Gata3 Acts Downstream of β-Catenin Signaling to Prevent Ectopic Metanephric Kidney Induction

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Abstract
Metanephric kidney induction critically depends on mesenchymal–epithelial interactions in the caudal region of the nephric (or Wolffian) duct. Central to this process, GDNF secreted from the metanephric mesenchyme induces ureter budding by activating the Ret receptor expressed in the nephric duct epithelium. A failure to regulate this pathway is believed to be responsible for a large proportion of the developmental anomalies affecting the urogenital system. Here, we show that the nephric duct-specific inactivation of the transcription factor gene Gata3 leads to massive ectopic ureter budding. This results in a spectrum of urogenital malformations including kidney adysplasia, duplex systems, and hydroureter, as well as ves deferens hyperplasia and uterine agenesis. The variability of developmental defects is reminiscent of the congenital anomalies of the kidney and urinary tract (CAKUT) observed in human. We show that Gata3 inactivation causes premature nephric duct cell differentiation and loss of Ret receptor gene expression. These changes ultimately affect nephric duct epithelium homeostasis, leading to ectopic budding of interspersed cells still expressing the Ret receptor. Importantly, the formation of these ectopic buds requires both GDNF/Ret and Fgf signaling activities. We further identify Gata3 as a central mediator of β-catenin function in the nephric duct and demonstrate that the β-catenin/Gata3 pathway prevents premature cell differentiation independently of its role in regulating Ret expression. Together, these results establish a genetic cascade in which Gata3 acts downstream of β-catenin, but upstream of Ret, to prevent ectopic ureter budding and premature cell differentiation in the nephric duct.

Introduction
In human, urinary tract anomalies rank among the most common birth defects, with an estimated occurrence of 1 in 250 live births [1]. Most of these oligoanomalies are classified as Congenital Anomalies of the Kidney and Urinary Tract (CAKUT) [2], which is a highly heterogeneous condition frequently diagnosed in combination with genital tract anomalies [3]. The most relevant clinical manifestations include absent, dysplastic or obstructed renal systems in infants as well as infertility, pregnancy complications, hypertension and chronic renal failure in adults [4].

The development of the urogenital system (UGS) begins with the formation of the nephric duct (or Wolffian duct) [5,6]. This epithelial duct is a central UGS component among all vertebrates and serves as the primordium for the ureter, kidney collecting duct system and male genital tract [7]. Upon its induction in the intermediate mesoderm at embryonic day E8.5 in the mouse, the nephric duct rapidly elongates caudally until it reaches the cloaca, a pouch from which the bladder and urethra later develop. At E10.5, the formation of the definitive [metanephric] kidney is initiated by sprouting of the ureteric bud from the nephric duct into the adjacent metanephric mesenchyme. The ureteric bud subsequently undergoes several branching cycles to form the collecting duct system, whereas the ureter tips induce nephron formation in the surrounding mesenchyme [8].

Ureteric bud outgrowth and positioning are among the most crucial steps of UGS development, since anomalies at the budding stage account for the majority of kidney and urinary tract developmental defects [9,10]. Extensive evidence has identified GDNF/Ret signaling as a central regulator of ureteric bud induction [11–16]. In this system, GDNF secretion in the metanephric mesenchyme activates the Ret receptor tyrosine kinase via its ligand binding GFRα1 co-receptor. In turn, Ret activation results in the initiation of intracellular signaling cascades, which mediate bud outgrowth, proliferation and subsequent ureter branching. Understandably, the activity of GDNF/Ret signaling is tightly regulated by various mechanisms to allow for the generation of a single ureter at the appropriate position [9]. In the mesenchyme surrounding the nephric duct, the forkhead transcription factor FoxC1 and the Sli2/Robo2 ligand-receptor pair repress the rostral expression of GDNF [17,18], while Bmp4 antagonizes its activity [19]. At the budding site,
Gata3 in Ureteric Bud Induction

Author Summary

In humans, kidney development originates during embryonic development by the sprouting of an epithelial bud—called the ureteric bud—from a simple epithelial structure—the nephric duct. The ureteric bud quickly grows and branches in a treelike fashion to form the kidney collecting duct system, while the emerging ureteric tips induce nephron differentiation. One of the most important steps during kidney development is the positioning of a single ureteric bud along the nephric duct, since mutations of genes implicated in this process lead to severe urogenital malformations. In this study, we identified the Gata3 protein as a crucial regulator of ureteric bud positioning by using genetically modified mice. Deleting the Gata3 gene in the mouse resulted in the development of multiple kidneys emerging at improper positions. We show that this defect was caused by a hypersensitivity of nephric duct cells in their response to local growth signals. Interestingly, this phenomenon was partly triggered by premature differentiation of a subset of nephric duct cells. Furthermore, we report a genetic pathway in which Wnt/β-catenin signaling activates the Gata3 gene, which in turn positively regulates the Ret gene. In summary, we introduce a mouse model system that can be used to study human birth defects affecting the urogenital system.

Gremlin releases GDNF inhibition by Bmp4 [20], thereby allowing ureteric bud outgrowth. In the nephric duct epithelium, Sprouty1 protein function is crucial to negatively modulate Ret signaling levels [21]. Of notice, gene mutations in any of these regulators of GDNF/Ret signaling result in CAKUT-like phenotypes in mice. In support of the clinical importance of these regulators of GDNF/Ret signaling result in CAKUT-like urogenital malformations. In this study, we identified the Gata3 gene in the mouse resulted in the development of multiple kidneys emerging at improper positions. We show that this defect was caused by a hypersensitivity of nephric duct cells in their response to local growth signals. Interestingly, this phenomenon was partly triggered by premature differentiation of a subset of nephric duct cells. Furthermore, we report a genetic pathway in which Wnt/β-catenin signaling activates the Gata3 gene, which in turn positively regulates the Ret gene. In summary, we introduce a mouse model system that can be used to study human birth defects affecting the urogenital system.

