GLI3 Repressor Controls Nephron Number via Regulation of Wnt11 and Ret in Ureteric Tip Cells

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Abstract
Truncating GLI3 mutations in Pallister-Hall Syndrome with renal malformation suggests a requirement for Hedgehog signaling during renal development. HH-dependent signaling increases levels of GLI transcriptional activators and decreases processing of GLI3 to a shorter transcriptional repressor. Previously, we showed that Shh-deficiency interrupts early inductive events during renal development in a manner dependent on GLI3 repressor. Here we identify a novel function for GLI3 repressor in controlling nephron number. During renal morphogenesis, HH signaling activity, assayed by expression of Ptc1-lacZ, is localized to ureteric cells of the medulla, but is undetectable in the cortex. Targeted inactivation of Smo, the HH effector, in the ureteric cell lineage causes no detectable abnormality in renal morphogenesis. The functional significance of absent HH signaling activity in cortical ureteric cells was determined by targeted deletion of Ptc1, the SMO inhibitor, in the ureteric cell lineage. Ptc1−/−UB mice demonstrate ectopic Ptc1-lacZ expression in ureteric branch tips and renal hypoplasia characterized by reduced kidney size and a paucity of mature and intermediate nephrogenic structures. Ureteric tip cells are remarkable for abnormal morphology and impaired expression of Ret and Wnt11, markers of tip cell differentiation. A finding of renal hypoplasia in Gli3−/− mice suggests a pathogenic role for reduced GLI3 repressor in the Ptc1−/−UB mice. Indeed, constitutive expression of GLI3 repressor via the Gli3<sup>1699</sup> allele in Ptc1−/−UB mice restores the normal pattern of HH signaling, and expression of Ret and Wnt11 and rescued the renal phenotype. Thus, GLI3 repressor controls nephron number by regulating ureteric tip cell expression of Wnt11 and Ret.

Introduction
Development of the permanent mammalian kidney (the metanephros) is dependent on growth and branching of the ureteric bud and its daughter branches, a process termed renal branching morphogenesis. At the onset of this process, the ureteric bud elongates towards and invades the metanephric mesenchyme before undergoing spatial specification into ‘ureteric stalk’ and ‘ureteric tip’ domains. Reciprocal inductive interactions between the ureteric tip and surrounding metanephric mesenchyme results in division of the ureteric tip, forming the first of a series of ureteric branches, which ultimately constitute the mature collecting duct system. Simultaneously, each ureteric bud tip induces adjacent metanephric mesenchyme cells to undergo a mesenchyme-epithelial transformation and form the epithelial components extending from the glomerulus to the distal tubule, a process known as nephrogenesis. The number of nephrons formed is directly related to the number of ureteric branches and their inductive capacity. Severe reductions in nephron number, characteristic of renal hypoplasia/dysplasia, are the leading cause of childhood renal failure. More subtle defects in nephron number have been associated with the development of adult-onset essential hypertension and chronic renal failure [1,2,3,4,5].

The Hedgehog-GLI signaling pathway plays critical roles during mammalian kidney development. GLI proteins function downstream of Hedgehog’s (HH), extracellular proteins, and Patched (PTC) and Smoothened (SMO), cell surface proteins. HH ligand signals upon binding to its receptor, PTC, relieving PTC-mediated inhibition of SMO, a transmembrane protein. In this state, SMO interacts with a molecular complex including Costal-2 (Cos2), Fused (Fu) and Suppressor of Fused (SuFu), ultimately resulting in translocation of GLI protein family members into the nucleus where they act as transcriptional activators. In the absence of HH ligand, PTC inhibits SMO and prevents its interaction with the Cos2-Fu-SuFu complex resulting in C-terminal cleavage of GLI protein, which translocates to the nucleus and acts as a transcriptional repressor. In vertebrates, three GLI family members, GLI1, GLI2 and GLI3, mediate HH signals. During murine embryogenesis, GLI1 and GLI2 are believed to function primarily as transcriptional activators while GLI3 is believed to function primarily as a transcriptional repressor [6,7].
HH Function in Ureteric Cells

The balance of GLI activator and repressor activities is critical during renal morphogenesis. Mutations that are predicted to generate a truncated protein similar in size to GLI3 repressor are observed in humans with Pallister-Hall Syndrome (PHS) and renal dysplasia [9]. The pathogenic role of constitutive GLI3 repressor activity during renal morphogenesis is further demonstrated by the renal dysplastic phenotype in mice engineered to express GLI3 repressor in a dominant manner [9] and in Shh-deficient mice [10]. Dysplastic kidney tissue in Shh-deficient mice is characterized by sustained GLI3 repressor expression in the face of decreased levels of GLI activators (GLI1, GLI2 and full-length GLI3), resulting in a shift in the balance of GLI activators and GLI repressors in favor of repressor [10]. Remarkably, genetic inactivation of GLI3 in the Shh null background restores expression of GLI activators and normalizes renal morphogenesis [10]. The expression of Shh in ureteric cells suggests that it may control renal development via direct effects in the ureteric cell lineage. While conditional inactivation of Shh in ureteric cells results in renal hypoplasia, characterized by reduced kidney size and glomerular number [11], the dependency of this pathogenic phenotype on Shh signaling in ureteric cells is unknown.

Here we define the specific function of HH signaling in the ureteric cell lineage during murine kidney development, in genetic models of deficient or constitutively active signaling. HH signaling activity is specifically restricted to the ureteric cells of the medulla and ureter but is absent from the ureteric cell tips of the renal cortex. Genetic inactivation of Smo in the ureteric cell lineage exerted no deleterious effects on renal morphogenesis. In contrast, genetic inactivation of Ptc1 in the ureteric cell lineage caused ectopic HH signaling activity in ureteric tip cells, impaired ureteric tip-specific gene expression and renal hypoplasia. Genetic inactivation of Gli3 alone, the primary GLI repressor, resulted in a similar phenotype suggesting a critical role for GLI3 repressor. Indeed, introduction of a constitutively active GLI3 repressor in a Ptc1-deficient background normalized the renal phenotype, restored the normal domain of HH signaling activity and rescued expression of genes specific to ureteric tip cells and required for their functions. We propose a model in which SHH-SMO signaling controls the spatial generation of GLI3 repressor, which is required in the cortical ureteric cells for ureteric tip cell-specific gene expression and cell function.

