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The cytoplasmic domain of neuropilin 1 is dispensable for angiogenesis, but promotes the spatial separation of retinal arteries and veins

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SUMMARY

Neuropilin 1 (NRP1) is a transmembrane glycoprotein that is essential for blood vessel development in vertebrates. Best known for its ability to bind members of the vascular endothelial growth factor (VEGF) and class 3 semaphorin families through its extracellular domain, it also has a highly conserved cytoplasmic domain, which terminates in a SEA motif that binds the PDZ protein synectin/GIPC1/NIP. Previous studies in zebrafish embryos and tissue culture models raised the possibility that the SEA motif of NRP1 is essential for angiogenesis. Here, we describe the generation of mice that express a form of NRP1 that lacks the cytoplasmic domain and, therefore, the SEA motif (*Nrp1*$^{cyto\Delta/\Delta}$ mice). Our analysis of pre- and perinatal vascular development revealed that vasculogenesis and angiogenesis proceed normally in these mutants, demonstrating that the membrane-anchored extracellular domain is sufficient for vessel growth. By contrast, the NRP1 cytoplasmic domain is required for normal arteriovenous patterning, because arteries and veins crossed each other at an abnormally high frequency in the *Nrp1*$^{cyto\Delta/\Delta}$ retina, as previously reported for mice with haploinsufficient expression of VEGF in neural progenitors. At crossing sites, the artery was positioned anteriorly to the vein, and both vessels were embedded in a shared collagen sleeve. In human eyes, similar arteriovenous crossings are risk factors for branch retinal vein occlusion (BRVO), an eye disease in which compression of the vein by the artery disrupts retinal blood flow, causing local tissue hypoxia and impairing vision. *Nrp1*$^{cyto\Delta/\Delta}$ mice may therefore provide a suitable genetic model to study the aetiology of BRVO.

KEY WORDS: Neuropilin, Angiogenesis, Artery, Vein, VEGF

INTRODUCTION

Organ development, homeostasis and repair rely on properly perfused blood vessel networks, which form during embryogenesis and postnatal development (Risau, 1997). The first blood vessels in the embryo are assembled from single cell precursors in a process known as vasculogenesis. This primitive plexus expands by sprouting angiogenesis and is remodelled into a hierarchical vascular tree containing capillaries, arterioles, venules, arteries and veins. A key molecule promoting all stages of vascular development is the vascular endothelial growth factor VEGF, which exists in four isoforms known as VEGF120, VEGF144, VEGF164 and VEGF188 in mice, and VEGF121, VEGF145, VEGF165 and VEGF189 in humans (Ruhrberg, 2003). All VEGF isoforms bind the receptor tyrosine kinases VEGFR1 (FLT1) and VEGFR2 (KDR, FLK1). VEGFR2 is the main signalling VEGF receptor in endothelial cells, but its activity is balanced by VEGFR1, which sequesters excess VEGF with its extracellular domain (Shibuya, 2006; Kappas et al., 2008).

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The neuropilins NRP1 and NRP2 are non-tyrosine kinase receptors for VEGF. NRP1 preferentially binds VEGF165, whereas NRP2 binds both VEGF165 and VEGF145 (Soker et al., 1998; Gluzman-Poltorak et al., 2000). A recent study suggests that VEGF121 also binds NRP1 in human endothelial cells that express KDR (Pan et al., 2007). NRP1-deficient mice die between embryonic day (E) 10.5 and 14.5 with defects in brain, spinal cord and yolk sac vasculature, and disrupted aortic arch remodelling (Kawasaki et al., 1999; Gerhardt et al., 2004; Schwarz et al., 2004; Jones et al., 2008), whereas NRP2-deficient mice survive postnatally with lymphatic, but not blood vascular defects (Yuan et al., 2002).

NRP1 has a large extracellular domain of 860 amino acid residues, a short transmembrane domain of 23 amino acids and an intracellular domain of 39 amino acids (Fujisawa, 2002; Schwarz and Ruhrberg, 2010). In neurons, NRP1 recruits plexins to transduce semaphorin signals independently of the NRP1 cytoplasmic tail (Nakamura et al., 1998). By analogy, it has been proposed that NRP1 recruits VEGFR1 or VEGFR2 to transduce VEGF signals in endothelial cells (e.g. Soker et al., 1998; Gluzman-Poltorak et al., 2001; Fantin et al., 2009). In agreement, NRP1 enhances VEGFR2 signalling by binding VEGF165 and promotes complex formation between the three molecules in cultured endothelial cells (Soker et al., 1998; Whitaker et al., 2001; Soker et al., 2002). VEGFR2/NRP1 interaction also requires the three C-terminal amino acids (SEA) of the NRP1 cytoplasmic domain, which confer binding to the PDZ protein synectin, also known as NIP or GIPC1 (De Vries et al., 1998; Cai and Reed, 1999; Gao et al., 2000; Prahst et al., 2008). The SEA motif promotes VEGF-dependent endothelial migration and fibronectin assembly in vitro (Wang et al., 2006; Valdembri et al., 2009).

