

Dosage-sensitive requirement for mouse *Dll4* in artery development

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Involvement of the Notch signaling pathway in vascular development has been demonstrated by both gain- and loss-of-function mutations in humans, mice, and zebrafish. In zebrafish, Notch signaling is required for arterial identity by suppressing the venous fate in developing artery cells. In mice, the Notch4 receptor and the Delta-like 4 (*Dll4*) ligand are specifically expressed in arterial endothelial cells, suggesting a similar role. Here we show that the *Dll4* ligand alone is required in a dosage-sensitive manner for normal arterial patterning in development. This implicates *Dll4* as the specific mammalian endothelial ligand for autocrine endothelial Notch signaling, and suggests that *Dll4* may be a suitable target for intervention in arterial angiogenesis.

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Results and Discussion

Gain- and loss-of-function mutations in humans, mice, and zebrafish have demonstrated the importance of Notch signaling in the regulation of vascular development (Shawber and Kitajewski 2004). In zebrafish, Notch signaling is required for arterial identity by suppressing the venous fate in developing artery cells (Zhong et al. 2001; Lawson et al. 2002). In mice, the Notch4 receptor (Villa et al. 2001) and the Delta-like 4 (*Dll4*) ligand (Shutter et al. 2000; Mailhos et al. 2001; Villa et al. 2001) are specifically expressed in arterial and not venous endothelial cells, suggesting a possible similar role. Knock-out of the Notch4 receptor gave no obvious phenotype alone, although *Notch1/Notch4* double mutant embryos did show severe vascular remodeling defects (Krebs et al.

2000), which were also observed, to a lesser degree, in *Hey1/Hey2* double mutants, the putative downstream targets of Notch signaling (Fischer et al. 2004). Here we show, in contrast, that the *Dll4* ligand alone is required in a dosage-sensitive manner for normal arterial patterning in development.

The *Dll4* gene was inactivated by targeted disruption in embryonic stem (ES) cells. The targeting vector was designed to replace the initiation codon and the first three coding exons with the β -galactosidase (*lacZ*) reporter gene (Fig. 1A). Electroporation of R1 ES cells with the targeting vector followed by G418 selection resulted in the derivation of four correctly gene-targeted ES cell lines. Chimeric mice were generated by aggregation between *Dll4*^{+/-} ES cells and ICR host embryos. When crossed with wild-type ICR females, five male chimeras produced F1 agouti pups at 100% frequency. Genotyping of F1 agouti mice by Southern blotting or polymerase chain reaction (PCR; Fig. 1B,C) identified *Dll4*^{+/-} individuals at only 21% frequency (55 out of 260), suggesting the death in utero of a proportion of the *Dll4*^{+/-} F1 embryos. Outcrossing these viable heterozygotes to ICR mice still resulted in a reduced number of heterozygotes in subsequent generations. This effect was dependent on the genetic background, as no live *Dll4*^{+/-} offspring were obtained when the 100% germ-line transmitter chimeric males were crossed with 129/Sv-CP females ($n = 65$).

To further characterize this incompletely penetrant haploinsufficiency, the phenotypes of *Dll4*^{+/-} embryos were investigated. Two different approaches were used to obtain heterozygous embryos: F1 embryos from ICR females crossed with 100% germ-line transmitting chimeric males ($n = 52$) and *Dll4*^{+/-} ES-derived embryos ($n = 59$) generated by aggregation between *Dll4*^{+/-} ES cells (from two different independent cell lines) and *Dll4*^{+/+}, EGFP-labeled tetraploid host embryos. The EGFP reporter was used to confirm the absence of tetraploid cells in the embryo proper of chimeric embryos. *Dll4*^{+/-} embryos obtained by either approach displayed similar phenotypic traits.

