

Materials and Methods

Generation of *sema3E*^{-/-} mutant mice

For details about the *sema3E* locus genomic structure, see Genebank NT 080431 and (1). Mouse genomic clones containing the *Sema3E* first exon were identified in a 129/Ola cosmid library (RZPD) using a probe corresponding to nucleotides 600-724 of *Sema3E* cDNA (Genebank #Z93947). To generate the targeting vector, we used a plasmid (*pEGFPΔNeo*) containing a *GFP-LoxP-PGK-Neo-LoxP* cassette, kindly provided by Stephane Nicolas (NMDA-IBDM, Marseille). A 5 kb SfiI-NcoI genomic fragment immediately 5' to the *Sema3E* start codon was cloned in the *EGFP* NcoI site. A 1.2 kb PCR fragment containing the intronic sequence just 3' to *Sema3E* first coding exon was generated with primers: 5'-TCCCCCGGGAGGTTACCTTTCCTTATCC-3' and 5'-CCCATCGATTTCTTTTCTGGCATCTTC-3', and cloned downstream of the *EGFPΔNeo* cassette using Xma I and Cla I sites. E14 ES cells were electroporated with the linearized targeting construct, selected with G418, and screened by PCR (1.4 kb product). Positive clones were further confirmed by Southern blot using a 3' 1 kb probe (Eco RI or Xma I digest). Recombinant ES cells were injected into C57BL/6 blastocysts. Chimeric founders were bred to C57BL/6 mice, and heterozygous mice were used to establish lines that were backcrossed into either a C57BL/6 or a CD1 genetic background. Offspring were genotyped by PCR using three primers: 5'-GACAGAAAGGCTTAGCGGATC-3' (WT sense), 5'-GGTTCGCCGAGTGACCTG-3' (WT antisense), 5'-CTTGCTACCATGGTGCCTG-3' (GFP antisense), revealing a 250 bp fragment for the wild-type allele and a 200 bp fragment for the mutant allele. Analysis of the *Sema3E*^{-/-} phenotype was performed on embryos derived from intercrosses between heterozygous animals. *Sema3E* mutant animals were viable and fertile and did not show any gross abnormality. Moreover, they were born at the frequency predicted for Mendelian transmission.

Generation of *plexin-D1* mutant mice

Mouse genomic clones were derived from a 129/Sv genomic library (Stratagene). A targeting vector was constructed using a 2 kb XhoI-SacI fragment containing 5' untranslated sequences and a 10 kb EcoRV-SalI fragment (SalI is derived from the phage DNA). A *LoxP-PGKneo-triple pA signal-LoxP* and IRES farnesylated *EGFP-pA* cassettes were inserted between the two arms. A linearized targeting construct was electroporated into 129Sv/Ev derived ES cells. Cells were selected with G418 and screened by Southern blot analysis using a 0.8 kb Xba I-Xho I fragment, generating a 12 kb wild-type and a 4 kb mutant band. Another probe outside the 3' long arm was also used to confirm 3' homologous recombination. The cells carrying the correct mutation were injected into C57BL/6J blastocysts. Chimeric offspring were mated with C57BL/6J mice. Germ-line transmission of the mutant allele was determined by Southern blot analysis of genomic DNA from tails of mice. Heterozygous F1 animals were intercrossed to obtain homozygous (*plexin-D1*^{-/-}) mice. Homozygosity for the *plexin-D1* knockout allele was confirmed by Southern blot analysis and in situ hybridization using a probe for 3' untranslated sequences. GFP expression was not observed in *plexin-D1* mutant mice. Mutant embryos were genotyped using the following oligonucleotides: 5'-ACCGCAGAACCGGTCACCGTGTT-3' (*Plexin-D1* 5'), 5'-GGTTAAGGTCGAAGGTGAAGAGCTT-3' (*Plexin-D1* 3'), 5'-

ATGGTGAGCAAGGGCGAGGA-3'(GFP 5'), and 5'-TACTTGTACAGCTCGTCCA-3'(GFP 3'). All experiments involved analysis of embryos derived from heterozygous 129Sv X C57BL/B6J intercrosses.

