Notch2, but not Notch1, is required for proximal fate acquisition in the mammalian nephron

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The Notch pathway regulates cell fate determination in numerous developmental processes. Here we report that Notch2 acts non-redundantly to control the processes of nephron segmentation through an Rbp-J-dependent process. Notch1 and Notch2 are detected in the early renal vesicle. Genetic analysis reveals that only Notch2 is required for the differentiation of proximal nephron structures (podocytes and proximal convoluted tubules) despite the presence of activated Notch1 in the nuclei of putative proximal progenitors. The inability of endogenous Notch1 to compensate for Notch2 deficiency may reflect sub-threshold Notch1 levels in the nucleus. In line with this view, forced expression of a γ-secretase-independent form of Notch1 intracellular domain drives the specification of proximal fates where all endogenous, ligand-dependent Notch signaling is blocked by a γ-secretase inhibitor. These results establish distinct (non-redundant), instructive roles for Notch receptors in nephron segmentation.

KEY WORDS: Notch, Rbp-J, Wnt4, Proximal tubule, Podocytes, Nephron segmentation, Mouse

INTRODUCTION

The kidney is an essential excretory organ that maintains osmotic, acid-base and electrolyte equilibrium. The crucial importance of the kidney makes it a common target of systemic diseases, developmental syndromes and drug toxicity. The functional unit of the mammalian kidney is the nephron; each nephron pattern along a proximodistal axis into distinct functional domains; proximal to distal there are the glomerulus, proximal tubule, loop of Henle and distal tubule. How this crucial regional structure is established is not well understood.

Nephrons form from a simple epithelial precursor, the renal vesicle (RV), itself a product of a Wnt-induced mesenchymal-to-epithelial transition in the outer cortex (Carroll et al., 2005). The RV begins a series of molecular changes reflected by a stereotyped set of morphological and molecular changes. Morphologically, the RV transitions through a comma-shaped, then an S-shaped body stage before fusing with the adjacent epithelium of the ureteric bud (UB)-derived collecting duct system to establish a continuous tubular network. Asymmetric expression of Brn-1 (Pou3f3 – Mouse Genome Informatics) (Nakai et al., 2003), E-cadherin (cadherin-1 – Mouse Genome Informatics) and cadherin 6 (Cho et al., 1998) provide some of the first evidence for polarization of the proximodistal axis (the future glomerulo-collecting duct axis), but it is not until the S-shaped body stage that the future proximodistal axis is readily distinguishable. At this time, Pax2 is highly expressed within the distal portion of the S-shaped body, including the region that fuses to the UB. Podocyte precursors, which differentiate into glomerular podocytes, reside in the proximal limb of the S-shaped body (the visceral epithelial cells) and express high levels of Wilms’ tumor-1 (Wt1) (Kreidberg et al., 1993). Adjacent to the podocyte in the cleft of the proximal limb, the vascular endothelial network of the future glomerular filtration apparatus starts to assemble. Although fate-mapping studies have not addressed the contribution of distinct regions within the S-shaped body to the future nephron, a cadherin-6-positive domain that lies between the presumptive podocytes and the Pax2(+) distal tubule progenitors is likely to give rise, at least in part, to the proximal convoluted tubule, a Lotus tetragonolobus lectin (LTL) (Laitinen et al., 1987) -binding epithelium in the mature nephron first visible at embryonic day (E) 14.5-15.5 in the mouse. The process of nephron formation continues at the periphery of the mouse kidney up to postnatal day 7; newborn kidneys thus contain nephrons at all stages of development.

Previous work examining the expression of Notch pathway components (Chen and Al-Awqati, 2005; Leimeister et al., 2003; Piscione et al., 2004) and modulating Notch signaling (Cheng et al., 2003; Wang et al., 2003) supported the argument for a Notch pathway activity in mammalian nephrogenesis. Notch genes encode single-transmembrane receptors that mediate short-range communication between cells. Receptor binding to ligand expressed on adjacent cells triggers the shedding of its extracellular domain and the subsequent cleavage of the transmembrane domain by the enzyme γ-secretase (for a review, see Mumm and Kopan, 2000). On γ-secretase-mediated proteolysis, the Notch intracellular domain (N1-ICD or N2-ICD) is released and translocates to the nucleus, where it associates with a DNA-bound REL-like protein (Cbf1/Rbp-J in vertebrates; Rbpsu – Mouse Genome Informatics) and promotes transcription of its targets (Fryer et al., 2004; Lubman et al., 2003). Notch1, Notch2, Dll1 and Jag1 mRNA are detected in the RV and its derivative; the expression domain of Notch1 partially overlaps with Notch2 in the S-shaped body (Chen and Al-Awqati, 2005). Notch2 and Jag1 are also expressed in the collecting duct. Humans haploinsufficient for Jagged 1 (Li et al., 1997) are prone to Alagille syndrome, one symptom of which can result in the development of renal abnormalities (McCright, 2003; Piccoli and Spinner, 2001), whereas abnormal glomerulogenesis is observed when Notch2 activity is reduced (McCright et al., 2001). Notch3 expression has

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been reported in the distal portion of the S-shaped body (Piscione et al., 2004); however, a lacZ knockin into the Notch3 locus indicates that only the glomerulus and blood vessels may express Notch3 (H.-T.C. and R.K., unpublished).

