

Non-canonical Wnt5a/Ror2 signaling regulates kidney morphogenesis by controlling intermediate mesoderm extension

Kangsun Yun¹, Rieko Ajima^{1,†}, Nirmala Sharma¹, Frank Costantini², Susan Mackem¹, Mark Lewandoski¹, Terry P. Yamaguchi¹ and Alan O. Perantoni^{1,*}

¹Cancer and Developmental Biology Laboratory, National Cancer Institute, National Institutes of Health, Frederick, MD 21702, USA and ²Department of Genetics and Development, Columbia University Medical Center, New York, NY 10032, USA

Received June 25, 2014; Revised and Accepted July 29, 2014

Congenital anomalies of the kidney and urinary tract (CAKUT) affect about 1 in 500 births and are a major cause of morbidity in infants. Duplex collecting systems rank among the most common abnormalities of CAKUT, but the molecular basis for this defect is poorly understood. In mice, conditional deletion of *Wnt5a* in mesoderm results in bilateral duplex kidney and ureter formation. The ureteric buds (UBs) in mutants emerge as doublets from the intermediate mesoderm (IM)-derived nephric duct (ND) without anterior expansion of the glial cell line-derived neurotrophic factor (*Gdnf*) expression domain in the surrounding mesenchyme. *Wnt5a* is normally expressed in a graded manner at the posterior end of the IM, but its expression is down-regulated prior to UB outgrowth at E10.5. Furthermore, ablation of *Wnt5a* in the mesoderm with an inducible Cre at E7.5 results in duplex UBs, whereas ablation at E8.5 yields normal UB outgrowth, demonstrating that *Wnt5a* functions in IM development well before the formation of the metanephros. In mutants, the posterior ND is duplicated and surrounding Pax2-positive mesenchymal cells persist in the nephric cord, suggesting that disruption of normal ND patterning prompts the formation of duplex ureters and kidneys. *Ror2* homozygous mutants, which infrequently yield duplex collecting systems, show a dramatic increase in incidence with the additional deletion of one copy of *Wnt5a*, implicating this receptor in non-canonical *Wnt5a* signaling during IM development. This work provides the first evidence of a role of *Wnt5a/Ror2* signaling in IM extension and offers new insights into the etiology of CAKUT and possible involvement of *Wnt5a/Ror2* mutations.

INTRODUCTION

Congenital anomalies of the kidney and urinary tract (CAKUT) are among the most common birth defects and a major cause of pediatric morbidity. Duplex kidney formation is included in the spectrum of malformations associated with CAKUT and is characterized by the development of two separate collecting systems with either bifid (merged) or fully duplicated ureters. While duplex kidneys are generally asymptomatic, patients bearing this abnormality may be prone to urinary tract infections (1,2).

The kidney develops from progenitors originating from the intermediate mesoderm (IM). Development is initiated by the invasion of nephric (Wolffian) duct (ND) progenitors along the A–P axis toward the cloaca in the IM and their conversion to an epithelium under the influence of signals provided at

least in part from surface ectoderm (3). Subsequently, the metanephric mesenchyme (MM) becomes delineated as a loose tissue mass at the posterior end of the IM adjacent to the ND. Signals emanating from the MM induce the outgrowth of single ureteric buds (UBs) from the ND, which arborizes in the MM beginning at E11.5 in the mouse. The rearranged during transfection (c-RET)/glial cell line-derived neurotrophic factor (GDNF) signaling pathway is the principal regulatory mechanism responsible for bud outgrowth, and its dysregulation results either in no bud outgrowth or supernumerary budding. Thus, several molecules involved in the regulation of c-RET/GDNF expression are critical for normal kidney development (4–6).

In addition to components of the c-RET/GDNF signaling pathway, mutations in molecules associated with the planar

*To whom correspondence should be addressed. Email: perantoa@mail.nih.gov

†Present address: Developmental Genetics Group, Graduate School of Frontier Biosciences, Osaka University, Osaka 565-0871, Japan.

cell polarity (PCP) pathway (7,8) also result in duplex kidneys (7,9). However, the mechanism of duplex kidney formation in these mutants has not been clearly investigated. *Wnt5a* is frequently implicated in non-canonical Wnt/PCP signaling (10). Its loss of function causes several defects in tissue outgrowth from truncation of the A–P axis to shortening of the tail, limbs, digits, face and genitals (11). Also, *Wnt5a* affects morphogenesis of several organs. Its deletion induces abnormal patterning of the pituitary gland (12), a shortened and bifurcated intestine (13), a shortened and widened cochlea (14), and abnormal outflow tract morphogenesis in the heart (15)—all through non-canonical Wnt/PCP signaling. *Ror2* is known to be a receptor for *Wnt5a* signaling. The phenotypes of *Ror2* null mice also show dwarfism, facial abnormalities, short limbs and tails like *Wnt5a* null mice (16), suggesting that *Wnt5a* may regulate the morphogenesis of several organs through *Ror2*.

In this study, we investigated the role of *Wnt5a* in the development of the metanephros and found that it regulates IM extension, affecting kidney morphogenesis. *Wnt5a* mutants form a shortened and broadened IM with duplicated NDs at the posterior end prior to UB outgrowth. Thus, the abnormal extension of the posterior ND alters the outgrowth of the UB from the ND, creating a double UB which causes duplex kidney formation. This occurs without a decrease in canonical Wnt activity in the ND. Furthermore, deletion of one *Wnt5a* allele in *Ror2*^{−/−} mutants causes haploinsufficiency for the duplex kidney phenotype, implicating *Ror2* as the receptor responsible for *Wnt5a* signaling during IM development. These findings suggest that a defect in the non-canonical *Wnt5a/Ror2* signaling pathway may be responsible for duplex kidney formation through dysgenesis of IM extension.

RESULTS

Ablation of *Wnt5a* using *T-Cre* results in duplex kidney formation

Since the kidney is derived from the IM, we conditionally inactivated *Wnt5a* using the *T-Cre* mouse, which expresses Cre recombinase in nascent primary mesoderm (17). The *Wnt5a* mutants from this cross showed a shortened A–P body axis, outgrowth defects in the limbs and a tail comparable to that of the *Wnt5a* null mouse (11). However, unlike the null mutants, the conditional *Wnt5a* mutant embryos exhibited normal facial structures (Fig. 1A). Since the observed phenotype was limited to mesoderm, it was not overly complicated by abnormalities involving non-mesoderm-derived organ development and the indirect consequences from such defects. The kidneys from mutant embryos exhibited uni- or bilateral renal abnormalities. By H&E staining, mutant kidneys were duplexed and associated with double ureters (Fig. 1B, *n* = 24/24). Also, whole-mount immunostaining for calbindin clearly showed double ureter formation in the *Wnt5a* mutant (Fig. 1C).

