

Sindhwani, S., Syed, A. M., Ngai, J., Kingston, B. R., Maiorino, L., Rothschild, J., MacMillan, P., Zhang, Y., Rajesh, N. U., Hoang, T., et al. (2020). The entry of nanoparticles into solid tumours. *Nat Mater.*
Song, W., Fhu, C. W., Ang, K. H., Liu, C. H., Johari, N. A., Lio, D., Abraham, S., Hong, W., Moss, S. E., Greenwood, J., et al. (2015). The fetal mouse metatarsal bone explant as a model of angiogenesis. *Nat Protoc* 10, 1459-1473.

Soriano, P., Montgomery, C., Geske, R. and Bradley, A. (1991). Targeted disruption of the c-src proto-oncogene leads to osteopetrosis in mice. *Cell* 64, 693-702.

Stenzel, D., Franco, C. A., Estrach, S., Mettouchi, A., Sauvaget, D., Rosewell, I., Schertel, A., Armer, H., Domogatskaya, A., Rodin, S., et al. (2011). Endothelial basement membrane limits tip cell formation by inducing Dll4/Notch signalling in vivo. *EMBO Rep* 12, 1135-1143.

Sun, Z., Li, X., Massena, S., Kutscher, S., Padhan, N., Gualandi, L., Sundvold-Gjerstad, V., Gustafsson, K., Choy, W. W., Zang, G., et al. (2012). VEGFR2 induces c-Src signaling and vascular permeability in vivo via the adaptor protein TSAd. *The Journal of experimental medicine* 209, 1363-1377.

Testini, C., Smith, R. O., Jin, Y., Martinsson, P., Sun, Y., Hedlund, M., Sainz-Jaspeado, M., Shibuya, M., Hellstrom, M. and Claesson-Welsh, L. (2019). Myc-dependent endothelial proliferation is controlled by phosphotyrosine 1212 in VEGF receptor-2. *EMBO Rep* 20, e47845.

Tzima, E., Irani-Tehrani, M., Kiosses, W. B., Dejana, E., Schultz, D. A., Engelhardt, B., Cao, G., DeLisser, H. and Schwartz, M. A. (2005). A mechanosensory complex that mediates the endothelial cell response to fluid shear stress. *Nature* 437, 426-431.

Wang, Y., Nakayama, M., Pitulescu, M. E., Schmidt, T. S., Bochenek, M. L., Sakakibara, A., Adams, S., Davy, A., Deutsch, U., Luthi, U., et al. (2010). Ephrin-B2 controls VEGF-induced angiogenesis and lymphangiogenesis. *Nature* 465, 483-486.

Weber, G. F., Bjerke, M. A. and DeSimone, D. W. (2011). Integrins and cadherins join forces to form adhesive networks. *J Cell Sci* 124, 1183-1193.

Weis, S., Cui, J., Barnes, L. and Cheresh, D. (2004). Endothelial barrier disruption by VEGF-mediated Src activity potentiates tumor cell extravasation and metastasis. *The Journal of cell biology* 167, 223-229.

Wessel, F., Winderlich, M., Holm, M., Frye, M., Rivera-Galdos, R., Vockel, M., Linnepe, R., Ipe, U., Stadtmann, A., Zarbock, A., et al. (2014). Leukocyte extravasation and vascular permeability are each controlled in vivo by different tyrosine residues of VE-cadherin. *Nat Immunol* 15, 223-230.

Westhoff, M. A., Serrels, B., Fincham, V. J., Frame, M. C. and Carragher, N. O. (2004). SRC-mediated phosphorylation of focal adhesion kinase couples actin and adhesion dynamics to survival signaling. *Mol Cell Biol* 24, 8113-8133.
Yamamoto, H., Ehling, M., Kato, K., Kanai, K., van Lessen, M., Frye, M., Zeuschner, D., Nakayama, M., Vestweber, D. and Adams, R. H. (2015). Integrin beta1 controls VE-cadherin localization and blood vessel stability. *Nature communications* **6**, 6429.