To date, no specific study has addressed the regional-specific action of the Notch pathway in nephron patterning, although the general, organ-wide inhibition of γ-secretase activity suggested that Notch activity is likely to be important, as podocytes and proximal tubules are lost when γ-secretase activity is abolished (Cheng et al., 2003; Wang et al., 2003). However, the observed phenotypes cannot be unequivocally attributed to loss of Notch signaling due to the existence of multiple other substrates of γ-secretase.

Here we have addressed the specific function of Notch1 and Notch2 by tissue-specific modulation of their activity. These studies reveal distinct roles for Notch1 and Notch2 in nephron development. Notch2 activity is essential for patterning of the proximal regions of the nephron. However, although Notch1 is normally activated, and when overactivated is capable of proximalizing the nephron, it is not sufficient for the development of proximal cell fates. Thus, local activation of Notch2 during renal tube morphogenesis is a central determinant of segmented pattern in the mammalian kidney.

**MATERIALS AND METHODS**

**Notch2, Rbp-J and Notch1 mutant animals**

We crossed Pax3-cre<sup>fl</sup>/*;N2f/f (<sup>flxed Notch2</sup>)/+ males (Engleka et al., 2005) to N2f/f females to obtain Pax3-cre<sup>fl</sup>/N2f/f embryos or pups. We crossed Pax2-cre<sup>fl</sup>/*;N1f/f (<sup>flxed Notch1</sup>)/+ females to obtain Pax2-cre<sup>fl</sup>/N1f/f embryos. We crossed Pax2-cre<sup>fl</sup>/*;Rbp-Jf/f embryos. Pax2-cre<sup>fl</sup>/Rbp-Jf/f (Ohyama and Groves, 2004; Tanigaki et al., 2002) males were crossed, Rbp-Jf/f females to produce Pax2-cre<sup>fl</sup>/N1f/f embryos. The kidneys or the cultured explants were fixed in Bouin's fixative or in 4% paraformaldehyde (PFA) for LTL and 5-Bromo-2'-deoxyuridine (BrdU) staining.

**Generation of Notch1<sup>-/-</sup> ↔ wild-type chimeric embryos**

The procedure is described in detail in Hadland et al. (Hadland et al., 2004). Briefly, compound heterozygotes for the Rosa26 locus and N1<sup>1/4</sup> females to obtain Rosa26<sup>+/f</sup>; Six2-GFP<sup>+</sup>/+. Female Six2-GFP<sup>+</sup> mice were used. Six2-GFP<sup>+</sup> mice were crossed to Rosa26<sup>+/f</sup> females to produce Six2-GFP<sup>+</sup>/N1f/f embryos. All mice used in this study were maintained on mixed backgrounds. Embryos were genotyped by standard PCR protocol. Noon of the day on which a vaginal plug was scored was designated as E0.5. The day when pups were born was designated as their first postnatal day (P1).

**β-gal staining to detect LacZ activity**

The kidneys were fixed in 4% PFA for 2 hours before whole-mount β-gal staining at room temperature overnight. The specimens were embedded in paraffin, sectioned and stained with antibody and/or counterstained with diluted Hematoxylin or Nuclear Fast Red (Vector Laboratories).

**Histology and immunohistochemistry**

The kidneys or the cultured explants were fixed in Bouin's fixative or in 4% paraformaldehyde (PFA) for LTL and 5-Bromo-2'-deoxyuridine (BrdU) analysis, embedded in paraffin and sectioned at 5 μm. The sections were then stained with Hematoxylin and Eosin (H&E) for histological analysis.

For immunohistochemistry, the sections were boiled in Tris-buffered saline (TBS) with 1% Triton X-100. The kidney was fixed in MEMFA (MOPS 0.1 mol/l pH 7.4, EGTA 2 mmol/l, MgSO4 1 mmol/l, formaldehyde 3.7%) for 1 hour before embedding in paraffin. Rehydrated sections were boiled in Tris-buffered saline (Cell Marque) for antigen retrieval. The antibodies and the lectins were diluted as follows: rabbit anti-mouse cdherin 6 (1:300; kindly provided by Dr Dressler) (Cho et al., 1998), Ck8 (TROMA1; 1:10; Developmental Studies Hybridoma Bank), E-cadherin (1:1000; Transduction Labs), Jag1 (1:200; Santa Cruz), N-Cam (1:300, Sigma), Pax2 (1:200; Covance), Wt1 (1:100, Santa Cruz), synaptophysin (1:40, gift from Dr P. Mundel), laminin α1 and fluorescein isothiocyanate-conjugated LTL (1:100; Vector Labs). Hoechst (0.5 μg/ml, Sigma) was used for nuclear staining. Fluorescein- and Cy3-conjugated anti-IgG corresponding to the species of the primary antibodies was used to visualize the antigen. For cadherin 6, E-cadherin and Jag1, we used horseradish peroxidase (HRP)-conjugated IgG followed by tyramide-conjugated FITC or Cy3 for better results. For Notch1 detection, metanephoepithelium were fixed in Bouin's fixative after 6 days in organ culture, dehydrated and embedded in paraffin wax. Sections (7 μm) were boiled in Tris-buffered saline for antigen retrieval, pre-blocked with PBS supplemented with 1% BSA, 0.2% skimmed milk, and 0.3% Triton X-100. A rabbit primary Notch1 antibody (1:200, abcam ab27526) was used, followed by a biotinylated anti-rabbit IgG (1:3000). The Vectastain ABC kit (Vector Laboratory, Inc.) and tyramide-conjugated Cy3 (TSA-Plus Cyanine 3, PerkinElmer, 1:100) were used to detect the signal.

