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Defects in development of the kidney, heart and eye vasculature in mice homozygous for a hypomorphic *Notch2* mutation

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SUMMARY

The Notch gene family encodes large transmembrane receptors that are components of an evolutionarily conserved intercellular signaling mechanism. To assess the in vivo role of the *Notch2* gene, we constructed a targeted mutation, *Notch2^{del1}*. Unexpectedly, we found that alternative splicing of the *Notch2^{del1}* mutant allele leads to the production of two different in-frame transcripts that delete either one or two EGF repeats of the Notch2 protein, suggesting that this allele is a hypomorphic *Notch2* mutation. Mice homozygous for the *Notch2^{del1}* mutation died perinatally from defects in glomerular development in the kidney. *Notch2^{del1}/Notch2^{del1}* mutant kidneys were hypoplastic and mutant glomeruli lacked a normal capillary tuft. The Notch ligand encoded by the *Jag1* gene was expressed in developing glomeruli in cells adjacent to *Notch2*-expressing cells. We show that mice heterozygous for both the *Notch2^{del1}* and *Jag1^{dDSL}* mutations exhibit a glomerular defect similar to, but less severe than, that of *Notch2^{del1}/Notch2^{del1}* homozygotes. The co-localization and genetic interaction of *Jag1* and *Notch2* imply that this ligand and receptor physically interact, forming part of the signal transduction pathway required for glomerular differentiation and patterning. *Notch2^{del1}/Notch2^{del1}* homozygotes also display myocardial hypoplasia, edema and hyperplasia of cells associated with the hyaloid vasculature of the eye. These data identify novel developmental roles for *Notch2* in kidney, heart and eye development.

Key words: Notch2, Glomerulogenesis, Kidney, Hypomorphic mutation, Persistent hyperplastic primary vitreous, Mouse

INTRODUCTION

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, sea urchins, nematodes and mammals. Genes of the Notch family encode large transmembrane receptors that interact with membrane-bound ligands encoded by Delta, Serrate and Jagged family genes. The signal induced by ligand binding is transmitted intracellularly by a process involving proteolysis of the receptor and nuclear translocation of the intracellular domain of the Notch protein (reviewed by Artavanis-Tsakonas et al., 1999; Weinmaster, 2000).

Proteins of the Notch family share several repeated peptide motifs. The extracellular domain of the protein contains a large number (>30) of tandemly repeated copies of an epidermal growth factor (EGF)-like motif. The extracellular domain of these proteins also contains three repeats of another motif, unique to this protein family, termed the Notch/lin-12 repeat. The intracellular domain of Notch family proteins contains six copies of another conserved motif termed the cdc10/ankyrin repeat, as well as nuclear localization signals and domains required for interaction with a number of cytoplasmic and nuclear proteins.

Four Notch genes have been identified in mammals (*Notch1*, *Notch2*, *Notch3* and *Notch4*). Analysis of both inherited human diseases and mutant mice has shown that some of these genes are essential for embryonic development and adult homeostasis (Swiatek et al., 1994; Conlon et al., 1995; Joutel et al., 1996; Hamada et al., 1999; Krebs et al., 2000). Similarly, genes encoding ligands for the Notch family of receptors are also

essential in both humans and mice (Hrabé De Angelis et al., 1997; Li et al., 1997; Oda et al., 1997; Sidow et al., 1997; Jiang et al., 1998; Kusumi et al., 1998; Xue et al., 1999; Bulman et al., 2000). The *Notch2* gene was the second of the mammalian Notch family receptors cloned (Weinmaster et al., 1992). Hamada et al. have constructed and analyzed a *Notch2* mutant allele in which the *Escherichia coli* β-galactosidase-coding region replaces all but one of the ankyrin repeats in the intracellular domain of the mouse Notch2 protein (Hamada et al., 1999). Embryos homozygous for this *Notch2* mutant allele die before 11.5 days of gestation.

In this study, we have constructed and analyzed another targeted mutation of the *Notch2* gene. Unexpectedly, alternative splicing of our *Notch2* mutant allele leads to the production of two different in-frame transcripts that delete either one or two EGF repeats of the Notch2 protein. Mice homozygous for this *Notch2* mutant allele die as neonates, owing to defects in kidney development, and also exhibit defects in development of the heart and eye vasculature. This probable *Notch2* hypomorphic allele has therefore permitted us to identify and analyze additional developmental decisions that are dependent on Notch2 function.

Our work indicates that Notch2 function is required for development of the kidney. The vertebrate kidney is formed by a series of inductive interactions between epithelial and mesenchymal tissues (Saxén, 1987). In mice, formation of the adult kidney, or metanephros, begins at embryonic day (E) 11 and is driven by reciprocal induction between the ureteric bud epithelium and the metanephric mesenchyme (for recent reviews, see Davies and Bard, 1998; Clark and Bertram, 1999; Kuure et al., 2000; Schedl and Hastie, 2000). The ureteric bud branches from the Wolffian duct and grows into the metanephric mesenchyme. Branching and growth of the ureteric bud is induced by signals from these mesenchymal cells. At the tips of the ureteric bud branches, the mesenchyme is induced by the epithelial cells of the ureteric bud to form aggregates. These aggregates then undergo a mesenchymal to epithelial transition to form an epithelial vesicle. The vesicles go through a series of well-characterized morphological stages, which have been termed the comma-shape stage, the S-shape stage, the capillary loop (or cup-shape) stage and the maturing glomerulus stage. At the S-shape stage, the region of the S-shape body destined to form the distal tubule fuses with the terminal tips of the ureteric bud derivatives, which will form the collecting ducts and ureters of the kidney; vascularization of the glomerulus also commences with the migration of endothelial cells into the glomerular cleft. These endothelial cells form a capillary loop, which branches to produce the complex capillary tuft of the mature glomerulus. Mesangial cells also populate the inner part of the glomerulus and maintain the looped configuration of the capillaries of the glomerular capillary tuft. Formation of the capillary tuft is accompanied by differentiation of glomerular epithelial cells into podocytes.

We report here that *Notch2* function is required for glomerulogenesis in the kidney, as well as for development of heart and eye vasculature. We also demonstrate by gene expression analyses and genetic interaction studies that the Notch ligand encoded by the *Jag1* gene is required for *Notch2* signaling in the developing glomerulus.

MATERIALS AND METHODS

Targeting vector construction

A *Notch2* genomic clone was isolated from a 129/Sv mouse genomic phage library. The genomic organization of a portion of the mouse *Notch2* locus was determined by restriction enzyme mapping, blot hybridization and nucleotide sequencing. To construct the targeting vector, a 2.8 kb *Sca*I-*Xba*I fragment of *Notch2* was subcloned upstream of a PGK-neo expression cassette (Soriano et al., 1991), and a 3.5 kb *Hind*III-*Xba*I *Notch2* fragment was subcloned downstream of the PGK-neo cassette. This resulted in the deletion of a 0.4 kb genomic fragment, deleting codons for 22 amino acids in EGF repeat 14 (amino acids 538-560 of the Notch2 protein), the splice donor site, and 0.3 kb of intron sequence. An HSV-tk cassette (Mansour et al., 1988) was introduced for negative selection. We refer to this allele as *Notch2*^{del1}.

Electroporation, selection and screening of ES cells and mouse genotyping

CJ7 embryonic stem (ES) cells were electroporated with 25 μg of linearized targeting vector, placed under positive-negative selection in G418 and FIAU, and screened for homologous recombination by Southern blot hybridization as previously described (Swiatek and Gridley, 1993). For Southern blot analysis, 10 μg of DNA was digested with *Hin*dIII, fractionated in 0.8% agarose gels, transferred to Zeta-Probe GT membranes (BioRad) and hybridized with a 1.0 kb *Cla*I-*Xba*I fragment. ES cells containing the expected recombination event were injected into blastocysts from C57BL/6J mice. Male chimeras were bred with C57BL/6J females, and germline transmission was obtained for three independently targeted ES cell clones. Animals were genotyped by Southern blot analysis or by PCR. PCR primers for the wild-type *Notch2* allele were sp3 (5′-CCAG-TGTGCCACAGGTAAGTG-3′), located in the deleted fragment in the *Notch2*^{del1} mutant allele, and sp4 (5′-TCTCCATATTGAT-GAGCCATGC-3′), located in the genomic region 3′ from the deletion in the mutant allele; the primers for the *Notch2*^{del1} allele were sp4 and sp6 (5′-TTCCTGACTAGGGGAGGAGTAG-3′), located in the neo cassette.

