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Notch pathway activation can replace the requirement for Wnt4 and Wnt9b in mesenchymal-to-epithelial transition of nephron stem cells

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SUMMARY

The primary excretory organ in vertebrates is the kidney, which is responsible for blood filtration, solute homeostasis and pH balance. These functions are carried out by specialized epithelial cells organized into tubules called nephrons. Each of these cell types arise during embryonic development from a mesenchymal stem cell pool through a process of mesenchymal-to-epithelial transition (MET) that requires sequential action of specific Wnt signals. Induction by Wnt9b directs cells to exit the stem cell niche and express Wnt4, which is both necessary and sufficient for the formation of epithelia. Without either factor, MET fails, nephrons do not form and newborn mice die owing to kidney failure. Ectopic Notch activation in stem cells induces mass differentiation and exhaustion of the stem cell pool. To investigate whether this reflected an interaction between Notch and Wnt, we employed a novel gene manipulation strategy in cultured embryonic kidneys. We show that Notch activation is capable of inducing MET in the absence of both Wnt4 and Wnt9b. Following MET, the presence of Notch directs cells primarily to the proximal tubule fate. Only nephron stem cells have the ability to undergo MET in response to Wnt or Notch, as activation in the closely related stromal mesenchyme has no inductive effect. These data demonstrate that stem cells for renal epithelia are uniquely poised to undergo MET, and that Notch activation can replace key inductive Wnt signals in this process. After MET, Notch provides an instructive signal directing cells towards the proximal tubule lineage at the expense of other renal epithelial fates.

KEY WORDS: Metanephric mesenchyme, MET, Notch, Mouse

INTRODUCTION

The nephron is the functional unit of the kidney, comprising more than 25 specialized epithelial cell types that regulate blood homeostasis. The formation of nephrons depends on a reciprocal interaction between the metanephric mesenchyme (MM) and the ureteric bud (UB) (for reviews, see Costantini and Kopan, 2010; Dressler, 2009). UB invasion divides the MM into two distinct compartments: the cap metanephric mesenchyme (CMM), which condenses around the bud; and the stromal mesenchyme (SM), a more loosely associated population at the periphery (Humphreys et al., 2008; Mugford et al., 2009). The CMM and SM share a common progenitor that temporal lineage analysis suggests can adopt either fate until UB invasion (Mugford et al., 2008). However, each nephron is derived from the CMM, which comprises a stem cell niche that arises in the mouse at midgestation and exists until shortly after birth when the full complement of nephrons has formed (Boyle et al., 2008; Hartman et al., 2007; Kobayashi et al., 2008). The SM contains progenitors for interstitium, smooth muscle and vasculature (Humphreys et al., 2008).

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Molecular crosstalk between the CMM and UB provides for the reiterative branching and elongation of the bud to form the collecting system and for the induction of new nephrons from the CMM at each UB tip. The CMM undergoes mesenchymal-to-epithelial transition (MET), followed by a stepwise progression that polarizes the emerging nephron epithelia, segments the nephron along a proximal-distal axis and specifies diverse cell types. Perturbation of this process has a wide variety of consequences in humans, including congenital kidney disease, pediatric malignancies and cardiovascular disease later in life (Schedl, 2007; Vize et al., 2002).

MET of the CMM is based on a balance between putative signals that promote maintenance of stem cells and those that induce epithelial differentiation. The molecular mechanisms that govern this balance are emerging. Stem cell maintenance depends on the transcription factor Six2, the removal of which results in mass differentiation of the CMM. Differentiation is specifically induced by canonical Wnt signaling. Induction occurs when UB-derived Wnt9b, directs a subset of CMM cells to form the pre-tubular aggregate (PTA) (Carroll et al., 2005). PTA cells express Wnt4, which acts cell-autonomously to promote formation of the renal vesicle (RV), where cells begin to acquire epithelial characteristics, forming junctions and organizing around a luminal space. In the absence of Wnt9b, the PTA never forms, Wnt4 is not expressed and epithelialization fails (Carroll et al., 2005). Epithelia rarely arise in Wnt4-null kidneys, with differentiation arresting at the PTA stage. In each case, the result is a severe or complete absence of nephrons and non-functional kidneys. Importantly, exogenous Wnt4 can rescue epithelialization of *Wnt9b*$^{-/-}$ cells, but not vice versa, indicating that Wnt4 is both necessary and sufficient for MET (Carroll et al., 2005). Without Wnt9b (and therefore Wnt4), Six2-deficient stem cells

persist for several days but do not differentiate (Karner et al., 2011; Kobayashi et al., 2008); Wnt9b-null stem cells are competent to respond to induction (Karner et al., 2011).

Once epithelia emerge, multiple factors cooperate to specify distinct nephron segments and cell types. Key among these is Notch signaling. In the absence of Notch2, CMM and UB interactions are preserved, branching morphogenesis and MET occur, and the PTA and RV form. Distal epithelia are specified normally but the RV never matures into a segmented S-shaped body (SSB), and the resulting nephrons lack the entire proximal domain, including proximal tubules (PT) and glomeruli (Cheng et al., 2007; Surendran et al., 2010). A role for Notch1 during nephrogenesis is also evident on a sensitized background (Surendran et al., 2010).

Curiously, ectopic and constitutive activation of Notch1 (Cheng et al., 2007) or of Notch2 (Fujimura et al., 2010) in the CMM results in mass differentiation of nephron progenitors and subsequent loss of the self-renewing stem cell population. The ability of Notch to promote differentiation of the CMM raises questions about the relationship between Notch and Wnt in the early nephron. In the zebrafish pronephros, Notch function is required upstream of Wnt4 to promote formation of the proximal domain (Naylor and Jones, 2009). Although phenotypes in the mouse do not suggest a strict conservation of this hierarchy, it is certainly possible that Notch activation in the CMM promotes differentiation either by inducing Wnt4 or by tipping the balance towards Wnt-dependent induction. To investigate the Wnt/Notch relationship during MET of stem cells in the kidney, we employed a Notch gain-of-function paradigm based on a novel method to mosaically delete conditional alleles in cultured kidneys. Using this system, we demonstrate that N1ICD-expressing CMM cells undergo premature MET, that they form polarized epithelia that adopt primarily the PT fate, and that stromal and duct cells are refractory to N1ICD. We provide evidence that, although Notch acts after Wnt4 in the developing mammalian nephron, it functions to promote MET and PT formation independently of Wnt4 and Wnt9b. We arrive at this conclusion by observing robust MET in response to N1ICD in CMM that lacks both Wnt4 and Wnt9b and that the fate choices of N1ICD-expressing cells are not affected by their absence. These data demonstrate that CMM cells are uniquely determined to undergo MET, and that a Wnt-independent permissive signal is sufficient to expose this potential. When this signal is provided by Notch, downstream targets either instruct PT or restrict cells from assuming other fates, demonstrating that this pathway is both necessary and sufficient for the formation of proximal epithelia.

MATERIALS AND METHODS

Mice

Animal studies were approved by the Washington University Division of Comparative Medicine, protocol #20080010. All mouse lines used in this study have been previously described: Rosa^{N1ICD} (Murtaugh et al., 2003), *Six2*$^{tgCre::GFP}$ (Kobayashi et al., 2008; Surendran et al., 2010), *FoxD1*^{Cre} (Humphreys et al., 2008), *Pax3*^{tgCre} (Grieshammer et al., 2005; Cheng et al., 2007), N2$^{f/f}$ (McCright et al., 2006), *Wnt4*$^{+/–}$ (Stark et al., 1994) and *Wnt9b*$^{+/–}$ (Carroll et al., 2005). Genotyping was performed using the universal PCR genotyping protocol (Stratman et al., 2003).

