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Notch is required for the formation of all nephron segments and primes nephron progenitors for differentiation

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ABSTRACT

Notch signaling plays important roles during mammalian nephrogenesis. To investigate whether Notch regulates nephron segmentation, we performed Notch loss-of-function and gain-of-function studies in developing nephrons in mice. Contrary to the previous notion that Notch signaling promotes the formation of proximal tubules and represses the formation of distal tubules in the mammalian nephron, we show that inhibition of Notch blocks the formation of all nephron segments and that constitutive activation of Notch in developing nephrons does not promote or repress the formation of a specific segment. Cells lacking Notch fail to form the S-shaped body and show reduced expression of *Lhx1* and *Hnf1b*. Consistent with this, we find that constitutive activation of Notch in mesenchymal nephron progenitors causes ectopic expression of *Lhx1* and *Hnf1b* and that these cells eventually form a heterogeneous population that includes proximal tubules and other types of cells. Our data suggest that Notch signaling is required for the formation of all nephron segments and that it primes nephron progenitors for differentiation rather than directing their cell fates into a specific nephron segment.

KEY WORDS: Notch, Nephron segmentation, Nephrogenesis, Kidney development, Wnt4, Six2, Mouse

INTRODUCTION

In mammals, nephrons are formed only during development (McMahon, 2016). At the cortex of the developing kidney, mesenchymal nephron progenitors reside adjacent to the branching tips of the collecting duct. At each branching event, a subset of nephron progenitors undergoes mesenchymal-to-epithelial transition (MET) to form a ball-like epithelial structure called the renal vesicle (RV). The RV becomes the comma-shaped body (CSB), which develops into the S-shaped body (SSB). Each SSB eventually gives rise to a nephron. The nephrogenesis process continues until nephron progenitors are depleted around birth, by 36 weeks of gestation in humans and by postnatal day (P) 4 in mice (Hartman et al., 2007; Hinchliffe et al., 1991; Rumballe et al., 2011). The absence of nephron progenitors thereafter prevents the generation of new nephrons, even after kidney injury (Humphreys et al., 2008). Undifferentiated mesenchymal nephron progenitors express *Six2*, which encodes a homeodomain transcription factor (Self et al., 2006). Six2 is required for the maintenance of nephron progenitors, blocking their premature depletion (Kobayashi et al., 2008; Self et al., 2006). Previously, we have shown that the differentiation of nephron progenitors requires the downregulation of *Six2*, and that Notch signaling downregulates *Six2*, thereby promoting nephrogenesis (Chung et al., 2016).

The nephron serves as the blood filtration unit of the kidney. Each nephron is composed of distinct segments (Desgrange and Cereghini, 2015). Podocytes and Bowman's capsule cells in the renal corpuscle originate from nephron progenitors (Kobayashi et al., 2008). Along the proximodistal axis of the nephron, the renal corpuscle is followed by the proximal tubule, loop of Henle, and distal tubule, all of which originate from a common pool of $Six2^+$ nephron progenitor cells. Different nephron segments are composed of specific types of epithelial tubule cells that perform distinct physiological functions. The developmental process of nephron segmentation is not well understood, especially in mammals. It is believed that Notch is a major signaling pathway involved in regulating mammalian nephron segmentation (Desgrange and Cereghini, 2015; Kopan et al., 2014; Park and Kopan, 2015). Activation of Notch signaling requires cell-to-cell interaction between a ligand-expressing cell and a Notch receptor-expressing cell, resulting in the cleavage of the intracellular domain (ICD) of the Notch receptor (Kopan and Ilagan, 2009). Subsequently, the Notch ICD released from the plasma membrane forms a complex with its DNA-binding partner Rbpj (also called CSL) and regulates the transcription of its target genes in the nucleus (Kopan and Ilagan, 2009).

It was previously thought that Notch signaling promoted the formation of proximal tubules and repressed the formation of other nephron segments, especially distal tubules (Cheng et al., 2007, 2003; Surendran et al., 2010). This model was based on several genetic studies of Notch signaling in mouse models. Reports showed that deletion of *Notch2* with $Pax3^{tm1(cre)Joe}$ (*Pax3cre*) inhibits the formation of proximal tubules (Cheng et al., 2007; Liu et al., 2015). However, since *Pax3cre* targets not only the nephron lineage but also the interstitial lineage in the kidney (Engleka et al., 2005), it is possible that the deletion of *Notch2* in the interstitial lineage might have contributed to the mutant phenotype. In fact, when *Notch2* was deleted by the nephron lineage-specific *Tg(Six2-GFP/cre)1Amc* (*Six2GFPcre*) (Kobayashi et al., 2008; Park et al., 2007), proximal tubules were still formed (Surendran et al., 2010). We have recently shown that deletion of *Notch1* and *Notch2* with *Six2GFPcre* arrests nephrogenesis largely at the RV stage and that in this mutant neither proximal nor distal tubules are formed (Chung et al., 2016). The nephron lineage-specific Notch loss-of-function (LOF) study does not therefore support the model that Notch signaling proximalizes the nephron. Although one report showed that constitutive expression of the Notch1 ICD with *Six2GFPcre* resulted in the ectopic formation of proximal tubules (Cheng et al., 2007), another reported that constitutive expression of the Notch2 ICD caused the depletion of $Six2^+$ nephron progenitors without the

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ectopic formation of proximal tubules (Fujimura et al., 2010). The model that Notch signaling proximalizes the nephron is only supported by the Notch1 gain-of-function (GOF) experiment, but not by the Notch2 GOF report. Thus, further investigation is required to determine whether Notch signaling promotes the formation of proximal tubules and represses the formation of distal tubules.

Removing Notch in cap mesenchyme progenitors with *Six2GFPcre* completely blocks nephrogenesis before nephron segmentation initiates (Chung et al., 2016). It is therefore necessary to remove Notch signaling at a later stage of nephrogenesis to study its possible role in segmentation. To this end, we employed *Wnt4*$^{tm3(EGFP/cre)Amc}$ (*Wnt4GFPcre*) (Mugford et al., 2009). Since *Wnt4* is one of the earliest genes to be activated during the differentiation of nephron progenitors (Park et al., 2007; Stark et al., 1994), *Wnt4GFPcre* allowed us to genetically manipulate Notch signaling during the differentiation of nephron progenitors. Here we show that Notch signaling is required for the formation of all nephron segments and does not promote the formation of any specific nephron segment during nephrogenesis. Furthermore, we show that Notch signaling regulates the expression of *Lhx1* and *Hnf1b*, two genes encoding key transcription factors required for proper nephron segmentation. Collectively, our data suggest that Notch signaling primes nephron progenitors for differentiation rather than directing their cell fates into proximal tubules. This work proposes a new model for the role of Notch in nephrogenesis.

