Notch signaling is essential for vascular morphogenesis in mice

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The Notch gene family encodes large transmembrane receptors that are components of an evolutionarily conserved intercellular signaling mechanism. To assess the role of the *Notch4* gene, we generated *Notch4*-deficient mice by gene targeting. Embryos homozygous for this mutation developed normally, and homozygous mutant adults were viable and fertile. However, the *Notch4* mutation displayed genetic interactions with a targeted mutation of the related *Notch1* gene. Embryos homozygous for mutations of both the *Notch4* and *Notch1* genes often displayed a more severe phenotype than *Notch1* homozygous mutant embryos. Both *Notch1* mutant and *Notch1/Notch4* double mutant embryos displayed severe defects in angiogenic vascular remodeling. Analysis of the expression patterns of genes encoding ligands for Notch family receptors indicated that only the *Dll4* gene is expressed in a pattern consistent with that expected for a gene encoding a ligand for the *Notch1* and *Notch4* receptors in the early embryonic vasculature. These results reveal an essential role for the Notch signaling pathway in regulating embryonic vascular morphogenesis and remodeling, and indicate that whereas the *Notch4* gene is not essential during embryonic development, the *Notch4* and *Notch1* genes have partially overlapping roles during embryogenesis in mice.

The Notch signaling pathway is an evolutionarily conserved intercellular signaling mechanism, and mutations in its components disrupt cell fate specification and embryonic development in organisms as diverse as insects, nematodes, and mammals. This signaling pathway was first studied in *Drosophila*. The *Notch* gene of *Drosophila* encodes a large transmembrane receptor that, at the extracellular surface of a cell, interacts with membrane-bound ligands encoded by the *Delta* and *Serrate* genes. The signal induced by ligand binding is then transmitted at the intracellular surface in a process involving proteolytic processing of the receptor and nuclear translocation of the intracellular domain of the Notch protein (for recent reviews of the Notch signaling pathway, see Gridley 1997; Weinmaster 1997; Chan and Jan 1998; Greenwald 1998; Artavanis-Tsakonas et al. 1999).

Four Notch genes have been described in mice. The *Notch4* gene was originally identified as a common viral

integration site (originally termed the int3 locus) in mouse mammary tumor virus (MMTV)-induced mammary tumors (Gallahan and Callahan 1987; Robbins et al. 1992). In these tumors, proviral integration leads to the production of a truncated *Notch4* transcript. This truncated transcript initiates within the 3′ MMTV long terminal repeat and includes the region encoding the intracellular domain of the Notch4 protein (Robbins et al. 1992; Gallahan and Callahan 1997), which is a constitutively active form of the Notch4 protein. Further work in transgenic mouse models and in tissue culture cells has demonstrated that unregulated expression of the cytoplasmic domain of the Notch4 protein inhibits mammary development (Smith et al. 1995; Uyttendaele et al. 1998). Cloning of the full-length *Notch4* cDNA revealed that it encodes a protein of ~200 kD with fewer epidermal growth factor (EGF)-like repeats and a shorter intracellular domain than the other mammalian Notch homologs (Uyttendaele et al. 1996; Gallahan and Callahan 1997; Shirayoshi et al. 1997). Somewhat surprisingly, expression analyses indicated that embryonic expression of the *Notch4* gene was largely restricted to vascular endothelial cells (Uyttendaele et al. 1996; Shirayoshi et al. 1997).

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Formation of the vascular system is one of the earliest and most important events during embryogenesis in mammals. During the early stages of vascular development in both the mammalian embryo and its extraembryonic membranes such as the yolk sac, endothelial cell precursors differentiate and coalesce into a network of homogeneously sized primitive blood vessels (the primary vascular plexus) in a process termed vasculogenesis (Risau and Flamme 1995). This primary vascular plexus is then remodeled by the process of angiogenesis, which involves the sprouting, branching, splitting, and differential growth of vessels in the primary plexus to form both the large and small vessels of the mature vascular system (Risau 1997; Gale and Yancopoulos 1999). A number of different intercellular signaling pathways have been implicated in the control of vasculogenesis and angiogenesis in mammals. These pathways include the vascular endothelial growth factor pathway, the transforming growth factor-β (TGF-β) pathway, the angiopoietin/Tie receptor pathway, and the ephrin/Eph receptor pathway (for reviews, see Folkman and D'Amore 1996; Hanahan 1997; Carmeliet and Collen 1999; Gale and Yancopoulos 1999; Neufeld et al. 1999).

A number of observations indicate that the Notch signaling pathway also plays a critical role in vascular development and homeostasis. In addition to the *Notch4* gene, the *Notch1* gene is expressed in endothelial cells in the embryonic vasculature (Franco del Amo et al. 1992; Reaume et al. 1992). Analysis of targeted mutations of genes encoding Notch ligands has shown that both *Jag1* (Xue et al. 1999) and *Dll1* (Hrabé de Angelis et al. 1997) homozygous mutant embryos die from vascular defects and hemorrhaging at approximately gestational day E10.5. Expression of the human *JAG1* gene is induced in an in vitro angiogenesis model, and administration of *Jag1* antisense oligonucleotides modulates in vitro angiogenesis (Zimrin et al. 1996). In adults, a role for the Notch pathway in vascular homeostasis has been suggested by the finding that the degenerative vascular disease CADASIL is caused by missense mutations in the *Notch3* gene (Joutel et al. 1996).

In this study we examined whether the *Notch4* gene plays an essential role during embryonic development in mice. *Notch4*-deficient mice were viable and fertile and exhibited no obvious mutant phenotype. However, the *Notch4* null mutation genetically interacted with a mutation we had constructed previously in the *Notch1* gene (Swiatek et al. 1994), indicating partial functional redundancy between the *Notch1* and *Notch4* genes during embryogenesis. Further characterization of the phenotypes of both $Notch1^{-/-}$ mutant and $Notch1^{-/-}$ $Notch4^{-/-}$ double mutant embryos revealed extensive defects in angiogenic vascular remodeling that affected the embryo, the yolk sac, and the placenta. Characterization of the expression patterns of the genes encoding Notch ligands revealed that only the newly isolated *Dll4* gene (Shutter et al. 2000) was expressed in a pattern consistent with that expected for a gene encoding a ligand for the Notch1 and Notch4 receptors in the early embryonic vasculature.