RT-PCR analysis

Total RNA was isolated from whole embryos (E10.5). A *Notch2*-specific primer, N2-3 (bases 2220-2199 of Notch2 cDNA, GenBank Accession Number D32210) and AMV reverse transcriptase (Promega) were used for the reverse transcription reaction. Primers N2-10 (bases 1337-1357) and N2-2 (bases 2156-2136) were used to generate the PCR product. The PCR products from the wild-type and mutant RNAs were then sequenced to determine the nucleotides present in the alternatively spliced *Notch2*^{del1} mRNA. Genomic DNA was sequenced in the deletion region to identify splice donor and acceptor sites.

Northern blot

Poly(A+) RNA was isolated from E14.5 embryos, and 2.5 μg of RNA was separated on a 1% formaldehyde gel and transferred to a Zeta-Probe GT membrane (Bio-Rad). An 800 bp fragment from the 3′ end of *Notch2* cDNA was used for generation of a ^{32}P-dCTP labeled probe. Hybridization was at 42°C in a buffer containing 50% formamide, 2× SSC, 5× Denhardt's, 100 μg/ml denatured salmon sperm DNA and 1% SDS.

Histological analysis

Embryos and organs for histological analysis were fixed in Bouin's solution. Fixed embryos were dehydrated through graded alcohols, embedded in paraffin wax or methacrylate, sectioned at 7 μm (paraffin wax) or 2 μm (methacrylate), and stained with Hematoxylin and Eosin or Toluidine Blue.

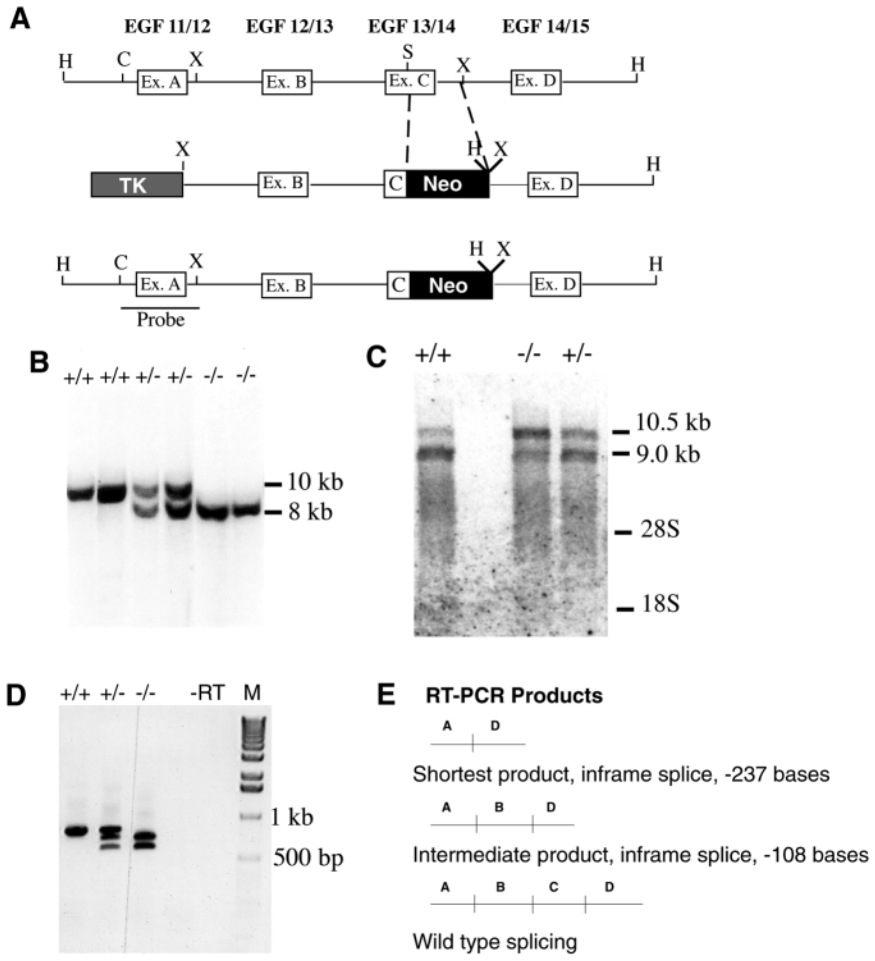

Fig. 1. Targeted disruption of the *Notch2* gene. (A) The upper line shows the genomic organization of a region of the *Notch2* gene. Exons are indicated by boxes. The middle line represents the structure of the targeting vector. A 0.4 kb deletion was created that disrupts and removes most of the exon encoding EGF repeat 14. Shown at the bottom is the predicted structure of the *Notch2* locus following homologous recombination of the targeting vector. The probe used for Southern blot analysis is indicated. Restriction enzymes: C, *Cla*I; H, *Hin*dIII; S, *Sca*I; X, *Xba*I. (B) DNA isolated from embryos of the intercross of *Notch2*^{del1}/+ heterozygous mice was digested with *Hin*dIII, blotted and hybridized with the indicated probe. Genotypes of progeny are indicated at the tops of the lanes. (C) Northern blot of mRNA isolated from E14.5 embryos. Two transcripts of approximately 9.0 and 10.5 kb were observed for each genotype. (D) RT-PCR analysis of RNA isolated from E10.5 embryos. A sample of *Notch2*^{del1}/*Notch2*^{del1} RNA subjected to RT-PCR without reverse transcriptase is indicated by –RT. Two alternatively spliced transcripts were present in both heterozygous and mutant mRNA. (E) Alternative splicing of the *Notch2*^{del1} mutant allele. RT-PCR products were sequenced and compared with the genomic DNA sequence. Nucleotide sequence analysis revealed that the alternative transcripts are the products of splicing between native splice donor and acceptor sites. The intermediate-sized RT-PCR product removes exon C, which results in an in-frame 108 base pair deletion, while the smallest RT-PCR product removes exons B and C, which results in an in-frame 237 base pair deletion.

Non-radioactive in situ hybridization

Embryonic kidneys were dissected and fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) overnight at 4ºC, washed with PBS, then equilibrated in PBS containing 30% glycerol. Samples were embedded in OCT, sectioned on a cryostat at 10-14 μm and mounted on Superfrost Plus (Fisher) slides. Tissue sections were permeabilized with 1 μg/ml proteinase K and 0.5% Triton X-100 before hybridization. 300 ng/ml of digoxigenin-labeled antisense RNA was hybridized to slides at 60 ºC in a buffer containing 5% dextran sulfate, 50% formamide, 2× SSC, 5× Denhardt's, 75 μg/ml yeast tRNA and 0.5% SDS. After washing off excess probe, the slides were blocked with 10% goat serum in 50 mM Tris pH 8.0, 200 mM NaCl and 0.05% Tween 20 (TTBS). Alkaline phosphatase-conjugated anti-digoxigenin (Roche) was diluted 1:500 in 2% goat serum/TTBS, and used to detect the digoxigenin-labeled antisense RNA. After washing, developing solution containing NBT and BCIP was added and the slides were allowed to develop 6-18 hours. Probes used for RNA in situ were Jag1 (Mitsiadis et al., 1997); Dll1 (Bettenhausen et al., 1995); Dll3 (Dunwoodie et al., 1997); Notch2, bases 6437-7289 of D32210; Bmp7 (Dudley et al., 1995);Vegf, nucleotides 517-864 of IMAGE clone M95200; Wt1, nucleotides 2449-3042 of IMAGE clone M55512; Pdgfrb (Lindahl et al., 1998); Wnt4 (Stark et al., 1994); Pax2 (Dressler et al., 1990).

Immunohistochemistry

Kidneys from embryos at E18.5 were dissected, fixed, and cryosectioned as for in situ hybridization. The sections were then permeabilized with 0.5% Triton X-100 and endogenous peroxidase activity was quenched in 3% H_2O_2/10% methanol. Blocking was carried out with 10% goat serum/TTBS. Antibody to mouse platelet endothelial cell-adhesion molecule 1 (PECAM, Pharmingen) was used in 2% goat serum/TTBS at a dilution of 1:100. A peroxidase-conjugated donkey anti-rat IgG secondary antibody (Jackson ImmunoResearch), at a 1:100 dilution in TTBS, was used to detect PECAM and to develop the DiaminoBenzidine (DAB, Sigma) substrate with 0.2% NiCl added. Polyclonal antibody to desmin (Sigma) was used at 1:100 dilution in 2% goat serum/TTBS and was detected using a peroxidase-conjugated donkey anti-rabbit IgG secondary antibody, at a 1:100 dilution in TTBS (Jackson ImmunoResearch).