Gata3 is a transcription factor of the Gata Zn-finger family, which perform important functions during organogenesis [27]. In humans, GATA3 haploinsufficiency causes hypoparathyroidism, sensorineural deafness and renal anomalies (HDR) syndrome [28]. The urogenital defects of HDR patients closely resemble CAKUT in combination with genetic tract anomalies and include renal aplasia, dysplasia, hypoplasia and vesicoureteral reflux [29,30]. Gene ablation studies in mice further revealed a critical role for Gata3 in the development of several tissues [31,32]. In the urogenital system, Gata3 is necessary for proliferation control and guidance of the nephric duct [33]. Accordingly, it is the only Gata factor expressed in this tissue prior to E12.5 [34].

Here we report the conditional inactivation of Gata3 specifically in the nephric duct, at a stage past the developmental defects observed in germline knockout embryos. These mice display multiple UGS malformations affecting the kidney, ureter and genital tracts. The detailed analysis of this phenotype reveals a genetic cascade whereby β-catenin promotes Gata3 expression in the nephric duct, which in turn activates Ret expression, maintains an undifferentiated epithelial cell state and prevents the inappropriate response to signaling pathways promoting ureter budding.

Gata3ND−/− Embryos Show Multiple Urogenital System Abnormalities

We initially characterized whole dissected urogenital systems (UGS) of Gata3ND−/− embryos at embryonic day (E)18.5. This gross analysis revealed a broad variety of malformations affecting the kidneys and genital tracts. Kidney defects included agenesis (15%), aplasia (20%) and severe dysplasia (65%) (Figure 2A–G). Moreover, one third of the Gata3ND−/− embryos displayed duplex kidneys (arrows in 2F). Histological analysis further revealed that the dysplastic kidneys were associated with hydronephrosis and hydroureter (Figure 2H,I). As expected from embryos with such poor kidney endowment, no Gata3ND−/− pups could be recovered after birth.

In addition to these renal defects, over 80% of male mutant genital tracts displayed a massive enlargement of the vas deferens in comparison to control embryos (Figure 2A,B,G). In female embryos, Gata3 inactivation in the nephric duct resulted in a complete loss of uterus in over 85% of UGSs examined. The oviduct, however, was still present in these embryos (Figure 2C,D,L,M). Hence the conditional inactivation of Gata3 leads to a broad spectrum of urogenital defects, including a high incidence of hydronephrotic kidneys and hydroureters.

Multiple Ectopic Buds Are Observed in Gatas3ND−/− Embryos

The combination of kidney hydronephrosis, hydroureter and duplex kidneys seen in Gata3ND−/− embryos pointed to a primary defect at the level of ureter budding. In order to easily visualize nephric duct cells, we crossed the Rosa26FLTP-LacZ allele [31] into control and Gata3ND−/− genetic backgrounds and stained the UGSs for β-Galactosidase activity.

During normal development, a localized swelling of the caudal portion of the nephric duct indicates the site of ureteric bud outgrowth at E10.5 (Figure 3A). The bud quickly emerges and undergoes the first dichotomous branching event at E11.5, forming the T-stage kidney (Figure 3C). Subsequently, the ureter lengthens and multiple branching cycles mark the development of the metanephric kidney (Figure 3E). In Gata3ND−/− mutant embryos at E10.5, nephric duct swelling was sometimes observed (Figure 3B), but normal ureteric bud formation failed in most embryos analyzed. In addition, the nephric duct looked more

Results

Conditional Inactivation of Gata3 in the Urogenital System

The strong mesonephric phenotype observed in Gata3−/− embryos [33] precludes the study of Gata3 function later during urogenital system development. To investigate the role of Gata3 in ureteric bud formation, we first generated a Gata3 conditional loss of function allele (Figure 1A,B). For this, the parental Gata3 allele (Gata3floxlox) [33] was crossed with a transgenic strain expressing FLIP in the germline [33] to excise the GFP-neo reporter cassette, thereby generating a conditional Gata3 allele with loxP sites flanking exon 4 (Gata3flx). These mice were subsequently bred with the More-Cre germline deleter strain [36] to generate the Gata3ND allele in which exon 4 is removed. The splicing from exon 3 to exon 5, expected from this modification, would generate a frameshift leading to protein truncation just upstream of the first zinc-finger DNA-binding domain. This gene mutation is therefore predicted to be null, like other previously reported Gata3 mutant alleles [32,33,37]. To delete Gata3 specifically in the mural nephric duct and derived collecting duct system, we crossed Gata3flx mice with the HoxB7-Cre mouse strain [38] to generate HoxB7−/−; Gata3flx/+ and HoxB7−/−; Gata3flx/flx embryos. Both genotypes had the same phenotype and are subsequently referred to as Gata3−/− embryos. Gata3flx/flx , Gata3flx/+ and HoxB7−/−; Gata3flx/flx embryos failed to show any overt phenotype and were used as controls.
sinuous in appearance (Figure 3B). Strikingly, at E11.5, ectopic epithelial buds formed along the entire length of the nephric duct, with a preferential accumulation in the middle segments of the duct (Figure 3D). By E12.5, the majority of the ectopic buds started to regress, while some buds expanded to form ectopic kidneys at a position far more rostral than the normal kidney induction site (Figure 3F). Following ectopic bud regression, the male nephric duct started to enlarge (Figure 4A,B). This was accompanied by a slight increase of about 50% in cell proliferation index, as determined by phospho-histone H3 immunolabelling at E13.5 (Figure 4C,D). In females, in situ hybridization with cRNA probes against Emx2 and Wnt4, staining the Müllerian duct epithelium and mesenchyme, respectively, revealed a block in female genital tract elongation in the region where most ectopic ureteric buds occurred (Figure 4E–H). Since the expression of the key Müllerian duct regulators Wnt4, Emx2, Wnt9b and Lim1 was

![Figure 1. Generation of a conditional Gata3 knockout allele.](https://www.plosgenetics.org/article/f1000316/g001)

