Notch signaling promotes nephrogenesis by downregulating Six2

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Summary statement:
Chung et al. found that, during nephrogenesis, Notch signaling is necessary and sufficient for downregulation of Six2, a transcription factor required for nephron progenitor maintenance, and that nephron progenitors lacking Notch signaling fail to differentiate into any nephron segments.
ABSTRACT

During nephrogenesis, multipotent mesenchymal nephron progenitors develop into distinct epithelial segments. Each nephron segment has distinct cell types and physiological function. In the current model of kidney development, Notch signaling promotes the formation of proximal tubules and represses the formation of distal tubules. Here, we present a new role of Notch in nephrogenesis. We show that differentiation of nephron progenitors requires downregulation of Six2, a transcription factor required for progenitor maintenance, and that Notch signaling is necessary and sufficient for downregulation of Six2. Furthermore, we find that nephron progenitors lacking Notch signaling fail to differentiate into any nephron segments, not just proximal tubules. Our results demonstrate how cell fates of progenitors are regulated by a transcription factor governing progenitor status and a differentiation signal in nephrogenesis.
INTRODUCTION

In mammals, nephrons are formed only during development with nephrogenesis stopping at 36 weeks gestation for humans and by the fourth postnatal day in mice (Hartman et al., 2007; Hinchliffe et al., 1991; Rumballe et al., 2011). Nephron progenitors residing at the cortex of the developing kidney undergo mesenchymal-to-epithelial transition (MET) and give rise to all of the epithelial cells of the nephron (McMahon, 2016). In order to generate a sufficient number of nephrons, it is critical to balance self-renewal and differentiation of nephron progenitors before they are depleted around birth.

Undifferentiated nephron progenitors express Six2, a homeobox transcription factor whose expression is gradually lost as these progenitors undergo differentiation (Kobayashi et al., 2008; Park et al., 2012; Self et al., 2006). Deletion of Six2 disrupts the balance between self-renewal and differentiation of nephron progenitors, resulting in premature depletion of nephron progenitors accompanied by ectopic nephrogenesis (Kobayashi et al., 2008; Self et al., 2006). This illustrates the pivotal role of Six2 in the maintenance of progenitors and implies that downregulation of Six2 is a critical step for the differentiation of nephron progenitors.

In addition to Six2, the Wnt/β-catenin and Notch signaling pathways are known to regulate cell fate decisions during nephrogenesis (McMahon, 2016). Activation of Wnt/β-catenin signaling blocks degradation of β-catenin, allowing β-catenin to regulate expression of its target genes in the nucleus (Clevers and Nusse, 2012). Wnt/β-catenin is required for both self-renewal and differentiation of nephron progenitors (Carroll et al., 2005; Karner et al., 2011; Park et al., 2007). During differentiation, Wnt/β-catenin signaling activates key differentiation genes, such as Fgf8 and Wnt4, whose expression is required for nephrogenesis (Grieshammer et al., 2005; Park et al., 2012; Park et al., 2007; Perantoni et al., 2005; Stark et al., 1994). Notch signaling is believed to act downstream of Wnt/β-catenin signaling (Boyle et al., 2011; Park et al., 2012). Activation of Notch signaling leads to release of the intracellular domain (ICD) of the Notch receptor from the membrane. Notch ICD forms a complex with its DNA binding partner, Rbpj, to regulate expression of its target genes (Park and Kopan, 2015). In the current model of nephrogenesis, it is
thought that Notch signaling is dispensable for the initiation of nephrogenesis and that it promotes the formation of the proximal segment of the nephron and represses the formation of the distal segment (Cheng et al., 2007; Cheng et al., 2003; Park and Kopan, 2015).

Here we show definitively that downregulation of Six2 is required for differentiation of nephron progenitors and that Notch is necessary and sufficient for downregulating Six2. Furthermore, our lineage analysis shows that Notch signaling is required for the formation of all segments of the nephron. This work reveals a novel role of Notch signaling in nephrogenesis.

