

RESEARCH ARTICLE

Hedgehog-Gli signaling in *Foxd1*-positive stromal cells promotes murine nephrogenesis via TGF β signaling

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ABSTRACT

Normal kidney function depends on the proper development of the nephron: the functional unit of the kidney. Reciprocal signaling interactions between the stroma and nephron progenitor compartment have been proposed to control nephron development. Here, we show that removal of hedgehog intracellular effector smoothened (*Smo*-deficient mutants) in the cortical stroma results in an abnormal renal capsule, and an expanded nephron progenitor domain with an accompanying decrease in nephron number via a block in epithelialization. We show that stromal-hedgehog-*Smo* signaling acts through a Gli3 repressor. Whole-kidney RNA sequencing and analysis of FACS-isolated stromal cells identified impaired TGF β 2 signaling in *Smo*-deficient mutants. We show that neutralization and knockdown of TGF β 2 in explants inhibited nephrogenesis. In addition, we demonstrate that concurrent deletion of *Tgfb2* in stromal and nephrogenic cells *in vivo* results in decreased nephron formation and an expanded nephrogenic precursor domain similar to that observed in *Smo*-deficient mutant mice. Together, our data suggest a mechanism whereby a stromal hedgehog-TGF β 2 signaling axis acts to control nephrogenesis.

KEY WORDS: Gli, Hedgehog, Stroma, Nephron, Mouse, Crosstalk, TGF β

INTRODUCTION

The mammalian kidney contains hundreds of thousands of nephrons that each connect to a collecting system composed of collecting ducts, calyces, the renal pelvis and the ureter (Nyengaard and Bendtsen, 1992). These structures are supported by renal stromal cells that originate during embryonic organogenesis (Hatini et al., 1996; Mendelsohn et al., 1999). Nephrons and collecting system elements are derived from the metanephric mesenchymal SIX2⁺ cells and *Hoxb7*⁺ ureteric bud cells, respectively, each of which is derived, in turn, from *Osr1*⁺ intermediate mesoderm (Mugford et al., 2008). Reciprocal inductive interactions between ureteric and nephrogenic cells result in iterative branching of the ureteric bud (to form the collecting system) and induction of nephron formation by mesenchymal cells adjacent to ureteric bud tips, respectively

(Grobstein, 1953; reviewed by O'Brien and McMahon, 2013; Kopan et al., 2014; Dressler, 2009; Costantini and Kopan, 2010; Blake and Rosenblum, 2014). Nephrons form via sequential morphological changes in mesenchymal aggregates that progress to epithelial vesicles, comma- and S-shaped bodies and, finally, to mature nephrons.

Although the classical theory of mammalian nephron formation focuses on ureteric bud-metanephric mesenchyme tissue interactions, nephron formation is also controlled by *Foxd1*⁺ stromal cells that also derive from *Osr1*⁺ intermediate mesoderm (Mugford et al., 2008). During mouse kidney development, *Foxd1*⁺ cells can be identified as early as E11-E11.5 at the periphery of the metanephric mesenchyme (Hatini et al., 1996; Yallowitz et al., 2011; Hum et al., 2014). Thereafter, these cells come to surround the kidney and migrate between nephrogenic elements in the renal cortex (Yallowitz et al., 2011; Kobayashi et al., 2014). Homozygous deficiency of *Foxd1* or genetic ablation of *Foxd1*⁺ cells disrupts renal development, leading to: abnormal formation and cellular composition of the renal capsular stroma; disorganization of renal patterning; and decreased nephron formation (Hatini et al., 1996; Levinson et al., 2005; Hum et al., 2014; Das et al., 2013). Furthermore, deficiency of stromal genes, including *Pbx1*, *Pod1*, *Sall1* and *Fat4* cause similar perturbations, including expansion of SIX2⁺ cells in the condensing mesenchyme (Schnabel et al., 2003; Ohmori et al., 2015; Bagherie-Lachidan et al., 2015; Das et al., 2013; Quaggin et al., 1999). Together, these studies suggest that stromal cells interact with nephrogenic precursors to control nephron formation. Yet the identity of the factors that mediate these cell-cell interactions is largely undefined.

Hedgehog signaling is a highly conserved pathway that controls cell specification, cell-cell interactions and tissue patterning during embryonic development (Jiang and Hui, 2008; Ingham and McMahon, 2001). In mammals, there are three known hedgehog (HH) ligands, although only sonic hedgehog (SHH) is known to have a role in renal development (Yu et al., 2002; Hu et al., 2006). In the absence of SHH ligand, the patched (PTCH) family of cell-surface receptors bind to smoothened (SMO) and allow for the formation of a proteolytic complex that cleaves full-length Gli transcriptional activators Gli2 and Gli3 to transcriptional repressors (reviewed by Briscoe and Thérond, 2013; Robbins et al., 2012). When SHH ligand is present, it binds to PTCH, which in turn alleviates PTCH-mediated inhibition of SMO; this proteolytic complex is inhibited, allowing for the accumulation of full-length Gli transcriptional activators (Bai et al., 2002; Park et al., 2000). Thus, the genetic inactivation of SMO leads to obligate processing of Gli transcription factors. The ratio of Gli activators to Gli repressor determines the overall cellular response to HH stimulation. In the mammalian kidney, SHH controls early morphogenetic events, including ureteric bud growth and metanephric induction by controlling formation of the Gli3 repressor (Gli3R) (Hu et al., 2006; Blake et al., 2016). In addition, Gli-dependent signaling is crucial for expression of genes required

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DOI: 10.1242/dev.159947

for ureteric branching and the development of pacemaker activity by the ureter (Cain et al., 2009; Cain et al., 2011).

We demonstrate that HH-GLI signaling in *Foxd1*⁺ stromal cells controls the formation of capsular stromal cells in a cell autonomous fashion and nephron development in a non-cell autonomous manner. We further demonstrate that HH signaling is active in *Foxd1*⁺ cortical stromal cells of the embryonic kidney and that CRE-mediated deficiency of *Smo* in *Foxd1*⁺ stromal cells disrupts the formation of the renal capsule and decreases nephron number by impairing the formation of renal vesicles. RNA sequencing in *Smo*-deficient kidney tissue identified *Tgfb2* as a downstream target of Hedgehog-GLI signaling. Neutralization and knockdown of TGFβ2 in embryonic kidney explants decreased nephron formation. CRE-mediated deletion of the TGFβ receptor *Tgfr2* in *Foxd1*⁺ stromal cells disrupted stromal patterning and expanded *Six2*⁺ nephrogenic precursors in the condensing mesenchyme, partially recapitulating the *Smo*-deficient phenotype. In addition to these abnormalities, deletion of *Tgfr2* in both *Foxd1*⁺ and *Six2*⁺ cells causes nephron deficiency and renal hypoplasia. Together, these results implicate HH signaling as a regulator of TGFβ2 signaling between stromal cells and nephrogenic compartments.

RESULTS

A murine model of HH signaling deficiency in the renal cortical stroma

Previous work using a *Ptch1* reporter allele *in vivo* suggested that HH signaling activity is limited to the ureter and medullary domain of the embryonic kidney (Cain et al., 2009). We further investigated the spatial domain of HH signaling activity using two distinct approaches.

In situ hybridization demonstrated expression of *Gli1*, a downstream HH signaling target, in the kidney cortex, including the developing kidney capsule and the nephrogenic zone (Fig. 1A). *Gli1* expression was further examined in mice in which *lacZ* expression was activated at the onset of ureteric branching (E11.5) via a tamoxifen-inducible *Gli1Cre^{ERT2}* allele. X-gal staining demonstrated β-galactosidase activity in the cortical stroma surrounding the ureteric bud and nephrogenic precursors, indicating HH signaling activity in this domain, consistent with previous observations in newborn mice (Fabian et al., 2012) (Fig. 1B). To investigate the role of HH signaling in the cortical stroma, we generated mice with conditional *Smo* deficiency targeted to the stromal lineage using *Foxd1Cre-eGFP* mice (*Smo*-deficient mutants; *Foxd1Cre;Smo^{loxP/+}*). *In situ* hybridization for *Gli1* in E13.5 *Smo*-deficient mutants confirmed a decrease in HH signaling, specifically in the outer cortex (Fig. 1C). To further verify deletion of *Smo*, we used eGFP reporter expression driven by the *Foxd1* promoter to isolate *Foxd1*⁺ cells using fluorescence-activated cell sorting (FACS) (Fig. S1). Quantitative RT-PCR (qRT-PCR) analysis of E13.5 mutant FACS-isolated stromal cells demonstrated an 86% decrease in *Smo* mRNA expression, as well as decreased expression of the HH target genes *Ptch1* and *Gli1* (Fig. 1D, Fig. S2A). Decreased *Smo*, *Gli1* and *Ptch1* expression, measured in FACS-isolated stromal cells, persisted at E15.5 (Fig. S2B).

Smo deficiency in *Foxd1*-positive stromal cells disrupts development of the cortical stroma and reduces nephron number

FOXD1 is crucial to development of the renal capsular stroma and stromal-nephrogenic cell interactions (Hatini et al., 1996; Levinson