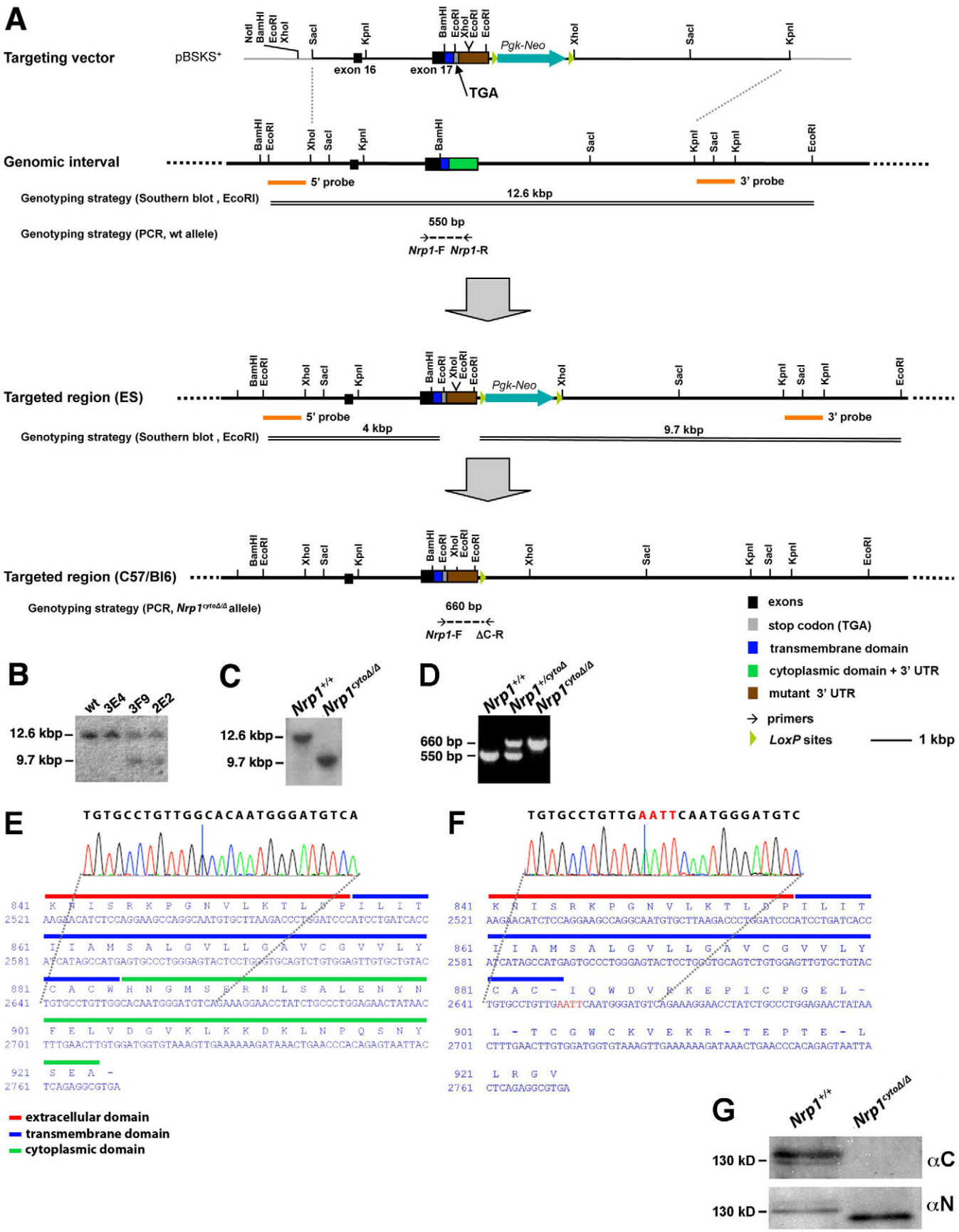

Fig. 1. See next page for legend.

