Distal ureter morphogenesis depends on epithelial cell remodeling mediated by vitamin A and Ret

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Almost 1% of human infants are born with urogenital abnormalities, many of which are linked to irregular connections between the distal ureters and the bladder. During development, ureters migrate by an unknown mechanism from their initial integration site in the Wolffian ducts up to the base of the bladder in a process that we call ureter maturation. *Rara*$^{-/-}$ *Rarb2*$^{-/-}$ mice display impaired vitamin A signaling and develop syndromic urogenital malformations similar to those that occur in humans, including renal hypoplasia, hydronephrosis and mega-ureter, abnormalities also seen in mice with mutations in the proto-oncogene *Ret*. Here we show that ureter maturation depends on formation of the 'trigonal wedge', a newly identified epithelial outgrowth from the base of the Wolffian ducts, and that the distal ureter abnormalities seen in *Rara*$^{-/-}$ *Rarb2*$^{-/-}$ and *Ret*$^{-/-}$ mutant mice are probably caused by a failure of this process. Our studies indicate that formation of the trigonal wedge may be essential for correct insertion of the distal ureters into the bladder, and that these events are mediated by the vitamin A and Ret signaling pathways.

Introduction

Urinary-tract abnormalities comprise a complex syndrome of malformations that include some of the most common birth defects in humans[1,2]. In many cases, renal malformations occur in conjunction with incorrectly positioned distal ureters that join the lower urogenital tract outside the normal integration site in the bladder. These abnormal connections are thought to be the cause of several conditions, including vesico-ureteral reflux, mega-ureter and ureteroceles, depending on the site where abnormal termination of the ureter occurs[3–5]. Despite the frequent occurrence of these distal ureter abnormalities, little is known about their cause or about the events that normally control ureter maturation.

Development of the urogenital tract begins with Wolffian ducts, which are paired epithelial tubes that form in both sexes but persist only in males, where they differentiate into the vas deferens, seminal vesicles and epididymis. Wolffian ducts extend along the anterior–posterior embryonic axis, and at embryonic day 9 (E9) integrate into the primitive urogenital sinus—the primordium of the bladder and urethra. The ureteric bud, an epithelial outgrowth that sprouts from the base of the Wolffian ducts, forms at E10. The ureteric bud tips invade the metanephric blastema on E11, where they differentiate into the renal collecting duct system; the portion of the ureteric bud that lies outside the kidney becomes the ureter—the tube that will connect the kidney with the bladder. At this stage, the upper ureter joins the kidney and the lower (distal) ureter is in an immature configuration attached to the Wolffian duct. Mature connections are established during ureter maturation, a poorly understood process in which the distal ureters detach from the Wolffian ducts and migrate up to their final integration site at the base of the bladder.

Studies in rodents have shown that vitamin A is essential for morphogenesis of most fetal tissues[6–9]. During gestation, impaired vitamin A signaling or maternal vitamin A deficiency induces syndromic urogenital-tract malformations similar to those seen in humans[10]. Mammals obtain vitamin A from the diet in an inactive form (retinol) that is transported to fetal tissues and subsequently metabolized to the biologically active form, retinoic acid. Retinoic acid is a highly potent signaling molecule that functions by binding to and activating retinoic acid receptors (Rars), transcription factors that belong to the nuclear receptor superfamily. Mice deficient in two members of the Rar family, Rara and Rarb2 (*Rara*$^{-/-}$ *Rarb2*$^{-/-}$ mutants), develop urinary-tract abnormalities, including renal hypoplasia, incorrectly positioned distal ureters, hydronephrosis and mega-ureter[11]. Previously, we showed that renal hypoplasia in *Rara*$^{-/-}$ *Rarb2*$^{-/-}$ mutants is caused by impaired branching morphogenesis and that vitamin A normally regulates branching morphogenesis through the receptor tyrosine kinase Ret[12], which is required for ureteric bud growth and branching[13,14].

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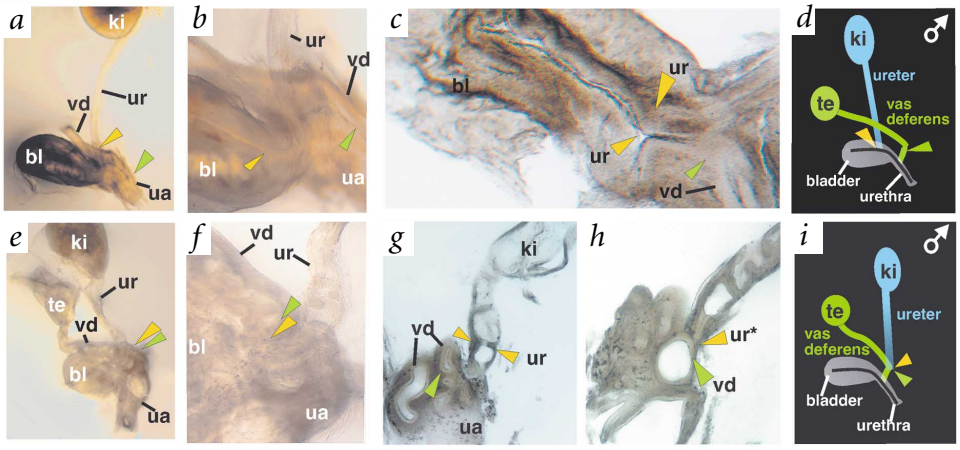