Purification of Tat-Cre

Recombinant TAT-Cre was expressed and purified as previously described (Peitz et al., 2002). Briefly, *Escherichia coli* TUNER(DE3)pLacI (Novagen) containing pTriEx-His-TAT-NLS-Cre was grown in LB with 100 mg/ml ampicillin and 34 mg/ml chloramphenicol at 37°C until OD_{600} reached 0.9-1.0. Protein expression was induced for 4 hours with 0.5 mM IPTG. For lysis, cells were pelleted, resuspended in phosphate buffer [50 mM Na_2HPO_4, 5 mM Tris (pH 7.8), 500 mM NaCl; 3 ml/gram wet weight of bacterial pellet] with 1 mg/ml of lysozyme (Sigma) and benzonase (Novagen), followed by sonication (2 minutes, 0.5-second pulses). An equal volume of tartartic salt buffer (1× phosphate buffer with 20 mM imidazole and 2 M L-Tartaric acid) was added, membranes were removed by centrifugation and supernatant was passed through 0.22 μM filter. TAT-Cre protein was purified from the supernatant using Ni-NTA matrix (Qiagen, 2 ml slurry/6 ml cleared lysate). The matrix was washed extensively with 1× phosphate buffer containing 20 mM imidazole and eluted with phosphate buffer containing 250 mM imidazole. Protein-containing fractions were pooled and dialyzed against 600 mM NaCl, 20 mM HEPES and subsequently against 600 mM NaCl, 20 mM HEPES and 50% glycerol to concentrate.

Organ culture

Kidneys were isolated, cultured and stained as described (Barak and Boyle, 2011). Briefly, kidney rudiments were dissected from E12.5 embryos and grown in defined medium on transwell filters at the air-liquid interface. For Tat-Cre studies, 1 μm purified Tat-Cre was added to the culture medium overnight. After washing once with PBS, medium was replaced and changed every 24 hours. Cultures were maintained for an additional 72 hours (except where indicated) before staining.

Antibody staining

Cultures were fixed in 4% PFA in PBS at 4° for 1 hour, washed in PBS + 0.1% Tween-20 (PBST) and blocked in PBS + 1% BSA + 0.2% Milk + 0.3% Triton-X100 (PBS-BB) + 10% normal donkey serum (NDS) for 1 hour. Primary antibodies were diluted in PBS-BB and incubated overnight at 4°C as follows: chick α-GFP (Aves, 1:500); rabbit α-cadherin 6 (Cho et al., 1998) (1:400); rabbit α-Aqp1 (Millipore, 1:500); rat α-cytokeratin 8 (Troma-1, DSHB, 1:50); rabbit α-Wt1 (Santa Cruz, 1:200); rabbit αClcnkb (Alomone Labs, 1:250); Biotinylated-LTL (Vector Labs, 1:200); rabbit α-laminin (Sigma, 1:250); mouse α-Zo1 (Invitrogen, 1:250); mouse α-E-cadherin (BD, 1:350); mouse α-CD28k (Sigma, 1:200); and rabbit α-Tamm Horsfall protein (Biomedical Technologies, 1:250). Kidneys were washed extensively in PBST (minimum 3×1 hour at room temperature; the final wash was routinely left overnight at 4°C). Cultures were incubated with secondary antibodies in PBS-BB for 1 hour at room temperature as follows: donkey α-chick DyLight 488 (Jackson ImmunoResearch, 1:500); donkey α-rabbit DyLight 549 (Jackson ImmunoResearch, 1:500); donkey α-mouse DyLight 549 (Jackson ImmunoResearch, 1:500); donkey α-Rat DyLight 649 (Jackson ImmunoResearch, 1:250); and AMCA Avidin-D (Vector Labs, 1:200). Following secondary antibody application, kidneys were washed three times for 30 minutes in PBST and mounted with Vectashield (Vector Labs). Images were acquired with a Zeiss Axioimage.Z1 equipped with an Apotome for optical sectioning. For quantification of cell types, Tat-Cre treated kidneys from indicated genetic backgrounds were grown for 72 hours and stained for GFP, LTL and Wt1. For each genotype, two 20× fields from three different kidneys were scored for GFP$^+$/LTL$^+$ cells and GFP$^+$/Wt1^{high} cells.

RT-PCR

For RT-PCR analysis, wild-type and *Wnt9b*$^{–/–}$ explants were grown on separate filters to prevent cross-contamination. RNA was collected using RNAeasy Micro Kit (Qiagen). For reverse transcription, 150 ng of total RNA/20 μl reaction was primed with random hexamers and transcribed according the SuperScript 2 protocol (Invitrogen). cDNA (2 μl) was used per PCR, with 1 μM primers and KlenTaq-LA. Primer sequences are listed in Table S1 in the supplementary material, all spanning intron/exon boundaries. Annealing temperature was 58°C, and minimum detectable cycle numbers were determined for each gene using wild-type RNA, listed in Table S1 in the supplementary material.

RESULTS

Constitutive activation of Notch1 using Tat-Cre induces epithelialization in a subset of cell types in the developing kidney

To examine the competence of different cell types within the developing kidney to undergo differentiation in response to Notch activation, and to determine whether MET specifically requires Wnt signals, we adapted a system to recombine the conditional

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Rosa$^{N1ICD::GFP}$ locus (Murtaugh et al., 2003) in cultured metanephroi using TAT-Cre. TAT is an HIV-derived peptide that can mediate direct cellular uptake of fused proteins (Wadia and Dowdy, 2003; Wadia et al., 2004) and TAT-mediated delivery has been used to overexpress genes in cultured kidneys (Plisov et al., 2005). With this allele, cells taking up TAT-Cre will experience Cre-mediated recombination and express a constitutively active, GFP-fused version of the Notch1 intracellular domain (N1ICD). We anticipated that this unbiased delivery method would target multiple cell types but leave most cells unaffected, allowing us to analyze the fate of a few cells expressing N1ICD in relatively intact metanephroi.

To examine the suitability of the TAT-Cre delivery system, E12.5 kidneys were isolated from Rosa$^{N1ICD::GFP}$ mice and cultured overnight in defined medium with or without 1 μM of purified TAT-Cre. After 72 hours, explants were stained with antibodies against GFP to identify cells that experienced Cre-mediated recombination, cadherin 6 (Cdh6) (Cho et al., 1998) to determine whether they had undergone differentiation into PT and cytokeratin 8 (Ck8) to label the collecting system (Fig. 1). We observed widespread expression of N1ICD::GFP in Tat-Cre-treated kidneys (Fig. 1B), but not in controls (Fig. 1A). Whereas only limited recombination was achieved in the collecting system, mesenchymal cells were readily transduced by Tat-Cre (Fig. 1B,C).

The response to N1ICD was variable within explants. N1ICD::GFP$^+$ cells in the collecting duct retained their morphology, were Ck8 positive and did not express Cdh6 (Fig. 1C, concave arrowheads). Similarly, many dispersed cells at the periphery with mesenchymal morphology were N1ICD::GFP$^+$ but Cdh6 negative (Fig. 1B,C, arrowheads). By contrast, multiple tight clusters of GFP$^+$, Cdh6$^+$ cells were detected adjacent to the Ck8$^+$ collecting ducts, indicative of commitment to the PT fate (Fig. 1B-D, arrows) and consistent with the consequences of constitutive Notch activation in the CMM in vivo. Although N1ICD-expressing cells could be incorporated into normal, elongated PT (not shown), most of the ectopic clusters (Cdh6$^+$/GFP$^+$) existed outside of nephrons (Cdh6$^+$/GFP$^–$, Fig. 1B). The overall branching of the UB was attenuated in Tat-Cre treated metanephroi (compare Fig. 1A″ with 1B″), presumably because many CMM cells differentiated in response to N1ICD, depleting GDNF signals crucial for proper branching as was observed in vivo (Cheng et al., 2007; Fujimura et al., 2010).