Fig. 1. Generation of mouse mutants lacking the NRP1 cytoplasmic domain. (**A**) The targeting construct contains a stop codon after the sequence encoding the transmembrane domain. (**B**,**C**) Southern blot of *Eco*RI-digested genomic DNA from neomycin-resistant ES cell clones (B) and gene-targeted mice after removal of the neomycin cassette (C) with the 3′ probe (orange). (**D**) PCR analysis of genomic mouse DNA with the oligonucleotides indicated in A. (**E**,**F**) cDNA sequencing of E9.5 embryos; sequencing traces in the mutated area and the predicted amino acid sequence are shown above the nucleotide sequence. Wild-type cDNA (E) encodes an extracellular (red; only the C-terminal end is shown), a transmembrane (blue) and a cytoplasmic (green) domain. Mutant cDNA (F) contains a 4 bp insertion (AATT, red), which introduces a stop codon after the transmembrane domain and shifts the reading frame. (**G**) Immunoblot analysis of protein from wild-type and *Nrp1*$^{cyto\Delta/\Delta}$ primary cardiac endothelial cells with a NRP1 cytoplasmic domain antibody (top panel; αC); the blot was stripped and reprobed with an antibody for the NRP1 extracellular domain (bottom panel; αN).

Morpholino-induced *Nrp1* knockdown in zebrafish impairs intersomitic vessel (ISV) growth, which is rescued by the forced expression of full-length, but not SEA-deficient NRP1 (Wang et al., 2006). Synectin knockdown in zebrafish also inhibits ISV sprouting and additionally impairs dorsal aorta formation (Chittenden et al., 2006). In mice, however, synectin is not required for developmental angiogenesis, but instead promotes mural cell recruitment to retinal blood vessels by regulating PDGF signalling (Paye et al., 2009) and arterial branching in pathological tissue ischemia by enhancing VEGFR2 signalling (Chittenden et al., 2006; Lanahan et al., 2010). By contrast, it has not previously been determined whether the NRP1 cytoplasmic domain is essential for vascular development in mice.

Here, we describe the generation of mice that express a form of NRP1 that lacks the cytoplasmic domain (*Nrp1*$^{cyto\Delta/\Delta}$). *Nrp1*$^{cyto\Delta/\Delta}$ mutants undergo normal vasculogenesis and angiogenesis, demonstrating that membrane-anchored extracellular NRP1 is sufficient for both processes. However, retinal arteries and veins crossed each other at an abnormally high frequency, as previously reported for mice with haploinsufficient expression of neural progenitor-derived VEGF (Haigh et al., 2003). The phenotypic similarity of mutants with reduced VEGF levels and mutants lacking the NRP1 cytoplasmic domain suggests that NRP1 signalling conveys VEGF signals to implement the spatial separation of arteries and veins. We further show that arteriovenous crossings in *Nrp1*$^{cyto\Delta/\Delta}$ mice are similar to those at sites of branch retinal vein occlusion (BRVO), a human eye pathology in which blockage of blood flow in a branch of the central retinal vein causes loss of vision.

MATERIALS AND METHODS

A targeting construct introducing a stop codon and a neomycin cassette into *Nrp1* after the transmembrane domain was inserted into BACPAC clone RP23-298G15 (Children's Hospital Oakland Research Institute, California) by ET recombineering (Angrand et al., 1999). Homologously recombined embryonic stem (ES) cell clones were identified by Southern blotting and aggregated with CD1 mouse embryos. A germline-transmitting male was mated to a female *Hprt*^{Cre} carrier to delete the neomycin cassette. Offspring was backcrossed into C57/Bl6 (Charles River Laboratories) and genotyped by PCR with the oligonucleotides 5′-CCTTTTGATGGACATGTGAC-CTGTAGC-3′ (*Nrp1*-F), 5′-CACCAGGTCTGATTGAAGAGAAGG-3′ (*Nrp1*-R) and 5′-ATGGTACCTTGAGCATCTGACTTCTG-3′ (*Nrp1*-ΔC). Protein lysates from primary cardiac endothelial cells (Allport et al., 2002) were immunoblotted with rabbit anti-mouse (Cell Signalling) and anti-human NRP1 (Epitomics), followed by goat anti-rabbit horseradish peroxidase-coupled secondary antibodies (DAKO). Immunostaining, imaging and quantitations were performed as described previously (Vieira et al., 2007; Fantin et al., 2010), but including anti-NG2 (Millipore). Confocal *z*-stacks of arteriovenous crossings, obtained with the LSM710 microscope (Zeiss), were subjected to three-dimensional surface rendering with Imaris (Bitplane). Animal research was conducted with United Kingdom Home Office and local ethical approval.