The UB in the *Wnt5a* mutant extends from the ND as a doublet without anterior expansion of the *Gdnf* expression domain.

To explore the mechanism responsible for double ureter formation, we first examined the outgrowth of the UB. The UB extends

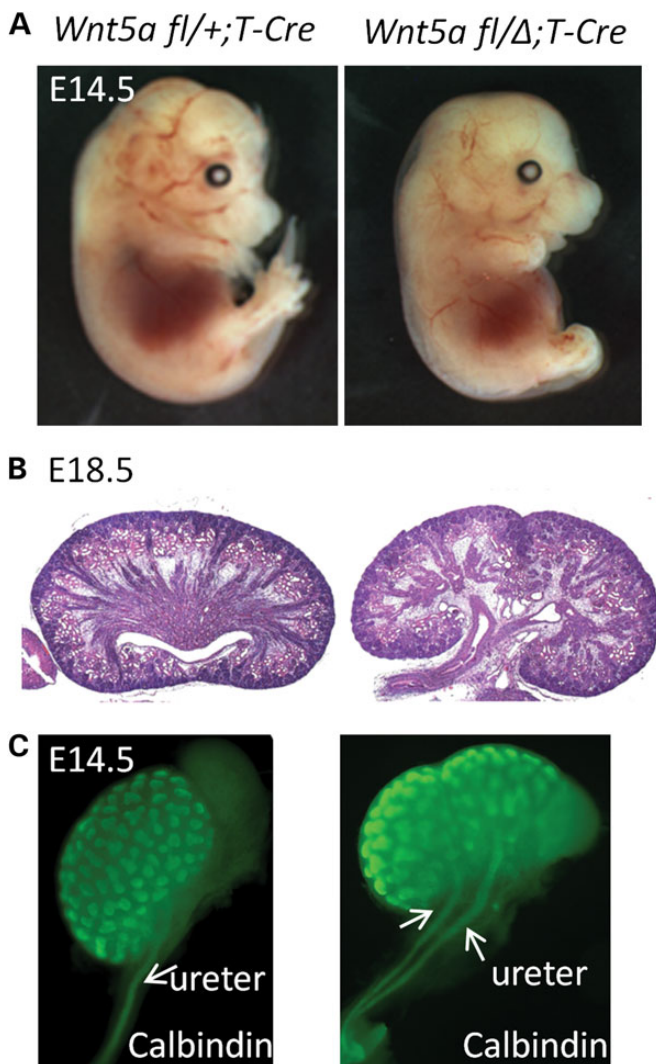
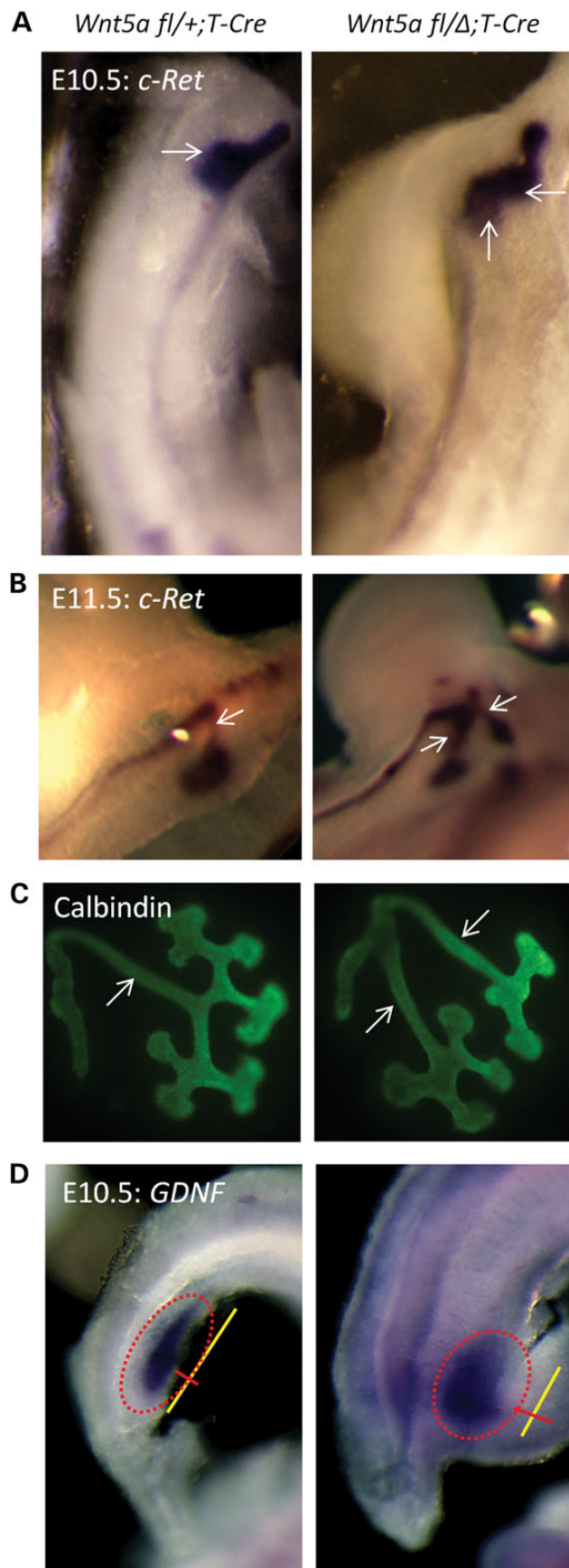


Figure 1. Ablation of *Wnt5a* using *T-Cre* results in bilateral duplex kidney formation. (A) left: *Wnt5a* *fl/+*; *T-Cre* embryo at E14.5; right: *Wnt5a* *fl/Δ*; *T-Cre* embryo at E14.5. (B) H&E staining of sagittal sections from *Wnt5a* *fl/+*; *T-Cre* (left) and *Wnt5a* *fl/Δ*; *T-Cre* (right) kidneys at E18.5. (C) Calbindin staining of *Wnt5a* *fl/+*; *T-Cre* and *Wnt5a* *fl/Δ*; *T-Cre* kidneys at E14.5.