RESULTS

Downregulation of Six2 is essential for differentiation of nephron progenitors

In order to test if downregulation of Six2 is required for differentiation of nephron progenitors, we performed a Six2 gain-of-function (GOF) study. We generated a transgenic mouse line carrying 3xFLAG-tagged Six2 and IRES-EGFP under the control of the tetracycline operator promoter (tetO-Six2-IRES-EGFP). We used a nephron-lineage specific Six2GFPcre to activate Rosa26-LNL-tTA, a floxed transcription stop cassette followed by a tetracycline-controlled (tet-off) transactivator (Kobayashi et al., 2008; Park et al., 2007; Wang et al., 2008). The combination of these three transgenes results in the expression of both FLAG-tagged Six2 and EGFP in the nephron lineage in the absence of tetracycline (Fig. 1A). Since this transgenic expression of Six2 from tetO transgene is persistent even after endogenous expression of Six2 is turned off, FLAG or EGFP can serve as a lineage tracer. We confirmed that 3xFLAG-tagged Six2 is functionally equal to untagged Six2 in both transcriptional activation and repression (Fig. S1).

In the control kidney, GFP+ cells were found in developing nephrons (Fig. 1B, first column). By contrast, in the Six2 GOF mutant kidney, GFP+ or FLAG+ cells were restricted to the cap mesenchyme, failing to escape from the progenitor niche (Fig. 1B, second column). The absence of GFP+ nephrons suggests that nephron progenitors constitutively expressing Six2 do not go on to form nephrons. A more
A detailed analysis of the Six2 GOF mutant kidney showed that, similar to the control kidney, the mutant nephron progenitors in the cap mesenchyme expressed Wt1 and Pax2, two key transcription factors required for proper nephrogenesis (Fig. 1C). Six2 and proliferation marker expression in the Six2 GOF mutant kidney were also similar to those of the control kidney (Fig. S2). However, the mutant nephron progenitors failed to differentiate into Lhx1+ nephron tubules or Cdh1+ epithelial cells, as shown by the lack of GFP lineage tracer in Lhx1+ or Cdh1+ cells (Fig. 1C, right panels). This result demonstrates that persistent expression, rather than overexpression, of Six2 prevents mesenchymal nephron progenitors from differentiating into epithelial nephron tubules, strongly suggesting that downregulation of Six2 is required for nephrogenesis. Interestingly, some nephron tubules do form in the Six2 GOF mutant kidney (Fig. 1C). However, these nephron tubules were negative for both FLAG and GFP, indicating that these cells were descendants of progenitors that escaped Six2GFPcre-mediated recombination and failed to express transgenic Six2. This was likely caused by mosaic expression of Six2GFPcre (Surendran et al., 2010).

**Notch signaling is necessary and sufficient for downregulation of Six2**

Combined with previous Six2 loss-of-function (LOF) studies (Kobayashi et al., 2008; Self et al., 2006), our Six2 GOF analysis suggests that expression of Six2 needs to be tightly regulated in order to balance self-renewal and differentiation of nephron progenitors. Six2 expression is gradually lost during differentiation of nephron progenitors (Park et al., 2012). We observed that downregulation of Six2 coincided with expression of Jag1, the major Notch ligand in the process of nephrogenesis (Fig. 2). Expression of Jag1 was polarized in the developing nephron as previously reported (Cheng et al., 2007; Georgas et al., 2009; Park et al., 2012). In the aggregate and renal vesicle (RV), Jag1 was detected in the distal part that is adjacent to the tip of the collecting duct. At the same time, downregulation of Six2 also occurred at the same distal part of the aggregate and RV. Six2 was completely lost when the expression domain of Jag1 expanded to the entire comma-shaped body (Fig. 2). This result showed an inverse correlation of Jag1 and Six2 expression and raised the possibility that Notch signaling downregulates Six2 in aggregates and RVs.
To investigate if Notch signaling regulates the expression of Six2, we performed Notch GOF and LOF studies by utilizing Six2GFPcre to specifically target nephron progenitors and their descendants (Kobayashi et al., 2008; Park et al., 2007). We found that expression of an active form of Notch1 (Notch1 ICD) in nephron progenitors completely abolished the expression of Six2 (Fig. 3A). Our RT-qPCR analysis of several specific cap mesenchyme markers showed that Osr1 was still expressed in the Notch GOF mutant kidney while other markers including Six2 were downregulated (Fig. 3B). This result suggests that downregulation of Six2 in the Notch GOF mutant kidney is not simply due to depletion of nephron progenitors. We found that deletion of either Rbpj, which is required for Notch signaling, or two Notch receptor genes, Notch1 and Notch2, caused a defect in downregulation of Six2 (Fig. 4). In these mutants, Six2+ cells were found deeper at the medullary side of the tips of the collecting duct, most likely due to lack of Six2 downregulation. Despite the apparent defect in Six2 downregulation, some nephron tubules appeared to form in these mutants, albeit at a reduced frequency. This is caused, at least in part, by mosaic expression of Six2GFPcre. We found that Lhx1+ nephron tubules formed in these mutant kidneys were often not labeled with lineage tracer (Fig. S3), suggesting that intact Notch signaling allowed the formation of Lhx1+ nephron tubules. Taken together, our Notch LOF and GOF data show that Notch signaling is necessary and sufficient for downregulation of Six2.