The *Dll4*^{+/-} embryos were dissected at embryonic day 7.5–10.5 (E7.5–E10.5) and stained with X-gal or immunostained for PECAM. In pre-somite stages, *lacZ* expression was exclusively detected in trophoblast giant cells (data not shown). Around the two-somite stage (E8.0), the *lacZ* reporter was expressed in the cardiac crescent and the primordia of the dorsal aortae (Fig. 2A). At the five- to 10-somite stage (E8.5), it was expressed in the heart, paired dorsal aortae, branchial arch arteries, internal carotid arteries, umbilical artery, vitelline artery, and in the posterior region of the yolk sac where the arteries are formed (Fig. 2B,C). This expression pattern is consistent with previous reports that *Dll4* is an early marker for arterial endothelial cells (Shutter et al. 2000; Mailhos et al. 2001; Villa et al. 2001).

At E8.5 there were no obvious differences between wild-type and *Dll4*^{+/-} embryos (data not shown). As development proceeded, however, some heterozygous embryos started to show developmental delay. At E9.5 only 31% of the *Dll4*^{+/-} embryos ($n = 32$) were normal in body size and somite number (mean somite number \pm S.D.: 22.0 ± 1.5 ; $n = 10$), and the rest showed growth retardation (mean somite number 19.5 ± 1 ; $n = 22$) compared

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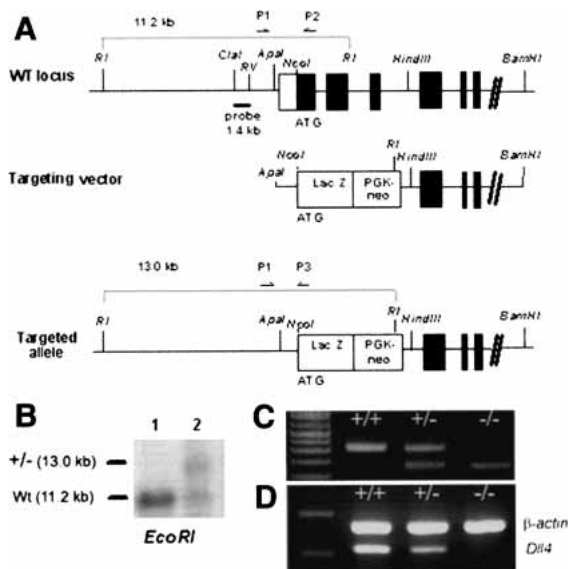


Figure 1. Generation of *Dll4* mutant mice. (A) Schematic representations of the wild-type *Dll4* locus, targeting vector, and mutant allele. The probe for Southern blot analysis (*Clal*-*EcoRV* genomic fragment) and the PCR primers (P1–P3) used for genotyping are indicated. (B) Southern blot analysis of control (lane 1) and targeted (lane 2) ES cells. Genomic DNA was digested with *EcoRI* and hybridized with the *Clal*-*EcoRV* probe. (C) PCR genotyping of wild-type (+/+), heterozygous (+/-), and *Dll4* homozygous null (-/-) mice with primers P1, P2, and P3. (D) RT-PCR analysis of *Dll4* gene expression in E9.5 wild-type (+/+), heterozygous (+/-), and double mutant embryos (-/-).

with the wild-type littermates. At E10.5, two types of *Dll4*^{+/-} conceptuses (*n* = 34) were found: normal-sized embryos with reduced vitelline circulation (*n* = 11), and severely retarded embryos with reduced vitelline circulation and enlarged pericardial space, indicative of embryonic circulatory defects (Fig. 2D–F; *n* = 23). The caliber of the major vitelline arteries and the arterial branching on the yolk sac was reduced in all of the heterozygous embryos (Fig. 2I,J). The umbilical artery and placental blood vessels were also reduced in size in some *Dll4*^{+/-} embryos (Fig. 2G,H). The dependence of the embryo on the vitelline circulation for gas and nutrient exchange is only transient until the placental circulation is established. The variable phenotype of the placental vascular bed probably explains why some *Dll4*^{+/-} embryos could survive to term, despite the poor vitelline circulation in all heterozygotes.