from the ND into the adjacent mesenchyme at E10.5, mainly through the interaction of *Gdnf*, which is secreted by MM cells, with its receptor *c-Ret*, which is elaborated by the ND (4,6). In other models of duplex kidney development, mutants generally display multiple budding from the ND as a result of dysregulation of *c-Ret/Gdnf* signaling (1,4,18). We performed whole-mount *in situ* hybridization (WISH) for *c-Ret* to evaluate budding at the initiation of kidney development. At E10.5, the ND develops in the mutant as a tubular structure without supernumerary budding, except at the site of normal bud outgrowth (Fig. 2A). Twin buds formed in the mutants, creating double stalks at E11.5 (Fig. 2B). Moreover, these observations were reproducible in explant culture using metanephric rudiments from embryos at E11.5. *Wnt5a* mutant explants exhibited early signs of duplex kidney formation with double UB outgrowth (Fig. 2C). We also assessed the *Gdnf* expression domain, as some mutants with a duplex kidney phenotype have an



anterior expansion of this domain (1,18). We could not observe an anterior expansion of the *Gdnf* expression domain in *Wnt5a* mutants. The dimensions of the domain, however, did reflect the posterior truncation in the embryo, being shorter and wider than the control, but overall, the domain was comparable to that of normal embryos and complemented the pattern observed for *c-Ret* expression (Fig. 2D). We further evaluated the expression of molecules that regulate UB outgrowth, including *Bmp4* (19), *Gremlin1* (19) and *Sprouty1* (20). Mutants showed a shortened IM and ND, reflecting the truncated A–P body axis, but we found none with expression patterns that were altered from control littermates other than ND marker *Sprouty1*, which expressed two domains as observed for *c-Ret* at the posterior end of the ND (Supplementary Material, Fig. S1).

***Wnt5a* ablation during early IM development induces duplex kidney formation**

Since the *Wnt5a* mutant showed outgrowth of the UB as a doublet at the initiation of kidney development, we analyzed the *Wnt5a* expression pattern in early kidney development. Although *Wnt5a* is strongly expressed normally in the tail and limb buds, its expression was not demonstrable in the area of UB outgrowth at the time of bud initiation (Supplementary Material, Fig. S2B, yellow ellipse). Even at E11.5, *Wnt5a* expression was barely detected in the metanephros and then only after lengthy incubations in WISH substrate (Supplementary Material, Fig. S2C, yellow ellipse). This observation is supported by semi-quantitative RT-PCR in which the detection of *Wnt5a* expression in normal isolated E11.5 MMs or UBs required several more cycles of amplification than for *Gdnf* or *Ret* (Supplementary Material, Fig. S2D). These findings suggest that *Wnt5a* expression at the initiation of metanephric development is not responsible for the suppression of duplex kidney formation.

In addition to the dysregulation of *c-Ret/Gdnf* expression as a possible cause of duplex kidney formation, some mutants for genes implicated in the PCP signaling pathway, e.g. *Ror2* (9) or *Fat4* (7), also form duplex kidneys. These mutants, like the *Wnt5a* mutants, display abnormal A–P body axis extension (7,8) as well. Given that *Wnt5a* has been demonstrated to function in non-canonical Wnt/PCP signaling (10), defects in this signaling pathway may also be involved in duplex kidney formation. Since the kidney arises from the IM, we hypothesized that the observed defect in *Wnt5a* mutants may result from the aberrant extension of the IM along the defective A–P axis. Because the ND reaches the cloaca around E10.0, we analyzed embryos for *Wnt5a* expression during IM elongation at E9.5. *Wnt5a* was expressed at the posterior end of the IM in a graded manner (Supplementary Material, Fig. S2A)—a pattern comparable to that observed in the tail. This suggests that *Wnt5a* may

Figure 2. The UB in the *Wnt5a* mutant grows out as a doublet without anterior expansion of the *Gdnf* expression domain. (A) WISH for *c-Ret* using E10.5 embryos. left: *Wnt5a fl/+;T-Cre*; right: *Wnt5a fl/Δ;T-Cre*. (B) WISH for *c-Ret* using E11.5 embryos. left: *Wnt5a fl/+;T-Cre*; right: *Wnt5a fl/Δ;T-Cre*. (C) Calbindin staining of E11.5 explants of metanephric rudiments cultured for 24 h. left: *Wnt5a fl/+;T-Cre*; right: *Wnt5a fl/Δ;T-Cre*. (D) WISH for *Gdnf* using E10.5 embryos. left: *Wnt5a fl/+;T-Cre*; right: *Wnt5a fl/Δ;T-Cre*. White arrows label the UB (A) or UB stalk (B and C). A red-dotted ellipse marks *Gdnf* expression (D) with a yellow line for length and red line for width.

function earlier in urogenital development to regulate posterior IM extension and thus cause duplex kidney formation.

To test whether duplex kidney formation in *Wnt5a* mutants arises from the abnormal development of the IM, we ablated *Wnt5a* in a time-dependent and tissue-specific manner using a well-characterized tamoxifen-inducible *T-CreER^{T2}* line (21). Tamoxifen was injected at E7.5 or E8.5 and embryos harvested at E11.5 for the formation of double ureters. While tamoxifen injected at E7.5 induced recombination along the entire IM, at E8.5 tamoxifen induced only posterior IM recombination (Supplementary Material, Fig. S3). All treatments activated a *Rosa26-YFP* reporter allele by E10.5 in the posterior IM from which the UB emanates. *Wnt5a* ablation during early IM development by tamoxifen injection at E7.5 induced duplex kidney formation bilaterally ($n = 5/6$) or unilaterally ($n = 1/6$) (Fig. 3B), whereas *Wnt5a* ablation by tamoxifen injection at E8.5 failed to elicit the phenotype (Fig. 3A, $n = 7/7$). This finding suggests that the cause of double UB outgrowth in *Wnt5a* mutants is associated with early IM morphogenesis.

The IM is shortened and broadened, and the ND is duplicated at its caudal terminus in the *Wnt5a* mutant at E9.5

Since *Wnt5a* is expressed at the posterior end of the IM at E9.5, we examined its role in ND morphogenesis using the

Hoxb7-myr-venus-YFP reporter. The reporter is active in the ND but not the surrounding mesenchyme in the nephric cord as shown by the coincidental expression of E-cadherin that outlines the epithelial cells of the ND (Supplementary Material, Fig. S4). We also examined IM morphogenesis with staining for Pax2, which is expressed in both the ND and nephric cord (Supplementary Material, Fig. S5). The domain of Pax2 expression is significantly shortened and broadened in the mutant (Fig. 4A and Supplementary Material, Fig. S6). The ND itself, as shown with the *Hoxb7-myr-venus-YFP* reporter, is thickened even in the middle of the mutant IM (Supplementary Material, Fig. S7). Moreover, the posterior end of the duct is especially abnormally thickened, i.e. hypercellular, in the mutant (Fig. 4B and C, and Supplementary Material, Fig. S8). The combination of *Hoxb7-myr-venus-YFP* reporter activity with Pax2 staining in transverse sections of the posterior end of the E9.5 embryo revealed that, in contrast to the normal ND, the mutant duct is duplicated, showing two foci of *Hoxb7-myr-venus-YFP* activity (Fig. 4D and E, and Supplementary Material, Fig. S8). The duplication is most often observed as bilateral fusions, such as demonstrated in mutants in Supplementary Material, Fig. S8; however, it may also manifest at the caudal end as distinct ND structures (Fig. 4D). These findings are consistent with our hypothesis that the abnormality precedes the outgrowth of the UB in the *Wnt5a* mutant.