Zaidel-Bar, R., Milo, R., Kam, Z. and Geiger, B. (2007). A paxillin tyrosine phosphorylation switch regulates the assembly and form of cell-matrix adhesions. *J Cell Sci* **120**, 137-148.

Zimnicka, A. M., Husain, Y. S., Shajahan, A. N., Sverdlov, M., Chaga, O., Chen, Z., Toth, P. T., Klomp, J., Karginov, A. V., Tiruppathi, C., et al. (2016). Src-dependent phosphorylation of caveolin-1 Tyr-14 promotes swelling and release of caveolae. *Mol Biol Cell* **27**, 2090-2106.
Figure 1. c-Src is required for developmental angiogenesis.

(A) Endothelial cells were isolated from lungs of tamoxifen treated c-Src^{flox/flox} and c-Src^{flox/flox}; Cdh5-CreERT2 mice and c-Src protein knockdown was assessed by western blot.

(B) A reduction in c-Src protein was observed from c-Src^{flox/flox}; Cdh5-CreERT2 mice compared to c-Src^{flox/flox}. n > 6.

(C-D) Vasculature of tracheas from P6 mice immunostained for CD31. Scale bar, 100 μm.

(E-H) Vasculature of retinas from P6 mice immunostained for Isolectin B4. Scale bar, 1000 μm E, F, 100 μm G, H.

(I) c-Src-deficient tracheas display reduced capillaries crossing cartilage rings of tracheas, n>8, n is number of mice.
(J-M) c-Src-deficient retinas display reduced vascular area, outgrowth from the optic nerve and number of branch points, but no change in number of tip cells. n > 6, n is number of retinas.
*p< 0.05, **p< 0.01, ****p< 0.0001. Error bars represent mean ± SEM. Statistical significance was determined using a Mann-Whitney test.
Figure 2. c-Src regulates filopodial extension but not tip cell identity.

(A, B) Metatarsals from E16.5 embryos were cultured for 14 days and blood vessels were immunostained for CD31.

(C, D) c-Src-deficient metatarsals displayed significantly reduced total vessel growth and branch points in vasculature from metatarsals. n > 13, n is number of metatarsals.

(E-F) c-Src\textsuperscript{lox/lox}; mTmG; Cdh5-CreERT2 metatarsals, where recombined cells express GFP and non-recombined cells are immunostained for CD31. Scale bar, 1000 μm E, 100 μm F.
(G, H) c-Src\textsuperscript{flox/flox}, mTm; Cdh5-CreERT2 retinas, where recombined cells express GFP and non-recombined cells are immunostained for Isolectin B4. Scale bar, 1000 µm G, 50 µm H.

(I, J) Quantification of recombined c-Src-deficient cells at the tip, normalised to overall contribution of cells to the endothelium. Statistical significance was determined by comparing the proportion of c-Src-deficient (green) cells at the tip with the total proportion of c-Src-deficient cells. No significant changes were observed. n=12, n is number of metatarsals (I) or retinas (J).

(K, L) c-Src\textsuperscript{flox/flox}; Lifeact and c-Src\textsuperscript{flox/flox}; Lifeact; Cdh5-CreERT2 retinas were analysed for filopodial protrusions at the vascular front of P6 retinas. Red dots denote sites of filopodial termination. Scale bar, 25 µm.

(M, N) c-Src-deficient retinas display reduced number of filopodia and average length of filopodia. The number of filopodia was quantified from a standardised length of the vascular front (1024 µm). n > 4, n is number of retinas.

**p< 0.01. Error bars represent mean ± SEM. Statistical significance was determined using a Mann-Whitney test.
Figure 3. c-Src controls vascular density through vessel stability.

(A, B) Proliferating cells in c-Src^flox/flox and c-Src^flox/flox; Cdh5-CreERT2 retinas at P5 were detected by Ki67 immunostaining. Arrows highlight Ki67-positive cells. Scale bar, 100 μm.