Nephron progenitors lacking Notch signaling fail to differentiate into any nephron segments

Thus far, we demonstrated that downregulation of Six2 is required for the formation of nephron tubules and that Notch signaling is necessary and sufficient for this downregulation. These results suggest that Notch signaling is required for the formation of all nephron segments. This is inconsistent with the current model of nephrogenesis, where Notch signaling promotes the formation of proximal tubules and podocytes but represses the formation of distal tubules (Cheng et al., 2007; Cheng et al., 2003; Park and Kopan, 2015). To address this discrepancy, we examined which segments of the nephron could be formed from nephron progenitors lacking Notch1 and Notch2 receptors by lineage analysis. We used Wt1 and LTL to mark podocytes and proximal tubules, respectively. In addition, we used Slc12a1
and Slc12a3 to label loop of Henle and distal tubules, respectively, because they have been reported to be specifically expressed in those segments (Lee et al., 2015). In the control kidneys, cells originating from Rosa GFP reporter-labeled nephron progenitors formed podocytes (Wt1+), proximal tubules (LTL+), loop of Henle (Slc12a1), and distal tubules (Slc12a3+) (Fig. 5, left panels). In contrast, in the Notch double mutant kidneys, the Rosa GFP reporter-labeled nephron progenitors failed to form any segments of the nephron (Fig. 5, right panels). While each segment of the nephron was present in the Notch double mutant kidneys, they were in significantly lower numbers. Importantly, most of these cells were not labeled with the Rosa GFP reporter, suggesting that these cells originated from progenitors that had escaped recombination by Six2GFPcre and thus expressed Notch genes. We also performed the same lineage analysis in the Rbpj LOF mutant kidneys. We found that the Rbpj LOF mutant nephron progenitors poorly differentiate into any nephron segments (Fig. S4). Our data strongly suggest that Notch signaling is required for the formation of all segments of the nephron.

Our lineage analysis showed that differentiation of nephron progenitors is severely defective in the absence of Notch signaling. It led us to examine if Notch signaling was required for MET of nephron progenitors. In the control kidneys, we found significant overlap of Cdh1 (a pan-epithelial marker) and the Rosa GFP reporter, indicating expected MET of nephron progenitors (Fig. 6A, left panel). We also found that some nephron tubules (Cdh1+ Cytokeratin-) were not marked with the Rosa GFP reporter, showing again that expression of Six2GFPcre was mosaic. Interestingly, in the Notch double mutant kidney, most of the Rosa reporter-labeled cells remained at the cortex of the kidney, unable to differentiate into Cdh1+ nephron tubules (Fig. 6A, right panel). The mutant kidneys formed some nephron tubules (Cdh1+ Cytokeratin-) but most of these tubules were not labeled with GFP (marked with white arrows in Fig. 6A, right panel), suggesting that these nephron tubules arose from escapers of Cre recombination. Although most of the Rosa GFP reporter labeled cells in the Notch double mutant kidney failed to differentiate into Cdh1+ nephron tubules, we found that the mutant kidney had Cdh1-negative RV-like structures (marked by white arrowheads in Fig. 6A, right panel) but no S-shaped bodies (SSB). It appeared that RVs did form in the Notch double mutant kidney but they failed to develop into SSB. Since RV is considered as epithelial cells, we
conclude that Notch signaling is not required for MET of nephron progenitors, consistent with previous reports (Cheng et al., 2007; Cheng et al., 2003; Wang et al., 2003).