To detect cleaved Notch1 in metanephoepithelium, the following modified method was used. After incubation of the primary antibody V1744 (1:500, Cell Signaling Technology, a division of New England Biolabs), the sections were treated with HRP-conjugated anti-rabbit IgG (1:1000; Jackson ImmunoResearch), tyramide-conjugated FITC (NEN, PerkinElmer, 1:1000), and then HRP-conjugated anti-fluorescein antibody. The antigen was then visualized with fluorescein-conjugated Cy3 (Cy3 plus, NEN, 1:400).

Detection of Lim1 was done with anti-Lim1 antibody (Chemicon International) (Karavanov et al., 1996) with slight modification to the manufacturer's protocol. The kidney was fixed in MEMFA (MOPS 0.1 mol/l pH 7.4, EGTA 2 mmol/l, MgSO4 1 mmol/l, formaldehyde 3.7%) for 1 hour before embedding in paraffin. Rehydrated sections were boiled in Tris-buffered saline (Cell Marque) for antigen retrieval, and incubated with anti-Lim1 antibody at 5 mg/ml (1:200 of stock). To visualize the antigen, it is necessary to apply HRP-conjugated anti-rabbit IgG followed by tyramide-conjugated Cy3. This allows double staining with other primary rabbit antibodies like anti-Pax2.

For whole-mount staining, metanephoepithelium were fixed in 4% PFA, washed in PBS and incubated in the blocking solution (MABT: 100 mmol/l maleic acid pH 7.5, 150 mmol/l NaCl, 0.1% Tween-20; plus 2% Blocking Reagent; Boehringer Mannheim) for one hour before adding anti-Ck8 antibody (1:10) for overnight incubation at room temperature. After extensive wash in MABT, the specimen was incubated with the Cy3-conjugated anti-rat IgG (1:1000). After this step, the metanephoepithelium were incubated with FITC-LTL (1:200) for 1 hour at room temperature and then washed by PBS.

Quantification of BrdU-labeling of cells

BrdU solution containing 5-Fluoro-2'-deoxyuridine (10% of the BrdU concentration) was injected intraperitoneally in pregnant mice 2 to 3 hours before kidney harvest. The samples were prepared and sectioned as described above before being incubated with mouse anti-mouse BrdU antibody (1:200) (Becton and Dickinson). It was visualized by Cy3 following HRP-conjugated IgG incubation. The sections were then subjected to staining with another antibody (Jag1 or Pax2) and then Hoechst nuclear stain. The single-color images were merged into one RGB file magnified in Adobe Photoshop. For BrdU-labeled Pax2 cells, we counted the number of BrdU-stained Pax2-expressing cells and the number of Pax2-
expressing cells within the Pax2-expressing Rvs or early nephrons from one wild-type and one mutant kidney (19 Rvs or early nephrons from each sample; each sample contains multiple sections). The data were presented as percentage of BrdU-positive Pax2 cells within the Pax2-expressing cells. For BrdU labeling index of Jag1 cells, sections from three different wild-type and three different mutant kidneys were included and 15 Jag1 clusters were counted in each kidney sample. Within each cluster we counted the number of BrdU-stained Jag1-expressing cells and the number of Jag1-expressing cells; and calculated the percentage of BrdU-labeled Jag1 cells in the Jag1-expressing cells. Student’s t-test was used in the first comparison, and one-way ANOVA in the second comparison. P<0.01 was considered statistically significant.

In situ hybridization
Briefly, kidney samples were fixed in 4% PFA in PBS for 24 hours at 4°C and processed for OCT embedding. Frozen blocks were sectioned at 16 μm thickness and air dried. Slides were post-fixed in 4% PFA for 10 minutes, followed by three PBS washes, 3 minutes each. Slides were treated with Proteinase K, acetylated and rinsed and dehydrated before use. Digoxigenin-labeled riboprobes were made and column purified according to the manufacturer’s instructions (Roche Applied Science). Hybridization was performed in a humidified chamber with a 200 μl probe and a parafilm coverslip at 68°C overnight. After hybridization, non-specific signal was removed by SSC washes and RNaseA digestions (detailed protocol is available upon request). Slides were then washed in MBST (100 mmol/l maleic acid, 150 mmol/l NaCl, 0.1% Tween-20, pH to 7.5) and blocked with 5% heat inactivated sheep serum (HISs) in 2% BMB (Roche Applied Science) in MBST, before adding anti-digoxigenin-AP antibody (1:4000 dilution) in 1% HISs, 2% BMB in MBST and incubated overnight in a humidified chamber at 4°C. Following extensive washes, signal was developed using BM Purple for 1 to 6 days in a humidified chamber at room temperature.

RESULTS
Conditional deletion of Notch2 in the kidney causes complete loss of glomeruli and proximal tubules
To investigate the in vivo function of Notch receptors during nephrogenesis, we employed a Cre-mediated knockout strategy to disrupt these genes in the kidney mesenchyme using the Pax3-cre line (Li et al., 2000), which induces recombination in the metanephric mesenchyme (Grieshammer et al., 2005; Perantoni et al., 2005). Kidneys from Pax3-cre<sup>+/</sup>; Rosa26<sup>R<sub>tg</sub>/+</sup> embryos (Soriano, 1999) display LacZ expression in all metanephric mesenchyme-derived tissues, including comma- and S-shaped body and stromal tissue, while the UB derivatives remain unlabeled. The early and broad recombinase activity assures an early and complete recombination of alleles that is specific to the mesenchymal compartment and their epithelial derivatives (see Fig. S1A,B in the supplementary material) (see also Grieshammer et al., 2005; Perantoni et al., 2005).

