

FIG. 4 Secondary axis formation. *a*, Incidence of ectopic somite and notochord formation in embryos injected with *Xlim-1* mutant *3m*, *gsc*, or both. Open bars, ectopic somite; grey bars, one ectopic notochord; black bar, two ectopic notochords. The *3m*-induced secondary axes also contained a high incidence of neural tissue and cement gland (not shown). The total number examined was 28 (globin), 100 (3m), 72 (gsc) and 39 (3m/gsc). *b*, Example of two ectopic notochords formed in an embryo coinjected with *3m* and *gsc* RNAs, as visualized by whole-mount staining with the notochord-specific antibody Tor70²⁷ at stage 27/28. Dorsal view, anterior is up; arrows indicate ectopic notochords.

METHODS. Embryos were injected with RNA into two blastomeres at the 4-cell stage in the ventral equatorial region. The amount of RNA was 0.5–1.75 (globin), 0.5–1.0 (3m) and 0.38–0.75 (gsc) ng per embryo. In coinjection of *3m* and *gsc*, the ratio of *3m* to *gsc* was 1 to 0.75. At tailbud stages, embryos were analysed by whole-amount immunostaining²⁸ after bleaching with 10% H₂O₂. In some injected embryos, blastopore closure was incomplete, probably due to gastrulation arrest; such embryos were discarded.

are displayed by *gsc* when its mRNA is injected into the ventral equatorial region^{3,14}, but not in animal ectodermal cells (ref. 15 and our results). Cells expressing *3m* send inducing signals to adjacent cells (Fig. 2); therefore, it is likely that LIM domain mutants of *Xlim-1* activate genes encoding neural- and muscle-inducing factors. These putative molecules appear to be distinct from noggin or follistatin (Fig. 3*a*), implicating new signalling factors in organizer function. □

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Epithelial transformation of metanephric mesenchyme in the developing kidney regulated by *Wnt-4*

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THE kidney has been widely exploited as a model system for the study of tissue inductions regulating vertebrate organogenesis^{1,2}. Kidney development is initiated by the ingrowth of the Wolfian duct-derived ureteric bud into the presumptive kidney mesenchyme. In response to a signal from the ureter, mesenchymal cells condense, aggregate into pretubular clusters and undergo an epithelial conversion generating a simple tubule. This then undergoes morphogenesis and is transformed into the excretory system of the kidney, the nephron. We report here that the expression of *Wnt-4*, which encodes a secreted glycoprotein, correlates with, and is required for, kidney tubulogenesis. Mice lacking *Wnt-4* activity fail to form pretubular cell aggregates; however, other aspects of mesenchymal and ureteric development are unaffected. Thus, *Wnt-4* appears to act as an autoinducer of the mesenchyme to epithelial transition that underlies nephron development.

Wnt-signalling is thought to play diverse roles, regulating pattern, cell fate choices and mitotic activity in different embryonic tissues. To address the possibility that *Wnt*-family members may participate in development of the vertebrate kidney, we examined expression of 12 mouse *Wnt* genes by *in situ* hybridization (data not shown). One of these, *Wnt-4*, was expressed in the kidney mesenchyme and its derivatives. *Wnt-4* messenger RNA was detected in condensed mesenchymal cells on both sides of the stalk of the ureter at 11.5 days postcoitum (d.p.c.) and correlated to the site where the first pretubular cell aggregates form² (Fig. 1*a–c*). During subsequent development, this expression pattern was repeated in newly forming aggregates, as well as their simple epithelial tubular derivatives (Fig. 1*d–f*).

Wnt-4 expression was also detected in comma-shaped bodies, but later became restricted in descendent S-shaped bodies to the region where epithelial fusion was occurring with the collecting duct, and was lost after fusion was completed (Fig. 1*e, f* and data not shown). At later stages, *Wnt-4* expression became confined to the periphery of the kidney where new tubules were forming (Fig. 1*f*). Interestingly, in both the chick (Fig. 1*g*) and mouse³ (data not shown), the dorsal spinal cord, a potent inducer of kidney tubule differentiation^{1,2}, also expressed *Wnt-4*.

Development of the metanephric kidney is preceded by the formation of a mesonephric kidney. In the mouse, this is a vestigial structure, whereas in avian species, it is an elaborate functional organ during embryonic life. *Wnt-4* was expressed throughout mesonephric tubulogenesis from the aggregating mesenchyme until fusion of the epithelial tubules with the Wolfian (mesonephric) duct (Fig. 1*g, h*). Thus, it is likely that the role of *Wnt-4* is conserved in the development of the mesonephric and metanephric kidneys.