We then asked whether N1ICD is simply activating Cdh6 in some cells, inducing sorting and aggregation, or whether MET has actually occurred in these clusters. To determine whether the Cdh6$^+$/GFP$^+$ cells were polarized epithelia, we examined expression of the tight junction protein Zo1 and the basement membrane component laminin, two hallmarks of epithelia. Single plane and three-dimensional reconstruction of *z*-stack images demonstrates that GFP$^+$ clusters formed stereotypical tight junctions between cells, visualized as Zo1 'rings', and deposited a stratified basement membrane (Fig. 1E,F). Based on these findings, we conclude that a subset of cells have undergone epithelialization in response to N1ICD. Together, these experiments demonstrate the utility of Tat-Cre to recombine conditional alleles in metanephric explants, and show that we can use the Notch1 gain-of-function paradigm to address questions about MET in the developing kidney.

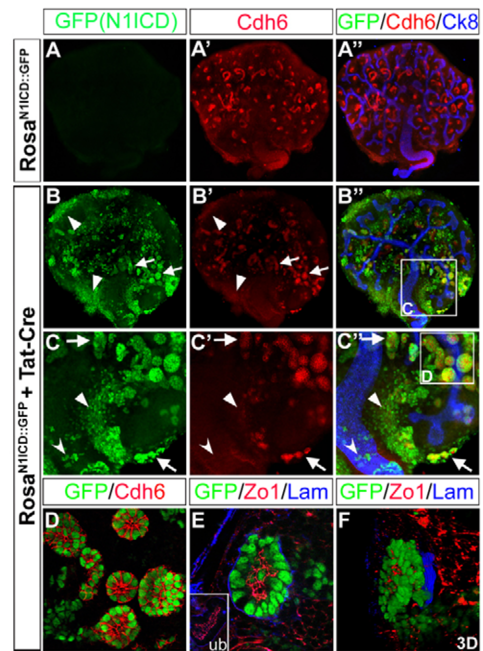

Fig. 1. Tat-Cre mediates efficient recombination in cultured kidneys and a subset of cells undergo MET in response to N1ICD. (**A-B″**) E12.5 Rosa$^{N1ICD::GFP}$ kidneys cultured with vehicle (A-A″) or 1 μM Tat-Cre (B-B″) and grown for 72 hours. GFP (green) marks cells that have undergone recombination: Cdh6 (red) indicates PT differentiation and Ck8 (blue) labels the collecting system. GFP$^+$ cells are only detected in Tat-Cre treated kidneys. (**C-C″**) High-power image of area boxed in B″. A subset of GFP$^+$ cells forms Cdh6$^+$ clusters in response to N1ICD (arrows in B-C″), whereas others do not (arrowheads in B-C″). The collecting duct is largely resistant to Tat-Cre delivery, but Ck8$^+$, GFP$^+$, Cdh6$^+$ cells were never observed (C-C″, concave arrowheads). (**D**) High-power image of area boxed in C″ demonstrating morphology GFP$^+$, Cdh6$^+$ clusters. (**E**,**F**) Single plane (E) and 3D-reconstruction (F) images indicate presence of Zo1-expressing tight junctions (red) and deposition of a stratified laminin basement membrane (blue), demonstrating that GFP$^+$ clusters are epithelial cells. Inset in E shows staining of duct epithelium in the same kidney.

Notch activation restricts cells to the PT and early podocyte fates

Having established that a subset of N1ICD cells undergo MET, we wanted to determine the range of cell types produced in response to Notch1 activation. For this, we examined the expression of terminal differentiation markers in N1ICD::GFP$^+$ clusters. These included, Wt1 (low in CMM and high in committed podocytes), synaptopodin (mature podocytes), lotus tetraglobus lectin (LTL, mature proximal tubule), aquaporin 1 (Aqp1, mature proximal tubule and loop of Henle), Tamm-Horsfall protein (THP, thick ascending limb), E-cadherin and chloride channel 2 (Cdh1 and Clcknb, distal tubule), calbindin DK28 (Cdk28, connecting tubule) and Ck8 (collecting duct). Additional markers of these nephron segments were examined by in situ hybridization (see Fig. S1 in the supplementary material). These analyses demonstrated that the majority of N1ICD-expressing clusters progressed to mature PT cells (Aqp1 and LTL positive, Fig. 2A-C). We did not detect clusters that were Aqp1$^+$,LTL$^–$, indicative of the loop of Henle (Fig. 2C). Clusters of Wt1^{high}, *Cdh6*$^–$ were also identified (Fig. 2D; see Fig. S1C-F in the supplementary material), indicating podocyte commitment, but these never developed the crescent morphology indicative of glomerular maturation, nor did they expressed terminal markers such as Mafb or synaptopodin (Fig. 2D; see Fig. S1A,B,E,F in the supplementary material). Although some clusters have detectable E-Cadherin expression (Fig. 2F, arrows), we were unable to find evidence of conversion into thick-ascending limb

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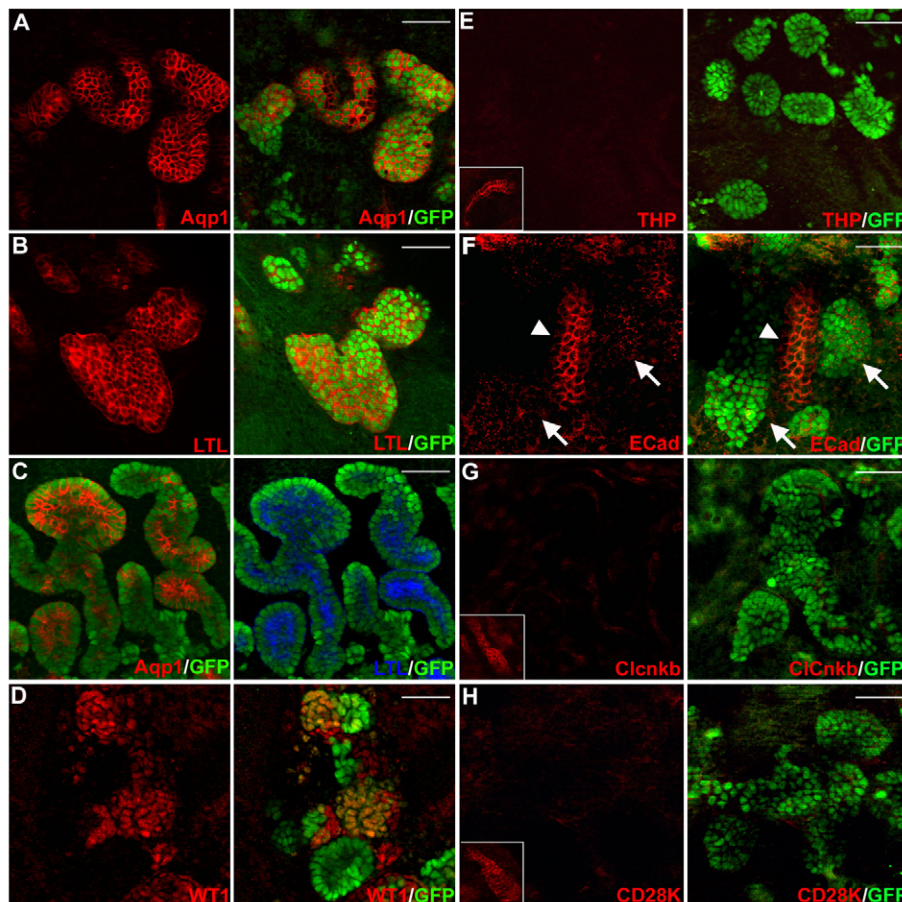

Fig. 2. Expression of N1ICD limits potential to PT and early podocyte lineages. N1ICD clusters were stained with markers of differentiated nephron segments. (**A**,**B**) Examples of Aqp1- (A) and LTL- (B) positive cells indicate fully differentiated PT. (**C**) Clusters consistently co-expressed Aqp1 and LTL, indicative of PT, but not the loop of Henle (Aqp1$^+$,LTL$^-$). (**D**) Wt1^{high} clusters were found at much lower frequency, and indicate presence of immature podocytes. No glomerular organization was observed in these clusters. (**E-H**) GFP$^+$ cells did not express markers of more distal nephron segments, including thick ascending limb (E, Tamm Horsfall protein), distal tubule (F, E-cadherin; G, Clcknb) or connecting segment (H, Cd28K). Insets in E,F,H demonstrate the fidelity of antibodies in the same explants. Scale bars: 50 µm. Arrows indicate low levels of E-cadherin in N1ICD::GFP-expressing clusters; arrowheads indicate level and localization normally seen in duct.