AP-fusion protein binding to cells and tissue sections

AP-fusion protein binding to COS-1 cells and quantitative cell surface binding were done essentially as described (2). AP-fusion protein binding to tissue sections was performed as previously described (3).

COS-7 cell morphology assay

COS-7 cells were transfected with 0.5 µg of EGFP together with either 2 µg of vector or 2 µg of Plexin-D1. After 48 hours, cells were incubated with AP-Sema3E (0.1-0.5 nM), Sema3A (1-2 nM), or control medium for 30 min at 37⁰C. Cells were fixed with 4% paraformaldehyde (PFA) and visualized under fluorescence microscopy. At least 200 EGFP-positive cells were scored from each well, and the percentage of EGFP-positive cells with an area less than 1600 µm² was calculated (4).

Whole-mount immunostaining and immunohistochemical procedures

Embryos were fixed in PBS containing 4% PFA and whole-mount staining was performed as described (3) using primary monoclonal antibodies against PECAM-1 (PharMingen, clone MEC13.3, isotype rat, 1:500 dilution) and peroxidase-conjugated secondary antibodies (BD Biosciences PharMingen, San Diego, CA). For analysis of the vasculature, E13.5 embryos were fixed for 8 hrs in PBS containing 4% PFA and cryoprotected overnight in 30% sucrose. Horizontal brain cryosections (20µm) were stained with peroxidase-conjugated isolectin B4 (1:100, Sigma) and developed with 3,3'-diaminobenzidine (DAB) in PBS as described previously (3).

Whole-mount In Situ Hybridization

In situ hybridization was performed as described (5).

Chick embryo preparation and in Ovo Electroporation

Chick eggs (Spafas, Truslow Farms) were incubated and staged (6). In ovo electroporation was done at E3 as described previously (7).

Intravital lectin perfusion and visualization of chick blood vessels

Rhodamine conjugated lectin LCA (Lens Culinaris Agglutinin) (Vector Laboratories, Burlingame, CA) was diluted in PBS and intracardiac injection was performed in E5 embryos as described previously (8). After perfusion, embryos were fixed in 4% PFA overnight at 4⁰C and embryos were sectioned at 200µm thickness using a vibratome. The staining was visualized by confocal microscopy. Quantification of the staining was performed using the OpenLab program (Improvision).

Supplemental Figure Legends

Supplemental Figure 1. Complementary expression patterns of *sema3E* in somites and *plexin-D1* in intersomitic blood vessels in E10.5 mouse embryos.

A. *Sema3E* in situ hybridization of a sagittal section of an E10.5 embryo. **B.** *Plexin-D1* in situ hybridization of a sagittal section of an E10.5 embryo. . Scale bar: 150 μ m.

Supplemental Figure 2. *Sema3C*, *Sema3D* and *Sema3F* bind to *Npn-1* or *Npn-2*, but not to *plexin-D1*.

COS-1 cells were transfected with vector alone or an expression vector encoding *plexin-D1* (B,E,H), *Npn-1* (C,F) or *Npn-2* (I). Then, cells were incubated with medium containing AP-*Sema3C* (A-C), AP-*Sema3D* (D-F) or AP-*Sema3F* (G-I) (0.5 nM). Note that while AP-*Sema3C*, and AP-*Sema3D* bind to *Npn-1*-expressing cells, and AP-*Sema3F* binds to *Npn-2*-expressing cells, none of these ligands binds to cells expressing *plexin-D1*. Scale bar = 50 μ m.

Supplemental Figure 3. Targeted disruption of *plexin-D1*

A. Schematic illustration of wild-type genomic DNA, targeting vector, and targeted locus of the *plexin-D1* gene. Exon 1 of the *plexin-D1* is shown as a closed box. **B.** The 5' external probe as shown in (A) was used for Southern blot analysis to detect homologous recombinants. Tail DNA from F1 progeny of two heterozygote intercrosses were digested with Bgl II and subjected to Southern blot hybridization. The genotype for the *plexin-D1* locus is indicated above each lane. Sizes of the DNA fragments are indicated on the right. **C.** Absence of *plexin-D1* mRNA in P0 *plexin-D1* mutant mouse, as assessed by in situ hybridization. Scale bar: 250 μ m.