Fig. 1 Distal ureters in *Rara*$^{-/-}$ *Rarb2*$^{-/-}$ mice do not join the bladder. Yellow and green arrowheads indicate the respective positions where the distal ureters and the vas deferens join the lower urinary tract. ***a***, Whole-mount urogenital tract from an E18 wildtype male. ***b***, Higher magnification view of *a*, showing the point where the distal ureters connect to the bladder lumen. ***c***, A vibratome section of an E18 wildtype male, showing the ureters and the vas deferens connecting with the bladder and urethra, respectively. ***d***, Representation of images shown in *a–c*. ***e***, Whole-mount urogenital tract from an E18 *Rara*$^{-/-}$ *Rarb2*$^{-/-}$ male, showing renal hypoplasia, mega-ureter and hydronephrosis. ***f***, Higher magnification view of *e*, showing the ureter joining the vas deferens. ***g,h***, Serial vibratome sections (100 μm) of an E18 *Rara*$^{-/-}$ *Rarb2*$^{-/-}$ male, showing abnormal connections between the dilated ureters and the vas deferens. ***i***, Representation of images shown in *e–h*. Kidney and ureters are shown in blue, bladder and urethra in gray, and testes and vas deferens in green. Black line represents the lumen of the urogenital sinus. bl; bladder; ki, kidney; te, testes; ua, urethra; ur, ureter; vd, vas deferens. Original magnification, ×100 (*a,e,g,h*), ×200 (*b,f,c*).

Here we show that distal ureter maturation also depends on vitamin A and Ret, and that this process occurs in three distinct stages. First, the ureter buds from the Wolffian duct at a site that is rostral to the primitive bladder. Second, the segment of the Wolffian duct lying between the ureter and the bladder, the common nephric duct, disappears, which allows the ureter to come into contact with the primitive bladder. Last, the distal ureter moves to its mature integration site in the bladder by migrating rostrally, dorsally and laterally away from the Wolffian duct, in conjunction with outgrowth of an epithelial wedge that is probably a precursor of the trigone—a muscular component of the bladder thought to be derived from the terminal Wolffian duct. In *Rara*$^{-/-}$ *Rarb2*$^{-/-}$ mice, lateral displacement is impaired and outgrowth of the trigonal wedge is blocked. We show that *Ret* is normally expressed in epithelial cells that form the wedge, and that loss of *Ret* expression in these cells is a probable cause of impaired lateral displacement in *Rara*$^{-/-}$ *Rarb2*$^{-/-}$ mice. Thus, vitamin A and Ret are probably important for remodeling epithelial cells at distinct stages of urogenital-tract formation, by controlling branching morphogenesis in the embryonic kidney and regulating the outgrowth of epithelial cells that form the trigonal wedge during ureter maturation.

Results

Distal ureters end outside the bladder in *Rara*$^{-/-}$ *Rarb2*$^{-/-}$ mice

Analysis of *Rara*$^{-/-}$ *Rarb2*$^{-/-}$ mutants at birth showed that the distal ureters terminated aberrantly in both sexes (Fig. 1). In wildtype males, ureters join the bladder and the vas deferens joins the urethra (Fig. 1*a–d*). In *Rara*$^{-/-}$ *Rarb2*$^{-/-}$ males, however, instead of joining the bladder, dilated ureters were connected directly to the vas deferens, which was also dilated (Fig. 1*e–i*). *Rara*$^{-/-}$ *Rarb2*$^{-/-}$ females showed similar malformations, with the distal ureters joining the uterus or vagina instead of the bladder (ref. 1 and see below). Hydronephrosis and mega-ureter are almost always associated with incorrectly positioned distal ureters in *Rara*$^{-/-}$ *Rarb2*$^{-/-}$ mice, which may be caused by obstruction linked to abnormal connections[11].

Ureter maturation involves remodeling of epithelial cells

In normal development, distal ureters undergo ureter maturation, which involves moving from a primary integration site in the Wolffian duct to the base of the bladder, where mature connections are established. At E18 in *Rara*$^{-/-}$ *Rarb2*$^{-/-}$ mice, ureters were still in an immature configuration, which suggested that some aspect of ureter maturation was abnormal, although little is known about how maturation normally occurs. Therefore, to identify the cause of malformations in *Rara*$^{-/-}$ *Rarb2*$^{-/-}$ mutants, we first characterized ureter maturation in normal embryos. Distal ureter morphogenesis was visualized *in vivo* in *Hoxb7–GFP* mice, a transgenic line that expresses green fluorescent protein in epithelia of the fetal excretory system, including the kidneys, ureters and Wolffian ducts[15].

Ureter maturation preceded sexual differentiation and thus occurred in a similar manner in both sexes. This process could be divided into three stages: ureteric bud formation, vertical displacement and lateral displacement. The primary ureteric bud, an epithelial outgrowth from the base of the Wolffian ducts, formed at E10 (Fig. 2*a–c*). During the next 24 h, ureteric bud tips grew out and invaded the renal mesenchyme, and distal ureters remained attached to the Wolffian ducts. At this stage, the distal ureters were separated from the primitive bladder by a terminal Wolffian duct segment, the common nephric duct (Fig. 2*b–f*). During the next 48 h, vertical displacement occurred. The distal ureters descended, coming into contact with the urogenital sinus, the primordium of the bladder and the urethra (Fig. 2*d–f*). Vertical displacement seemed to be linked to morphogenetic changes in the common nephric duct (the terminal Wolffian duct segment), which was prominent at E12 but barely visible by E13 (Fig. 2*d–f*). It was not apparent from this analysis whether the common nephric duct regressed or was absorbed into the expanding urogenital sinus.

The third stage of ureter maturation, lateral displacement, occurred between E12 and E14 (Fig. 2*g–m*). At this stage, the distal ureters separated from the Wolffian ducts and moved along the urogenital sinus to a position at the base of the bladder. These rearrangements were facilitated by the formation of a prominent epithelial outgrowth at the base of the Wolffian ducts. This 'epithelial wedge' seemed to be formed from the common nephric duct, caudal Wolffian ducts or both, and its continued expansion physically separated the distal ureters from the Wolffian ducts (Fig. 2*g–m*).