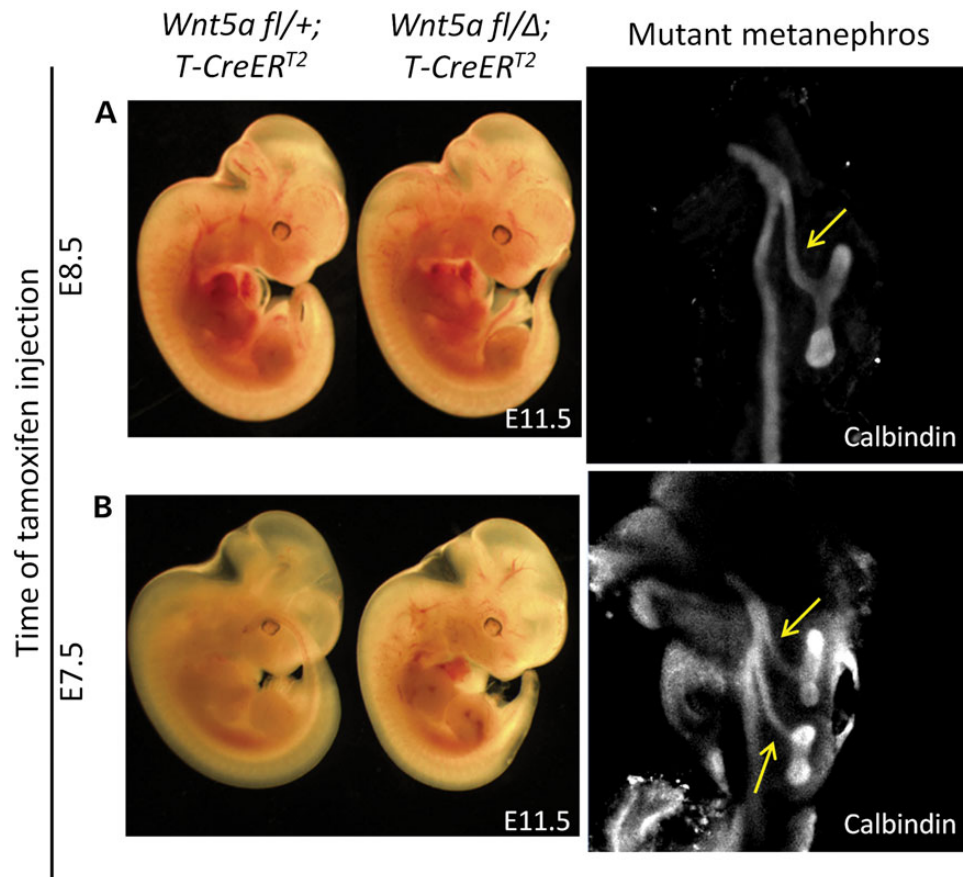


Figure 3. *Wnt5a* ablation in the early IM induces duplex kidney formation. (A) Tamoxifen was injected at E8.5 and embryos were harvested at E11.5. left: *Wnt5a fl/+; T-CreER^{T2}*; middle: *Wnt5a fl/delta; T-CreER^{T2}*; right: metanephros from mutant embryo were stained with calbindin antibody. (B) Tamoxifen was injected at E7.5 and embryos were harvested at E11.5. left: *Wnt5a fl/+; T-CreER^{T2}*; middle: *Wnt5a fl/delta; T-CreER^{T2}*; right: metanephros from mutant embryo were stained with calbindin antibody. The yellow arrows label the UB stalk.

