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Pax2/8-regulated Gata3 expression is necessary for morphogenesis and guidance of the nephric duct in the developing kidney

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The mammalian pro- and mesonephros are transient embryonic kidneys essential for urogenital system development. The nephric (Wolffian) duct, which is a central constituent of both structures, elongates caudally along a stereotypical path to reach the hindlimb level where it induces metanephros (adult kidney) formation, while the remaining duct gives rise to the male genital tract (epididymis, vas deferens). The transcription factors Pax2 and Pax8 are essential for the initiation of pro- and mesonephros development. In a cDNA microarray screen for genes specifically expressed in the pro/mesonephros and regulated by Pax proteins, we identified *Gata3*, a transcription factor gene associated with hypoparathyroidism, deafness and renal anomaly (HDR) syndrome. *Gata3* is already expressed in the pronephric anlage, together with *Pax2* and *Pax8*, suggesting that it may be a direct Pax2/8 target gene. Inactivation of *Gata3* by insertion of an *Ires-GFP* reporter gene resulted in a massive increase in nephric duct cellularity, which was accompanied by enhanced cell proliferation and aberrant elongation of the nephric duct. Interestingly, however, the nephrogenic cord extended, with delayed kinetics, along the entire caudal path up to the level of the hindlimb bud, indicating that extension of the nephric duct and cord is controlled by different guidance cues. At the molecular level, the nephric duct of $Gata3^{-/-}$ embryos is characterized by the loss of Ret expression and signaling, which may contribute to the guidance defect of the nephric duct. Together, these results define Gata3 as a key regulator of nephric duct morphogenesis and guidance in the pro/mesonephric kidney.

KEY WORDS: Pax2, Pax8, Gata3, Mesonephros, Nephric (Wolffian) duct, Duct guidance, Kidney development, Mouse

INTRODUCTION

The formation of tubular epithelia from mesenchymal cells or pre-existing ducts is a common feature of organ development. In the mammalian kidney, tubulogenesis first occurs during the formation of the pronephros. This primary embryonic kidney generates a single nephric (Wolffian) duct that elongates caudally towards the cloaca (Saxen, 1987). The nephric duct subsequently forms the second embryonic kidney, the mesonephros, by induction of mesonephric tubules in the adjacent mesenchymal nephric cord. When the nephric duct reaches the metanephric mesenchyme at the level of the hindlimb, interactions between both tissues initiate metanephros development by inducing budding and invasion of the ureter from the duct into the metanephric mesenchyme. The newly formed ureter branches and induces mesenchymal-epithelial transitions in the surrounding mesenchyme, thereby initiating the first of numerous cycles of nephron formation. Later during development, the nephric duct is transformed into the male genital tract (epididymis and vas deferens) or degenerates in female embryos. Hence, unraveling the molecular mechanisms of nephric duct morphogenesis is essential for our understanding of urogenital system development.

Despite the central role of the pro- and mesonephros (referred to as pro/mesonephros) for kidney and genital tract formation, very few genes have so far been found to control the development of these two structures in the mouse. Notably, the *Pax2* and *Pax8* genes are both necessary and sufficient for the formation of the pronephros and all subsequent kidney structures (Bouchard et al., 2002). *Pax2,Pax8* double-mutant embryos fail to specify the nephric lineage, as they neither undergo the initial epithelial-mesenchymal transitions leading to nephric duct formation nor activate early nephric marker genes (Bouchard et al., 2002). Pax2 and Pax8 are closely related members of the Pax family of sequence-specific transcription factors (Chi and Epstein, 2002). $Pax8^{-/-}$ embryos have a severe defect in thyroid development but form normal kidneys (Mansouri et al., 1998). By contrast, *Pax2* is necessary for metanephros development (Torres et al., 1995), as it controls the survival of the ureter and late nephric duct (Ostrom et al., 2000; Porteous et al., 2000; Torres et al., 1995; Bouchard, 2004) and induces the mesenchymal-epithelial transitions leading to nephron formation (Rothenpieler and Dressler, 1993). At the molecular level, Pax2 regulates the expression of important nephrogenic molecules such as *Wt1* (Dehbi et al., 1996) and *Gdnf* (Brophy et al., 2001). These genes are expressed in the metanephric mesenchyme, and are necessary for metanephros induction and growth (Kuure et al., 2000). These data therefore point to a role of Pax2 in late mesonephros and metanephros development. However, the observation that $Pax2^{-/-}Pax8^{-/-}$ embryos show a more severe phenotype than single-mutant embryos underscores not only the functional redundancy among the two Pax genes (Bouchard et al., 2000; Bouchard et al., 2002), but also suggests an early role for Pax2 and Pax8 in regulating key target genes involved in pro/mesonephros formation (Bouchard et al., 2002; Bouchard, 2004).

In an attempt to identify these effector molecules, we have searched for mesonephros-specific, Pax2/8-regulated genes by cDNA microarray analysis of FACS-sorted mesonephric cells from wild-type and $Pax2^{-/-}$ embryos. These experiments identified the transcription factor gene *Gata3* as an early Pax2/8-regulated gene.

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In humans, *Gata3* has been associated with the HDR syndrome (Van Esch et al., 2000), a genetic disease characterized by hypoparathyroidism, sensorineural deafness and renal dysplasia (Bilous et al., 1992). In the mouse, homozygous inactivation of *Gata3* results in a complex phenotype including the failure to form the metanephros (Lim et al., 2000; Pandolfi et al., 1995). Here, we show that *Gata3* is a genetic target of *Pax2* and *Pax8* in the nephric duct of the pro/mesonephric kidney and that *Gata3* is required for the morphogenesis and guidance of the nephric duct along the anteroposterior axis of the embryo.

MATERIALS AND METHODS

Mice

The $Pax2^{lacZ}$ and $Pax8^{neo}$ alleles and the $Pax2^{GFP}$ BAC transgene (#30) were maintained on the C3H/He genetic background and genotyped as described (Bouchard et al., 2000; Bouchard et al., 2002; Pfeffer et al., 2002).