RESULTS AND DISCUSSION

Generation and validation of mouse mutants lacking the NRP1 cytoplasmic domain

We introduced a stop codon into the last exon of mouse *Nrp1*, immediately downstream of the transmembrane domain (Fig. 1A). Southern blotting identified targeted ES cells (Fig. 1B) and confirmed gene targeting in mice derived from these cells (Fig. 1C). PCR genotyping (Fig. 1D) established that wild type, heterozygotes (*Nrp1*$^{+/cyto\Delta}$) and homozygotes (*Nrp1*$^{cyto\Delta/\Delta}$) were present at the expected Mendelian ratio (see Table S1 in the supplementary material). Mutant mice were viable, fertile and appeared anatomically normal. Sequencing reverse-transcribed cDNA from mutants and wild-type littermates with oligonucleotide primers for *Nrp1* confirmed that a 4 bp insertion in the mutants introduced a premature stop codon at the end of the transmembrane domain and shifted the mRNA reading sequence out of frame, which prevented translation of the cytoplasmic domain (Fig. 1F). Immunoblotting of proteins from primary cardiac endothelial cells also confirmed deletion of the cytoplasmic domain in the mutants. Thus, an antibody for the NRP1 cytoplasmic domain (αC) detected a doublet of 130-135 kDa in wild types, as expected (e.g. Soker et al., 1998; Gu et al., 2002), but failed to bind NRP1 in mutants (Fig. 1G). By contrast, an antibody for the extracellular domain (αN) detected NRP1 in wild types and mutants (Fig. 1G). Mutant NRP1 migrated at a lower molecular weight, as predicted by the C-terminal 40 amino acid deletion (Fig. 1F,G). The cDNA and protein analyses established that the cytoplasmic domain was successfully deleted in *Nrp1*$^{cyto\Delta/\Delta}$ mice and that mutant NRP1 protein was stably expressed.

The NRP1 cytoplasmic domain is dispensable for embryonic vasculogenesis and angiogenesis

We compared vascular development in E9.5 *Nrp1*$^{cyto\Delta/\Delta}$ and wild-type littermates by immunofluorescence (Fig. 2A-F). The dorsal aorta and pharyngeal arch arteries, formed by vasculogenesis, and microvessel networks, which arise through angiogenesis, appeared normal in mutants (Fig. 2A,D). Higher magnification images also showed normal remodelling of head vessels (Fig. 2B,E) and appropriate branching of intersomitic and other trunk vessels in mutants (Fig. 2C,F). Double immunostaining of mutants and wild types with the NRP1 extracellular domain antibody demonstrated similar NRP1 localisation to blood vessels (Fig. 2B′,C′,E′,F′) and the trigeminal ganglia (arrowheads in Fig. 2B″,E″), known to express NRP1 at this developmental stage (Schwarz et al., 2008). The lack of obvious vascular or other morphological defects at E9.5 suggests that the requirement for NRP1/synectin signalling in fish (Wang et al., 2006) is not conserved in mammals.

NRP1-deficiency disrupts angiogenesis in the central nervous system, including the hindbrain (Gerhardt et al., 2004; Kawasaki et al., 1999). Thus, vessels invading the brain from the pial