Viable, normal Pax3-cre<sup>+/</sup>; N2<sup>ff</sup> newborns were obtained at Mendelian ratios (data not shown). However, despite feeding successfully (data not shown), Pax3-cre<sup>+/</sup>; N2<sup>ff</sup> animals died 24 to 48 hours after birth. Gross anatomical examination revealed that Pax3-cre<sup>+/</sup>; N2<sup>ff</sup> had smaller kidneys than Pax3-cre<sup>+/</sup>; N2<sup>ff</sup> siblings (Fig. 1A-D), and a small bladder suggested failure to produce urine (black arrows, Fig. 1A,C). During postnatal day 2 (P2), Notch2-deficient kidneys appeared to have lost vascular integrity (Fig. 1D); We observed hemorrhage into the interstitial spaces in Pax3-cre<sup>+/</sup>; N2<sup>ff</sup> P1 kidney (Fig. 1G, circle). The renal pelvis was collapsed, the papilla was flattened (see Fig. S2A,B in the supplementary material) and the collecting ducts (turquoise arrow, Fig. 1E-H) were less extensively branched than those of wild-type or heterozygote Pax3-cre<sup>+/+</sup>; N2<sup>ff</sup> littermates, even though N2<sup>ff</sup> remains intact in the collecting duct network of the mutant. The nephrogenic zone, where the nephron initiating mesenchymal-to-epithelial transition takes place, appeared similar in thickness in both heterozygote and mutant kidneys (blue arrows, Fig. 1E,G), indicating a normal progression of the epithelialization process. By contrast, S-shaped bodies (yellow arrows), convoluted renal epithelia (green arrows) and glomeruli (red arrows), were not histologically distinguishable (Fig. 1E-H). Thus, the cause of death was renal failure due to the absence of a filtration apparatus. Heterozygote kidneys were indistinguishable from wild type in their morphological and histological features; hence we used ‘wild type’ throughout to encompass both genotypes, although the exact genotype is detailed in the figures.

To address the state of nephrogenesis, we used immunohistochemical methods to examine the residual renal tubules that were present in the mutant kidneys (green arrowheads in Fig.
Epithelial ductal labeling with anti-cytokeratin 8 (Ck8; Krt8 – Mouse Genome Informatics) antibodies (specific for UB derivatives) (Hemmi and Mori, 1991) confirmed that a branched collecting duct was present as expected (Fig. 2C,E). Expression of Wt1, a zinc-finger-containing transcription factor, expressed at low levels in the metanephric mesenchyme (MM) and at high levels in podocyte progenitors from the S-shaped body stage (Fig. 2A), was detected only in the MM surrounding the tips of the UB (Fig. 2B). Furthermore, whereas LTL, a marker specific for mature proximal convoluted tubules (PCT), labeled numerous tubules in wild type at E16.5 (Fig. 2D), no LTL-positive structures were detectable in the mutants (Fig. 2C). Thus, the organization of proximal fates was clearly compromised by Notch2 removal. By contrast, a comprehensive analysis of E-cadherin and Ck8 indicated that distal nephrons were Notch2-independent. Both RV-derived epithelia and collecting duct epithelium express E-cadherin, whereas Ck8 is expressed only in the collecting ducts. Many E-cadherin-positive, Ck8-negative tubular structures were detected in mutant kidneys. Several of these were continuous with the UB tips (Fig. 2E,E'). Whereas proximal tubules also express E-cadherin, the lack of LTL-binding activity, the continuity with the duct, their smaller size and the regular-shaped lumen (see Fig. 1H and see Fig. S2E in the supplementary material) indicate that renal tubules formed in the absence of Notch2 and most proximal fates were compromised.

Fig. 2. Notch2-deficient kidneys (N2) develop distal tubules without formation of podocytes or proximal tubules. (A,B) Wild-type (A) kidney contains cells expressing high levels of Wt1 in glomerular podocytes and S-shaped bodies. The only cells that express low levels of Wt1 in the mutant (B) are mesenchymal cells surrounding cytokeratin-8-expressing ureteric buds (red). (C,D) LTL-stained proximal tubules found in wild type (D) are absent in the mutant (C). (E,F) Mutant kidney (E) develops numerous E-cadherin-positive, cytokeratin-8-negative distal tubules, some of which are connected to cytokeratin-8-positive ducts (dashed line in enlarged view E'). The wild-type proximal tubules, judged by morphology, also express E-cadherin (arrowhead in F). N2, Notch2 mutant; Wt, wild type. Scale bars: 0.1 mm in B for A,B,E'/H11032; in C for C,D.

Fig. 3. Notch2-deficient mesenchyme undergoes normal epithelialization but the newly formed nephron fails to resolve into the S-shaped body seen in wild type. (A-E) Wild type (A) showing S-shaped bodies (arrowheads). In the Notch2 mutant, one nephron, expressing N-Cam, can be identified at each ureteric bud tip (arrowheads in B). These synthesize laminin α1 (arrowheads in D), as do the S-shaped bodies (arrowheads in C). (E) Three progressive stages during nephrogenesis in the mutant are marked as 1, 2 and 3. (F-J) Expression of molecular markers in each of the three segments in the S-shaped body (see text). Distal tubule precursors are Pax2\textsuperscript{high}, and E-cadherin-positive (F,H). Jag1 is localized in the middle segment in the position of proximal tubule precursors (G). Wt1 marks the podocyte precursors (I). N1-ICD is detectable in both the proximal and podocyte precursors (J). Scale bars: 0.05 mm in A for A-D and in J for F-J; 0.1 mm in E.
likely comprise only distal tubule segments of the nephron. Together, these data suggest that Notch2 is essential for the establishment of podocyte and PCT cell fates during nephron segmentation.