Results

### Mice homozygous for a targeted mutation of the Notch4 gene are viable and fertile

To analyze the role of the *Notch4* gene, we created a deletion allele by gene targeting. A *Notch4* targeting vector was constructed that deleted a 1.0-kb genomic fragment containing exons 21 and 22 encoding part of the extracellular domain of the protein (Fig. 1A). We refer to this allele as $Notch4^{d1}$. Germ-line transmission of the $Notch4^{d1}$ mutant allele was obtained for two targeted clones (Fig. 1B). Mice heterozygous for the $Notch4^{d1}$ mutation appeared normal and were fertile.

To examine whether mice homozygous for the $Notch4^{d1}$ mutation were viable, heterozygous F_1 animals were intercrossed and the genotypes of F_2 progeny were determined 2–3 weeks after birth. Mice homozygous for the $Notch4^{d1}$ mutation (designated $Notch4^{-/-}$) were recovered at the expected frequency and by morphological and histological analyses did not differ from heterozygous and wild-type littermates. Genomic PCR analysis confirmed that the expected genomic fragment was deleted in the $Notch4^{-/-}$ mutant homozygotes (Fig. 1C). Mammary glands from the $Notch4^{-/-}$ mutant homozygotes were examined in particular detail, because the *Notch4* gene was initially identified as the *int3* gene, a common integration site for MMTV-induced mammary tumors (Robbins et al. 1992), and unregulated expression of the cytoplasmic domain of the Notch4 protein inhibits mammary development (Smith et al. 1995; Uyttendaele et al. 1998). However, morphological and histological analyses of mammary glands isolated from virgin, pregnant, and lactating females did not reveal any defects in the $Notch4^{-/-}$ homozygotes (data not shown).

We analyzed RNA isolated from $Notch4^{-/-}$ homozygous mutant adult animals for the presence of *Notch4* transcripts by RT-PCR with several different primer sets (Fig. 1D), and analyzed $Notch4^{-/-}$ homozygous mutant embryos by whole-mount in situ hybridization with a *Notch4* antisense riboprobe encoding the entire intracellular domain of the Notch4 protein (Fig. 1E). No *Notch4* transcripts were detected in the homozygotes by either technique. Thus, unlike mouse embryos homozygous for targeted mutations in the *Notch1* (Swiatek et al. 1994; Conlon et al. 1995) and *Notch2* (Hamada et al. 1999; T. Gridley unpubl.) genes, embryos homozygous for a *Notch4* null mutation develop normally. We next examined whether we could detect genetic interactions between the $Notch4^{d1}$ mutation and a null mutation we had made previously in the *Notch1* gene (the $Notch1^{in32}$ mutation; Swiatek et al. 1994).

Notch1$^{+/-}$ Notch4$^{-/-}$ mutant mice are viable but show growth retardation

To examine possible genetic interactions between the *Notch1* and *Notch4* targeted mutations, we crossed mice heterozygous for the $Notch1^{in32}$ mutation (designated $Notch1^{+/-}$) to mice homozygous for the $Notch4^{d1}$ mutation ($Notch4^{-/-}$). Double heterozygous ($Notch1^{+/-}$
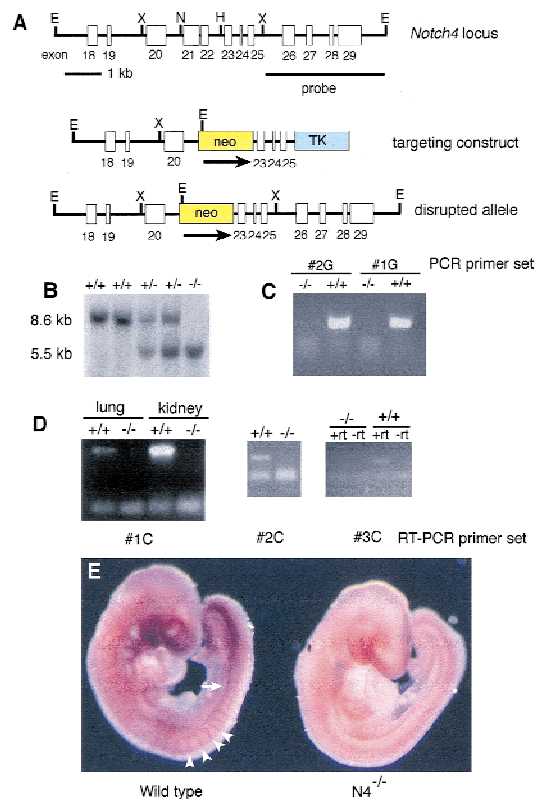
Figure 1. Targeted disruption of the *Notch4* gene. (*A*) Targeting scheme. (*Top*) The genomic organization of a portion of the *Notch4* gene. Exons are indicated by white boxes. (*Middle*) The structure of the targeting vector. The deleted exons 21 and 22 encode amino acids 1249–1434 in the extracellular domain of the Notch4 protein. (*Bottom*) The predicted structure of the *Notch4* locus following homologous recombination of the targeting vector. (E) *Eco*RI; (H) *Hind*III; (N) *Nco*I; (X) *Xba*I. (*B*) DNA isolated from embryos of the intercross of *Notch4+/−* heterozygous mice was digested with *Eco*RI, blotted, and hybridized with the indicated probe. Sizes of hybridizing fragments are indicated. Genotypes of progeny are indicated at the top of the lane. (*C*) Genomic PCR analysis with primers located in region deleted in the *Notch4d1* mutant allele. Genotypes are indicated at *top*. (*D*) RT–PCR analysis. RT–PCR primer sets are indicated at the *bottom* of each panel. Primer set #1C flanks the deleted region; primers sets #2C and #3C are located at the 3′ end of the *Notch4* cDNA. Genotypes are indicated at the *top* of the lane. (+rt) Plus reverse transcriptase; (−rt) without reverse transcriptase. (*E*) Whole mount in situ hybridization of a wild-type and a *Notch4−/−* embryo with an antisense *Notch4* riboprobe encoding the intracellular domain of the *Notch4* protein. *Notch4* expression in the wild-type embryo is observed in intersomitic blood vessels (arrowheads) and the dorsal aorta (arrow). No *Notch4* expression is observed in the mutant embryo.