Generation of *Gata3* mutant mice

The Gata3 targeting vector was assembled in the pSP64 vector with a modified polylinker containing the appropriate restriction sites. Both recombination arms were cloned by PCR from the BAC clone RP23-136M6 with the following primers: long arm (5′ fragment), 5′-GCGCTCG-AGCTCCAACCTAAACAAACACAG-3′ and 5′-CTGGCTACATGCT-CACTCCCT-3′; long arm (3′ fragment), 5′-CAAGGCAGCACAGT-ATGGAGT-3′ and 5′-CACAGGCCGGCCAAACTCTTACGACTGAG-GA-3′; short arm, 5′-GCGGAGCTCTTAGCAACATCAGAAACCACT-3′ and 5′-GCGTTAATTAAAGGCAATCATTACTCAAGA-3′. The long arm was assembled using a *Drd*I site common to both fragments, located 2616 bp from the 5′ end. Exon 4 was cloned by PCR from the same BAC clone with the following primers: 5′-CACAGGCCGGCCAATAACTTCGTA-TAATGTATGCTATACGAAGTTATTAAGTGCTTTTGGTTTTATTTT-3′ (containing a loxP site) and 5′-GCGGGTTACACAGTGGTTCTG-ATGTTGCTA-3′. A 3660 bp *Kpn*I-*Sac*I fragment from the pk11-*iresGFP* vector was inserted into the modified pSP64 vector. The pk11-*iresGFP* was generated by modifying the pk11 vector (a gift from Gail Martin), containing a loxP site followed by the pgk neo selection cassette flanked by Frt sites. The modification of pk11 was made by insertion of a *Cla*I-*Eco*RI PCR fragment containing the splice acceptor of *Pax5* exon 2 modified to contain stop codons in all three reading frames, a 1360 bp *Eco*RI-*Sal*I fragment from the pBV-IRES-GFP1 vector containing the *Ires-GFP* sequences and a 150 bp *Xho*I-*Sal*I PCR fragment from the pRK7 vector, containing a *SV40* polyA sequence. These three fragments were added to pk11 using the *Cla*I-*Hin*dIII restriction sites located between the first Frt site and the neo gene. The primers used for these modifications are as follow: *Pax5* splice acceptor, 5′-GCGATCGATTTATGTCATCCGCATGATTGA-3′ and 5′-GCGGAATT-CATTTAATCACCCAAGCTGATTCACTCCTCC-3′; *SV40* polyA, 5′-GCGCTCGAGAACTTGTTTATTGCAGCTTAT-3′ and 5′-GCGAAGC-TTGATCCAGACATGATAAGATACA-3′. The negative selection cassettes were added as a 1940 bp *Not*I-*Sal*I fragment from the pBS-hsv-tk vector and a 1150 bp *Hin*dIII-*Not*I fragment from the p64-DT vector. HM1 ES cells were electroporated with 15 μg of *Not*I-linearized plasmid and selected with 250 μg/ml G418 and 2 μM gancyclovir. The selected clones were screened by PCR followed by confirmation by Southern blot analysis on the positive clones using a 705 pb PCR-generated probe located outside of the long arm. Two independent targeted ES cell lines were used to generate parental $Gata3^{ex4GFP}$ mice. These mice were subsequently mated to more-*Cre* transgenic mice (C57BL/6 background) (Tallquist and Soriano, 2000) to induce *Cre*-mediated germline deletion of *Gata3* exon 4 ($Gata3^{GFP}$) (Fig. 2A). The $Gata3^{GFP}$ mice (used in this study) were backcrossed in a C3H/He background for more than five generations.

Isolation of pro/mesonephric cells

The pro/mesonephric cells were isolated essentially as described for mid-hindbrain cells sorting (Bouchard et al., 2005). Briefly, $Pax2^{lacZ/+}$ $Pax2^{GFP}$ mice were intercrossed to generate Pax2 mutant and control embryos carrying the transgene. The tail region of GFP$^+$ embryos containing the pro/mesonephros was dissected and the tail tip removed. The isolated tissue was dissociated in 1% trypsin in PBS at 37°C for 15 minutes and the reaction stopped in cold DMEM without phenol red containing 10% fetal calf serum (DMEM-Rfree-FCS). The cells were washed once and resuspended in DMEM-Rfree-FCS containing 1 μg/ml propidium iodide. GFP$^+$ and GFP$^-$ live cells were sorted by flow cytometry directly in Trizol Reagent (Gibco-BRL), vortexed briefly and stored at –80°C. Typically, this procedure yielded 2000 to 10,000 GFP$^+$ cells per embryo.

Linear amplification and cDNA microarray analysis

Total RNA preparation, linear amplification and cDNA microarray analysis procedures were performed as described (Bouchard et al., 2005; Hoffmann et al., 2003). Linear amplification was performed on material from one or two embryos of identical somite-stage corresponding to a minimum of 5000 cells.

In situ hybridization

Embryo isolation and in situ hybridization using digoxigenin-labeled RNA probes was performed as described (Henrique et al., 1995). Hybridization was carried out with probes for *Gata3* (George et al., 1994), *Pax2* and *Pax8* (Bouchard et al., 2002), *Ret* (Pachnis et al., 1993), *Wnt11* (Majumdar et al., 2003), *Wt1* (Buckler et al., 1991), *Brn1* (Bouchard et al., 2005), and *Emx2* (Yoshida et al., 1997).

Immunohistochemistry and TUNEL staining

Embryos were dissected and processed for immunohistochemistry as described (Bouchard et al., 2000). The following antibodies were used: rabbit anti-Pax2 (1:200; Covance), mouse anti-Wt1 (1: 150, Dako), rat anti-E-cadherin (1:400, Zymed Laboratories), rabbit anti-GFP (1:1000, Abcam) and rabbit anti-phospho-H3 (1:200, Upstate Biotechnology). Secondary detection was performed using anti-mouse, anti-rat or anti-rabbit secondary antibodies labeled with Alexa488 or Alexa568 (1:200, Molecular Probes). TUNEL assay was performed using the In Situ Cell Death Detection Kit according to manufacturer's instruction (Roche). Counterstaining was obtained with DAPI at 50 μg/ml in SlowFade Light mounting medium (Molecular Probes).

Confocal imaging

Confocal analysis of the developing mesonephros was performed on dissected trunks of $Pax2^{GFP}Gata3^{+/+}$ and $Pax2^{GFP}Gata3^{-/-}$ embryos at E9.5. The samples were fixed for 20 minutes in 4% paraformaldehyde, washed in PBS, equilibrated in Slowfade buffer and finally mounted in Slowfade light reagent (Molecular Probes). The GFP emission signal was detected on a Zeiss LSM510 confocal microscope.

RESULTS

Regulation of *Gata3* expression by Pax2 and Pax8 in early kidney development

To identify important molecules regulated by Pax genes during pro/mesonephros development, we used a novel procedure (Bouchard et al., 2005) to isolate *Pax2*-expressing mesonephric cells from wild-type and $Pax2^{-/-}$ embryos for subsequent microarray analysis. For this purpose, a BAC transgene expressing *GFP* from the *Pax2* locus ($Pax2^{GFP}$) (Pfeffer et al., 2002) was used to label pro/mesonephric cells, which were purified by FACS sorting from the remaining cells of the dissected trunk regions of wild-type or *Pax2* mutant embryos at 13-, 16- and 20-somite stages. Importantly, these stages correspond to the early period of pro/mesonephros formation, prior to the tissue degeneration observed in *Pax2* mutant embryos (Bouchard, 2004; Bouchard et al., 2002; Torres et al., 1995). Sorted GFP$^+$ cells (2000 to 10,000 cells per embryo) and control GFP$^-$ cells were subjected to linear RNA amplification and microarray analysis. Pax2-regulated genes were identified as transcripts: (1) that were differentially regulated by Pax2 in the pro/mesonephros at all three stages; (2) that were upregulated between 13 to 16 somites and 13 to 20 somites in the pro/mesonephros; and (3) that were expressed in the pro/mesonephros but not in the surrounding GFP$^-$ tissue at the 13-