Results

Spatial Restriction of HH Signaling Activity to the Renal Medulla and Ureter

To further investigate the role of SHH signaling during renal morphogenesis, we examined the expression of Ptc1 utilizing the Ptc1-lacZ reporter mouse [12]. Since Ptc1 is a downstream target of HH signaling, Ptc1-lacZ expression is indicative of the site of HH signaling activity [12,13,14]. In the WT (Ptc1+/+2) kidney at E13.5, Ptc1-lacZ is strongly localized to cells surrounding the presumptive ureter and the presumptive medullary stroma (Figure 1A,B), consistent with the pattern of Ptc1 mRNA expression [11]. Ptc1-lacZ is also weakly localized to the epithelium of the presumptive ureter and the distal or medullary collecting ducts (Figure 1A–C). Interestingly, Ptc1-lacZ expression is not observed in any structures of the presumptive renal cortex, suggesting that HH signaling activity is restricted to the ureter and medullary regions of the developing kidney (Figure 1B,D). At a later stage (E18.5) of kidney development, a similar pattern of expression is maintained in the cells surrounding the ureter and medullary stroma (Figure S1A–C). However, at E18.5, Ptc1-lacZ expression is not observed in any epithelial structures (Figure S1A–C). Taken together, Ptc1-lacZ expression in both ureteric and metanephric mesenchyme-derived structures suggests a role for SHH function in both the ureteric bud and metanephric mesenchyme lineages of the early developing kidney but only in the presumptive ureter and medullary regions.

SHH-SMO-Dependent Signaling is Not Required in the Urteric Cell Lineage

We began to investigate the possible autocrine functions of SHH-SMO-dependent signaling by generating a loss of function model for HH signaling in the ureteric cell lineage. Smoothened is required for the transduction of all HH signals. Similar to Shh inactivation, inhibition of SMO with a steroidal alkaloid, cyclopamine, results in sustained GLI3 repressor in the absence of GLI activators [10]. Homozygous germline deficiency of Smo in the mouse results in embryonic lethality prior to the commencement of metanephric development [15]. Therefore, we utilized Hoxb7creEGFP mice to generate a murine model in which Smo is genetically inactivated in the ureteric cell lineage [16,17], thereby eliminating SHH-SMO-dependant signaling.

Targeted deficiency of Smo in the ureteric cell lineage did not adversely affect survival since mutants were recovered in the near expected Mendelian ratios (Table S1). To confirm that Smo is abolished in Smo+/−UB kidneys, we performed quantitative real-time PCR using ureteric bud tissue isolated at E13.5, a stage that closely follows metanephric induction. Interestingly, Smo mRNA transcripts were decreased by ~80% in Smo+/−UB ureteric cells compared to WT (WT vs. Smo+/−UB; 0.60±0.12 vs. 0.12±0.06, p<0.05) (Figure 1E). Consistent with a loss of Smo, analyses of Ptc1-lacZ in Smo+/−UB kidneys at E13.5 revealed a marked reduction in HH signaling activity in the ureteric cells of the ureter and medullary collecting ducts (Figure 1F,G). Examination of the gross anatomical and histological features of newborn Smo+/−UB kidneys revealed no major differences compared to WT kidneys (Figure 1H,1I,1L,1M). Consistent with this observation, a more detailed analysis of nephrogenic and ureteric structures using immunofluorescence microscopy and RNA in situ hybridization revealed that glomerulogenesis, nephrogenesis, nephron segmentation, ureteric branching morphogenesis and ureteric tip cell-specific gene expression was normal in Smo+/−UB kidneys (Figure S2). In order to determine if HH-SMO-dependant signaling is required in the mature kidney, we also examined Smo−/−UB kidneys at Pn30. No histological abnormalities were detected in the Smo−/−UB kidneys (Figure 1J,K,N,O). Taken together, these results demonstrate that HH-SMO-dependent signaling is not required in the ureteric cell lineage and suggest that SHH has no autocrine function [in the ureteric cells] during renal morphogenesis.

A Model of Cortical HH Signaling Activity in the Embryonic Kidney

Our results demonstrate that HH signaling activity is absent from the renal cortex. We determined the importance of this spatial restriction of HH signaling activity by generating a gain-of-function model in which HH signaling is ectopically activated in the cortical ureteric epithelium.

Patched1 is a negative regulator of the HH signaling pathway. In the absence of Ptc1, repression of HH target genes is alleviated, even in the absence of SHH ligand. Homozygous germline deficiency of Ptc1 in the mouse results in embryonic lethality prior to the commencement of metanephric development [12]. Therefore, we
utilized Hoxb7creEGFP mice to generate a murine model in which $Ptc1$ is genetically inactivated in the ureteric cell lineage [16,17] (refer to Methods). To confirm that inactivation of $Ptc1$ results in a constitutively active HH signaling pathway in the ureteric cell lineage, we analyzed $Ptc1$-lacZ expression in $Ptc1^{2/2}$ UB kidneys. While $Ptc1$-lacZ expression is maintained in the cells surrounding the presumptive ureter and presumptive medullary stroma (Figure 1A,B), $Ptc1$-lacZ is markedy upregulated in the epithelium of the presumptive ureter and distal collecting ducts in $Ptc1^{1/1}$ UB kidneys (Figure 1C vs. Figure 2C). Remarkably, $Ptc1$-lacZ is ectopically expressed throughout the cortical or proximal collecting ducts and in the ureteric bud tips (Figure 2A,B,D), albeit in a mosaic pattern (Figure S1F). A similar pattern of upregulated and ectopic ureteric bud epithelial $Ptc1$-lacZ expression was also observed at a later stage (E18.5) of kidney development (Figure S1D-F). To confirm that $Ptc1$ inactivation occurs by an early stage in renal morphogenesis, we assayed expression of $Ptc1$ mRNA, a surrogate marker of HH signaling activity, in ureteric bud tissue isolated from E11.5 kidney. Quantitative real-time PCR using primers designed for an undisrupted region of the mutant $Ptc1$ transcript demonstrated a 50-fold increase in $Ptc1$ mRNA transcripts in $Ptc1^{2/2}$ UB compared to WT ureteric cells (WT vs. $Ptc1^{2/2}$ UB: 2.22 ± 0.02 vs. 108.51 ± 6.47, p < 0.001) (Figure S3H). Together, these results show that genetic elimination of PTC1 in the ureteric cell lineage leads to increased and ectopic HH signaling activity in the developing kidney.