Notch4+/−) animals were crossed with *Notch4−/−* mice to generate animals with the genotype *Notch1+/− Notch4−/−*. Mice with this genotype appeared smaller than their *Notch1+/+ Notch4−/−* littermates. Breeding studies indicated that *Notch1+/− Notch4−/−* adult mice were fertile. We therefore crossed *Notch1+/− Notch4−/−* mice with *Notch4−/−* mice and followed the growth rates of the progeny.

This analysis revealed postnatal growth retardation in the *Notch1+/− Notch4−/−* double mutant animals. At birth, no clear differences in the weight of the pups could be detected. By weaning age (around 3 weeks), however, the average weight of the *Notch1+/− Notch4−/−* animals was ~80% that of their *Notch1+/+ Notch4−/−* littermates (Fig. 2). Although we have not determined the cause of the growth retardation in the *Notch1+/− Notch4−/−* mice, this analysis clearly revealed a genetic interaction between the *Notch1* and *Notch4* mutations.

Synergistic effects in Notch1−/− Notch4−/− double homozygous mutant embryos

We next analyzed whether a genetic interaction between the *Notch1* and *Notch4* mutations was detectable in double homozygous mutant embryos. Because *Notch1−/−* embryos arrest in development at or shortly before E9.5 (Swiatek et al. 1994; Conlon et al. 1995), we isolated embryos at E9.5 from the intercross of *Notch1+/− Notch4−/−* mice (Fig. 3). Typically, *Notch1−/−* mutant embryos arrest in development after forming 16–20 somites, completing embryonic turning and completing closure of the anterior neural tube (Fig. 3C) (Swiatek et al. 1994; Conlon et al. 1995). Of *Notch1−/− Notch4−/−* double mutant embryos ~50% were more severely affected than a typical *Notch1−/−* mutant embryo. These severely affected *Notch1−/− Notch4−/−* double mutant embryos had formed fewer somites, had not completed embryonic turning, and had open neural tubes (Fig. 3A,B). The remaining double mutant embryos appeared phenotypically like *Notch1−/−* mutant embryos. These data indicated that, despite the fact that the *Notch4* gene is not essential for embryonic development, the *Notch1* and *Notch4* genes play partially redundant roles during embryogenesis.

Defects in angiogenic vascular remodeling in Notch1−/− and Notch1−/− Notch4−/− mutant embryos

Both the *Notch1* (Franco del Amo et al. 1992; Reaume et
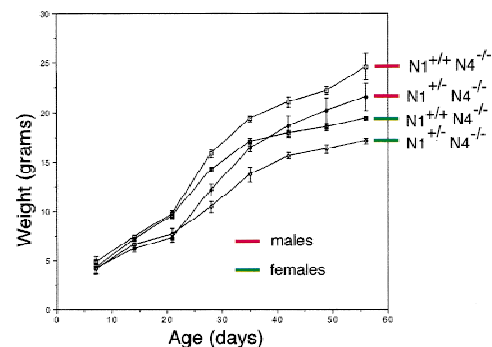
Figure 2. Genetic interactions of *Notch1* and *Notch4* mutations. Growth curve of *Notch1+/− Notch4−/−* (♦, ◇) vs. *Notch1+/+ Notch4−/−* (■, □) littermates. The weights of *Notch1+/− Notch4−/−* and *Notch1+/+ Notch4−/−* mutant animals are plotted against age. Males (red) and females (green) are plotted separately. Data presented are from four mice in each group. Error bars indicate the S.D..

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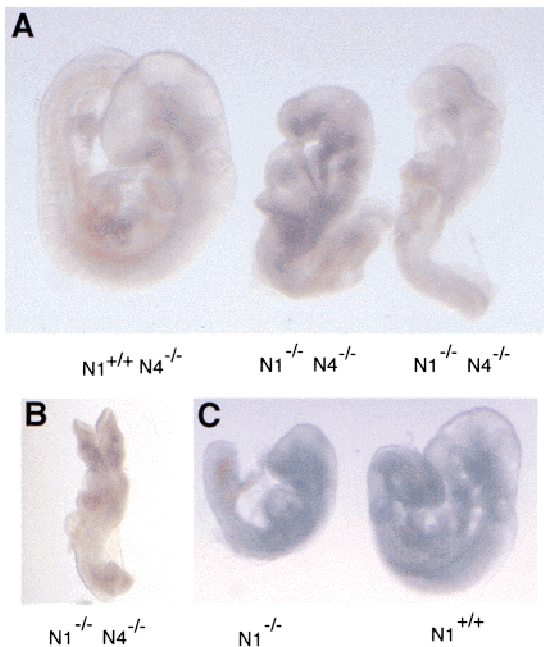
Figure 3. Synergistic effects in *Notch1*^{−/−} *Notch4*^{−/−} double mutant embryos. (*A*) Two *Notch1*^{−/−} *Notch4*^{−/−} double homozygous mutant embryos and littermate control isolated at E9.5. (*B*) Severely affected *Notch1*^{−/−} *Notch4*^{−/−} double homozygous mutant embryo. The double homozygous mutant embryos in both *A* and *B* have not completed embryonic turning and have open neural tubes. (*C*) A typical *Notch1*^{−/−} embryo and control littermate at E9.5. The *Notch1*^{−/−} mutant embryo has completed turning and has a closed neural tube.

al. 1992) and *Notch4* (Uyttendaele et al. 1996; Shirayoshi et al. 1997; also see Fig. 1E) genes are expressed in vascular endothelial cells, and the *Notch1*^{−/−} mutant and *Notch1*^{−/−} *Notch4*^{−/−} double mutant embryos exhibit defects (e.g., pericardial edema) suggestive of a failure to maintain vascular homeostasis. We therefore examined vascular development in both the *Notch1*^{−/−} mutant and *Notch1*^{−/−} *Notch4*^{−/−} double mutant embryos. This analysis revealed substantial defects in angiogenic vascular remodeling in both the *Notch1*^{−/−} mutant and *Notch1*^{−/−} *Notch4*^{−/−} double mutant embryos.