(C, D) Apoptosis in c-Src^flox/flox and c-Src^flox/flox; Cdh5-CreERT2 retinas at P5 were detected by Cleaved Caspase 3 immunostaining. Arrows highlight apoptotic cells. Scale bar, 100 μm.

(E) No changes were detected in endothelial cell proliferation upon loss of c-Src. n > 4, n is number of retinas.
(F) No changes were detected in endothelial cell apoptosis upon loss of c-Src. n > 4, n is number of retinas.

(G, H) Pericyte coverage in c-Src$^{\text{flox/flox}}$ and c-Src$^{\text{flox/flox}}$; Cdh5-CreERT2 retinas at P5 was assessed by NG2 immunostaining. Scale bar, 100 μm.

(I) No changes were detected in pericyte coverage upon loss of c-Src. n > 4, n is number of retinas.

(J, K) Visualisation of empty collagen IV sleeves in c-Src$^{\text{flox/flox}}$ and c-Src$^{\text{flox/flox}}$; Cdh5-CreERT2 retinas at P5. Arrowheads indicate empty sleeves in the retinal vasculature. Scale bar, 50 μm.

(L) c-Src$^{\text{flox/flox}}$; Cdh5-CreERT2 retinas at P5 display increased number of empty collagen IV sleeves in the retinal vasculature compared to c-Src$^{\text{flox/flox}}$ littermates. n > 6, n is the number of retinas.

(M, N) Empty collagen IV sleeves in both c-Src$^{\text{flox/flox}}$ and c-Src$^{\text{flox/flox}}$; Cdh5-CreERT2 retinas lack Icam2 expression, suggesting c-Src is not required for initial vessel lumen formation, but for lumen maintenance and stability. Arrowheads indicate empty collagen IV sleeves. Scale bar, 50 μm.

(O, P) Visualisation of macrophages by F4/80 immunostaining in c-Src$^{\text{flox/flox}}$ and c-Src$^{\text{flox/flox}}$; Cdh5-CreERT2 retinas. Arrowheads indicate macrophages. Scale bar, 50 μm.

(Q) c-Src$^{\text{flox/flox}}$; Cdh5-CreERT2 retinas at P6 display no difference in the number of macrophages within the vasculature or associated with empty collagen IV sleeves compared to c-Src$^{\text{flox/flox}}$ littermates. n > 6, n is the number of retinas.

**p< 0.01. Error bars represent mean ± SEM. Statistical significance was determined using a Mann-Whitney test.
Figure 4. VEGF-A induced c-Src activation does not significantly alter VE-cadherin patterning.

(A-D) High magnification images of the sprouting front of retinas. B and D are heat maps of the VE-cadherin (VEC) morphology of retinas in A and C, respectively. Scale bar, 25 μm.

(E, F) Representative patches of VE-cadherin morphology, from active (warm colours) to intermediate to inactive (cool colours).
(G) Quantification of A-D. The VE-cadherin morphology in each patch was manually classified using a scale from active (red, serrated line, and bright interior) to inactive (blue, straight line, and dark interior). n=4, n is number of retinas, >150 patches quantified per retina.

(H) Endothelial cells were isolated from lungs of $c-Src^{\text{fl}ox/\text{fl}ox}$ and $c-Src^{\text{fl}ox/\text{fl}ox}; Cdh5-\text{CreERT2}$ mice and protein expression was assessed by western blot.

(I) No significant decrease in p-VE-cadherin at either Y658 and Y685 was observed by western blot. n>6 mice.

(J-M) High magnification images of the sprouting front of retinas immunostained for VE-cadherin and phospho-VE-cadherin Y658 (pVECY658). K and M are single channel images of pVECY658 in J and K, respectively. Scale bar, 25 $\mu$m.

(N) Quantification of pVECY658 staining area normalised to total VEC immunostaining. n>12, n is the number of retinas.