Terminal transferase-mediated dUTP nick end labeling (TUNEL) assay

Kidneys from E15.5 embryos were dissected, fixed in 4% paraformaldehyde, embedded in paraffin and sectioned. Cell death was detected using the In Situ Cell Death Detection Kit (Boehringer Mannheim), according to the manufacturer's instructions. The average number of TUNEL-positive cells was determined by making 25 measurements on sections of both wild-type and homozygous mutant kidneys. The field size used for counting the TUNEL-positive cells was 0.04 mm^2, and measurements were taken for both medullary and cortical regions of the kidneys. Four individual wild-type and four *Notch2*^{del1}/*Notch2*^{del1} mutant kidneys at E15.5 were used for this analysis.

Bromodeoxyuridine (BrdU) labeling of proliferating cells

Pregnant mice were injected intraperitoneally with 3 mg of BrdU 2-3 hours before their embryos were removed for processing. Embryo

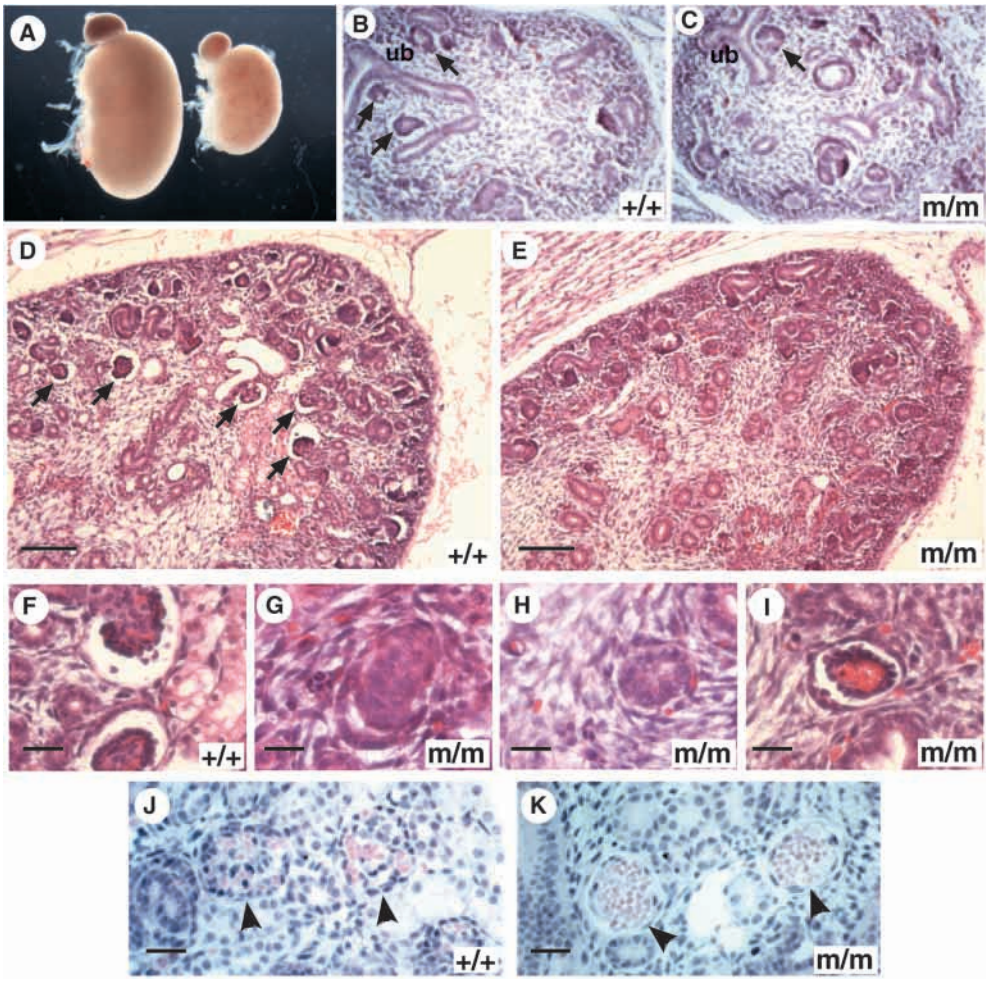

Fig. 2. Defects in glomerulogenesis in *Notch2*^{del1}/*Notch2*^{del1} homozygotes. (A) Kidney and adrenal gland isolated from *Notch2*^{del1}/*Notch2*^{del1} homozygous mutant (right) and wild-type littermate control (left) at P0. Mutant kidneys are hypoplastic and exhibit vascular lesions near the cortical surface. Mutant adrenal glands appear unaffected. (B-K) Histological analysis of *Notch2*^{del1}/*Notch2*^{del1} homozygous mutant (C,E,G,H,I,K) and wild-type littermate (B,D,F,J) kidneys. (B,C) Kidneys at E13.5. Both wild type (B) and *Notch2*^{del1}/*Notch2*^{del1} mutant (C) kidneys exhibit ureteric bud (ub) branching and formation of metanephric vesicles (arrows). (D-I) Kidneys at E16.5. (D,E) Numerous morphologically normal glomeruli (arrows) are present in the wild-type kidney (D), while in the *Notch2*^{del1}/*Notch2*^{del1} kidney (E) no mature glomeruli are present. Ureteric bud branching, mesenchymal condensations and collecting ducts are present in both the mutant and wild type kidneys. (F) Wild-type glomerulus. (G-I) Examples of *Notch2*^{del1}/*Notch2*^{del1} mutant glomeruli. (G,H) These examples represent the end stage of the majority of glomeruli in *Notch2*^{del1}/*Notch2*^{del1} mutant kidneys. The wild-type glomerulus (F) has entered the capillary loop stage and has become vascularized, but the mutant glomeruli have not and are instead made up of a disorganized clump of cells. (I) In a subset of abnormal glomeruli in the *Notch2*^{del1}/*Notch2*^{del1} mutants, the glomerular capillary tuft was replaced by a capillary aneurysm-like structure that filled the region of Bowman's capsule with red blood cells. (J,K) Kidneys at P0. Methacrylate sections of wild type (J) and *Notch2*^{del1}/*Notch2*^{del1} mutant (K) kidneys. Glomeruli (arrowheads) of wild-type kidneys have morphologically normal capillary tufts, while some glomeruli of mutant kidneys exhibit capillary aneurysms. Scale bars: 100 μm in D,E; 25 μm in F-K.

kidneys were fixed in 4% paraformaldehyde and processed into paraffin blocks as for histological analysis. 7 μm sections were then rehydrated and peroxidase activity was quenched in 3% H_2O_2/10% methanol. Tissue was permeabilized with 20 μg/ml proteinase K and the DNA was denatured using 2N HCl. An anti-BrdU monoclonal antibody (Pharmingen) was used at 1:100 dilution in 2% goat serum/TTBS. It was detected with a peroxidase-conjugated donkey anti-mouse IgG secondary antibody at a 1:100 dilution in TTBS (Jackson ImmunoResearch). Diaminobenzidine (DAB, Sigma) substrate with 0.2% NiCl added was used for detection. Quantitation was performed as described above for detection of TUNEL-positive cells.

Generation and analysis of double heterozygous mice

Official nomenclature and references for the mutant alleles used in these studies are *Jag1*^{dDSL} (Xue et al., 1999): *Jag1*^{tm1Grid}; *Notch2*^{del1} (this report): *Notch2*^{tm1Grid}; and *Dll1*^{tm1Go} (Hrabé de Angelis et al., 1997). Heterozygous *Notch2*^{del1} mice were mated with either *Jag1*^{dDSL} or *Dll1*^{tm1Go} heterozygous mice to generate double heterozygotes. Kidneys were dissected at postnatal day 6 and were processed for histological analysis as described above. Double heterozygous, single heterozygous and wild-type kidneys were analyzed.

RESULTS

Targeted disruption of the *Notch2* gene

To analyze the in vivo role of the *Notch2* gene, a targeting vector was constructed that deleted 0.4 kb of genomic sequence of the *Notch2* gene (Fig. 1A). The deleted fragment interrupts the exon encoding EGF repeat 14 in the extracellular domain of the protein, deleting codons for 22 amino acids in EGF14, the splice donor site, and 0.3 kb of intron. We refer to this allele as *Notch2*^{del1}. The linearized targeting vector was electroporated into ES cells and germline transmission of the *Notch2*^{del1} mutant allele was obtained for three independently targeted clones (Fig. 1B). Mice heterozygous for the *Notch2*^{del1} mutant allele appeared normal and were fertile.