Defects in vascular structures were also seen within the *Dll4*^{+/-} embryos, most notably the reduction of the caliber of the dorsal aortae. At E9.0 the dorsal aortae were already thinner than normal in 60% of the embryos (*n* = 15), a feature which was accentuated at E9.5 (*n* = 32) and E10.5 (*n* = 34). The constriction of the dorsal aortae was primarily observed rostrally between the cardiac level and the intersections with the branchial arch arteries (Fig. 2K–P), but some embryos exhibited longer zones of constriction extending caudally (data not shown). At both E9.5 and E10.5 (*n* = 66), 90% of the embryos displayed some degree of aortic constriction. The degree of constriction was variable among embryos and also between the dorsal aortae of any specific embryo. Interestingly, when the aortic lesions were asymmetric (observed in 50% of the embryos), the left dorsal aorta was

always more severely affected. The potential impact of these arterial defects on the development of the venous system was also investigated. Reduced caliber and disorganization of the anterior and posterior cardinal veins were observed in the *Dll4*^{+/-} embryos, but only in those cases where the dorsal aortae were severely affected, suggesting that the venous defect is a secondary response to a primary arterial restriction (Fig. 2, cf. L,O and M,P).

Surviving *Dll4*^{+/-} mice were apparently normal, suggesting that in these animals the major defects caused by reduction of *Dll4* are transient. Subtle defects in the ability of the adult mice to undertake neovascularization associated with gametogenesis and pregnancy are not impaired. This allowed us to intercross *Dll4*^{+/-} mice to examine the homozygous null phenotype. The embryos were collected at E8.5–E10.5, genotyped by PCR of yolk

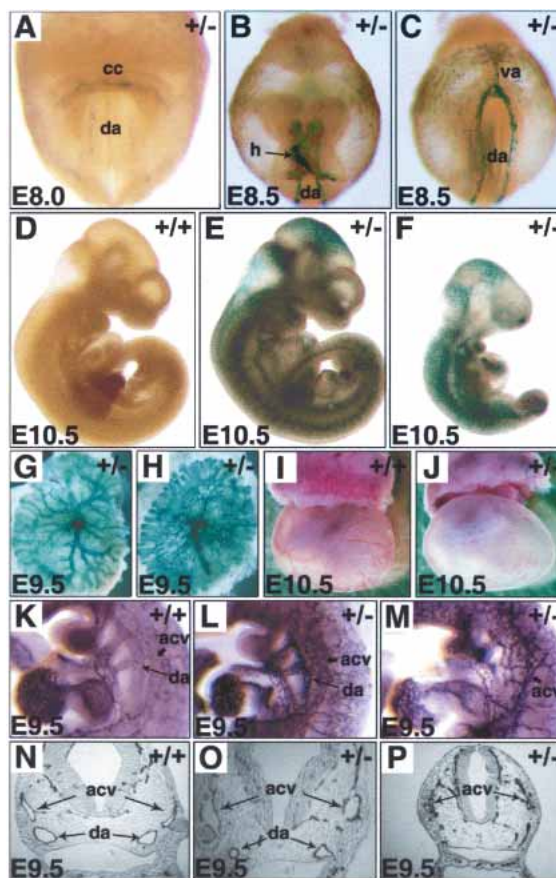


Figure 2. *Dll4* haploinsufficiency. (A–H) Whole-mount X-gal staining. (K–P) PECAM immunostaining: whole-mount (K–M) and sections (N–P). (I,J) Unstained specimens. (A) *Dll4* expression at two-somite stage in the cardiac crescent (cc) and dorsal aortae (da) primordia. (B,C) At E8.5, *Dll4* expression is observed in all arterial vessels (da, dorsal aortae; va, vitelline arteries) and in the endothelial lining of the heart (h). (D–H) Variable penetrance of the hemizygous phenotype: littermate embryos (E10.5, D–F) and arterial vessels of the placenta (E9.5, G,H). (I,J) Morphology of E10.5 yolk sacs. Note the absence of large vitelline vessels and the poor circulation in the *Dll4*^{+/-} yolk sac compared with wild-type. (K–P) At E9.5 two types of *Dll4*^{+/-} embryos can be found: with reduced dorsal aorta but without defects in the anterior cardinal vein (L,O) and with more severe defects, with a very reduced dorsal aorta, enlarged heart, and the anterior cardinal vein reduced to a disorganized venous plexus (M,P).