**DISCUSSION**

Notch signaling was previously thought to be dispensable for the early differentiation of nephron progenitors. Instead it was believed to play a later role in nephron segmentation, with Notch required for the formation of proximal but not distal segment of the nephron (Cheng et al., 2007; Cheng et al., 2003). Here we show that downregulation of Six2 is required for differentiation of nephron progenitors and that Notch signaling is necessary and sufficient for Six2 downregulation. The finding that Notch is involved in the downregulation of Six2 suggests that Notch signaling has a profound impact on the gene regulatory network governing the maintenance of nephron progenitors. We observed that the Six2+ cell population expanded in the Rbpj or Notch LOF mutant kidneys (Fig. 4) and that Six2 expression was abolished in the Notch GOF mutant kidney. (Fig. 3). Our results showing that Notch-mediated downregulation of Six2 is required for nephrogenesis predicted that Notch signaling would be required for the formation of all nephron segments regardless of different cell fates along the proximal-distal axis of the nephron. This prediction was inconsistent with the current model, in which Notch signaling promotes the formation of the proximal segment and represses the formation of the distal segment of the nephron (Cheng et al., 2007; Cheng et al., 2003). Strikingly, we found that nephron progenitors lacking both Notch1 and Notch2 failed to form not only the proximal tubule but also the distal tubule, loop of Henle, and podocytes (Fig. 5). We also found largely similar results in the Rbpj LOF mutant kidney. Unlike loss of Notch receptors, loss of Rbpj did not completely block nephron segmentation (Fig. S4). This may be due to the fact that, in the Rbpj LOF mutant kidney, a small minority of cells labeled with lineage tracer still express Rbpj (Fig. S5). We suspect that this was a result of either incomplete removal of floxed Rbpj or the persistence of Rbpj protein. Nonetheless, the Rbpj LOF mutant nephron progenitors poorly differentiate into any specific nephron segment. Our results strongly support a new model, in
which Notch signaling promotes nephrogenesis by downregulating Six2 expression (Fig. 6B).

The previous model for the role of Notch signaling in nephrogenesis was, at least in part, based on the characterization of Notch2 LOF mutant kidneys (Cheng et al., 2007). In that study, when Notch2 was deleted with Pax3Cre which targets both nephron and interstitium lineages in the kidney (Engleka et al., 2005), nephrogenesis was severely defective. However, in another study, when the nephron lineage specific Six2GFPcre was used to delete Notch2, the mutant kidney showed no nephron segmentation defect although it generated fewer nephrons (Surendran et al., 2010). Deletion of both Notch1 and Notch2 receptors with Six2GFPcre phenocopies deletion of Rbpj with Six2GFPcre, causing a severe defect in nephrogenesis, which indicates that Notch1 and Notch2 act redundantly in the nephron lineage (Surendran et al., 2010). In order to address Notch function in nephrogenesis, we removed both Notch1 and Notch2 with the nephron-specific Six2GFPcre and performed extensive characterization of the mutant kidney with lineage analysis.

Our data show that the Notch double mutant kidneys formed RV that fails to develop into SSB. This is consistent with previous reports demonstrating that pharmacological inhibition or genetic removal of γ-secretase activity allowed the formation of RV, but not SSB (Cheng et al., 2003; Wang et al., 2003). The notion that the distal tubules are still formed in the absence of Notch signaling was in part due to the use of Cdh1 (E-cadherin) as a distal tubule marker (Cheng et al., 2007; Cheng et al., 2003). Although the distal and median segments of SSB have higher expression of Cdh1 than the proximal segment (Barker et al., 2012; Cheng et al., 2007), most of the segments of the nephron tubules in the developing kidney express Cdh1, except for the Bowman’s capsule and podocytes (Fig. 6A). Thus, Cdh1 is not an adequate marker to distinguish proximal and distal tubules of the nephron. We observed that, in the Notch double mutant kidney, a small number of Rosa reporter-positive cells did become Cdh1+ epithelial cells (Fig. 6A, yellow arrowhead). Unlike nephron tubules in the wild-type kidney, these cells are not elongated and do not express any nephron segmentation markers, suggesting that they failed to differentiate into mature nephron tubules. Despite the fact that RVs are formed in the Notch double
mutant kidney, the paucity of Cdh1+ nephron tubules in the mutant kidney suggests that Notch signaling is required for the differentiation of nephron progenitors (Fig. 6B).