Table 1. Identification of *Gata3* as a Pax2-regulated gene by microarray analysis of pro/mesonephric cells

	Genotype comparison [wild type versus Pax2$^{-/-}$ GFP (+) cells]			Upregulation in time [GFP (+) cells]		Pro/mesonephros expression [GFP (+) versus GFP (–) cells]	
	13 s	16 s	20 s	13 s versus 16 s	13 s versus 20 s	13 s	20s
Gata3	1.7	2.1	1.7	1.7	1.8	2.6	4.5
β2-microglobulin	1.1	0.9	1.0	1.0	1.2	1.0	1.0

Ratios are expressed as control/mutant, late/early or GFP$^+$/GFP$^-$. The embryonic stages are defined by somite number (s).

and 20-somite stages (Table 1). By applying a stringent cut-off of ⩾1.7-fold activation in all seven microarray experiments, we identified a single Pax2-regulated transcript out of 26,000 expressed sequence tags (ESTs corresponding to 17,000 UniGene clusters; Table 1). This EST (BG080090) corresponds to a cDNA transcript of the *Gata3* gene (UniGene cluster Mm.313866). By contrast, the control house-keeping gene β2-microglobulin failed to show differential expression in any of the seven microarray experiments (Table 1).

We next analyzed the expression pattern of *Gata3* by in situ hybridization of wild-type embryos to investigate whether this gene is expressed within the known *Pax2* and *Pax8* expression domains during kidney development. In accordance with our microarray data, we detected strong *Gata3* expression in the pronephros anlage at the 10-somite stage (E8.5; Fig. 1A). This early onset of expression identifies *Gata3* as the earliest known marker gene of pronephros development that follows *Pax2* and *Pax8* expression (Bouchard et al., 2002). At E9.5, *Gata3* was detected only in the nephric duct of the mesonephros (Fig. 1B); by E13.5 to E18.5, *Gata3* mRNA was present in the ureter tips and collecting duct system of the metanephros, but was absent from the developing nephrons (Fig. 1C,D). This expression pattern is consistent with, and extends, previously published data (George et al., 1994; Manaia et al., 2000). Importantly, the expression domains of *Gata3* in the nephric duct and ureter correspond to a subset of the *Pax2/Pax8*-expressing cells (Bouchard et al., 2002; Dressler et al., 1990) (Fig. 2A,C), suggesting that *Gata3* is regulated by Pax2 and Pax8.

To directly investigate this possibility, we examined the expression of *Gata3* in embryos of a *Pax2,Pax8* mutant allelic series. Whereas *Pax2*$^{-/-}$*Pax8*$^{-/-}$ embryos fail to specify the nephric lineage, *Pax8* expression is still observed in the developing kidney of *Pax2*$^{-/-}$*Pax8*$^{+/-}$ embryos, indicating that one functional *Pax8* allele is sufficient to induce pro/mesonephros development (Bouchard et al., 2002) (Fig. 2D). In situ hybridization analysis of tissue sections revealed that *Gata3* was similarly expressed in wild-type and *Pax2*$^{-/-}$ embryos (Fig. 1A, data not shown). By contrast, *Gata3* expression was virtually undetectable in the *Pax2*$^{-/-}$*Pax8*$^{+/-}$ mesonephros (Fig. 1B), demonstrating that Pax2 and Pax8 together regulate the *Gata3* gene. Some weak *Gata3* expression could be detected in the most rostral region of the mesonephros (data not shown), which is probably due to increased Pax8 expression in this region (Bouchard et al., 2002). In support of *Gata3* regulation by Pax2 and Pax8 proteins, two genomic elements conserved in the mouse, human and chick *Gata3* region were found to contain Pax2/5/8 consensus binding sites (data not shown). Together, these data identify *Gata3* as a Pax2/8-regulated gene that is already expressed at the onset of pro/mesonephros development.

Gata3 is necessary for mesonephros formation

The results presented above suggest that *Gata3* may be an important effector of Pax gene function during pro/mesonephros development. To investigate this possibility, we inactivated the *Gata3* locus in embryonic stem (ES) cells by deletion of exon 4, coding for the first DNA-binding zinc finger, and by simultaneous insertion of an *Ires-GFP* minigene (Fig. 3A). Heterozygous *Gata3*$^{GFP/+}$ mice express

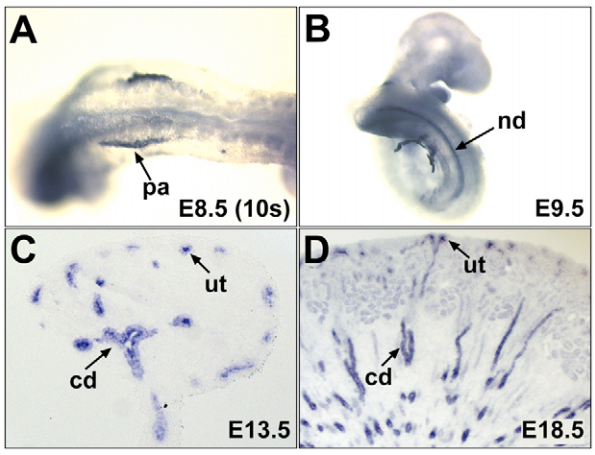

Fig. 1. *Gata3* expression in wild-type nephric tissues. A *Gata3* cRNA probe was used for in situ hybridization on whole-mount embryos (**A**,**B**) or metanephros sections (**C**,**D**) at the indicated stages. *Gata3* expression is detected in the pronephros anlage (pa; A), in the nephric duct (nd) of the mesonephros (B), and in the collecting duct (cd) and ureter tip (ut) of the metanephros (C,D).

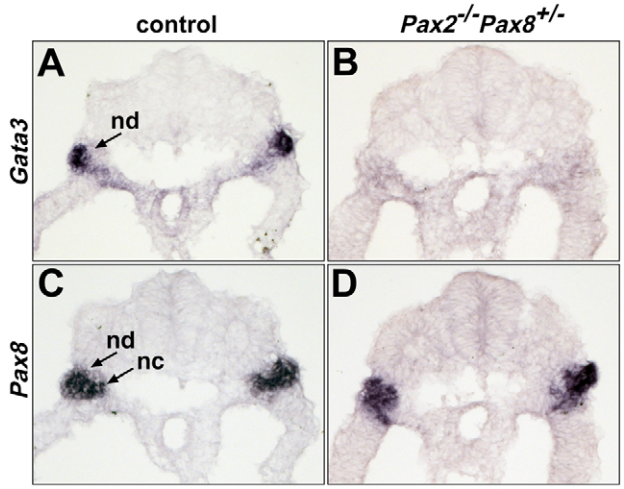

Fig. 2. Regulation of *Gata3* by Pax transcription factors in the pro/mesonephros. Wild-type (A,C) and *Pax2*$^{-/-}$*Pax8*$^{+/-}$ (B,D) embryos were analyzed at the 15-somite stage by in situ hybridization of *Gata3* (**A**,**B**) and *Pax8* (**C**,**D**) cRNA probes on adjacent sections. *Gata3* expression is specifically lost in the *Pax2*$^{-/-}$*Pax8*$^{+/-}$ nephric duct. nc, nephric cord; nd, nephric duct.