Vascular defects can first be observed in the yolk sacs of the mutant embryos. When viewed in whole mount at E9.5, yolk sacs from wild type (not shown), *Notch1*^{+/−} (Fig. 4A), and *Notch4*^{−/−} (Fig. 4B) embryos all exhibited large vitelline blood vessels. All *Notch1*^{−/−} mutant and *Notch1*^{−/−} *Notch4*^{−/−} double mutant embryos had pale yolk sacs lacking obvious blood vessels (Fig. 4C,D). We then visualized the vascular network of mutant embryos and littermate controls by staining with a monoclonal antibody to platelet endothelial cell adhesion molecule-1 (PECAM-1), a specific marker for vascular endothelial cells (Baldwin et al. 1994). In the yolk sacs of both *Notch1*^{−/−} mutant and *Notch1*^{−/−} *Notch4*^{−/−} double mutant embryos, the primary vascular plexus appeared to form normally, indicating that there were no apparent defects in vasculogenesis in the mutants. However, the

mutant embryos failed to remodel the primary vascular plexus to form the large and small blood vessels of the mature yolk sac (Fig. 4E–G). Histological analysis of PECAM-1-stained yolk sacs demonstrated that both small capillaries and large vitelline collecting vessels differentiated in control yolk sacs (Fig. 4H), whereas both *Notch1*^{−/−} (Fig. 4I) and *Notch1*^{−/−} *Notch4*^{−/−} (not shown) yolk sacs exhibited a disorganized, confluent vascular plexus.

Vascular defects were also observed in the labyrinthine layer of the placentas of the mutant embryos (Fig. 4J,K; data not shown). In both *Notch1*^{−/−} mutant and *Notch1*^{−/−} *Notch4*^{−/−} double mutant embryos at E9.5,
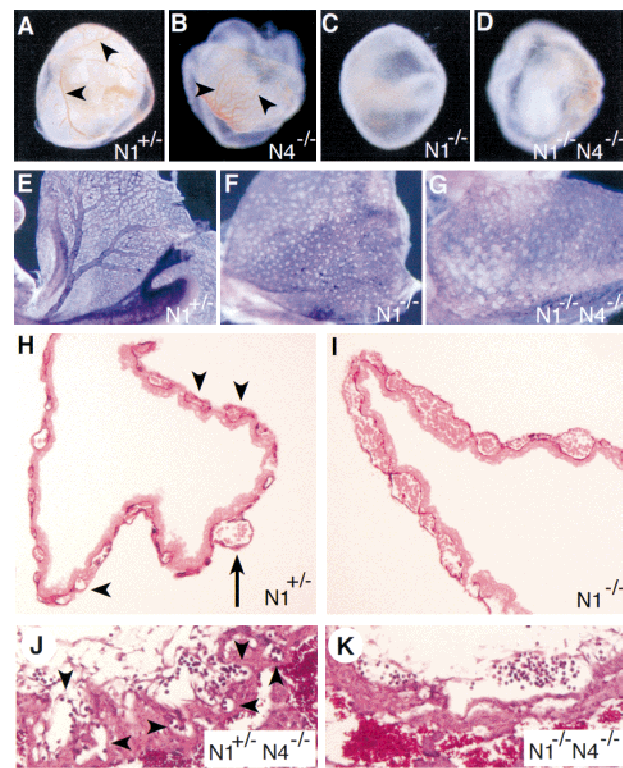

Figure 4. Yolk sac and placental defects in mutant embryos. (*A–D*) Morphology of embryos in their yolk sacs at E9.5. Large vitelline blood vessels (arrowheads) are observed in the *Notch1*^{+/−} and *Notch4*^{−/−} yolk sacs, but not in the *Notch1*^{−/−} and *Notch1*^{−/−} *Notch4*^{−/−} mutants. (*E–G*) PECAM-1-stained yolk sacs. The yolk sacs of the *Notch1*^{−/−} and *Notch1*^{−/−} *Notch4*^{−/−} mutant embryos are at the primitive vascular plexus stage and have not undergone vascular remodeling to form the large and small blood vessels of the mature yolk sac. (*H,I*) Histological sections of PECAM-1-stained yolk sacs. The *Notch1*^{+/−} yolk sac (*H*) has differentiated both small capillaries (arrowheads) and large vitelline collecting vessels (arrow). The *Notch1*^{−/−} yolk sac (*I*) exhibits a disorganized, confluent vascular plexus. (*J,K*) Histological sections of placentas at E9.5. In the Notch1^{+/−} *Notch4*^{−/−} control embryo (*J*), embryonic blood vessels containing nucleated erythrocytes (arrowheads) have invaded the labyrinthine layer of the placenta. In the *Notch1*^{−/−} *Notch4*^{−/−} mutant embryo (*K*), embryonic blood vessels are present at the edge of the placenta but have not invaded the labyrinthine layer.

the allantois of the mutant embryos fused with the chorionic plate, which is an initiating step in the formation of a functional chorioallantoic placenta. However, in the control embryos (e.g., the $Notch1^{+/-}$ $Notch4^{-/-}$ embryo shown in Fig. 4J, which is phenotypically wild type), blood vessels containing embryonic nucleated erythrocytes could be observed invading and interdigitating into the labyrinthine trophoblast layer of the developing placenta. In $Notch1^{-/-}$ mutant and $Notch1^{-/-}$ $Notch4^{-/-}$ double mutant embryos, embryonic blood vessels containing nucleated erythrocytes remained at the periphery of the trophoblast layer and did not invade the labyrinthine layer (Fig. 4K). The failure of mutant endothelial cells to efficiently invade the labyrinthine region provides further evidence of defects in angiogenesis in the $Notch1^{-/-}$ mutant and $Notch1^{-/-}$ $Notch4^{-/-}$ double mutant embryos.