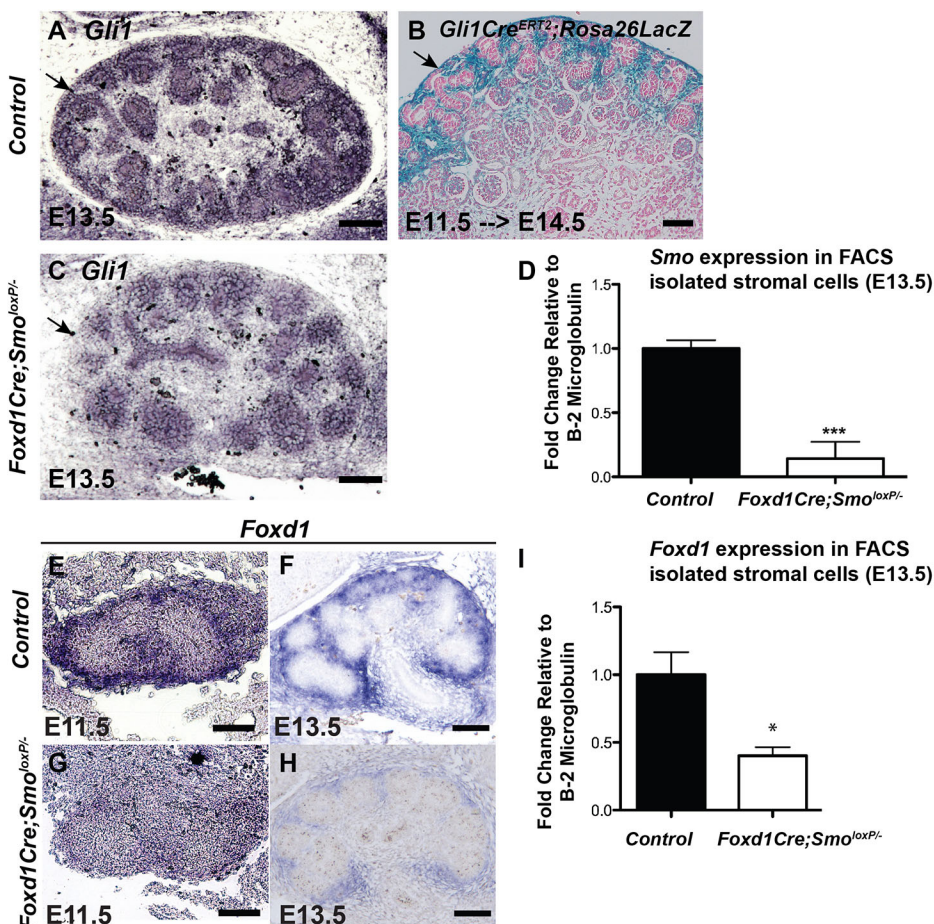


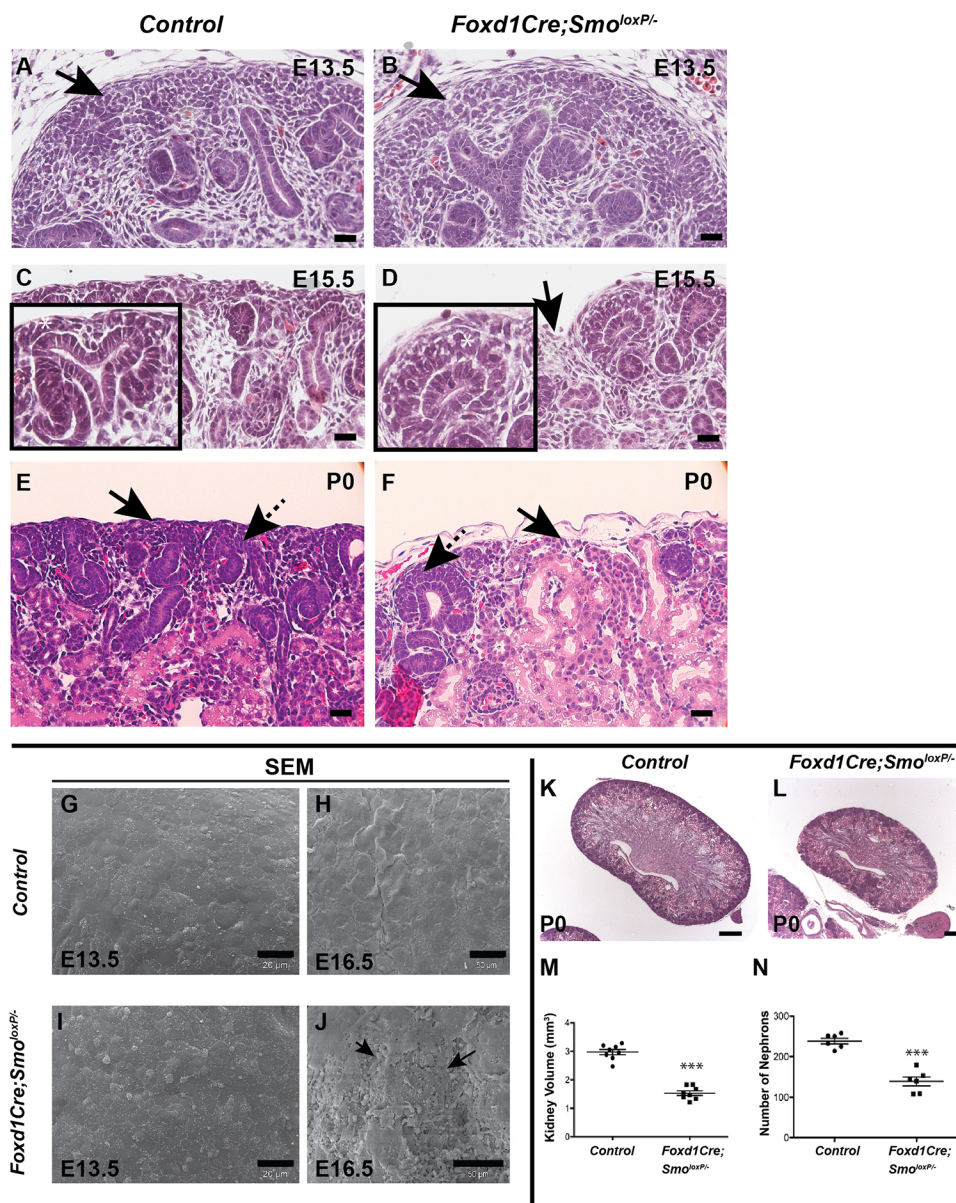
Fig. 1. A model of HH signaling deficiency in the renal cortical stroma. (A-D) *In situ* hybridization of *Gli1* shows expression in the cortical stroma and capsule (arrows). (B) Active HH signaling in the cortex is shown using inducible *Gli1Cre* expression. Conditional deletion of *Smo* from the cortical stroma using *Foxd1Cre* results in a loss of *Gli1* (compare C with A) and *Smo* expression at E13.5 (D, 1:0.14, *n*=4 *Foxd1Cre;Smo^{loxP/+}* and *n*=7 mutants, ****P*<0.0001). (E-H) *In situ* hybridization for stromal cell identity markers *Foxd1* shows decreased expression at multiple embryological time points. (I) Quantitative RT-PCR for *Foxd1* demonstrates a significant decrease in E13.5 mutant FACS-isolated stromal cells compared with control stromal cells (1:0.40, *n*=4 *Foxd1Cre;Smo^{loxP/+}* and *n*=5 mutants, **P*=0.025). Data are mean±s.e.m. Scale bars: 100 μm in A,C,E,H; 200 μm in B.

et al., 2005; Hum et al., 2014; Fetting et al., 2014). Accordingly, we first investigated *Foxd1* expression in *Smo*-deficient mice. *In situ* hybridization at multiple embryonic stages demonstrated decreased *Foxd1* expression beginning at E11.5 (Fig. 1E-H). Loss of *Foxd1* expression in *Smo*-deficient mice was confirmed by qRT-PCR in FACS-isolated stromal cells. *Foxd1* mRNA in cells from E13.5 *Foxd1Cre;Smo^{loxP/-}* mice was decreased by 60% compared with that in cells from *Foxd1Cre;Smo^{loxP/+}* mice (Fig. 1I). Together, these data suggest that HH signaling in cortical stroma is required for the expression of the key stromal gene *Foxd1*.

Since *Foxd1Cre* is a 'knock-in' allele that causes heterozygous expression of *Foxd1*, we first established that *Foxd1Cre;Smo^{loxP/+}* mice do not exhibit renal histological abnormalities at P0 (Fig. S3). In contrast, histological analysis of *Smo*-deficient kidney tissue revealed mis-patterning of the renal cortex as early as E15.5 (Fig. 2). Although stromal cells and cortical structures were normally patterned at E13.5 (Fig. 2A,B, arrows), by E15.5, the capsular stromal layer was disrupted (arrow in Fig. 2D) and nephrogenic precursors were expanded in number (Fig. 2C,D, asterisk in inset).

At P0, *Smo*-deficient mutants exhibit severe patterning defects in the nephrogenic zone with almost complete absence of the capsular stromal layer (solid arrows in Fig. 2), an increased number of nephrogenic precursor cells in the condensing mesenchyme (dotted arrows in Fig. 2), and tubules projecting into the kidney surface (Fig. 2E,F). *Smo*-deficient mice died within a few hours of birth, precluding postnatal analysis of kidney structure and function.

The morphology of the renal capsule was further analyzed by scanning electron microscopy (SEM). Consistent with histological analysis (Fig. 2A-B), E13.5 mutant tissue was characterized by a honeycomb-like appearance (Fig. 2G,I), as observed in control mice. However, by E16.5, mutant mice were characterized by a discontinuous renal capsule (Fig. 2H,J, see arrows). To further characterize the state of the capsule and cortical stroma, we analyzed expression of stromal markers RALDH2 and PBX1 at both E13.5 and E15.5. Immunostaining demonstrated the presence of RALDH2⁺ and PBX1⁺ stromal cells at E13.5 in *Smo*-deficient mice. However, by E15.5, staining of RALDH2 was decreased in intensity and there were gaps in the continuity of RALDH2⁺/PBX1⁺ cells in



the renal capsule (Fig. S4). This apparent loss in stromal cells was not associated with decreased proliferation or increased apoptosis at E13.5 (Fig. S5A-F). However, at E15.5, *Smo*-deficient stromal cells were characterized by a small (6%), but significant, decrease in cortical stromal proliferation, and a marked increase in apoptosis (2.4-fold). The vast majority of cortical stromal cells that were TUNEL⁺ in *Smo*-deficient mice were located in the space between tubule and nephrogenic elements (Fig. S5G-L).

Finally, by P0, kidney size was markedly decreased in mutant mice, associated with a 48% decrease in volume (Fig. 2K-M). Consistent with this hypoplastic phenotype, the number of nephrons at E18.5 was decreased by 42% in mutant mice (Fig. 2N). Taken together, these data demonstrate that *Smo* deficiency in stromal cells disrupts development of the renal stromal capsule and causes renal hypoplasia with nephron deficiency.

***Smo* deficiency in *Foxd1*-positive stromal cells decreases formation of nephrogenic precursor structures while expanding the number of nephron progenitor cells**

Histological analysis suggested that the pool of cells that give rise to nephrons is expanded in mutant mice, despite a deficiency of nephrons in the mature kidney (Fig. 2). To further investigate this apparent paradox, we analyzed the population of SIX2⁺ cells that give rise to nephrons (Fig. 3). Qualitative analysis of tissue sections stained with anti-SIX2 antibody suggests the number of SIX2⁺ cells in the condensing mesenchyme surrounding each ureteric tip is increased at both E13.5, when this change was not apparent by

histology, and at E15.5 (Fig. 3A,B,D,E). Quantitative analysis of mid-sagittal sections demonstrated a 1.6-fold increase in the number of SIX2⁺ cells per ureteric tip at E15.5 (Fig. 3C). To investigate whether the expanded SIX2⁺ domain was a result of increased proliferation, we used immunostaining with anti-phospho-histone H3 (PHH3) to mark SIX2⁺ cells in an active state of cell division. *Smo*-deficient mutants exhibited a 42% increase in SIX2⁺ cell proliferation compared with that in controls (Fig. 3D-F). These data demonstrate that stromal-HH signaling acts in a non-cell autonomous manner to restrict the number of nephrogenic cells.