Two Notch GOF studies have been reported using Six2GFPcre to activate the expression of Notch ICD, the active form of Notch, from the Rosa26 locus. Expression of Notch1 ICD lacking the PEST domain caused ectopic formation of proximal tubules (Cheng et al., 2007) while expression of the full-length Notch2 ICD with the PEST domain caused premature depletion of nephron progenitors without ectopic formation of proximal tubules (Fujimura et al., 2010). It was also shown that, similar to the Six2 LOF mutant kidney, the Notch2 GOF mutant kidney ectopically expresses Wnt4, a key differentiation gene in nephrogenesis. Since the PEST domain is involved in degradation of Notch (Chiang et al., 2006; Fryer et al., 2004), Notch ICD lacking the PEST domain is believed to be more stable, hence more potent, than full-length Notch ICD (Murtaugh et al., 2003). It is unclear whether the ectopic formation of proximal tubules is due to the potency of the presumably more stable Notch ICD or whether the difference comes from the type of Notch receptor. Nonetheless, premature depletion of nephron progenitors was common in both Notch GOF mutants, consistent with our finding that Notch signaling downregulates Six2.

We found that constitutive expression of Six2 prevents nephron progenitors from differentiating into nephron tubules, suggesting that downregulation of Six2 is required for nephrogenesis. We have previously reported that Six2 and β-catenin share common transcriptional targets in nephron progenitors (Park et al., 2012). Among their common targets are Wnt4 and Fgf8, both of which are required for nephrogenesis (Grieshammer et al., 2005; Perantoni et al., 2005; Stark et al., 1994) and are activated by β-catenin (Park et al., 2012; Park et al., 2007). We showed that β-catenin forms a complex with Six2 in vitro and in vivo (Park et al., 2012). It is known that β-catenin is required for differentiation of nephron progenitors (Park et al., 2007), and in this report we show that constitutive expression of Six2 can block nephrogenesis. These results suggest that Six2 may block expression of β-catenin targets that drive differentiation of nephron progenitors. This idea is supported by the fact that Six2 can repress β-catenin-mediated transcriptional activation (Fig. S1B).
On the other hand, it was shown that β-catenin is required for nephron progenitor maintenance (Karner et al., 2011). It remains to be determined how Six2 antagonizes β-catenin-mediated initiation of nephrogenesis but not β-catenin-mediated nephron progenitor maintenance.

The inverse correlation of Six2 and Jag1 expression during nephrogenesis (Fig. 2) and Six2 expression patterns in the Notch LOF and GOF mutant kidneys (Fig. 3 and Fig. 4) provide compelling evidence supporting the idea that Notch signaling downregulates Six2. Consistent with this, it was recently shown that pharmacological inhibition of Notch signaling promotes self-renewal of Six2+ nephron progenitors in vitro (Tanigawa et al., 2016; Yuri et al., 2015). Expression of Six2 appears to be regulated by multiple factors. We have previously shown that an enhancer located at 60kb upstream of the Six2 gene was capable of driving a transgenic reporter in Six2+ cells (Park et al., 2012). This enhancer was bound by both Six2 and β-catenin. Since treatment of nephron progenitors with a GSK inhibitor causes repression of Six2, we concluded that β-catenin contributed to repression of Six2. However, it was recently reported that Wnt/β-catenin signaling is required for the maintenance of nephron progenitors in vivo and in vitro (Brown et al., 2015; Karner et al., 2011; Tanigawa et al., 2016). A low level of β-catenin may be required for maintenance of Six2 expression and a high level of β-catenin may contribute to downregulation of Six2. It is possible that Wnt/β-catenin and Notch signals coordinate to regulate Six2 expression.

Our findings have implications for cell replacement therapy of the kidney. Much effort has been focused on in vitro generation of nephron tubules by directed differentiation of embryonic stem cells (Morizane et al., 2015; Takasato et al., 2015; Tanigawa et al., 2016). In most cases, Wnt/β-catenin signaling is employed to initiate differentiation of nephron progenitors even though Wnt/β-catenin signaling is also important for the maintenance of nephron progenitors (Brown et al., 2015; Karner et al., 2011). Our work suggests that Notch signaling may serve as a more efficient trigger for differentiation of nephron progenitors into nephron tubules.
MATERIALS AND METHODS