In addition to the vascular defects observed in the yolk sac and placenta, extensive defects in vascular morphogenesis were observed in the mutant embryos themselves (Fig. 5). A prominent feature in the $Notch1^{-/-}$ mutant and $Notch1^{-/-}$ $Notch4^{-/-}$ double mutant embryos was malformation of the large blood vessels in the anterior of the embryo. The primary vessels for arterial flow to the head of the mammalian embryo are the dorsal aortae, and the primary vessels for venous return are the anterior cardinal veins. In the $Notch4^{-/-}$ (phenotypically wild type) PECAM-1-stained embryo shown in Figure 5A, the anterior cardinal vein (arrowhead) was easily visualized as a large vessel running in an anterior–posterior direction just beneath the surface ectoderm of the embryo. In the $Notch1^{-/-}$ embryo shown in Figure 5B, the anterior cardinal vein (arrowhead) had formed, but was smaller than in the control. Two $Notch1^{-/-}$ $Notch4^{-/-}$ double mutant embryos at E9.5 are shown in Figure 5, C and D. In the less severely affected double mutant embryo, discontinuous remnants of the anterior cardinal vein were observed (arrowheads in Fig. 5C). In the more severely affected $Notch1^{-/-}$ $Notch4^{-/-}$ double mutant embryo, the anterior cardinal vein appeared to be entirely missing (Fig. 5D). In the trunk of wild-type embryos at E9.5, intersomitic blood vessels were apparent along the boundaries between adjacent somites (Fig. 5E). On the dorsal side of the embryo, the intersomitic vessels branch to form a highly anastomosed capillary network. In both $Notch1^{-/-}$ mutant (Fig. 5F) and $Notch1^{-/-}$ $Notch4^{-/-}$ double mutant (Fig. 5G) embryos, intersomitic vessels were severely disorganized.

Histological analyses of PECAM-1-stained embryos confirmed that dorsal aortae and anterior cardinal veins were frequently atretic in the mutant embryos (Fig. 5H–J). In the section of the $Notch1^{+/-}$ (phenotypically wild type) embryo shown in Figure 5H, both the dorsal aortae and the anterior cardinal veins had open lumens that were lined with a continuous layer of PECAM-1-positive endothelial cells. In the $Notch1^{-/-}$ embryo shown in Figure 5I, the anterior cardinal veins appeared relatively normal, but both dorsal aortae had a collapsed appearance. In the more severely affected $Notch1^{-/-}$ $Notch4^{-/-}$ double mutant embryo (Fig. 5J), endothelial cells were
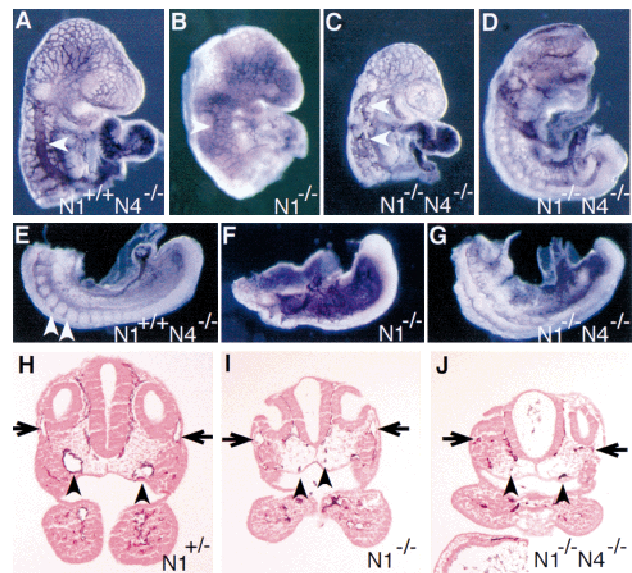

Figure 5. Defects in vascular remodeling in $Notch1^{-/-}$ and $Notch1^{-/-}$ $Notch4^{-/-}$ mutant embryos. (*A–G*) PECAM-1-stained whole mount embryos. (*A–D*) Defective morphogenesis of the main trunk of the anterior cardinal vein (arrowhead) in $Notch1^{-/-}$ (*B*) and $Notch1^{-/-}$ $Notch4^{-/-}$ mutant embryos (*C,D*). The double mutant embryo in *D* is more severely affected than the embryo in *C*. (*E–G*) In the $Notch1^{+/-}$ $Notch4^{-/-}$ control embryo (*E*), intersomitic vessels (arrowheads) differentiate, whereas these vessels are not observed in the $Notch1^{-/-}$ (*F*) and $Notch1^{-/-}$ $Notch4^{-/-}$ (*G*) mutant embryos. (*H–J*) Histological sections of PECAM-1-stained embryos at the level of the otic vesicle. In the $N1^{+/-}$ control embryo (*H*), both the dorsal aortae (arrowheads) and the anterior cardinal veins (arrows) have open lumens and normal morphology. In the $Notch1^{-/-}$ $Notch4^{-/-}$ mutant embryo (*J*), endothelial cells have differentiated but both the dorsal aortae and the anterior cardinal veins have an abnormal, collapsed morphology. In the less-severely affected $Notch1^{-/-}$ mutant embryo (*I*), the anterior cardinal veins still have an open lumen but the dorsal aortae are collapsed.

present at the correct positions of the anterior cardinal veins and dorsal aortae, but no vessel lumens were apparent.

The Dll4 gene encodes the probable ligand for the Notch1 and Notch4 receptors in the early embryonic vasculature