(Fig. 2E), mature DT (Fig. 2G), connecting segment (Fig. 2H) or collecting duct (Ck8; not shown), or co-expression of proximal and distal markers. These data indicate that N1ICD provides a signal instructing PT and early podocyte (POD) differentiation, but blocks terminal differentiation of podocytes. Consistent with these results, POD and PT are missing in Notch loss-of-function mutants (Cheng et al., 2007), and podocytes cannot tolerate persistent Notch activation (Niranjan et al., 2008; Waters et al., 2008).

The cap mesenchyme is uniquely poised to undergo MET

It was obvious from the distribution of N1ICD::GFP$^+$ cells in our initial experiments that many mesenchymal cells within the peripheral (non-duct) populations did not respond by forming epithelia. We hypothesized that this reflected differential competence in the related CMM and SM progenitor lineages. Both probably arise from a common progenitor (Osr1$^+$) (Mugford et al., 2008); the renal interstitium progenitors (FoxD1$^+$) segregate away from the CMM (Six2$^+$) to form a distinct cell layer immediately outside of the cap after invasion of the UB. Importantly, lineage studies have shown that as late as E10.5, some Osr1$^+$ progenitors with dual cap/stromal potential persist (Mugford et al., 2008). To test this hypothesis directly, we crossed cap and stromal specific Cre lines with Rosa$^{N1ICD::GFP}$ mice to activate Notch in distinct progenitor compartments [*Six2*$^{Cre::GFP}$ (Kobayashi et al., 2008; Surendran et al., 2010) and FoxD1^{Cre} (Humphreys et al., 2008), respectively]. Kidneys were collected at E15.5 and examined for maintenance of the CMM by Cited1 staining (Boyle et al., 2007) and the differentiation status of N1ICD-expressing cells by Cdh6 and Wt1 staining (Fig. 3). As reported previously, in *Six2*$^{Cre::GFP}$, *Rosa*$^{N1ICD::GFP}$ animals, the stem cell compartment (Cited1$^+$) was completely exhausted (compare Fig. 3C with 3A) and all cells converted to proximal nephron fates. The majority of cells were Cdh6$^+$ (Fig. 3E), with smaller percentage of cells marked by *Wt1*^{high}, *Cited1*$^-$ indicating commitment to the POD lineage (Fig. 3F). Again, glomerular structures were never detected. By contrast, when we expressed N1ICD in the stromal mesenchyme using *FoxD1*^{Cre}, the CMM remained intact, stromal morphology was preserved and the vast majority of GFP$^+$ cells remained negative for Cdh6 and Wt1 (Fig. 3G-I). Very rarely, we observed a small Cdh6$^+$ cluster embedded in N1ICD-expressing GFP$^+$ stroma (Fig. 3H, inset). These genetic experiments and the results obtained in Tat-Cre-treated kidneys demonstrate that N1ICD is not sufficient to instruct all intermediate mesenchyme-derived cells to make epithelia: once Osr1-expressing progenitors are separated into distinct lineages, the CMM is uniquely poised to undergo MET, whereas the FoxD1 linage is not responsive to N1ICD expression.

Notch does not promote MET and nephron segmentation by activating Wnt4

The ability of Notch to trigger MET of CMM cells may permit or enhance the action of Wnt proteins, similar to loss of Six2 (Self et al., 2006). Wnt4 has been shown to be both necessary and sufficient for epithelialization of the CMM (Kispert et al., 1998; Stark et al., 1994), and in zebrafish Wnt4 is functionally

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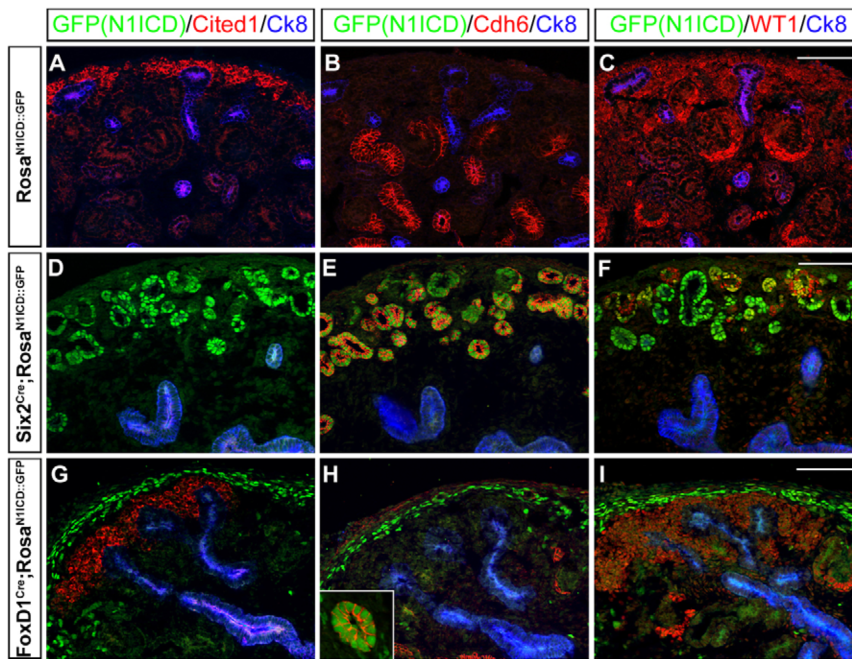

Fig. 3. Only cap mesenchyme undergoes MET in response to N1ICD. N1ICD was expressed in vivo in cap (Six2^{Cre}) and stromal (FoxD1^{Cre}) mesenchyme to assess response. (**A-C**) Expression of CMM marker Cited1 (A), PT marker Cdh6 (B) and CMM/early podocyte marker Wt1 (C) in wild-type kidneys. (**D**) Expression of N1ICD in the CMM exhausts the Cited1^{+} stem cell pool. (**E**,**F**) CMM responding to N1ICD adopt the PT (Cdh6, E) and early podocyte (Wt1^{high}, F) fates. (**G**) Cited1-expressing stem cells (red) are maintained when N1ICD is expressed in the stromal mesenchyme. (**H**) The vast majority of stromal cells do not undergo MET in response to N1ICD. Small Cdh6^{+} clusters are rarely detected in the stroma (inset). (**I**) N1ICD^{+} stromal cells do not express Wt1. Scale bars: 100 μm.