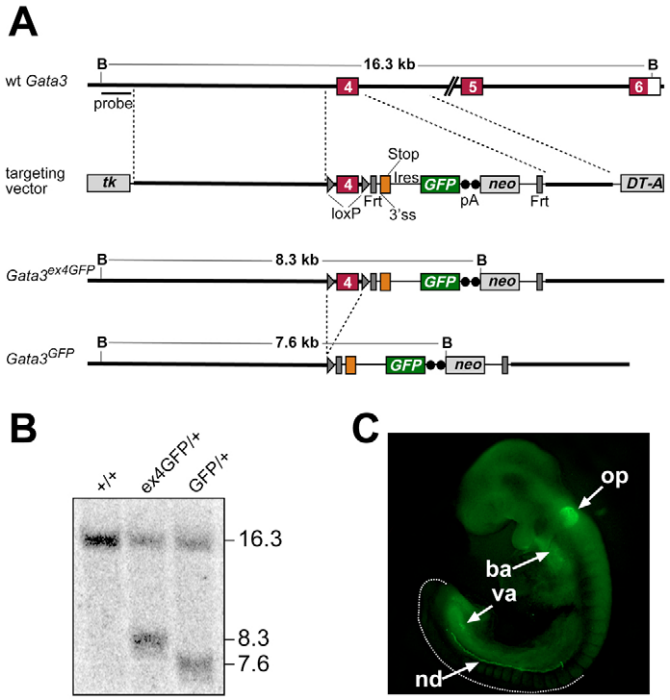

Fig. 3. Inactivation of the *Gata3* gene. (**A**) The *Gata3* gene was targeted by insertion of *loxP* sites (grey triangles) flanking exon 4 (encoding the first zinc finger), followed by a cassette, flanked by *frt* sites (grey rectangles), containing a translation stop codon, an internal ribosome entry site (Ires) driving the *GFP* gene, and the neomycin selection gene (*neo*). The *GFP* and *neo* genes were followed by SV40 polyadenylation signals. The targeting construct additionally contained the thymidine kinase (*tk*) and dyphteria toxin A (*DT-A*) genes at the 5′ and 3′ ends, respectively, for selection against random genomic integration. Mice with the original targeted allele (*Gata3*^{ex4GFP}) were mated with the *More-cre* germline deleter strain (Tallquist and Soriano, 2000) to excise exon 4 and generate the *Gata3*^{GFP} allele (referred to as *Gata3*$^{-}$ allele). The different alleles were detected by Southern blot analysis of *Bam*HI-digested genomic DNA with the indicated probe. The exons are numbered according to Pandolfi et al. (Pandolfi et al., 1995). (**B**) Southern blot analysis of wild-type (+/+), *Gata3*^{ex4GFP} (ex4GFP/+) and *Gata3*^{GFP} (GFP/+) tail DNA digested with BamHI. (**C**) GFP expression of the targeted *Gata3* gene in *Gata3*$^{GFP/+}$ embryo at E9.5. Broken line indicates the contour of the embryo. ba, branchial arches; op, otic placode; nd, nephric duct; va, vitelline artery.

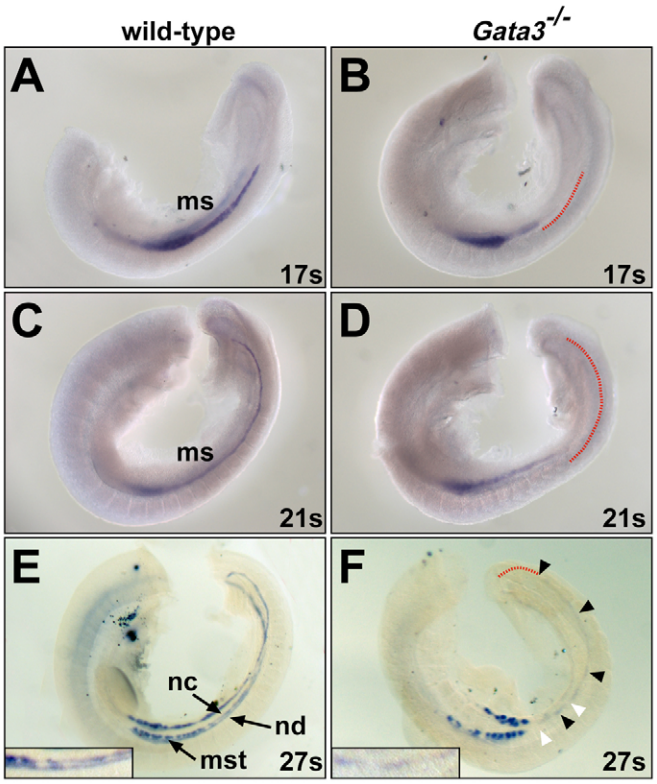

Fig. 4. Mesonephric defects in *Gata3*$^{-/-}$ embryos. *Pax2* expression was detected in wild-type (A,C,E) or *Gata3*$^{-/-}$ (B,D,F) embryos at 17 somites (A,B), 21 somites (C,D) and 27 somites (E,F). (**A**-**D**) A delay is observed in the caudal extension of *Pax2*$^{+}$ mesonephric cells (broken red lines in B,D,F) of *Gata3*$^{-/-}$ embryos. (**E**,**F**). The distinct Pax2 expression domains in the nephric cord and duct of wild-type embryos (insert in E) were replaced by a single stream of *Pax2*$^{+}$ cells (black arrowheads, insert in F) with regions of discontinuity (white arrowheads) in *Gata3*$^{-/-}$ embryos. ms, mesonephros; mst, mesonephric tubules; nc, nephric cord; nd, nephric duct.

GFP in all endogenous *Gata3* expression domains, including the nephric duct of the pro/mesonephros (Fig. 3C). Homozygous *Gata3*$^{GFP/GFP}$ embryos died around E11.0 (data not shown), consistent with previously published *Gata3* mutant mice (Lim et al., 2000; Pandolfi et al., 1995). Hence, the *Gata3*^{GFP} allele corresponds to a null allele, which we subsequently refer to as *Gata3*$^{-}$.

To study the role of *Gata3* in the kidney, we initially looked at the progression of mesonephros development in *Gata3* mutant embryos at E9.0-E9.5 by whole-mount in situ hybridization with a *Pax2* probe. At the 17-somite stage, the mesonephros of wild-type embryos extends over 12 somites (Fig. 4A). At the same stage, the mesonephros of *Gata3*$^{-/-}$ embryos showed, however, a marked delay in caudal extension (Fig. 4A,B), which became even more prominent at the 21-somite stage (Fig. 4C,D). By 27 somites, the wild-type nephric duct has reached the cloaca, while a second row of *Pax2*-positive cells was apparent in the adjacent nephric cord (Fig. 4E, insert). In *Gata3*$^{-/-}$ embryos, a single and discontinuous row of *Pax2*-positive cells extended caudally, which eventually reached the tip of the tail (Fig. 4F, insert). Hence, the absence of *Gata3* causes a severe growth defect of the mesonephros.