Supplemental Figure 4. Targeted disruption of *sema3E*

A. Targeting strategy. The first coding exon of mouse *Sema3E* encodes the signal peptide. In the targeting vector, a DNA fragment encoding the green fluorescent protein EGFP is inserted exactly at the start codon of the *sema3E* gene. The vector also contains a Neo cassette flanked by LoxP sites for ES cell selection. This vector was used to target the *sema3E* locus by homologous recombination in ES cells to generate the mutant locus. PCR primers and Southern probe used to screen recombinant clones are indicated respectively by arrowheads and a bar. **B.** Isolation of recombinant ES clones. Homologous recombination events were screened by PCR (1.7 kb product, see primer location in A). Selected clones were confirmed by Southern blot. Recombination resulted in the generation of a 5 kb EcoRI fragment and a 2.9 kb XbaI fragment, (see probe and restriction sites location in A). **C.** Absence of *sema3E* mRNA in E11.5 *sema3E* mutant mouse, as assessed by in situ hybridization. Scale bar: 1.2 mm.

Supplemental Figure 5. Semaphorin-neuropilin signaling is not essential for development of the forebrain vasculature or intersomitic vascular patterning

Mice doubly homozygous for a null mutation in *npn-2* and a knock-in mutation in *npn-1* (*npn-1^{Sema-}*), which abolishes secreted semaphorin binding sites but not VEGF binding sites in *Npn-1* (3, 9), were generated by double heterozygous intercrosses. The vasculature was visualized following whole-mount PECAM staining of E10.5 control (A,

A') and mutant (C, C') embryos, which reveals the integrity of the intersomitic vasculature, or by isolectin staining of horizontal brain sections from E13.5 control (B, B') and mutant embryos (D, D'). In both assays, the vasculature of *npn-1^{Sema-}; npn-2^{-/-}* double mutant mice was indistinguishable from that of their control littermates. n = 3 for all genotypes presented. Black arrow, intersomitic vessels. Scale bar: (A, C), 0.5 mm; (A', C'), 0.1 mm; (B, D), 350 μ m; (B', D'), 88 μ m.

Supplemental Figure 6. Plexin-D1 is required for intersomitic vascular patterning in the E10.5 mouse embryo.

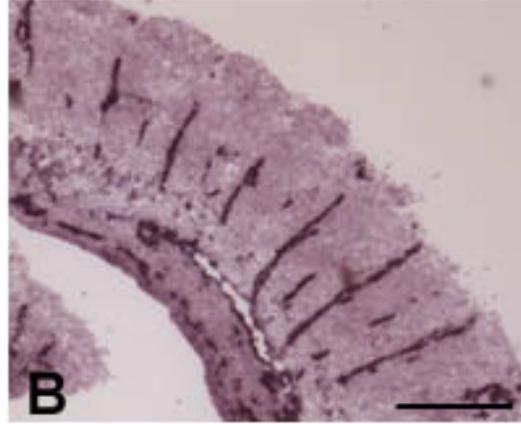
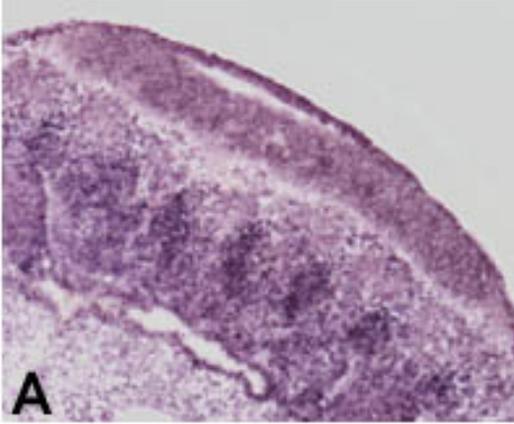
A, B. Whole-mount PECAM staining of E10.5 wild-type (A) and *plexin-D1* (B) mutant embryos showing dramatic disorganization of intersomitic vasculature in the absence of plexin-D1 at E10.5 **A', B'**. High magnification views of E10.5 wild-type control (A') and *plexin-D1^{-/-}* mutant (B') embryos. The vascular phenotype was observed in all *plexin-D1^{-/-}* mice (n = 4) but not in wild-type littermates (n = 4). Scale bar: 1.2 mm (A, B); 0.6 mm (A', B').