downstream of Notch (Naylor and Jones, 2009). Furthermore, it has been suggested that N2ICD promotes differentiation of the CMM through downregulation of Six2 and augmentation of Wnt4 expression (Fujimura et al., 2010). Together, these observations raise the possibility that Notch may induce MET by activating Wnt4, and that loss of proximal epithelia in Notch-deficient kidneys (Cheng et al., 2007) is due to inactivation or improper maintenance of Wnt4. To test the later hypothesis, we performed in situ hybridization on Pax3^{tgCre}; N2$^{f/f}$ kidneys that lack Notch2 in both nephron and interstitial progenitors. *Wnt4* mRNA was detected in Notch2-deficient renal vesicles (RV) at levels comparable with controls (Fig. 4A,B), indicating that induction of Wnt4 expression in the RV was independent of a Notch2 signal. To determine whether loss of Notch somehow affected Wnt secretion or activity, we provided exogenous Wnt4 to E12.5 kidneys isolated from *Pax3^{tgCre}; N2$^{+/f}$* and *Pax3^{tgCre}; N2$^{f/f}$* mice and asked if it could rescue differentiation of the proximal nephron. The ability of Wnt4-expressing cells (Kispert et al., 1998) to induce tubulogenesis in isolated MM and in *Wnt4$^{-/-}$* explants confirmed their inductive activity (see Fig. S2 in the supplementary material). Control kidneys contain numerous LTL^{+} PT (Fig. 4C,E) and Wt1^{+}/synaptopodin^{+} glomeruli (Fig. 4D,F) when cultured on top of either 3T3-*lacZ* cells or 3T3-Wnt4 cells. Consistent with our previous report, Notch2 mutant kidneys grown on 3T3-*lacZ* cells are completely devoid of both PT and POD (Fig. 4G,H). When Notch2 mutants were grown on Wnt4 feeders, the phenotype was unchanged (Fig. 4I,J), indicating that proximal specification defects in Notch loss-of-function kidneys are not a consequence of Wnt4 misregulation. As previously reported, distal epithelia formed normally in all contexts (not shown). Identical results were obtained when mutant kidneys were grown in the presence of lithium chloride, which activates Wnt signaling by inhibiting Gsk3β (not shown). Taken together, these results indicate that the normal function of Notch during the transition from RV to SSB is not mediated by influencing Wnt4 expression or activity.

N1ICD induces MET independently of canonical Wnt signals

Next, we asked whether the activity of inductive Wnt signals was required for cells to undergo MET in response to N1ICD. For this, we used Tat-Cre to express N1ICD in *Rosa$^{N1ICD::GFP}$*; *Wnt4$^{-/-}$* metanephric explants and assayed for the emergence of GFP^{+}, Cdh6^{+} cells. Untreated Wnt4 null kidneys have attenuated UB branching, the CMM fails to undergo MET and no nephrons form (Fig. 5A, compare to Fig. 1A). As observed by others (Kobayashi et al., 2005) small clusters of epithelia can infrequently be found in *Wnt4$^{-/-}$* kidneys (Fig. 5A″, inset). Strikingly, when *Rosa$^{N1ICD::GFP}$; Wnt4$^{-/-}$* kidneys were treated with Tat-Cre, numerous Cdh6^{+} clusters emerged (Fig. 5B,C). These structures were primarily at the tips of the branching UB, where the CMM is located (Fig. 5B, arrows). As observed in wild type, no Cdh6^{+} clusters arose in the stroma (Fig. 5B, arrowheads). *Wnt4$^{-/-}$* clusters were morphologically indistinguishable from wild type: they expressed Zo1, deposited a basement membrane (Fig. 5D), and expressed markers of mature PT or immature podocytes (Fig. 5E,F). These data demonstrate that Wnt4 is dispensable for MET and PT maturation in N1ICD-expressing CMM cells.

This shows that it is possible for nephron stem cells to make epithelia without Wnt4, but does not address whether induction of the CMM by Wnt9b is required for MET. To test this, we expressed N1ICD in *Rosa$^{N1ICD::GFP}$; Wnt9b$^{-/-}$* explants using Tat-Cre. Cdh6^{+} cells are never found in *Wnt9b$^{-/-}$* kidneys on any background (Fig. 6A). However, when N1ICD is expressed in these kidneys, numerous GFP^{+}, Cdh6^{+} clusters appear (Fig. 6B,C). Like *Wnt4$^{-/-}$* clusters, these cells expressed Zo1 and laminin, indicating formation of polarized epithelia (Fig. 6D). N1ICD-expressing, Wnt9b-null cells progressed to form mature PT (Aqp1^{+}, LTL^{+}; Fig. 6E and not shown) or early podocytes (Fig. 6F). This demonstrates that the CMM can form epithelia in the absence of primary induction by Wnt9b. Counting of LTL^{+} PT cells and Wt1^{high} immature podocytes in wild-type, *Wnt4$^{-/-}$* and *Wnt9b$^{-/-}$* *Rosa$^{N1ICD::GFP}$* kidney cultures treated with Tat-Cre demonstrated

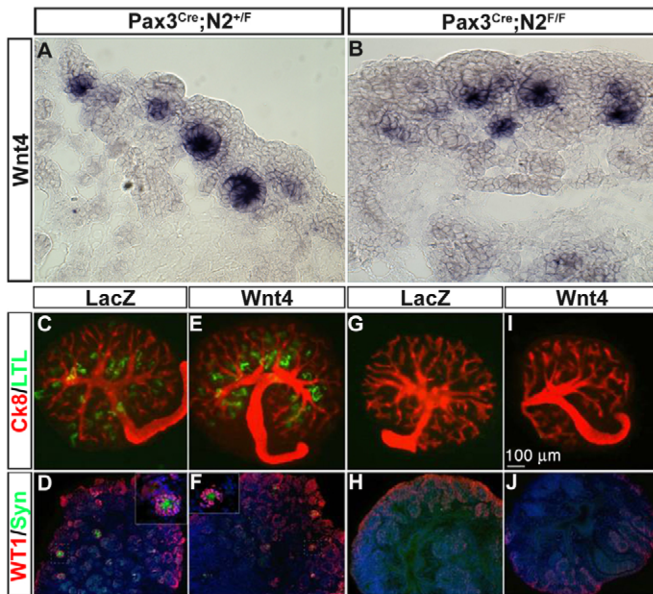

Fig. 4. Proximal differentiation defects in Notch mutants are not due to loss of Wnt4. (**A**,**B**) In situ hybridization for Wnt4 mRNA in E15.5 control (A, Pax3^{tgCre}; N2$^{+/f}$) and Notch loss-of-function kidneys (B, Pax3^{tgCre}; N2$^{f/f}$). Wnt4 expression is maintained in the absence of Notch2. (**C-F**) Control E12.5 explants grown on either 3T3-*lacZ* (C,D) or 3T3-Wnt4 cells (E,F) readily form LTL^{+} PT (C,E, green) and synaptopodin^{+} (D,F, green), Wt1^{+} (D,F, red) glomeruli. (**G-J**) Exogenous Wnt4 does not rescue proximal fates in Notch mutants. PT (G,I) and glomeruli (H,J) are absent in Pax3^{Cre},Notch2$^{f/f}$ explants, demonstrated by absence of LTL and synaptopodin/glomerular Wt1 in kidneys cultured on control (G,H) and Wnt4-expressing cells (I,J).