**Figure 1.** $Ptc1$-lacZ expression and site of HH signaling activity in the developing murine kidney. (A–D) Whole mount X-gal staining of kidney tissue isolated from E13.5 Ptc1-lacZ reporter mice reveals strong localization of HH signaling activity to the cells surrounding the presumptive ureter (ur), medullary stroma (ms) and weakly to the epithelium of the presumptive ureter and distal collecting ducts (dc). (D) Ptc1-lacZ is not observed in any structures of the presumptive renal cortex. (E) Quantitative real-time PCR of isolated E11.5 ureteric buds (inset) demonstrates decreased $Smo$ mRNA transcripts in $Smo^{2/2}$ UB kidneys compared to WT littermates (WT vs. $Smo^{2/2}$ UB: 0.60 ± 0.12 vs. 0.12 ± 0.08, p < 0.05). (F,G) Ptc1-lacZ expression in $Smo^{2/2}$ kidneys at E13.5. $Ptc1$-lacZ expression is maintained in the cells surrounding the ureter and medullary stroma but is markedly reduced in the ureteric cells of the ureter and distal collecting ducts. (H,L) Macroscopic and histological analysis of newborn $Smo^{2/2}$ kidneys is comparable to WT littermates. (J,K,N,O) Analysis of mature $Smo$-deficient kidneys at PN30 demonstrates no histological abnormalities. c = cortex, m = medulla, p = papilla, pc = proximal collecting duct, arrowhead = mature glomerulus. doi:10.1371/journal.pone.0007313.g001
Figure 2. Renal hypoplasia in mice with Ptc1-deficiency in the ureteric cell lineage. (A–D) In Ptc1 mutant kidneys (Ptc1\(^{−/−}\)UB), Ptc1-lacZ expression is maintained in the cells surrounding the presumptive ureter (ur) and medullary stroma (ms). (A–C) HH signaling activity is upregulated in the epithelium of the presumptive ureter and distal collecting ducts (dc). (D) Ectopic HH signaling activity is observed in the proximal collecting ducts (pc) and weakly in ureteric bud tips. (E–L) Histological and immunofluorescence analysis of WT and Ptc1\(^{−/−}\)UB kidneys at E18.5. Ptc1-deficient mice exhibit small kidneys, reduced density of medullary epithelial tubules (mt) and a paucity of mature glomeruli (g) and nephrogenic intermediate structures (n) and sparsity of the nephrogenic zone (arrows). (G,K) PAX2 (green) positive nephrogenic structures are reduced in the Ptc1\(^{−/−}\)UB kidneys. (H,L) LTL (green) positive proximal tubules are markedly reduced in Ptc1\(^{−/−}\)UB kidneys. (M) Kidney volume in Ptc1\(^{−/−}\)UB mutants is reduced by 45% in comparison to WT littermates (p<0.01). (N) Ptc1\(^{−/−}\)UB kidneys demonstrate a 45% reduction in mature glomerular number compared to WT littermates (p<0.01). (O,P) Histological analysis also revealed a sparsity of the nephrogenic zone (arrows) and reduction in the number of nephrogenic structures in Ptc1\(^{−/−}\)UB compared to WT kidneys at E15.5. (Q,R) Analysis of ureteric branching morphogenesis in WT and Ptc1\(^{−/−}\)UB kidneys at E12.5. The number of ureteric branches was comparable between WT and Ptc1\(^{−/−}\)UB kidneys. However, Ptc1\(^{−/−}\)UB kidneys exhibited abnormal ureteric tip morphology (arrowhead). (S–V) Whole mount GFP immunofluorescence of control and mutant kidneys. Kidney size and branching is mildly reduced at E13.5 (S,T) and is more pronounced at E15.5 (U,V).

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Ectopic HH Signaling Activity in the Proximal Epithelium Causes Renal Hypoplasia

Viable neonatal PTC1-deficient pups could not be recovered (Table S2). However, live \textit{Ptc1}^{-/-}\textit{UB} mutant embryos were recovered in expected Mendelian ratios at all embryonic time points analyzed (Table S2). Macroscopic analyses of \textit{Ptc1}^{-/-}\textit{UB} embryos revealed severe exencephaly, with 100% penetrance. Otherwise, \textit{Ptc1}^{-/-}\textit{UB} embryos were indistinguishable from \textit{WT} littermates (Figure S3). It is therefore likely that \textit{Ptc1}^{-/-}\textit{UB} embryos reach term and die and/or are cannibalized immediately following birth.