sac DNA, and analyzed by X-gal staining and PECAM immunostaining. Homozygous mutant embryos were found at normal Mendelian frequencies at E9.5 (wild-type, 27.1%; *Dll4*^{+/-}, 47.9%; *Dll4*^{-/-}, 25.0%; *n* = 239) but at E10.5 no viable *Dll4*^{-/-} embryos were found (*n* = 48). In addition, no *Dll4*^{-/-} offspring was obtained (wild-type, 62.5%; *Dll4*^{+/-}, 37.5%; *n* = 39). As expected, RT-PCR analysis demonstrated the complete absence of *Dll4* mRNA in the null embryos (Fig. 1D).

The *Dll4*^{-/-} embryos showed more severe and precocious vascular defects than heterozygotes. Mutant embryos were morphologically normal until E8.5 (data not shown), and LacZ expression from the *Dll4* locus was similar in intensity to the heterozygotes. The correct migration and aggregation of the angioblasts occurred to form the dorsal aortae, showing no disruption of the onset of vasculogenesis. However, the dorsal aortae already showed a clear reduction in diameter by E8.75 (Fig. 3A,B). By E9.0 the homozygous null embryos were highly delayed and abnormal, with severe pericardial swelling, and drastically reduced dorsal aortic diameter

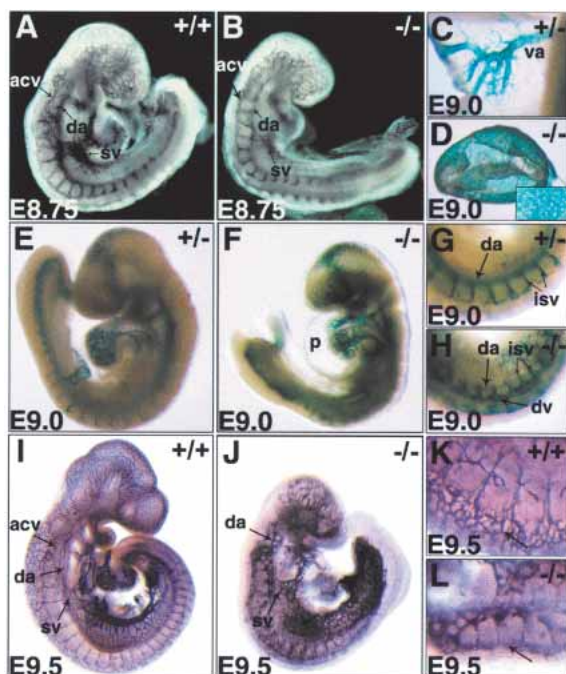


Figure 3. Defective arterial and venous remodeling in *Dll4*^{-/-} embryos. Whole-mount PECAM (A,B,I-L) and X-gal staining (C-H) of control and *Dll4*^{-/-} embryos. At E8.75, *Dll4*^{-/-} embryos display reduced dorsal aorta (da), anterior cardinal vein (acv), and sinus venosus (sv). (C,D) Presence of vitelline arteries (va) in the yolk sac of a E9.0 *Dll4*^{-/-} embryo contrasts with *Dll4*^{+/-} yolk sac vessels, which appear stalled at the primary capillary plexus stage (inset); in *Dll4*^{-/-} embryos the *Dll4-lacZ* reporter expression is observed throughout the yolk sac vessels. (E-H) By E9.0, *Dll4*^{-/-} embryos exhibit growth retardation and arrested heart development with pericardial edema (p); the dorsal aorta appears further reduced, and there is an abnormal accumulation of lacZ-positive cells in the apical portion of the intersomitic vessels (isv) forming an enlarged dorsal vessel (dv). By E9.5, the *Dll4*^{-/-} embryos are severely retarded with extremely reduced dorsal aortae, a reduced and almost indistinguishable venous structure, sinus venosus, and anterior cardinal vein. (K,L; higher magnification of I,J, respectively) In the more dorsal region, instead of an intricately branched network between arterial and venous intersomitic vessels, the *Dll4*^{-/-} embryos display a single fused vessel that did not undergo angiogenic remodeling.

in the anterior region (Fig. 3E-H). Not only were the major arteries abnormal; branching morphogenesis was also affected. *Dll4*^{-/-} embryos showed an abnormal accumulation of lacZ⁺ endothelial cells in the apical portion of the intersomitic vessels and an abnormally dilated dorsal vessel in this region (Fig. 3, cf. G and H). This defect would result in abnormal blood circulation in the embryo, with misdirection from the dorsal aorta to the lateral vessels.