We used confocal microscopy to visualize this wedge and its connections to ureters and Wolffian ducts in three dimensions (Web Fig. A online). The wedge was tightly associated with the

urogenital sinus, which was not labeled with GFP and thus was not visible in the microscopy images. The central portion of the wedge was composed of a sheet of cells that were distinct from the tubular epithelia of the adjoining Wolffian ducts and ureters. The ends of this sheet were bordered on the left side by the ureter, with its orifice visible, and on the right side by the Wolffian duct orifice, which was greatly enlarged. The position and timing of this epithelial outgrowth suggest that the wedge may be the primordium of the trigone, a muscular portion of the bladder that is thought to be derived from the terminal Wolffian duct[16–18].

Vitamin A is necessary for lateral displacement

Having established a sequence of events for ureter maturation in wildtype embryos, we examined *Rara*$^{-/-}$ *Rarb2*$^{-/-}$ mice to identify the underlying cause of distal ureter malformations at birth. In some animal models, particularly in those where supernumerary ureters form, distal ureter abnormalities have been linked to ectopic sprouting of the primary ureteric bud branch on the Wolffian duct. At the stage of primary ureteric bud formation, however, we did not observe many differences between *Rara*$^{-/-}$ *Rarb2*$^{-/-}$ embryos and wildtype littermates. In both mutant and wildtype littermates, a single ureteric bud branched from the Wolffian duct bilaterally at the appropriate position, opposite somite 25/26, and had invaded the renal mesenchyme by E11 (n = 4, data not shown)[11]. Thus, the incorrectly positioned distal ureters seen in *Rara*$^{-/-}$ *Rarb2*$^{-/-}$ mutants were probably linked to abnormalities at later stages.

Although analysis of vertical displacement in *Rara*$^{-/-}$ *Rarb2*$^{-/-}$ embryos did not reveal substantial abnormalities, lateral displacement was markedly impaired (Fig. 3). In E14 wildtype embryos, the distal ureters had descended to the level of the urogenital sinus and had separated from the Wolffian ducts (Fig. 3*a*,*b*,*d*). In *Rara*$^{-/-}$ *Rarb2*$^{-/-}$ mutant littermates, the distal ureters and Wolffian ducts had descended to the region of the urogenital sinus, but separation had not occurred; thus, the distal ureters remained attached to the Wolffian ducts and did not join the bladder directly (Fig. 3*e*,*f*,*h*). Of 17 *Rara*$^{-/-}$ *Rarb2*$^{-/-}$ mutants examined by whole-mount and histological analysis, 14 showed abnormal distal ureter connections bilaterally and the remaining 3 showed abnormal connections on one side.

Analysis of ureter maturation *in vitro* confirmed that vitamin A was necessary for lateral displacement. In urogenital explants from E12 wildtype embryos cultured in medium containing vitamin A, the distal ureters separated from Wolffian ducts and the trigonal wedge formed in between (Fig. 3*c*,*d*). But in explants cultured without a source of vitamin A, formation of the wedge was impaired and, as a consequence, the distal ureters did not join properly to the bladder and retained immature connections