Supplemental Figure 7. Semaphorin-neuropilin signaling is not required for intersomitic vascular patterning in the E11.5 mouse embryo.

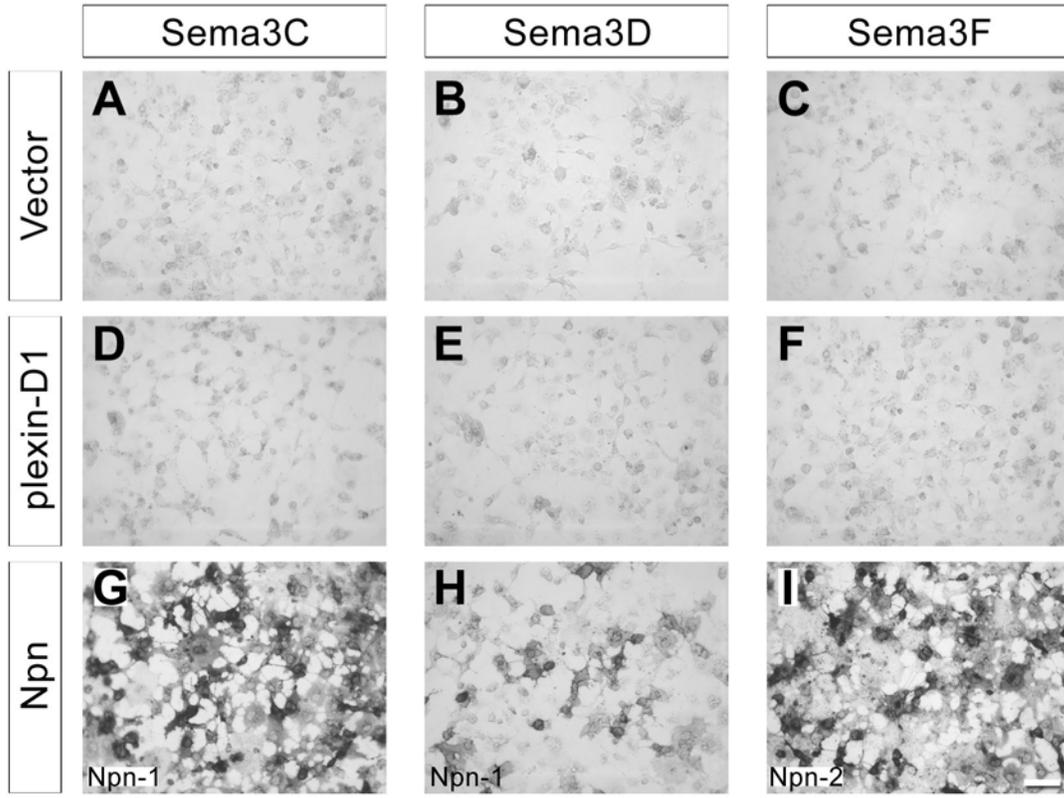
A, B. Whole-mount PECAM staining of E11.5 *npn-1^{Sema+/+}; npn-2^{+/+}* control mice (A) and *npn-1^{Sema-}; npn-2^{-/-}* double mutant embryos (B) showing normal intersomitic vasculature in the absence of Sema3-Npn signaling. **A', B'**. High magnification views of E11.5 control (A') and *npn-1^{Sema-}; npn-2^{-/-}* double mutant (B') embryos. Scale bar: 0.5mm (E,G); 0.1mm (E',G').