RESULTS

Wnt4GFPcre targets early developing nephrons

In order to determine when and where *Wnt4GFPcre* becomes active during nephrogenesis, we performed lineage analysis by examining *Wnt4GFPcre*-activated Rosa reporter (*Rosa26*^{lacZ}) in early developing nephrons. We used *Jag1* expression to determine the stages of developing nephrons (Georgas et al., 2009; Park et al., 2012). Jag1 marked the distal part (closer to the tip of the collecting duct) of the RV (Fig. 1A). *Jag1* was expressed in the comma head portion of the CSB but little Jag1 was detected in the tail of the CSB (Fig. 1B). In the SSB, *Jag1* expression was strongest in the median segment (Fig. 1C). *Wnt4GFPcre* expression was also dynamic during nephrogenesis. *Wnt4GFPcre* was expressed in the entire RV (Fig. 1A, GFP), in the comma head of the CSB (Fig. 1B, GFP), and in the median segment of the SSB (Fig. 1C, GFP). Despite the dynamic expression of *Wnt4GFPcre* during early nephrogenesis, the entire RV, CSB and SSB were marked with Rosa reporter (Fig. 1, β-gal), demonstrating that *Wnt4GFPcre* targets developing nephrons as early as the RV stage.

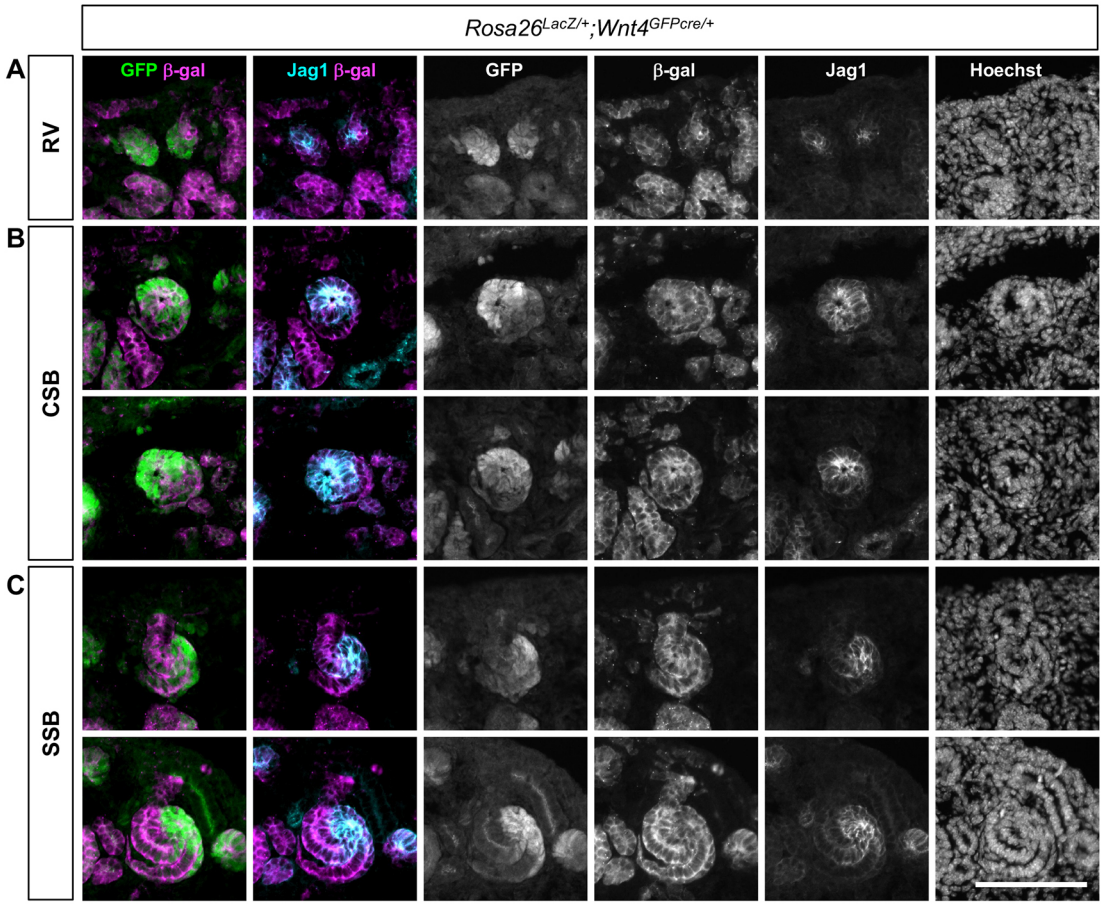

Fig. 1. *Wnt4GFPcre* targets developing nephrons. Rosa reporter (*Rosa26*^{lacZ}) activated by *Wnt4GFPcre* was examined in the renal vesicle (RV) (A), the comma-shaped body (CSB) (B) and the S-shaped body (SSB) (C). *Jag1* expression was used to determine the stages of nephrogenesis. *Jag1* was expressed in the distal part of the RV (A). The *Jag1* expression domain was expanded in the CSB (B). *Jag1* was expressed in the median segment of the SSB (C). *Wnt4GFPcre* was expressed in the entire RV (A). In CSB and SSB, expression of *Wnt4GFPcre* largely overlaps with that of *Jag1* (B,C). Expression of the Rosa reporter indicates that *Wnt4GFPcre* can target the RV and its derivatives despite its dynamic and polarized expression during early nephrogenesis. (A-C) Mouse kidneys at E16.5 are shown. Images are representative of two independent experiments. Scale bar: 100 µm.

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Deletion of Notch with *Wnt4GFPcre* inhibits the formation of all nephron segments

By employing *Wnt4GFPcre*, which targets early developing nephrons, we carried out a Notch LOF study. Since it is known that *Notch1* and *Notch2* act redundantly during nephrogenesis (Surendran et al., 2010), we deleted both *Notch1* and *Notch2* with *Wnt4GFPcre*. In order to trace only those nephron progenitors in which *Wnt4GFPcre*-mediated recombination occurred, we included a lineage tracer (EYFP) in our genetic analysis. It is likely that most of the *Wnt4GFPcre*-activated Rosa EYFP reporter-positive cells also experienced the deletion of conditional alleles of Notch genes. First, we tested how *Wnt4GFPcre*-mediated deletion of Notch genes affects nephron segmentation. It was previously thought that Notch signaling promoted the formation of proximal tubules while repressing the formation of distal tubules (Cheng et al., 2007, 2003; Surendran et al., 2010). If this model is correct, blocking Notch signaling in developing nephrons should allow the formation of distal tubules and inhibit the formation of proximal tubules. We examined nephron segmentation in the Notch mutant kidney by immunofluorescence analysis. We used Wt1 (podocyte), *Lotus tetragonolobus* lectin (LTL) (proximal tubule), Slc12a1 (loop of Henle) and Slc12a3 (distal tubule) to mark specific nephron segments (Fig. 2A). In the control kidney, Rosa EYFP reporter-positive cells could differentiate into all segments of the nephron (Fig. 2A, left). By contrast, in the Notch double-mutant kidneys, the Rosa EYFP reporter-labeled cells failed to form any segment of the nephron (Fig. 2A, right).

In order to quantify defects of nephron segmentation in the Notch double-mutant kidneys, we performed quantitative reverse-transcription PCR (RT-qPCR) of genes expressed in the specific segments of the nephron (Fig. 2B). In the nephron lineage, *Nphs2* and *Slc34a1* are expressed specifically in podocytes and proximal tubules, respectively (Lee et al., 2015; Moeller et al., 2003). *Slc12a1* and *Slc12a3* are expressed specifically in the loop of Henle and distal tubules, respectively (Lee et al., 2015). Consistent with our immunofluorescence analysis (Fig. 2A), the Notch double-mutant kidneys showed severe defects in the formation of all nephron segments (Fig. 2B). Contrary to the previous model of mammalian nephron segmentation, our data showed that Notch signaling is required for the formation of all nephron segments, not just for the proximal tubule segment.