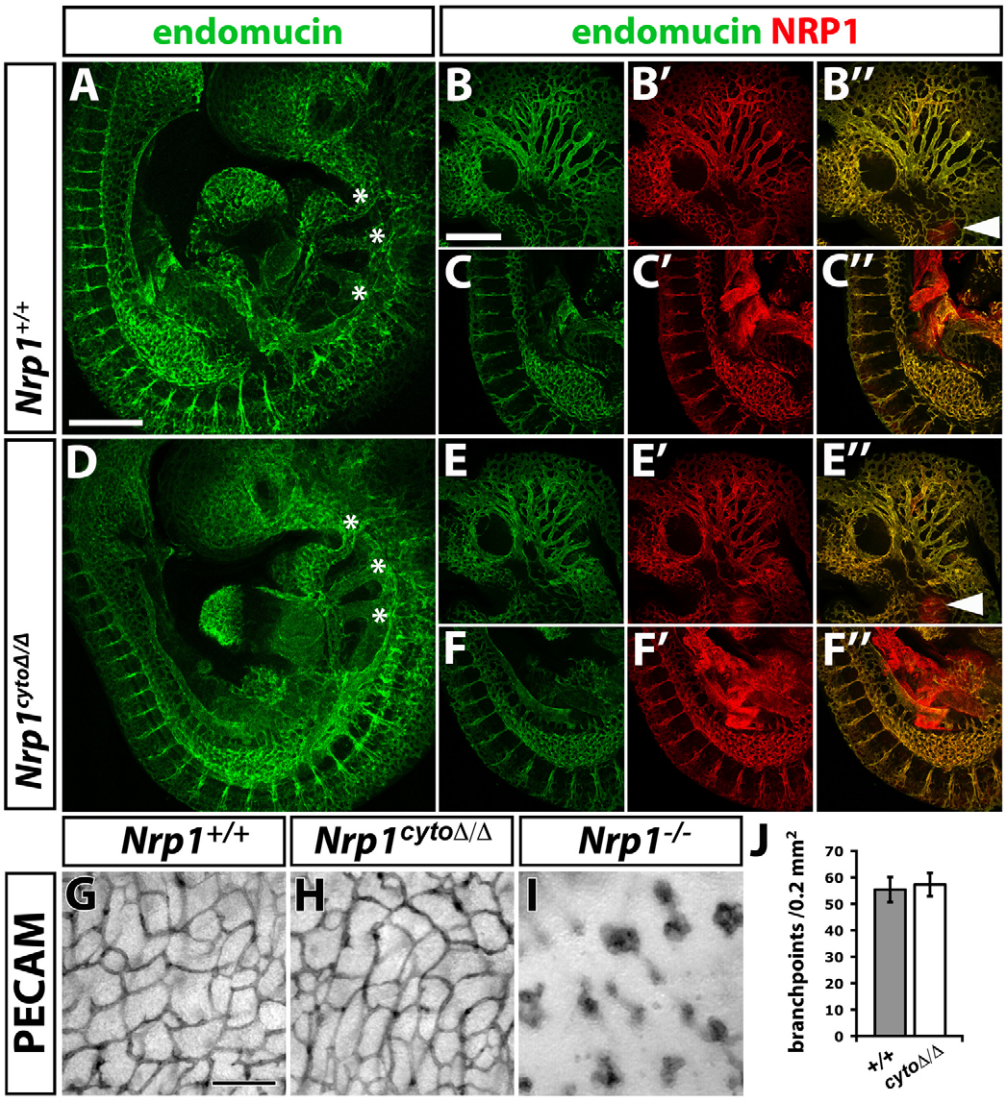

Fig. 2. The NRP1 cytoplasmic domain is not essential for embryonic vasculogenesis or angiogenesis. (**A-F″**) Immunostaining of E9.5 littermates with endomucin (green) reveals microvessels and pharyngeal arch arteries (asterisks in A,D); higher magnification images show head vessels (B,E), ISVs and microvessels at forelimb level (C,F); an antibody for the NRP1 extracellular domain stains blood vessels (endomucin/NRP1-double positive vessels appear yellow) and trigeminal ganglia (red; arrowheads in B″,E″). (**G-I**) PECAM immunohistochemistry of E12.5 hindbrains expressing (G) or lacking (H) the NRP1 cytoplasmic domain or lacking NRP1 entirely (I). Scale bars: 500 μm in A,D; 200 μm in B-C″,E-F″; 100 μm in G-I. (**J**) Quantitation of vascular density in the E12.5 hindbrain SVP of $Nrp1^{cytoΔ/Δ}$ and wild-type littermates; $P>0.05$; $n\geq4$. Data are mean±s.d.

surface normally fuse into the subventricular vascular plexus (SVP) that supplies the neural progenitors by E12.5 (Fig. 2G) (Ruhrberg et al., 2002), but *Nrp1*-null mutants lack the SVP (Fig. 2I). In $Nrp1^{cytoΔ/Δ}$ mutants, the SVP formed (Fig. 2H) and contained a normal number of branchpoints (Fig. 2J; wild type 55.39±4.74 versus $Nrp1^{cytoΔ/Δ}$ 57.29±4.40; mean±s.d.; $n\geq4$ each). Together with the E9.5 analysis, these findings show that membrane-anchored NRP1 is sufficient for vasculogenesis and angiogenesis.

The NRP1 cytoplasmic domain is dispensable for angiogenesis and mural cell recruitment, but regulates arteriovenous patterning in the retina

To determine whether the NRP1 cytoplasmic domain regulates postnatal vascular development, we compared retinal angiogenesis in $Nrp1^{cytoΔ/Δ}$ and their wild-type littermates on perinatal day (P) 7, when vessels extend from the centre to the periphery, and at P21, when angiogenesis is largely complete in this organ (Fruttiger, 2007). In both genotypes, vessels had extended similarly towards the periphery at P7 (Fig. 3A-C) and into the deeper retinal layers to form three plexi at P21 (see Fig. S1A-F in the supplementary material). Endothelial cell growth and migration in the retina therefore proceeds normally in mice lacking the NRP1 cytoplasmic domain, as reported for mice deficient in synectin (Paye et al., 2009).

Synectin knockout mice show defective mural cell recruitment to retinal vessels (Paye et al., 2009). By contrast, staining of $Nrp1^{cytoΔ/Δ}$ mutants for α-smooth muscle actin (SMA) showed normal smooth muscle cell (SMC) coverage of arteries at P7 (Fig. 3A′,B′,D) and P21 (Fig. 3G,H). Staining for the pericyte marker NG2 (Ozerdem et al., 2001) also showed a normal distribution of mural cells along arteries, veins and capillaries in mutants (see Fig. S2 in the supplementary material). $Nrp1^{cytoΔ/Δ}$ mice therefore do not phenocopy synectin knockouts with respect to mural cell recruitment, consistent with the idea that reduced PDGF, rather than VEGF signalling, is responsible for mural cell defects in synectin knockouts (Paye et al., 2009).