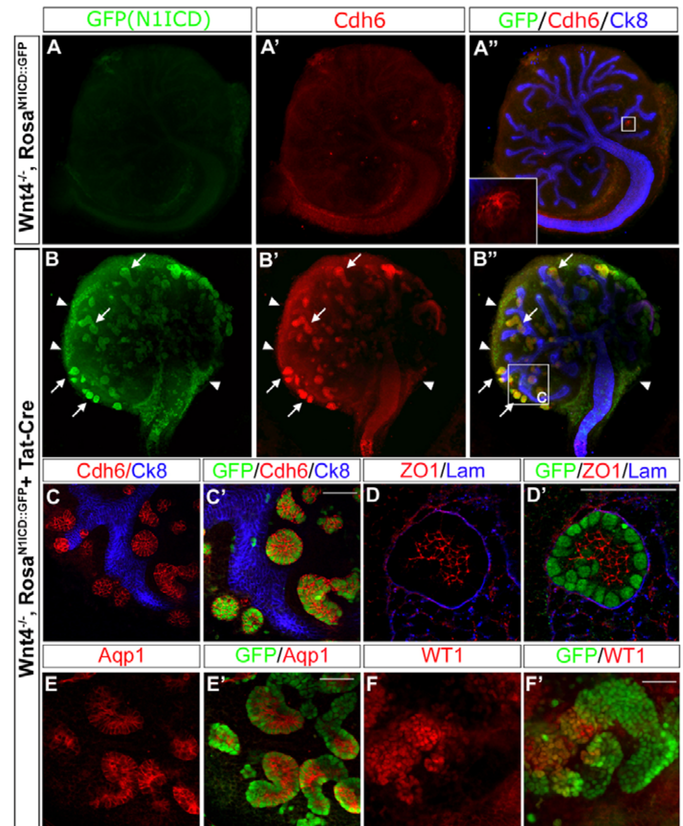

Fig. 5. Wnt4 is not required for MET in response to N1ICD. (**A-A″**) CMM in *Wnt4*$^{-/-}$ kidneys does not undergo MET. An occasional Cdh6^{+} cluster can be detected (A″, inset), but no functional nephrons are made. (**B-B″**) N1ICD rescues MET in *Wnt4*$^{-/-}$ kidneys. Numerous GFP^{+}/Cdh6^{+} clusters are found near branched UB tips (arrows). Like wild type, the stroma does not respond (arrowheads). (**C**) High-power image of boxed area in B″. (**D-D′**) N1ICD clusters in *Wnt4*$^{-/-}$ explants are epithelia; they form Zo1^{+} tight junctions (red) and deposit basement membrane (blue). (**E-F′**) N1ICD cells in *Wnt4*$^{-/-}$ explants progress to Aqp1^{+} PT epithelia (E) and Wt1^{high} immature podocytes (F). Scale bars: 50 µm.

GFP^{+} clusters selected between PT/POD fates at approximately a 3:1 ratio (Fig. 6G). No significant difference in this ratio was observed when Wnt4 or Wnt9b was removed. Thus, constitutive activation of Notch1 is sufficient to induce MET in the absence of Wnt4 or Wnt9b and limits the potential of CMM-derived cells regardless of mutant background.

CMM cells responding to N1ICD undergo a molecularly normal pattern of differentiation

Normally, the cap mesenchyme can differentiate into all the epithelial components of the nephron (Boyle et al., 2008; Kobayashi et al., 2008). We have shown here that N1ICD is sufficient to drive MET of the CMM but limits potential to PT and immature POD. Given that this occurred independently of Wnt4 or Wnt9b, we wanted to determine whether CMM cells responding to N1ICD in the absence of Wnt-mediated induction followed a normal, stepwise program of differentiation from PTA to PT. To address this, we performed semi-quantitative RT-PCR analysis on transcripts isolated from wild-type and *Wnt9b*$^{-/-}$ kidney explants in the presence or absence of Tat-Cre. Wnt9b-null tissue is an ideal control for this analysis because the CMM is intact, but MET never occurs. We selected markers representing uninduced CMM [Six2, GDNF and Wt1 (also expressed in podocyte precursors)]; PTA [Fgf8, Pax8 and Wnt4 (also expressed in early PT)]; RV [Lxh1, Brn1, Delta-like 1 (Dll1)]; SSB [Hes1, Hes5, Papps2]; podocytes [Greb1, podocin and Wt1]; PT [Cdh6 (also expressed in RV), Aqp1]; distal tubule (Clcnkb). GFP, which should only be expressed in Tat-Cre treated kidneys, and GAPDH, which is expressed in all samples, served as controls. As predicted, control *Rosa*$^{N1ICD::GFP}$ kidney explants expressed the entire panel of markers with or without Tat-Cre treatment (Fig. 6H, lanes 1 and 2). GFP mRNA was found only in Tat-Cre treated kidneys, and no-RT reactions using wild-type RNA controls failed to produce bands (Fig. 6H, lane 6). As expected, only CMM transcripts were detected in untreated *Wnt9b*$^{-/-}$ kidneys (Fig. 6H, lane 3). For analysis of epithelial differentiation in response to N1ICD, we collected mRNA 24 and 48 hours after removal of Tat-Cre from *Rosa*$^{N1ICD::GFP}$, *Wnt9b*$^{-/-}$ kidneys. We detected transcripts that indicated intermediate stages of epithelialization, including PTA markers Fgf8 and Wnt4 (Pax8 was not detected), RV transcripts (Lxh1, Brn1 and Dll1) and markers of the middle (putative proximal) domain of the SSB (Hes1, Hes5 and Papss2). In agreement with analyses reported here and elsewhere (Fig. 2) (Cheng et al., 2007)], most cells acquired PT markers, and DT differentiation markers were absent. PT maturation occurred over time; Cdh6 was detectable at both time points, whereas Aqp1 was robustly detected only after 72 hours. Immature podocytes also formed, as evidenced by higher levels of Wt1 in Tat-Cre treated