Analysis of \textit{Ptc1}^{-/-}\textit{UB} kidneys at E18.5 revealed renal hypoplasia, characterized by a 45% reduction in kidney volume (\textit{WT} vs. \textit{Ptc1}^{-/-}\textit{UB}: 163.67 ± 38.67 × 10^{-6} mm³ vs. 90.72 ± 24.05 × 10^{-6} mm³, \textit{p} < 0.01) and reduced density of medullary epithelial tubules (Figure 2E,I,M). The extent of reduction in \textit{Ptc1}^{-/-}\textit{UB} kidney size was variable, exhibiting a 1.7 fold range, but was 100% penetrant. Histological analyses of the renal cortex of \textit{Ptc1}^{-/-}\textit{UB} kidneys revealed severe exencephaly, with 100% penetrance. Histological analyses of the renal cortex of \textit{Ptc1}^{-/-}\textit{UB} kidneys indicated that PTC1-deficiency leads to deficient nephrogenesis. Determination of ureteric tip cell fate is dependent on \textit{Gdnf}/\textit{Ret} signaling [19]. Furthermore, \textit{Gdnf}/\textit{Ret} signaling is required for the maintenance of \textit{Wnt11} expression, also in the ureteric bud tip cells [20,21]. Conversely, \textit{Wnt11} promotes \textit{Gdnf} expression in the surrounding metanephric mesenchyme suggesting that \textit{Gdnf}, \textit{Ret} and \textit{Wnt11} participate in an autoregulatory feedback loop to regulate ureteric branching morphogenesis [20]. We examined the effect of ectopic HH signaling activity on ureteric tip cell-specific gene expression using \textit{in situ} hybridization. In contrast to \textit{WT} kidneys, expression of \textit{Ret} and \textit{Wnt11} was markedly reduced in the majority of ureteric bud tips in \textit{Ptc1}^{-/-}\textit{UB} kidneys at E13.5 (Figure 3E-H). Consistent with the autoregulatory feedback loop, expression of \textit{Gdnf} in the surrounding metanephric mesenchyme was also markedly reduced in the \textit{Ptc1}^{-/-}\textit{UB} kidneys (Figure 3L). The specificity of decreased \textit{Gdnf} expression in \textit{Ptc1}^{-/-}\textit{UB} kidneys is demonstrated by normal expression of \textit{Osr1}, \textit{Six2}, and \textit{CITED1}, each of which marks mesenchymal precursor cells [22,23,24,25,26]; \textit{Wnt4}, a marker of proximal tubules [27]; and \textit{Wnt9b} which is required for the earliest inductive response in metanephric mesenchyme and acts upstream of \textit{Wnt4} [28] (Figure S6). To further investigate the ontogeny of abnormal ureteric tip cell gene expression in \textit{Ptc1}^{-/-}\textit{UB} mice, we assayed \textit{Ret} and \textit{Wnt11} expression in isolated ureteric bud tissue using quantitative real-time PCR. At E11.5, a stage that immediately follows induction of the metanephric mesenchyme by the ureteric bud, expression of \textit{Ret} was comparable between \textit{WT} and \textit{Ptc1}^{-/-}\textit{UB} ureteric cells (\textit{WT} vs. \textit{Ptc1}^{-/-}\textit{UB}: 1.65 ± 0.37 vs. 1.50 ± 0.61, \textit{p} = 0.78) (Figure 3M). In contrast, \textit{Wnt11} expression was reduced by ~70% in \textit{Ptc1}^{-/-}\textit{UB} ureteric cells (\textit{WT} vs. \textit{Ptc1}^{-/-}\textit{UB}: 1.79 ± 0.26 vs. 0.51 ± 0.27, \textit{p} < 0.05) (Figure 3N). Taken together, these results demonstrate that ectopic HH signaling activity in the proximal ureteric epithelium specifically impairs the expression of \textit{Wnt11} and \textit{Ret} in ureteric tip cells.

Our results indicate that HH signaling activity is normally restricted to the distal ureteric cells of the ureter and medulla.
Given the impairment of tip cell gene expression, we investigated the possibility that HH signaling activity biases ureteric cells towards a distal cell fate. To determine whether Ptc1−/−UB tips cells had adopted characteristics of the ureteric stalk we performed DBA-lectin whole mount immunofluorescence microscopy on WT and Ptc1−/−UB kidneys at E13.5. DBA is a marker of the ureteric stalk but not the ureteric tip [29]. DBA expression was observed in ureteric tips in Ptc1−/−UB kidneys in a mosaic pattern but rarely in WT kidneys (Figure 3K,L and Figure S5E–H). The expression of Wnt7b, another marker of ureteric stalk that is absent from the ureteric tips [30], was comparable between WT and Ptc1−/−UB kidneys. Since ectopic HH signaling activity is also observed in proximal collecting ducts in mutant kidneys, we next investigated the possibility that HH signaling activity biases cortical collecting ducts towards a more medullary/distal cell fate. Expression of uroplakin III, a marker of the transitional epithelium of the ureter and renal pelvis, was unchanged in the Ptc1−/−UB kidneys (Figure S7A–F). Similarly, the localization of αSMA, a marker of the smooth muscle population surrounding the ureter, was also unaltered (Figure S7G–I). Together, these results suggest that...
ectopic HH signaling activity in the proximal epithelium does not induce a distal ureteric bud cell fate. Furthermore, increased HH signaling activity in the distal epithelium (ureter and distal collecting ducts) has no deleterious effects on these structures.

**Reduced Levels of GLI Repressor in the Renal Cortex Result in Renal Hypoplasia**

The spatial restriction of HH signaling activity from the renal cortex during normal renal morphogenesis suggests that the cortex is a GLI repressor-dominant domain. We hypothesized that the deleterious effects of ectopic HH signaling in the proximal ureteric cells in Ptc1−/−UB kidneys is due to a reduction in local GLI repressor levels. We addressed this hypothesis by analyzing kidneys in Gli3-deficient embryos.

Gli3 is the primary source of GLI repressor in mammalian cells [31]. Mice with homozygous deficiency in Gli3 die soon after birth or in late gestation [32]. We were able to recover viable Gli3-deficient embryos as late as E18.5. Gli3-deficient embryos exhibited polysyndactyly and occasional exencephaly but were similar in size to WT littermates (data not shown). Gli3−/− mice kidneys were indistinguishable from WT littermates (data not shown). Analysis of Gli3−/− kidneys at E18.5 revealed mild hypoplasia, characterized by a 15% decrease in kidney volume (p<0.05) and 15% reduction in glomerular number (p<0.05) compared to WT littermates (Figure 4). Otherwise, nephrogenesis, nephron segmentation, and smooth muscle and urothelium differentiation was normal in Gli3−/− kidneys (Figure S8). These results are consistent with a functional role for GLI repressor in the renal cortex during renal morphogenesis.