Notch signaling is required for the formation of the SSB and for robust expression of *Lhx1* and *Hnf1b*

In order to investigate mechanisms underlying nephron segmentation defects seen in the Notch double-mutant kidney, we examined early nephrogenesis. First, we tested whether the deletion of Notch genes with *Wnt4GFPcre* still allows nephron progenitors to undergo MET, using Cdh1 (E-cadherin) as an epithelial marker (Hay, 2005). We found that EYFP$^+$ Notch double-mutant cells could become Cdh1$^+$ epithelial cells (Fig. 3A) but that these EYFP$^+$ cells failed to form the SSB (Fig. 3B, Fig. S1). Whereas in the control kidney (Fig. 3B, left) there was abundant adjacent expression of *Jag1* and *Wt1* marking the SSB (arrows), these structures were missing in the mutant kidney (Fig. 3B, right), suggesting the absence of SSB in the Notch mutant. The mutant kidney did contain some Wt1$^+$ or Jag1$^+$ cells (arrows in Fig. 3B, right) in developing nephrons but these were largely negative for EYFP, meaning that they had escaped *Wnt4GFPcre*-mediated recombination and were likely to have intact Notch. Our data suggest that, during nephrogenesis, Notch signaling is required for the formation of the SSB. Although *Wnt4GFPcre* targeted a subset of mesenchymal nephron progenitors (Fig. 3A), genetic manipulation of Notch signaling with *Wnt4GFPcre* did not affect the expression of *Six2*, *Pax2* and *Wt1* in the cap mesenchyme (Fig. S2A,B), which suggests that the nephrogenesis

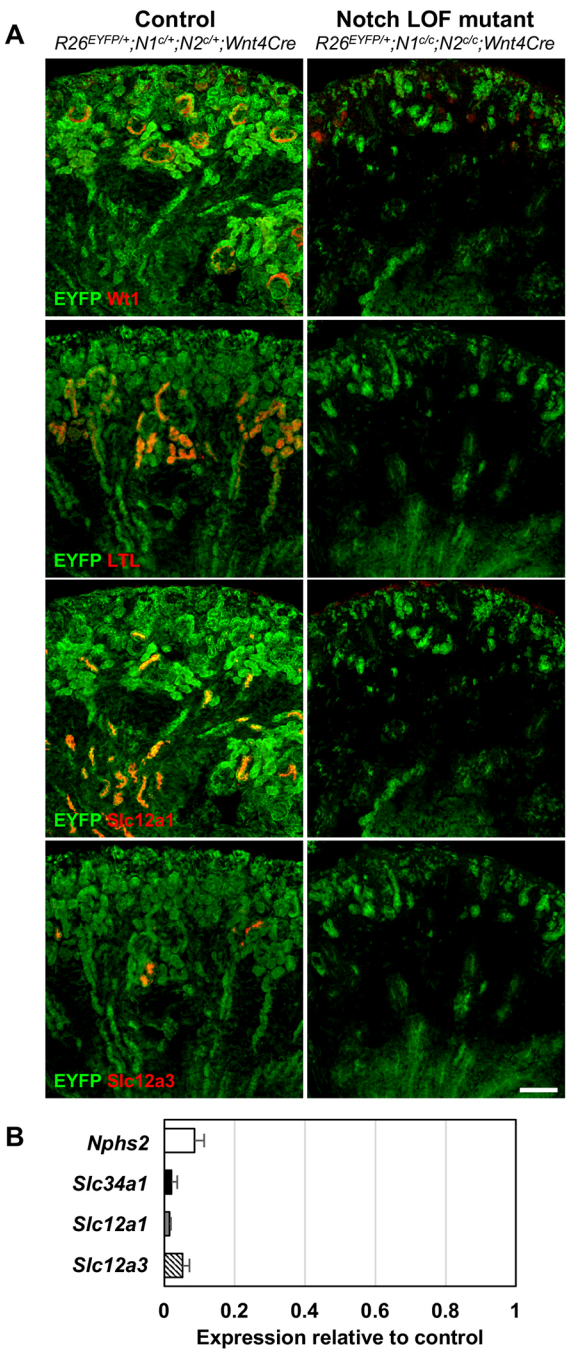

Fig. 2. Deletion of Notch with *Wnt4GFPcre* inhibits the formation of all nephron segments. (A) Lineage analysis of Wnt4$^+$ cells shows that the nephron segmentation process is blocked in the *Notch1* and *Notch2* double-mutant kidney. In the control kidney (left), Rosa EYFP reporter-positive cells form Wt1$^+$ podocytes, LTL$^+$ proximal tubules, Slc12a1$^+$ loop of Henle, and Slc12a3$^+$ distal tubules. In the Notch double-mutant kidney (right), EYFP$^+$ cells fail to develop into any segment of the nephron. *N1^c* and *N2^c* indicate conditional alleles of *Notch1* and *Notch2*, respectively. Kidneys at E18.5 are shown. Images are representative of two independent experiments. Scale bar: 100 µm. (B) RT-qPCR of genes expressed in specific segments of the nephron shows that all nephron segments are poorly formed in the Notch double-mutant kidney at E18.5. *Nphs2* and *Slc34a1* are specifically expressed in podocytes and proximal tubules, respectively. Error bars indicate s.d., *n*=4.

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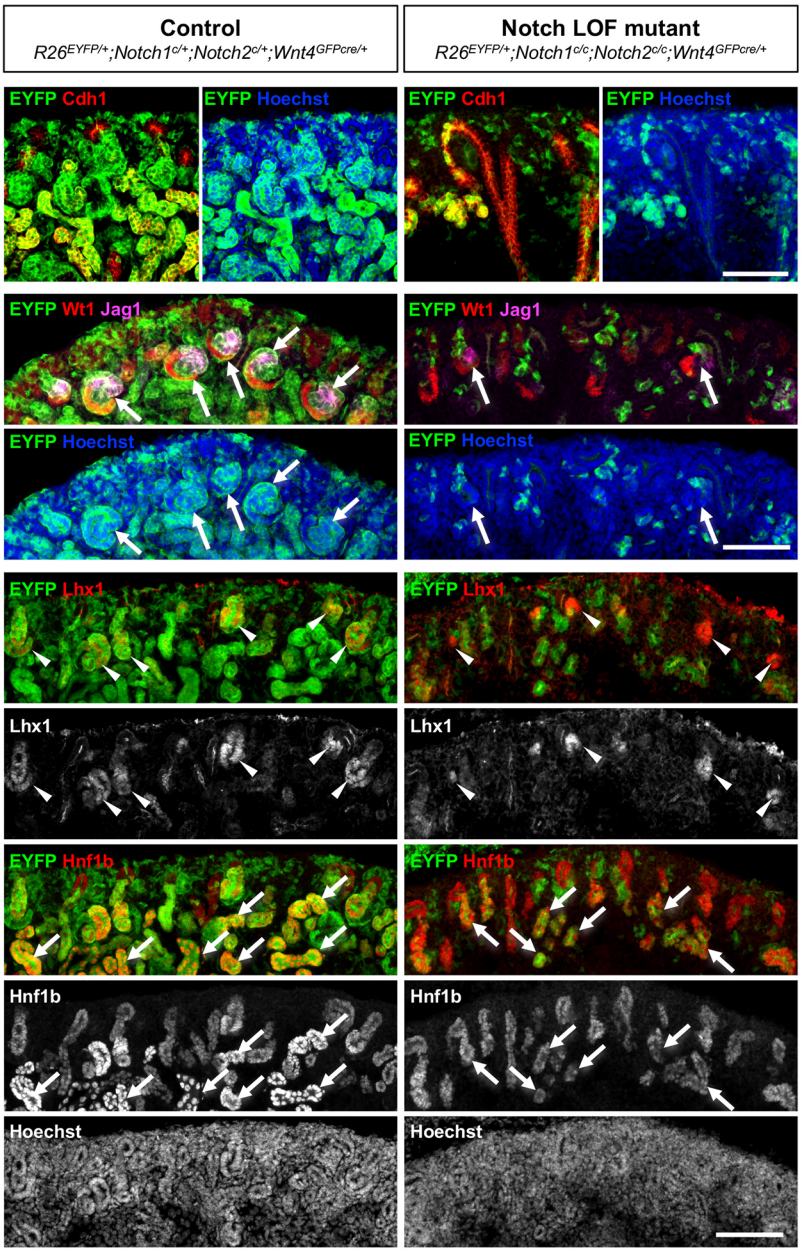