**Figure 1. Generation of a conditional Gata3 knockout allele.** (A) The conditional Gata3 knockout allele was generated by crossing Gata3<sup>ex4GFP</sup> mice with FLPe germline deleter mice. The FLPe recombinase removed the ires-GFP-neo cassette, thereby generating a Gata3 allele with loxP sites flanking exon 4 (Gata3<sup>flox</sup>). Subsequent crosses of Gata3<sup>flox</sup> mice with More-Cre germline deleter mice resulted in Gata3-exon 4 excision and the creation of a null allele (Gata3<sup>D</sup>). (B) Representative genotyping by PCR for wild-type (+/+/), Gata3<sup>flox/flox</sup> (flox/flox) and Gata3<sup>D/+</sup> (D+/+) mice prepared from tail DNA. doi:10.1371/journal.pgen.1000316.g001

![Figure 2. Gata3<sup>ND</sup>/ND embryos display multiple urogenital system abnormalities.](https://www.plosgenetics.org/article/f1000316/g002)

**Figure 2. Gata3<sup>ND</sup>/ND embryos display multiple urogenital system abnormalities.** Urogenital systems were dissected at E18.5 and either processed as whole mount (A–Q) or subjected to H&E analysis on tissue sections (H–M). (A) Wild-type male UGS showing normal kidneys (k) and vas deferens (vd). (B) UGS of a Gata3<sup>ND</sup>−/− male embryo, showing unilateral kidney agenesis (*), an aplastic kidney (ak) and bilateral vas deferens dilations (white arrowheads). (C) Wild-type female UGS harboring a normal uterus (ut). (D) Female Gata3<sup>ND</sup>−/− UGS, with dysplastic kidneys (dk) and bilateral agenesis of the uterus (white arrowheads point to remaining connective tissue). (E) Wild-type kidney and ureter. (F) Duplex kidneys and ureters (white arrowheads) and (G) hydroureret, hydrenephrosis and kidney dysplasia are frequently observed in conditional Gata3<sup>ND</sup>/ND embryos. (H, I) H&E histological analysis of wild-type (H) and Gata3<sup>ND</sup>−/− (I) kidneys reveals severe kidney dysplasia and hydrenephrosis affecting the collecting duct system and all nephron segments. (J, K) Histological analysis of wild-type (J) and Gata3<sup>ND</sup>−/− (K) male genital tracts highlighting the dilated and fluid filled vas deferens in mutant embryos. (L, M) H&E staining of wild-type (L) and Gata3<sup>ND</sup>−/− (M) female genital tract, confirming uterus agenesis (*) and showing normal morphology of the ovaries (ov) and oviducts (od) in mutant embryos. cd, collecting duct; dt, distal tubule; g, glomerulus; pt, proximal tubule.

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of Müllerian duct elongation (red arrowhead in F, H) in morphogenesis in control embryos. In Gata3ND metanephric kidney (k) undergoes its third round of branching frequently fail to induce the primary bud (*). (E, F) At E12.5 the kidney. In embryos invades the metanephric mesenchyme to form a T-stage and Gata3ND duct (nd) at E10.5 marks the ureteric bud induction site (ub) in control embryonic stages. (A, B) Swelling of the caudal portion of the nephric buds in male embryos were stained for Wolffian duct hyperplasia in Figure 4. Genital tract anomalies in Gata3ND−/− embryos. (A–F) Dissected urogenital systems of control (HoxB7-Cre; Gata3fl/fl; Rosa26-loxPloxP2a+) and Gata3ND−/− (HoxB7-Cre; Gata3fl/fl; Rosa26-loxPloxP2a+) embryos were stained for β-Galactosidase activity at the indicated embryonic stages. (A, B) Swelling of the caudal portion of the nephric duct (nd) at E10.5 marks the ureteric bud induction site (ub) in control and Gata3 mutant embryos. (C, D) At E11.5, the ureteric bud of control embryos invades the metanephric mesenchyme to form a T-stage kidney. In Gata3ND−/− embryos, ectopic buds form along the entire length of the nephric duct (arrowheads). Gata3-deficient embryos frequent fail to induce the primary bud (*). (E, F) At E12.5 the metanephric kidney (k) undergoes its third round of branching morphogenesis in control embryos. In Gata3ND−/− embryos, some ectopic buds give rise to ectopic kidneys (ek) showing deficient branching morphogenesis, whereas the most rostral buds start to regress. Note the aberrant branch point of the ectopic kidneys (red arrowhead). doi:10.1371/journal.pgen.1000316.g003

Figure 3. Ectopic ureteric budding in Gata3ND−/− embryos. (A–F) Dissected urogenital systems of control (HoxB7-Cre; Gata3fl/fl; Rosa26-loxPloxP2a+) and Gata3ND−/− (HoxB7-Cre; Gata3fl/fl; Rosa26-loxPloxP2a+) embryos were stained for β-Galactosidase activity at the indicated embryonic stages. (A, B) Swelling of the caudal portion of the nephric duct (nd) at E10.5 marks the ureteric bud induction site (ub) in control and Gata3 mutant embryos. (C, D) At E11.5, the ureteric bud of control embryos invades the metanephric mesenchyme to form a T-stage kidney. In Gata3ND−/− embryos, ectopic buds form along the entire length of the nephric duct (arrowheads). Gata3-deficient embryos frequently fail to induce the primary bud (*). (E, F) At E12.5 the metanephric kidney (k) undergoes its third round of branching morphogenesis in control embryos. In Gata3ND−/− embryos, some ectopic buds give rise to ectopic kidneys (ek) showing deficient branching morphogenesis, whereas the most rostral buds start to regress. Note the aberrant branch point of the ectopic kidneys (red arrowhead). doi:10.1371/journal.pgen.1000316.g003

normal in these embryos (Figure 4E–H and data not shown), it is possible that this elongation defect is simply caused by a physical obstruction by ectopic ureteric buds. Together, these results indicate that the urogenital defects observed in Gata3ND−/− embryos are largely caused by the emergence of ureteric buds at aberrant positions along the nephric duct.