Previous work has shown that both *Jag1* and *Dll1* homozygous mutant embryos die from vascular defects and hemorrhaging (Xue et al. 1999; Hrabé de Angelis et al. 1997). However, in both these cases, embryonic lethality does not occur until E10.5, at least a day later than in $Notch1^{-/-}$ and $Notch1^{-/-}$ $Notch4^{-/-}$ mutant embryos. We therefore examined the RNA expression pattern of all the genes encoding Notch ligands at E8.5 and E9.5 (Fig. 6; data not shown). Only the *Dll4* gene (Shutter et al. 2000) was expressed in a spatial and temporal pattern that suggested it could encode the ligand for the Notch1 and Notch4 receptors during early vascular development. At E8.5, *Dll4* expression was observed in the dor-
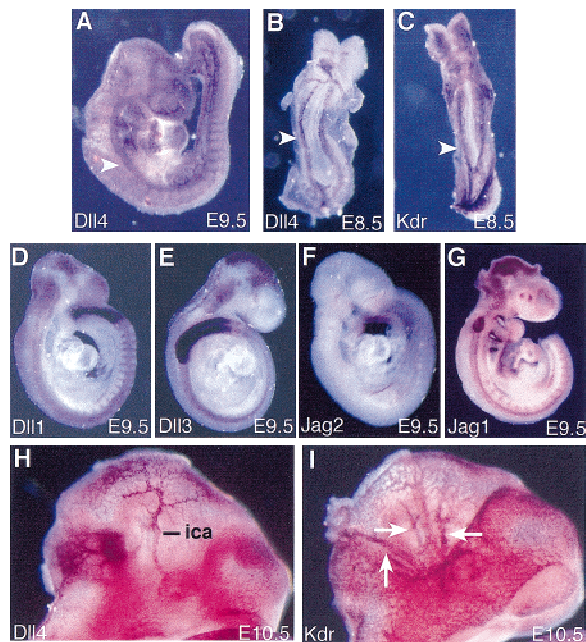
Figure 6. Expression pattern of the *Dll4* gene suggests that it encodes the ligand for the Notch1 and Notch4 receptors in the embryonic vasculature. Whole-mount in situ hybridization with the indicated probes of embryos isolated at E8.5 (*B,C*), E9.5 (*A,D–G*), and E10.5 (*H,I*). *Dll4* expression is observed in the dorsal aorta (arrowheads) and, at E9.5, the intersomitic vessels (*A*). The other genes encoding Notch ligands do not exhibit obvious expression in the vasculature at E9.5 (*D–G*). At E10.5, *Dll4* expression is observed in the internal carotid artery (ica) (*H*), but is not observed in branches of the primary head vein (arrows in *I*), which express *Kdr*.

sal aortae and the endocardium (Fig. 6B). Comparision of *Dll4* expression with *Kdr* (formerly *Flk1*) expression (Fig. 6C), which is expressed in all endothelial cells and their precursors (Yamaguchi et al. 1993), indicated that *Dll4* was expressed in only a subset of endothelial cells. Analysis of *Dll4* expression at E9.5 and E10.5 (Fig. 6A,H) demonstrated that *Dll4* was preferentially expressed in endothelial cells of the arterial system. In contrast, none of the other genes encoding Notch ligands exhibited obvious expression in the vascular system at E8.5 or E9.5 (Fig. 6D–G; data not shown), indicating that these ligands most likely are not involved in activation of the Notch1 and Notch4 receptors at these stages. We also examined *Dll4* and *Kdr* expression in *Notch1*$^{-/-}$ mutant and *Notch1*$^{-/-}$ *Notch4*$^{-/-}$ double mutant embryos. *Dll4* expression was not down-regulated in the mutants, but the expression patterns of both *Dll4* and *Kdr* revealed disruptions in vascular morphogenesis similar to those revealed by PECAM-1 staining of mutant embryos (Fig. 7).

Discussion

The Notch4 *gene is not essential, but the* Notch4 *mutation genetically interacts with a* Notch1 *mutation*

To determine the biological role of the *Notch4* gene in mice, we constructed and analyzed animals homozygous

for a deletion that removed exons 21 and 22, which encode 186 amino acids of the extracellular domain of the Notch4 protein. The two closest remaining exons, exon 20 and exon 23, are out of frame with respect to each other. Therefore, a partially functional Notch4 protein that contains the intracellular (i.e., signal-transducing) domain of the Notch4 protein could not be produced by alternative splicing from exon 20 to exon 23. We also used RT-PCR and in situ hybridization analyses to assay for alternatively spliced *Notch4* transcripts that would contain the region of the *Notch4* cDNA encoding the intracellular domain of the Notch4 protein. We found no evidence of such transcripts in the *Notch4*$^{-/-}$ homozygous mutant mice. These results suggest that the *Notch4*^{d1} allele causes a null mutation in the *Notch4* gene. Our results reveal that the *Notch4* gene is dispensable for apparently normal embryonic growth in mice, and that *Notch4*$^{-/-}$ adults are viable and fertile. In contrast, both the *Notch1* (Swiatek et al. 1994; Conlon et al. 1995) and *Notch2* (Hamada et al. 1999; T. Gridley, unpubl.) genes are essential for embryonic development. It should be noted that all Notch ligands for which either spontaneous or targeted mutations have been isolated are also essential for proper embryonic development in mice. This includes mutations in the *Jag1* (Xue et al. 1999), *Jag2* (Sidow et al. 1997; Jiang et al. 1998), *Dll1* (Hrabé de Angelis et al. 1997), and *Dll3* (Kusumi et al. 1998) genes.

The *Notch4* gene is primarily expressed in vascular endothelial cells during early embryogenesis in mice (Uyttendaele et al. 1996; Shirayoshi et al. 1997). Because the *Notch1* gene is expressed in endothelial cells at the same stages as the *Notch4* gene (Franco del Amo et al.
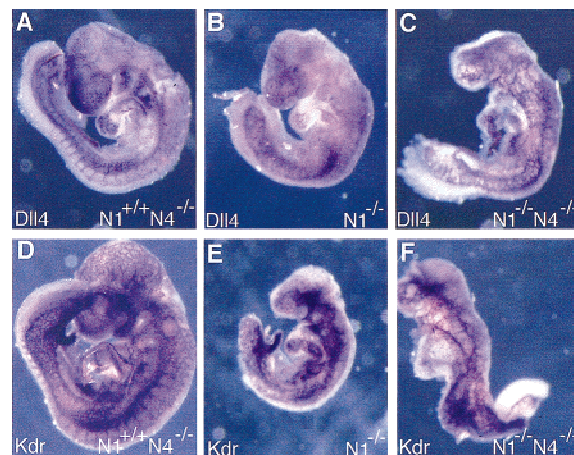
Figure 7. *Dll4* and *Kdr* expression in mutant embryos. Embryos of the indicated genotypes were hybridized with riboprobes for *Dll4* (*A–C*) and *Kdr* (*D–F*). Note the more severe dysmorphogenesis of the *Notch1*$^{-/-}$ *Notch4*$^{-/-}$ double mutant embryos (*C,F*) compared with the *Notch1*$^{-/-}$ single mutant embryos (*B,E*). *Dll4* expression was not downregulated in either the *Notch1*$^{-/-}$ or *Notch1*$^{-/-}$ *Notch4*$^{-/-}$ mutant embryos. However, the expression patterns of both *Dll4* and *Kdr* reveal disruptions in vascular morphogenesis in the mutant embryos.