Mouse Strains

tetO-Six2-IRES-EGFP and tetO-IRES-EGFP transgenic lines were generated by pronuclear injections at CCHMC transgenic animal and genome editing core. Six2 was tagged with 3xFLAG at their C-terminus. Founders for each line were outcrossed to Swiss Webster. BAC transgenic Six2GFPcre (Kobayashi et al., 2008; Park et al., 2007), Rosa26-Notch1ICD-IRES-GFP (Murtaugh et al., 2003), Rosa26-LNL-tTA (Wang et al., 2008), Rosa26-EGFP (Srinivas et al., 2001), Rbpj c/c (Tanigaki et al., 2002), Notch1 c/c (Yang et al., 2004), and Notch2 c/c (McCright et al., 2006) mice were described previously. All mice were maintained in the Cincinnati Children's Hospital Medical Center (CCHMC) animal facility according to animal care regulations. Animal Studies Committee of CCHMC approved the experimental protocols (IACUC2013-0105).

Immunofluorescence

Embryonic or P0 kidneys were fixed with 4% PFA/PBS, incubated in 30% sucrose/PBS at 4°C overnight, and imbedded in OCT. 10 μm cryosections were incubated overnight with 5% heat-inactivated sheep serum/PBST containing primary antibodies. The antibody information is in Supplemental Experimental Procedures. Fluorophore-labeled secondary antibodies were used for indirect visualization. Images were taken with Carl Zeiss ApoTome or Nikon Ti-E SpectraX Widefield microscopes.

Quantitative PCR

Control or Notch GOF embryonic kidneys were dissected out and total RNA was extracted using the Qiagen RNeasy Micro kit according to manufacturer’s instructions for microdissected tissue. With approximately 1μg of total RNA, reverse transcription was done using the RevertAid cDNA Synthesis kit (Thermo Scientific...
cat. no. K1621) to obtain cDNA. Quantitative PCR was performed on Applied Biosystems StepOne Plus (Thermo) using Power SYBR Green PCR Master Mix (Thermo #4368706). Oligonucleotide primers used are as follows:

GAPDH (CAACTTTGTCAAGCTCATTTCCTG and CCTCTCTTGCTCAGTGCTCTT),
Six2 (ACATGAGGGCGTAAAATGGGA and CACCTCGCTGGTTCTTCTCT),
Meox2 (GTGCGGGCAAATGTCTGTATTT and GCTTTGTTTGGCACTTGGTT),
Eya1 (ATGGCAACACAAAGACCACA and AGGATGATGGGAGAAACACA),
Cited1 (AGCAGCCAGAGGGAAAATCT and GGATGAGGAGGTGCTGATGT),
Osr1 (CCGGAAGGAAACTGCATT and CGGAGTTTTCGTGTTGTTG).

Two biological replicates each of control and Notch1ICD expressing E13.5 kidneys were used. Fold change calculations were done using the ΔΔCt method.
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Competing Interests
No competing interests declared.

Author Contributions
E.C. performed most experiments. P.D., S.M., and J.S. contributed several figures. E.C. and J.-S.P. contributed to the concept, experimental design, data interpretation, and co-wrote the manuscript.

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Figures

A

\[\text{loxp} \quad \text{Rosa26} \quad \text{Neo-Stop} \quad \text{ITA} \quad \text{loxp} \quad \text{SIX2-GFP-cre}\]

\[\text{loxp} \quad \text{Rosa26} \quad \text{ITA} \quad \text{tetO} \quad \text{SIX2-3xFLAG} \quad \text{IRES} \quad \text{EGFP}\]

\[\text{SIX2} \quad \text{EGFP}\]

B

\[\text{R}^{26\text{TA/+}}\]

\[\text{SIX2-GFP-cre}\]

\[\text{tetO-IRES-EGFP} \quad \text{tetO-SIX2-3xFLAG-IRES-EGFP}\]

\[\text{FLAG} \quad \text{GFP}\]

\[\text{GFP}\]

\[\text{FLAG}\]

\[\text{Hoechst}\]