We next determined the cellular identity of the *Pax2*-positive cells observed in the caudal *Gata3*$^{-/-}$ mesonephros. In wild-type E9.5 embryos, Pax2 is expressed in cells of both the nephric duct and cord. E-cadherin expression is, however, restricted to the nephric duct, whereas Wt1 expression is excluded from the duct, but is present in the intermediate mesoderm (including cord cells; Fig. 5A,C). Strikingly, E-cadherin^{+} nephric duct cells were absent in *Gata3*$^{-/-}$ embryos, while the caudal mesonephric cells were located exclusively in the Pax2^{+} Wt1^{+} nephric cord (Fig. 5B,D). The loss of the nephric duct was further confirmed by the absence of laminin staining in this region of E9.5 *Gata3*$^{-/-}$ embryos (data not shown). The absence of the nephric duct led to increased apoptosis in the nephric cord (Fig. 5E,F). This cell death may be responsible for the discontinuity in *Pax2* expression, which is observed in *Gata3*$^{-/-}$ embryos at 27 somites (Fig. 4E,F). By E10.5, the nephric duct and ureteric bud were missing at the level of the metanephric mesenchyme in *Gata3*$^{-/-}$ embryos, as revealed by the absence of expression of *Pax2* and the epithelial marker genes *Emx2*, *Brn1* (*Pou3f3* – Mouse Genome Informatics) and *Sim1* (Fig. 5G,H,I,J, data not shown). Owing to the absence of a caudal nephric duct,

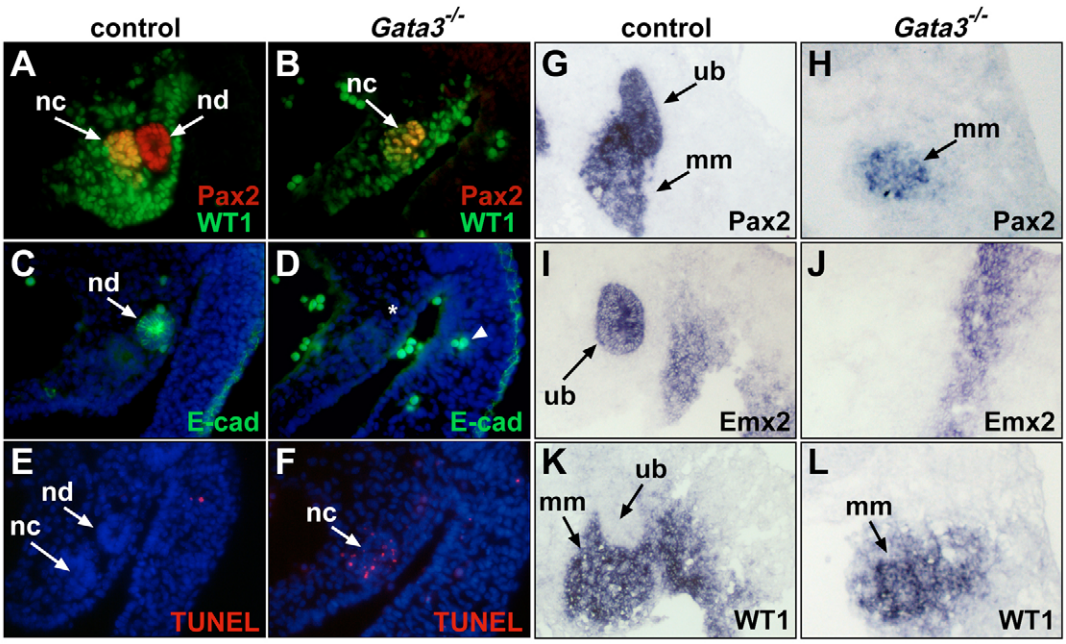

Fig. 5. Nephric cord extension in *Gata3*$^{-/-}$ embryos. Control *Gata3*$^{+/+}$ or *Gata3*$^{+/-}$ embryos (A,C,E,G,I,K) and *Gata3*$^{-/-}$ embryos (B,D,F,H,J,L) were analyzed for marker expression on sections of caudal E9.75 mesonephros (A-F) and E10.5 metanephros (G-L). (**A**,**B**) Pax2 (red) and Wt1 (green) antibody staining reveals the presence of Pax2$^+$ Wt1$^+$ nephric cord cells (nc; yellow), but an absence of Pax2$^+$ Wt1$^-$ nephric duct cells in *Gata3*$^{-/-}$ embryos. (**C**,**D**) Immunostaining of the epithelial marker E-cadherin (green) further reveals the absence of a nephric duct in *Gata3*$^{-/-}$ embryos (asterisk). Some non-specific signal caused by blood cell autofluorescence is apparent and reflects Gata3-dependent hemorrhages (white arrowhead) (Pandolfi et al., 1995). (**E**,**F**) TUNEL signals (red) indicated an increase in apoptosis in *Gata3*$^{-/-}$ nephric cord cells. Sections C-F were counterstained with DAPI (blue). (**G**,**H**) In situ hybridization with a *Pax2* cRNA probe showed the presence of reduced levels of *Pax2* expression in the metanephric mesenchyme (mm) in the absence of a ureteric bud (ub). (**I**,**J**) The lack of a ureteric bud at the hindlimb level of *Gata3*$^{-/-}$ embryos was confirmed by the absence of *Emx2* mRNA expression. *Emx2* expression in surrounding tissues was unaffected. (**K**,**L**) *Wt1* mRNA expression in the metanephric mesenchyme is still detected in *Gata3*$^{-/-}$ embryos.

metanephros induction could never be detected in these embryos (Fig. 5G-L, data not shown). Surprisingly, however, cells expressing *Wt1*, *Eya1* and *Pax2* were still detected in the region of the metanephric mesenchyme, indicating that the expression of these genes does not depend on inductive signals from the nephric duct (Fig. 5G,H,K,L, data not shown). We therefore conclude that *Gata3* is critically important for the caudal extension of the nephric duct but not of nephric cord cells.