Our observation of decreased nephron number in *Smo*-deficient mice, despite an expansion in the number of nephron progenitors, raised the possibility that nephrogenic precursor cells are inhibited while undergoing a mesenchymal-epithelial transformation. To determine this, we analyzed the expression of *Wnt9b*, a ureteric cell-derived factor that is required to induce SIX2⁺ mesenchyme condensation in conjunction with *Wnt4* (Park et al., 2012; Self et al., 2006; Park et al., 2007). Expression analysis using *in situ* hybridization demonstrated no apparent difference in *Wnt9b* or *Wnt4* expression at E15.5 (Fig. S6). Furthermore, quantitation of the number of pretubular aggregates, identified by NCAM staining (Fig. 3G,H), demonstrated no significant difference between mutant and control mice (Fig. 3I). As the number of ureteric branches is also a determinant of nephron number, we examined this process. We observed no difference in the number of ureteric branches generated in mutant mice (Fig. S7A-C) nor in the expression of genes involved in ureteric branching, including *Ret*, *Gdnf* and *Wnt11* (Fig. S7D-I).

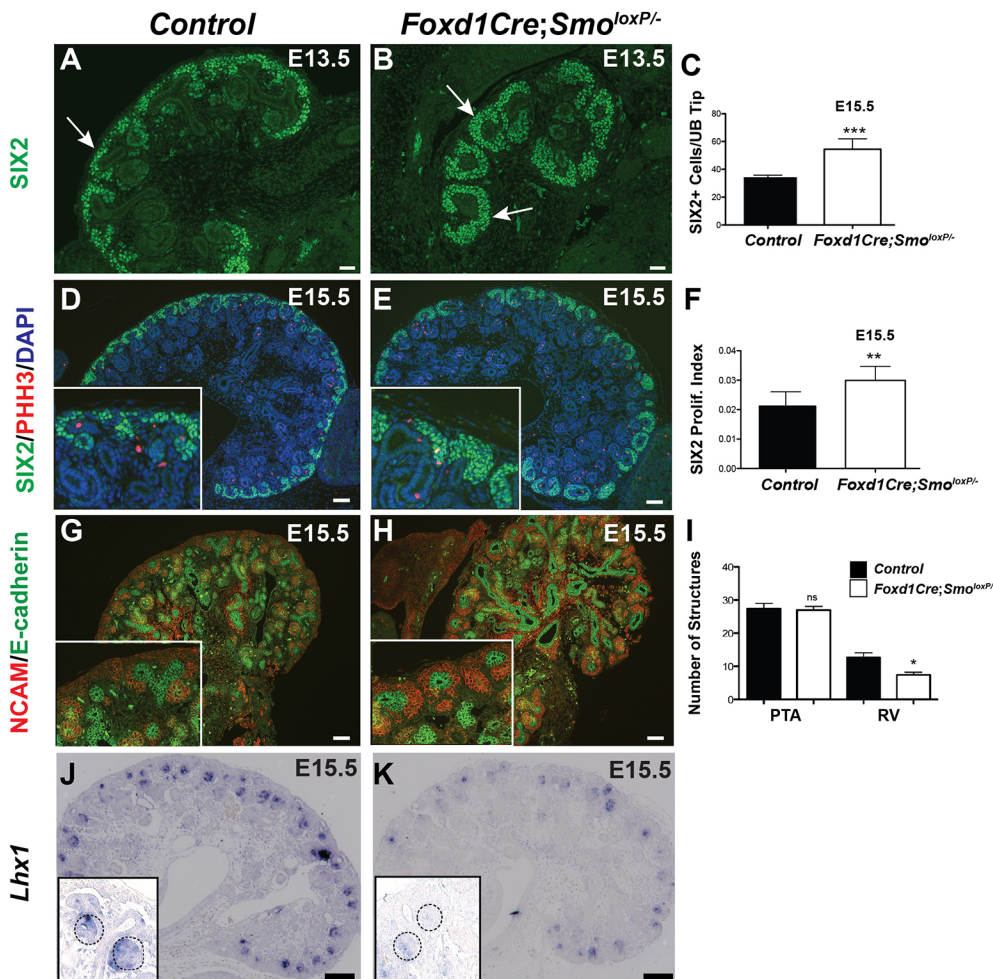


Fig. 3. *Smo*-deficient mice demonstrate an increased number of nephron progenitors and decreased MET. (A,B,D,E) Mutant kidneys have an apparent increase in nephrogenic precursor cells, marked by SIX2 expression, throughout development. (C,F) The increased number of SIX2⁺ nephrogenic precursor cells in mutant mice (C, 1.6-fold increase, ***P<0.0001) have an increased proliferative index at E15.5 (F, 42% increase; mutant versus control; 0.029 versus 0.021, **P=0.001). (G-I) In contrast to the increased nephrogenic precursor cells, mutants exhibit a 41% (control versus mutant, 12.7 versus 7.4, n=6, *P=0.01) decrease in renal vesicle structures (RV, marked with NCAM and E-cadherin) compared with a similar number (control versus mutant, 27.4 versus 26.9, n=6, P=0.82) of pretubular aggregates (PTA, marked with NCAM). (J,K) *Lhx1*, a marker of renal vesicles, is markedly decreased at E15.5 in mutants by *in situ* hybridization. Insets show higher resolution imaging of renal structures. Data are mean±s.e.m. Scale bars: 100 µm in A,B,J,K; 200 µm in D,E, G,H; 500 µm in insets in G,H; 400 µm in insets in D,E; 100 µm in insets in J,K.

Next, we investigated the expression of *Lhx1*, which controls formation of renal vesicles from pretubular aggregates, an essential step in the process of mesenchymal-epithelial transformation during nephrogenesis (Kobayashi et al., 2005). In contrast to control mice, *Lhx1* expression in apparent renal vesicles and comma-shaped bodies was markedly reduced in *Smo*-deficient kidneys at E15.5 (Fig. 3J,K). Reduction in *Lhx1* is consistent with the hypothesis that signals other than Wnt4 regulate *Lhx1*, as *Lhx1* expression is maintained, albeit at lower levels, in *Wnt4*-null embryos (Kobayashi et al., 2005). Quantitation of the number of renal vesicles, marked by E-cadherin, demonstrated a 41% decrease in *Smo*-deficient mutants (Fig. 3I). Together, these results indicate that loss of SMO-dependent signaling in stromal cells impairs the formation of nephrogenic epithelial structures to a degree consistent with the final decrement in nephron number.

***Smo* deficiency exerts its deleterious effects in the renal stroma via the *Gli3* repressor**

HH ligands signal via GLI-dependent and -independent mechanisms. Previously, we have identified GLI3 as the GLI family member that controls renal development in cell lineages (ureteric, metanephric mesenchyme) other than stromal cells (Cain et al., 2009, 2011; Blake et al., 2016). Here, we have investigated the contribution of *Gli3* in renal stroma by crossing *Smo*-deficient mice with mice deficient in *Gli3* (*Foxd1Cre;Smo^{loxP/-};Gli3^{-/-}*). Removal of *Gli3* in the *Smo*-deficient background rescued tissue patterning and capsular defects (Fig. 4A-C,E-G). Immunostaining for SIX2 confirmed that the expanded condensing mesenchyme phenotype observed

in *Smo*-deficient mutants was rescued by homozygous deletion of *Gli3* (Fig. 4I-K). Furthermore, *Gli3* deficiency partially rescued both kidney volume and nephron number, demonstrating that GLI3 is the effector by which HH non-cell autonomous effects are observed (Fig. 4M,N). GLI3 plays dual roles in HH signaling as it exists as a full-length transcriptional activator (GLI3A) and truncated transcriptional repressor (GLI3R). The relative amount of GLI3A versus GLI3R is regulated by HH-SMO signaling. Phenotypic rescue by generating *Gli3* deficiency in *Smo*-deficient mice suggested that GLI3R is pathogenic in this context. This possibility was further tested by introducing an obligate GLI3R targeted to stroma using *Foxd1Cre (Foxd1Cre;Gli3Tflag^{+/+};Δ699⁺)*. Compound mutant mice phenocopied *Smo*-deficient mutants as shown by renal hypoplasia, capsule deficiency and expansion of condensing mesenchyme (Fig. 4D,H,L). Taken together, these data demonstrate that HH signaling in stromal cells exerts cell-autonomous and non-cell autonomous effects by controlling levels of GLI3R.

RNA sequencing of *Smo*-deficient kidneys identifies TGFβ2 as a downstream target of HH-GLI signaling

We hypothesized that a HH-dependent secreted factor from the stroma controls adjacent nephrogenic cells, thus controlling the epithelialization of these cells. To identify possible effectors, we performed RNA sequencing on E13.5 wild-type and *Smo*-deficient whole kidneys. Of the dysregulated genes, the vast majority were downregulated in mutant mice (Fig. 5A). Key genes are listed in Table 1. *Smo*, *Ptch1* and *Gli1* expression were decreased in mutant kidneys, consistent with *Smo* deficiency and decreased HH signaling.