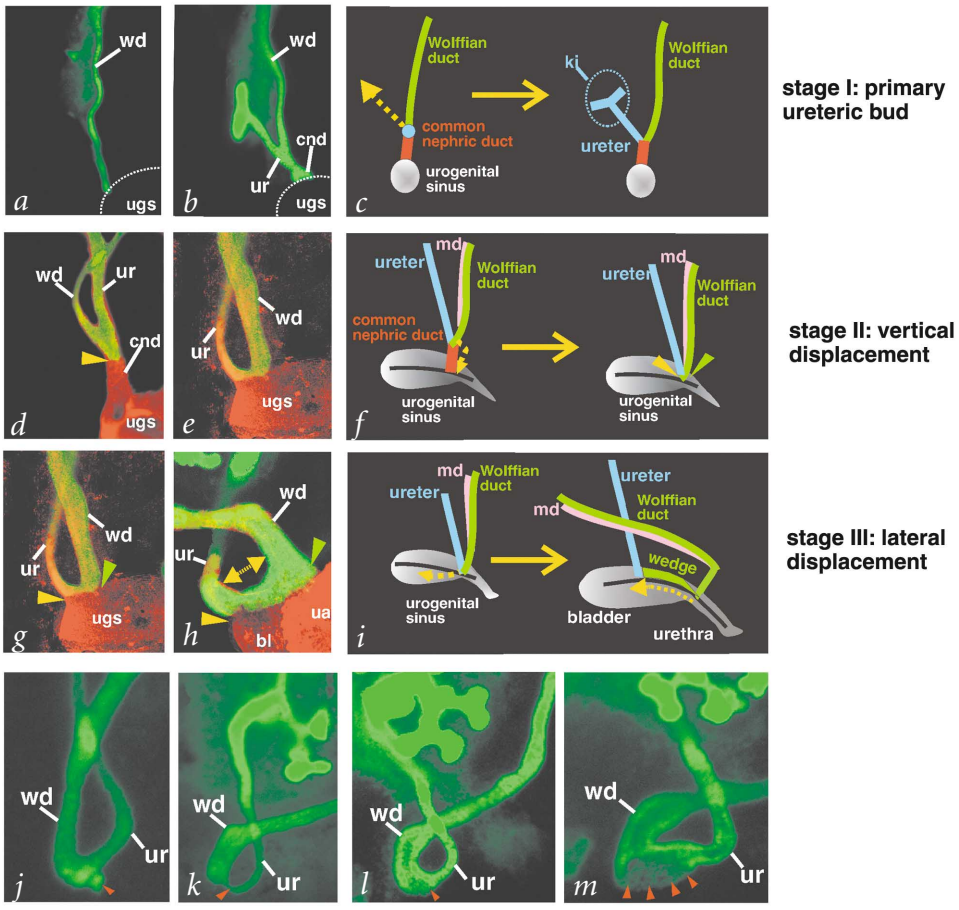

Fig. 2 The three stages of ureter maturation. *a–c*, Primary ureteric bud formation. ***a***, Urogenital tract from an E10 *Hoxb7–GFP* embryo. ***b***, Urogenital tract from an E11 *Hoxb7–GFP* embryo after ureteric bud outgrowth. ***c***, Representation of images shown in *a* and *b*. Broken yellow arrow indicates the primary ureteric bud branch. *d–f*, Vertical displacement. ***d***, Whole-mount urogenital tract from an E12 *Hoxb7–GFP* embryo (green) stained with cytokeratin (red). Yellow arrow indicates the common nephric duct. ***e***, Whole-mount urogenital tract from an E13 *Hoxb7–GFP* embryo stained with cytokeratin (red). ***f***, Representation of images shown in *d* and *e*. Broken yellow arrow shows downward movement of the ureter towards the urogenital sinus. Yellow and green arrowheads indicate the positions of the distal ureters and Wolffian ducts after vertical displacement. *g*,*h*, Lateral displacement. ***g***, Whole-mount urogenital tract from an E13 *Hoxb7–GFP* embryo stained with cytokeratin. Yellow and green arrowheads mark the position of the distal ureters and Wolffian ducts. ***h***, Whole-mount urogenital tract from an E14 *Hoxb7–GFP* embryo stained with cytokeratin (red). Yellow and green arrowheads indicate the new positions of the distal ureter and Wolffian duct, which have separated. Double-headed yellow arrow indicates the epithelial wedge, whose growth has separated the ureters and Wolffian ducts. ***i***, Representation of images shown in *g* and *h*. Broken yellow arrow indicates the movement of the distal ureters to the base of the bladder as the trigonal wedge grows out from the base of the Wolffian ducts. Although both sex ducts are present by E12, the Müllerian duct (female duct) is not fully formed caudally and connections in males and females are made through the Wolffian ducts. *j–m*, Expression of the *Hoxb7-GFP* transgene during ureter maturation. Red arrowheads indicate the common nephric duct or terminal Wolffian duct segment. ***j***, Urogenital tract from an E12 *Hoxb7–GFP* embryo. ***k***, Urogenital tract from a *Hoxb7–GFP* embryo isolated early on E13. ***l***, Urogenital tract from a *Hoxb7–GFP* embryo isolated late on E13. ***m***, Urogenital tract from an E14 *Hoxb7–GFP* embryo. Color code for *c*,*f*,*i*: Wolffian duct and trigonal wedge are shown in green, urogenital sinus in gray, Müllerian duct in pink, common nephric duct in red, and the kidney and ureters in blue. bl, bladder; cnd, common nephric duct; md, Müllerian duct; ur, ureter; ugs, urogenital sinus; wd, Wolffian duct. Original magnification, ×200 (*a*,*b*,*j*), ×100 (*d*,*e*,*g*,*h*,*k–m*).

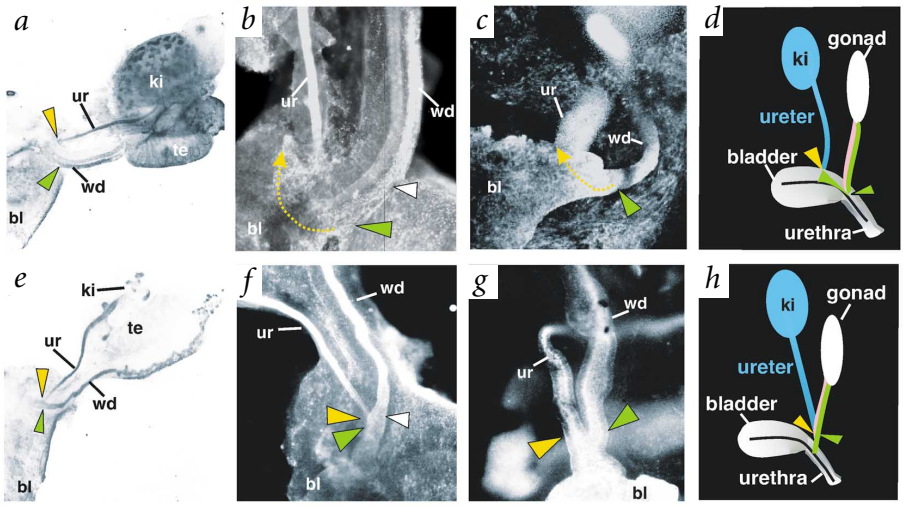

Fig. 3 Vitamin A is necessary for lateral displacement. Yellow and green arrowheads indicate the respective positions where ureters (yellow) and Wolffian ducts (green) join the urogenital sinus. ***a***, Whole-mount urogenital tract from an E14 wildtype male embryo stained with cytokeratin. ***b***, Higher magnification view of *a*. Broken yellow arrow indicates the movement of the ureter end to the mature position; white arrow points to the junction between the Wolffian duct and the urogenital sinus. ***c***, Urogenital tract from an E12 *Hoxb7–GFP* embryo cultured for 48 h in medium with added vitamin A and stained with cytokeratin. Broken yellow arrow indicates the region at the base of the Wolffian duct where the trigonal wedge forms. ***d***, Representation of images shown in *a–c*. ***e***, Whole-mount urogenital tract from an E14 *Rara*$^{-/-}$ *Rarb2*$^{-/-}$ male stained with cytokeratin. ***f***, Higher magnification view of *e*. White arrow points to the junction between the Wolffian duct and the urogenital sinus. ***g***, Whole-mount urogenital tract from an E12 *Hoxb7–GFP* embryo cultured for 48 h in medium without added vitamin A and stained for cytokeratin. ***h***, Representation of images in *e–g*. Kidney and ureters are shown in blue, gonad in white, Wolffian duct in green and Mullerian duct in pink. bl, bladder; ua, urethra; ur, ureter; wd, Wolffian duct. Original magnification, ×100 (*a*,*c*,*e*,*g*), ×200 (*b*,*f*).

with the Wolffian ducts (Fig. 3*g*,*h*). These studies suggest that vitamin A signaling is important for ureter maturation and for controlling formation of the trigonal wedge. Disruption of this process may therefore be a cause of the abnormalities in the distal ureters in *Rara*$^{-/-}$ *Rarb2*$^{-/-}$ mice.