To examine *Wnt-4* function, we used gene targeting in mouse embryonic stem (ES) cells to replace the third coding exon of the *Wnt-4* gene with a selection cassette containing neomycin phosphotransferase (neo) (Fig. 2), thereby generating a likely

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null allele. Adult mice heterozygous for this disrupted allele showed no obvious phenotype. However, homozygous pups died within 24 h of birth. As *Wnt-4*^{-/-} embryos were recovered at the expected mendelian frequency at late stages of gestation (data not shown), *Wnt-4* is not essential for *in utero* development. All neonatal and 18.5 d.p.c. *Wnt-4*^{-/-} embryos contained small agenic kidneys (compare with adjoining adrenal gland in Fig. 3a, b), consisting of undifferentiated mesenchyme interspersed with branches of collecting duct epithelium (Fig. 3c-f). Thus, death of homozygous pups is almost certainly due to the lack of kidney function.

To address the role of *Wnt-4*, we analysed kidney development in mutant embryos. As expected, initial development of the primary condensate around the tips of the ureteric bud appeared histologically normal (Fig. 3g, h), suggesting that induction of kidney mesenchyme by the ureter was unaffected. Early on the 15th day of pregnancy, no gross difference was observed in the size of kidneys between *Wnt-4*^{+/+} and *Wnt-4*^{-/-} embryos (Fig. 3i-l). Like their normal litter mates, mutant embryos had undergone considerable branching of the ureteric epithelium. However, the kidney mesenchyme remained morphologically undifferentiated, lacking pretubular aggregates and more developed tubules (Fig. 3j, l). Later on the same day, *Wnt-4*^{-/-} kidneys were clearly growth retarded (Fig. 3m, n) and the mesenchyme persisted in a morphologically undifferentiated state (data not shown). Three out of ten *Wnt-4*^{-/-} embryos

examined on the 15th day of pregnancy showed no histological evidence of mesenchymal aggregation. In the remainder, a few poorly developed aggregates were observed (less than 10% of the number of aggregates scored in *Wnt-4*^{+/+} or *Wnt-4*^{-/-} litter mates). We never observed comma-shaped or S-shaped bodies.

Kidney development was further investigated by the analysis of a variety of molecular markers. Presumptive and induced kidney mesenchyme, as well as differentiating glomerulae, express the *Wilms tumor* (*WT-I*) before mesenchymal condensation^{4,5}. Initial expression of *WT-I* was unchanged in *Wnt-4*^{-/-} embryos (Fig. 4a, b), persisting until at least 14.5 d.p.c. However, throughout this period, expression was only observed in the mesenchyme (Fig. 4c, d). *N-Myc*, an early marker of tubule induction, is first detected in a subpopulation of condensed mesenchyme and is then upregulated in epithelial structures which presumably arise from these cells⁶. *Pax2* has also been suggested to regulate induction and epithelial conversion of the kidney mesenchyme^{7,8}. *N-Myc* and *Pax2* were expressed until at least 14.5 d.p.c. in *Wnt-4*^{-/-} embryos; however, no expression was detected in tubules (Fig. 4e-l). These data suggest that the mesenchymal cells fated to form tubules were induced but failed to convert into epithelial structures.

Failure of pretubular aggregation is also supported by analysis of *Wnt-4* and *Pax8* expression. Although *Wnt-4* expression appears to precede that of *Pax8* (data not shown) both genes share an identical distribution in the pretubular aggregate and