**Gli3 Repressor is Required for Ureteric Tip Cell Expression of Wnt11 and Ret**

We further tested the contribution of GLI repressor in the proximal epithelium by reinstating GLI3 repressor levels in the Ptc1−/−UB mutants. We generated mice with both Ptc1-deficiency targeted to the ureteric cells and with one allele of WT Gli3 replaced with Gli3−/−, a constitutively active repressor form of Gli3. To determine what effect, if any, the reinstatement of Gli3 repressor had on HH signaling activity we analyzed Ptc1−/−UB, Gli3−/−/+ kidneys in the Ptc1−/−UB, Gli3−/−/+ kidneys. In the Ptc1−/−UB, Gli3−/−/+ kidney at E13.5, Ptc1−/−UB expression is maintained in the cells surrounding the presumptive ureter and medullary stroma, consistent with the expression in WT and Ptc1−/−UB kidneys (Figure 5A,C,E). However, in contrast to ectopic Ptc1−/−UB expression throughout the proximal ureteric cells in Ptc1−/−UB kidneys, the proximal collecting ducts and ureteric cell tips are devoid of Ptc1−/−UB expression in Ptc1−/−UB, Gli3−/−/+ kidneys (Figure 5A-F). These results suggest that reinstatement of GLI3 repressor in Ptc1−/−UB kidneys, restores the normal pattern of HH signaling activity to the medullary and ureter domains and eliminates ectopic HH signaling activity in the renal cortex. Next we analyzed ureteric tip cell gene expression in Ptc1−/−UB, Gli3−/−/+ kidneys. Remarkably, expression of Ret and Wnt11 was restored to comparable levels of that observed in WT and Gli3−/−/+ kidneys (Figure 3E,H, Figure S9). Furthermore, consistent with a rescue of Ret and Wnt11 expression, Gdnf expression was also restored by the reinstatement of GLI3 repressor (Figure 3I,J, Figure 5K,L, Figure S9). Moreover, macroscopic analysis of Ptc1−/−UB, Gli3−/−/+ kidneys at E18.5 demonstrated a rescue in kidney size, comparable to that observed in WT littermates (Figure 5M,O,Q). In fact, kidney volume was significantly restored from 55% in Ptc1−/−UB kidneys alone, to 92% in Ptc1−/−UB, Gli3−/−/+ kidneys in comparison to WT kidney volume (WT vs. Ptc1−/−UB, Gli3−/−/+ p>0.05; Ptc1−/−UB vs. Ptc1−/−UB, Gli3−/−/+ p<0.05) (Figure 5S). Remarkably, histological analyses of the renal cortex also revealed a rescue in nephrogenesis in Ptc1−/−UB, Gli3−/−/+ kidneys (Figure 5N,P,R). In contrast to a 45% deficit in Ptc1−/−UB kidneys, glomerular number in Ptc1−/−UB, Gli3−/−/+ kidneys was significantly restored to 85% of that of WT littermates (WT vs. Ptc1−/−UB, Gli3−/−/+ p>0.05; Ptc1−/−UB vs. Ptc1−/−UB, Gli3−/−/+ p<0.05) (Figure 5T). Taken together, these results demonstrate a requirement for GLI3 repressor for ureteric tip cell gene expression and function.

**Discussion**

Disruption of renal development in humans with Pallister-Hall Syndrome and truncating GLI3 mutations [33] and mice with elevated levels of GLI3 repressor [9,10] provides compelling evidence in favor of a critical role for GLI3-dependent signaling during mesenchymal-epithelial interactions during early stages of renal morphogenesis. Evidence is beginning to accumulate that GLI repressor is not merely a suppressor of HH signaling during renal morphogenesis, but that it is a necessary driving force essential for the development of the renal cortex.
Figure 5. GLI3 repressor is required for ureteric tip cell-specific gene expression and function. (A–F) Normal of HH signaling activity is restored in Ptc1−/−;Gli3−/− kidneys at E13.5. In contrast to Ptc1−/− kidneys, Ptc1-lacZ expression is absent from the cortical collecting ducts and ureteric bud tips in Ptc1−/−;Gli3−/− kidneys. (G–L) Expression of Ret, Wnt11 and Gdnf was restored in Ptc1−/−;Gli3−/− kidneys at E13.5. Analysis of Ptc1−/−;Gli3−/− kidneys at E18.5 revealed a restoration in kidney size (M,O,Q) and density of the nephrogenic zone (N,P,R). Kidney volume (S) and glomerular number (T) is rescued in Ptc1−/−;Gli3−/− kidneys to comparable levels to WT littermates.

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metanephric development. However, the functions of HH signaling during subsequent morphogenic events including nephrogenesis are unknown.

Here, we demonstrate that domains of GLI-dependent activator and repressor function are spatially patterned during renal morphogenesis. We investigated the functional significance of these domains in the ureteric cell lineage using genetic murine models of deficient or constitutively active HH signaling. Smo-deficiency targeted to the ureteric cell lineage does not disrupt kidney development, demonstrating HH-dependent GLI activators are not required for ureteric cell function. The absence of HH signaling in the renal cortex of Wt mice suggests that the cortex is a zone of low GLI activator and high GLI repressor levels. We determined the importance of exclusion of HH signaling activity from the cortical collecting ducts in mice with Ptc1-deficiency targeted to the UB lineage. Absence of ureteric cell Ptc1, a negative regulator of the HH signaling pathway, results in ectopic HH signaling activity in the cortical collecting ducts and ureteric bud tips. Ectopic HH signaling activity in the ureteric bud tips, which under normal circumstances is a domain of GLI repressor function, leads to decreased expression of Ret and Wnt11. These changes result in disruption of ureteric branching morphogenesis and nephrogenesis resulting in renal hypoplasia, likely due to impaired tip function. Remarkably, constitutive GLI3 repressor expression in the Ptc1−/−UB background, restores ureteric tip cell-specific gene expression and normalizes renal morphogenesis, demonstrating a spatial requirement for GLI3 repressor in the proximal ureteric cells. Together, these results demonstrate a requirement for GLI3 repressor-dependent regulation of nephron number via ureteric tip cell Wnt11- and Ret-dependent functions.