Notch2-deficient RVs initiate the segmentation process but fail to establish the proximal fates

To examine the early patterning of RV derivatives that preceded establishment of mature fates, we analyzed expression of a number of key reporters of these events. In both wild-type and mutant kidneys, a mesenchymal Pax2 domain forms around the UB tip (Fig. 3A,B and data not shown). Pax2<sup>High</sup> mesenchymal cells were congregated around the tips of the UB (compare with Wt1 staining in Fig. 2B). Neural cell adhesion molecule (N-Cam; Ncam1 – Mouse Genome Informatics) is expressed in the mesenchymal cells and in their derivatives, including the nascent renal vesicle. In wild type, N-Cam-positive cell clusters with elevated Pax2 expression appeared adjacent to the UB tip (Fig. 3A). In Notch2 mutants, we detected similar groups of amorphic Pax2<sup>High</sup>, N-Cam-positive, Ck8-negative structures located close to the UB tips (Fig. 3B,E). These clusters were positive for two signature processes of epithelialization: cellular polarization, indicated by formation of a laminin α1-positive basal lamina deposition (Abrahamson et al., 1989) (Fig. 3C,D), and the synthesis of epithelial adhesion molecules (E-cadherin, Fig. 3E). Therefore, a robust mesenchymal condensation and mesenchymal-epithelial transition occurs normally in Notch2-deficient metanephric mesenchyme.

The analysis of E-cadherin expression revealed three types of epithelial structures in the Notch2 mutant kidneys. First, the aforementioned early RVs: small cell clusters located just below the tips of the UB that expressed Pax2<sup>High</sup> (#1 in Fig. 3E); the second, larger epithelial clusters of Pax2 positive, E-cadherin-positive cells (#2 in Fig. 3E), a structure potentially analogous to comma- or S-shaped bodies or a ‘transitional’ structure between the early RV and S-shaped body; and a third structure that was tubular in shape, Pax2<sup>Low</sup> and E-cadherin-positive (#3 in Fig. 3E).

We examined the expression of Wt1, cadherin 6 and E-cadherin for any evidence of putative podocyte or proximal tubule precursors in the Notch2-deficient renal epithelia. As was the case with LTL, cadherin 6, an adhesion molecule thought to be expressed in the precursors of PCTs in S-shaped bodies, was not detected in Notch2-deficient kidneys (Fig. 4A,B). Further, Wt1<sup>High</sup> podocyte precursors were also absent (Fig. 4A,B'). On close examination, the data suggested that proximal segmentation initiated, but failed to establish independent proximal identities. During early stages of nephrogenesis preceding formation of the S-shaped body, Pax2 is expressed in all epithelia and is required to initiate expression of Wt1 (Dehbi et al., 1996); upregulation of Wt1 inhibits Pax2 expression (Dehbi et al., 1996; Ryan et al., 1995). As in the wild type (Fig. 4A', A''), we observed Wt1 expression in Notch2-deficient nephrons that appeared to segregate from Pax2, such that cells containing lower levels of Wt1 displayed relatively higher levels of Pax2 and vice versa (Fig. 4B', B''). Further evidence of polarity comes from analysis of Lim1 (Lhx1 – Mouse Genome Informatics); Lim1 expression is restricted to cells within the RV that are closest to the UB tip (Fig. 4C), and this is observed in Notch2 mutant kidney (Fig. 4D). Thus, the initiation of RV polarity appears to be Notch2-independent, but the establishment of stable regional identities in distinct proximal and distal regions of the developing nephron is defective and proximal fates are absent from the S-shaped body.

To address Notch pathway activation in normal nephron segmentation, we analyzed the spatial and temporal expression domain of Notch pathway proteins, in conjunction with segment-specific markers. Jag1 protein was first detected in clusters of Pax2<sup>Low</sup>-expressing epithelial cells in early RVs (Fig. 4E). In the absence of a good antibody to Dll1, we monitored the presence of LacZ knocked into the Dll-1<sup>LacZ</sup> gene in Dll1<sup>1lacZ</sup> heterozygotes (Hrabe de Angelis et al., 1997). Dll1<sup>1lacZ</sup> was also present in a subset of RV cells (Fig. 4G) that also contained activated Notch1 (Fig. 4H,I) and Lim1 (Fig. 4C). Jag1 expression and N1-ICD accumulation became...
elevated on morphogenesis of the RV to the S-shaped body (Fig. 3G, I, Fig. 4C, G). Although the cell fates have not been mapped to date, these observations suggest that activated Notch1, Jag1 and Dll-1LacZ expression are likely to mark more proximal precursor populations within RVs and their early derivatives and are thus among the first markers of segmentation. Distal precursors within the S-shaped body expressed E-cadherin and high levels of Pax2 (Pax2<sup>High</sup>) (Ryan et al., 1995), but no Jag1 or N1-ICD (Fig. 3F, H, I). Podocyte precursors were Wt1-positive, Pax2<sup>Low</sup>- and Jag1-negative; some contained N1-ICD (Fig. 3F, Fig. 4F; see Fig. S3C in the supplementary material). In the S-shaped body, Dll-1LacZ expression overlapped with, but was broader than, the jag1 domain in most S-shaped bodies. Whether this reflects real differences or the possible perdurance of β-galactosidase activity is unclear. Dll1LacZ was detected in a few E-cadherin-expressing distal precursors and in some podocyte precursors (see Fig. S4D in the supplementary material). In this respect, the Dll-1LacZ expression domain resembled the pattern of Notch1 activation better than the Jag1 domain (see Fig. S4G, D in the supplementary material). We suggest that Jag1-positive, Dll-1LacZ-expressing, N1-ICD-containing, Pax2<sup>Low</sup> cells probably define proximal regional fates, and Jag1 is likely to be a better marker of PCT precursors than Dll-1 (the functional significance of Dll-1 is discussed below). Unfortunately, while a detailed in situ expression analysis has been published for Notch receptors (Chen and Al-Awqati, 2005; Leimeister et al., 2003; Piscione et al., 2004), the absence of a suitable antibody has prevented mapping of Notch2 protein distribution.