C

\[\text{R}^{26\text{TA/+}}\]

\[-\quad \text{SIX2-GFP-cre}\]

\[-\quad \text{tetO-SIX2-3xFLAG-IRES-EGFP}\]

\[\text{GFP}\]

\[\text{Wt1}\]

\[\text{Lhx1}\]

\[\text{Pax2 GFP Cdh1}\]

\[\text{GFP}\]

\[\text{Pax2}\]

\[\text{Cdh1}\]
Fig. 1. Downregulation of Six2 is required for differentiation of nephron progenitors. (A) Generation of Six2 gain-of-function (GOF) mutant. We generated a transgenic line carrying tetO-regulated Six2 followed by IRES-EGFP. When combined with Rosa26-LNL-tTA and Six2GFPcre, it expresses 3xFLAG-tagged Six2 and EGFP permanently in nephron progenitors and their descendants. (B) Constitutive expression of Six2 restricts nephron progenitors at the cap mesenchyme. IRES-GFP serves as a lineage tracer and FLAG expression indicates expression of Six2 from tetO-Six2 transgene. In the control kidney (first column), GFP is expressed in the entire nephron lineage. In the Six2 GOF mutant kidney (second column), GFP+ cells are restricted to the cap mesenchyme. (C) The Six2 GOF mutant cells fail to differentiate. Six2 GOF mutant cells (GFP+) fail to express the differentiation marker Lhx1 or the epithelial marker Cdh1 (E-cadherin) while they still express Wt1 and Pax2 in the cap mesenchyme. Embryonic kidneys at E14.5 were examined. Images are representative of two independent experiments. Scale bars, 100 μm.
Fig. 2. Inverse correlation of Jag1 and Six2 expression. Expression of Jag1 in the aggregate and the renal vesicle coincides with the downregulation of Six2. As the expression domain of Jag1 expands in the comma-shaped body, little or no Six2 is detected. E16.5 kidneys. Images are representative of two independent experiments. Scale bars, 100 μm.
Fig. 3. Notch signaling is sufficient for downregulation of Six2. (A) Expression of an active form of Notch in nephron progenitors depletes Six2+ cells. (B) RT-qPCR analysis of cap mesenchyme markers in the Notch GOF mutant kidney. All cap mesenchyme markers are downregulated in the Notch GOF mutant kidney, except for Osr1. E13.5 kidneys. Results are representative of two independent experiments.
Fig. 4. Notch signaling is required for downregulation of Six2. Deletion of Rbpj (A) or Notch receptors (B) causes expansion of Six2+ cells. Notably, in these mutants, Six2+ cells are found deeper into the medullary side of tips of the collecting duct because downregulation of Six2 is defective. P0 kidneys. Images are representative of three independent experiments. Scale bars, 100 µm.
Fig. 5. Nephron progenitors lacking Notch receptors fail to form any nephron segments. Lineage analysis of Six2+ cells shows that nephrogenesis is blocked in the Notch1 and Notch2 double mutant kidney. In the control kidney, Rosa GFP reporter positive cells form Wt1+ podocytes (A), LTL+ proximal tubules (B), Slc12a1+
loop of Henle (C), and Slc12a3+ distal tubules (D). In the Notch double mutant kidney, GFP positive cells fail to develop into any segment of the nephron. P0 kidneys. Images are representative of two independent experiments. Scale bars, 100 µm.
Fig. 6. Notch double mutant kidneys form renal vesicles that fail to develop into Cdh1+ nephron tubules (A) Lineage analysis shows significant overlap of Cdh1 (E-cadherin) and GFP in the control kidney (left panel), indicating the formation of epithelial nephron tubules from nephron progenitors. In the Notch double mutant kidney (right panel), Cdh1+ epithelial cells are either recombination escapers (EGFP-, marked by white arrows) or collecting duct cells (Cytokeratin+). The Notch double
mutant kidney forms RV-like structures that are Cdh1 negative (marked by white arrowheads). A rare GFP and Cdh1 double positive structure in the Notch double mutant kidney was marked with a yellow arrow. P0 kidneys. Images are representative of three independent experiments. Scale bars, 100 μm. (B) Model of Notch-mediated regulation of nephrogenesis. Notch-mediated downregulation of Six2 is required for nephrogenesis. Since downregulation of Six2 occurs early in nephrogenesis likely prior to nephron segmentation, Notch signaling is required for the formation of all segments of the nephron.
Supplemental Experimental Procedures