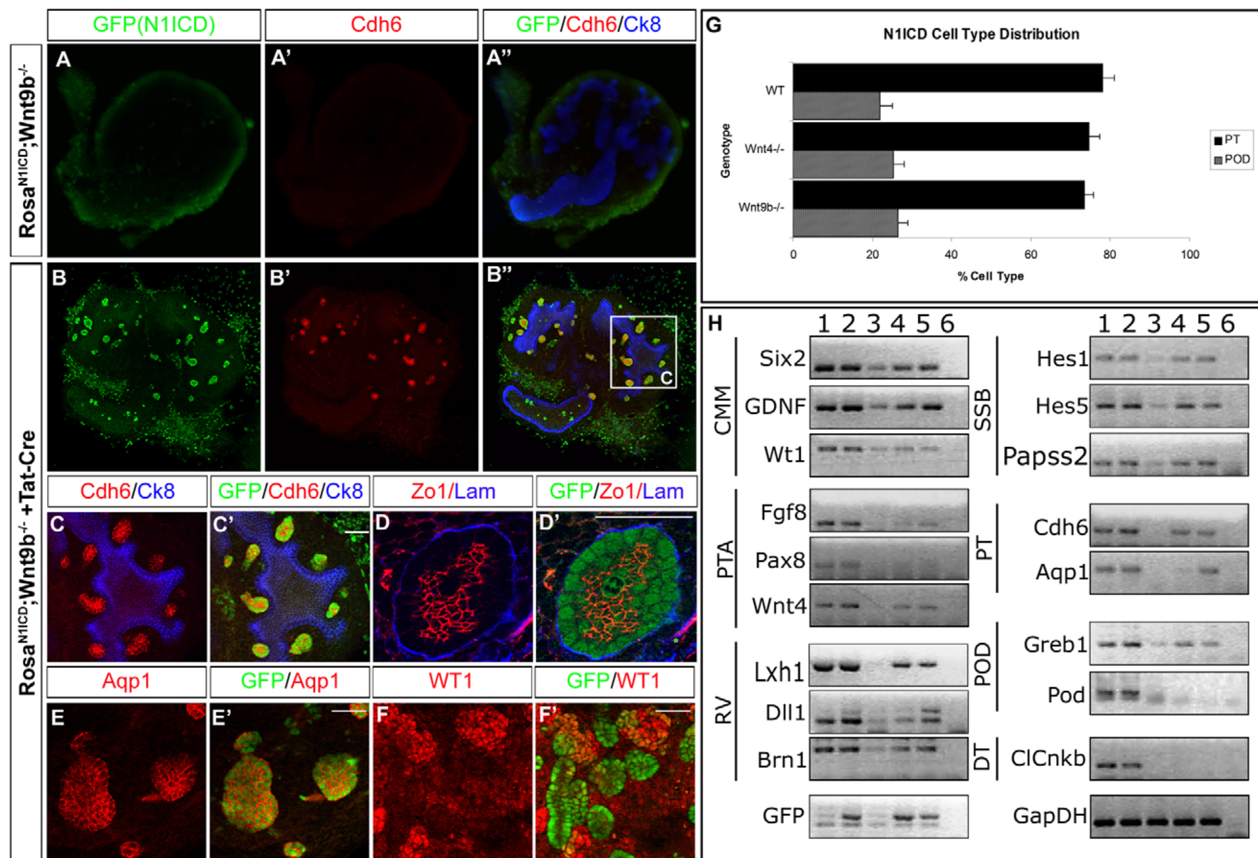

Fig. 6. N1ICD can induce MET independently of Wnt9b. (**A-A″**) No epithelia are formed in control *Wnt9b*$^{-/-}$ kidneys. (**B-B″**) MET is restored in Tat-Cre treated Rosa^{N1ICD}, *Wnt9b*$^{-/-}$ kidneys, as demonstrated by numerous GFP^{+}/Cdh6^{+} clusters around UB tips. (**C**,**C′**) High-magnification image of boxed area in B″. (**D**,**D′**) N1ICD cells in *Wnt9b*$^{-/-}$ explants are epithelia; they form Zo1^{+} tight junctions (red) and deposit basement membrane (blue). (**E-F′**) N1ICD cells in *Wnt9b*$^{-/-}$ make Aqp1^{+} PT epithelia (E) and Wt1^{high} immature podocytes (F). Scale bars: 50 μm. (**G**) Distribution of fate decisions among cells responding to N1ICD in wild-type, *Wnt4*$^{-/-}$ and *Wnt9b*$^{-/-}$ kidneys. Data are mean+s.e.m. (**H**) Semi-quantitative RT-PCR on kidneys from wild-type and *Wnt9b*$^{-/-}$ explants. Lane 1, wild-type control; lane 2, wild-type Tat-Cre treated; lane 3, *Wnt9b*$^{-/-}$ control; lane 4, *Wnt9b*$^{-/-}$ Tat-Cre treated (24 hours); lane 5, *Wnt9b*$^{-/-}$ Tat-Cre treated (48 hours); lane 6, wild-type Tat-Cre treated (no-RT). Markers of all transitional and mature cell types are detected in both wild-type and wild-type Tat-Cre-treated kidneys. *Wnt9*$^{-/-}$ kidneys express only markers of CMM. *Wnt9b*$^{-/-}$ kidneys treated with Tat-Cre express markers indicative of normal PT development, including Fgf8, Wnt4 (PTA), Lhx1, Brn1, Dll1 (RV), Hes1, Hes5 and Papss2 (SSB). Expression of PT markers has a temporal component, Cdh6 is detected at 24 hours, but Aqp1 not until 48 hours. The podocyte marker Greb1 is expressed, but not the mature marker podocin. Treated and untreated explants do not make distal epithelium (Clcnkb).

kidneys and expression of Greb1 mRNA. However, the terminal markers podocin and synaptopodin (see Fig. S1 in the supplementary material) were absent. These data demonstrate that cells responding to N1ICD undergo a relatively normal sequence of epithelial differentiation. Notably, we observed that Wnt4 was re-expressed in *Wnt9b*$^{-/-}$ metanephroi following treatment with Tat-Cre.

MET in response to N1ICD occurs in the absence of Wnt9b and Wnt4

The temporal resolution of the RT-PCR method described above did not allow us to determine whether Wnt4 was expressed during the initial (RV-like) stages of MET or whether it was a consequence of cells adopting the PT fate. Given that exogenous Wnt4 can rescue *Wnt9b*$^{-/-}$ MM (Carroll et al., 2005), and that *Wnt4* is detected in the early PT in vivo (Brunskill et al., 2008), we tested directly whether MET and PT induction in Wnt9b-null kidneys occurs as a consequence of Wnt4 expression. To achieve this, we treated *Rosa*^{N1ICD}, *Wnt4*$^{-/-}$; *Wnt9b*$^{-/-}$ kidneys with Tat-Cre. No differentiation was ever detected in untreated double mutant kidneys and UB branching was severely impaired (Fig. 7A). By contrast, MET occurred and Cdh6^{+} epithelial clusters formed after Tat-Cre-mediated N1ICD activation (Fig. 7B-D). Again, these cells could progress to Aqp1^{+} PT and Wt1^{high} POD precursors (Fig. 7E,F, arrows). Together, these data demonstrate conclusively that CMM cells are poised to undergo MET, that this property is unique to the stem cell niche and that this process requires only a permissive signal that can be provided either by Wnt signaling or by the activation of the Notch pathway, independent of Wnt4 and Wnt9b activity.

DISCUSSION

Kidney development is one of a handful of developmental processes where mesenchymal to epithelial transition occurs. Even more unusual, a mesenchymal stem cell pool in the CMM responds to paracrine and autocrine factors that regulate whether cells remain within the niche or undergo MET. For conversion to occur, an external cue (‘inducer’) directs some cells to exit the niche and

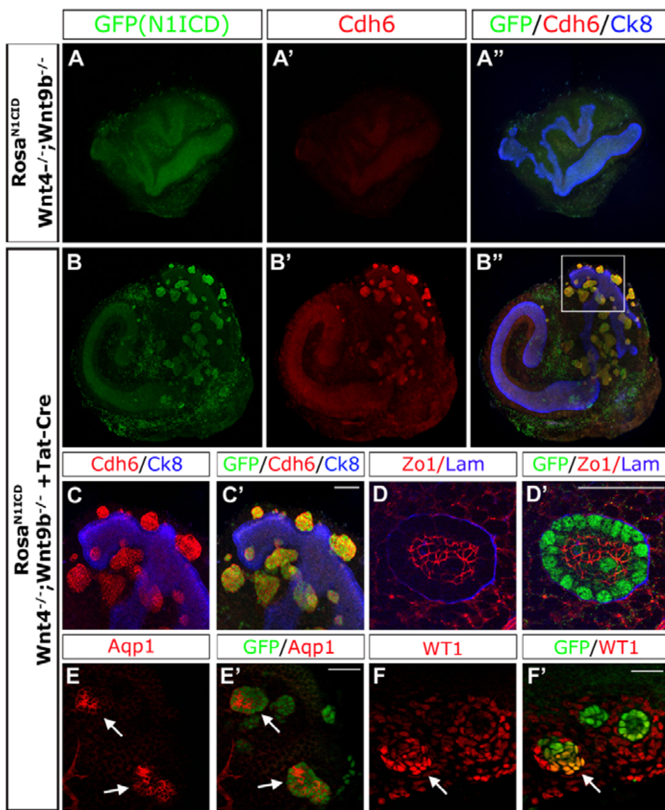

Fig. 7. Wnt4/Wnt9b double mutants make epithelia in response to N1ICD. (**A-A″**) MET does not occur in *Wnt9b*$^{-/-}$,*Wnt4*$^{-/-}$ kidneys. (**B-B″**) MET occurs in Tat-Cre treated Rosa^{N1ICD},*Wnt9b*$^{-/-}$,*Wnt4*$^{-/-}$ kidneys, as evidenced by many GFP+/Cdh6$^+$ clusters around UB tips. (**C**,**C′**) High magnification image of boxed area in B″. Position of GFP$^+$/Cdh6$^+$ cells is consistent with MET of CMM in the absence of both Wnt9b and Wnt4. (**D**,**D′**) N1ICD cells in *Wnt9b*$^{-/-}$,*Wnt4*$^{-/-}$ explants are epithelia; they form Zo1$^+$ tight junctions (red) and deposit basement membrane (blue). (**E-F′**) N1ICD cells in double mutants can make Aqp1+ PT epithelia (E, arrows) and Wt1^{high} immature podocytes (F, arrows). Scale bars: 50 μm.

epithelialize. Several pathways have been shown to act as MET inducers in isolated MM in vitro, including Fgf (Perantoni et al., 1995; Plisov et al., 2001), LIF (Barasch et al., 1999), HGF (Santos et al., 1994) and TGFβ1 (Rogers et al., 1993). However, none of these pathways is required in vivo (Kulkarni et al., 1993; Schmidt et al., 1995; Shull et al., 1992; Stewart et al., 1992; Zhou et al., 1998). It is well established in vivo that molecules in the Wnt (Park et al., 2007), FGF and BMP pathways (Dudley et al., 1999; Grieshammer et al., 2005; Perantoni et al., 1995; Perantoni et al., 2005) are important for this process, but the primary inductive balance appears to rely on Wnt9b and Six2. Thus far, Wnt9b, secreted from the UB, is the only molecule shown to be essential in vivo for induction of the CMM. Conversion to epithelia requires expression of Wnt4 (Carroll et al., 2005). *Six2*, which is expressed in the CMM, is the only molecule whose loss forces cells to exit the niche and differentiate via MET (Self et al., 2006) in a Wnt9b-dependent manner (Kobayashi et al., 2008), further solidifying the central role of Wnt9b (and its target, Wnt4) in triggering MET. Recently, it has been shown that the CMM also receives a Wnt9b signal and integrates it (probably via Six2) to further regulate this balance within the niche (Karner et al., 2011).