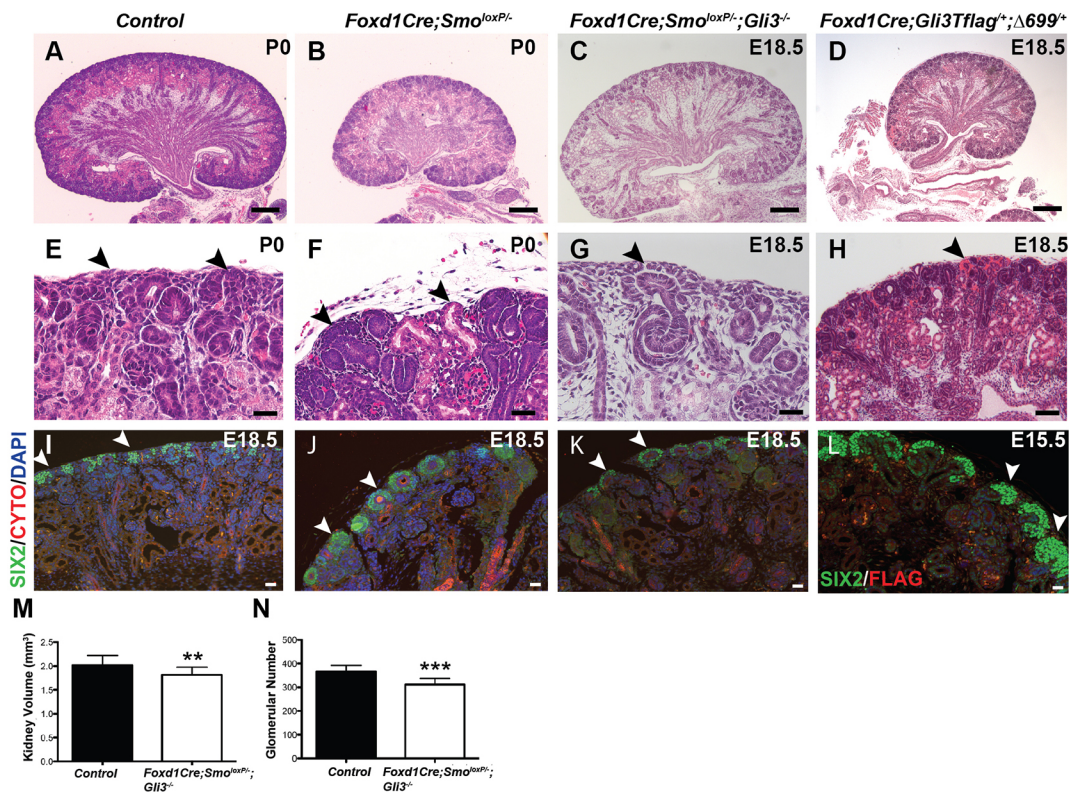


Fig. 4. *Smo* deficiency in the renal stroma is dependent on GLI3R. (A-H) Hematoxylin and Eosin-stained images reveal that deletion of *Gli3* rescues nephrogenic zone patterning and capsular defects seen in *Smo*-deficient mutants (A-C,E-G), whereas introduction of GLI3R phenocopies the *Smo*-deficient mutants (D,H). Arrowheads indicate the renal capsule (E-H). (I-L) Immunostaining for SIX2 shows that deletion of *Gli3* rescues the expanded condensing mesenchyme (K) and obligate stromal GLI3R causes an expansion in condensing mesenchyme (L, arrowheads). (M,N) Removal of *Gli3* partially, but not completely, rescues kidney volume (M; 10% decrease, control versus mutant, $n=6$, $**P=0.0081$) and nephron number (N; 15% reduction, control versus mutant, $n=6$, $***P<0.001$) at E18.5. Data are mean±s.e.m. Scale bars: 1000 μm in A-D; 20 μm in E-H; 100 μm in I-L.

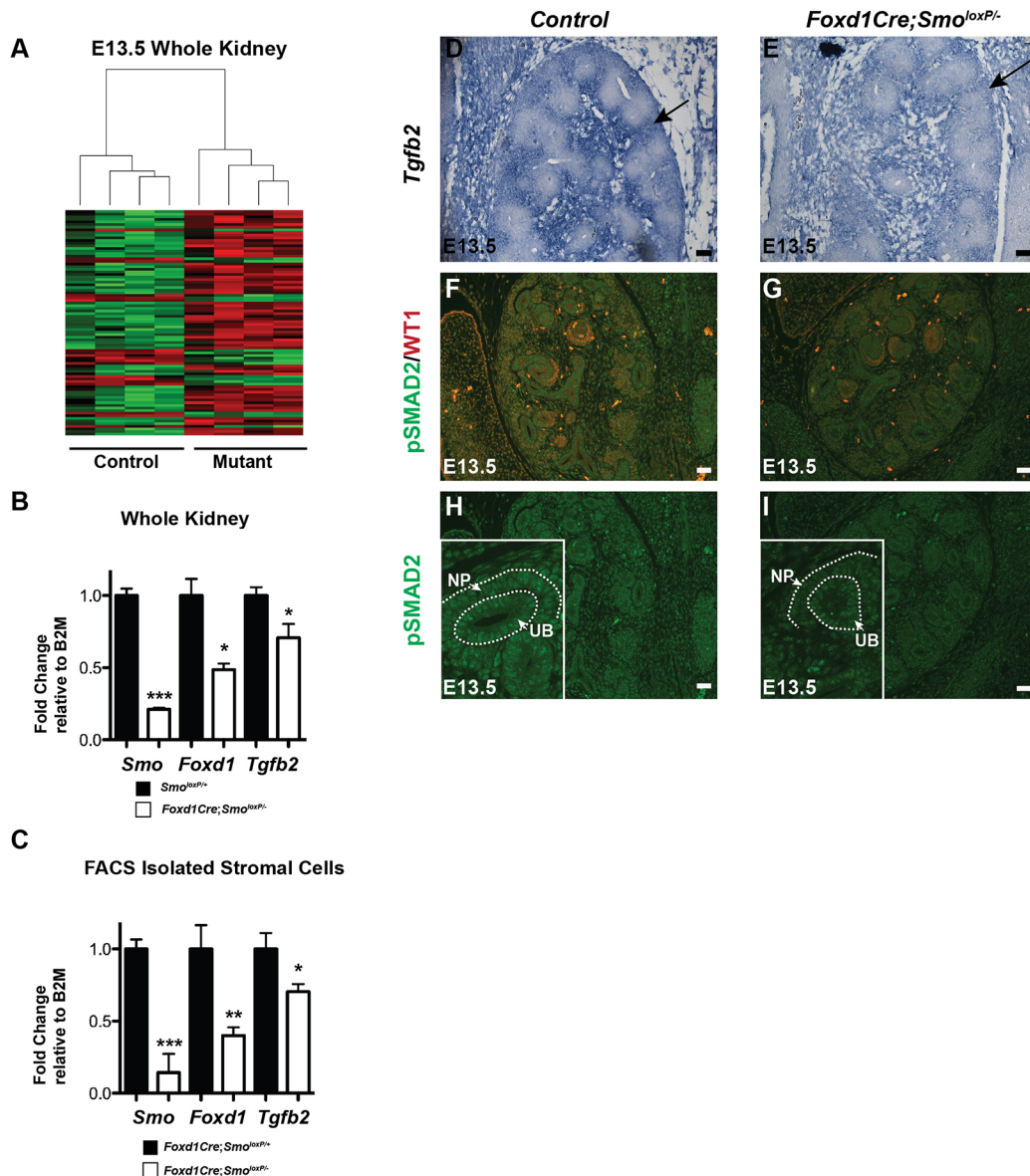


Fig. 5. RNA sequencing identifies downstream gene targets perturbed in *Smo*-deficient mice. (A) Heat-map representation of RNA sequencing of E13.5 control and mutant whole kidneys reveals differences in gene expression in mutant tissue (green, upregulated; red, downregulated). (B,C) Quantitative RT-PCR validation of select candidates in E13.5 whole kidney tissue (B) (control versus mutant; *Smo*, 1:0.212, $n=4$, $***P<0.01$; *Foxd1*, 1:0.485, $n=4$, $**P=0.005$; *Tgfb2*, 1:0.71, $*P=0.025$, $n=6$ wild type and $n=7$ mutants) and FACS isolated stromal cells (C) (control versus mutant; *Smo*, 1:0.14, $n=4$ controls and $n=7$ mutants, $***P<0.0001$; *Foxd1*, 1:0.40, $n=4$ controls and $n=5$ mutants, $**P=0.001$; *Tgfb2*, 1:0.70, $*P=0.01$, $n=4$ controls and $n=7$ mutants). (D,E) *Tgfb2* expression, by *in situ* hybridization, in stromal cells of wild-type E13.5 kidney tissue (arrows) is diminished in *Smo*-deficient mutants. (F-I) Phospho-SMAD2 staining (green) is decreased in *Smo*-deficient kidneys, specifically in nephrogenic precursor cells (see inset in I). NP, nephrogenic precursor; UB, ureteric bud. Data are mean $\pm$ s.e.m. Scale bars: 100 μ m; 300 μ m in insets.

Expression of *Foxd1* was decreased consistent with *in situ* hybridization and analysis of RNA expression in FACS-sorted *Foxd1*-positive cells (Table 1, Fig. 1). Expression of *Six2* was increased consistent with our previous analyses. To identify candidate genes that could mediate stromal-nephrogenic cell interactions, the population of dysregulated genes was filtered for factors expressed in the stroma and secreted, and with a known role in epithelialization. Among the genes decreased in *Smo*-deficient kidney tissue (Table 1), *Tgfb2* best fits these criteria in that it is secreted, expressed in the cortical stroma (Pelton et al., 1991) and is reported to have a role in epithelialization (Plisov et al., 2001). Furthermore, *Tgfb2*-KO mice exhibit renal agenesis with incomplete penetrance, with reduced nephron endowment (Sanford et al., 1997). Downregulation of

Tgfb2, as well as *Smo* and *Foxd1*, was verified using qRT-PCR in E13.5 whole kidneys (Fig. 5B). *Tgfb2* expression was further investigated in FACS-isolated *Foxd1*⁺ cells from E13.5 *Foxd1Cre;Smo^{loxP/+}* (control) and *Foxd1Cre;Smo^{loxP/-}* (mutant) mice. qRT-PCR analysis demonstrated a significant decrease in *Tgfb2*, as well as *Smo* and *Foxd1*, in mutant stromal cells (Fig. 5C). This finding was further confirmed by *in situ* hybridization (Fig. 5D,E, arrows). In addition, immunostaining for pSMAD2 demonstrated a decrease in nuclear staining within nephrogenic precursor cells, but not in ureteric bud cells, in *Smo*-deficient mice compared with controls (Fig. 5F-I). Together, these results demonstrate that SMO-dependent signaling in stromal cells controls expression of *Tgfb2* and downstream signaling events.