Regulation of Ureteric Tip Cell Differentiation by GLI3 Repressor

We have established that loss of GLI3 repressor impairs ureteric tip cells by reducing Ret and Wnt11 expression. The precise mechanism by which GLI1-dependent signaling may control Ret and Wnt11 is unclear. Decreased expression of Wnt11 precedes a decrease in Ret expression in the ureteric bud tips of Ptc1−/−UB kidneys. Wnt11 maintains Gdnf expression in the mesenchyme [20]. Thus, it is probable that reduced Gdnf expression in Ptc1−/−UB mice is secondary to decreased Wnt11 expression in ureteric tip cells. Consistent with this, Wnt11 null mice exhibit mild renal hypoplasia and a reduction in Gdnf expression [20] almost identical to that observed in Ptc1−/−UB mutants.

The mosaic expression of Ptc1-lacZ expression in the ureteric tips suggests that not all tip cells were exposed to ectopic HH signaling. This explains, in part, why Ret and Wnt11 expression is not completely abolished from the ureteric tips and why expression levels are variable among tips within the same kidney. It is tempting to speculate that the extent of ‘chimerism’ of a ureteric tip may influence the efficiency of its function. The ability of ‘mutant’ tips to speculate that the extent of ‘chimerism’ of a ureteric tip may influence the efficiency of its function. The ability of ‘mutant’ tips to contribute to renal development demonstrates that HH signaling activity is not required in the ureteric bud lineage for normal renal morphogenesis. This is likely due to a number of reasons. Firstly, analysis of kidneys in patients with GCPS or NBCCS has not been performed. Our results provide a basis for analyses of kidney size in affected individuals. Secondly, the degree of haploinsufficiency in humans with Ptc1 mutations may be insufficient to result in a renal phenotype. Ptc1 heterozygous mutant mice exhibit phenotypes similar to those observed in humans with BNCCS [12], yet we did not observe any renal abnormalities in these mice. Furthermore, Ptc1 homozygous null mice are embryonic lethal suggesting that Ptc1 homozygous null mutations in humans are also incompatible with life [12]. Further, mutational analysis in humans exhibiting sporadic renal hypoplasia for genes involved in HH signaling, including GLI3 and Ptc1, is also warranted, as done for other genes [43,44].

SHH Does Not Signal in a Autocrine Manner During Renal Morphogenesis

We show that HH signaling activity is not required in the ureteric bud lineage for normal renal morphogenesis. This is consistent with previous results demonstrating metanephric mesenchyme as the primary target of SHH signaling [11]. Analysis of mice deficient in Shh the ureteric bud lineage revealed that Shh secreted by the epithelium of the ureter and distal collecting ducts acts on the surrounding mesenchyme to promote cell proliferation and regulate the timing and patterning of smooth muscle progenitor differentiation [11]. Since we have genetically eliminated Smo in the ureteric bud only, Shh is still capable of paracrine signaling, acting on the surrounding stroma and
mesenchyme. In addition, recent in vitro and in vivo data in the pancreas, has suggested additional, non-canonical, mechanisms of GLI activation, downstream of SMO, via two HH-unrelated pathways, RAS and TGFβ [45,46,47]. Whether non-canonical GLI activation occurs during renal morphogenesis remains unknown.

A Model of Spatial GLI Activator and Repressor Functional Domains

We propose a model whereby distinct SHH-dependent medullary GLI activator domain and cortical repressor domain functions are critical for normal renal ureteric patterning and function (Figure 6). It is likely these domains are established by a SHH gradient, emitted by the ureteric cells of the medullary collecting ducts. While SHH signaling is not required in the medullary collecting ducts themselves, the absence of signal in cortical ureteric cells, contributed to by diminishing SHH concentration and/or pathway inhibitors, is critical for ureteric tip cell gene expression required for ureteric branching and nephron induction. Identification of GLI3 repressor gene targets will provide novel insights into this novel mechanism of ureteric tip cell regulation and function. The presence of renal agenesis/dysplasia in humans with Pallister-Hall syndrome and GLI3 repressor dominant murine models [9,10] suggests that a fine spatial and lineage-specific balance of GLI activator and GLI repressor is critical for ureteric tip cell-specific gene expression and subsequent nephron induction (B). In the absence of cortical GLI repressor, such as in Ptc1

**Materials and Methods**

**Ethics Statement**

Experiments using mice were approved in advance by the Animal Ethics Committee at The Hospital for Sick Children and were carried out in accordance with the ‘Canadian Council of Animal Care.’

**Mice**

Hoxb7creEGFP mice [17] were mated to Ptc1+/−, Ptc1+/lacZ [12] or Smo+/− [15] mice to generate Hoxb7creEGFP;Ptc1+/−, Hoxb7creEGFP;Ptc1+/lacZ and Hoxb7creEGFP;Smo+/− males. These males were mated to homozygous Ptc1 conditional (Ptc1−/−) [48] or Smo conditional (Smo−/−) [49] females to generate Hoxb7creEGFP;Ptc1−/−, Hoxb7creEGFP;Ptc1−/−, and Hoxb7creEGFP;Smo−/− progeny in which Ptc1 or Smo was specifically removed from the UB lineage. These kidneys were referred to as ‘Ptc1−/−/UB’ or ‘Smo−/−/UB’ in the text. Hoxb7creEGFP;Ptc1−/−, Hoxb7creEGFP;Ptc1−/−, and Hoxb7creEGFP;Ptc1−/−, Smo−/− mutant kidneys were indistinguishable, displaying the same phenotype. Gli3XtJ/+ mice [9] were mated to Ptc1−/−/+ mice to ultimately generate Ptc1−/−/+;Gli3−/−/+ mice. Hoxb7creEGFP;Ptc1−/− or Hoxb7creEGFP;Ptc1−/− males were mated to Ptc1−/−/+;Gli3−/−/+ females to generate Hoxb7creEGFP;Ptc1−/−, Hoxb7creEGFP;Ptc1−/−, and Hoxb7creEGFP;Ptc1−/−, Gli3−/−/+ progeny, referred to as Ptc1−/−/+, UB;Gli3−/−/+ in the text. Gli3−/−/+ heterozygote mice [32] were intercrossed to generate Gli3−/−/+ embryos.