The Formation of the Ectopic Buds Depends on GDNF/Ret as well as Fgf Signaling

To determine the cause of ectopic ureter budding in Gata3ND−/− embryos, we first looked at Ret expression by in situ hybridization. Consistent with the previous analysis of Gata3 germline knockout embryos [33], most Gata3ND−/− nephric duct cells lost Ret expression. Curiously, however, the ectopic buds of Gata3ND−/− embryos remained positive for Ret (Figure 5A,B). In situ hybridization with a cRNA probe against Gata3 exon 4 (which is excised in the Gata3 conditional allele) confirmed that the bud cells still expressed Gata3 (Figure 5C,D), whereas nephric duct cells had already lost Gata3 expression (Figure 5C–F). The remaining Ret expression therefore resulted from the incomplete action of Cre in the nephric duct at this stage. Interestingly, these ectopic buds consisted of Gata3+/Ret+ and Gata3−/Ret− cells segregated from each other (Figure 5A–D). The continuous inactivation of Gata3 as well as the downregulation of Ret expression was confirmed in dysplastic Gata3ND−/− metanephric kidneys derived from such ectopic buds (Figure 5G–J). The severity of kidney dysplasia in these embryos correlated well with the amount of remaining Ret expression (Figure 5J insert, data not shown). As expected, these dysplastic kidneys additionally showed severely impaired nephron differentiation, as evidenced by the strong reduction in Fgf8-positive nephron precursors in comparison to control embryos (Figure 5K,L).

Since Ret expression was specifically localized in the ectopic ureteric buds of Gata3ND−/− embryos (Figure 5A,B), we hypothesized that Ret signaling might be causally involved in the budding process. To investigate whether the ectopic buds required GDNF/Ret signaling, we performed organ culture experiments with dissected Gata3ND−/− and control UGSs starting at E10.5. After 42 hours in culture, their development progressed, forming either T-stage metanephric kidneys in control UGSs or multiple ectopic buds in Gata3ND−/− UGSs (Figure 5K,L). Importantly, addition of the Ret tyrosine kinase inhibitor SU5416 [39] efficiently suppressed ectopic budding in Gata3ND−/− UGSs in culture.

Figure 4. Genital tract anomalies in Gata3ND−/− embryos. (A, B) β-Galactosidase staining of E14.5 genital ridges marks the beginning of Wolffian duct hyperplasia in male Gata3ND−/− embryos (red arrowhead in B), it is possible that this elongation defect is simply caused by a physical obstruction by ectopic ureteric buds. Together, these results indicate that the urogenital defects observed in Gata3ND−/− embryos are largely caused by the emergence of ureteric buds at aberrant positions along the nephric duct.

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Morphogenesis and nephron differentiation in Galactosidase activity (D insert). (E, F) The caudal nephric duct efficiently deleted Gata3 (B) In Gata3ND/− embryos, Ret expression is lost in most nephric duct cells except for some cells located in the ectopic ureteric bud tips (arrows). (C,D) In situ hybridizations with a Gata3 cRNA probe specific for exon 4 confirm that the Ret expressing cells in the ectopic buds still express Gata3. In these embryos, Gata3+ cells segregate from Gata3− cells. A similar tendency is observed in Gata3ND/− embryos stained for β-Galactosidase activity (D insert). (E, F) The caudal nephric duct efficiently deleted Gata3 already at this stage. (G–L) Deficient branching morphogenesis and nephron differentiation in Gata3ND/− hypodysplastic kidneys. (G,H) In situ hybridization with the Gata3-exon 4 cRNA probe confirms Gata3 inactivation in Gata3ND/− metanephric kidneys. (I, J) Ret expression becomes strongly downregulated in highly dysplastic Gata3-mutant kidneys, in contrast to control kidneys. (J insert) Milder kidney dysplasia showing a pattern of Ret expression intermediate between wild-type and highly dysplastic kidneys. (K,L) Fgf8 in situ hybridizations reveal impaired nephron induction in Gata3ND/− kidneys. Tissue sections in D (insert) and I–L are counterstained with nuclear fast red. nd, nephric duct; cl, cloaca; ut, ureter tip; rv, renal vesicle.

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Figure 5. Gata3 is necessary for maintenance of Ret expression. (A–F) Gata3 maintains Ret expression in the nephric duct at E11.5. (A) Whole mount in situ hybridization of control UGS with a Ret cRNA probe shows the smooth gradient of Ret expression along the entire length of the nephric duct. (B) In Gata3ND/− embryos, Ret expression is lost in most nephric duct cells except for some cells located in the ectopic ureteric bud tips (arrows). (C,D) In situ hybridizations with a Gata3 cRNA probe specific for exon 4 confirm that the Ret expressing cells in the ectopic buds still express Gata3. (E) In Gata3ND/− embryos, Ret expression is lost in most nephric duct cells except for some cells located in the ectopic ureteric bud tips (arrows). (C,D) In situ hybridizations with a Gata3 cRNA probe specific for exon 4 confirm that the Ret expressing cells in the ectopic buds still express Gata3. (E, F) The caudal nephric duct efficiently deleted Gata3 already at this stage. (G–L) Deficient branching morphogenesis and nephron differentiation in Gata3ND/− hypodysplastic kidneys. (G,H) In situ hybridization with the Gata3-exon 4 cRNA probe confirms Gata3 inactivation in Gata3ND/− metanephric kidneys. (I, J) Ret expression becomes strongly downregulated in highly dysplastic Gata3-mutant kidneys, in contrast to control kidneys. (J insert) Milder kidney dysplasia showing a pattern of Ret expression intermediate between wild-type and highly dysplastic kidneys. (K,L) Fgf8 in situ hybridizations reveal impaired nephron induction in Gata3ND/− kidneys. Tissue sections in D (insert) and I–L are counterstained with nuclear fast red. nd, nephric duct; cl, cloaca; ut, ureter tip; rv, renal vesicle.