sema3E ISH

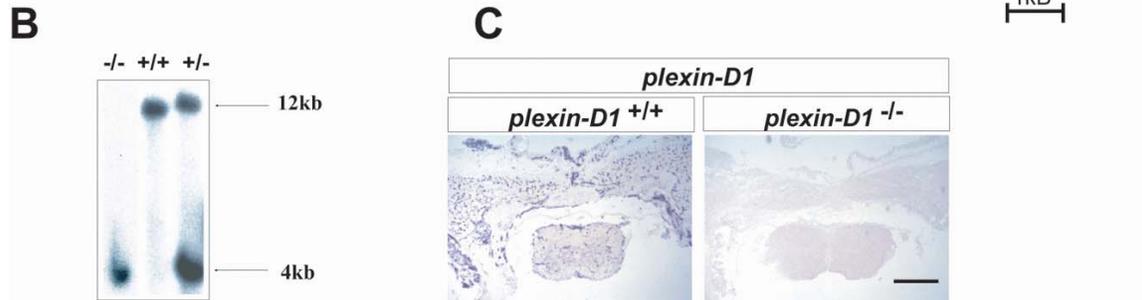
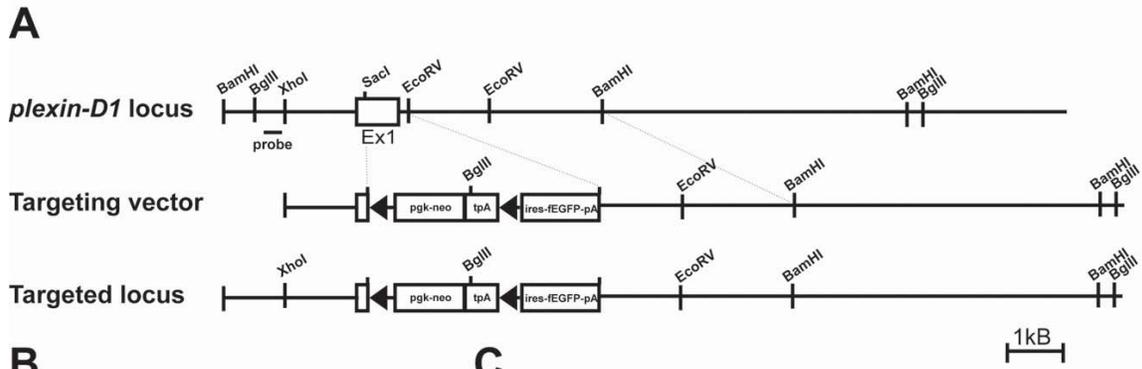
plexin-D1 ISH