Notch2^{del1} is a hypomorphic allele of the *Notch2* gene

Hamada et al. recently described construction of a targeted mutation of the *Notch2* gene (Hamada et al., 1999). They observed completely penetrant embryonic lethality by E11.5 in

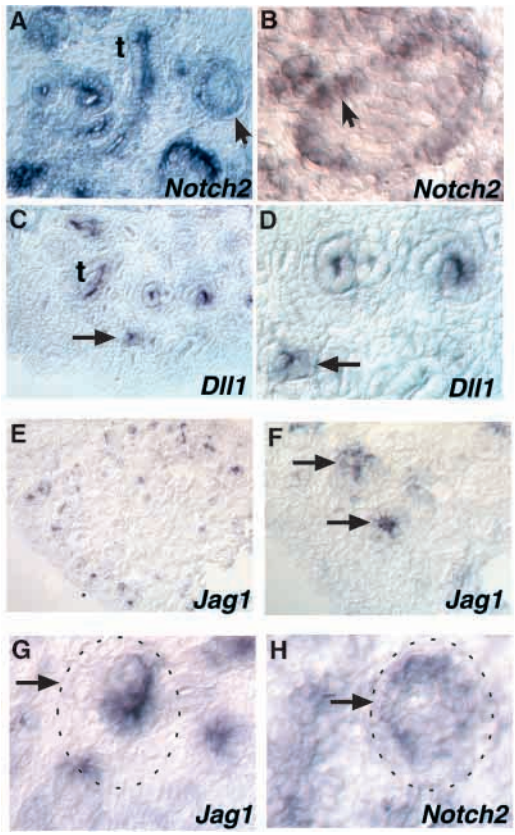

Fig. 3. Expression of the *Notch2* gene and genes encoding Notch family ligands during metanephric kidney development. (A,B) *Notch2* expression is observed in comma-shape bodies and tubules (t). In more mature glomeruli, expression is concentrated around the outer layer of cells where podocytes are differentiating (arrows). (C,D) E16.5 kidney showing *Dll1* expression in comma-shape bodies (arrows) and tubules (t) but not in vascularized glomeruli. (E,F) *Jag1* is expressed in the early glomerular structures (arrows) and in collecting ducts of E16.5 kidneys. (G,H) Comparison of *Jag1* expression (G) and *Notch2* expression (H) at E18.5 in glomeruli which are at the beginning of vascularization at the late S- to capillary loop stage. The boundaries of the glomerulus are indicated by the broken line. Note that *Jag1* expression is centered in the middle of the glomerulus and *Notch2* expression is at the periphery.

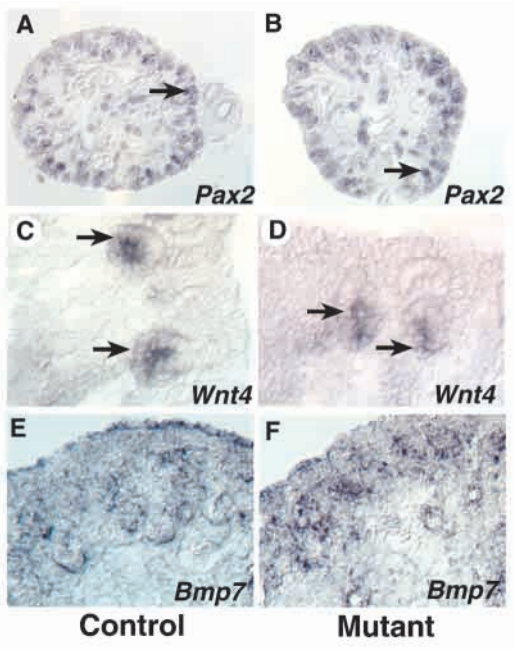

Fig. 4. Analysis of molecular markers for metanephric mesenchyme and pretubular aggregates. (A,B) *Pax2* is expressed in the same pattern in both wild type (A) and *Notch2*^{del1}/*Notch2*^{del1} homozygous mutant (B) kidneys at E16.5. *Pax2* expression is present in the condensed mesenchyme and comma shape bodies. (C,D) The *Wnt4* expression pattern is similar in both wild type (C) and mutant (D). Both express *Wnt4* in the epithelial vesicles and comma shape bodies. (E,F) *Bmp7* is expressed in metanephric mesenchyme and in condensing aggregates in both wild-type (E) and mutant (F) kidneys at E16.5.

mouse embryos homozygous for their targeted allele. However, when we set up timed matings of *Notch2*^{del1} heterozygous mice, we observed survival of *Notch2*^{del1}/*Notch2*^{del1} homozygous mutant embryos to gestational ages later than E11.5 (see below). We therefore examined whether any potentially functional *Notch2* transcripts could be transcribed from the *Notch2*^{del1} mutant allele. Northern blot hybridization using a probe from the 3′ end of the Notch2 cDNA identified similar transcripts present in homozygous *Notch2*^{del1}/*Notch2*^{del1} embryos and in wild-type embryos (Fig. 1C). In addition, in situ hybridization analyses of homozygous *Notch2*^{del1}/*Notch2*^{del1} embryos using the same 3′ Notch2 probe demonstrated the presence of *Notch2* transcripts in the mutant embryos (data not shown). Since transcription termination and polyadenylation signals are present in the neo selection cassette contained in the *Notch2*^{del1}-targeted allele, the presence of *Notch2* transcripts containing sequences 3′ of the targeted region suggested that there may be splicing around the neo cassette in the *Notch2*^{del1} allele. Therefore, we performed a series of RT-PCR analyses to determine the nature of the *Notch2*^{del1} transcript (Fig. 1D). RT-PCR analysis of *Notch2*^{del1} RNA and subsequent DNA sequencing of the RT-PCR products identified two alternative transcripts that are the result of in-frame splicing around both the deleted region of the *Notch2*^{del1} allele and the neo cassette (Fig. 1D,E). The alternatively spliced transcripts should result in the deletion of either one or two EGF repeats from the Notch2 protein. The shorter protein isoform joins the first half of EGF12 to the second half of EGF14, while the longer isoform joins the first half of EGF13 to the second half of EGF14 (Fig. 1E). The levels of these alternative transcripts are similar to the amount of wild-type transcript. These data demonstrate that the *Notch2*^{del1} mutant allele is not a null allele, and most likely is a hypomorphic allele of the *Notch2* gene.

Defects in glomerulogenesis in *Notch2*^{del1}/*Notch2*^{del1} mice

To examine whether mice homozygous for the *Notch2*^{del1} mutation were viable, heterozygous F1 animals were intercrossed. We observed that some of the progeny of intercrosses of *Notch2*^{del1} heterozygous mice died within the first 24 hours of birth. Genotypic analyses revealed that these dying neonatal animals were homozygous for the *Notch2*^{del1} mutant allele. Gross anatomical analysis of the *Notch2*^{del1}/*Notch2*^{del1} neonates revealed that their kidneys were

hypoplastic and exhibited vascular lesions near the cortical surface (Fig. 2A).

Histological analysis revealed defects in glomerulogenesis in the *Notch2*^{del1}/*Notch2*^{del1} kidneys. At E13.5, mutant and control kidneys were similar in size, and both exhibited ureteric bud growth and branching (Fig. 2B,C). In both mutant and control kidneys, at the tips of the bud branches the metanephric mesenchyme condensed and formed epithelial vesicles. However, by E16.5, kidneys from the *Notch2*^{del1}/*Notch2*^{del1} embryos were smaller than those of wild-type and heterozygous littermate controls, and while ureteric bud branches and metanephric vesicles appeared morphologically normal in the mutants, no morphologically normal glomeruli could be observed in the *Notch2*^{del1}/*Notch2*^{del1} homozygous mutant kidneys (Fig. 2D-K).

Two types of abnormal glomeruli were observed in the mutant kidneys. Most commonly, differentiation of the mutant glomeruli appeared to arrest just before the capillary loop stage. In these mutant glomeruli, the capillary tuft of the normal mature glomerulus was absent and the glomerulus appeared to be a disorganized clump of cells (Fig. 2G,H). In the second type of abnormal mutant glomeruli, the glomerular capillary tuft was replaced by a capillary aneurysm-like structure that filled the region of Bowman's capsule with red blood cells (Fig. 2I,K). Similar structures have been observed in mice mutant for the platelet-derived growth factor gene (*Pdgfb*; Levéen et al., 1994; Lindahl et al., 1998) or the PDGFβ receptor gene (*Pdgfrb*; Soriano, 1994). It has been demonstrated for the *Pdgfb* and *Pdgfrb* mutants that these glomerular capillary aneurysms arise due to defects in mesangial cell differentiation.