We next addressed whether the Jag1-expressing proximal region precursors within the RV and early RV derivatives, the first to downregulate Pax2 (Fig. 3F, G), contain N1-ICD in Notch2-deficient kidneys. A small group of Jag1-expressing cells were detected in virtually every Notch2-deficient early epithelial clusters (Fig. 4F, I), consistent with the possibility that initiation of proximal patterning is initially independent of Notch2. N1-ICD was detectable in these Jag1-expressing cells (Fig. 4I inset), indicating that Notch1 signaling was apparently active in these cells. Clearly, Notch1 activity could not compensate for the loss of Notch2 in subsequent patterning steps. This observation leaves open the possibility that Notch1 may contribute to the establishment of the earliest polarity pathway. Note that Pax2 expression in Jag1-positive, Notch2-deficient cell clusters in the RV derivatives remained ubiquitous and high, consistent with impairment in acquisition of proximal and podocyte fates.

In conclusion, segregation of Wt1, Pax2 and Lim1 expression initiated and was accompanied by transient acquisition of a proximal precursor fate (Jag1-positive, N1-ICD present). However, podocyte precursors (Wt1<sup>High</sup>) never formed. This is because Notch2-deficient epithelial cells could not resolve proximal from distal fates, as evident from the simultaneous expression of markers typical for proximal and distal differentiation at the S-shaped stage. Notch2-deficient cells either die or adopt a Notch2-independent distal fate. We thus conclude that the transitional epithelial structures marked as #2 in Fig. 3E represent defective comma- or S-shaped bodies in which segmentation has initiated but the specification of appropriate regional identities has failed.

**Notch2-deficient proximal precursors have reduced capacity to proliferate**

Three hypotheses can explain why morphologically distinct comma- and S-shaped bodies fail to form in the Notch2-deficient kidneys. First, cells with dual identity (i.e. expressing both Jag1 and Pax2) die, resulting in failure to form comma- and S-shaped bodies. Second, due to global proliferation defects in the early renal epithelium, abnormal epithelial structure forms. Third, Jag1-positive proximal tubule precursors are unable to expand, and, in their absence, typical S-shaped bodies fail to form.

The first possibility was addressed by examining the distribution of active caspase3, an early marker of apoptosis; no significantly enhanced apoptosis was observed (data not shown). The second explanation was addressed by pulse-labeling S-phase cells with BrdU and the fraction of Pax2<sup>High</sup> cell examined in renal epithelia close to the UB tips; we observed a BrdU-positive fraction of ~50% in both wild-type and Notch2 mutant metanephroi (54 versus 48%, P<0.01; Fig. 5). Thus, a general proliferative defect does not underlie abnormal tubule morphogenesis in Notch2 mutants. When proliferation was scored specifically in Jag1-expressing cells, the subpopulation of Notch2-deficient, Pax2<sup>High</sup>, Jag1-expressing cells entered the cell cycle twofold less frequently than their wild-type counterparts (F<sub>(6, 15)</sub>=8.697, P<0.001; Fig. 5). Therefore, Notch2 activity is required for normal proliferation of proximal regional precursors.

**Notch1 is not required for cell fate determination during early nephron formation**

To test if Notch2 was sufficient on its own for nephron segmentation in the absence of any Notch1 input, we generated Pax3-cre<sup>Lox<sup>o</sup></sup>; N1<sup>fl</sup> embryos. However, these failed to survive beyond E9.5 (data not shown) and consequently were uninformative. Two alternative strategies were adopted. First, we used chimera analysis with ES cells deficient for Notch1 (Fig. 6). Second, we used Pax2-cre transgenic mice (Fig. 7) (Ohyama and Groves, 2004). We examined chimeric kidney embryos generated by injecting N1<sup>Δ/Δ</sup>; Rosa26-lacZ<sup>Lox</sup> ES cells (see Hadland et al., 2004; Nichols et al., 2004) into wild-type blastocysts. N1<sup>Δ</sup> contains a large deletion of the locus (Conlon et al., 1995). We analyzed seven chimeric
mice with Rosa26^{Rosa26}\textsuperscript{tg/+}, N1\textsuperscript{Δ/Δ} cells and four with matched Rosa26^{Rosa26}\textsuperscript{tg/+} wild-type controls. In all, lacZ-positive cells contributed extensively to normal-looking RVs, S-shaped bodies and elongating nephrons, consistent with the absence of an early function for Notch1 (Fig. 6B). Many glomeruli were composed entirely from Notch1-deficient podocytes. The number of N1\textsuperscript{Δ} podocytes that surrounded a wild-type capillary tuft were within the normal range (Fig. 6F). Further, the contribution of the Notch1-deficient cells to the LTL\textsuperscript{+} proximal convoluted tubules was also extensive (Fig. 6D,D'). Thus, there is no cell-autonomous requirement for Notch1 activity to establish proximal renal tubule fates; however, we could not rule out a non-autonomous contribution from Notch1-expressing cells intermingled with N1\textsuperscript{Δ−} cells.