Table 1. Summary of select targets of RNA sequencing of E13.5 *Smo*-deficient mutants

Gene	Fold change	P value (adjusted)
<i>Smo</i>	0.24	<0.00001
<i>Ptch1</i>	0.71	0.011
<i>Gli1</i>	0.63	<0.00001
<i>Foxd1</i>	0.44	<0.001
<i>Tgfb2</i>	0.65	<0.0001
<i>Six2</i>	1.27	0.001

Neutralization and knockdown of TGFβ2 results in fewer nephrons *ex vivo*

We investigated the functional contribution of *Tgfb2* to nephron formation in cultured embryonic kidney explants. Neutralizing antibody against TGFβ2 was added to culture media of E12.5 wild-type kidney explants for 3 days after which time the explants were imaged. Treated explants were smaller and appeared abnormal compared with controls (Fig. 6A). Quantification of nephrons, marked by WT1, revealed a 65% decrease in anti-TGFβ2 treated explants compared with controls (Fig. 6B). Nephron number was decreased in a dose-dependent manner. The possibility of antibody-mediated toxicity was ruled out by the lack of inhibitory effects in control

explants treated with an equivalent concentration of IgG (Fig. 6A,B). *Tgfb2* function was further tested by *in vivo* morpholino-mediated knockdown using a splice-inhibiting morpholino (MO) specific for TGFβ2 and a control (non-targeting) MO (Eisen and Smith, 2008; Hartwig et al., 2010; Kann et al., 2015; Jacobi et al., 2013; Motamed et al., 2014; Rudigier et al., 2017). *Tgfb2* knockdown by splice-inhibiting MO was verified using qRT-PCR on treated explants (Fig. S8). Treatment with a TGFβ2 splice-inhibiting MO resulted in similar effects to that observed with neutralizing antibodies. TGFβ2 splice MO-treated explants exhibited fewer nephrons (33%) compared with control MO-treated explants (Fig. 6C,D). Together, our data provide evidence of a functional, dose-dependent role for TGFβ2 signaling in the normal differentiation of nephron progenitor cells, consistent with *Tgfb2*-KO mouse models.

Deficiency of TGFβ receptor II (*Tgfr2*) in both stromal and nephrogenic cells causes abnormalities similar to those in *Smo*-deficient mice

Next, we investigated which cell population is targeted by stroma-mediated TGFβ2 signaling, resulting in the deleterious effects on nephrogenesis. We generated mice in which deficiency of the TGFβ-specific receptor, *Tgfr2*, was generated in *Hoxb7*⁺, *Six2*⁺

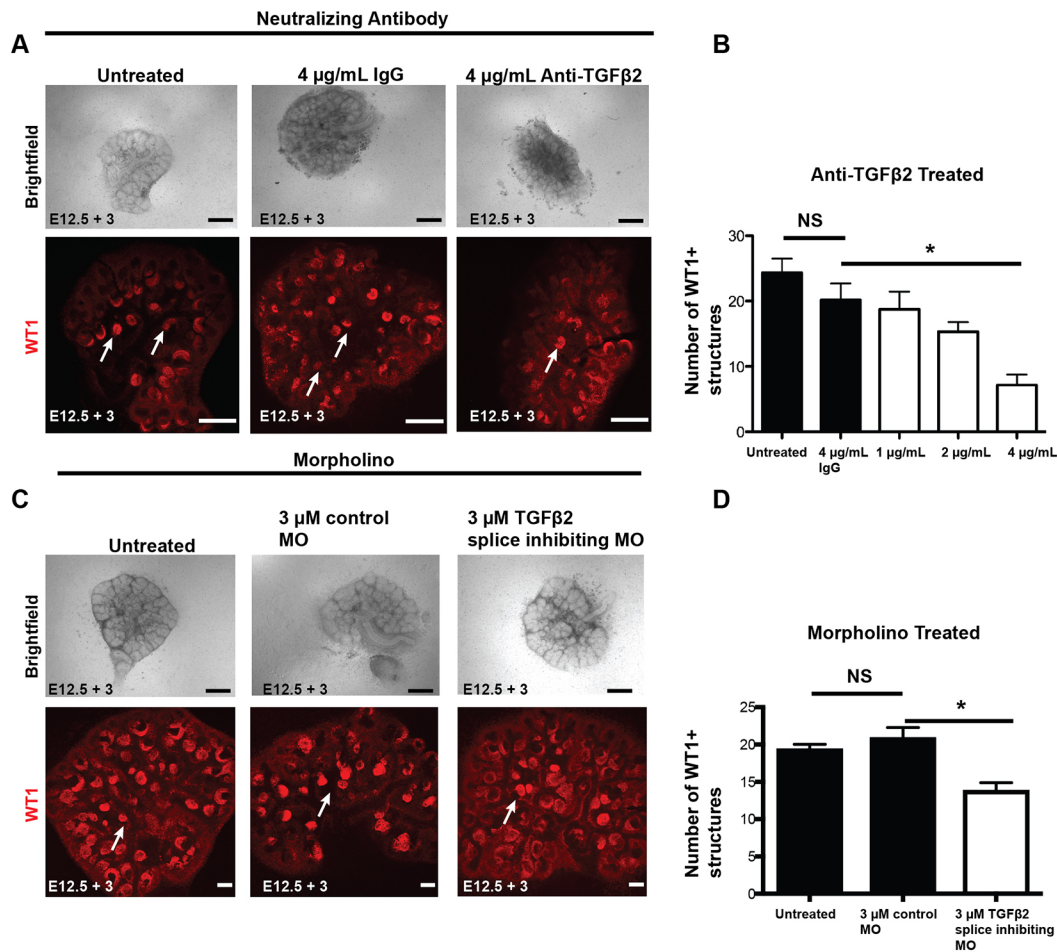


Fig. 6. Neutralization and knockdown of TGFβ2 results in fewer nephrons *ex vivo*. (A,B) Neutralization of TGFβ2 results in fewer nephrons quantified by WT1 staining [number of nephrons (arrows in A) quantified in B; 4 μg/ml IgG versus 4 μg/ml anti-TGFβ2: 20.2 versus 7.2, *n*=3, **P*=0.017]. (C,D) Knockdown of TGFβ2 using *in vivo* morpholinos results in a decrease in the number of WT1⁺ structures, similar to neutralization [number of nephrons (arrows in C) quantified in D; control MO treated versus splice inhibiting MO treated: 20.9 versus 13.8, *n*=3 controls MO and *n*=4 splice inhibiting MO treated, **P*=0.015]. Each biological replicate (*n*) was an average of untreated or treated explants from a different litter. Data are mean±s.e.m. Scale bars: 1000 μm in bright-field images; 220 μm in immunofluorescent images.

or *Foxd1*⁺ cells in a CRE-dependent manner. Deletion of *Tgfb2* in *Hoxb7*⁺ cells did not adversely affect renal development (Fig. 7A-C). *Tgfb2* deficiency in *Six2*⁺ cells caused a small but statistically significant decrease (18%) in nephron number (Fig. 7D,E). *Tgfb2* deficiency in *Foxd1*⁺ cells (Fig. S9) did not affect nephron number, but interfered with capsule formation (Fig. 7F,G, see inset in F). Remarkably, *Tgfb2* deficiency in both *Foxd1*⁺ and *Six2*⁺ cells concurrently disrupted capsule formation accompanied by fewer nephrons (Fig. 7H,I, inset in H). Although deletion of *Tgfb2* in *Hoxb7*⁺ or *Six2*⁺ cells did not affect the number of SIX2⁺ nephrogenic progenitors per UB tip (Fig. 8A-E), *Tgfb2* deficiency in *Foxd1*⁺ cells or in both *Foxd1*⁺ and *Six2*⁺ cells concurrently resulted in a 28% and 20% increase in the number of SIX2⁺ cells per UB tip, respectively (Fig. 8F-I). Thus, *Tgfb2* deficiency in both *Foxd1*⁺ and *Six2*⁺ cells produced a phenotype highly similar to that observed in *Smo*-deficient mice. Our results *in vivo* and *ex vivo* support the hypothesis that there is TGFβ2-dependent crosstalk between stromal and nephrogenic compartments, and that HH-GLI signaling in the stromal compartment serves as the master regulator of this interaction.

DISCUSSION

Although the embryonic *Foxd1*⁺ renal stroma has been shown to modulate both nephrogenesis and branching morphogenesis, the molecular mechanisms that control stromal cells themselves and that mediate stromal interactions with adjacent cells are largely undefined. Here, we demonstrate that stromal HH-SMO signaling acts in a cell-autonomous manner to control renal capsule formation and, in a non-cell autonomous fashion, to control epithelialization of nephrogenic precursor cells and final nephron number. The role of GLI-dependent signaling downstream of SMO was demonstrated by a rescue of the *Smo*-deficient phenotype by homozygous deletion of *Gli3* in *Smo*-deficient mice. Furthermore, targeted expression of GLI3R in *Foxd1*⁺ stromal cells phenocopied the hypoplastic phenotype of *Smo*-deficient mutants. Analysis of *Smo*-deficient mutants revealed that HH signaling controls expression of *Tgfb2*. The functional contribution of TGFβ2 was demonstrated through *ex vivo* experiments in which neutralization and knockdown of TGFβ2 resulted in a decrease in nephrons. These results were further substantiated by genetic experiments *in vivo*, in which

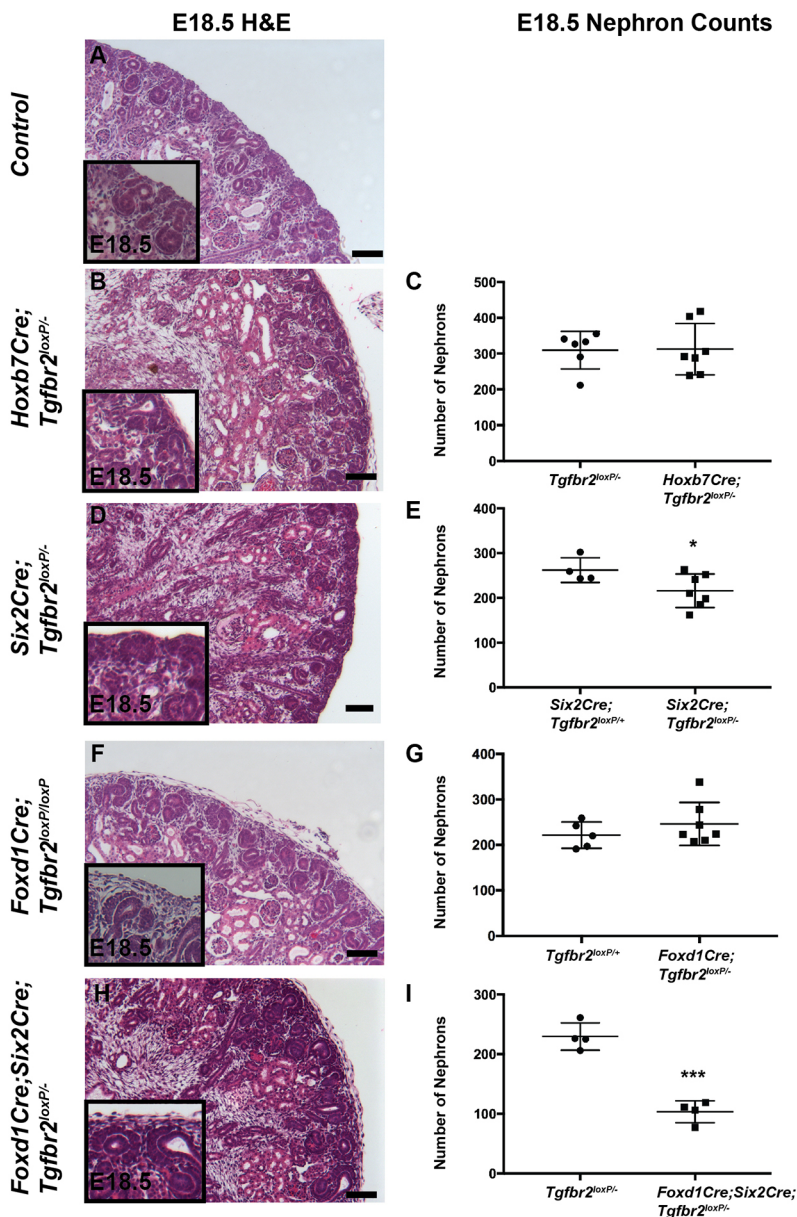


Fig. 7. Nephron number is decreased in *Six2Cre;Tgfb2^{loxPI/-}* and *Foxd1Cre;Six2Cre;Tgfb2^{loxPI/-}* mutant mice.