(O-R) High magnification images of the sprouting front of retinas immunostained with VE-cadherin and phospho-VE-cadherin Y685 (pVECY685). P and R are single channel images of pVECY685 in O and Q, respectively. Scale bar, 25 $\mu$m.

(S) Quantification of pVECY685 staining area normalised to total VEC immunostaining. n>12, n is the number of retinas.

Error bars represent mean ± SEM. Statistical significance was determined using a Mann-Whitney test.
Figure 5. c-Src is required for vessel growth but not for the velocity of sprout movement.

(A-A''', B-B''') Metatarsals from c-Src<sup>flx/flx</sup>; Lifeact and c-Src<sup>flx/flx</sup>; Lifeact; Cdhs5-CreERT2 E16.6 embryos were imaged over 4 hours at 37 degrees, an image for every hour is shown. Red dots indicate filopodia. Scale bar, 25 μm.

(C) c-Src deficient sprouts displayed reduced forward movement.

(D) Velocity of total sprout movement over 4h. c-Src deficient sprouts displayed no changes in overall sprout velocity.

(E) Directional velocity of total sprout movement over 4h, positive values are forward movement, negative values are backwards movement. c-Src deficient sprouts displayed reduced forward sprout velocity.

(F) Directional velocity over 30-minute increments. c-Src deficient sprouts had both forward and backwards movement, demonstrating erratic movements. WT sprouts moved in a largely consistent forward direction.

n > 14, n is number of sprouts from 3 independent experiments.

**p< 0.01. Error bars represent mean ± SEM. Statistical significance was determined using a Mann-Whitney test.
Figure 6. VEGF-A/VEGFR2 activation does not alter c-Src phosphorylation.

(A) HUVECs transfected with Control or c-Src siRNA, or with overexpression of c-Src-mKate2 were treated with VEGF-A for 0, 2, 5 or 20 minutes. Activation of proteins was assessed using phospho-specific antibodies and western blotting.

(B-D) Total VEGFR2 levels (normalised to GAPDH loading control) decreased upon activation by VEGF-A, shown by phosphorylation on Y951 and Y1175 (normalised to total VEGFR2 expression).

(E-G) Upon VEGF-A stimulation c-Src did not become phosphorylated at Y416 or Y527 (normalised to total c-Src expression).

(H) Total VE-cadherin levels (normalised to GAPDH loading control) did not significantly change upon stimulation with VEGF-A, c-Src depletion or overexpression.

(I-J) Total FAK levels (normalised to GAPDH loading control) and activated Y576 FAK, (normalised to total FAK) are not responsive to VEGF-A stimulation. c-Src-mK2 overexpression increased FAK activation at Y576.
(K-L) Total Paxillin levels (normalised to GAPDH loading control) and activated Y118 Paxillin, did not increase with VEGF-A stimulation. c-Src-mK2 overexpression increased Paxillin activation at Y118.

n=3 from 3 independent experiments. Error bars represent mean ± SEM.
Figure 7. c-Src mediates Paxillin phosphorylation and subsequent focal adhesion formation.

(A) Endothelial cells were isolated from lungs of c-Src^{flox/flox} and c-Src^{flox/flox}, Cdh5-CreERT2 mice and protein expression was assessed by western blot.

(B) A decrease in phospho-Paxillin at Y118 was observed. n>5 mice.

(C) HUVECs transfected with siRNA for Control or c-Src, or with overexpression of empty-mKate2 or c-Src-mKate2 cultured on fibronectin coated coverslips were immunostained for p-Paxillin-Y118 (pPaxY118), c-Src (for siRNA treated cells), VE-cadherin and DAPI. c-Src-
mKate2 is visualised with mKate2 signal. Single nel images of pPaxY118 are shown on the right, mKate2-positive cells are shown with dashed lines. Scale bar, 20 μm.

(D-F) Focal adhesion number, density and size based on pPaxY118 staining was significantly increased upon c-Src-mKate2 overexpression. n>30 cells in total, from 3 independent experiments.