Fig. 3. Deletion of Notch with *Wnt4GFPcre* causes defects in early nephrogenesis. (A) In both control (left) and mutant (right), Rosa EYFP reporter-positive cells differentiate into Cdh1$^+$ epithelial cells, suggesting that the Notch mutant cells can undergo MET. (B) In the control kidney, Rosa EYFP reporter-positive cells form the SSB (arrows). The SSB exhibits a characteristic Jag1$^+$ median segment and Wt1$^+$ proximal segment. In the Notch mutant kidney, EYFP$^+$ cells fail to form SSB. Most of the Wt1$^+$ proximal segment of the SSB in the mutant kidney is negative for EYFP, suggesting that these Wt1$^+$ cells have intact Notch. (C) In the control kidney, EYFP$^+$ cells develop into Lhx1$^+$ cells (arrowheads). In the Notch mutant kidney, most of the Lhx1$^+$ cells (arrowheads) are negative for EYFP, suggesting that Notch signaling is required for expression of *Lhx1*. In addition, expression of *Hnf1b* in the EYFP$^+$ cells is significantly reduced in the Notch mutant kidney (arrows), suggesting that Notch signaling is required for robust expression of *Hnf1b*. (A-C) Kidneys at E18.5 are shown. Images are representative of two independent experiments. Scale bars: 100 µm.

defect in the Notch LOF mutant kidney was not caused by premature depletion of mesenchymal nephron progenitors.

In Notch LOF and GOF studies employing *Six2GFPcre*, which targets undifferentiated nephron progenitors, we have previously shown that Notch signaling downregulates *Six2* (Chung et al., 2016). Although *Wnt4GFPcre* acts later than *Six2GFPcre*, both *Cre* lines appear to cause similar Notch LOF mutant phenotypes. This suggests that Notch signaling plays additional roles, such as activating key differentiation genes, in developing nephrons after *Six2* is downregulated. We tested if Notch signaling is required for the activation of *Lhx1* and *Hnf1b*, two transcription factors required for proper nephron segmentation. In the *Lhx1* mutant, nephron progenitors are known to be arrested at RV during nephrogenesis (Kobayashi et al., 2005) and *Hnf1b* mutant nephron progenitors fail to develop into the SSB (Heliot et al., 2013; Massa et al., 2013). In kidneys at embryonic day (E) 18.5, *Lhx1* appears to be expressed in the nephron lineage but *Hnf1b* is expressed in both the collecting duct and developing nephrons. Since *Wnt4GFPcre* targets mostly the nephron lineage, EYFP$^-$ Hnf1b$^+$ cells are either collecting duct cells or nephron lineage cells that escaped Cre-mediated recombination. In the control kidney, the wild-type nephron progenitors marked with EYFP could differentiate into Lhx1$^+$ cells or Hnf1b$^+$ cells, as expected (Fig. 3C, left). When both *Notch1* and *Notch2* were deleted by *Wnt4GFPcre*, Notch LOF mutant (EYFP$^+$) cells could also differentiate into Lhx1$^+$ or Hnf1b$^+$ cells (Fig. 3C, right), although expression levels of *Lhx1* and *Hnf1b* were significantly lower in the Notch LOF compared with control cells (Fig. 3C, right versus left). The Notch LOF mutant kidney contained some cells that express *Lhx1* at higher levels (arrowheads in Fig. 3C, right) but most of these were negative for EYFP, suggesting that they had escaped Cre-mediated deletion of Notch genes and that Notch signaling is intact in these cells. In the control kidney (Fig. 3C, left), expression of *Hnf1b* tended to be higher in EYFP$^+$ Lhx1$^-$ cells (arrows) than in EYFP$^+$ Lhx1$^+$ cells (arrowheads), suggesting that expression of *Hnf1b* was upregulated where *Lhx1* was downregulated. However, in the Notch LOF mutant kidney (Fig. 3C, right), expression of *Hnf1b* was comparable in Lhx1$^+$

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and Lhx1^{-} cells. Collectively, these results suggest that Notch signaling is required for robust expression of *Lhx1* and *Hnf1b*.

Constitutive activation of Notch signaling by *Wnt4GFPcre* does not promote the formation of a specific segment of the nephron

The results of our Notch LOF study were inconsistent with the previous model, in which Notch signaling proximalizes the mammalian nephron (Cheng et al., 2007, 2003; Surendran et al., 2010). To address this discrepancy, we performed Notch GOF studies. The fact that *Wnt4GFPcre*-mediated deletion of Notch blocks nephron segmentation suggests that *Wnt4GFPcre* becomes active before nephron segmentation occurs. We activated the expression of an active form of Notch1 (NICD) in developing nephrons with *Wnt4GFPcre*. If Notch signaling promotes the formation of proximal tubule and represses the formation of other nephron segments, then the NICD-expressing cells should preferentially differentiate into proximal tubules. In order to trace only those nephron tubules with constitutive activation of Notch signaling, we included the Rosa EYFP reporter in the analysis. Interestingly, we found that EYFP^{+} cells with constitutive activation of Notch signaling could differentiate into any nephron segment (Fig. 4A). Quantitation of nephron segmentation by RT-qPCR analysis showed that the Notch GOF mutant kidney exhibited largely normal nephron segmentation without increase or decrease in any specific nephron segment (Fig. 4B). Our data strongly suggest that constitutive activation of Notch signaling during the differentiation of nephron progenitors does not affect nephron segmentation. Despite largely normal nephron segmentation, the Notch GOF mutant kidneys were glomerulocystic (Fig. 4C, Fig. S3B).