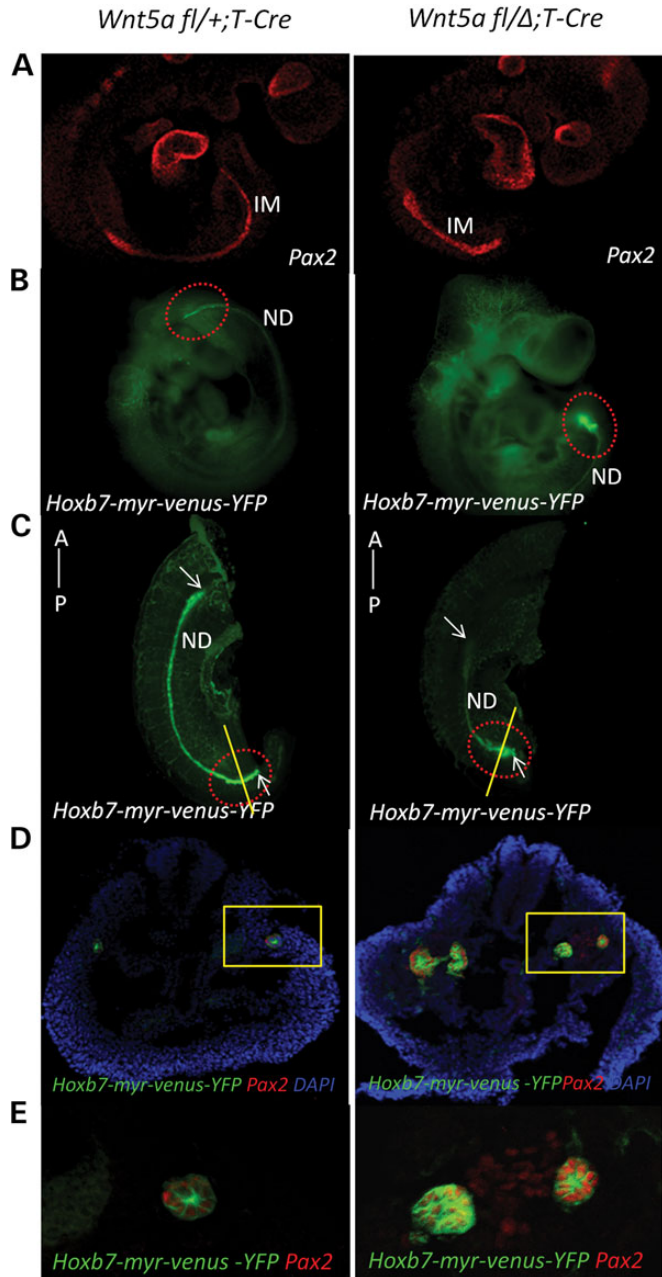


Figure 4. The IM is shortened and broadened in the E9.5 *Wnt5a* mutant and manifests a duplicated ND at its caudal terminus. (A) Embryos at E9.5 were stained with Pax-2 antibody. left: *Wnt5a fl/+;T-Cre*; right: *Wnt5a fl/Δ;T-Cre*. (B) left: *Wnt5a fl/+;T-Cre;Hoxb7-myr-venus-YFP* embryo at E9.5; right: *Wnt5a fl/Δ;T-Cre;Hoxb7-myr-venus-YFP* embryo at E9.5. A red-dotted ellipse marks the posterior end of the embryos. (C) Embryo images without the head using light sheet microscopy. A red-dotted ellipse marks the posterior end of the embryos. White arrows mark the anterior and posterior ends of the ND. (D) Transverse sections of the posterior end of the IM in E9.5 embryos were stained for Pax-2 expression. left: *Wnt5a fl/+;T-Cre;Hoxb7-myr-venus-YFP* embryo at E9.5; right: *Wnt5a fl/Δ;T-Cre;Hoxb7-myr-venus-YFP* embryo at E9.5. A yellow rectangle marks the IM. (E) Enlarged image of the IM in (D).

Non-canonical *Wnt5a/Ror2* signaling regulates IM extension

Since *Wnt5a* can also activate β -catenin-dependent canonical Wnt signaling (22,23), we analyzed mutant and normal embryos

for T cell factor (TCF) activation using the *BATLacZ* mouse (24). β -Catenin activity was detected in both ND structures found in the abnormal posterior of the mutant (Supplementary Material, Fig. S9). However, TCF-dependent activity was not decreased in the *Wnt5a* mutant (Supplementary Material, Fig. S9), indicating that *Wnt5a* signaling in the IM is likely through a β -catenin-independent non-canonical Wnt mechanism. The orphan receptors, *Ror1* and *Ror2*, are established *Wnt5a* receptors and involved in the non-canonical Wnt/PCP signaling pathway (10). The phenotypes of *Ror2* null mice are similar to those of *Wnt5a* null mice showing dwarfism, facial abnormalities, short limbs and tails (16), suggesting that *Ror2* is also a receptor for *Wnt5a* signaling during embryonic development. Moreover, mutations in both *Ror2* (25) and *Wnt5a* (26) have been implicated in the rare genetic disease, Robinow syndrome, which exhibits some similar defects as found in the *Wnt5a* null mouse such as dwarfism and genital abnormalities (27). Therefore, *Ror2* may serve as a receptor for *Wnt5a* in IM morphogenesis. To test this, we generated mice that were null for *Ror2* and heterozygous for *Wnt5a*. In controls that were heterozygous for both *Wnt5a* and *Ror2*, single ureters were always observed; however, one-third of *Ror2* null mice already showed a duplex kidney phenotype (Fig. 5 and Table 1). With the additional deletion of a single copy of *Wnt5a*, the occurrence of the duplex kidney phenotype increased to >90% (Fig. 5B and Table 1). Furthermore, *Wnt5a* $+/-$; *Ror2* $-/-$ embryos at E9.5 manifested the same shortened IM (Fig. 6A) with abnormal posterior ND as the *Wnt5a* mutants (Fig. 6B and C). These results are consistent with the established relationship between *Wnt5a* and *Ror2* in the development of other mesodermal tissues, e.g. limb patterning (28).

DISCUSSION

Wnt5a controls the morphogenesis of several tissues by providing spatial limitations on cell positioning during tissue elongation. Its loss therefore typically yields shorter and wider structures due, at least in part, to the dysregulation of the non-canonical Wnt/PCP pathway (11,13,14). In the current study, *Wnt5a* ablation resulted in a shorter and wider ND, consistent with dysfunctional non-canonical Wnt signaling. The bifurcated ND affects subsequent metanephric development presumably through the permissive outgrowth of double UBs, resulting in duplex kidney formation and twin ureters. In other words, *Wnt5a* regulates not only outgrowth from the primary body axis (11), but also outgrowth of the UB from the ND by controlling IM extension. The exact mechanism of its activity is a major problem in the field. In our particular model, the dysmorphic posterior ND could stem from an aberrant orientation of cells during cell division, abnormal cell migration in the IM or abnormal intercalation of cells in the ND, behaviors consistent with a defective non-canonical Wnt/PCP pathway (29). These possibilities are currently under investigation.

During preparation of this manuscript, Nishita *et al.* (30) published an article with the title 'Role of *Wnt5a-Ror2* signaling in morphogenesis of the metanephric mesenchyme during ureteric budding'. Like us, they investigated a similar phenotype, i.e. duplicated ureters in the *Wnt5a*- and *Ror2* null mice. They suggested that dysregulated positioning of the MM causes a spatio-temporally aberrant interaction between the MM and ND,

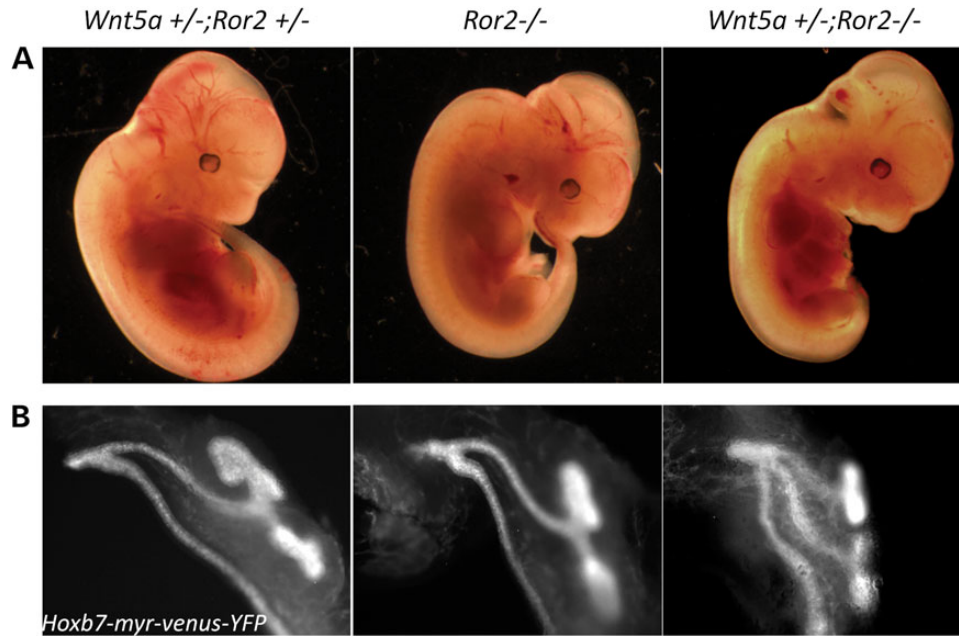


Figure 5. *Ror2* mediates *Wnt5a* signaling in duplex kidney formation. Embryos at E11.5 (A) and metanephros from the embryos (B) left: *Wnt5a* +/-;*Ror2* +/-; *Hoxb7-myr-venus-YFP*; middle: *Ror2* -/-;*Hoxb7-myr-venus-YFP*; right: *Wnt5a* +/-;*Ror2* -/-;*Hoxb7-myr-venus-YFP*.