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Molecular Characterization of Ectopic Ureteric Bud Formation in Gata3ND/− Embryos

To study ectopic bud formation at the molecular level, we looked at the expression of key components of the GDNF/Ret signaling cascade in E10.5 and E11.5 embryos. Already at E10.5, prior to budding, Ret expression was lost in a subset of nephric duct cells in Gata3ND/− embryos (Figure 7A,B). Staining of adjacent sections with a GDNF in situ probe revealed that the remaining Ret-expressing cells were concentrated near the mesenchymal source of GDNF (Figure 7C,D). At E11.5, only the caudal ectopic buds that could maintain GDNF and Fgfr2 expression in the surrounding mesenchyme were able to develop into ectopic kidneys (Figure 7E,F and data not shown). In some Gata3ND/− embryos, the forming buds induced more Wnt11 expression than the presumptive ureteric bud induction site in control embryos, pointing to an elevated GDNF/Ret signaling response in those cells (Figure 7G,H). By E12.5, the expression of Wnt11 and Ret was lost in the regressing ectopic buds of Gata3ND/− embryos (data not shown). Surprisingly, assessing the expression of the GDNF/Ret modulators Spry1, Spry2, Spry4, Sizz2, Robo2, Foxc1, Foxc2, Bmp4 and Grem1, only revealed a modest downregulation of Spry1 and Sizz2 expression in Gata3-mutant nephric duct cells at E10.5, while the other markers remained unaffected (Figure 7I–L and data not shown). In order to further support the involvement if Fgfs in ectopic bud formation, we additionally probed control and Gata3ND/− embryos with the soluble Fgfr2-Fc fusion protein, which recognizes several Fgfs [44]. This experiment revealed strong and similar Fgf expression levels surrounding the nephric duct in both Gata3-mutant and control embryos at E11.5 (Figure 7M,N). This protein localization was consistent with the expression of one of the known Fgfr2 ligands, Fgf10 (Figure 7O,P). Hence, the molecular marker analysis reveals no obvious mesenchymal defects, pointing to a primary defect in the response of the nephric duct to GDNF and Fgf signals.
Figure 6. Ectopic ureter budding in Gata3\(^{3\Delta/3\Delta}\) embryos depends on GDNF/Ret and Fgf signaling. (A, B) E10.5 UGS organ cultures recapitulate the phenotype of both control and Gata3\(^{3\Delta/3\Delta}\) embryos observed in vivo. (C, D) Treatment of cultures with the Ret kinase inhibitor SU5416 efficiently inhibits both primary (*) and ectopic budding in control and Gata3\(^{3\Delta/3\Delta}\) UGSs. (E, F) Supplementing the organ cultures with recombinant GDNF induces ectopic ureteric budding in control UGS cultures (arrows in E) and enhances ectopic bud growth in Gata3\(^{3\Delta/3\Delta}\) UGS cultures (arrows in F). (G, H) Organ culture of control or Gata3-deficient UGSs treated with a GDNF blocking antibody suppresses primary and ectopic bud formation, respectively. (I, J) Soluble FgfR2-Fc fusion protein has no major effect on primary budding in control UGSs, but successfully inhibits ectopic budding in Gata3\(^{3\Delta/3\Delta}\) UGSs. ub, ureteric bud; *, primary bud agenesis. doi:10.1371/journal.pgen.1000316.g006

Figure 7. Molecular characterization of ectopic ureteric bud formation in Gata3\(^{3\Delta/3\Delta}\) embryos. (A, B) At E10.5 in Gata3\(^{3\Delta/3\Delta}\) embryos, the Ret+ nephric duct cells (nd) segregate from Ret− cells prior to budding. (C,D) Adjacent sections show that Ret+ cells are localized next to GDNF expressing cells in the metanephric mesenchyme (mm). (E, F) Ectopic kidneys (ek) maintain mesenchymal GDNF expression in the metanephric mesenchyme at E11.5. (G, H) Induced ectopic ureteric buds in Gata3-deficient embryos strongly upregulate the expression of Wnt11 at E10.5. (I-L) Slit2 and Spry1 are slightly downregulated in Gata3 mutant (nd) cells at E10.5. (M,N) FgfR2-Fc staining shows a strong but unaffected expression of Fgf ligands in both control and Gata3\(^{3\Delta/3\Delta}\) embryos at E11.5. (O,P) Fgf10 is expressed in the mesenchymal cells adjacent to the nephric duct in both wild-type and Gata3\(^{3\Delta/3\Delta}\) embryos. nd k: kidney, ur ureter. doi:10.1371/journal.pgen.1000316.g007
β-Catenin Signaling Regulates Gata3 Expression in the Nephric Duct

The developmental defects of Gata3ND−/− embryos described above show striking similarities with the phenotypes resulting from the conditional inactivation of β-catenin (Ctnnb1) in the nephric duct [45]. The fact that both animal models have the same spectrum of genitral tract and kidney defects, prompted us to verify whether Gata3 and β-catenin act in the same genetic pathway. For this, we first performed in situ hybridization against Gata3 in E11.5 HoxB7-Cre, Ctnnb1lox−/− embryos (Ctnnb1ND−/−) and found a strong downregulation of Gata3 expression in the nephric duct in comparison to control embryos (Figure 8A,B). Accordingly, in situ hybridizations with a Ret cRNA probe revealed a loss of Ret expression in Ctnnb1ND−/− embryos (Figure 8C,D), which mimics the loss of Ret expression in Gata3ND−/− embryos (Figure 5). Staining of adjacent tissue sections with in situ probes for the canonical Wnt target genes Axin2, Sp5 and Daple [46,47] confirmed the loss of β-catenin transcriptional response in Ctnnb1ND−/− embryos (Figure 8E,F and data not shown). In order to clarify the genetic hierarchy between Gata3 and β-catenin, we next stained Gata3ND−/− embryos with antibodies against β-catenin and phospho-β-catenin. These experiments failed to show any modification of β-catenin expression levels or activity following Gata3 inactivation (Figure 8G,H and data not shown). In support of this, the expression of the canonical Wnt-signaling target genes Axin2, Daple and Sp5 was unchanged in Gata3ND−/−/− embryos (Figure 8I−L and data not shown). From these data, we conclude that Gata3 acts genetically downstream of β-catenin but upstream of Ret in the nephric duct. To further characterize the molecular basis of the β-catenin-Gata3-Ret pathway, we first performed a bioinformatics analysis of the 50 kb genomic region upstream of the mouse and human Gata3 genes. Highly conserved sequences shared by 11 of the 11 conserved regions 50 kb upstream of the Ret-ATG (Figure 9B). The potential of β-catenin to regulate endogenous Gata3 expression was further evaluated in mouse IMCD3 collecting duct-derived cells. Using the GSK3β inhibitor BIO to stabilize the β-catenin protein [49], we observed a significant increase of Gata3 expression levels in those cells (Figure 9C). In order to assess the activity of Gata3 on the Ret regulatory region, we took advantage of the fact that one of the Gata3 binding sites mapped to a region previously reported to drive reporter gene expression in the zebrafish pronephros (Figure 9B, large asterisk) [50]. To test whether Gata3 acts on this site, we isolated the 1.2 kb conserved fragment and introduced a specific point mutation in the Gata3 binding site (Figure 9B). The wild-type and mutated fragments cloned upstream of a β-Gal reporter construct were transfected in IMCD3 cells stably expressing Gata3. The inactivation of the Gata3 binding site led to a significant downregulation of β-Gal expression relative to the wild-type control (Figure 9D), thereby suggesting that Gata3 may regulate Ret expression directly from this binding site. Together these data are consistent with a β-catenin-Gata3-Ret genetic cascade in the nephric duct.