Yolk sac circulation was also highly abnormal. The vitelline artery was drastically reduced, and the yolk sac vascular plexus showed an angiogenic remodeling defect with persistence of the primary capillary bed. Interestingly, whereas in the heterozygous embryos the lacZ reporter was strictly expressed in the arterial (posterior) region of the yolk sac vasculature, in the null embryos, lacZ was expressed in all endothelial cells of the yolk sac plexus (Fig. 3C,D). Although vitelline arteries fail to form, *Dll4* expression is clearly activated in the yolk sac precursors, consistent with it being upstream of the genes that specify artery fate.

By E9.5 these phenotypic traits were accentuated, with more pronounced growth retardation and arterial atrophy, the dorsal aortae being absent or reduced to a rudimentary capillary plexus (Fig. 3I,J). The hearts in these embryos showed reduced atrial and ventricular chambers, and the ventricular trabeculation was markedly reduced (data not shown). The head vasculature consisted of a simple plexus of disorganized and fused vessels (Fig. 3J). Venous development was also impaired in the null embryos. At E8.75 the anterior cardinal vein already appeared to have a reduced caliber and ectopic branching at some points, and the sinus venosus appeared smaller (Fig. 3A,B). By E9.0 the anterior cardinal vein was further reduced, and by E9.5, a distinct anterior cardinal vein was absent and the embryos showed a very reduced sinus venosus (Fig. 3I,J). Therefore, although the major arteries and veins of the embryo form in the absence of *Dll4*, their later development is severely disrupted. As *Dll4* expression is artery-specific, the venous defects are likely secondary to arterial patterning and growth problems.

In zebrafish, Notch signaling has been implicated in the specification of arterial endothelial cells by suppressing the venous cell fate (Lawson et al. 2001, 2002; Zhong et al. 2001). To investigate whether the disruption of vascular development in *Dll4* mutants could be at least partially attributable to abnormal identity of the vascular endothelial cells, we carried out RNA in situ hybridization and immunostaining to determine the expression of arterial and venous markers. In *Dll4*^{-/-} embryos with residual intact dorsal aortae, the VEGF receptor, Flk1, was normally expressed in both arteries and veins, and the *Dll4-lacZ* reporter was expressed in the arteries. However none of the downstream arterial markers studied (EphrinB2, Connexin37, and Connexin40) were expressed in the endothelium (Fig. 4A-F; data not shown). This is consistent with the proposed pathway from zebrafish, where VEGF signaling upstream of Notch signaling promotes arterial cell fate (Lawson et al. 2002). In addition to loss of arterial markers, the venous marker EphB4 was ectopically expressed in the dorsal aortae as well as the cardinal veins (Fig. 4J). In some null embryos, from E9.0, the dorsal aorta fused with the anterior cardinal vein at the level of the sinus venosus (Fig. 4J), consistent with possible loss of separate identity of the two