Ectopic formation and aberrant guidance of the nephric duct in *Gata3*$^{-/-}$ embryos

We next followed nephric duct formation in *Gata3* mutant embryos at E9.0-E9.5 by *Pax2* whole-mount in situ hybridization and confocal imaging. In wild-type embryos, the nephric duct was visible as a single tube extending towards the tail region, with the mesonephric tubules forming in the adjacent nephric cord (Fig. 6A,I). By contrast, *Gata3*$^{-/-}$ embryos consistently showed abnormal pro/mesonephros morphogenesis starting at E9.25, when swellings of the nephric duct were observed (Fig. 6B). By E9.5 to E10.5, most *Gata3*$^{-/-}$ mesonephros (~60%, *n*=52) contained clusters of multiple disorganized nephric ducts, which were located in the intermediate mesoderm adjacent to the mesonephric tubules (Fig. 6J). In these embryos, the ectopic ducts grew in the direction of the surface ectoderm (Fig. 6A-D,I,J; data not shown). This result was confirmed by immunohistochemistry with an anti-E-cadherin antibody, which stained the duct but not the mesonephric tubules at this stage. In contrast to the single epithelial duct observed in wild-type embryos (Fig. 6E), *Gata3*$^{-/-}$ embryos harbored a large cluster of E-cadherin$^+$ ductal structures (Fig. 6F, data not shown). These E-cadherin$^+$ cells also expressed the GFP protein from the targeted *Gata3* locus, further confirming their nephric duct origin (Fig. 6G,H). Interestingly, in about 65% of *Gata3*$^{-/-}$ mesonephros (*n*=52), the nephric duct initiated caudal extension but moved away from the nephric cord into an aberrant elongation path (Fig. 6K-N). As for the multiple disorganized ducts (Fig. 6C-J), all misguided ducts in these embryos extended to and fused with the surface ectoderm, as revealed by the expression of the epithelial marker gene *Brn1* (Fig. 6O,P). Importantly, despite the observed variability in mesonephros dysgenesis, *Gata3*$^{-/-}$ nephric ducts invariably failed to reach the cloaca region. Together, these results unequivocally demonstrate an important role for *Gata3* in controlling the morphogenesis and guidance of the nephric duct.

Altered proliferation and signaling in the nephric duct of *Gata3* mutant embryos

The massive increase in nephric duct tissue suggested a possible role for Gata3 in cell proliferation control. To test this hypothesis, we determined the cellularity and mitotic index of the E-cadherin$^+$ nephric duct cells in wild-type and *Gata3*$^{-/-}$ embryos. The mitotic index was determined as the ratio of phospho-histone H3 signals per 50 nephric duct cells (E-cadherin$^+$). These analyses revealed a 2.6-fold increase in the number of nephric duct cells and a 4.6-fold increase in cell proliferation in the mesonephros of *Gata3*$^{-/-}$ embryos compared with wild-type controls (Table 2, Fig. 7A,B).

Table 2. Cellularity and mitotic index of control and *Gata3*$^{-/-}$ nephric ducts

	Average nd cells per section (s.d.)	Average mitotic cells per 50 duct cells (s.d.)
Control (*n*=4)	11* (3.5)	0.7** (0.7)
Gata3$^{-/-}$ (*n*=6)	29* (7.0)	3.1** (1.3)

*P<0.002, **P<0.01 (Student's *t*-test).
Nephric duct cells are defined by E-cadherin staining.
s.d., standard deviation.

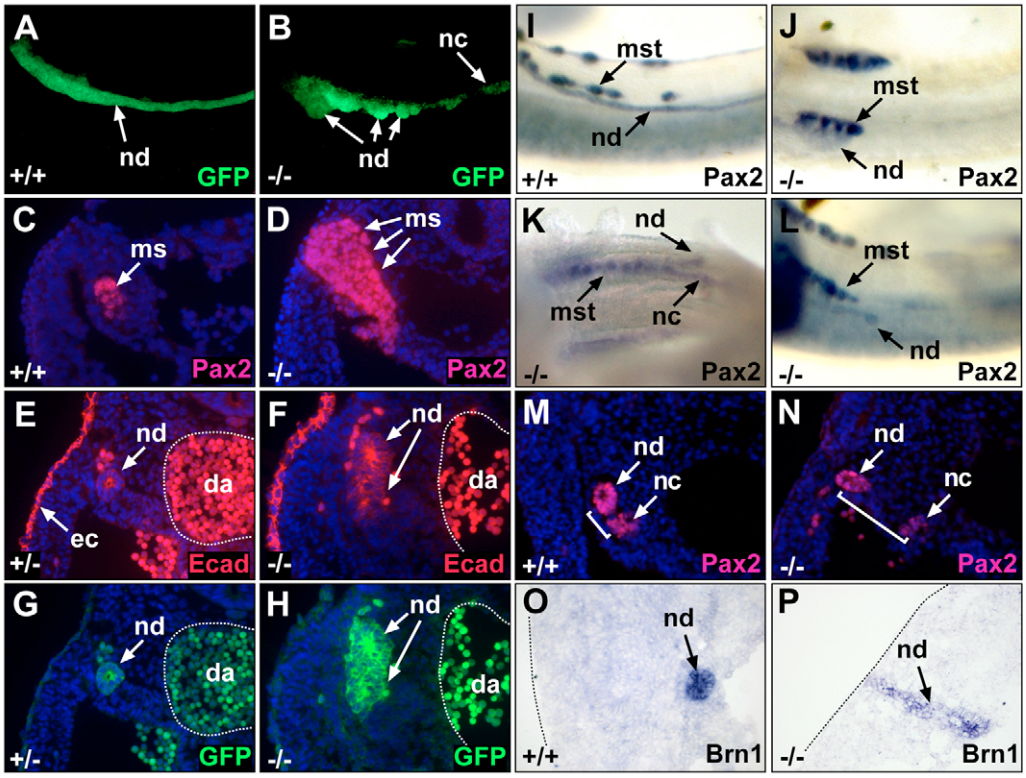

Fig. 6. Ectopic tubulogenesis and guidance defects in the *Gata3*$^{-/-}$ mesonephros. Control *Gata3*$^{+/+}$ (+/+) or *Gata3*$^{+/-}$ (+/–) embryos (A,C,E,G,I,M,O) and *Gata3*$^{-/-}$ (–/–) (B,D,F,H,J,K,L,N,P) embryos were analyzed for nephric duct defects at E9.25 (A,B,K), E9.5 (C-J,L-N) and E10.5 (O,P). (**A**,**B**) Confocal analysis of GFP expression from the *Pax2*^{GFP} BAC transgene shows the initiation of aberrant nephric duct morphogenesis. (**C**,**D**) Extreme case of mesonephros hypercellularity revealed by immunohistochemistry with an anti-Pax2 antibody. (**E**,**F**) *Gata3*$^{-/-}$ ectopic ducts expressed the nephric duct marker E-cadherin. (**G**,**H**) Same section as in E,F, showing the colocalization of E-cadherin with GFP expressed from the targeted *Gata3* locus. (**I**,**J**) Whole-mount in situ hybridization with a *Pax2* cRNA probe reveals the presence of multiple nephric ducts (nd) growing towards the ectoderm at the level of the mesonephric tubules (mst) in *Gata3*$^{-/-}$ embryos. (**K**,**L**) *Gata3*$^{-/-}$ embryos in which a nephric duct grew caudally showed a guidance defect, as revealed by whole-mount in situ hybridization with a *Pax2* cRNA probe. (**M**,**N**) Immunohistochemistry with an anti-Pax2 antibody showed an abnormally large distance between the nephric duct and nephric cord in *Gata3*$^{-/-}$ embryos. (**O**,**P**) *Brn1* cRNA in situ staining of a representative E10.5 embryo in which the duct turned abruptly in the direction of the surface ectoderm (broken line) and fused with it. da, dorsal aorta; ec, ectoderm; ms, mesonephros; mst, mesonephric tubules; nc, nephric cord; nd, nephric duct.