Constitutive activation of Notch signaling by *Six2GFPcre* does not convert all nephron progenitors into proximal tubules

It was previously reported that constitutive expression of NICD in undifferentiated nephron progenitors with *Six2GFPcre* promoted the formation of proximal tubules while inhibiting the formation of distal tubules and podocytes (Cheng et al., 2007). This conclusion was, in part, based on the observation of ectopic formation of LTL-stained (LTL^{+}) proximal tubules in the Notch GOF mutant kidney by *Six2GFPcre* (Cheng et al., 2007). However, it has not been definitively addressed whether other types of cells are present in the Notch GOF kidney. We generated the same Notch GOF mutant kidney with *Six2GFPcre* and included a Rosa reporter (EYFP or β-galactosidase) to label Notch GOF cells in the nephron lineage (*Rosa26*$^{EYFP/NICD}$*;Six2GFPcre* or *Rosa26*$^{lacZ/NICD}$*;Six2GFPcre*).

We found that only a subset of Cdh1^{+} epithelial cells were positive for LTL staining and that most of the epithelial cells were negative for LTL (white arrows in Fig. 5A, first row) in the mutant kidney, suggesting that constitutive activation of Notch signaling does not convert all nephron progenitors into proximal tubules. The Notch GOF mutant kidney formed Wt1^{+} Mafb^{+} podocytes, which adopted the typical crescent configuration found in glomeruli (yellow arrow in Fig. 5A, second row). Similar to the Notch GOF mutant kidneys generated with *Wnt4GFPcre* (Fig. 4C, Fig. S3B), the glomeruli formed in the Notch GOF mutant kidneys with *Six2GFPcre* were also cystic (Fig. 5A, second row, and Fig. S4B). These results suggest that nephron progenitors with constitutive activation of Notch signaling can differentiate into podocytes and Bowman's capsule. Strikingly, there were large clusters of Rosa reporter-positive Notch GOF mutant cells that were positive for Wt1 and negative for Cdh1 and Mafb (white arrowheads in Fig. 5A, second and third rows). These cells form clusters without forming a lumen, suggesting that they did not complete MET. Taken together, these data (Fig. 5A) suggest that constitutive activation of Notch signaling in mesenchymal nephron progenitors generates a heterogeneous population of various cell

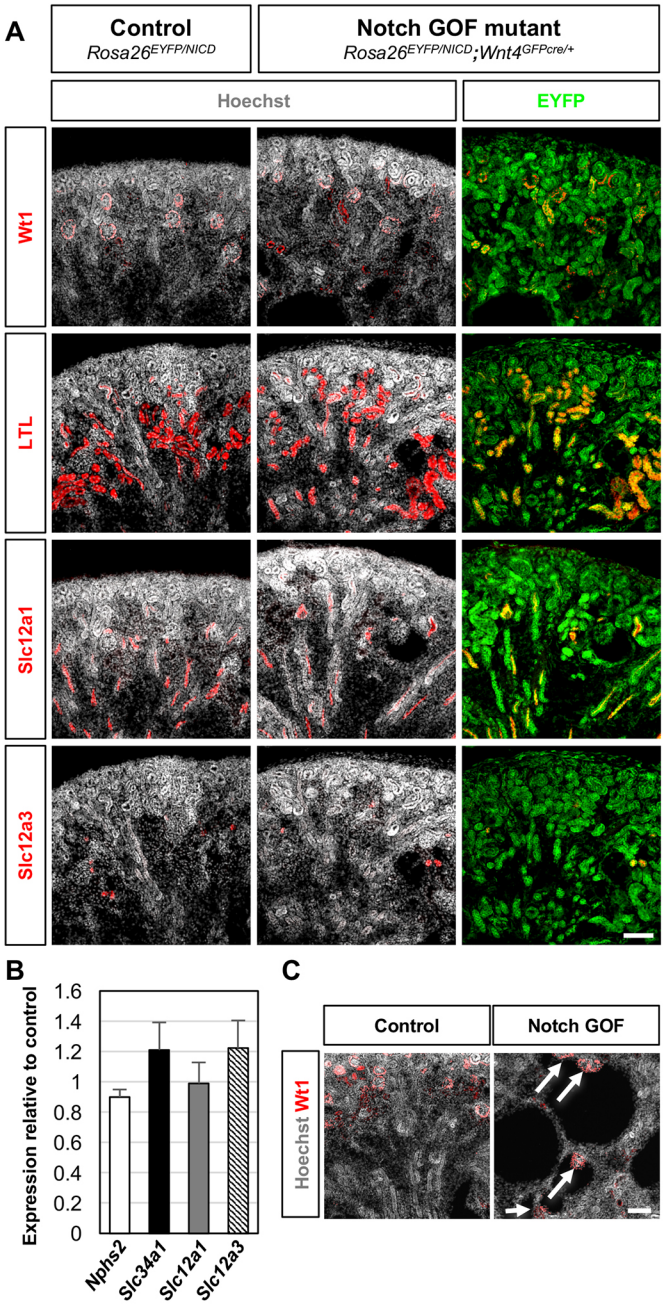

Fig. 4. Constitutive activation of Notch signaling by *Wnt4GFPcre* does not promote the formation of a specific segment of the nephron. (A) Lineage analysis of Wnt4^{+} cells shows that Notch gain-of-function (GOF) mutant cells undergo normal nephron segmentation. Rosa EYFP reporter-positive Notch GOF cells form Wt1^{+} podocytes, LTL^{+} proximal tubules, Slc12a1^{+} loop of Henle, and Slc12a3^{+} distal tubules. Since *Wnt4GFPcre* is absent in the control kidney and present in the mutant kidney, the Rosa EYFP is active only in the Notch GOF kidney. (B) RT-qPCR of nephron segmentation marker genes shows that constitutive activation of Notch signaling by *Wnt4GFPcre* does not promote the formation of a specific segment of the nephron. Error bars indicate s.d., *n*=4. (C) Activation of Notch signaling by *Wnt4GFPcre* causes cystic dilation of Bowman's capsule. Wt1^{+} podocytes in glomerulocysts are marked with arrows. (A-C) Kidneys at E18.5 are shown. Images are representative of two independent experiments. Scale bars: 100 µm.