Table 1. Frequency of the duplex kidney phenotype in *Ror2* mutants

Genotype	<i>Wnt5a</i> +/-; <i>Ror2</i> +/-	<i>Ror2</i> -/-	<i>Wnt5a</i> +/-; <i>Ror2</i> -/-
No. of embryos with duplex kidney(s)/no. of total embryos	0/53	8/24	24/26
Percentage (%)	0	33.3	92.3

providing the ND with inappropriate *Gdnf* signaling together with reduced proliferation in mutant MM, and thus resulting in ectopic UB induction. However, we found that early IM extension, especially abnormal duplication of the posterior ND, is the likely cause of duplex kidney formation in the *Wnt5a* mutants. As mentioned earlier, we also examined *Wnt5a* expression by RT-PCR of isolated UBs and MMs from the E11.5 metanephros. This was done because *Wnt5a* WISH data revealed almost no expression of *Wnt5a* in the metanephric region during initial UB formation, compared with its strong expression in the tail and limbs in a *gradient* manner. *Wnt5a* was very weakly expressed based on results from PCR amplification in the E11.5 metanephros in both UB and MM, in agreement with our WISH data. We could detect a *Wnt5a* PCR product at 43 reaction cycles, while *Gapdh* was detected at 27 reaction cycles using the same cDNA (Supplementary Material, Fig. S2). To determine if this expression is sufficient to induce double ureter formation, we produced *Wnt5a* mutants using several Cre lines which ablate *Wnt5a* in both the MM and UB, e.g. D111-Cre. However, *Wnt5a* mutants generated with D111-Cre do not show a double ureter phenotype, but their kidneys form normally except that they develop a hydronephrotic kidney phenotype at E16.5 (unpublished data). It was in fact this observation that prompted us to focus on earlier IM extension prior to

UB outgrowth. Furthermore, our time-dependent analysis of *Wnt5a* deletion clearly showed that only early deletion of *Wnt5a* can cause double ureter formation. *Wnt5a* mutants with tamoxifen injection at E7.5 formed duplex kidneys. However, *Wnt5a* mutants with tamoxifen injection even at E8.5 did not yield a duplex kidney phenotype (Fig. 3)—a finding consistent with abnormal IM morphogenesis.

The IM develops along the A–P axis, and *Wnt5a* is expressed both in the tail bud and in the posterior end of the IM, where we observed that it is distributed in a graded manner. Since the *Wnt5a* null mutant is truncated along the A–P axis, *Wnt5a* is regarded as a significant factor in A–P axis extension (11). We observed that the duplex kidney phenotype is always accompanied by truncation of the A–P axis in the *Wnt5a* mutants, suggesting that IM extension is controlled together with A–P axis extension. It is likely that both are affected by a *Wnt5a* gradient from early embryonic development, and that an IM extension defect is the result of an accumulation of dysmorphic events that may be maximal at the time ND reaches the cloaca, thus causing the abnormal ND duplication in the *Wnt5a* mutants. The relationship between the A–P axis and IM extension, however, needs further clarification.

In conclusion, our studies establish a role for *Wnt5a* in the regulation of IM extension and suggest that duplex kidneys result from the dysmorphogenesis of the posterior ND.

MATERIALS AND METHODS

Animal and tissue culture

Generation, maintenance and genotyping of *Wnt5a* *flox/flox* mice (*Wnt5a*^{tm1.1Tpy}) (31), *T-Cre* (17) mice, *Hoxb7/myr-Venus-YFP* (32), *BATLacZ* (24), *Ror2* (8) and *T-CreER*^{T2} (21) mice have been described previously (33). Noon on the day of vaginal

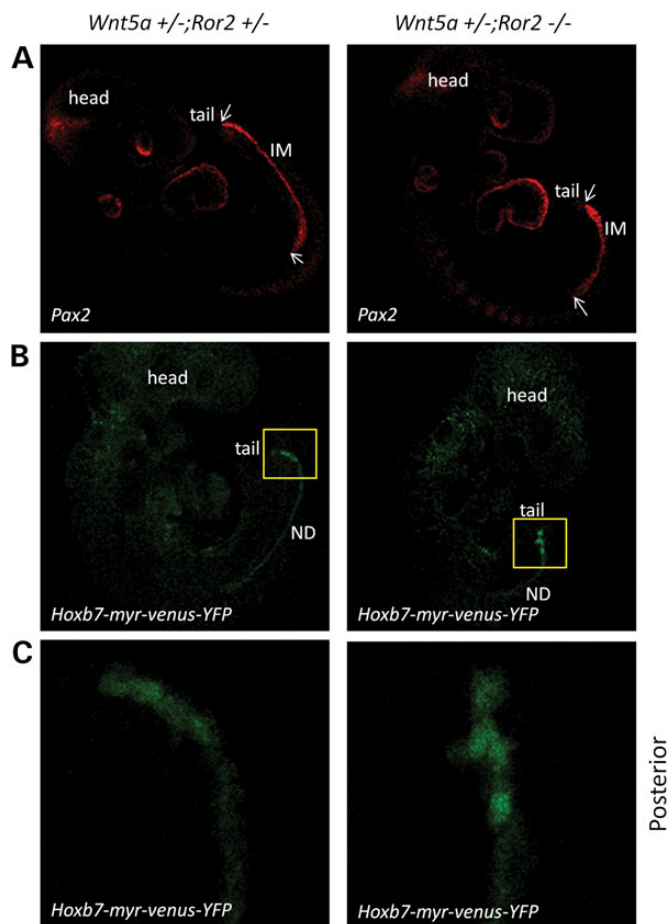


Figure 6. *Ror2* mutants also show abnormal IM and ND extension. (A) Embryos at E9.5 were stained with Pax-2 antibody. left: *Wnt5a*^{+/-};*Ror2*^{+/-}; right: *Wnt5a*^{+/-};*Ror2*^{-/-}. White arrows mark the anterior and posterior ends of the IM. (B) Embryos at E9.5. left: *Wnt5a*^{+/-};*Ror2*^{+/-};*Hoxb7-myr-venus-YFP*; right: *Wnt5a*^{+/-};*Ror2*^{-/-};*Hoxb7-myr-venus-YFP*. A yellow rectangle marks the posterior ends of the embryos. (C) Enlarged image of posterior ND in (B).

plug detection was designated E0.5. Mice were managed according to the NIH guidelines for the care and use of laboratory animals. Metanephric rudiments were dissected from E11.5 mouse embryos and were cultured on type IV collagen-coated filters, as previously described (17), in DMEM/F12 (50 : 50) with 10% fetal calf serum in 5% CO₂ at 37°C. For *BATLacZ* mice, X-gal staining was performed as previously described (24).