Antibodies

| Antigen | Vendors            | Catalog #   | Host     | dilution |
|---------|--------------------|-------------|----------|----------|
| Six2    | Proteintech        | 11562-1-AP  | rabbit   | 1:500    |
| GFP     | Aves               | GFP-1020    | chick    | 1:500    |
| FLAG    | Sigma-Aldrich      | F3165       | mouse    | 1:500    |
| Jag1    | DSHB               | TS1.15H     | rat      | 1:20     |
| Calbindin | Abcam            | ab82812     | mouse    | 1:500    |
| Lhx1    | DSHB               | 4F2         | mouse    | 1:20     |
| Pax2    | Covance            | PRB-276P    | rabbit   | 1:200    |
| Wt1     | Santa Cruz         | sc-192      | rabbit   | 1:200    |
| biotin-LTL | Vector Laboratories | B-1325    |          | 1:900    |
| Slc12a1 | Proteintech       | 18970-1-AP  | rabbit   | 1:500    |
| Slc12a3 | Sigma-Aldrich      | HPA028748   | rabbit   | 1:300    |
| Cdh1    | Santa Cruz         | sc-59778    | rat      | 1:500    |
| Cytokeratin | Sigma-Aldrich         | C2562    | mouse    | 1:200    |
| pHH3    | Cell Signaling Tech.| 9701S    | rabbit   | 1:100    |
| Rbpj    | Cosmo bio USA      | SIM-2ZRPBP1| rat      | 1:1000   |
| β-gal   | MPbio              | 559761      | rabbit   | 1:15000  |

Luciferase assay

Six2 promoter-driven reporter assays were done as previously described (Brodbeck et al., 2004). Human embryonic kidney HEK293 cells were plated on a 24-well dish to near confluency and transiently transfected with the firefly luciferase reporter plasmid pSix2p-luc (p524) and SV40-renilla luciferase plasmid (p184) using Lipofectamine 2000 (Invitrogen), according to manufacturer’s instructions. SuperTopFlash cells (Xu et al., 2004) were plated as described above. In addition to reporters, we transfected plasmids expressing untagged Six2 (p420) or 3xFLAG-tagged Six2 (p416) or empty vector. Duplicate or triplicate wells of transfected cells were processed for firefly and renilla luciferase assays using the Dual-Glo Luciferase Assay System (Promega). Firefly luciferase activities were divided by renilla luciferase activity for calculation of normalized fold activity.
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**Fig. S1 3xFLAG-tagged Six2 is functional** (A) Both untagged and 3xFLAG-tagged Six2 can activate the Six2 promoter-driven luciferase reporter. (B) Both untagged and 3xFLAG-tagged Six2 can repress β-catenin-mediated activation of SuperTopFlash reporter.
Fig. S2 Characterization of the Six2 gain-of-function (GOF) mutant kidney (A) Six2 expression levels in the Six2 GOF mutant and control kidneys are similar. (B) Proliferation of nephron progenitors in the Six2 GOF mutant and control kidneys is similar. Phospho-histone H3 (pHH3) was used as a proliferation marker.
Fig. S3 Expression of a differentiation marker Lhx1 in the loss-of-function (LOF) mutant kidneys of Rbpj and Notch. Lineage tracer β-gal (A and B) or EGFP (C and D) was used to follow LOF cells. (A) In the control kidney, β-gal+ nephron progenitors differentiate into Lhx1+ cells. (B) In the Rbpj mutant kidney, Lhx1 expression is reduced. (C) In the Notch double mutant kidney, some Lhx1+ cells are seen that are not labeled with EGFP (yellow arrow), meaning that these cells have escaped Cre-mediated recombination. (D) In the Notch double mutant kidney, some EGFP+ cells (white arrow) differentiate into Lhx1+ cells. These cells are often adjacent to EGFP- Lhx1+ wild type cells (yellow arrow).
Fig. S4 The nephron progenitors in the Rbpj LOF mutant kidney differentiate into nephron segments inefficiently Lineage analysis of Six2+ cells shows that nephrogenesis is almost blocked in the Rbpj mutant kidney. In the control kidney, the Rosa GFP reporter positive cells form Wt1+ podocytes (A), LTL+ proximal tubules (B), Slc12a1+ loop of Henle (C), and Slc12a3+ distal tubules (D). In the Rbpj mutant kidney, GFP positive cells poorly differentiate into any segment of the nephron. P0 kidneys. Images are representative of two independent experiments. Scale bars, 100 mm.
Fig. S5 Expression of Rbpj in the Rbpj LOF mutant kidney (A) In the control kidney, expression of Rbpj is high in the cap mesenchyme and collecting duct. Rbpj expression is reduced after nephron progenitors undergo differentiation. (B) In the Rbpj mutant kidney, while Rbpj is largely absent in β-gal+ cells, still a small minority of β-gal+ cells are Rbpj+ (white arrowhead).