Because NRP1 is more highly expressed in developing arteries than veins, we next examined arteriovenous patterning in the $Nrp1^{cytoΔ/Δ}$ retina. In the mutant retina, arteries and veins radiated from the optic nerve head in the centre towards the periphery in their usual alternating pattern (Fig. 3A,B), and arteries and veins were present at a normal number (Fig. 3I; arteries: $Nrp1^{+/+}$ 5.38±0.92 versus $Nrp1^{cytoΔ/Δ}$ 5.58±0.79; veins: $Nrp1^{+/+}$

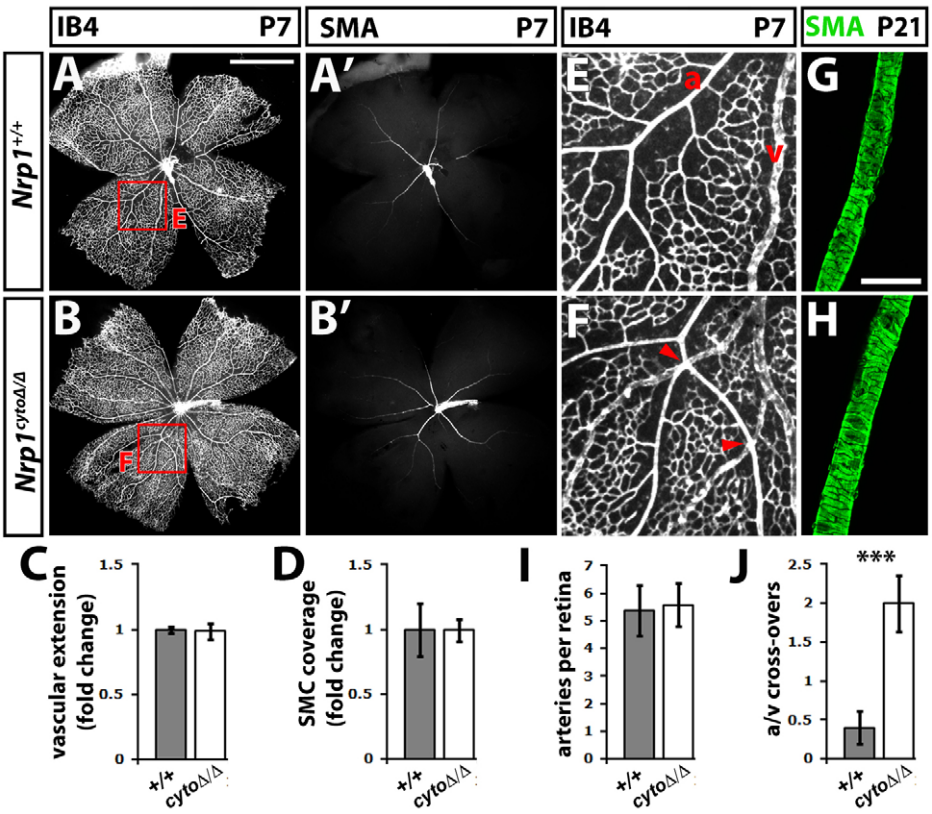

Fig. 3. Increased incidence of arteriovenous crossing points in the *Nrp1*$^{cytoΔ/Δ}$ retina.
(**A-B′**,**E-H**) Immunostaining of retinas for IB4 and SMA at P7 (A,B) or for SMA at P21 (G,H); boxed areas are shown at higher magnification in E,F. Scale bars: 1 mm in A,B; 50 μm in G,H. (**C**,**D**) Quantitation of vascular extension and SMC coverage, expressed as fold change relative to wild-type littermates; $n \geq 8$ in C, $n \geq 4$ in D; $P > 0.05$. (**I**) Quantitation of artery number; $n \geq 8$; $P > 0.05$. (**J**) Quantitation of artery/vein crossings; $n \geq 14$; ***$P < 0.001$. Data are mean±s.d.

5.38±1.3 versus *Nrp1*$^{cytoΔ/Δ}$ 5.58±1.08; mean±s.d., $n \geq 8$ each). Unexpectedly, the mutant retina showed a significant increase of artery/vein crossing points, which are rare in wild types (Fig. 3F,J; Fig. 4; *Nrp1*$^{+/+}$ 0.4±0.83 versus *Nrp1*$^{cytoΔ/Δ}$ 2.0±1.36; mean±s.d.; $n \geq 14$ each; $P < 0.001$). Similar arteriovenous crossings have previously been identified in the retina of mice with haploinsufficient expression of VEGF in neural progenitors (Haigh et al., 2003), consistent with the idea that NRP1 conveys VEGF signals during vascular development. It is not known whether arteriovenous crossings are restricted to the retina with its unique two-dimensional radial array of arteries and veins, or are also present in other organs of *Vegfa* or the *Nrp1*$^{cytoΔ/Δ}$ mutants.