Premature and Ret-Independent Differentiation of Gata3 Mutant Cells

Another aspect of β-catenin-loss in the nephric duct is a premature differentiation of the affected cells [45]. To test whether this phenotype was also present in Gata3ND−/−/− embryos, we stained mutant and control embryos with the differentiation markers DBA and Zo1+. In the developing metanephric kidney, DBA and Zo1+ are strongly expressed in the distal collecting duct and downregulated in Ret+ ureter tip cells [45,51]. At E11.5, DBA and Zo1+ expression were almost undetectable in the nephric duct of control embryos (Figure 10A,B). However, Gata3-deficient cells located at −110 kb [48], but failed to detect any conserved TCF/Lef binding sites in this element. A similar bioinformatics analysis of the Ret regulatory region revealed putative Gata3 binding sites in 4 of the 11 conserved regions 30 kb upstream of the Ret-ATG (Figure 9B). The potential of β-catenin to regulate endogenous Gata3 expression was further evaluated in mouse IMCD3 collecting duct-derived cells. Using the GSK3β inhibitor BIO to stabilize the β-catenin protein [49], we observed a significant increase of Gata3 expression levels in those cells (Figure 9C). In order to assess the activity of Gata3 on the Ret regulatory region, we took advantage of the fact that one of the Gata3 binding sites mapped to a region previously reported to drive reporter gene expression in the zebrafish pronephros (Figure 9B, large asterisk) [50]. To test whether Gata3 acts on this site, we isolated the 1.2 kb conserved fragment and introduced a specific point mutation in the Gata3 binding site (Figure 9B). The wild-type and mutated fragments cloned upstream of a β-Gal reporter construct were transfected in IMCD3 cells stably expressing Gata3. The inactivation of the Gata3 binding site led to a significant downregulation of β-Gal expression relative to the wild-type control (Figure 9D), thereby suggesting that Gata3 may regulate Ret expression directly from this binding site. Together these data are consistent with a β-catenin-Gata3-Ret genetic cascade in the nephric duct.

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defect in embryos (Figure 10A,B and E,F). To quantify the differentiation levels could be observed between wild-type and Ret-deficient embryos, we generated a conditional knockout allele of Ret [33], we generated a conditional knockout allele of Ret. From these data, we conclude that Gata3 acts independently of Ret to maintain a precursor state expression in the nephric duct. The only other regulators of genes at this stage [33]. The recent identification of a Gata3-regulatory element [50] and enhancer active in the nephric duct may help clarify some aspects of the question of the transcriptional control of Gata3 expression in the nephric duct. Using Ret expression is not sufficient for Ret expression in the absence of Gata3. The identification of β-catenin acting upstream of Gata3 also raises the question of the transcriptional control of Gata3 expression in the nephric duct. The only other regulators of Gata3 identified in this tissue are the transcription factors Pax2 and Pax8 [33]. It is thus possible that Pax2/8-mediated activation of Gata3 acts through the β-catenin pathway. Alternatively, Pax2/8 and β-catenin may act independently to regulate Gata3 expression in the nephric duct either together or as activation and maintenance factors, respectively. We favor the latter model based on the observation that the canonical Wnt signaling readouts Axin2 and Dapple are not yet expressed in 18-somite stage embryos [E9;0, D.G., M.B. unpublished results], whereas Gata3 is already under the control of Pax genes at this stage [33]. The recent identification of a Gata3 kidney enhancer active in the nephric duct may help clarify some aspects of Gata3 regulation in this tissue [48].

Mechanisms of Ectopic Ureteric Bud Formation

Defects in ureteric bud branching account for most UGS defects in humans and mice. The underlying cause is typically a deregulation of GDNF/Ret signaling [9]. Accordingly, our data show a direct implication of GDNF/Ret signaling in the formation of Gata3ND−/−-ectopic buds. Recombinant GDNF treatment indeed sustained ectopic bud growth in organ culture, while blocking the signaling pathway with a GDNF blocking antibody or a chemical inhibitor against Ret was sufficient to prevent ectopic ureter budding. We also demonstrated that the emerging buds consist of Ret+ cells, which respond to mesenchymal GDNF by upregulating Wnt11 expression. Importantly, however, we show that Ret expression was lost in Gata3-mutant nephric duct cells and that these Gata3−/− Ret− cells failed to contribute to ectopic ureteric bud formation. This suggests that ectopic ureteric buds form as a consequence of Gata3 loss in to specifically address the later role of Gata3 in urogenital system morphogenesis, we inactivated Gata3 in the nephric duct using the HoxB7-Cre transgenic line (Gata3ND−/−). This resulted in severe malformations of the urogenital system including kidney agenesis, aplasia, dysplasia, duplex systems, ureteric agenesis and vesicoureteric reflux. This spectrum of malformations overlaps with the urogenital phenotypes observed in HDR syndrome patients (heterozygous for GATA3) and is generally reminiscent of human congenital anomalies of the kidney and urinary tract (CAKUT). Interestingly, most of these phenotypes can be either directly or indirectly attributed to ectopic ureteric budding observed in Gata3ND−/− embryos. Furthermore, our results identify Gata3 as a critical mediator of β-catenin signaling, which regulates both cell differentiation and Ret expression in the nephric duct.