Tamoxifen injections and embryo analyses

About 1.0 mg of tamoxifen (Sigma, T-5648) was administered at either E7.5 or E8.5 by IP injection as previously described (34) without progesterone. The tamoxifen-injected mice were harvested at E11.5 and their kidneys were immunostained for calbindin, which labels the ureter and UB/collecting duct.

In situ hybridization and immunohistochemistry

Embryos were fixed and processed for WISH as described (35). Also, embryos were fixed and processed for paraffin section as

described (36). Deparaffinized tissues were probed with Pax-2 antibody (Abcam) at 1 : 50 and visualized using a Dako Cytomation kit according to the manufacturer's instructions.

Immunostaining

Calbindin staining of kidneys or kidney explant cultures was performed according to a protocol for immunostaining described in Kitagaki *et al.* (33) with some modifications. Briefly, dissected embryonic kidneys were fixed in ice-cold 100% methanol for 10 min followed by two brief washes in phosphate-buffered saline containing 0.1% Triton X-100 (PBST). All subsequent washes and incubations were carried out in PBST. The samples were blocked in 10% heat-inactivated sheep serum (Sigma, St Louis, MO, USA) for 2 h at room temperature, washed and incubated with a 1 : 1000 dilution of calbindin polyclonal antibody (Millipore, Billerica, MA, USA) overnight at 4°C. After three washes, they were incubated with Alexafluor 488 secondary antibodies (Invitrogen) at 1 : 2000 dilution overnight at 4°C. After extensive washes, the embryonic kidneys were photographed by fluorescence microscopy (Zeiss Axio Observer). Likewise, Pax2 staining of embryos or embryo transverse sections was performed with a 1 : 100 dilution of Pax2 polyclonal antibody (Invitrogen) and Alexafluor 568 secondary antibodies (Invitrogen) at 1 : 2000 dilution.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

ACKNOWLEDGEMENTS

We thank members of the Perantoni lab for their critical review of the manuscript. We also thank Dr Y. Minami (Department of Physiology and Cell Biology, Graduate School of Medicine, Kobe University, Japan) for providing the *Ror2* mouse line. We are grateful to C. Hubbard (SAIC) for excellent mouse husbandry. We thank Dr J. Zhu (Cancer and Developmental Biology Laboratory, NCI-Frederick) for help with studies involving tamoxifen-induced Cre activation. We thank OMAL for help with confocal microscope imaging and Zeiss for light sheet microscope imaging.

Conflict of Interest statement. None declared.

FUNDING

This work was supported by the National Institutes of Health, National Cancer Institute and Center for Cancer Research.

REFERENCES

1. Kume, T., Deng, K. and Hogan, B.L. (2000) Murine forkhead/winged helix genes *Foxc1* (*Mf1*) and *Foxc2* (*Mfh1*) are required for the early organogenesis of the kidney and urinary tract. *Development*, **127**, 1387–1395.
2. Whitten, S.M. and Wilcox, D.T. (2001) Duplex systems. *Prenat. Diagn.*, **21**, 952–957.
3. Obara-Ishihara, T., Kuhlman, J., Niswander, L. and Herzlinger, D. (1999) The surface ectoderm is essential for nephric duct formation in intermediate mesoderm. *Development*, **126**, 1103–1108.