To determine whether any requirement for Notch1 exists, a Pax2-cre\textsuperscript{tg} transgene was used to remove Notch1 function from the metanephric kidney. Cre-mediated recombination is catalyzed in the condensing metanephric mesenchyme and in UB derivatives (see Fig. S3A-B in the supplementary material) (Ohyma and Groves, 2004). Compound heterozygote Pax2-cre\textsuperscript{tg}; N1\textsuperscript{Δ/Δ} embryos were normal at E12.5 but died at E13.5 from unrelated vascular failure and hemorrhage in the internal organs (data not shown). We therefore removed the E12.5 metanephros from Pax2-cre\textsuperscript{tg}; N1\textsuperscript{Δ/Δ} embryos and littermate controls (some of which also carried the Dll1lacZ allele) and examined their development.

Notch1 protein is detected in the plasma membranes of duct and renal epithelial cells (inset in Fig. 7C; see Fig. S3E-F in the supplementary material). Intense apical staining indicates accumulation of Notch1 in S-shaped bodies (inset in Fig. 7C; see Fig. S3E-F in the supplementary material). Staining for Notch1 was also extensive (Fig. 6D,D). Intense apical staining indicates renal epithelial cells (inset in Fig. 7C; see Fig. S3E-F in the supplementary material). Staining for Notch1 in S-shaped bodies (inset in Fig. 7C; see Fig. S3E-F in the supplementary material). Staining for Notch1 was also extensive (Fig. 6D,D).

was detected in renal epithelia of Pax2-cre\textsuperscript{tg}; N1\textsuperscript{Δ/Δ} embryos (see Fig. S3C-D in the supplementary material). By contrast to Notch2, Notch1-deficient metanephros appeared morphologically and histologically normal; they contained LTL-positive proximal tubules (Fig. 7B), E-cadherin-positive and CK8-negative distal tubules (not shown) and Wt1\textsuperscript{High}, synaptopodin-positive (Mundel et al., 1997) podocytes (Fig. 6D). By contrast, Pax2-cre\textsuperscript{tg}; N2\textsuperscript{β/β} embryos developed kidneys lacking proximal tubule and podocytes (data not shown), indicating that Pax2-cre\textsuperscript{tg} used in this study removes Notch alleles before the critical window described previously (Cheng et al., 2003). Thus, Notch1 is not required for regional organization of distinct cell fates.

**Rbp-J deletion mimics the effect of Notch2 deficiency on nephron formation**

One possible explanation for the crucial role of Notch2 and the failure of active Notch1 signaling to normally complement Notch2 action would be that Notch2 has acquired a unique, Rbp-J-independent activity. To address this issue, we generated Pax2-cre\textsuperscript{tg}; Rbp-Jf/f mice (Tanigaki et al., 2002). Pax2-cre\textsuperscript{tg}; Rbp-J\textsuperscript{f/f} embryos were normal at E12.5 but died at E13.5; when cultured at E12.5, metanephros from Pax2-cre\textsuperscript{tg}; Rbp-J\textsuperscript{f/f} embryos branched properly but failed to produce LTL-positive proximal convoluted tubules or Wt1\textsuperscript{High} podocytes (Fig. 7E-H). Thus, it appears that Notch2 acts in a conventional, Rbp-J-dependent pathway during nephron segmentation.

**Notch1 can stimulate proximal fates and inhibit distal ones when ectopically activated in nephron precursors**

A second possible explanation for the failure of Notch1 signaling to compensate for Notch2 could be a requirement for distinct levels of signaling inputs; the existence of activation thresholds for the Notch...
targets Hes1 and Hes5 was recently demonstrated in organ culture (Ong et al., 2006). If so, higher amounts of N1-ICD may be able to compensate for Notch2 in activating its targets and promoting formation of proximal pattern.

To test this hypothesis, we used a metanephric mesenchyme-specific line Six2-GFP::Cre. This Cre strain will be described more fully elsewhere. Importantly, Six2-GFP::Cre is active in the cap stage, slightly later than the Pax2-Cre but before RV formation (Xu et al., 2003). Hence, a stable recombination results in a genetic modification of the MM and its derivatives. To elevate N1-ICD levels, we created RosaNotch/+; Six2-GFP::Cre tg/+ mice. In these animals Six2-GFP::Cre excises a ‘stop’ cassette and constitutive expression of N1-ICD activates Notch1 signaling (Murtaugh et al., 2003). Pups with this genotype were born in the correct Mendelian ratio but displayed severely hypoplastic kidneys (Fig. 8A) in which the UB underwent a single branching event (Fig. 8B). The absence of Six2-producing cells at E13.5 in the RosaNotch/+; Six2-GFP::Cre tg/+ kidney suggested that lack of branching was secondary to the loss of glial cell line-derived neurotrophic factor-producing MM cells (data not shown).