Regulation of Gata3 by β-Catenin

The UGS malformations observed in Gata3ND−/− embryos were strikingly similar to the ones reported recently for Ctnnb1ND−/− embryos [45]. Gene expression analyses in both genotypes revealed a genetic cascade whereby Gata3 acts downstream of β-catenin in the nephric duct to maintain Ret expression and prevent premature epithelial differentiation. These genetic interactions were further supported by promoter analyses and cell culture assays. Hence, our results identify Gata3 as a crucial mediator of β-catenin activity in the nephric duct. Using Ret−/− embryos, we could further determine that the premature differentiation phenotype observed in Ctnnb1 and Gata3 mutant embryos is not mediated by Ret, thereby establishing at least two distinct cellular functions regulated by the β-catenin/Gata3 pathway (Figure 11A). The possibility remains that β-catenin also has a Gata3-independent effect on Ret expression. However, our data indicate that, if present, this effect is not sufficient for Ret expression in the absence of Gata3. The identification of β-catenin acting upstream of Gata3 also raises the question of the transcriptional control of Gata3 expression in the nephric duct. The only other regulators of Gata3 identified in this tissue are the transcription factors Pax2 and Pax8 [33]. It is thus possible that Pax2/8-mediated activation of Gata3 acts through the β-catenin pathway. Alternatively, Pax2/8 and β-catenin may act independently to regulate Gata3 expression in the nephric duct either together or as activation and maintenance factors, respectively. We favor the latter model based on the observation that the canonical Wnt signaling readouts Axin2 and Dapple are not yet expressed in 18-somite stage embryos [E9;0, D.G., M.B. unpublished results], whereas Gata3 is already under the control of Pax genes at this stage [33]. The recent identification of a Gata3 kidney enhancer active in the nephric duct may help clarify some aspects of Gata3 regulation in this tissue [48].

Discussion

We previously reported the critical role played by Gata3 in proliferation control and nephric duct guidance in the pro/mesonephros [33]. To circumvent this early renal phenotype and the embryonic lethality of Gata3−/− embryos at midgestation [31–33], we generated a conditional knockout allele of Gata3. In order
neighboring nephric duct cells. It further implies that Ret-mediated signaling played a role in the segregation of Gata3+/Ret+ from Gata3−/Ret− cells. Interestingly, a biased contribution of Ret+ and Ret− cells was also observed in the ureteric bud and metanephric kidney of embryos chimeric for the Ret gene [40]. However, the precise mechanism leading to Gata3+/Ret+ and Gata3−/Ret− cell segregation remains unclear.

Importantly, a number of observations argue against a simple GDNF/Ret-based budding mechanism. Among them are 1) the different sensitivity of ectopic buds towards GDNF-blocking antibody concentration and 2) the fact that ectopic buds emerge in all directions whereas GDNF is expressed in the inner intermediate mesoderm only. Together, these observations point to the existence of a parallel pathway to GDNF/Ret promoting ectopic ureter budding in Gata3−/Ret− embryos. Using a soluble recombinant Fgf receptor 2, we identified Fgf signaling as a crucial component of this alternative pathway. In support of this, Fgfs are able to induce ectopic budding in organ culture [41,42]. Of notice, the RTK inhibitor (SU5416) we used to inhibit Ret signaling in culture was also reported to have an effect on Fgfrs [39], which may have enhanced its activity against ectopic ureteric bud formation.

The genetic regulation of Ret by Gata3 corroborates our previous observations in germline Gata3 mutant embryos [33], but raises the intriguing question of the relationship between Gata3+/Ret+ budding cells and their surrounding Gata3−/Ret− cells in ectopic ureteric bud formation. Remarkably, the expression of the GDNF/Ret signaling regulators, Fox1, Fox2, Robo2, Bmp4, Gremlin1 and GDF-5 was not significantly affected in Gata3−/Ret− embryos. The modest downregulation of Spry1 and Slit2 expression was additionally restricted to non-budding Gata3-mutant cells and might be secondary to the loss of Ret expression. Hence, none of the major known mechanisms of GDNF/Ret modulation are likely to cause ectopic ureter budding in Gata3−/Ret− embryos. Instead, this suggests that the juxtaposition of nephric duct cells with different Gata3/Ret status is central to the ectopic budding phenotype. Hence, the simplest model to explain ectopic ureteric bud formation would be that the combined expression of GDNF and Fgf along the defective Gata3−/Ret− nephric duct at E10.5 is
Gata3 in Ureteric Bud Induction

Materials and Methods

**Mice**

Conditional and germ-line Gata3 knockout mice were generated by crossing Gata3α/α mice [33] with Actb::LFp [35] and Mouse-Cre [36] transgenic mice. The Gata3βα and Gata3ββ alleles were generated using the primers 5'-TATACCGGGTTCATCTACGAGC and 5'-TGTCAGGAGTCGAGGCGATT. HoxB7-Cre and Rosa26mTmG mice [38,34] were purchased from The Jackson Laboratory. All mice were kept in a pure C57BL/6 genetic background. Mutant mice for Cimbh [45] and Ret [16] were generated as described. The HoxB7-Venus line will be reported elsewhere.