Expression of *Notch2* and of genes encoding Notch ligands during kidney development

The *Notch2* gene is expressed widely in mouse embryos and adults (Weinmaster et al., 1992; Lardelli and Lendahl, 1993; Swiatek et al., 1994; Williams et al., 1995; Lindsell et al., 1996). To study the *Notch2*^{del1}/*Notch2*^{del1} kidney phenotype in more detail, the expression patterns of *Notch2* and the genes encoding its potential ligands were assayed by RNA in situ hybridization. Since metanephric kidney development does not proceed synchronously, examination of kidneys during late embryogenesis permits examination of multiple developmental stages (Saxén, 1987). *Notch2* is expressed in both the ureteric bud-derived collecting duct system, and in condensations and vesicular structures (such as metanephric vesicles and comma- and S-shape bodies) derived from the metanephric mesenchyme (Fig. 3A). In maturing glomeruli *Notch2* is expressed primarily at the periphery, where glomerular epithelial cells are differentiating into podocytes (Fig. 3A,B,H).

Several genes encoding Notch ligands were also expressed in the developing kidney. The *Dll1* gene was expressed in tubules and in comma- and S-shape bodies at E16.5 (Fig. 3C,D; see also Beckers et al., 1999). The *Jag1* gene was expressed in the mesenchymal condensations and vesicular structures of the developing glomeruli (Fig. 3E-G). *Jag1* expression was primarily observed in the inner region of the developing glomerulus, in a position consistent with expression in differentiating mesangial and/or endothelial cells (Fig. 3G). The *Jag1* and *Notch2* genes appeared to be expressed in adjacent cells in the developing glomerulus (Fig. 3G,H). *Jag1* expression was also detected in the collecting ducts. The *Dll4* gene, which encodes a recently identified Notch ligand, is expressed in endothelial cells of the glomerulus (Shutter et al., 2000). The *Jag2* gene was expressed at low levels (much lower than the expression levels of *Dll1* and *Jag1*) throughout the kidney at E16.5, and we did not detect expression of the *Dll3* gene in the kidney (data not shown).

Notch2 is not required for mesenchymal condensation and epithelialization, but is essential for glomerular differentiation and patterning

To define the *Notch2*^{del1}/*Notch2*^{del1} kidney phenotype in further detail, and to determine at which step in development the mutants deviate from the normal pathway, we analyzed expression of a panel of molecular markers. Both the *Pax2* (Dressler et al., 1990) and *Wnt4* (Stark et al., 1994) genes are expressed in the condensing mesenchyme and pretubular aggregates of the developing metanephric kidney. By in situ analysis, both *Pax2* (Fig. 4A,B) and *Wnt4* (Fig. 4C,D) were expressed normally in these structures in *Notch2*^{del1}/*Notch2*^{del1} kidneys. The *Bmp7* gene is expressed early during nephrogenesis in the ureteric bud, and expression is subsequently observed in the metanephric mesenchyme and in the pretubular aggregates (Dudley et al., 1995; Luo et al., 1995). In *Notch2*^{del1}/*Notch2*^{del1} kidneys, *Bmp7* expression in the metanephric mesenchyme and the condensing aggregates was normal (Fig. 4E,F). Thus, the early stages of metanephric kidney development, including ureteric bud growth and branching and mesenchymal condensation and vesicle formation, occurred relatively normally in the *Notch2*^{del1}/*Notch2*^{del1} mutant kidneys. However, there appeared to be a quantitative difference in these processes in the *Notch2*^{del1}/*Notch2*^{del1} mutants, in that far fewer condensations and vesicles formed in the mutants than in wild-type and heterozygous littermate controls.

We assessed differentiation and patterning of the glomerulus using molecular markers for the three primary cell types of the mature glomerulus: endothelial cells, mesangial cells and podocytes. Endothelial cell differentiation was assayed using a monoclonal antibody to PECAM1 (platelet-endothelial cell adhesion molecule 1) (Baldwin et al., 1994). This analysis revealed that few endothelial cells were present in the abnormal mutant glomeruli (Fig. 5A,B). However, some endothelial cells could be observed migrating into the cleft of the comma- and S-shape bodies in the mutants (Fig. 5B). The migration of endothelial cells into the cleft of the comma- and S-shape bodies is consistent with the development of capillary aneurysm-like structures in a subset of mutant glomeruli (Fig. 2I,K).

Mesangial cell differentiation was assayed using an antibody to desmin (Lindahl et al., 1998; Miner and Li, 2000) and an in situ hybridization probe for *Pdgfrb* (Seifert et al., 1998; Lindahl et al., 1998). This analysis showed there were no glomeruli that contained desmin-positive cells in the mutant kidneys (Fig. 5C,D). *Pdgfrb* expression was also not observed in the mutant glomeruli (Fig. 5F). In the wild-type and heterozygous control kidneys, *Pdgfrb* expression was present in the center of cup-staged glomeruli, consistent with its expression in mesangial cells (Fig. 5E). These data reveal the absence of mesangial cells in the abnormal glomeruli of the *Notch2*^{del1}/*Notch2*^{del1} mutants.

Podocyte differentiation was assayed using in situ hybridization probes for the vascular endothelial growth factor

A (*Vegf*) gene (Breier et al., 1992; Kitamoto et al., 1997; Tufró, 2000) and the Wilms' Tumour (*Wt1*) gene (Kreidberg et al., 1993). In wild-type and heterozygous littermate control kidneys, both genes were expressed in the cup-shaped semicircle of glomerular epithelial cells that are differentiating into podocytes (Fig. 5G,I). In *Notch2*^{del1}/*Notch2*^{del1} homozygous mutant kidneys, *Vegf*- and *Wt1*-positive cells were present in the abnormal mutant glomeruli, but the cells expressing these markers were clumped together in the center of the glomerulus, and did not form the cup-shaped epithelial layer observed in the controls (Fig. 5H,J).

Analysis of cell death and proliferation in *Notch2*^{del1}/*Notch2*^{del1} kidneys

Since by the later stages of embryogenesis the kidneys of *Notch2*^{del1}/*Notch2*^{del1} mutant embryos were significantly smaller than kidneys of control littermate embryos, we analyzed cell proliferation and cell death in developing kidneys. Cell proliferation was analyzed by BrdU labeling of kidneys isolated at E15.5. Cell proliferation was approximately equal in both wild-type and mutant kidneys in the highly proliferative cortex of the kidney (Fig. 6A,B). There were 51.1±5.8 (mean±s.d.) BrdU-positive cells/0.04 mm^2 in wild-type sections compared with 47.2±8.1 BrdU-positive cells/0.04 mm^2 in mutant sections in this region. However, while proliferating cells were observed inside the morphologically normal glomeruli of wild-type and heterozygous littermate controls, no proliferating cells were observed inside the abnormal glomeruli that were present in the *Notch2*^{del1}/*Notch2*^{del1} mutant kidneys.

Apoptotic cell death was assessed by TUNEL assay. We detected an increased amount of cell death in *Notch2*^{del1}/*Notch2*^{del1} mutant kidneys (Fig. 6C,D). There were 9.1±3.4 TUNEL-positive cells/0.04 mm^2 in wild-type sections compared with 17.0±4.8 TUNEL-positive cells/0.04 mm^2 in mutant sections. Higher numbers of TUNEL-positive cells were detected at the periphery of the mutant kidneys, immediately beneath the renal capsule. These results suggest that increased cell death may contribute to the growth retardation observed in the *Notch2*^{del1}/*Notch2*^{del1} mutant kidneys.

Notch2^{del1}/*Jag1*^{dDSL} double heterozygous mice exhibit glomerular defects

Phenotypes sensitive to gene dose are a common feature of Notch pathway mutants in both vertebrates and invertebrates (Artavanis-Tsakonas et al., 1991; Li et al., 1997; Oda et al., 1997; Xue et al., 1999; Krebs et al., 2000). Since the *Notch2* and *Jag1* genes were expressed in adjacent regions of the developing glomerulus, we examined kidneys from mice heterozygous for both the *Notch2*^{del1} and *Jag1*^{dDSL} (Xue et al., 1999) mutant alleles. These double heterozygous mice exhibited kidney defects (Fig. 7). The kidneys of the double heterozygotes (n=6) were about half the size of kidneys from the littermate controls (which consisted of wild-type mice, and *Notch2*^{del1} and *Jag1*^{dDSL} single heterozygotes) (Fig. 7A). The number of glomeruli was greatly reduced in the double heterozygotes, and about a quarter of the glomeruli present lacked glomerular capillary tufts and exhibited the capillary aneuryisms similar to those observed in *Notch2*^{del1}/*Notch2*^{del1} homozygous mutant kidneys (compare Fig. 7C with Fig. 2I,K). These results demonstrate that *Notch2*^{del1}/+ *Jag1*^{dDSL}/+ double heterozygotes have a glomerular defect similar to, but less severe than, those of the *Notch2*^{del1}/*Notch2*^{del1} homozygous mutants.