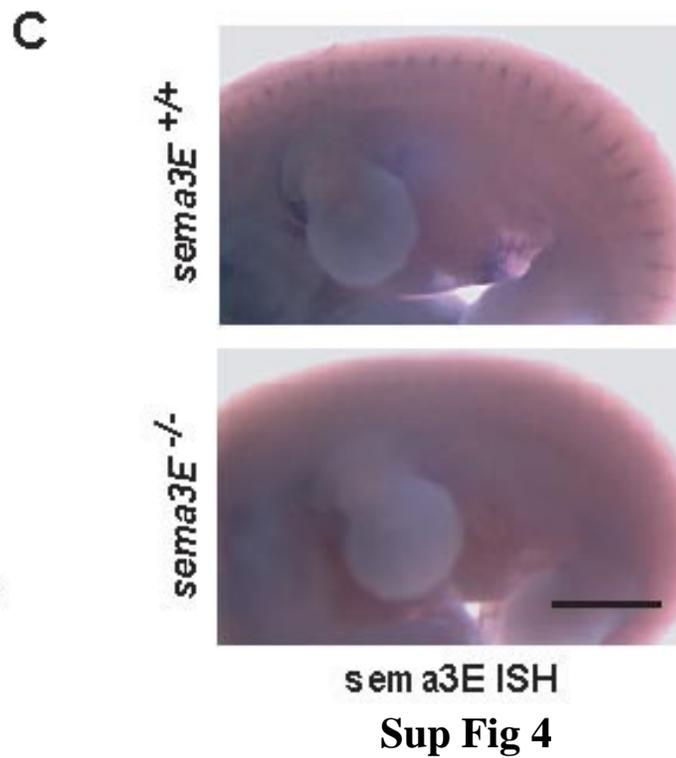
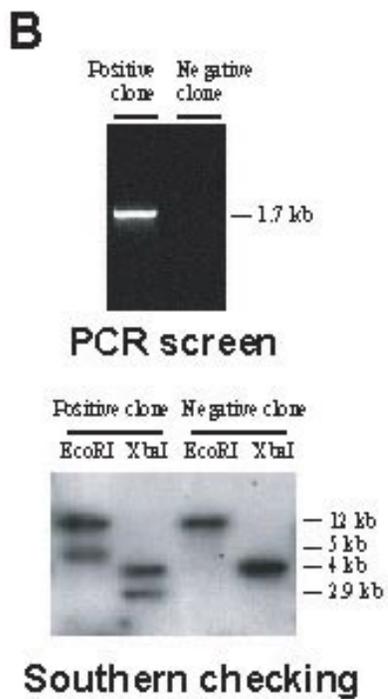
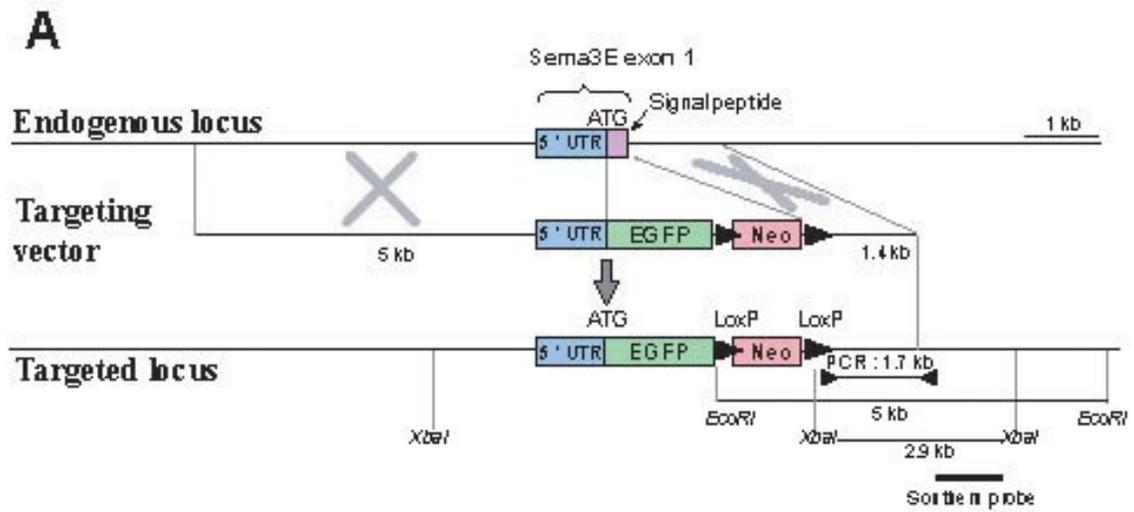
Sup Fig 1