(G, H) HUVECs transfected with siRNA for Control or c-Src cultured on vitronectin coated coverslips were stained for p-Paxillin-Y118 (pPaxY118), VE-cadherin and DAPI. Single panel images of pPaxY118 are shown on the right. Scale bar, 20 μm.

(I-K) Focal adhesion number and density based on pPaxY118 staining was significantly decreased upon c-Src depletion, while the size was not significantly changed. n>15 cells in total, from 3 independent experiments.

(L-O) c-Src<sup>flox/flox</sup>; mTmG and c-Src<sup>flox/flox</sup>; mTmG; Cdh5-CreERT2 retinas were immunostained with pPaxY118, H and J show single channel pPaxY118. Boxed inserts show higher magnification of sprouting front in dashed box. Scale bar, 25 μm.

(P) A significant decrease in the pPaxY118 staining intensity of the vasculature (normalised to pPaxY118 staining of the non-vascular retina) was observed upon loss of c-Src. Scale bar, 50 μm. n>15, n is number of retinas.

*p< 0.05, ***p< 0.005, ****p< 0.001. Error bars represent mean ± SEM. Statistical significance was determined using a Wilcoxon matched-pairs signed rank test (D-F) or Mann-Whitney test (B, I-K, P).
Supplemental Information

Figure S1. Analysis of global c-Src knockout mice.

(A, B) WT and c-Src-/− mice were analysed for retinal defects at P6. Scale bar, 200 µm.

(C) Moderate but not significant defects were observed in total vascular area.

(D) c-Src-/− retinas at P6 display increased number of empty collagen IV sleeves in the retinal vasculature compared to WT littermates. n > 10, n is the number of retinas.

** p < 0.01. Error bars represent mean ± SEM. Statistical significance was determined using a Mann-Whitney test.
Figure S2. Generation of c-Src floxed mice.

(A) c-Src-floxed mice were generated through homologous recombination and insertion of a FRT-SA-IRES-LacZ-loxP cassette targeted to the 5’ upstream of exon 7 in the Src (AW259666; pp60c-src) gene (gene ID:20779). A single loxP site was inserted downstream of exon 9.

(B) To render the mouse conditional, the KO mice were crossed with FLP deleted mice, causing global excision of the FRT sites, with two loxP sites remaining. The deletion results in deletion of exon 7,8,9 and a frame shift mutation in exon 10.

(C) Western blot assessment of c-Src protein expression in endothelial cells isolated from lungs of c-Src<sup>floxed/flox</sup> and c-Src<sup>floxed/flox; Cdh5-CreERT2</sup> mice showed no presence of predicted truncated 15.7 kDa c-Src protein after induction of c-Src deletion.
Figure S3. Analysis of c-Src\textsuperscript{floox/floox}; Cdh5-CreERT2 mice.

(A) Endothelial cells were isolated from lungs of c-Src\textsuperscript{floox/floox} and c-Src\textsuperscript{floox/floox}; Cdh5-CreERT2 mice and protein expression was assessed by western blot.

(B, C) While no significant change was detected in Yes expression, Fyn expression decreased in c-Src deficient mice. \(n>5\) mice.

(D, E) Vasculature of retinas from P23 mice immunostained with Isolectin B4. Scale bar, 1000 \(\mu\)m.

(F) c-Src-deficient retinas display no reduced vascular area. \(n>6\), \(n\) is the number of retinas.

(G-L) Vasculature of c-Src\textsuperscript{floox/floox} and c-Src\textsuperscript{floox/floox}; Cdh5-CreERT2 retinas from P23 mice
immunostained with Isolectin B4, Collagen IV and Icam2 at different depths showed no
difference in the fully remodelled retinal vasculature. Scale bar, 100 µm.

(M-P) Hyaloid vasculature of c-Src\textsuperscript{flox/flox} and c-Src\textsuperscript{flox/flox}; \textit{tdTomato}; \textit{Cdh5-CreERT2} mice at
P5. Scale bar, 500 µm in M and O, 100 µm in N and P. Images are representative of \textit{n}>6 mice.