Since the *Dll1* gene is expressed in the developing glomerulus, we also generated mice heterozygous for both the *Notch2*^{del1} and *Dll1*^{tm1Go} (Hrabé de Angelis et al., 1997) mutant alleles. However, we observed no kidney defects in *Notch2*^{del1}/*Dll1*^{tm1Go} double heterozygous mice (n=3).

Mice homozygous for the *Notch2*^{del1} mutation exhibit defects in development of the hyaloid vasculature of the eye

In addition to hypoplastic kidneys, other defects were observed in *Notch2*^{del1}/*Notch2*^{del1} mutant embryos and neonates. Gross anatomical analysis of *Notch2*^{del1}/*Notch2*^{del1} neonates revealed that all mutants displayed bilateral microphthalmia (Fig. 8A). Histological analysis of the eyes of *Notch2*^{del1}/*Notch2*^{del1} neonates typically revealed an aberrant bulbous structure at the terminus of the hyaloid artery (Fig. 8B). Numerous small capillaries were observed emanating from this structure (Fig. 8C). At earlier embryonic stages, a pronounced retrolenticular hyperplasia was evident in sections of eyes from the *Notch2*^{del1}/*Notch2*^{del1} embryos (Fig. 8D,E). Eyes of the *Notch2*^{del1}/*Notch2*^{del1} embryos exhibited a pronounced asymmetry (Fig. 8F,G), which was apparently caused by the retrolenticular hyperplasia.

Heart defects in Notch2^{del1}/Notch2^{del1} embryos

Quantitative analysis of intercross progeny from *Notch2*^{del1} heterozygous mice revealed that, at gestational stages later than E16.5, only 12% of the progeny were *Notch2*^{del1}/*Notch2*^{del1} homozygous mutants (Table 1). We therefore isolated mutant embryos at earlier stages in order to determine the cause of embryonic lethality prior to E16.5. Through E10.5 we detected no obvious mutant phenotype in *Notch2*^{del1}/*Notch2*^{del1} embryos. However, at E11.5, approximately 40% of the homozygous mutant embryos exhibited growth retardation, pericardial effusion and widespread hemorrhaging (Fig. 9A and Table 1). Mutant embryos that survived this stage displayed a variety of vascular defects, including myocardial hypoplasia, hemorrhaging, and edema (Fig. 9B-E, and Table 1). Beginning at E13.5, edema and hemorrhaging vessels near the surface of the skin were observed in approximately 50% of *Notch2*^{del1}/*Notch2*^{del1} homozygous mutant embryos (Fig. 9C, and data not shown). Myocardial hypoplasia and reduced myocardial trabeculation were observed in all mutant embryos E12.5 and older (Fig. 9D,E). We analyzed *Notch2* expression in the heart by in situ hybridization to determine whether the observed phenotype, particularly the myocardial hypoplasia, correlated with a domain of *Notch2* expression. We detected no *Notch2* message in the heart at either E11.5 or E14.5. However, we did detect *Notch2* expression in the outflow tract of the heart at these stages (data not shown).

DISCUSSION

The *Notch2*^{del1} allele causes a probable hypomorphic mutation due to alternative splicing

The *Notch2*^{del1} allele described here is a probable hypomorphic allele, owing to unexpected in-frame alternative splicing. This results in the production of novel *Notch2* mRNAs that can

encode Notch2 proteins that may be partially functional. The novel mRNAs have excised the coding sequence for either one or two of the 36 EGF repeats present in the Notch2 protein. The shorter protein isoform joins the first half of EGF12 to the second half of EGF14, while the longer isoform joins the first half of EGF13 to the second half of EGF14. These excised EGF repeats may hinder Notch2 ligand-binding ability owing to their proximity to the EGF11 and EGF12 repeats, which are essential for ligand binding by the *Drosophila* Notch and

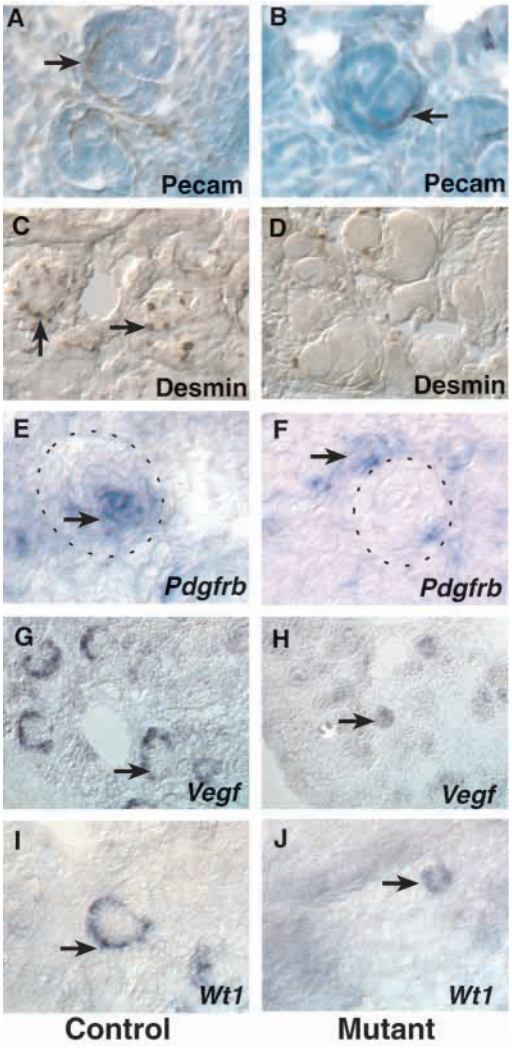

Fig. 5. Analysis of molecular markers for glomerular differentiation and patterning. (A,B) PECAM1 immunohistochemistry of E18.5 wild-type (A) and *Notch2*^{del1}*/Notch2*^{del1} mutant (B) kidneys. Some PECAM1-positive endothelial cells can be seen migrating into the glomerular cleft in both wild-type and mutant S-shape bodies. (C,D) Desmin immunohistochemistry of E18.5 wild-type (C) and mutant (D) kidneys. Wild-type glomeruli contain desmin-expressing mesangial cells (arrows) while no desmin-positive cells were expressed in mutant glomeruli. (E,F) *Pdgfrb* RNA expression in E16.5 kidneys. In the wild-type kidney, as the glomerulus reaches the capillary loop stage, the center becomes populated with *Pdgfrb* expressing mesangial cells (arrows). In the mutant, *Pdgfrb* expressing cells remain on the outside of the glomerulus. Boundaries of the glomerulus are indicated by broken lines. (G,H) *Vegf* RNA expression in E16.5 kidneys. In the wild-type kidney, *Vegf*-expressing podocytes formed a cup-shaped semicircle (arrow in G). In the mutant, kidney, *Vegf*-expressing cells were clustered (arrow in H). (I,J) *Wt1* RNA expression in E16.5 kidneys. The *Wt1* expression pattern resembled the *Vegf* expression pattern in both wild-type (I) and mutant (J) kidneys.

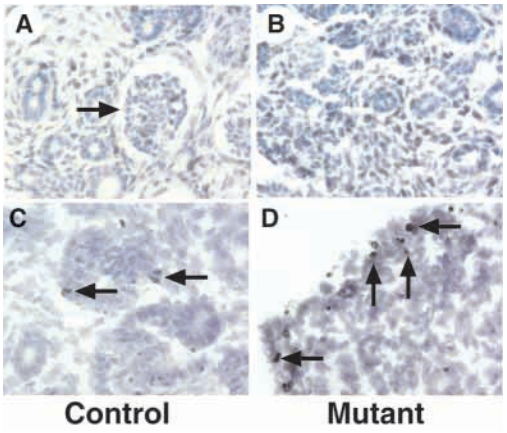

Fig. 6. Cell proliferation and cell death in *Notch2*^{del1}*/Notch2*^{del1} kidneys. (A,B) Cell proliferation was assayed by detecting incorporation of BrdU into newly synthesized DNA. Proliferating cells have brown nuclei. Large numbers of proliferating cells were detected in both wild-type (A) and *Notch2*^{del1}*/Notch2*^{del1} mutant (B) kidneys at E15.5. (C,D) Cell death was assayed by TUNEL assay to identify apoptotic cells in E15.5 kidneys. TUNEL-positive cells have brown nuclei. In wild-type kidneys (C), few TUNEL-positive cells were detected (arrows). In mutant kidneys (D), large numbers of TUNEL-positive cells (arrows) were detected. TUNEL-positive cells were present in higher numbers at the periphery of the kidney, immediately beneath the renal capsule.