Vitamin A controls *Ret* expression in the trigonal wedge

Our results suggested that lateral displacement involves the formation of an epithelial outgrowth at the base of the Wolffian ducts—a process that shares similarities with other events that are important for urinary-tract development, including formation of the ureteric bud and renal branching morphogenesis. Budding of the ureter involves a local proliferation and outgrowth of epithelial cells from the Wolffian duct, and branching morphogenesis involves a local proliferation and outgrowth of epithelial cells at the tips of the ureteric bud in the fetal kidney.

Several molecular pathways that mediate these events have been identified, including that of the proto-oncogene *Ret*, which is required for formation of the ureteric bud and for its subsequent growth and branching in the embryonic kidney[13,14]. In the urogenital system, Ret signaling is activated by the Ret ligand Gdnf (refs 19–21) and the co-receptor Gfra1 (refs 22–24). Vitamin A–dependent signals from the mesenchyme are important in branching morphogenesis because they maintain expression of *Ret* in the ureteric bud epithelium of the renal collecting-duct system[12,25]. Thus, it seemed plausible that vitamin A and Ret might also regulate lateral displacement by controlling epithelial expansion at the base of the Wolffian ducts. If so, then (i) Ret and its signaling partners should be localized in or near the caudal Wolffian ducts when expansion occurs, (ii) *Ret* expression at this site should depend on vitamin A and (iii) inactivation of Ret should cause distal ureter malformations similar to those seen in *Rara*$^{-/-}$ *Rarb2*$^{-/-}$ mice.

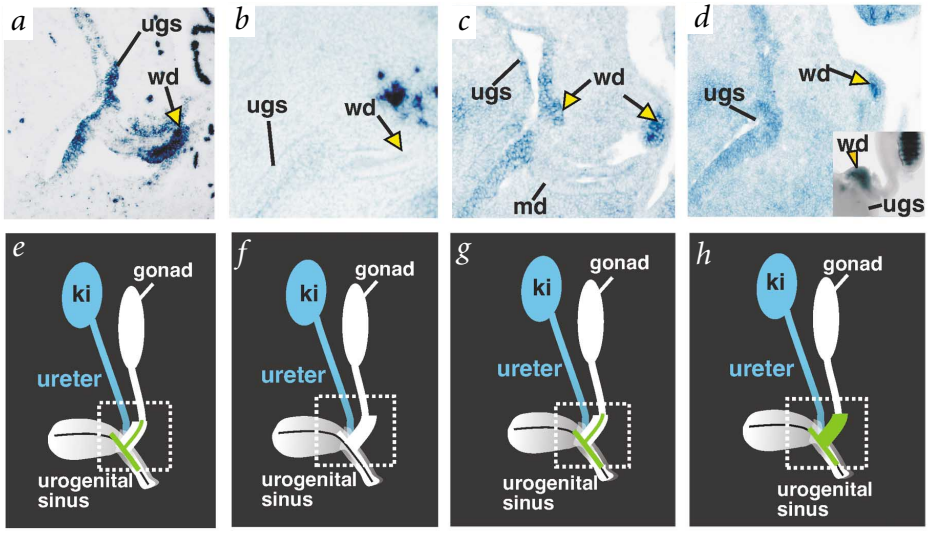

Fig. 4 A new domain of Ret signaling in the distal urinary tract. ***a***, *In situ* hybridization of an E13 wildtype embryo with a *Ret* probe. ***b***, *In situ* hybridization of an E13 *Rara*$^{-/-}$ *Rarb2*$^{-/-}$ embryo with a *Ret* probe. Ret expression was maintained in neuronal precursors, but not in the Wolffian duct or sinus epithelia. ***c***, Expression of *Gfra1* encoding the Ret co-receptor Gfra1, at E13 in a wildtype embryo. ***d***, *Gdnf* expression in the caudal Wolffian duct and urogenital sinus of an E13 wildtype embryo. Inset, *Gdnf* promoter activity in the testes and caudal Wolffian duct of an E12 *Gdnf–lacZ* embryo[26]. ***e***, Representation of image in *a*, showing distribution of Ret in the urogenital tract at E13 (green). ***f***, Representation of image in *b*, showing lack of *Ret* expression in the Wolffian duct of an E13 *Rara*$^{-/-}$ *Rarb2*$^{-/-}$ embryo. ***g***, Representation of image in *c*, showing expression of *Gfra1* in the distal Wolffian ducts (green). ***h***, Representation of image in *d*, showing expression of *Gdnf* (green). Kidney and ureters are shown in blue, sex ducts and gonad in white, and urogenital sinus in gray. md, Mullerian duct; ugs, urogenital sinus; wd, Wolffian duct. Original magnification, ×20 (*a–d*).