These results clearly implicate Gata3 in the regulation of mesonephric cell proliferation. The ectopic nephric structure also showed a mild increase in apoptosis, possibly reflecting the abnormal environment of these cells (data not shown).

We next investigated the molecular basis for the nephric duct elongation defect of *Gata3*$^{-/-}$ embryos. Several mechanisms have been implicated in duct guidance during embryogenesis. These include extracellular matrix attachment, axon-like guidance and morphogen signaling (Drawbridge et al., 2003; Eichmann et al., 2005; Myat, 2005). We thus studied the composition of the extracellular matrix and expression of integrin receptors during mesonephros development by immunostaining for pan-laminin, laminin B2 (γ1), β1 integrin, α3 integrin, α6 integrin and α5β1 integrin (fibronectin receptor) expression. Surprisingly, none of the proteins showed any significant difference in expression between *Gata3*$^{-/-}$ and wild-type embryos (data not shown). We then investigated the expression of axon guidance molecules known to be present in the kidney. These included Gdnf, Ret, Slit2, neuropilin, ephrins and Eph proteins. Although most of these molecules were normally expressed in the *Gata3*$^{-/-}$ mesonephros (data not shown), transcripts of the Gdnf receptor gene *Ret* were undetectable in the nephric duct of *Gata3*$^{-/-}$ embryos in contrast to control embryos (Fig. 7C,D). *Gdnf* itself remained, however, expressed in the mesenchyme adjacent to the *Gata3*$^{-/-}$ duct (data not shown). Importantly, *Wnt11* expression, which is regulated by Ret signaling (Majumdar et al., 2003; Grieshammer et al., 2004), was also lost in the *Gata3*$^{-/-}$ nephric duct (Fig. 7E,F). Hence, these data indicate that Gata3 controls Ret expression and signaling in the nephric duct.

DISCUSSION

The pro/mesonephros and its major constituent, the nephric duct, are central components of both kidney and genital system development (Capel, 2000; Saxen, 1987). The molecular mechanisms regulating nephric duct formation are, however, largely unknown. We have previously shown that the transcription factors Pax2 and Pax8 are both necessary and sufficient for the initiation of nephric duct formation by facilitating epithelial-mesenchymal transitions in the intermediate mesoderm (Bouchard et al., 2002). To date, no other mouse mutant was shown to regulate the early onset of pro/mesonephros morphogenesis, while most gene mutations with a strong kidney phenotype affect the later process of ureteric bud formation and subsequent metanephros development (Kuure et al., 2000; Bouchard, 2004). Here, we have used FACS sorting and cDNA microarray analysis of mesonephric cells combined with expression validation in wild-type and *Pax2,Pax8* mutant embryos for the identification of genes that are regulated by the two Pax proteins during pro/mesonephros formation. These experiments identified the transcription factor gene *Gata3* as the earliest known genetic target of Pax proteins in kidney development. Gene inactivation in the mouse revealed a crucial role for Gata3 in controlling nephric duct morphogenesis and guidance. The nephric ducts of *Gata3*$^{-/-}$ embryos are hypercellular and fail to elongate along the normal rostrocaudal path, thereby preventing metanephros induction. These defects may partly reflect the role of Gata3 in nephric duct cell proliferation and activation of the receptor gene *Ret*, which is an essential component of the GDNF signaling pathway involved in ureteric bud formation and nephric duct

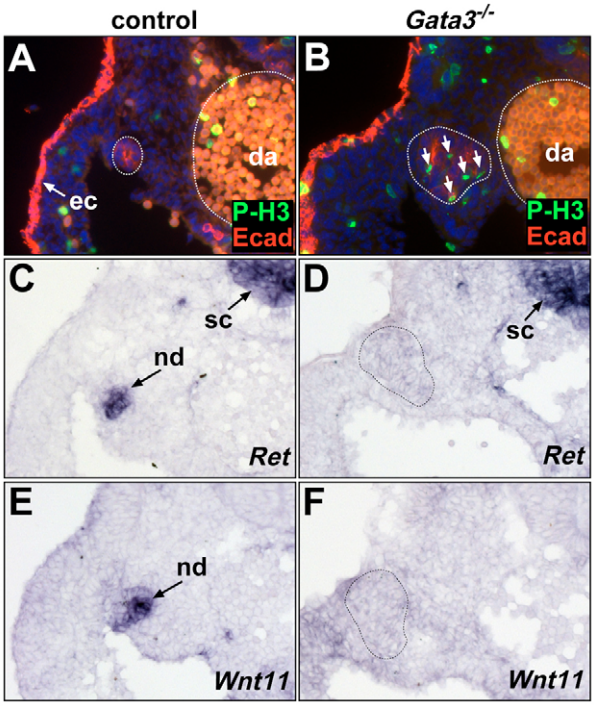

Fig. 7. Regulation of cell proliferation and gene expression by Gata3. Control wild-type or *Gata3*$^{+/-}$ embryos (A,C,E) and *Gata3*$^{-/-}$ embryos (B,D,F) were analyzed by immunohistochemistry with anti-phosphorylated-histone H3 and anti-E-cadherin antibodies (A,B) and by in situ hybridization with *Ret* (C,D) and *Wnt11* (E,F) cRNA probes. (**A**,**B**) The number of mitotic cells (white arrows) and the cellularity (broken lines) of the nephric duct were significantly higher in *Gata3*$^{-/-}$ compared with control embryos. Sections A,B were counterstained with DAPI (blue) (**C**,**D**) *Ret* expression was absent from *Gata3*$^{-/-}$ hypercellular nephric ducts. Strong *Ret* expression is still present in the *Gata3*$^{-/-}$ spinal cord (sc). (**E**,**F**) Expression of the Ret-regulated *Wnt11* gene was completely lost in *Gata3*$^{-/-}$ nephric ducts in contrast to control embryos. Broken lines in A,B,D,F demarcate the epithelial nephric duct, as defined by E-cadherin staining. da, dorsal aorta; ec, ectoderm; nd, nephric duct; sc, spinal cord.

guidance (Schuchardt et al., 1994; Drawbridge et al., 2000). These findings establish a transcriptional cascade whereby Pax proteins activate *Gata3* to regulate specific aspects of the pro/mesonephros developmental program.