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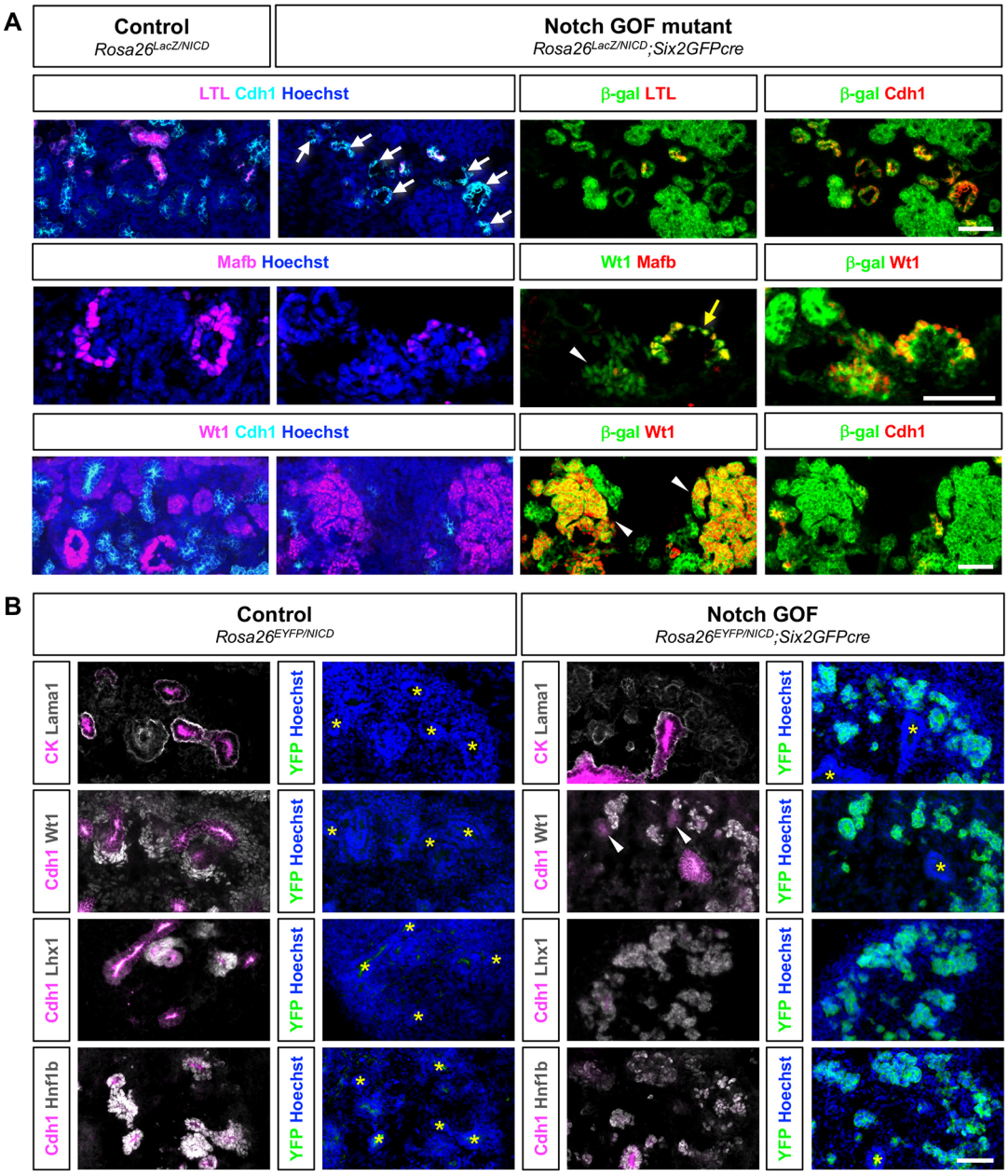

Fig. 5. Constitutive activation of Notch signaling by *Six2GFPcre* primes nephron progenitors for differentiation and leads to the formation of a heterogeneous population of cells. (A) New born (P0) kidneys are shown. Notch GOF mutant cells are labeled with the Rosa26 reporter (β-galactosidase) and epithelial cells are marked with Cdh1. Only a subset of epithelial cells is positive for LTL staining, with the majority negative for LTL (white arrows, first row), suggesting that not all Notch GOF mutant cells develop into proximal tubules. Notch GOF mutant cells can form $Wt1^+$ $Mafb^+$ podocytes (yellow arrow, second row). Most of the Notch GOF mutant cells are $Wt1^+$ $Cdh1^-$ and form clusters (white arrowheads, third row), and these cells do not appear to form a lumen. (B) Kidneys at E13.5. In the control kidney, *Lama1*, *Cdh1* and *Hnf1b* are expressed in the developing nephrons and collecting duct but these genes are not expressed in the cap mesenchyme. The collecting duct is positive for cytokeratin (CK) and the developing nephrons are positive for Lhx1. Wt1 is weakly expressed in the cap mesenchyme and highly expressed in the proximal segment of the SSB. Notch GOF mutant cells are labeled with YFP and most of them express *Lama1*, *Lhx1* and *Hnf1b*, similar to developing nephrons. YFP^+ $Wt1^-$ cells express Cdh1 (arrowheads), suggesting that the Notch GOF cells have completed MET. The lumens of the collecting ducts are labeled with asterisks. (A,B) Images are representative of two independent experiments. Scale bars: 100 µm.

types, rather than converting all nephron progenitors exclusively into proximal tubules.

Constitutive activation of Notch signaling by *Six2GFPcre* causes ectopic expression of *Lhx1* and *Hnf1b* in nephron progenitors

To investigate the direct effect of activation of Notch signaling in mesenchymal nephron progenitors, we examined the Notch GOF mutant kidney by *Six2GFPcre* at an earlier stage (E13.5). Since the results of our Notch LOF study (Fig. 3C) suggest that Notch signaling is required for robust expression of *Lhx1* and *Hnf1b*, two transcription factors required for proper nephron segmentation, we tested whether constitutive activation of Notch signaling in mesenchymal nephron progenitors causes ectopic expression of *Lhx1* and *Hnf1b*. We have previously shown that constitutive expression of Notch1 ICD with *Six2GFPcre* downregulates *Six2* at E13.5 (Chung et al., 2016). Since *Six2* is absent in the Notch GOF mutant kidney at this stage, the YFP signal seen in Fig. 5B is likely

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to be from the Rosa EYFP reporter rather than from *Six2GFPcre*. In the control kidney (Fig. 5B, left), developing nephrons expressed *Lama1*, *Lhx1* and *Hnf1b*. These genes were also expressed in the collecting duct but not in the cap mesenchyme. *Wt1* was weakly expressed in the cap mesenchyme but highly expressed in the proximal segments of the SSB. Most of the EYFP$^+$ Notch GOF mutant cells were positive for Wt1 and negative for Cdh1 (Fig. 5B, right, second row); only a subset of Notch GOF cells became Cdh1$^+$ epithelial cells (arrowheads in Fig. 5B, right, second row). We found that most of the EYFP$^+$ Notch GOF mutant cells were positive for *Lama1*, *Lhx1* and *Hnf1b*, showing early signs of differentiation (Fig. 5B, right). These data (Fig. 5B) suggest that activation of Notch signaling primes nephron progenitors for differentiation by activating expression of *Lhx1* and *Hnf1b*.

DISCUSSION

We have previously shown that Notch signaling promotes nephrogenesis by downregulating the expression of *Six2*, a key transcription factor required for the maintenance of nephron progenitors (Chung et al., 2016). In that study, we performed Notch LOF and GOF analyses with *Six2GFPcre*, which targets undifferentiated nephron progenitors (Kobayashi et al., 2008; Park et al., 2007). Since *Six2GFPcre*-mediated deletion of Notch causes the differentiation of nephron progenitors to be arrested largely at RV, it does not allow us to study the role of Notch signaling in nephron segmentation. Here, to explore the role of Notch during nephron segmentation, we employed *Wnt4GFPcre*. *Wnt4* is one of the earliest genes to be activated during the differentiation of nephron progenitors (Park et al., 2007; Stark et al., 1994). We have previously shown that Wnt/β-catenin signaling initiates the differentiation of nephron progenitors and that *Wnt4* is directly upregulated by Wnt/β-catenin signaling (Park et al., 2012, 2007). Our lineage analysis showed that *Wnt4GFPcre* targeted early developing nephron structures, including RV, CSB and SSB, where Notch signaling is active (Fig. 1). This allowed us to investigate the roles of Notch signaling in nephron segmentation.