Using defined genetic backgrounds and a novel system to activate a conditional Notch1 gain-of-function allele, we demonstrate here that there is no intrinsic requirement for Wnt9b or Wnt4 activity in MET induction. Once separated from the stromal lineage, CMM cells are uniquely poised to undergo MET and will do so in response to a variety of permissive signals, including Notch. It was assumed that this occurred because these signals were somehow promoting Wnt-mediated induction. Our data clearly demonstrate that this cue can be delivered independently of Wnt9b and Wnt4.

The Wnt and Notch pathways have a long history of context-specific entanglement (Blair, 1996; Cagan, 2003; Cossu et al., 1996; Gridley, 2006; Hayward et al., 2008; Kopan and Turner, 1996; Radtke et al., 2006). In some cells, Notch directly induces Wnt (Blair, 1996), whereas it antagonizes β-catenin (Sanders et al., 2009) or Wnt targets (Kopan and Turner, 1996) in others. Notch and Wnt can collaborate, as they do in intestinal stem cells (Radtke and Clevers, 2005). We therefore presumed the ability of N1ICD and N2ICD to promote epithelial differentiation of nephron stem cells reflected a relationship between Notch and Wnt. Unexpectedly, both MET and PT differentiation occurred to the same extent in the presence or absence of Wnt9b and Wnt4. How this relates to the actual relationship of Wnt and Notch during induction is unclear. It is possible that the primary function for Wnt4 in the PTA is to regulate expression of Notch pathway components. Transcription of the Notch ligand Dll1 is regulated by Wnt signals (Hofmann et al., 2004) and Dll1 is significantly downregulated in Wnt4 mutants (Valerius and McMahon, 2008). Jagged1 is a Wnt target in other tissues (Chen et al., 2010; Estrach et al., 2006; Modder et al., 2011; Pannequin et al., 2009). As Dll1 and Jag1 mRNA are only detected in the RV (GUDMAP; see Fig. S3 in the supplementary material) this places the establishment and activation of Notch signaling as a consequence of Wnt4 function. Notch, however, is not epistatic to Wnt during MET because Notch is not required for epithelia to form in response to Wnt; PTA, RV and distal epithelia all arise normally in the absence of Notch signaling (Cheng et al., 2007; Surendran et al., 2010).

The precise functions of Notch signaling in the developing nephron are continuing to emerge. In addition to a role in nephron segmentation, the data presented here show that Notch1 signals are sufficient to instruct stem cells into the adoption of PT fates. This is consistent with the site of high Notch1 activation (Cheng et al., 2003; Cheng et al., 2007), the absence of PT in Notch loss-of-function mutants (Cheng et al., 2007; Surendran et al., 2010) and the normal sequence of molecular events during PT differentiation of N1ICD clusters. We therefore conclude that Notch activity is both necessary and sufficient for specification of the proximal tubule fate. By contrast, Notch signaling is required for formation of podocyte precursors (Cheng et al., 2007), and consistent with previous reports, downregulation of Notch signaling is necessary for maturation (Niranjan et al., 2008; Waters et al., 2008).

Based on gain-of-function studies, it has recently been proposed that the role of Notch2 is to regulate the balance between differentiation and self-renewal by turning off Six2 and augmenting Wnt4 expression in CMM cells on the cusp of differentiation, thereby either ‘selecting’ cells to undergo MET or expelling them from the niche (Fujimura et al., 2010). The authors argue that this represents a unique quality of the N2ICD, which accounts for its dominance in the early nephron. Based on our previous work (Cheng et al., 2003; Cheng et al., 2007) and on the data presented here, we do not feel the available data support this model. We show

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here that early deletion of Notch2 from the MM does not alter Wnt4 expression, and that the inability of cells to form proximal nephron segments is not due to misregulation of Wnt4. The data also argue against a model whereby Notch promotes CMM differentiation through direct downregulation of Six2, exposing cells to Wnt-mediated induction. First, although Notch2 protein can be detected in the PTA and CMM, the ligands required for receptor activation, Dll1 and Jag1, are not expressed until the RV stage (see Fig. S3 in the supplementary material). Second, the CMM is intact and exit from the niche occurs normally in Notch2-null kidneys. RVs and truncated S-shaped bodies form, eventually differentiating into distal epithelium (Cheng et al., 2007). Moreover, there is no evidence for impairment of CMM to PTA transition in RBPjk or γ-secretase null kidneys, which lack all Notch signaling (Cheng et al., 2003; Cheng et al., 2007; Wang et al., 2003).

Whether activated Notch can repress Six2 remains to be determined; however, Six2-null cells cannot undergo MET without Wnt9b, whereas Notch1 expressing CMM cells readily undergo MET in the absence of Wnt9b and Wnt4. Thus, Six2 inhibition alone cannot explain the MET observed in NICD-expressing CMM cells. Instead, the data presented support a model of Notch activity in which signals are used to instruct or to inhibit cell fate choices in the early nephron, rather than mediate exit from the niche. Although there may be some differences in cell fate decisions mediated by activated Notch1 versus Notch2 in the CMM, we believe they essentially have the same effect on the stem cell pool: providing a permissive signal that promotes MET of cells poised to become epithelia. The precise differences between NICD1 and NICD2 in vivo will need to be addressed in an appropriate genetic model where these domains are exchanged.

Another interesting issue raised by these studies is the nature of the committed pre-epithelial state of the CMM following the split of the $Osr1^{+}$ population into distinct cap and stromal domains. The ability of N1ICD to convert CMM into PT indicates that segment-specific fate choices can be made at this stage, and fits with a model of renal epithelial differentiation whereby poised or bivalent regions in the genome are progressively restricted into chromatin that is either accessible or inaccessible to the transcription machinery (Surendran and Kopan, 2011). Our studies do not address whether this state is actively established in the CMM or whether it is repressed in the stromal compartment. Experiments designed to turn on N1ICD earlier in the common pool (i.e. using $Osr1^{Cre}$ at E10) could address this issue. Furthermore, these differences could represent a unique epigenetic landscape in each population, established by other signaling pathways (such as BMP) at the time of separation.

We do see an occasional $Cdh6^{+}$ cluster in the FoxD1-N1ICD-expressing population, raising the possibility that either some cells in the stroma retain the potential to undergo MET or that some CMM cells express FoxD1. Interestingly, we see rare incorporation of FoxD1-Cre-labeled cells into renal epithelia (A.P.M., unpublished). The significance of a small pool of progenitors that can adopt either interstitial or nephron fates is unclear.

In conclusion, we demonstrate here that the CMM exists in a poised pre-epithelial state, ready to undergo MET in response to a permissive signal. A number of pathways can elicit this response in vitro, regardless of their actual involvement in MET in vivo. Although Wnt9b and Wnt4 are genetically required in vivo, we show that they are not providing specific inductive information for mesenchyme to become epithelia. These findings shed new light on the earliest lineages in the developing kidney and raise the interesting question of the epigenetic state of the CMM that permits many stimuli to trigger MET. These insights should improve our understanding of how to achieve and control directed differentiation of renal stem cells for therapeutic purposes.

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Competing interests statement
The authors declare no competing financial interests.

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