(A,B,D) Hematoxylin and Eosin staining demonstrated no obvious abnormalities in the nephrogenic zone of *Hoxb7Cre;Tgfb2^{loxPI/-}* or *Six2Cre;Tgfb2^{loxPI/-}* mutant mice. Insets show the nephrogenic zone. (C,E) In contrast, there was a modest decrease in the number of nephrons in *Six2Cre;Tgfb2^{loxPI/-}* mutant mice [control versus *Hoxb7Cre;Tgfb2^{loxPI/-}* (C): 310 versus 312, *n*=6 controls, *n*=7 mutants, *P*=0.94; control versus *Six2Cre;Tgfb2^{loxPI/-}* (E): 262 versus 216, *n*=4 controls, *n*=7 mutants, **P*=0.047]. (F,H) Both *Foxd1Cre;Tgfb2^{loxPI/oxP}* and *Foxd1Cre;Six2Cre;Tgfb2^{loxPI/-}* mutants exhibited similar defects, including a disrupted capsule (insets). (G,I) However, only *Foxd1Cre;Six2Cre;Tgfb2^{loxPI/-}* compound mutants exhibited a decrease in nephron number [control versus *Foxd1Cre;Tgfb2^{loxPI/-}* (G): 222 versus 246, *n*=5 controls and *n*=7 mutants, *P*=0.29; control versus *Foxd1Cre;Six2Cre;Tgfb2^{loxPI/-}* (I): 230 versus 103, *n*=4 controls and mutants, ****P*=0.0002]. All mutant genotypes were compared with individual littermate controls from the same cross. Data are mean±s.e.m. Scale bars: 200 μm; 400 μm in insets.

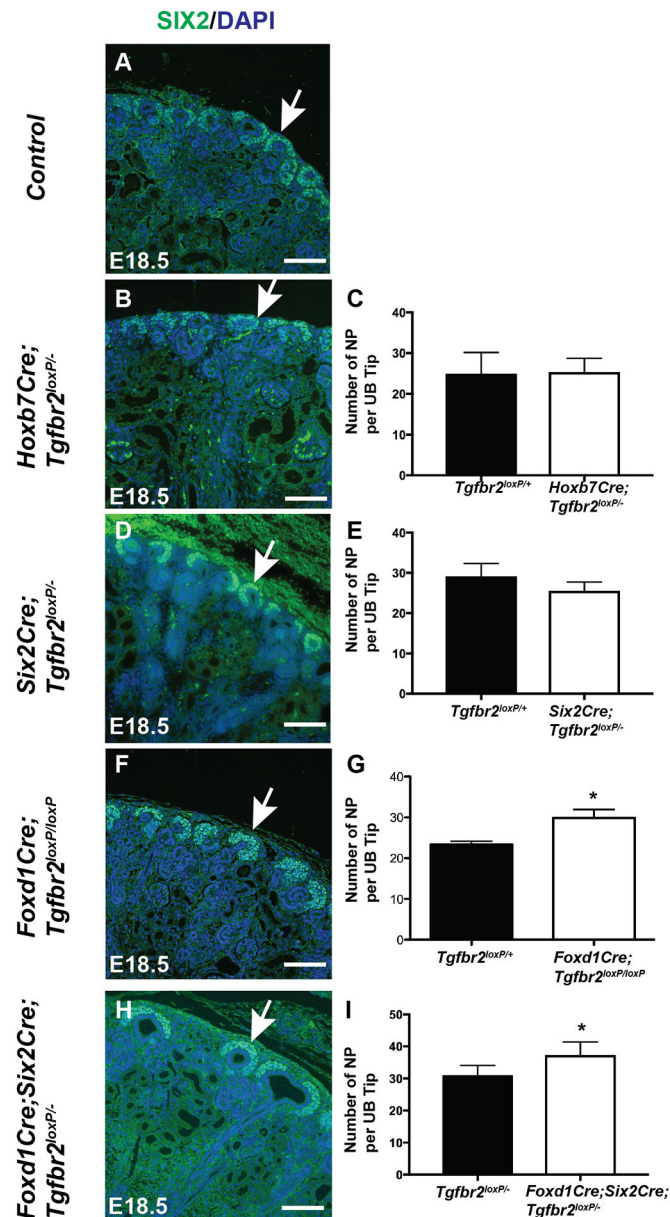


Fig. 8. The nephrogenic precursor domain is expanded in *Foxd1Cre; Tgfb2^{loxPI/loxP}* and *Foxd1Cre; Six2Cre; Tgfb2^{loxPI/-}* mutant mice. (A,B,D,F) Compared with controls (A), *Hoxb7Cre; Tgfb2^{loxPI/-}* (B) and *Six2Cre; Tgfb2^{loxPI/-}* (D) mutant mice, *Foxd1Cre; Tgfb2^{loxPI/loxP}* mutants (F) exhibit an expansion in SIX2⁺ nephrogenic precursor cells similar to *Smo*-deficient mutants. (C,E) When quantified, *Hoxb7Cre; Tgfb2^{loxPI/-}* (C) and *Six2Cre; Tgfb2^{loxPI/-}* (E) mutants do not exhibit any changes in the number of SIX2⁺ cells per ureteric bud tip (control versus *Hoxb7Cre; Tgfb2^{loxPI/-}*: 24.9 versus 25.3, *n*=3, *P*=0.93; control versus *Six2Cre; Tgfb2^{loxPI/-}*: 29.1 versus 25.5, *n*=3 controls and *n*=6 mutants, *P*=0.18). (F,G) *Foxd1Cre; Tgfb2^{loxPI/loxP}* mutants exhibit a 28% increase in the number of SIX2⁺ cells per ureteric bud tip compared with controls (control versus *Foxd1Cre; Tgfb2^{loxPI/loxP}*: 23.3 versus 29.8, *n*=8, *P*=0.017). (H,I) Likewise, *Foxd1Cre; Six2Cre; Tgfb2^{loxPI/-}* compound mutants exhibit a 20% increase in the number of SIX2⁺ cells per ureteric bud tip (control versus *Foxd1Cre; Six2Cre; Tgfb2^{loxPI/-}*: 31.1 versus 37.3, *n*=5 *Tgfb2^{loxPI/-}* controls, *n*=6 mutants, *P*=0.016). All mutant genotypes were compared with individual littermate controls from the same cross. Arrows indicate SIX2⁺ nephrogenic precursor cells. Data are mean±s.e.m. Scale bars: 100 μm.

deficiency of *Tgfb2* in *Foxd1*⁺ and *Six2*⁺ cells generated deleterious effects on both the cortical stroma (disrupted capsule and disorganized cortical stroma) and nephrogenic elements (expanded

nephrogenic precursor population and fewer nephrons). Together, our results indicate that stromal HH-SMO-GLI3 signaling controls renal capsule development and nephrogenesis in part through TGFβ2.

Cell-autonomous function of HH-GLI3-TGFβ signaling on renal capsule development

Progressive loss of the renal capsule in *Smo*-deficient mutants suggests a role for HH signaling in regulating the renal capsule. Development of the renal capsule is poorly understood, in part due to the lack of reliable and specific capsule markers. Kobayashi and colleagues demonstrated that the capsule is derived from *Foxd1*⁺ stromal progenitors (Kobayashi et al., 2014). Deficiency of *Foxd1* does not abrogate the capsular stroma (Levinson et al., 2005). Rather, *Foxd1* helps to specify the molecular identity of capsular stroma. In mice with homozygous deficiency of *Foxd1*, capsular cells are present but ectopically express genes including *Bmp4* (Levinson et al., 2005). Our data define a novel molecular pathway that is required for specification of the cortical signaling niche of the kidney. Here, we show that HH-GLI3 dependent signaling acts upstream of *Foxd1* and controls maintenance of capsule stromal cells in a TGFβ2-TGFβR2-dependent manner. Our findings of decreased *Foxd1* in *Smo*-deficient stromal cells suggest that disruption of the capsule in the more mature kidney is due to failure to generate a sufficient number of cells to fully constitute a capsule, a modest decrease in stromal proliferation and increased apoptosis. The difference in capsule phenotype between *Smo*-deficient and *Foxd1*-deficient mice suggests that HH signaling controls additional mechanisms that control capsule formation. Indeed, we show that loss of HH signaling decreases *Tgfb2* in stromal cells. Furthermore, our *in vivo* analyses show that deficiency of *Tgfb2* in stromal cells also disrupts formation of a complete capsule. These results provide a basis for further studies aimed at exploring the cortical signaling niche and its involvement in renal capsule formation.

Non-cell autonomous functions of HH-dependent signaling on crosstalk between stromal cells and SIX2⁺ nephrogenic cells

A prominent phenotypic feature *Smo*-deficient mice is the increase in proliferation and number of SIX2⁺ cells in the condensing mesenchyme preceding the decrease in nephron number. An increase in the number of SIX2⁺ cells in the condensing mesenchyme is a consistent finding in mice with stromal deficiency of *Fat4* or *Sall1*, as well as mice with germline deficiency of *Foxd1*, *Pod1* or *Pbx1* (Schnabel et al., 2003; Bagherie-Lachidan et al., 2015; Das et al., 2013; Ohmori et al., 2015; Levinson et al., 2005; Quaggin et al., 1999). These observations, together with our data, suggest a complex signaling environment between the stroma and adjacent SIX2⁺ mesenchyme.