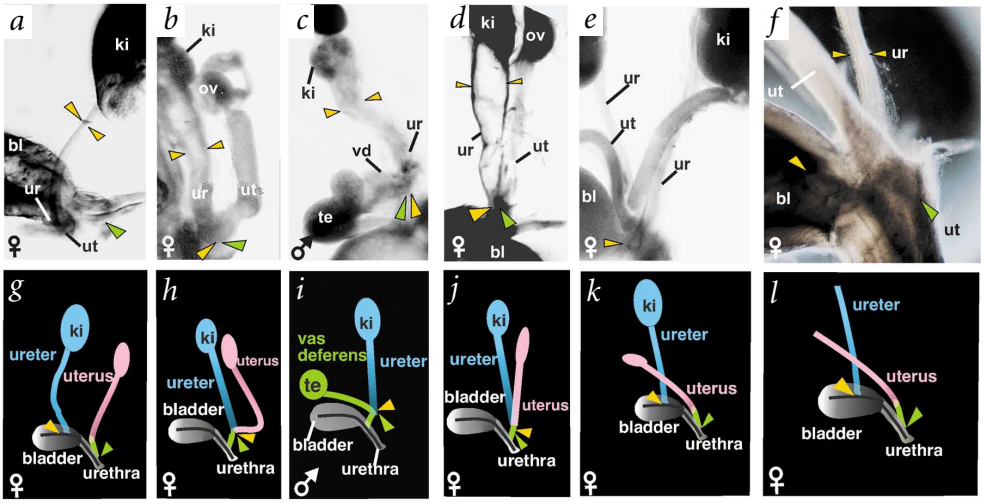

Fig. 5 Ret is required for distal ureter morphogenesis. Yellow arrowheads indicate ureters and their distal ends; green arrowheads indicate the points where the uterine horns join the urogenital sinus or vas deferens. ***a***, Urogenital tract from an E18 wildtype female. ***b***, Whole-mount urogenital tract from an E18 *Ret*$^{-/-}$ female. ***c***, Whole-mount urogenital tract from an E18 *Ret*$^{-/-}$ male. ***d***, Whole-mount urogenital tract from an E18 *Rara*$^{-/-}$ *Rarb2*$^{-/-}$ female. ***e***, Whole-mount urogenital tract from an E18 *Rara*$^{-/-}$ *Rarb2*$^{-/-}$ *Hoxb7–Ret* fetus, showing morphologically normal ureters and normal connections with the bladder. ***f***, Higher magnification view of *e*, showing normal morphology and position of the distal ureters and the uterus in rescued mutants. ***g–l***, Representation of images in *a–e*, respectively. Kidney and ureters are shown in blue, uterus in pink, testes and vas deferens in green, and bladder and urethra in gray and white. Panels *b* and *c* were reproduced from ref. 30. ki, kidney; te, testes; ur, ureter; ut, uterus; vd, vas deferens. Original magnification, ×40 (*a–e*), ×100 (*f*).

Analysis of wildtype embryos at E12, when lateral displacement begins, showed that *Ret* was indeed expressed in epithelial cells at the base of the Wolffian ducts—the region that subsequently expands to form the trigonal wedge. Expression of *Ret* persisted in this region as lateral displacement progressed (Fig. 4*a*,*e* and data not shown). The Ret signaling partners Gfra1 and Gdnf were localized in the same area: *Gfra1* was expressed predominantly in epithelia, and *Gdnf* was expressed in both epithelial and mesenchymal cells (Fig. 4*c*,*f*,*g*,*h*). Analysis of *Gdnf* promoter activity in urogenital tracts from *Gdnf–lacZ* mice[26] confirmed these findings and showed an intense and localized region of *lacZ* expression throughout the caudal Wolffian ducts (Fig. 4*d*, inset). As cells in the region where the trigonal wedge forms expressed *Ret* and its signaling partners, Ret might promote outgrowth or remodeling of the trigonal wedge during ureter maturation. Notably, *Ret*, *Gdnf* and *Gfra1* were also expressed in the epithelium of the dorsal urogenital sinus, indicating that Ret signaling might also be important for development of the bladder or urethra.

Vitamin A modulates the expression of *Ret* in several types of cell, including the ureteric bud epithelium, where loss of Ret signaling leads to impaired branching morphogenesis[25,27]. Vitamin A signaling depends on the availability of retinoic acid and retinoic acid receptors. In wildtype embryos, Rara and Rarb2, retinoic acid receptors that are required for lateral displacement, are colocalized in the mesenchyme that surrounds the Wolffian ducts[28]. Retinaldehyde dehydrogenase-2, an enzyme required for embryonic retinoic acid synthesis[29], was also highly expressed in these mesenchymal cells in wildtype embryos (data not shown). Thus, *Ret* expression might be maintained normally by vitamin A–dependent signals from the surrounding mesenchyme. To assess this, we analyzed *Ret* expression in urogenital tracts from *Rara*$^{-/-}$ *Rarb2*$^{-/-}$ embryos and wildtype littermates. At E12 and at subsequent stages, *Ret*, which was abundantly expressed in wildtype embryos, was downregulated in epithelia at the base of the

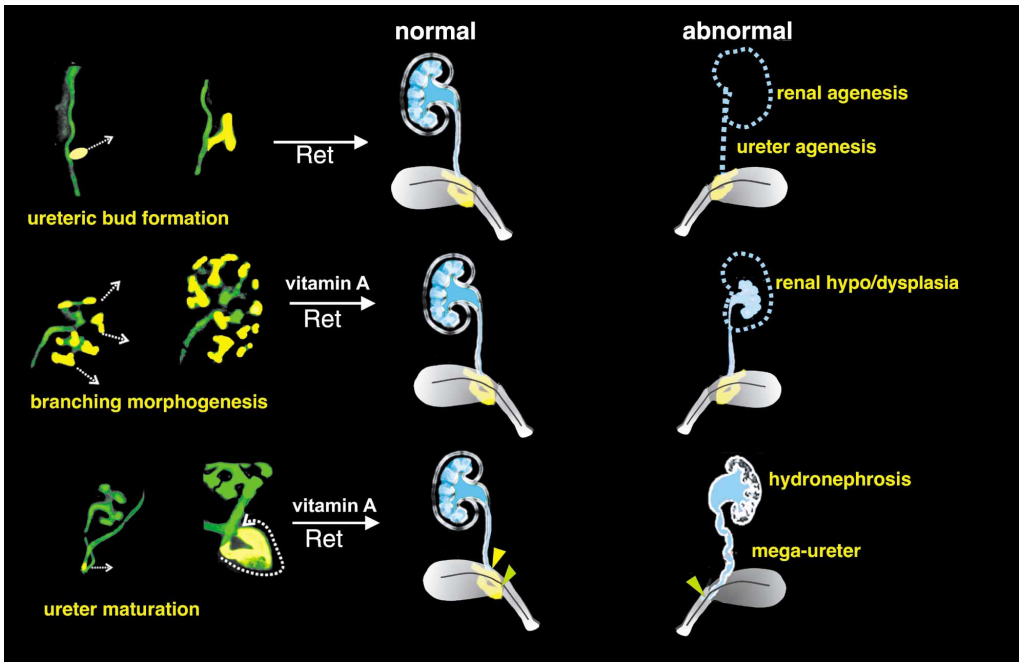

Fig. 6 Model showing how pleiotropic urogenital malformations may be linked to disruption of vitamin A and Ret signaling. Left, three processes requiring vitamin A and Ret: branching of the primary ureteric bud, branching morphogenesis in the embryonic kidney, and ureter maturation. Middle, representation of the development of the urinary tract in wildtype mice at birth. Right, the expected phenotypes in mutants in which ureteric bud formation (top), branching morphogenesis (middle) and ureter maturation (bottom) are disrupted.