4. Dressler, G.R. (2009) Advances in early kidney specification, development and patterning. *Development*, **136**, 3863–3874.
5. Costantini, F. (2012) Genetic controls and cellular behaviors in branching morphogenesis of the renal collecting system. *Wiley Interdiscip. Rev. Dev. Biol.*, **1**, 693–713.
6. Costantini, F. and Kopan, R. (2010) Patterning a complex organ: branching morphogenesis and nephron segmentation in kidney development. *Dev. Cell*, **18**, 698–712.
7. Saburi, S., Hester, I., Fischer, E., Pontoglio, M., Eremina, V., Gessler, M., Quaggin, S.E., Harrison, R., Mount, R. and McNeill, H. (2008) Loss of Fat4 disrupts PCP signaling and oriented cell division and leads to cystic kidney disease. *Nat. Genet.*, **40**, 1010–1015.
8. Takeuchi, S., Takeda, K., Oishi, I., Nomi, M., Ikeya, M., Itoh, K., Tamura, S., Ueda, T., Hatta, T., Otani, H. *et al.* (2000) Mouse Ror2 receptor tyrosine kinase is required for the heart development and limb formation. *Genes Cells*, **5**, 71–78.
9. Lyashenko, N., Weissenbock, M., Sharir, A., Erben, R.G., Minami, Y. and Hartmann, C. (2010) Mice lacking the orphan receptor ror1 have distinct skeletal abnormalities and are growth retarded. *Dev. Dyn.*, **239**, 2266–2277.
10. Kikuchi, A., Yamamoto, H., Sato, A. and Matsumoto, S. (2012) Wnt5a: its signalling, functions and implication in diseases. *Acta Physiol. (Oxf.)*, **204**, 17–33.
11. Yamaguchi, T.P., Bradley, A., McMahon, A.P. and Jones, S. (1999) A Wnt5a pathway underlies outgrowth of multiple structures in the vertebrate embryo. *Development*, **126**, 1211–1223.
12. Cha, K.B., Douglas, K.R., Potok, M.A., Liang, H., Jones, S.N. and Camper, S.A. (2004) WNT5A signaling affects pituitary gland shape. *Mech. Dev.*, **121**, 183–194.
13. Cervantes, S., Yamaguchi, T.P. and Hebrok, M. (2009) Wnt5a is essential for intestinal elongation in mice. *Dev. Biol.*, **326**, 285–294.
14. Qian, D., Jones, C., Rzadzinska, A., Mark, S., Zhang, X., Steel, K.P., Dai, X. and Chen, P. (2007) Wnt5a functions in planar cell polarity regulation in mice. *Dev. Biol.*, **306**, 121–133.
15. Sinha, T., Wang, B., Evans, S., Wynshaw-Boris, A. and Wang, J. (2012) Disheveled mediated planar cell polarity signaling is required in the second heart field lineage for outflow tract morphogenesis. *Dev. Biol.*, **370**, 135–144.
16. Oishi, I., Suzuki, H., Onishi, N., Takada, R., Kani, S., Ohkawara, B., Koshida, I., Suzuki, K., Yamada, G., Schwabe, G.C. *et al.* (2003) The receptor tyrosine kinase Ror2 is involved in non-canonical Wnt5a/JNK signalling pathway. *Genes Cells*, **8**, 645–654.
17. Perantoni, A.O., Timofeeva, O., Naillat, F., Richman, C., Pajni-Underwood, S., Wilson, C., Vainio, S., Dove, L.F. and Lewandoski, M. (2005) Inactivation of FGF8 in early mesoderm reveals an essential role in kidney development. *Development*, **132**, 3859–3871.
18. Grieshammer, U., Le, M., Plump, A.S., Wang, F., Tessier-Lavigne, M. and Martin, G.R. (2004) SLIT2-mediated ROBO2 signaling restricts kidney induction to a single site. *Dev. Cell*, **6**, 709–717.
19. Michos, O., Goncalves, A., Lopez-Rios, J., Tiecke, E., Naillat, F., Beier, K., Galli, A., Vainio, S. and Zeller, R. (2007) Reduction of BMP4 activity by gremlin 1 enables ureteric bud outgrowth and GDNF/WNT11 feedback signalling during kidney branching morphogenesis. *Development*, **134**, 2397–2405.
20. Basson, M.A., Akbulut, S., Watson-Johnson, J., Simon, R., Carroll, T.J., Shakya, R., Gross, I., Martin, G.R., Lufkin, T., McMahon, A.P. *et al.* (2005) Sprouty1 is a critical regulator of GDNF/RET-mediated kidney induction. *Dev. Cell*, **8**, 229–239.
21. Anderson, M.J., Naiche, L.A., Wilson, C.P., Elder, C., Swing, D.A. and Lewandoski, M. (2013) TCreERT2, a transgenic mouse line for temporal control of Cre-mediated recombination in lineages emerging from the primitive streak or tail bud. *PLoS ONE*, **8**, e62479.
22. van Amerongen, R., Fuerer, C., Mizutani, M. and Nusse, R. (2012) Wnt5a can both activate and repress Wnt/beta-catenin signaling during mouse embryonic development. *Dev. Biol.*, **369**, 101–114.
23. Najdi, R., Proffitt, K., Sprowl, S., Kaur, S., Yu, J., Covey, T.M., Virshup, D.M. and Waterman, M.L. (2012) A uniform human Wnt expression library reveals a shared secretory pathway and unique signaling activities. *Differentiation*, **84**, 203–213.
24. Nakaya, M.A., Biris, K., Tsukiyama, T., Jaime, S., Rawls, J.A. and Yamaguchi, T.P. (2005) Wnt3a links left-right determination with segmentation and anteroposterior axis elongation. *Development*, **132**, 5425–5436.
25. Afzal, A.R., Rajab, A., Fenske, C.D., Oldridge, M., Elanko, N., Ternes-Pereira, E., Tuysuz, B., Murday, V.A., Patton, M.A., Wilkie, A.O. *et al.* (2000) Recessive Robinow syndrome, allelic to dominant brachydactyly type B, is caused by mutation of ROR2. *Nat. Genet.*, **25**, 419–422.
26. Person, A.D., Beiraghi, S., Sieben, C.M., Hermanson, S., Neumann, A.N., Robu, M.E., Schleiffarth, J.R., Billington, C.J. Jr, van Bokhoven, H., Hooeboom, J.M. *et al.* (2010) WNT5A mutations in patients with autosomal dominant Robinow syndrome. *Dev. Dyn.*, **239**, 327–337.
27. Patton, M.A. and Afzal, A.R. (2002) Robinow syndrome. *J. Med. Genet.*, **39**, 305–310.
28. Gao, B., Song, H., Bishop, K., Elliot, G., Garrett, L., English, M.A., Andre, P., Robinson, J., Sood, R., Minami, Y. *et al.* (2011) Wnt signaling gradients establish planar cell polarity by inducing Vangl2 phosphorylation through Ror2. *Dev. Cell*, **20**, 163–176.
29. Gray, R.S., Roszko, I. and Solnica-Krezel, L. (2011) Planar cell polarity: coordinating morphogenetic cell behaviors with embryonic polarity. *Dev. Cell*, **21**, 120–133.
30. Nishita, M., Qiao, S., Miyamoto, M., Okinaka, Y., Yamada, M., Hashimoto, R., Iijima, K., Otani, H., Hartmann, C., Nishinakamura, R. *et al.* (2014) Role of Wnt5a-Ror2 signaling in morphogenesis of the metanephric mesenchyme during ureteric budding. *Mol. Cell. Biol.*, **34**, 3096–3105.
31. Miyoshi, H., Ajima, R., Luo, C.T., Yamaguchi, T.P. and Stappenbeck, T.S. (2012) Wnt5a potentiates TGF-beta signaling to promote colonic crypt regeneration after tissue injury. *Science*, **338**, 108–113.
32. Chi, X., Hadjantonakis, A.K., Wu, Z., Hyink, D. and Costantini, F. (2009) A transgenic mouse that reveals cell shape and arrangement during ureteric bud branching. *Genesis*, **47**, 61–66.
33. Kitagaki, J., Ueda, Y., Chi, X., Sharma, N., Elder, C.M., Truffer, E., Costantini, F., Lewandoski, M. and Perantoni, A.O. (2011) FGF8 is essential for formation of the ductal system in the male reproductive tract. *Development*, **138**, 5369–5378.
34. Nguyen, M.T., Zhu, J., Nakamura, E., Bao, X. and Mackem, S. (2009) Tamoxifen-dependent, inducible Hoxb6CreERT recombinase function in lateral plate and limb mesoderm, CNS isthmus organizer, posterior trunk neural crest, hindgut, and tailbud. *Dev. Dyn.*, **238**, 467–474.
35. Wang, H., Yang, Y., Sharma, N., Tarasova, N.I., Timofeeva, O.A., Winkler-Pickett, R.T., Tanigawa, S. and Perantoni, A.O. (2010) STAT1 activation regulates proliferation and differentiation of renal progenitors. *Cell. Signal.*, **22**, 1717–1726.
36. Yun, K., Choi, Y.D., Nam, J.H., Park, Z. and Im, S.H. (2007) NF-kappaB regulates Lef1 gene expression in chondrocytes. *Biochem. Biophys. Res. Commun.*, **357**, 589–595.