Analysis of HH signaling activity was achieved by mating Ptc1−/−/+ mice [12] to Hoxb7creEGFP mice or Ptc1−/−/+ mice to generate Hoxb7creEGFP;Ptc1−/−/+ or Ptc1−/−/+ females. Hoxb7creEGFP;Ptc1−/−/+ males were mated to Ptc1−/−/+ females to generate Hoxb7creEGFP;Ptc1−/−/+ (Ptc1−/−/UB) progeny that contain the Ptc1−/−/+ reporter allele and in which Ptc1 is specifically removed from the UB lineage. Hoxb7creEGFP;Smo−/−/+ females were mated to Smo−/−/+;Ptc1−/−/+ females to generate Hoxb7creEGFP;Smo−/−/+ (Ptc1−/−/UB) progeny that contain the Ptc1−/−/+ reporter allele and in which Smo is eliminated from the UB lineage. PCR genotyping for each allele was performed as previously described [12,15,17,48,49]. Gli3−/−/+ heterozygote and homozygous mice were genotyped according to their characteristic limb phenotypes [32,50].

![Figure 6. Model for HH signaling function in the ureteric epithelium.](Image)

(A) During normal renal morphogenesis, Shh secreted from the medullary collecting ducts (purple) establishes a gradient of HH signaling resulting in distinct medullary GLI activator (blue) and cortical GLI repressor domains. While HH signaling activity has no functional requirement in the ureteric cell lineage, the absence of HH signal, and thereby function of GLI3 repressor is critical for ureteric tip cell-specific gene expression and subsequent nephron induction (B). In the absence of cortical GLI repressor, such as in Ptc1−/−/UB mice, ureteric tip cell-specific gene expression is impaired resulting in reduced nephrogenesis and renal hypoplasia.

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β-galactosidase staining

Whole kidneys were briefly fixed in lacZ fix solution (25% gluteraldehyde, 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 lacZ staining solution (25 mg/ml X-gal, potassium ferrocyanide, potassium ferricyanide) at 37°C overnight in the dark. Once staining had occurred the reaction was terminated in wash buffer and post-fixed in 10% buffered formalin at 4°C. Whole kidneys were photographed using a Leica EZ4D dissecting microscope and processed for embedding in paraffin wax and sectioned at 5 μm. Sections were counterstained with eosin or nuclear fast red.

Histology and immunohistochemistry

Paraffin-embedded kidney sections were analyzed by histology after generating 4 μm tissue sections and staining with haematoxylin and eosin. Immunofluorescence was performed on formalin-fixed, paraffin-embedded kidney sections using anti-Pas2 (Govan, Berkeley, CA, 1:100 dilution), anti-pan cytokeratin (Sigma, St Louis, MO, 1:100 dilution), Lotus Tetragonolobus lectin (LTL) (Vector Laboratories, Burlingame, CA, 1:100 dilution), anti-NCAM (Sigma, 1:50 dilution), anti-WT1 (Santa Cruz, Santa Cruz, CA, 1:500 dilution), anti-α smooth muscle actin (αSMA) (Sigma, 1:500 dilution), anti-u-ropalin III (Progen Biotechnik, Heidelberg, Germany, 1:10 dilution), anti-Cited1 (Labvision, 1:500 dilution). Alexa 488 goat anti-mouse and Alexa 568 goat anti-rabbit (Molecular Probes, Carlsbad, CA, 1:1000 dilution) were used as secondary antibodies. Whole mount immunofluorescence was performed as described [51] with anti-Calbindin-D28K (DBA)-lectin (Vector Laboratories, 1:100 dilution), anti-WT1 (Santa Cruz, CA), NCAM (Sigma, 1:50 dilution), anti-WT1 (Santa Cruz, Santa Cruz, CA, 1:1000 dilution) and Dolichos Biflorus Agglutinin (DBA)-lectin (Vector Laboratories, 1:200 dilution).

In situ mRNA hybridization

Whole embryos were fixed in 4% PFA in PBS for 16 h at 4°C. In situ hybridization was performed on paraffin-embedded sections (4 μm) using DIG-labeled cDNA probes encoding Ret, Wt11, Gdnf, Wt4, Six2, Wt76, Wt9b and Osr1 as previously described [52].

In situ TUNEL assay, BrdU incorporation assay

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) was performed using formalin fixed paraffin-embodied tissue sections as described in the manufacturer’s instructions (Promega, Madison, WI). Cell proliferation was assayed in formalin fixed paraffin-embedded kidney tissue by incorporation of 5-bromo-2-deoxyuridine (BrdU, Roche Diagnostics) containing 10% FCS and 0.2% Collagenase-B (Roche Diagnostics) for 10 min at 37°C. Kidneys were washed in ice-cold media containing 10% FCS and ureteric buds and metanephric mesenchyme were isolated by microdissection using 30 g needles. Ureteric buds were stored in RNAlater RNA stabilization reagent (Qiagen) and RNA was then isolated using the RNAqueous-Micro RNA Isolation kit (Ambion Inc.). cDNA was generated using First Strand cDNA Synthesis (Invitrogen) from total RNA. Real-time PCR reaction mixture contained 1 ng of each cDNA sample, SYBR green PCR Mix (Applied Biosystems) and 500 nM of each primer to a total volume of 25 μL. Primers for Smo (Exon 1), Ptc1 (Exon 3), Ret and Wnt11 were designed using Primer 3 software and verified using the UCSC genome bioinformatics website (genome.ucsc.edu). Real-time PCR Amplification was performed using the Applied Biosystems 7900 HT Fast RT-PCR system. Relative levels of mRNA expression were determined using the standard curve method. Individual expression values were normalized by comparison to β-2 Microglobulin.