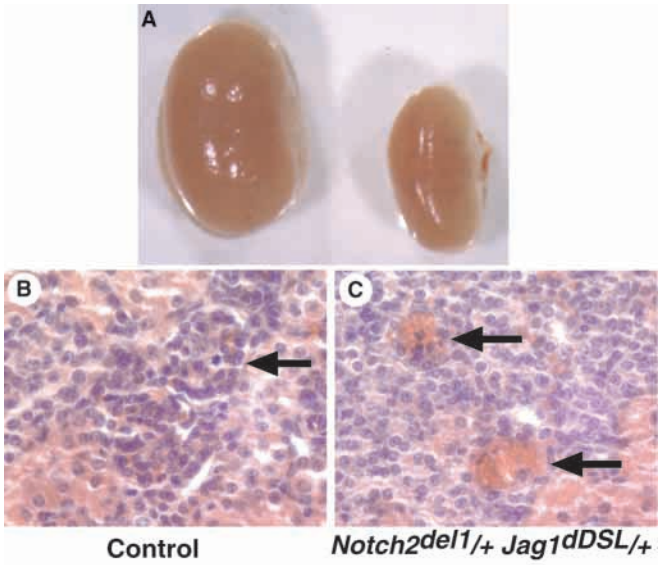

Fig. 7. Kidney defects in *Notch2*^{del1}*/+ Jag1*^{dDSL}*/+* double heterozygotes. Kidneys were isolated at P6 from wild-type and *Notch2*^{del1}*/+ Jag1*^{dDSL}*/+* double heterozygous mice. (A) The double heterozygous kidney (right) is smaller than the wild-type littermate kidney (left). While the control kidney glomeruli exhibit a normal capillary tuft (arrow in B), some double heterozygous glomeruli (arrows in C) exhibit capillary aneurysm-like structures similar to those observed in *Notch2*^{del1}*/Notch2*^{del1} homozygotes.

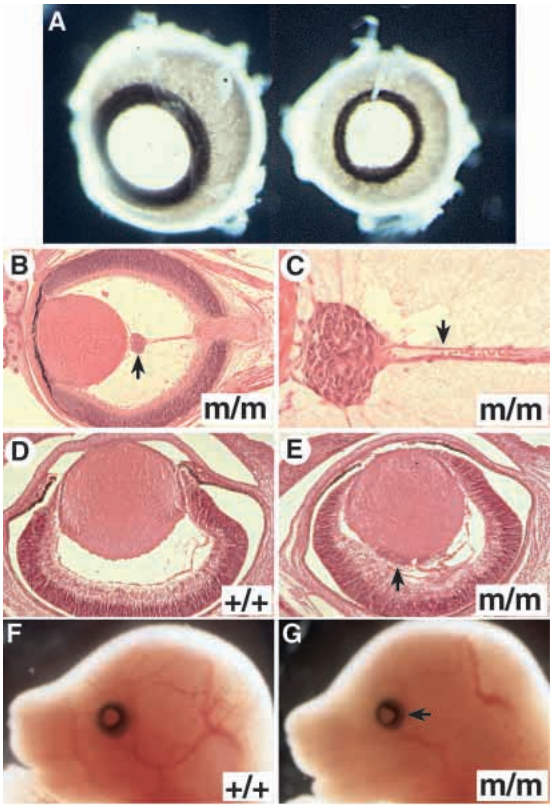

Fig. 8. Microphthalmia and eye vasculature defects in *Notch2*^{del1}/*Notch2*^{del1} homozygotes. (A) Wild-type (left) and *Notch2*^{del1}/*Notch2*^{del1} littermate (right) eyes at P0. The mutant eye is microphthalmic. (B-E) Histological analysis of *Notch2*^{del1}/*Notch2*^{del1} (B,C,E) and wild-type (D) eyes. (B,C) At E18.5, mutant eyes exhibit an aberrant bulbous structure (arrow) just dorsal to the lens. This structure, which has numerous small capillaries emanating from it, is attached to the hyaloid vessel (arrow in C). (D,E) At E16.5, eyes of *Notch2*^{del1}/*Notch2*^{del1} embryos (E) exhibit a pronounced retrolenticular hyperplasia (arrow) compared with wild type (D). (F,G) Wild-type (F) and *Notch2*^{del1}/*Notch2*^{del1} (G) embryos at E17.5. The mutant embryo exhibits an asymmetry in the eye. Note the constriction at the dorsal side of the eye (arrow) of the mutant embryo.

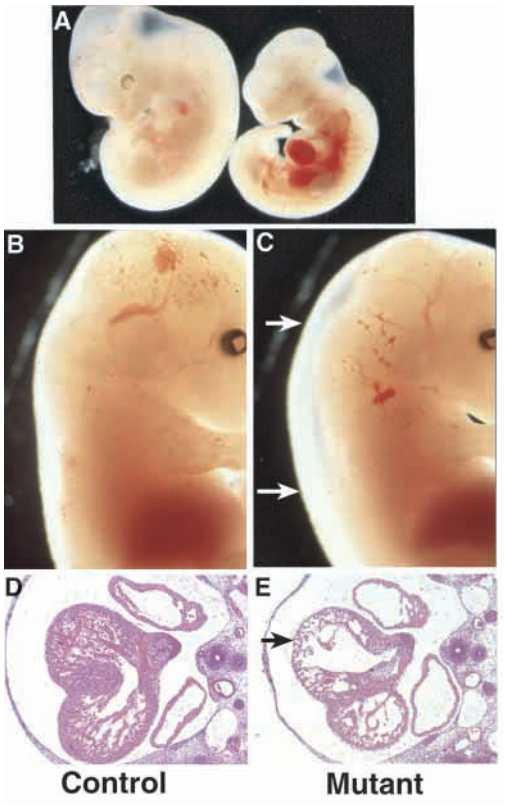

Fig. 9. Heart and vascular defects in *Notch2*^{del1}/*Notch2*^{del1} mutant embryos. (A) E11.5 *Notch2*^{del1}/*Notch2*^{del1} homozygote (right) and wild-type littermate (left). The mutant is growth retarded and exhibits pericardial edema with hemorrhaging. (B,C) E14.5 embryos. The *Notch2*^{del1}/*Notch2*^{del1} mutant (C) exhibits edema along the back of the embryo (arrows), indicating cardiovascular dysfunction. (D,E) Transverse sections of embryos at E12.5. The mutant heart (E) exhibits a thinning of the myocardial wall (arrow) and reduced myocardial trabeculation.

Table 1. Genotypes and phenotypes of *Notch2*^{del1} mutant embryos at various gestational ages

	+/+	+/–	–/–
E10.5	32	50	27*
E11.5	48	113	41‡
E12.5-E15.5	88	159	37§ (22)
E16.5-birth	61	96	21¶

+/+, wild type; +/–, +/*Notch2*^{del1} heterozygotes; –/–, *Notch2*^{del1}/*Notch2*^{del1} mutants.

*E10.5 *Notch2*^{del1}/*Notch2*^{del1} mutant embryos were all normal in appearance.

‡18 out of 41 E11.5 *Notch2*^{del1}/*Notch2*^{del1} embryos exhibited growth arrest and hemorrhaging in the heart sac.

§13 out of 37 E12.5 to E15.5 *Notch2*^{del1}/*Notch2*^{del1} embryos exhibited edema and 22 necrotic embryos were also present (in parentheses).

¶Five out of 21 of the E16.5 and older *Notch2*^{del1}/*Notch2*^{del1} embryos had widespread hemorrhaging and one exhibited edema.

Eye and kidney phenotypes were 100% penetrant.

Xenopus Notch1 proteins (Rebay et al., 1991). We hypothesize that the phenotypes we observe in *Notch2*^{del1}/*Notch2*^{del1} mutants are most likely caused by a decrease in *Notch2* signaling. An alternative possibility is that the phenotypes we observe in *Notch2*^{del1}/*Notch2*^{del1} mutants are due to deleterious indirect effects such as ligand sequestering. However, the fact that *Notch2*^{del1}/+ heterozygotes have no visible phenotype argues against this hypothesis.