** p < 0.01. Error bars represent mean ± SEM. Statistical significance was determined using
a Mann-Whitney test.
**Figure S4.** phospho-c-Src Y416 in migrating HUVECs.

(A) HUVECs migrating for 3 hours after scratching were segmented into 4 zones of 50 µm starting at the migration front.

(B) HUVECs were immunostained with phospho-c-Src Y416 to show active c-Src, VE-cadherin and DAPI. Scale bar, 50 µm.

(C, D) Higher magnification images of boxed areas in B show subcellular distribution of phospho-c-Src Y416 at the vascular front (C) and at junctions (D). Scale bar, 20 µm.

(E) c-Src activity based on phospho-c-Src Y416 intensity in the 4 zones in A showed a significant increase in staining intensity in the second and third zone compared to the migration front in zone 1. n=21 images from 2 independent experiments.

* p < 0.0180, **** p < 0.0001. Error bars represent mean ± SEM. Statistical significance was determined using a one-way ANOVA with Holm-Sidak’s multiple comparisons test.
Figure S5. Lifeact-EGFP is highly expressed in endothelial cells.

(A) Lifeact-EGFP vessels in the retina co-stained with Isolectin B4 and VE-cadherin, confirming their endothelial identity. Scale bar, 25 μm.

(B) Lifeact-EGFP vessels in metatarsals co-stained with CD31, confirming their endothelial identity. Scale bar, 25 μm.
Figure S6. No haemorrhage is observed in c-Src-deficient retinas.

(A-D) c-Src^{flox/flox} and c-Src^{flox/flox}; Cdh5-CreERT2 retinas after dissection showed no bleeding at P6. Scale bar, 200 µm.

(E, F) c-Src^{flox/flox} and c-Src^{flox/flox}; Cdh5-CreERT2 retinas immunostained for Ter119 (red) to visualise red blood cells (indicated with white arrows) and Isolectin B4 (green) at P6. Scale bar, 100 µm.

(G) No red blood cells were detected outside of the vasculature in c-Src^{flox/flox} or c-Src^{flox/flox}; Cdh5-CreERT2 retinas. n>4, n is the number of retinas. Error bars represent mean ± SEM. Statistical significance was determined using a Mann-Whitney test.
Figure S7. Empty collagen IV sleeves at sprouting front.
(A, B) Representative images of empty Collagen IV sleeves in c-Src<sup>fl/fl</sup> and c-Src<sup>fl/fl</sup>, Cdh5-CreERT2 retinas at the sprouting front. CollIV empty sleeves are abundant at the vascular front. Arrowheads indicate empty Collagen IV sleeves. Scale bar, 50 µm.
Figure S8. c-Src protein knockdown after siRNA.

(A) A significant decrease in c-Src expression upon transfection with siRNA based on c-Src staining intensity. n>30 cells in total, from 3 independent experiments. Intensity measurements from cells used in Figure 7 C-F.

(B-D) Western blotting of HUVECs used in Figure 7L-P showed no significant decrease in p-Paxillin Y118, while c-Src protein expression was significantly reduced upon siRNA transfection. n=4 from 4 independent experiments.

* p< 0.05, *** p < 0.001. Error bars represent mean ± SEM. Statistical significance was determined using a Mann-Whitney test.
Figure S9. Fibronectin deposit by Src depleted HUVECs.

(A) HUVECs transfected with control or c-Src siRNA were cultured on non-coated glass and stained with phospho-c-Src Y416, fibronectin and DAPI. Scale bar, 100 µm.

(B) Quantification of fibronectin deposit area showed no significant difference between control and c-Src depleted cells. Area was determined by thresholding fibronectin staining and measuring using ImageJ. n= 30 images from 3 independent experiments.

** p < 0.01. Error bars represent mean ± SEM. Statistical significance was determined using a Mann Whitney test.