As we reported previously (Brunskill et al., 2014), undifferentiated nephron progenitors in the cap mesenchyme were mosaically labeled with *Wnt4GFPcre*-activated Rosa reporter (Fig. 1). Although a subset of the cap mesenchymal cells was targeted by *Wnt4GFPcre*, cells differentiated further when Notch genes were deleted with *Wnt4GFPcre* than with *Six2GFPcre*, suggesting that *Wnt4GFPcre* acts later than *Six2GFPcre*. We have previously shown that, when Notch genes were deleted with *Six2GFPcre*, differentiation of the Notch mutant cells was arrested largely at the RV, which is negative for Cdh1 (Chung et al., 2016). However, when *Wnt4GFPcre* was used, more Cdh1$^+$ epithelial cells were formed (Fig. 3A, right), although these epithelial cells failed to differentiate into the SSB (Fig. 3B, Fig. S1B) or mature nephron segments (Fig. 2). These results suggest that Notch signaling is required for proper nephron segmentation. Considering that *Wnt4GFPcre* acts later than *Six2GFPcre*, the fact that deletion of Notch genes by either *Wnt4GFPcre* or *Six2GFPcre* inhibits nephron segmentation suggests later roles for Notch signaling after *Six2* is downregulated. Consistent with this, we found that Notch signaling regulates the expression of *Lhx1* and *Hnf1b*, two genes encoding key transcription factors required for proper nephron segmentation.

It has been reported that different parts of the RV exhibit differential gene expression (Cho et al., 1998; Georgas et al., 2009), possibly setting the stage for nephron segmentation. The RV was formed regardless of whether Notch genes were deleted with either *Six2GFPcre* or *Wnt4GFPcre*. However, the proximodistal axis of the RV was affected by the Cre used. When Notch genes were deleted with *Six2GFPcre*, *Lhx1* was expressed in the entire RV (Fig. S5), suggesting that this mutant kidney failed to establish a proper proximodistal axis at the RV. When Notch genes were deleted with *Wnt4GFPcre*, the distal part of the RV expressed *Jag1* and the proximal part expressed *Wt1* (arrowheads in Fig. S1B), suggesting that the proximodistal axis of the RV was established properly. This discrepancy is likely to be due to the fact that *Wnt4GFPcre* acts later than *Six2GFPcre*. Taken together, these results suggest that Notch signaling is required for the establishment of the proximodistal axis at the RV.

The previous model of mammalian nephrogenesis suggested that Notch signaling promotes the formation of proximal tubules and represses the formation of distal tubules (Cheng et al., 2007, 2003; Surendran et al., 2010). According to this model, the deletion of *Notch1* and *Notch2* with *Wnt4GFPcre* should have blocked the formation of proximal tubules, while still allowing the formation of distal tubules. However, our results showed that the Notch mutant cells failed to develop into any type of nephron segment, not just proximal tubules (Fig. 2). Consistent with this, the Notch LOF mutant cells also failed to form the SSB, which is thought to be a key intermediate structure for proper nephron segmentation (Fig. 3B, Fig. S1B). Our data suggest that, contrary to the previous model, Notch signaling is required for the formation of all nephron segments, not just proximal tubules.

We found that *Wnt4GFPcre*-mediated activation of Notch signaling in differentiating nephron progenitors did not promote the formation of the proximal tubule exclusively (Fig. 4). Considering that *Wnt4GFPcre* can target the RV and its derivatives (Fig. 1), activation of Notch ICD with *Wnt4GFPcre* should have caused differentiating nephron progenitors to experience constitutive activation of Notch signaling, instead of the regionalized activation of Notch signaling seen during normal differentiation of nephron progenitors. Surprisingly, constitutive activation of Notch signaling with *Wnt4GFPcre* neither increased nor decreased any specific nephron segment (Fig. 4), suggesting that Notch signaling does not favor the proximal tubule cell fate over other cell fates during nephron segmentation. Consistent with this, the Notch GOF mutant kidney generated with *Wnt4GFPcre* formed the SSB properly (Fig. S1C). Our data definitively showed that nephron tubules with constitutive activation of Notch signaling could form all nephron segments, suggesting that Notch signaling does not promote or repress the formation of a specific nephron segment.

Two Notch GOF studies have previously reported the targeting of undifferentiated nephron progenitors with *Six2GFPcre*. One study reported that expression of Notch1 ICD promoted the formation of proximal tubules (Cheng et al., 2007). The other study reported that expression of Notch2 ICD caused the depletion of Six2$^+$ nephron progenitors without the ectopic formation of proximal tubules (Fujimura et al., 2010). The *Notch1* ICD allele encodes a truncated Notch1 ICD lacking the PEST domain, while the *Notch2* ICD allele encodes the full-length Notch2 ICD containing the PEST domain (Fujimura et al., 2010; Murtaugh et al., 2003). The PEST domain is involved in the degradation of Notch (Chiang et al., 2006; Fryer et al., 2004). It was previously thought that differences in either the specific type of Notch or the stability of the two Notch ICDs might have caused the different phenotypes. Despite the discrepancy between these two Notch GOF studies, the long-held view was that, during nephrogenesis, activation of Notch signaling promotes proximal tubule formation while repressing the formation of other

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nephron segments (Park and Kopan, 2015). However, we show here that constitutive expression of Notch1 ICD with *Six2GFPcre* results in a phenotype similar to that observed in the previous Notch2 GOF study, namely that both LTL^+ and LTL^- epithelial cells were formed as well as glomerulocysts. Although it is not known if clusters of $Wt1^+$ $Cdh1^-$ cells are present in the Notch2 GOF mutant kidney, it was shown that expression of Notch2 ICD in mesenchymal nephron progenitor cells downregulates *Six2*, but not *Wt1*, at E11.5 (Fujimura et al., 2010). Our data show that the phenotype caused by the expression of Notch1 ICD in $Six2^+$ cells is more similar to that caused by the expression of Notch2 ICD than previously thought. The fact that *Six2* is downregulated by the expression of either Notch1 ICD or Notch2 ICD is consistent with the notion that the ICDs of Notch1 and Notch2 are functionally equivalent (Liu et al., 2015). It appears that ectopic proximal tubule formation occurs only when the Notch1 GOF mutant kidneys were cultured *in vitro* as explants (Boyle et al., 2011; Cheng et al., 2007). It is possible that the *in vitro* culture condition for kidney explants may have promoted the formation of proximal tubules.

Contrary to a previous report (Cheng et al., 2007), we found that a significant portion of nephron progenitors expressing Notch1 ICD remained positive for Wt1 and negative for Cdh1 at P0 (Fig. 5A, third row). It is difficult to define the exact nature of these cells because they show features of both developing nephrons and mesenchymal nephron progenitors. Similar to developing nephrons, these cells expressed *Lhx1*, *Hnf1b* and *Lama1*, but they also expressed *Wt1*, a key transcription factor expressed in nephron progenitors. We have previously shown that constitutive expression of Notch1 ICD in mesenchymal nephron progenitors with *Six2GFPcre* downregulates several key mesenchymal genes, including *Six2*, while not affecting *Osr1* expression (Chung et al., 2016). One possibility is that persistent expression of *Wt1* or *Osr1* or both prevents these cells from completing MET despite constitutive activation of Notch signaling. Consistent with this idea, *Wt1* was downregulated in the small portion of Notch GOF nephron progenitors that did become epithelialized at E13.5 (Fig. 5B). Another possibility is that these $Wt1^+$ $Cdh1^-$ cells might express different cadherins. It has been shown that the proximal segment of the SSB expresses *Wt1* and *Cdh6* (Cho et al., 1998).