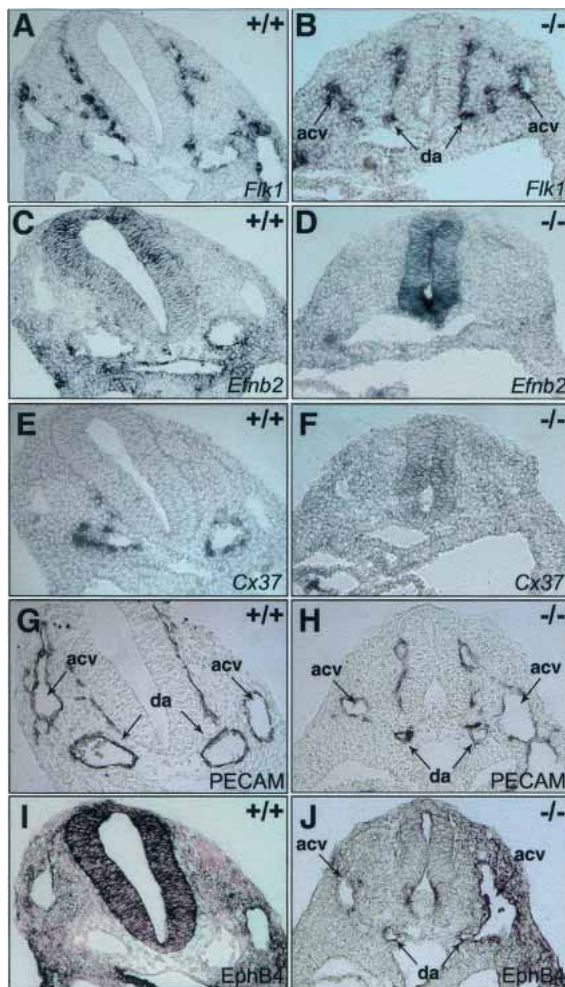


Figure 4. Loss of arterial identity in *Dll4*^{-/-} endothelial cells. In situ hybridization (A–F) and immunostaining (G–J) of adjacent cryosections from E9.0 embryos. (A,B) *Flk-1* mRNA is present in the endothelial cells of both dorsal aorta (da) and anterior cardinal vein (acv). (C,F) Endothelial cells in *Dll4*^{-/-} embryos fail to express arterial-specific *EphrinB2* (*Efnb2*) and *Connexin37* (*Cx37*) mRNAs. (G–J) The endothelial cells (shown by PECAM immunostaining in G,H) lining the dorsal aortae in *Dll4*^{-/-} embryos express the venous-specific EphB4 receptor; notice also the fusion between the dorsal aorta and the anterior cardinal vein on the right side of the picture (J).

vessels. These data strongly suggest an involvement of the Notch signaling pathway, mediated through the Dll4 ligand in a cell-autonomous manner, in the establishment of the endothelial arterial cell phenotype in mice. The similarity of the *Dll4*^{-/-} phenotype to that of the *Notch1/4* double mutants (Krebs et al. 2000) is consistent with this, as is the contrasting effect of endothelial-specific activated Notch expression (Uyttendaele et al. 2001). Loss of arterial vascular identity, in *Dll4*^{-/-} mutants, in turn, could cause angiogenic defects leading to a generalized disruption of the vasculature and embryonic death. However, this loss of arterial endothelial identity is probably not sufficient to explain all the vascular defects observed, such as reduction in aortic caliber and complete absence of arterial organization in the yolk sac. Notch signaling, in addition to regulating the endothelial cell fate, may also regulate the growth of arteries, through the regulation of endothelial cell proliferation

and/or the maintenance of a pool of undifferentiated angioblasts.