Calculation of kidney volume and the number of glomeruli

Kidney volume and glomerular number was measured according to Bertram et al. [54] with the following modifications: kidneys embedded in paraffin were exhaustively sectioned at 5 μm, collected at 100 μm intervals and stained with Haematoxylin and Eosin. The area of the tissue section was measured with AxioVision 4.6.3-SP1 (Zeiss) and multiplied by the section thickness. Total kidney volume is the sum of volumes for each section. Glomeruli were identified by the presence of both a podocyte layer and Bowman’s capsule.

Data analysis

Statistical analysis was performed using GraphPad Prism software (Version 3.01) (GraphPad Software Inc., San Diego, CA). Data were analyzed using a Student’s t-test (two tailed). A probability of less than 0.05 was considered to indicate statistical significance. Values are given as means±SD or SEM.

Supporting Information

Figure S1  HH signaling activity in developing murine kidney, Ptc1-lacZ expression and thereby HH signaling activity at E18.5. (A–C) In WT kidneys, Ptc1-lacZ is strongly localized cells surrounding the ureter (not shown) and the medullary stroma (ms). No Ptc1-lacZ activity is observed in the distal collecting ducts (dc) or any structures of the renal cortex. (D–F) In Ptc1+/−/−B kidneys, in addition to strong localization of Ptc1-lacZ to the cells surrounding the ureter and the medullary stroma, Ptc1-lacZ is ectopically expressed in the epithelium of the distal collecting ducts, proximal collecting ducts (pc) and in a mosaic pattern in the ureteric bud tips (tip). n = nephrogenic structure. Found at: doi:10.3713/journal.pone.0007313.s001 (2.47 MB TIF)

Figure S2  HH signaling is not required in the ureteric cell lineage. (A–H) Immunofluorescence analysis of newborn Smo−/−/− deficient kidneys demonstrated no difference in podocyte differentiation (pod) (A,E), normal patterning of the nephrogenic zone (B,F)[green], and comparable densities of proximal tubules (C,G)[green] and collecting ducts (D,H)[red]. (L,M) Ureteric branching morphogenesis is comparable between Smo−/−/−B and WT littermates at E12.5. (J,K,L,N,O,P) mRNA in situ hybridization demonstrates normal expression of Ret and Wt11 in the ureteric bud tips (arrowhead) and Wt4 in the developing nephrogenic structures, in Smo−/−B kidneys at E13.5.
Figure S3  Exencephaly in Ptc1-deficient mutants. (A–F) Macroroscopic analysis of Ptc1-deficient mice at all embryonic time points examined demonstrates severe exencephaly (arrowhead), with 100% penetrance. (G) No difference in body weights was detected between WT and Ptc1−/−UB embryos (WT vs. Ptc1−/−UB; 1.49±0.13 vs. 1.57±0.13, p>0.05). (H) Quantitative real-time PCR of E11.5 isolated ureteric buds. Ptc1 mRNA transcripts are increased 50-fold in Ptc1−/−UB ureteric cells (WT vs. Ptc1−/−UB: 2.23±0.2 vs. 108.51±6.47, p<0.001).

Figure S4  Quantitation of Ptc1-deficient kidneys. (A,B) No histological differences were observed between WT and Ptc1−/−UB kidneys at E13.5. (C,D) NCAM (red) positive nephrogenic structures in WT and Ptc1-deficient kidneys. (E) Quantitation of nephrogenesis at E13.5 demonstrates no significant difference in the number of NCAM positive nephrogenic structures in WT and Ptc1-deficient kidneys. (F) Quantitation of ureteric branching morphogenesis at E12.5 demonstrates no significant difference in branch number in WT and Ptc1−/−UB kidneys. n = nephrogenic intermediate structure, ub = ureteric epithelium.

Figure S5  Ptc1-deficiency does not effect metanephric cell survival. (A–D) Analysis of apoptosis in E13.5 kidney tissue using the TUNEL. TUNEL-positive cells (brown color) are rarely detected in the ureteric bud (ub) of WT or Ptc1−/−UB kidneys. There is no observable difference in TUNEL-positive cells in the mesenchyme (mes) between WT and Ptc1-deficient kidneys. (C,D) Quantitative analysis of ureteric bud and mesenchymal apoptosis. (C) Ureteric cell apoptosis, quantitated as the percent of TUNEL-positive ureteric cells, was not significantly altered in Ptc1−/−UB kidneys. (D) Mesenchymal cell apoptosis, quantitated as the number of TUNEL-positive cells per mm² of renal tissue was comparable in WT and Ptc1−/−UB kidneys (WT vs. Ptc1−/−UB; 0.32±0.13 vs 0.41±0.28, p = 0.78). (D) Mesenchymal cell apoptosis, quantitated as the number of TUNEL-positive cells per mm² of renal tissue was comparable in WT and Ptc1−/−UB kidneys (WT vs. Ptc1−/−UB; 0.23±0.03 vs 0.19±0.04, p = 0.46). n = nephrogenic structure. (E–H) Whole mount Calbindin-D28k and DBA-lectin immunofluorescence at E13.5. Calbindin-D28k is expressed in both ureteric stalks and tips in WT and Ptc1−/−UB kidneys (E,F). DBA-lectin localizes predominantly to the ureteric stalk in WT kidneys and is excluded from the ureteric tips (G). In Ptc1−/−UB kidneys DBA-lectin is observed throughout the ureteric tips and ureteric stalks (H). (I,J) Wnt7b is expressed in the ureteric stalks (arrow) but is absent from ureteric tips (arrowhead) in both WT and Ptc1−/−UB kidneys.

Figure S6  Ptc1-deficiency does not effect the nephron progenitor population. (A,D) RNA in situ hybridization demonstrates normal expression of Osr1 and Six2 in the mesenchymal precursor population of Ptc1-deficient kidneys. (E,F) Cited-1 immunofluorescence is comparable between WT and Ptc1−/−UB kidneys. (G, H) RNA in situ hybridization demonstrates a reduced number of developing nephrogenic structures in Ptc1−/−UB kidneys but those present exhibit normal expression of Wnt4. (I,J) Wnt16b expression is comparable between WT and Ptc1−/−UB kidneys.

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