We believe that our characterization of the Notch GOF mutant kidney by *Six2GFPcre* at an early developmental stage (E13.5) better elucidates the direct effect of Notch signaling. Most of the Notch GOF mutant progenitor cells at E13.5 showed ectopic expression of *Lhx1* and *Hnf1b* (Fig. 5B). Our data suggest that the major role of Notch signaling is to prime nephron progenitors for differentiation by downregulating *Six2* (Chung et al., 2016) and upregulating *Lhx1* and *Hnf1b* rather than dictating their cell fates into a specific segment of the nephron. Lack of robust expression of *Lhx1* and *Hnf1b* may be responsible for poor nephron segmentation in the Notch LOF mutant kidney (Fig. 3C).

During nephrogenesis, developing nephrons are positioned adjacent to the collecting duct and stroma. These neighboring cells may provide important signals that are required for proper elongation of the RV to form nephron tubules. Since the Notch GOF mutant kidney by *Six2GFPcre* is severely defective in branching of the collecting duct (Cheng et al., 2007), the precise positioning of different types of cells surrounding the developing nephrons is disrupted, resulting in a failure to provide the correct developmental cues for proper elongation of the RV into nephron tubules (Fig. 5A, Fig. S4). By contrast, the nephron tubules derived from the Notch GOF mutant cells by *Wnt4GFPcre* appeared to elongate normally (Fig. 4A, Fig. S3B).

Our results presented here collectively suggest that Notch signaling is required for the formation of all nephron segments and that Notch signaling does not promote the formation of a specific nephron segment during mammalian nephrogenesis. Our finding that Notch signaling does not proximalize the mammalian nephron is consistent with the model for pronephros segmentation in the zebrafish. In the zebrafish pronephros, which shares a remarkably similar segmentation pattern with mammalian nephrons (Desgrange and Cereghini, 2015; Naylor and Davidson, 2017), Notch signaling does not promote the formation of the proximal tubule segment. Rather, Notch signaling regulates the binary cell fate decision between multi-ciliated cells and transporting cells, resulting in the salt-and-pepper distribution pattern of these two types of cells along the pronephros (Liu et al., 2007). It remains to be further investigated how Notch-mediated binary cell fate decisions apply to mammalian nephrogenesis.

Our findings provide crucial insight into how to generate nephron tubules *in vitro* for potential cell replacement therapy. We have previously shown that Notch signaling in nephron progenitors can be activated by transient activation of Wnt/β-catenin signaling (Park et al., 2012). To take advantage of this endogenous activation of Notch signaling for the generation of nephron tubules *in vitro*, cell-to-cell interaction is required, which can be achieved by maintaining nephron progenitors in aggregates. Our findings predict that activation of Notch signaling is required for, and should be compatible with, the generation of all nephron segments *in vitro* and that, unless exogenous Notch input is provided, dispersed nephron progenitors will fail to form nephron tubules properly.

MATERIALS AND METHODS

Mouse strains

Wnt4$^{tm3(EGFP/cre)Amc}$ (*Wnt4GFPcre*) (Mugford et al., 2009), *Notch1*^{tm2Rko} (*Notch1*$^{c/c}$) (Yang et al., 2004), *Notch2*^{tm3Grid} (*Notch2*$^{c/c}$) (McCright et al., 2006), *Rbpj*^{tm1Hon} (*Rbpj*$^{c/c}$) (Tanigaki et al., 2002), *Gt(ROSA)26Sor*$^{tm1(Notch1)Dam}$ (*Rosa26*^{Notch1ICD}) (Murtaugh et al., 2003), *Gt(ROSA)26Sor*$^{tm1(EYFP)Cos}$ (*Rosa26*^{EYFP}, also known as R26R-EYFP) (Srinivas et al., 2001), *Gt(ROSA)26Sor*^{tm1Sor} (*Rosa26*^{lacZ}, also known as R26R) (Soriano, 1999), and *Tg(Six2-GFP/cre)1Amc* (*Six2GFPcre*) (Kobayashi et al., 2008; Park et al., 2007) mice were described previously. All mice were maintained in the Cincinnati Children's Hospital Medical Center (CCHMC) animal facility according to animal care regulations. The Animal Studies Committee at CCHMC approved the experimental protocols (IACUC2013-0105 and IACUC2017-0037). We adhere to the NIH Guide for the Care and Use of Laboratory Animals.

Immunofluorescence

Embryonic or newborn (P0) kidneys were fixed with 4% paraformaldehyde (PFA) in PBS for 10 min, incubated in 10% sucrose in PBS at 4°C overnight, and imbedded the following day in OCT (Fisher Scientific). We obtained 10 µm cryosections and incubated them overnight with 5% heat-inactivated sheep serum/PBST (PBS with 0.1% Triton X-100) containing primary antibodies (Table S1). Fluorophore-labeled secondary antibodies (Invitrogen or Jackson ImmunoResearch) were used for indirect visualization. Nuclei were stained using Hoechst 33342 (Invitrogen, H3570). Images were taken by wide-field microscopy with a Nikon TiE microscope with Andor Zyla 4.2 camera and Lumencor SpectraX light source housed at the Confocal Imaging Core (CIC) at CCHMC.

RT-qPCR

Control or Notch mutant kidneys at E18.5 were dissected out and total RNA was extracted using the Qiagen RNeasy Micro Kit according to the manufacturer's instructions for microdissected tissue. Starting with 1 µg total RNA, we obtained cDNA by reverse transcription using the RevertAid cDNA Synthesis Kit (Thermo Scientific, K1621). Quantitative PCR was

performed on an Applied Biosystems StepOne Plus device (Thermo Scientific) using Power SYBR Green PCR Master Mix (Thermo Scientific, 4368706). Oligonucleotide primers (5′-3′, forward and reverse) were: *Gapdh* (used as internal control), CAACTTTGTCAAGCTCATTTCCTG and CCTCTCTTGCTCAGTGTCCTT; *Nphs2*, CTCTGGCCCTAACAT-CTCCA and TTCAGTGAGCAAGCAACCAG; *Slc34a1*, TGCTGAGA-GACACTCCGTTG and TATTGGGGTGGCAAATTCTC; *Slc12a1*, AGCGGGCTCTCCTTAAGTTC and CTCAGGAGGCCAAGCAGAAT; *Slc12a3*, AGCTGGAGAAGAGGCTTCAA and TGCAACTTCAAGGT-CCAGAA. Biological replicates of control and Notch mutant kidneys were used as indicated in the figure legends. Fold change calculations were performed using the ΔΔCt method.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: E.C., J.-S.P.; Methodology: E.C., P.D., J.-S.P.; Formal analysis: E.C., J.-S.P.; Investigation: E.C., J.-S.P.; Resources: J.-S.P.; Data curation: E.C., P.D., J.-S.P.; Writing - original draft: E.C., J.-S.P.; Writing - review & editing: E.C., J.-S.P.; Supervision: J.-S.P.; Project administration: J.-S.P.; Funding acquisition: J.-S.P.

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Supplementary information
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