**In Situ Hybridization and Histology**

Embryo dissections and processing as well as in situ hybridization on whole mount embryos or tissue sections were performed as described [53,56]. The Gata-3-exon 4 probe was generated by PCR amplification of the coding sequence of Gata3 exon 4 and subsequent cloning into the pGEM-T-easy vector. The Daple (Ced3/ð3) in situ probe was transcribed from image clone 6408630 linearized at an internal Asp718 restriction site. The Ems2 [57] Wnt4 [58], Ret [59], Gata3 [60], Axin2 [61], Fgf8 [62], GDNF [63], Wnt11 [52], Slit2 [64], Spy1 [65] and Fgf10 [66] in situ probes have been reported previously. Hematoxylin and eosin stainings were performed on 6 μm-thick paraflin sections using standard procedures.

**Immunohistochemistry**

Frozen sections have been prepared for immunohistochemistry as described [67]. For immunostainings against β-catenin and ZO1+, an antigen retrieval step has been included [45]. The following antibodies and conjugates were used: rabbit anti-phospho-H3 (1:200, Upstate Biotechnology), rat anti-E-cadherin (1:400, Zymed Laboratories), mouse anti-β-catenin (1:1000, Sigma), rabbit anti-phospho-β-catenin (1:100, Cell signaling), rat anti-ZO1+ (1:400, Chemicon), rabbit anti-GFP (1:1000, Abcam) and biotinylated dolichos biflorus agglutinin (DBA) (1:500, Vector Laboratories). Secondary detection was performed, using Alexa488 or Alexa568 labeled anti-mouse, anti-rabbit or anti-rat antibodies (1:200, Invitrogen). DBA-lectin staining was visualized with FITC or Cy3 conjugated streptavidin (1:200, Zymed Laboratories). Fgfr2-α ligand detection was performed on 12 μm thick cryosections cut from freshly frozen unfixed embryos. After 3 washes in cold PBS, the slides were incubated for 1 hr at 4°C in blocking solution (10% normal goat serum, 2% BSA in PBS supplemented with 0.5 mM MgCl2 and 1 mM CaCl2). The slides were treated with 2.5 μg/ml mouse FgfR2-Fc (R&D systems) or DMSO (0.01%, Fisher Scientific). The slides were fixed for 10' in 4% PFA. Subsequently, the samples were handled as for standard immunohistochemistry. Secondary detection was performed using a successive combination of biotinylated anti-human IgG (1:500, Vector Laboratories) and streptavidin-FITC conjugate (1:200, Zymed Laboratories). All slides were counterstained with 50 μg/ml DAPI and mounted with Slow Fade Gold mounting medium (Invitrogen).

**Organ Culture and β-Galactosidase Staining**

Mouse urogenital ridges were micro-dissected in cold PBS supplemented with 1% FBS, 1 mM CaCl2 and 0.5 mM MgCl2. The ridges were collected in 10% FBS/DMEM medium (Wistest) containing Penicillin/Streptomycin (Gibco) and L-Glutamin (Gibco), preincubated at 37°C (20 mg/ml, R&D systems) or DMSO (0.01%, Fisher Scientific). The ridges were treated with 2.5 μg/ml mouse FgfR2-Fc (R&D systems) in 0.5× blocking solution for 1 hr at 4°C. Following several washes in PBS, the slides were fixed for 10' in 4% PFA. Subsequently, the samples were handled as for standard immunohistochemistry. Secondary detection was performed using a successive combination of biotinylated anti-human IgG (1:500, Vector Laboratories) and streptavidin-FITC conjugate (1:200, Zymed Laboratories). All slides were counterstained with 50 μg/ml DAPI and mounted with Slow Fade Gold mounting medium (Invitrogen).

**Cell Culture**

Murine inner medullary collecting duct cells (IMCD3, kindly provided by Dr Paul Goodyer) were cultured in a 1:1 mix of DMEM
Galactosidase expression vector pTrap [68] using the unique

dimensions: 612.3x790.9

and HAM's F12 media (Wisent) supplemented with 10% fetal bovine serum. To induce the canonical Wnt-pathway, mMCD3 cells were stimulated with 3 μM BIO (22,23′-E)-6-Bromoindirubin-3'-oxime, EMD) or 0.3% DMSO for 4 hours. RNA was extracted (Rneasy Mini kit, Qagen) and reverse transcribed (Superscript III, Invitrogen) according to the manufacturer’s instructions. Quantitative PCR was performed using iQ SYBR Green Supermix (BioRad) in a RealPlex2
cycler (Eppendorf) using the following primers: Gata3 sense 5'-
CTCTGGAAGGAGACCTA-3' and antisense 5'-TTTGACCATTTTTCCGATGC-3', S16 sense 5'-GTACAGTT-
TACTTGAGCCGTITTTG-3' and antisense 5'-GCCCTTGAGATGGCCTGTCCGATGG-3'. For the production of mMCD3
cells, stably overexpressing Gata3, mouse Gata3 cDNA was cloned
into pMCSV-HA3-ires-GFP vector (kindly provided by Dr. Jerry
Pelletier). The Gata3-pMCSV vector was then co-transfected with
pVPack-GP and pVPack-VSV-G vectors (Stratagene) in HEK-293T
cells, for virus production. The virus containing supernatant was
harvested after 48 hrs by filtration and added to mMCD3 cells.
48 hours post-infection, the cells were sorted according to GFP
expression on an FACS Aria-sorter (BD-Bioscience). Gata3 expres-
sion on an FACS Aria-sorter (BD-Bioscience). Gata3 expres-
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sion on an FACS Aria-sorter (BD-Bioscience).

Bioinformatics

The sequence alignments were performed, using blast2seq
(NCBI). The transcription factor binding sites were identified with
Mac Vector.

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Gata3 conditional mutant mice. Thanks also to Melina Narlis for the
histology work.

Author Contributions

Conceived and designed the experiments: DG SKB MB. Performed the
experiments: DG SKB. Analyzed the data: DG SKB MB. Contributed
reagents/materials/analysis tools: CM XC FC TC. Wrote the paper: DG
SKB MB. Generated Gata3 conditional mutant mice: AS. Critically
reviewed the manuscript: CM XC FC TC.

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