1992; Reaume et al. 1992), we generated *Notch1/Notch4* double mutant mice and embryos to assess whether there were genetic interactions between these two mutations. These experiments revealed genetic interactions between the *Notch1* and *Notch4* mutations at two different stages. Adult $Notch1^{+/-}$ $Notch4^{-/-}$ double mutant mice were viable and fertile, but exhibited postnatal growth deficiency. Although $Notch1^{+/-}$ $Notch4^{-/-}$ double mutant mice were the same size as their $Notch1^{+/+}$ $Notch4^{-/-}$ littermates at birth, their postnatal growth deficiency quickly became apparent. This was clearly seen at weaning age, when the weights of $Notch1^{+/-}$ $Notch4^{-/-}$ double mutant mice averaged ~80% that of their littermates.

We also observed genetic interactions between the *Notch4* and *Notch1* mutations during embryonic development. Of $Notch1^{-/-}$ $Notch4^{-/-}$ double homozygous mutant embryos ~50% had a more severe phenotype than $Notch1^{-/-}$ mutant embryos. These severely affected $Notch1^{-/-}$ $Notch4^{-/-}$ double mutant embryos formed fewer somites than $Notch1^{-/-}$ mutant embryos and did not complete embryonic turning or neural tube closure. Vascular defects were frequently more severe in the $Notch1^{-/-}$ $Notch4^{-/-}$ double mutant embryos than in $Notch1^{-/-}$ single mutant embryos (e.g., see Fig. 5). We hypothesize that the more severe phenotype of the $Notch1^{-/-}$ $Notch4^{-/-}$ double mutant embryos is due to a more severely compromised vascular system than in $Notch1^{-/-}$ mutant embryos, thereby leading to more severe growth retardation in the double mutant embryos. This interaction is not completely penetrant, as only ~50% of the $Notch1^{-/-}$ $Notch4^{-/-}$ double homozygous mutant embryos exhibit the more severe phenotype. We do not know the cause of this incomplete penetrance. However, it may be due to the segregation of modifier loci in the mixed C57BL/6J × 129/SvImJ genetic background on which the mutant alleles are maintained.

Notch signaling is required for angiogenic vascular remodeling

Targeted mutations in components of a variety of signaling pathways (e.g., vascular endothelial growth factors, TGF-β1, angiopoietins, ephrins) have been shown to regulate vascular morphogenesis in mice (for review, see Folkman and D'Amore 1996; Hanahan 1997; Carmeliet and Collen 1999; Gale and Yancopoulos 1999; Neufeld et al. 1999). Our results demonstrate that another major intercellular signaling pathway, the Notch pathway, also regulates vascular morphogenesis and angiogenic vascular remodeling. We observed vascular defects in the placenta, yolk sac, and embryo proper of both $Notch1^{-/-}$ mutant and $Notch1^{-/-}$ $Notch4^{-/-}$ double mutant embryos. In the yolk sac of the mutant embryos, the primary vascular plexus formed normally, indicating that there were no apparent defects in vasculogenesis in the mutants. However, both $Notch1^{-/-}$ mutant and $Notch1^{-/-}$ $Notch4^{-/-}$ double mutant embryos failed to remodel the primary vascular plexus to form the large vitelline blood vessels, a process that occurs by angio-

genesis. Defects in angiogenesis were also apparent in the placenta, where embryonic blood vessels failed to invade the placental labyrinth. In the embryo proper, defects in angiogenesis and vascular remodeling were apparent as malformations of major vessels such as the dorsal aortae, the anterior cardinal veins, and the intersomitic blood vessels.

Our expression analysis of the genes encoding ligands for Notch-family receptors indicated that only the newly cloned *Dll4* gene (Shutter et al. 2000) was expressed in the embryonic vasculature early enough to encode the ligand responsible for activation of the Notch1 and Notch4 receptors. The preferential expression of *Dll4* in endothelial cells of the arterial system is strongly reminiscent of the differential expression of ephrin ligands and their Eph receptors in the arterial and venous systems (Wang et al. 1998; Adams et al. 1999; Gerety et al. 1999; for review, see Gale and Yancopoulos 1999). During early vascular development, the *ephrinB2* gene is expressed in arterial endothelium, whereas its receptor EphB4 is expressed in venous endothelium. Embryos homozygous for targeted mutations of ephrinB2 (Wang et al. 1998; Adams et al. 1999) and EphB4 (Gerety et al. 1999) exhibit defects in angiogenesis and vascular remodeling that are essentially identical to the vascular defects we observe in the $Notch1^{-/-}$ mutant and $Notch1^{-/-}$ $Notch4^{-/-}$ double mutant embryos. This suggests the intriguing possibility that the Notch pathway and the ephrin/Eph pathway functionally interact during the intercellular signaling events that must be involved in remodeling the embryonic vasculature. Further work, including genetic epistasis experiments with various Notch pathway and ephrin/Eph pathway mutants, will be required to distinguish whether these two contact-mediated intercellular signaling pathways interact, or whether they function independently, during vascular development and remodeling.

A role for the Notch pathway in vascular homeostasis in adult humans has been indicated by the finding that the degenerative vascular disease CADASIL is caused by missense mutations in the *Notch3* gene (Joutel et al. 1996). Our studies reveal a previously unappreciated role for the Notch signaling pathway in regulating angiogenic vascular remodeling during embryonic development in mice. Taken together, these results demonstrate that the Notch signaling pathway is essential both for vascular development in embryos and maintenance of vascular homeostasis in adults.