Wolffian duct in *Rara*$^{-/-}$ *Rarb2*$^{-/-}$ mice ($n = 6$, Fig. 4*a*,*b*,*e*,*f*). Loss of *Ret* expression is thus a potential cause of the impaired ureter maturation and distal ureter abnormalities seen at birth in *Rara*$^{-/-}$ *Rarb2*$^{-/-}$ mice.

Ureter maturation is impaired in *Ret* mutants

If Ret normally controls distal ureter morphogenesis by inducing formation of the trigonal wedge, then loss of Ret function should result in abnormalities of the ureter. We therefore investigated whether distal ureter malformations similar to those in *Rara*$^{-/-}$ *Rarb2*$^{-/-}$ mutants were present in *Ret*$^{-/-}$ mice. Previous studies have shown that there is a low frequency of ureter abnormalities in *Ret*$^{-/-}$ mice; however, these defects have not been further characterized[7,30].

Here we examined urogenital tracts from 14 *Ret*$^{-/-}$ mice at E13–E18 by whole-mount and histological analysis to determine the nature of the urogenital-tract malformations. As found previously[7,30], ureter agenesis and agenesis of the ipsolateral kidney were the prevalent urogenital-tract phenotypes in *Ret*$^{-/-}$ mice and were present at a frequency of 54% (15 out of 28 ureters, with two ureters analyzed per embryo). In 7 of 28 cases, ureters were truncated proximally (lacking the ipsolateral kidney) but terminated normally in the bladder (25%). In 6 of 28 cases (21%), the distal ureters did not join the bladder but instead joined the sex ducts, an abnormality also seen in *Rara*$^{-/-}$ *Rarb2*$^{-/-}$ mice (Fig. 5). Distal ureters ended in the uterus in affected *Ret*$^{-/-}$ females and in the vas deferens in their male counterparts (Fig. 5*a*–*c*,*g*–*i*). These findings suggest that Ret may be important for distal ureter morphogenesis.

Because (i) Ret signaling was localized in a region of the caudal Wolffian ducts that expanded to form the trigonal wedge during ureter maturation, (ii) expression of *Ret* in these cells was dependent on vitamin A and (iii) inactivation of Ret resulted in a set of distal ureter malformations similar to those seen in *Rara*$^{-/-}$ *Rarb2*$^{-/-}$ mice, it seemed likely that vitamin A might control lateral displacement through Ret. These two molecular pathways could be important for ureter maturation by controlling epithelial cell growth and remodeling during formation of the trigonal wedge. We therefore tested whether the restoration of *Ret* expression in epithelia at the base of the Wolffian duct that presumably form the wedge could rescue distal ureter malformations in *Rara*$^{-/-}$ *Rarb2*$^{-/-}$ mice. To direct *Ret* expression to this area, we used transgenic *Hoxb7–Ret* mice, in which *Ret* is expressed predominantly in epithelial cells of the urinary tract including the ureteric bud, ureters and Wolffian ducts[12,15,31]. We crossed *Hoxb7–Ret* mice with *Rara*$^{-/-}$ *Rarb2*$^{-/-}$ mutants to generate *Rara*$^{-/-}$ *Rarb2*$^{-/-}$ *Hoxb7–Ret* mice. In *Rara*$^{-/-}$ *Rarb2*$^{-/-}$ mice, ectopic ureters, hydronephrosis and mega-ureter were observed with nearly 100% penetrance (14 of 17 bilaterally; 3 of 17 unilaterally). Expression of the *Hoxb7–Ret* transgene in *Rara*$^{-/-}$ *Rarb2*$^{-/-}$ mice rescued distal ureter anomalies almost completely. Unlike *Rara*$^{-/-}$ *Rarb2*$^{-/-}$ mice, in which the ureters terminated in the urethra or sex ducts (Fig. 5*d*,*j*), however, ureters of *Rara*$^{-/-}$ *Rarb2*$^{-/-}$ *Hoxb7–Ret* embryos terminated normally in the bladder (Fig. 5*e*,*f*,*k*,*l*; $n = 11$ of 11).

Analysis at E14 confirmed that the distal ureters had integrated normally into the bladder in *Rara*$^{-/-}$ *Rarb2*$^{-/-}$ *Hoxb7–Ret* embryos, whereas they still joined the Wolffian ducts in their *Rara*$^{-/-}$ *Rarb2*$^{-/-}$ counterparts (data not shown). Thus, expression of the *Hoxb7–Ret* transgene in the Wolffian duct of *Rara*$^{-/-}$ *Rarb2*$^{-/-}$ mutants rescued lateral displacement. The *Hoxb7–Ret* transgene also rescued mega-ureter and hydronephrosis, which suggests that these abnormalities are secondary to incorrectly positioned distal ureters; however, with our current data, we cannot rule out other causes. Taken together, these results indicate that vitamin A and Ret are probably mediators of ureter maturation and control lateral displacement by inducing the formation of an epithelial wedge at the base of the Wolffian ducts, and that the disruption of this process can cause complex distal ureter abnormalities at birth.