Notch2 is required for glomerular morphogenesis and patterning

Mice homozygous for the *Notch2*^{del1} mutation exhibit a complex phenotype, with at least two stages of lethality. All *Notch2*^{del1}/*Notch2*^{del1} homozygous mutants that survive until birth die within the first 24 hours. These homozygotes probably die from renal insufficiency due to defects in glomerular differentiation and patterning, although heart defects (i.e. myocardial hypoplasia) may also contribute to the lethality observed in the *Notch2*^{del1}/*Notch2*^{del1} neonates.

In the majority of abnormal glomeruli in the *Notch2*^{del1}/*Notch2*^{del1} mutants, neither mesangial cells nor endothelial cells could be detected using molecular markers. Some mutant

glomeruli appeared to consist of a disordered group of podocytes, as the clustered cells expressed the podocyte markers *Vegf* and *Wt1*. However, the mutant podocytes had not formed the cup-shaped epithelial layer observed in the control littermate glomeruli. This phenotype is reminiscent of the defects in development of epithelial tissues in *Drosophila* embryos mutant for *Notch* or for other components of the Notch signaling pathway (Hartenstein et al., 1992; Goode et al., 1996). In the second type of abnormal glomeruli observed in *Notch2*^{del1}/*Notch2*^{del1} mutants, the glomerular capillary tuft was replaced by a capillary aneurysm-like structure similar to that observed in mice mutant for the *Pdgfb* gene (Levéen et al., 1994; Lindahl et al., 1998) or the *Pdgfrb* gene (Soriano, 1994). It has been demonstrated for the *Pdgfb* and *Pdgfrb* mutants that these glomerular capillary aneurysms arise because of defects in mesangial cell differentiation.

It is important to point out that there is also a substantial quantitative effect on glomerular differentiation in *Notch2*^{del1}/*Notch2*^{del1} mutants. The total number of glomeruli is markedly reduced in the mutants, compared with heterozygous and wild-type littermates. This suggests that *Notch2* signaling may be required at multiple stages during glomerular differentiation. *Notch2*-mediated signaling may be required for cellular differentiation of the mesenchymal vesicles, as well as for providing the proper signals for migration of endothelial and mesangial precursors into the glomerular cleft, and for vascularization and epithelialization of the developing glomerulus.

The Jag1 protein is a probable ligand for the Notch2 receptor during glomerulogenesis

Expression analysis indicated that the *Notch2* and *Jag1* genes were expressed in adjacent cells during glomerular differentiation. *Notch2* was expressed at the periphery of the glomerulus in differentiating podocytes, while *Jag1* was expressed in the interior of the glomerulus, in a position consistent with expression in differentiating mesangial and/or endothelial cells. In addition, *Notch2*^{del1}/+ *Jag1*^{dDSL}/+ double heterozygotes displayed a kidney phenotype similar to, although somewhat less severe than, that of *Notch2*^{del1}/*Notch2*^{del1} homozygotes. These data indicate that the *Jag1* gene encodes a physiological ligand for the Notch2 receptor during glomerulogenesis. In support of this model, in vitro cell culture studies have confirmed that the Jag1 protein can act as a signaling ligand for the Notch2 protein, although either protein may bind to other ligands or receptors as well (Nofziger et al., 1999; Shimizu et al., 1999; Shimizu et al., 2000).

Heart and vascular defects in *Notch2*^{del1}/*Notch2*^{del1} mice

The second stage of lethality observed in *Notch2*^{del1}/*Notch2*^{del1} embryos was from E11 through E16. Abnormal mutant embryos at these stages exhibited symptoms of cardiovascular dysfunction. These embryos were edematous, and histological analysis revealed myocardial hypoplasia and reduced myocardial trabeculation. Many of these embryos also exhibited hemorrhages of small blood vessels in the skin.

We did not detect *Notch2* RNA in the embryonic heart, and it has been reported by others that *Notch2* is not expressed in the heart (Weinmaster et. al., 1992; Hamada et al., 1999). We did observe *Notch2* expression in the cardiac outflow tract. *Jag1* expression is also observed in the cardiac outflow tract (Loomes et al., 1999), and various heart defects are observed in humans with Alagille syndrome, an inherited disease syndrome caused by *Jag1* haploinsufficiency (Li et al., 1997; Oda et al., 1997; Krantz et al., 1999).

It is intriguing that several phenotypes observed in *Notch2*^{del1}/*Notch2*^{del1} homozygotes are similar to phenotypes observed in mice homozygous for targeted mutations of *Pdgfb* (Levéen et al., 1994; Lindahl et al., 1998) and *Pdgfrb* (Soriano, 1994). These shared phenotypes include formation of glomerular capillary aneurysms, myocardial hypoplasia and hemorrhages of blood vessels in the skin. Since *Pdgfb* and *Pdgfrb* are not expressed in the same cells that express the *Notch2* gene during glomerulogenesis, any interaction between these two signaling pathways is probably indirect, at least with respect to glomerular differentiation. Future studies will examine in more detail possible interactions between these signaling pathways.

Defects in the eye vasculature of *Notch2*^{del1}/*Notch2*^{del1} homozygotes

The hyaloid artery forms part of the hyaloid vascular system, a transient network of intraocular blood vessels that nourish the growing lens during embryogenesis (Jack, 1972; Balazs et al., 1980). During late embryonic and early postnatal stages in wild-type mice, the hyaloid vessel system atrophies. The retrolenticular hyperplasia present in the eyes of *Notch2*^{del1}/*Notch2*^{del1} embryos resembles a congenital human disease syndrome termed persistent hyperplastic primary vitreous (PHPV) (Reese, 1955; Haddad et al., 1978). In individuals with PHPV, the hyaloid vascular system does not atrophy but instead persists postnatally. This can lead to retinal detachment, cataracts, glaucoma and degeneration of the eye. While most human cases of PHPV are unilateral and show no obvious hereditary predisposition, approx. 10% are bilateral, and some cases of familial inheritance have been reported (cited by Haddad et al., 1978; Stades, 1983). Moreover, a genetic basis for the development of PHPV is supported by the frequent occurrence of PHPV in two dog breeds, the Doberman pinscher (Stades, 1983; Boevé et al., 1988) and the Staffordshire bull terrier (Curtis et al., 1984; Leon et al., 1986). However, the genetic loci causing predisposition for development of PHPV has not been identified in either dog model of the disease. *Notch2*^{del1}/*Notch2*^{del1} mutant mice may provide a useful, genetically defined model for study of the retrolenticular hyperplasia associated with the early embryonic stages of PHPV.

What is the *Notch2* null phenotype?

It is clear that the phenotype we observe in *Notch2*^{del1}/*Notch2*^{del1} homozygous mutants does not represent the *Notch2* null phenotype. Hamada et al. constructed and analyzed a *Notch2* mutant allele (*Notch2*^{lacZ}) in which the *E. coli* β-galactosidase-coding region replaces all but one of the ankyrin repeats in the intracellular domain of the mouse Notch2 protein (Hamada et al., 1999). Embryos homozygous for the *Notch2*^{lacZ} mutant allele exhibit a more severe phenotype than *Notch2*^{del1}/*Notch2*^{del1} homozygotes. All *Notch2*^{lacZ}/*Notch2*^{lacZ} homozygous mutant embryos die before 11.5 days of gestation (Hamada et al., 1999). The design of the *Notch2*^{lacZ} mutant allele is similar to the design of constructs

that exhibit dominant-negative effects when expressed in *Drosophila*. Rebay et al. found that dominant negative phenotypes resulted from overexpression of a Notch protein lacking most intracellular sequences (Rebay et al., 1993). Hamada et al., argue that they are not observing dominant negative phenotypes in *Notch2*^{lacZ}*/Notch2*^{lacZ} homozygotes, since *Notch2*^{lacZ}*/Notch2*^{lacZ} embryos arrested at a slightly later stage than *Notch1*$^{-/-}$ mutant embryos (Swiatek et al., 1994; Conlon et al., 1995), and no abnormalities were observed in *Notch2*^{lacZ}/+ heterozygotes. However, it is not clear whether the phenotype of *Notch2*^{lacZ}*/Notch2*^{lacZ} homozygotes represents the *Notch2* null phenotype. While the *Notch2*^{lacZ} mutant allele probably prevents *Notch2*-mediated signaling, dominant negative effects of this allele (such as sequestration of Notch family ligands) could contribute to the phenotype observed in the *Notch2*^{lacZ}*/Notch2*^{lacZ} homozygotes. Only creation of another *Notch2* mutant allele that totally abrogates production of Notch2 protein will answer the question of what the *Notch2* null phenotype really looks like.

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