Functional evidence to define the key signaling mediators is limited, as many stromal models have explored the genetic deletion of transcription factors, which are neither secreted nor act as receptors and likely act indirectly to control stromal-nephrogenic crosstalk. Investigation of *Smo*-deficient mutants demonstrated that TGFβ2, a secreted factor expressed in the stroma, is expressed downstream of HH-SMO signaling. Evidence of signaling between Hedgehog and TGFβ is limited, but has been explored in other contexts, i.e. bone chondrocyte differentiation, where SHH acts upstream of *Tgfb2* (Alvarez et al., 2002). In renal development, *Tgfb2*-null mice exhibit incompletely penetrant renal aplasia, similar to *Shh*-null mice, suggesting similarities in the phenotypic mechanisms of these models (Sanford et al., 1997; Hu et al., 2006).

We show that kidney explants treated with TGFβ2 neutralizing antibody or targeted morpholino have fewer nephrons, which

further implicates TGF β 2 in nephrogenesis. Our analysis of genetic mouse models sheds further insight into how *Tgfb β 2*-dependent signaling may control nephrogenesis. Deletion of *Tgfb β 2* in *Foxd1*⁺ stromal cells results in an increase in the number of SIX2⁺ cells in the condensing mesenchyme, similar to that observed in *Smo*-deficient mutants. Yet, deficiency of *Tgfb β 2* in SIX2⁺ nephrogenic cells has no detectable effect on the number of SIX2⁺ cells in the condensing mesenchyme, suggesting that *Tgfb β 2*-dependent signaling is not sufficient to control SIX2⁺ cells (Fig. 9, red dotted arrow). Interestingly, removal of TGF β signaling from both stromal and nephrogenic cells concurrently results in the accumulation of SIX2⁺ cells in mesenchymal aggregates and low nephron number. These data suggest that the primary function of TGF β signaling in this niche is intrinsic to *Foxd1*⁺ cells; deficiency of TGF β signaling in stromal cells abrogates the release of one or more downstream factors that control SIX2⁺ cells (Fig. 9). Published work has demonstrated that the atypical cadherin *Fat4* with receptor *Dchs1*, as well as the secreted protein decorin, directly control crosstalk between stromal-nephrogenic cells (Mao et al., 2015; Bagherie-Lachidan et al., 2015; Das et al., 2013; Fetting et al., 2014). It is possible that HH-SMO-TGF β signaling is upstream of these other signaling pathways that links stromal and nephrogenic cells.

A novel role for TGF β signaling in the renal stroma

Our data provide a novel functional role for TGF β signaling in renal development. Previous research has implicated TGF β signaling in controlling branching morphogenesis and nephrogenesis *ex vivo* (Bush et al., 2004; Clark et al., 2001). TGF β 2 has been shown specifically to induce nephrogenesis *ex vivo*, although induction appeared to be dose dependent (Plisov et al., 2001). Investigation of *Tgfb2*^{+/-} mice has yielded conflicting results with respect to increasing or decreasing branching morphogenesis and nephrogenesis, whereas *Tgfb2*-null mice have fewer nephrons (Sanford et al., 1997; Sims-lucas et al., 2008; Short et al., 2013; Short et al., 2010). Our *ex vivo* studies confirm that TGF β 2 is required for nephrogenesis in a dose-dependent manner based on neutralization and knockdown at various concentrations. Furthermore, we provide new-found evidence through our genetic mouse models that TGF β -dependent signaling in stromal and nephrogenic cells is required to generate a

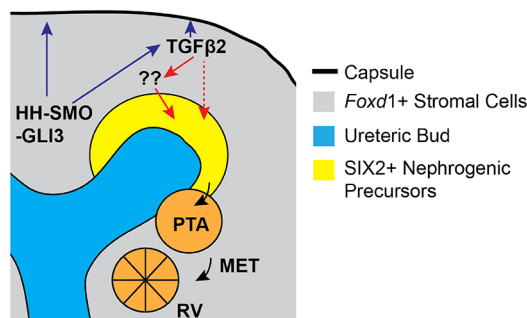


Fig. 9. A model of HH signaling function in the cortical stroma. *Foxd1*⁺ stromal cells (gray) are in an active state of HH-Gli3 signaling that acts to regulate development of the renal capsule (black line) in an autocrine manner (blue arrow). Stromal HH-Gli3 signaling also controls expression of TGFβ2 that, in turn, controls capsule development (blue arrow). TGFβ2, through TGFβRII, also acts non-autonomously to restrict nephrogenic precursor cell expansion directly (dotted red arrows) and through an as yet unidentified downstream factor (solid red arrow). PTA, pretubular aggregate; RV, renal vesicle; MET, mesenchymal-to-epithelial transition; blue arrows, cell-autonomous (stromal) signaling; red arrows, non-cell autonomous (stromal-nephrogenic) signaling.

full complement of nephrons. In addition, we also demonstrate that TGF β signaling is crucial in the formation of the renal capsule and for normal stromal patterning, although the mechanism behind this process remains to be defined.

Our model (Fig. 9) posits that canonical HH-SMO-GLI signaling in stromal cells controls expression of *Tgfb2* in the stroma. Our work adds to and emphasizes the complex signaling interactions that are present in the renal stroma and cortex of the embryonic kidney, and adds to the growing body of evidence that stromal cells influence nephrogenic cells through molecular crosstalk. Although HH-SMO-GLI signaling acts cell autonomously to control capsular integrity, it also acts non-cell autonomously to restrict the SIX2⁺ nephrogenic cell pool and control nephrogenesis, in large part through TGF β signaling. This body of work will aid in the understanding of how stromal cells signal, function and ultimately control the complex process of kidney development.

MATERIALS AND METHODS

Ethics statement

All experimental protocols involving mice were approved by the Animal Ethics Committee at the Hospital for Sick Children and were carried out in accordance with the regulations of the Canadian Council on Animal Care. All animal work was conducted at The Centre for Phenogenomics (TCP, Toronto, Canada) following all animal use protocols.

Mouse models

To generate mice in which *Smo* inactivation is restricted to the stroma (*Foxd1Cre;Smo^{loxP/-}*), *Foxd1Cre-eGFP* mice (herein labelled as *Foxd1Cre*) were mated with *Smo^{+/-}* mice to generate *Foxd1Cre;Smo^{+/-}* (Humphreys et al., 2010). These mice were subsequently mated with *Smo^{loxP/loxP}* mice (Zhang et al., 2001; Long et al., 2001). *Smo^{loxP/+}* mice were used as controls unless otherwise specified. *Foxd1Cre;Smo^{+/-}* and *Smo^{loxP/loxP}* mice were mated with *Gli3^{+/-}* to generate *Foxd1Cre;Smo^{+/-};Gli3^{+/-}* and *Smo^{loxP/loxP};Gli3^{+/-}* progeny, respectively (Litingtung et al., 2002). These two mouse lines were mated with one another to generate *Foxd1Cre;Smo^{loxP/-};Gli3^{-/-}* mice (Maynard et al., 2002). *Gli3^{Δ699/+}* mice were mated with *Foxd1Cre* mice to generate *Foxd1Cre;Gli3^{Δ699/+}* mice, which were subsequently mated with *RosaGli3TFlag^{+/c}* to generate *Foxd1Cre;RosaGli3TFlag^{+/+};Gli3^{Δ699/+}* (Vokes et al., 2004; Böse et al., 2002). *RosaGli3TFlag^{+/+}* mice were used as controls unless otherwise specified. To label *Gli1*-expressing cells, *Gli1Cre^{ERT2}* mice (Ahn and Joyner, 2004) were mated with *Rosa26^{LacZ}* reporter mice (Friedrich and Soriano, 1991). Pregnant dams were injected with tamoxifen at E11.5 and embryos dissected at E14.5. *Foxd1Cre* mice were mated with *Tgfb^{2loxP/loxP}* mice (Levéen et al., 2002) to generate *Foxd1Cre;Tgfb^{2loxP/+}* mice, which were bred with *Tgfb^{2loxP/loxP}* to generate *Foxd1Cre;Tgfb^{2loxP/loxP}* and *Foxd1Cre;Tgfb^{2loxP/-}* mutant mice. *Hoxb7Cre* mice (Zhao et al., 2004) and *Six2Cre* mice (Kobayashi et al., 2009) were mated with *Tgfb^{2+/-}* mice to generate *Hoxb7Cre;Tgfb^{2+/-}* and *Six2Cre;Tgfb^{2+/-}* mice. These mice were mated with *Tgfb^{2loxP/loxP}* mice to generate *Hoxb7Cre;Tgfb^{2loxP/-}* and *Six2Cre;Tgfb^{2loxP/-}* mutants. *Foxd1Cre;Six2Cre;Tgfb^{2loxP/-}* compound mutants were generated using the above crosses. Mice were genotyped using the primers listed in Table S1.

Tissue fixation and histology

Pregnant females were sacrificed using carbon dioxide. Embryonic (E) day 0.5 was set to noon the day the vaginal plug was found. Embryonic kidneys were dissected and fixed in 4% paraformaldehyde (PFA) overnight at 4°C. PFA-fixed kidneys were transferred to 70% ethanol the following day before paraffin wax embedding and sectioning. All tissue processing, sectioning and, Hematoxylin and Eosin staining was carried out by the CMHD Core Pathology Lab at TCP, Toronto, Canada.

β -Gal staining

Whole kidneys were briefly fixed in *lacZ* fix solution (25% glutaraldehyde, 100 nM EGTA, 1 M MgCl₂, 0.1 M sodium phosphate) and rinsed in wash buffer (0.1 M sodium phosphate buffer, 2% nonidet-P40, 1 M MgCl₂).

Kidneys were then placed in X-gal staining solution at 37°C overnight in the dark. Stained kidneys were then immersed in wash buffer and post-fixed in 10% buffered formalin at 4°C. Whole kidneys were photographed using a Leica EZ4D dissecting microscope, processed for embedding in paraffin wax, then sectioned at 5 µm. Sections were counterstained with nuclear Fast Red.

Immunofluorescence

Immunostaining was performed on PFA fixed, paraffin wax-embedded kidney sections. Sections (5 µm) were deparaffinized in xylene and rehydrated through an ethanol gradient. Antigen retrieval was performed in sodium citrate buffer (pH 6.0) in a microwavable pressure cooker.