Partial functional redundancy between the Notch1 and Notch4 genes

Notch genes form a multigene family in vertebrates, and our results provide the first evidence for functional redundancy in the Notch signaling pathway in mammals. Previous experiments in *Caenorhabditis elegans* had revealed functional redundancy in the Notch signaling pathway. Although only a single Notch gene is known in *Drosophila*, *C. elegans* contains two genes encoding Notch-family receptors, *lin-12* and *glp-1*. Lambie and

Kimble (1991) showed that the *lin-12* and *glp-1* genes were functionally redundant, as double homozygous mutant embryos exhibit a novel, and more severe, synergistic phenotype. The redundancy of *glp-1* and *lin-12* gene action was further supported by the finding that expression of *glp-1* coding sequences under control of *lin-12* regulatory sequences can rescue the *lin-12* null phenotype (Fitzgerald et al. 1993), and by the ability of a particular neomorphic *glp-1* allele to mimic dominant gain of function *lin-12* mutations (Mango et al. 1991). These results suggested that the Glp-1 and Lin-12 proteins are biochemically interchangeable, and that their distinct roles during development are due largely to differences in gene expression. Our results extend these findings to mammals and demonstrate that although the *Notch4* gene plays no essential role in laboratory mice, the *Notch1* and *Notch4* genes have partially overlapping roles during embryogenesis.

Materials and methods

Targeting vector construction

A *Notch4* genomic clone (Gallahan and Callahan 1997) isolated from a P$_1$ library was used for construction of the targeting vector. The sequence of the mouse *Notch4* gene is available in GenBank (accession no. AF030001). For construction of the targeting vector, 3.3-kb *Eco*RI–*Nco*I fragment of the *Notch4* gene was subcloned upstream of a PGK-*neo* expression cassette (Soriano et al. 1991), and a 1.1-kb *Hpa*I–*Xba*I *Notch4* fragment was subcloned downstream of the PGK-*neo* cassette. This resulted in the deletion of a 1.0-kb *Notch4* genomic fragment containing exons 21 and 22 of the *Notch4* gene. We refer to this allele as *Notch4^{d1}*. The official nomenclature for the alleles used in these studies are *Notch4^{d1}*: *Notch4^{tm1Grid}*; *Notch1^{in32}* (Swiatek et al. 1994): *Notch1^{tm1Grid}*. A HSV-*tk* cassette was introduced to allow negative selection against random integration of the targeting vector (Mansour et al. 1988).

Electroporation, selection, and screening of ES cells and mouse genotyping

CJ7 ES cells were electroporated with 25 µg of linearized targeting vector, selected, screened, and injected into blastocysts from C57BL/6J mice as described previously (Swiatek and Gridley 1993). For Southern blot analysis, a 2.4-kb *Xba*I–*Eco*RI fragment was used as probe. Germ-line transmission was obtained for two independently targeted ES cell clones. Animals were genotyped by PCR or by Southern blot analysis. PCR primers for the wild-type *Notch4* allele were (5′-CCTATGACCAGTACTGCCGAG-3′) and (5′-GAGGAATCCCTAGCTCCACTC-3′), yielding an amplification product of 312 bp; the primers for the *Notch4^{d1}* allele were (5′-GAGGAGTGTCTCTTTGATGGC-3′) and (5′-AAGCGCATGCTCCAGACTGCC-3′), yielding an amplification product of ~500 bp.

For confirmation that the expected fragment of the *Notch4* gene was deleted in the *Notch4^{d1}* homozygous mutant mice, genomic DNA was isolated from *Notch4^{d1}* adult homozygotes and wild-type controls and analyzed with PCR primers from the deleted region. Two primer sets were used: #1G forward (5′-CTTTCCCTTCCCACAGGTTTG-3′) and #1G reverse (5′-TTGGTTCTATCTCTCCCAGCGAGG-3′), which amplify a 257-bp product; #2G forward (5′-AGGTTTGTGGTAGTGATG-

GGAGTG-3′) and #2G reverse (5′-ACTGAATAGATGGGACAGGGGG-3′), which amplify a 322-bp product.

RT–PCR analysis

Total RNA was isolated from kidney and lung tissues of adult homozygous *Notch4^{d1}* mutants and wild-type littermates. For the RT–PCR analysis, cDNA was synthesized with oligo(dT) primer, and *Notch4* cDNAs were detected by PCR. Two RT–PCR primer sets were used. One primer set was chosen to flank the region deleted in the *Notch4^{d1}* mutant allele: #1C forward (5′-ACCCTGCTCCAATGGAGGAT-3′, encoding amino acids 1049–1055) and #1C reverse (5′-AGAACCTCCGATTCACACTCC-3′, encoding amino acids 1564–1570), generating an amplification product of 1.3 kb (Smith et al. 1995).

Additional primer sets were chosen to amplify the 3′ end of any potential alternatively spliced transcripts from the *Notch4^{d1}* mutant allele: #2C forward (5′-TGGAGCGGATAAAGATGCCC-3′, encoding amino acids 1752–1758) and #2C reverse (5′-AGCGTTAGCAGGTCCCAGTGAC-3′), encoding amino acids 1806–1813), generating an amplification product of 185 base pairs; #3C forward (5′-CCAAGAGATTCCCTTAAACTCGG-3′, encoding amino acids 1952–1958) and #3C reverse (5′-CCAGAGTTTAGGGATTCTCG-3′, located in the 3′ untranslated region), generating an amplification product of 260 bp. Because both primer #3C forward and #3C reverse are located in exon 29, the large 3′ terminal exon of the *Notch4* gene (Gallahan and Callahan 1997), total RNA was treated with ribonuclease-free DNase prior to synthesis of cDNA with oligo(dT) primer.

Histology, in situ hybridization, and immunohistochemistry

Organs from adult mice were fixed by immersion in Fekete's acid–alcohol–formalin and were processed as described (Relyea et al. 1999). Embryos were dissected and DNA was prepared from the yolk sacs or tails for genotyping by PCR or by Southern blot analysis. Embryos for whole-mount in situ hybridization were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) overnight at 4°C. Embryos for immunohistochemistry were fixed in 4% paraformaldehyde in PBS and stained with a monoclonal anti-PECAM-1 antibody (clone MEC 13.3; Pharmingen).

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