Discussion

The pleiotropic nature of urinary-tract abnormalities in humans has been puzzling for many years. Often, several renal and ureter abnormalities occur in the same individual[32]. Abnormal branching of the primary ureteric bud from the Wolffian duct, either above or below its normal branch site, has been considered to be an underlying cause of several of these abnormalities. This model is based on the idea that a misplaced ureteric bud sprout makes a poor contact with the kidney mesenchyme, thereby disrupting both renal morphogenesis and some aspects of ureter maturation owing to the abnormal position of the ureter on the Wolffian duct[33,34].

Although abnormal sprouting of the primary ureteric bud is probably a cause of malformations in the distal ureter, particularly those instances involving supernumerary ureters[35–37], our findings indicate that pleiotropic urogenital malformations also may result from the disruption of molecular pathways, such as vitamin A and Ret pathways, that carry out distinct developmental functions at different stages of urinary-tract formation (Fig. 6). Ret signaling is required for outgrowth of the ureteric bud from the Wolffian duct and for branching morphogenesis of the renal collecting duct system[13,14], where vitamin A signaling is necessary for expression of *Ret* (Fig. 6). Both events involve epithelial cell remodeling, which Ret signaling could promote by stimulating the proliferation or migration of epithelial cells[38,39]. Our studies suggest that Ret also regulates epithelial cell remodeling during ureter maturation, and that expression of *Ret* in epithelial cells that form the trigonal wedge depends on vitamin A. These findings indicate that the disruption of a single gene or signaling pathway with several functions may cause a multitude of urinary-tract abnormalities and might be a cause of complex urinary-tract malformations in humans.

Methods

Knockout and transgenic mice. We visualized *Hoxb7–GFP* embryos[15] by fluorescence microscopy. The *Rarb2*$^{-/-}$ mutant embryos were generated from crosses between *Rara*$^{+/-}$ *Rarb2*$^{+/-}$ females with *Rara*$^{+/-}$ *Rarb2*$^{-/-}$ males and genotyped as described[25]. The PCR conditions were 1 min of 94 °C, and then 30 cycles of 30 min at 94 °C, 30 min at 62 °C and 30 min at 72 °C. This generated a product of 370 bp. The primer sequences are available from C.M. on request. To generate *Rara*$^{-/-}$ *Rarb2*$^{-/-}$ *Hoxb7–Ret* mice, we crossed *Hoxb7–Ret* transgenic mice (expressing the *Ret9* isoform) with *Rara*$^{+/-}$ *Rarb2*$^{-/-}$ males and *Rara*$^{+/-}$ *Rarb2*$^{+/-}$ females to generate *Rara*$^{+/-}$ *Rarb2*$^{-/-}$ *Hoxb7–Ret* males and *Rara*$^{+/-}$ *Rarb2*$^{-/-}$ *Hoxb7–Ret* females. These mice were then intercrossed to generate *Rara*$^{-/-}$ *Rarb2*$^{-/-}$ *Hoxb7–Ret* embryos. For timed matings, the morning when vaginal plugs were visualized was taken to be E0.5. Note that this particular *Hoxb7–Ret* transgene does not generate dominant defects in either the kidney or the ureter[12]. The experimental design used in these studies has been approved by the institutional animal care and use committee at Columbia University.

***In situ* hybridization.** We carried out non-radioactive *in situ* hybridization of sections essentially as described[25]. The cDNA encoding *Ret* was linearized with *Sac*II, and T3 was used to produce a 3.3-kb riboprobe. The *Gfra1* cDNA was linearized with *Hin*dIII, and T7 was used to produce a 2-kb riboprobe. The *Gdnf* cDNA was linearized with *Hin*dIII, and SP6 was used to generate a 0.7-kb riboprobe.

Organ culture. Urogenital blocks from E12 wildtype embryos were dissected into ice-cold DMEM plus F12 medium and cultured on Transwell Clear filters (Costar) in serum-free (DMEM plus F12) medium with the

following additives: 5 μg per ml insulin, 5 μg per ml transferrin, 5 ng per ml selenium (Sigma), with Pen/Strep/Glu (Sigma). Rudiments were incubated at 37 °C in a 5% CO_2 atmosphere for up to 3 d. We added all-*trans*-retinoic acid (Sigma) and 9-*cis*-retinoic acid (Biomole) to the culture medium to a final concentration of 200 nM. All-*trans*-retinol (Sigma) was used at a final concentration of 1 μM.

Immunohistochemistry and histology. For cytokeratin staining, we fixed tissue samples in cold 100% methanol for 10 min and washed them in PBS plus 0.1% Triton X-100. Nonspecific staining was blocked by treatment with 2% horse serum for 2 h. Antibody against pan-cytokeratin (Sigma) was applied overnight at a 1:300 dilution. Samples were then washed with PBS and stained with secondary antibody (Cy3-conjugated donkey antibody against mouse IgG, Jackson Immunoresearch). We cut vibratome sections from tissue that had been fixed overnight in 4% paraformaldehyde. After fixation, tissue blocks were washed three times for 10 min in PBS, and then embedded in 3% agarose for sectioning.

Three-dimensional imaging. Whole dissected embryonic urogenital tracts were mounted in PBS with 10% glycerol and 100 mg –1 ml 1,4-diazabicyclo[2.2.2]octane (DABCO). The mounts were made from hanging drop slides and sealed with nail polish. We collected images with a Bio-Rad MRC 1024MP laser-scanning confocal/multiphoton scanner microscope equipped for two-photon confocal imaging with a titanium–sapphire laser (Spectra-Physics) attached to a Nikon Diaphot inverted microscope, which was available at the Indiana Center for Biological Microscopy and is supported by the Indiana Genomics Initiative. Images were collected at 0.5-μm steps at 860 nm using a 20×, 0.45 NA objective. We converted image stacks into three-dimensional volume-rendered images using Voxx image processing software[40]. Opacity, contrast and background parameters were manipulated independently to enhance visualization of the GFP-labeled structures.

Note: Supplementary information is available on the Nature Genetics website.

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Competing interests statement

The authors declare that they have no competing financial interests.

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Distal ureter morphogenesis depends on epithelial cell remodeling mediated by vitamin A and Ret

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