The artery is positioned anteriorly to the vein and both vessels share extracellular matrix at arteriovenous crossing points in the *Nrp1*$^{cytoΔ/Δ}$ retina

Confocal microscopy of *Nrp1*$^{cytoΔ/Δ}$ P7 retinas immunolabelled for IB4 and the venous marker NRP2 (Herzog et al., 2001), combined with 3D image rendering, established that the artery was positioned anteriorly to the vein in 13/13 crossings (Fig. 4A-A″). Thus, the vein became ‘sandwiched’ between the artery and neural retina. Immunolabelling for IB4 and NRP1, which is enriched in arteries (Herzog et al., 2001), revealed arteriovenous crossings also in mutants at P21 (Fig. 4B; high magnification of Fig. S1F in the supplementary material). Fluorescein angiography and infrared fundus microscopy identified arteriovenous crossings in the eyes of adult mutants (see Fig. S1G-J in the supplementary material). The presence of arteriovenous crossings at all postnatal stages examined suggests that they form during development and persist into adulthood.

A high frequency of arteriovenous crossings with an anterior position of the artery relative to the vein, combined with sharing of extracellular matrix by the crossed artery and vein, is a risk factor for BRVO (e.g. Weinberg et al., 1993; Zhao et al., 1993; Cahill and Fekrat, 2002). Immunolabelling with the vascular basement membrane/tunica media marker collagen IV (Megens et al., 2007) established that arteries and veins at sites of overlap in *Nrp1*$^{cytoΔ/Δ}$ mutants were embedded in a contiguous collagen IV-positive extracellular matrix, but that arterial and venous endothelium were segregated by a basement membrane at contact sites (Fig. 4C). The arteriovenous crossings present in the *Nrp1*$^{cytoΔ/Δ}$ retina are therefore highly similar to those present at sites of BRVO in humans.

NRP2 does not compensate for loss of the NRP1 cytoplasmic domain during angiogenesis

NRP2 is the closest relative to NRP1, with the cytoplasmic domain of the NRP2a isoform having a C-terminal SEA motif like NRP1 (Rossignol et al., 2000). Moreover, mice lacking both NRP1 and NRP2 have more severe vascular defects than mice lacking NRP1 alone (Takashima et al., 2002). To address whether the lack of angiogenesis defects in mutants lacking the NRP1 cytoplasmic domain was due to compensation by NRP2, we examined whether NRP2 was upregulated in *Nrp1*$^{cytoΔ/Δ}$ mutants, but found that this was not the case (see Fig. S2 in the supplementary material). Moreover, loss of the NRP1 cytoplasmic domain on a *Nrp2*-null background (Giger et al., 2000) did not impair angiogenesis in 3/3 E12.5 hindbrains or 10/10 P7 retinas from double homozygous mutants, and vascular extension and SMC coverage were also unaffected in double mutant retinas (see Fig. S3 in the supplementary material). NRP2 therefore does not compensate for loss of the NRP1 cytoplasmic domain during developmental angiogenesis or artery/vein specification.