Primary antibodies (Table S2) were incubated overnight at 4°C. Following PBS washes, slides were incubated in secondary antibody (Table S2), washed and mounted using VectaShield mounting medium (Vector Labs). Mounted slides were imaged using an epifluorescence microscope (Zeiss) or a Quorum Spinning Disk Confocal (Zeiss AxioVert 200 M). A minimum of three sections per kidney and at least three biological replicates were used in the assessments. Fixed explants were rehydrated in PBS before permeabilized using PBS with 1% Triton (PBST). Explants were then incubated with primary antibody overnight at 4°C. Following washes in PBST, explants were incubated in secondary antibody (1:500), washed in PBS and immediately imaged on a Quorum Spinning Disk Confocal (Zeiss AxioVert 200 M).

Quantification and proliferative index of *Six2*⁺ nephrogenic precursor cells

Following immunostaining, *SIX2*⁺ positive nephrogenic precursor cells were counted. Three to five representative fields of *SIX2*⁺ cells were counted per kidney; two kidneys were stained per animal (*n*=6 for control and mutants compared). Data were presented as the number of *SIX2*⁺ cells per ureteric bud tip. For proliferation analysis, double immunostaining for *SIX2* and phospho-histone-H3 was used (*n*=5 for control and mutants compared). A proliferative index of the nephrogenic precursor cells was determined by comparing the number of double positive *SIX2*⁺ cells to the total number of *SIX2*⁺ cells.

Quantification of stromal apoptosis

Quantification of apoptosis at E13.5 was performed using TdT In Situ Apoptosis Detection Kit (R&D Systems, 4810-30-K). Methyl Green was used as a counter stain. Staining was performed using a standard protocol supplied with the kit (Trevigen) and was performed by the CMHD Pathology Core at The Centre for Phenogenomics. TUNEL⁺ cortical stromal cells identified by brown DAB staining were counted and normalized to kidney area in equivalent, mid-sagittal sections. The area was measured using AxioVision 4.6.3-SP1 (Zeiss).

Quantification of stromal proliferation using BrdU

Pregnant dams were injected with 10 mg/ml solution of BrdU (Roche 10280879001), 10 µl per gram of body weight. Embryos were dissected out after 2 h and kidneys collected. Embryonic kidneys were fixed and sectioned as described above. Sections were stained with an anti-BrdU antibody (Table S2). An index of cortical stromal proliferation was determined by comparing the number of BrdU⁺; PBX1⁺ cortical stromal cells with the total number of PBX1⁺ cortical stromal cells.

In situ hybridization

Section *in situ* hybridization was performed as previously described (Ding et al., 1998). DIG-labeled riboprobes encoding *Gli1*, *Foxd1*, *Lhx1*, *Tgfb2*, *Ret*, *Gdnf*, *Wnt11*, *Wnt4* and *Wnt9b* were used.

Quantification of kidney volume and glomerular number

E18.5 kidneys were fixed, embedded in paraffin wax and sectioned in the sagittal plane. In order to quantify glomeruli number and estimate kidney volume, the samples were processed according to previous procedures (Bertram et al., 1992; Cain et al., 2009). For counting glomeruli, slides were stained with PAS, where the sum of all glomeruli in the sections making up

the kidney was used as an estimate of the total nephron number per kidney. For volumetric analysis, the area of tissue sections were measured with AxioVision 4.6.3-SP1 (Zeiss) and multiplied by 100 µm to calculate the volume of each representative section. Total kidney volume was calculated as the sum of volumes for each section.

Scanning electron microscopy

Whole E16.5 kidneys were harvested and fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3) overnight at 4°C. Fixed kidneys were given to The Advanced Bioimaging Centre at Mount Sinai Hospital to be dehydrated, dried and gold sputter coated. Prepared samples were imaged at the Bioimaging Centre with a FEI XL30 ESEM microscope.

Quantitative real time PCR (qRT-PCR)

RNA was extracted from either E13.5 whole kidney tissue or FACS isolated stromal cells using the RNeasy Micro RNA Extraction Kit (Qiagen). Following extraction, a standard amount of RNA from both control and mutant samples was reverse transcribed into cDNA using the SuperScript First Strand Synthesis System for RT-PCR (Invitrogen). Quantitative PCR was performed using gene specific cDNA primers and normalized to β -2 microglobulin expression before control and mutant samples were compared. Comparisons were performed using the $\Delta\Delta C_t$ method. At least three biological and three technical replicates were used per gene assayed.

Counting of pretubular aggregate and renal vesicle structures

Individual kidneys from three independent E15.5 litters were prepared and sectioned at 5 µm increments, starting from the midline of the kidney. To minimize section bias, equivalent sections from wild-type and mutant kidneys were immunostained for E-cadherin and NCAM. At least two sections per kidney (either wild type or mutant) were counted. Six wild-type and mutant biological replicates were used in the analysis across two individual separate experiments.

RNA sequencing

Biological replicates from four separate E13.5 litters were used for RNA sequencing analysis. E13.5 kidneys were dissected out of embryos and kept in RNAlater reagent. Two kidneys were pooled for each biological replicate (*n*=4 for control and mutant). RNA was isolated using the RNeasy Micro RNA Extraction Kit (Qiagen); RNA purity and yield was determined using the Agilent RNA 6000 Pico Lab Chip analysis prior to cDNA library preparation. All samples had RIN>9.4. RNA samples were submitted to the TCAG for RNA Sequencing analysis on the Illumina HiSeq 2500 system.

Following cDNA library preparation, whole kidney transcript reads were aligned to the mouse genome (mm10) using TopHat. HTSeq was used to determine the absolute number of read counts for genes. All sequence alignment and read counts were performed by TCAG. Subsequent analysis of differentially regulated genes was carried out using R/Bioconductor package using DESeq following a standard pipeline of normalization followed by differential comparison of wild-type and mutant samples (Anders et al., 2013). A full list of candidate genes from RNA sequencing is available in Table S3. All RNA sequencing data has been deposited in the Gene Expression Omnibus (GEO) repository, accession number GSE103923.

Fluorescence-activated cell sorting (FACS)

For comparative analysis, control (*Foxd1Cre*; *Smo*^{loxP/+} or *Foxd1Cre*; *Tgfb2*^{loxP/+}) stromal cells were collected and compared with mutant stromal cells (*Foxd1Cre*; *Smo*^{loxP/-} or *Foxd1Cre*; *Tgfb2*^{loxP/loxP}). Isolated kidneys were dissected in Dulbecco's Modified Eagle Medium (DMEM)/Hams-F12 culture media (Gibco) and digested in 2 mg/ml of collagenase B (Roche) in DEPC/Ringer's solution for 15 min at 37°C. After brief centrifugation, cells were re-suspended in PBS containing 1% heat-inactivated FCS and 1 mM EDTA. The cell suspension was filtered through a 40 µm nylon mesh cell strainer (BD Falcon). Propidium iodide (Sigma) was added at a final concentration of 0.5 mg/ml to label dead cells. FACS analysis was carried out using MoFlo Astrios BRVY (Beckman Coulter) or FACS Vantage (Becton Dickinson Immunocytometry Systems). Dead cells were excluded from live

FACS plots based on propidium iodide. GFP-positive cells were sorted directly into Buffer RLT (RNeasy Micro Kit, Qiagen) and stored at -80°C .

Kidney explant culture

E12.5 C57BL/6J kidneys were dissected out of embryos and placed onto transwell filters (0.4 μm , Falcon) in six-well plates immersed in 2 ml DMEM/F12 supplemented with FBS, 50 $\mu\text{g/ml}$ transferrin (Sigma) and either: 1, 2 or 4 $\mu\text{g/ml}$ anti-TGF β 2 neutralizing antibody (Abcam, ab10850); 4 $\mu\text{g/ml}$ rabbit IgG (Cell Signaling, 2729S); 3 μM control MO (5'CTACTAATTGTACAGCCGTCACGT) or 3 μM TGF β 2 splice inhibiting MO (5' CCCACAACCAGAGACAGGTTACCT). Explants were incubated at 37°C with 5% CO_2 and imaged on a light microscope (Zeiss). After treatment, explants were either fixed in ice-cold 4:1 methanol: DMSO for whole-mount immunofluorescence or flash frozen in lysis buffer for RNA extraction.

Statistical analyses

All statistical analyses were performed using GraphPad Prism software (Version 7.0). Student's *t*-test (two-tailed) was used to determine significance between mean differences in groups. $P \leq 0.05$ was considered to be statistically significant. Error bars indicate s.e.m.

Acknowledgements

We thank the staff at The Centre for Phenogenomics CMHD core for all histology work. We thank Dr Sunny Hartwig (University of Prince Edward Island) and Dr Jon Moulton (Gene Tools) for assistance in designing morpholinos. We also thank Dr Daniel Schramek (Mount Sinai Hospital) for the *Tgfb2* mice, Dr Carlton Bates (Children's Hospital of Pittsburgh) for the *Hoxb7Cre* mice, Dr C. C. Hui (The Hospital for Sick Children) for the *Gli1CreERT2* and *Gli3^{-/-}* mice, Dr Andrew McMahon (University of Southern California) for the *Six2Cre* mice and Dr Ulrich Ruthar (University of Dusseldorf) for the *Gli3^{Δ699}* mice. We also thank Dr Helen McNeill (Mount Sinai Hospital) for the *Tgfb2* *in situ* hybridization probe.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: N.D.R.; Methodology: C.J.R., W.L., H.M., N.D.R.; Validation: C.J.R., W.L., H.M., S.E., D.H., Y.-K.K., N.D.R.; Formal analysis: C.J.R., W.L., H.M., S.E.; Investigation: C.J.R., W.L., H.M., S.E., D.H., Y.-K.K., S.S.-D., J.M., J.B.; Resources: L.C., N.D.R.; Data curation: W.L., H.M., S.E.; Writing - original draft: C.J.R., N.D.R.; Writing - review & editing: C.J.R., W.L., H.M., S.E., Y.-K.K., S.S.-D., J.M., J.B., L.C., N.D.R.; Visualization: C.J.R.; Supervision: N.D.R.; Project administration: N.D.R.; Funding acquisition: N.D.R.

Funding

This work was supported by the Canadian Institutes of Health Research and a Tier I Canada Research Chair to N.D.R.; by Restrcomp trainee funding from the Hospital for Sick Children to C.J.R.; and by funding from the Natural Sciences and Engineering Research Council of Canada to W.L.

Data availability

All RNA sequencing data have been deposited in GEO under accession number GSE103923.

Supplementary information

Supplementary information available online at <http://dev.biologists.org/lookup/doi/10.1242/dev.159947.supplemental>

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