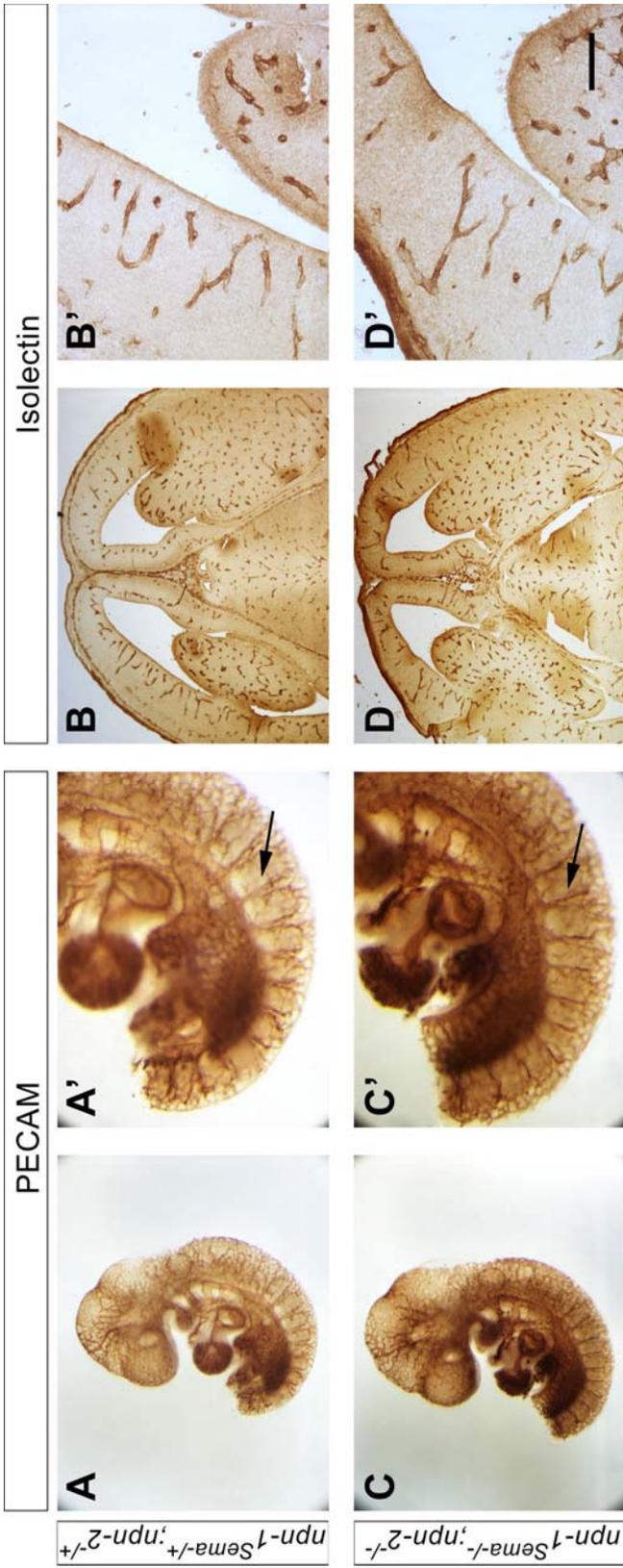


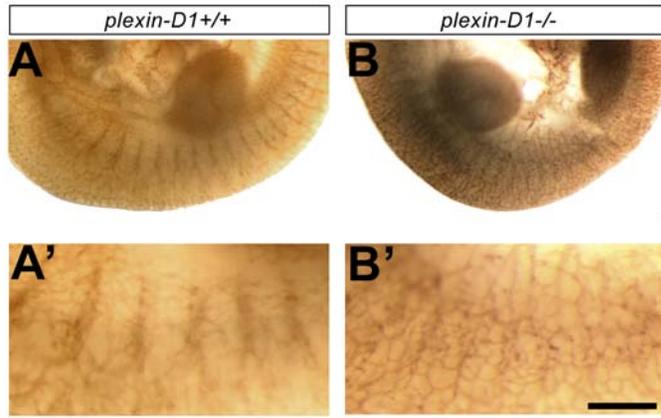
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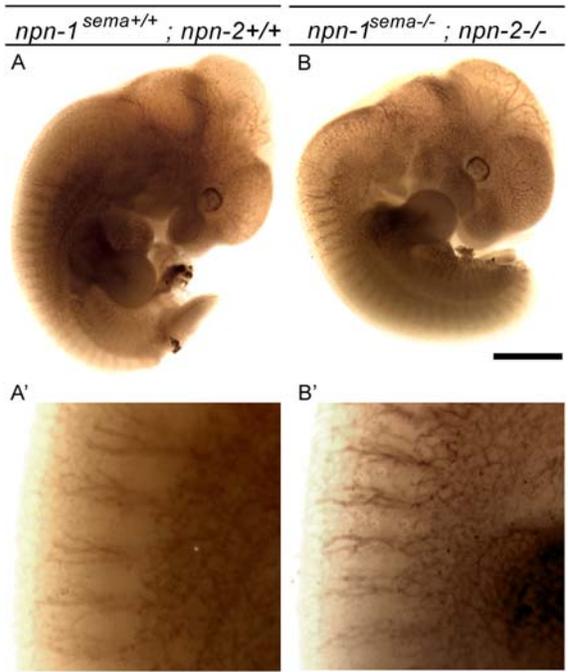
Sup Fig 3







Sup Fig 6



Sup Fig 7

Supplemental References and Notes

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