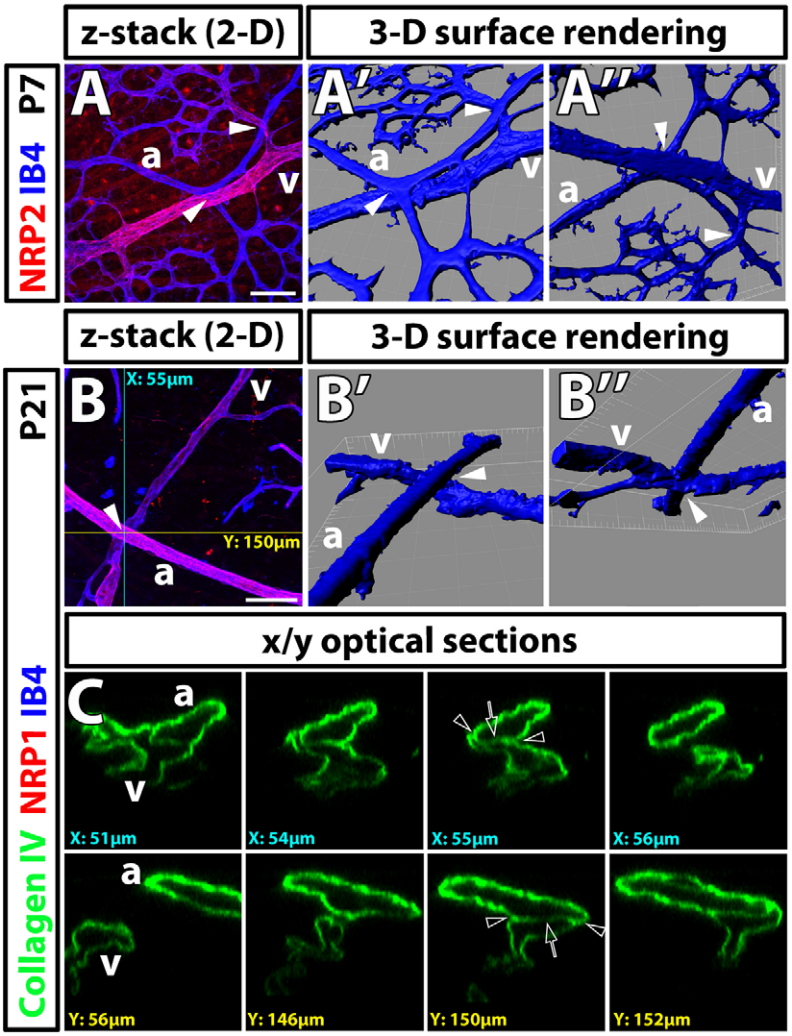

Fig. 4. Anatomy of arteriovenous crossings in *Nrp1*$^{cyto\Delta/\Delta}$ retinas. (**A**,**B**) Confocal *z*-stacks of arteriovenous crossings (solid arrowheads) in *Nrp1*$^{cyto\Delta/\Delta}$ retinas, stained for IB4 (blue) and NRP2 (red in A) at P7 or IB4, NRP1 (red in B) and collagen IV (not shown in B) at P21. The artery (a) is strongly positive for IB4 and NRP1, the vein (v) for IB4 and NRP2; axons underneath the vessel plexus are NRP2-positive. (**A′-B″**) Anterior (A′,B′) and posterior angle (A″,B″) snapshots of IB4 staining after three-dimensional surface rendering of *z*-stacks. (**C**) Selected *x* and *y* coordinates of optical cross-sections through the *z*-stack in B; only collagen IV is shown (green); arrows indicate basement membrane separating artery and vein; arrowheads indicate a contiguous extracellular matrix wrapping artery and vein. Scale bars: 50 µm.

Implications for future studies

Previous in vitro studies reported that the NRP1 cytoplasmic domain modulates VEGFR2 endocytosis in a mechanism that depends on synectin (Wang et al., 2003; Salikhova et al., 2008). These findings were thought to indicate an essential role in VEGF-mediated angiogenesis. However, the comparison of vascular defects in full NRP1 knockouts and *Nrp1*$^{cyto\Delta/\Delta}$ mutants implies that the essential angiogenic function of NRP1 involves its extracellular, rather than intracellular, domain. The interaction of the NRP1 cytoplasmic domain with VEGFR2 might therefore be important for endothelial functions other than angiogenesis. Because NRP1 is enriched in arteries and synectin promotes arteriogenesis in ischemic adult tissues, the NRP1 cytoplasmic domain may serve to link synectin and VEGFR2 to promote ERK signalling during adult arteriogenesis (Ren et al., 2010). Future work should therefore address whether *Nrp1*$^{cyto\Delta/\Delta}$ mutants have reduced arteriogenic VEGFR2 signalling.

The high frequency of arteriovenous crossings in *Nrp1*$^{cyto\Delta/\Delta}$ mutants raises the possibility that these mice could serve as an animal model to elucidate the poorly understood aetiology of BRVO. Thus, the high number of crossings with an anteriorly positioned artery positively increases the risk of developing BRVO in that eye (Weinberg et al., 1990), but it still needs to be determined how these anatomical abnormalities progress to become BRVO lesions. Current hypotheses propose the presence of additional, systemic risk factors such as advanced age, hypertension and cardiovascular disease (Cahill and Fekrat, 2002). For example, it has been hypothesised that an anteriorly placed artery stiffened by atherosclerosis compresses the underlying vein against the neural retina, reducing blood flow downstream of the crossing. By introducing atherosclerosis or other risk factors into *Nrp1*$^{cyto\Delta/\Delta}$ mutants and their wild-type littermates, it may be possible to determine their relative significance for lesion formation and devise medical strategies to prevent lesion establishment.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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Table S1. Wild type, heterozygotes (*Nrp1*$^{+/cyto\Delta}$) and homozygotes (*Nrp1*$^{cyto\Delta/\Delta}$) are present at the expected Mendelian ratio

Genotype	Total number of viable mice	Observed (%)	Predicted (%)
Nrp1$^{+/+}$	44	26	25
Nrp1$^{+/cyto\Delta}$	76	45	50
Nrp1$^{cyto\Delta/\Delta}$	49	29	25
Total	169	100	100