The strain-dependent haploinsufficiency of *Dll4* was surprising because no other component of the Notch pathway has shown such an effect on vascular development. Indeed, this is the first reported lethal haploinsufficiency for any Notch signaling component in mammals. However, vascular development-related haploinsufficiency has also been reported for the VEGF gene (Carmeliet et al. 1996; Ferrara et al. 1996), which lies upstream of Notch signaling in arterial development (Lawson et al. 2002). This suggests that the development and patterning of the arterial system may be controlled by levels of availability of critical ligands. Interestingly, both VEGF and Dll4 have been shown to be up-regulated by hypoxia (Shweiki et al. 1992; Mailhos et al. 2001), which is one of the environmental factors that can impinge on vascular patterning and growth. Presumably, exquisite sensitivity to ligand levels helps to ensure appropriate vascular responses to changing external environments. Sensitivity of the embryonic vasculature to Dll4 levels raises the possibility that Dll4 might be a good target for intervention in adult neovascularization.

Materials and methods

Generation of mice carrying a germ-line mutation of *Dll4*

Mouse *Dll4* genomic clones were isolated from a 129/Sv genomic library and characterized using standard protocols. In the targeting vector, a 2.0-kilobase (kb) DNA fragment encompassing the start codon and the first three exons of the *Dll4*-coding sequence was deleted and substituted by a *lacZ* and PGK-*neo* cassette, flanked by 1.5-kb 5' and 5-kb 3' homologous regions (Fig. 1A). This vector was linearized with *NotI* and electroporated into R1 ES cells. Correctly targeted clones were identified by PCR assays and confirmed by Southern blotting (Fig. 1B). Chimeric mice were generated by morula aggregation (Nagy et al. 1993), and males were crossed with ICR females.

Screening for germ-line transmission and genotyping was performed by PCR analysis of tail biopsy DNA (Fig. 1C) using the following primers: P1, 5'-GGGGAATCAGCTTTTCAGGAA-3'; P2, 5'-CGAACTCCTGCAGCCGAGCT-3'; P3, 5'-ACGACGTTGTAATACGAC-3' (Fig. 1A).

Generation of *Dll4*^{+/-} ES-derived embryos

Dll4^{-/-} ES cell lines were used in aggregations with EGFP-expressing tetraploid embryos as described (Hadjantonakis et al. 1998). Two-cell diploid embryos were fused to become tetraploid one-cell embryos, cultured to the four-cell stage, and aggregated with ES cells. Composite embryos were cultured overnight and then transferred to pseudopregnant recipients.

In situ hybridization, immunohistochemistry, and *lacZ* staining

Embryonic age was initially determined by the date of the formation of the copulation plug and confirmed by number of somites. Whole-mount immunohistochemistry and *lacZ* staining were carried out by standard techniques (Hogan et al. 1994). Antibodies were from Pharmingen (PECAM) and R&D Systems (EphB4). Digoxigenin-labeled RNA probes were transcribed from linearized templates, and in situ hybridization of cryosections was carried out as described (Henrique et al. 1997). Probe sizes were as follows: *EphrinB2*, 700 bp; *Flk1*, 800 bp; *Connexin37*, 978 bp; *Connexin40*, 1000 bp.

RT-PCR analysis

Individual yolk sacs and embryos from *Dll4*^{+/-} intercrosses were dissected and used to isolate total RNA from which first-strand cDNA was synthesized using a SuperScript Preamplification System kit (GIBCO-BRL). First-strand cDNA (0.1 µg) was used for PCR with specific primers for *Dll4* (forward, 5'-AGCTGGAAGTGGACTGTGGT-3'; reverse, 5'-TAGAGTCCCTGGGAGAGCAA-3') and, as a control, β -actin (forward, 5'-ACCGTGAAGATGACCCAG-3'; reverse, 5'-GCTGTGGTGGT

GAAGCTGTA-3'). PCR products were visualized by ethidium bromide staining [Fig. 1D].

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