The *Gata3* mutation causes two major defects in pro/mesonephros development. First, *Gata3*$^{-/-}$ embryos fail to form as a single nephric duct, but instead generate multiple disorganized ducts that accumulate in the intermediate mesoderm and frequently extend to the surface ectoderm. The molecular explanation for this phenotype is still unclear. One possibility is that the number of progenitors committed to the nephric lineage is increased in *Gata3*$^{-/-}$ embryos, resulting in the simultaneous formation of multiple pronephric ducts. Alternatively, the number of pronephric progenitors remains normal in the mutant, but the subsequent differentiation of these cells is defective. Finally, the initial specification and differentiation events are unaffected but the steps of tubulogenesis and cell proliferation are disturbed. We favor the last hypothesis based on the following evidence: (1) Pax2 and Pax8 regulate *Gata3* in a cell-autonomous manner and yet their mutation leads to a loss rather than an increase in nephric progenitors (Bouchard et al., 2002); (2) we could not detect any obvious increase in the cellularity or ectopic locations of the pronephros in the early *Gata3*$^{-/-}$ embryo; (3) *Gata3*$^{-/-}$ nephric duct cells showed normal expression of a number of cell fate and differentiation markers such as *Pax2*, *Pax8*, *Brn1*, *Wnt9b*, *Emx2*, *Sim1*, E-cadherin, laminin and various integrins; (4) the increased mitotic index observed in *Gata3*$^{-/-}$ mesonephros points to a defect in cell cycle control. In support of this notion, *Gata3* was recently shown to control the proliferation of epithelial cells and *Gata3* gene mutations were identified in breast tumors (Usary et al., 2004).

The second major aspect of the *Gata3*$^{-/-}$ phenotype is a defect in rostrocaudal guidance of the nephric duct. Most experiments addressing nephric duct guidance were performed in the amphibian Axolotl (*Ambystoma mexicanum*), which develops a functional pronephros and is amenable to classical tissue recombination experiments. Axolotl is an interesting model system as its pronephros shares a number of features with the mouse pro/mesonephros (Drawbridge et al., 2003). One of the guidance mechanisms identified in Axolotl depends on the deposition of an ectoderm-derived extracellular matrix containing laminin 1, which is recognized by the α6β1 integrin receptor of nephric duct cells (Drawbridge et al., 1995; Morris et al., 2003). However, we did not observe any significant difference in the expression of laminins and integrin receptors (α6β1, α3, α5β1) between wild-type and *Gata3*$^{-/-}$ embryos (data not shown). Hence, Gata3 is unlikely to control nephric duct guidance by regulating the expression of extracellular matrix components and integrins.

Another duct guidance cue identified in Axolotl is GDNF, which activates signaling through the dimeric GFRα1-Ret receptor (Drawbridge et al., 2000). GDNF-soaked beads were sufficient to attract the elongating duct in 81% of experimental animals, while subepidermal injection of GDNF inhibited duct extension, indicating that the duct cells move along a gradient of GDNF expression (Drawbridge et al., 2000). A similar GDNF gradient was recently identified in the mouse mesonephros, suggesting that GDNF is also a guidance cue for nephric duct extension in mammals (Grieshammer et al., 2004; Kume et al., 2000). Interestingly, GDNF signaling was also shown to act as a chemoattractant for kidney epithelial cells (Tang et al., 1998) and to have axon guidance properties in the CNS (Ledda et al., 2002). Consistent with these data, the loss of Ret expression and signaling may contribute to the misguidance of the nephric duct in *Gata3*$^{-/-}$ embryos. It is, however, important to note that the inactivation of components of the GDNF signaling pathway did not result in duct guidance defects in the mouse (Pichel et al., 1996; Schuchardt et al., 1994). Some redundancy in the GDNF signaling pathway may, however, exist as the loss of metanephros development was not fully penetrant in *Gdnf*, *Gfrα* or *Ret* mutant mice (Cacalano et al., 1998; Enomoto et al., 1998; Moore et al., 1996; Sanchez et al., 1996; Schuchardt et al., 1994). In addition to positive guidance cues, our observation that *Gata3*$^{-/-}$ nephric ducts invariably grew in the direction of the surface ectoderm reveals the presence of an attraction signal emanating from this tissue. In wild-type embryos, Gata3 would counteract this unknown signal, thereby allowing the nephric duct to follow its normal path. Taken together, the combination of positive and negative cues directing nephric duct elongation is highly reminiscent of axon guidance mechanisms and suggests that both processes may share similar molecular mechanisms.

Surprisingly, the Pax2^{+} E-cadherin^{-} nephric cord cells of *Gata3*$^{-/-}$ embryos still extended through the intermediate mesoderm along the entire rostrocaudal axis even in the absence of an elongating duct. The nephric cord cells do not express *Gata3* (Manaia et al., 2000) (this study) and were thought to follow the nephric duct by receiving

guidance cues and differentiation signals from the duct (Saxen, 1987). Our results unequivocally demonstrate that the two major cell populations of the mesonephros use independent guidance systems for caudal extension, although the mechanism underlying nephric cord formation is still elusive.

Expression of the *Pax2* gene was previously observed in the uninduced metanephric mesenchyme of *Ret* mutant embryos, which develop a nephric duct in the absence of ureteric bud formation (Brophy et al., 2001). However, it was still conceivable that the nephric duct could induce *Pax2* expression in the adjacent metanephric mesenchyme. The presence of *Pax2*$^{+}$ mesenchymal cells in *Gata3*$^{-/-}$ embryos demonstrates that metanephric mesenchyme cells can express *Pax2* in a ureteric bud- and nephric duct-independent manner. Hence, these Pax2^{+} cells are likely to originate from the early pro/mesonephros by caudal migration.

Transcriptional control of pro/mesonephros development

By identifying *Gata3* as a Pax2/8-regulated gene, we provide the first insight into the transcriptional cascade controlling early pro/mesonephros development. Our data do not allow a discrimination between direct or indirect regulation of *Gata3* by the two Pax proteins. Direct regulation would, however, be consistent with the fact that *Gata3* expression is activated in the pronephros anlage soon after the initiation of *Pax2* and *Pax8* transcription. A urogenital-specific regulatory element has been mapped to a region located between –35 and –150 kb upstream of the start codon of *Gata3* (Lakshmanan et al., 1999). Interestingly, a bioinformatic analysis of 185 kb *Gata3* genomic region revealed two elements containing putative Pax2/5/8-binding sites, which are conserved in mouse, human and chick. Functional analysis will be required to demonstrate that these conserved elements are involved in the Pax2/8-dependent regulation of *Gata3*.

In contrast to *Pax2*$^{-/-}$*Pax8*$^{-/-}$ embryos in which the whole nephric lineage fails to be specified, *Gata3*$^{-/-}$ embryos are competent to undergo mesenchymal-epithelial transitions to form the nephric duct, which in turn is competent to induce mesonephric tubule formation in the surrounding mesenchyme. On the one hand, these phenotypic differences indicate that *Pax* genes regulate other aspects of pro/mesonephros development, independently of *Gata3* regulation. Indeed, additional Pax2-regulated genes were identified by less stringent analysis of our microarray data (M.B., unpublished). On the other hand, a search for Gata3 target genes may lead to the identification of new molecules involved in the morphogenesis and guidance of the nephric duct.

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