Despite the branching deficit, multiple tubular epithelial structures formed from the Six2-GFP::Cre-negative cell clusters (Fig. 8Ba-c). These tubular epithelia expressed both LTL (Fig. 8Bb,8C) and Slc34a1 (not shown), characteristic of PCTs. To determine whether activated Notch1 accelerated the formation of proximal tubules, E11.5 metanephroi were cultured for 4 days. At this stage, there were very few LTL-positive tubules present in wild type; however, metanephroi that overexpressed N1-ICD had already developed numerous LTL-positive tubules (Fig. 8C,C/H11032). Furthermore, the activity of N1-ICD was independent of Notch2, as LTL-positive PCTs appeared even in the presence of DAPT (Fig. 9). In summary, N1-ICD can direct development of proximal nephron fates that are normally controlled by Notch2, consistent with a model in which N1-ICD is present at subthreshold levels during normal nephron patterning.

**DISCUSSION**

**Notch2 maintains or induces proximal fates in the developing nephron**

Mesenchymal cells in the metanephric blastema form renal epithelia in response to factors secreted by the UB tips (Carroll et al., 2005). The first epithelial structure (early RV stage) lacks distinctive
mammalian kidneys, and the two parallel inputs, one provided by Notch2 and another from Lim1. Therefore, the separation of the distal and proximal lineages and the differentiation of proximal tubule and podocyte precursors require two parallel inputs, one provided by Notch2 and another from Lim1.

**Notch1 and Notch2 have non-overlapping activities**
We demonstrate here that while direct activation of Notch1 is observed in proximal precursors of the S-shaped body, removal of Notch1 activity with Pax2-Cre had no impact on the establishment of proximal fates. Thus, Notch1 is clearly non-essential for this process and Notch2 is the only γ-secretase substrate that plays a significant role in these patterning events. As soon as Notch was identified as an X-linked locus in *Drosophila* (Welshons, 1958), it was realized that the wing phenotype associated with Notch mutations was due to haploinsufficiency. Notch1 is haploinsufficient in vertebrates as well: myelination in the mouse is slowed in Notch1 hypomorphic heterozygote animals (Givogri et al., 2002), and human tricuspid aortic stenosis results from haploinsufficiency of *Notch1* (Welshons, 1958), it
heart valve development and maintenance of valve flexibility throughout adult life require both alleles of Notch1 (Garg et al., 2005). Importantly, no kidney disease is reported in these kindred; however, as this manuscript underwent revisions, a human haploinsufficiency for Notch2 was reported to cause Alagille syndrome (McDaniell et al., 2006). This would not be possible if Notch1 and Notch2 played redundant roles in human nephron development; we infer from this data that in human, as in the mouse, Notch2 is the dominant receptor during nephron segmentation.

While Notch1 and Notch2 differ in their ability to activate targets (Ong et al., 2006), they have the same affinity to Rbp-J (Lubman et al., 2006). Notch-responsive promoters may respond differentially to similar nuclear concentrations of activated Notch receptors within the nephron, becoming inactive if this amount falls below a threshold (Ong et al., 2006). What then can be the mechanistic basis for these findings?

The observation that N1-ICD overexpression can promote the proximal fates when endogenous Notch processing (and thus signaling) is abolished supports a model whereby subthreshold levels of this protein in the normal renal epithelium fail to complement Notch2 deficiency. Thus, Notch1 may be a weak activator of key target(s) regulated normally by Notch2, or N1-ICD may fail to accumulate to sufficient levels in the normal kidney to functionally replace Notch2 deficiency. Another possibility is that N1-ICD is modified in a manner that decreases its association with Rbp-J. This will be predicted to prevent its degradation (Fryer et al., 2004), and ironically, facilitate its detection by immunohistochemistry. When overexpressed, some N1-ICD may escape modification, bind and activate crucial targets.

If differences existed between N1-ICD and N2-ICD that affected binding to Rbp-J or to other putative partners, where would they map within the intracellular domain? Domain swaps indicated that the divergent 426 amino acids that lie downstream of the ANK domain are not important: mice expressing a Notch2 hybrid containing this Notch1 domain (and thus deficient in the Notch2 C-terminal domain) lack a kidney phenotype (Kraman and McCright, 2005). Crystallographic analysis of the Notch1 ANK domain revealed a surface unique to each of the vertebrate Notch paralogs (Lubman et al., 2005). Therefore, the subtle differences in the ANK domain may be responsible for the lack of redundancy between these highly conserved receptors (see also Ong et al., 2006).

In conclusion, our study presents the first evidence for the crucial role of Notch2 in an intrinsic patterning mechanism that establishes proximodistal nephron polarity. We discovered a cell type in which two different Notch molecules are present in its nucleus yet only one is crucial (Notch2), a demonstration of unexpected complexity in this pathway, and provided evidence that detection of N1-ICD per se is not an unambiguous indicator of a functional role for Notch1 signaling. These results serve as an entry point to explore further details of the mechanisms controlling early nephron development. They establish that Notch2 acts to separate proximal and distal fates, and in this Notch signaling most likely plays an instructive role after an initial polarizing cue (Lim1?) acts in the RV. Thus, utilization of Notch proteins in the kidney appears to be quite different from their use in the skin, where all cellular identities emerge before, and independent of, Notch function (Kobayashi et al., 2005) and where Notch1 plays a dominant role (S. Demehri, Y. Pan and R.K., unpublished).

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Supplementary material
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