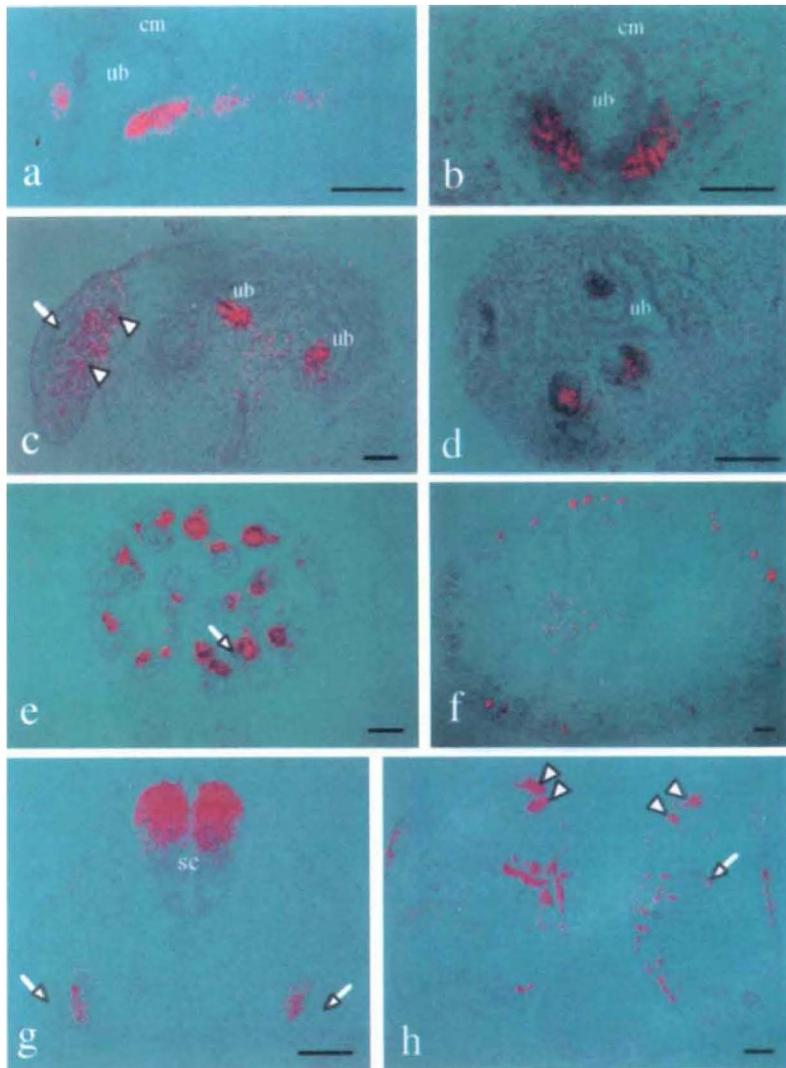


FIG. 1 Expression of *Wnt-4* during morphogenesis of the metanephric kidney of the mouse and mesonephric kidney of the chick. *In situ* hybridization with ³⁵S mRNA probes was used to detect mouse (a-f) and chick (g, h) *Wnt-4* mRNA. a, In the metanephric kidney *Wnt-4* expression is first seen at 11.5 d.p.c., in condensed mesenchymal (cm) cells on both sides of the invading ureteric bud (ub), preceding the formation of the first pretubular aggregates. b, A transverse section through a similar stage kidney to that shown in a. c, *Wnt-4* expression becomes restricted to pretubular aggregates on initiation of ureteric bud branching (12.5 d.p.c.). Expression is also seen in the mesonephric mesenchyme (arrowheads), adjacent to the mesonephric duct (arrow). d, By 13.5 d.p.c., *Wnt-4* expression is apparent in primitive tubular aggregates. e, On subsequent branching of the ureter (14.5 d.p.c.), *Wnt-4* expression is induced in the assembling aggregates. Expression is also seen in comma-shaped bodies (arrow), and more advanced stages of tubular morphogenesis. f, Later during development (16.5 d.p.c.) *Wnt-4* expression is confined to the cortex of the kidney where new tubular aggregates are still forming. g, In the stage 20 chick embryo, *Wnt-4* expression is induced in the mesonephric mesenchyme of the mesonephros, adjacent to the mesonephric duct (arrow). *Wnt-4* is also expressed in a graded distribution in the dorsal half of the spinal cord (sc). h, At stage 27–29, development of the mesonephric kidney is advanced and *Wnt-4* expression is apparent in early stage aggregates (arrowheads), as well as in more advanced stages. By the time that the tubules fuse with the mesonephric duct, *Wnt-4* expression becomes restricted to the tip of the tubule at the point of fusion (arrow) and is lost after fusion. Scale bars, 10 µm (a-c, g, h); 100 µm (f).

METHODS. *In situ* hybridization analysis was according to published procedures²⁴ using a *Wnt-4* mRNA probe³. The chick *Wnt-4* cDNA consisted of a roughly 400 base pair fragment generated by polymerase chain reaction (J. McMahon and A.P.M., unpublished data).

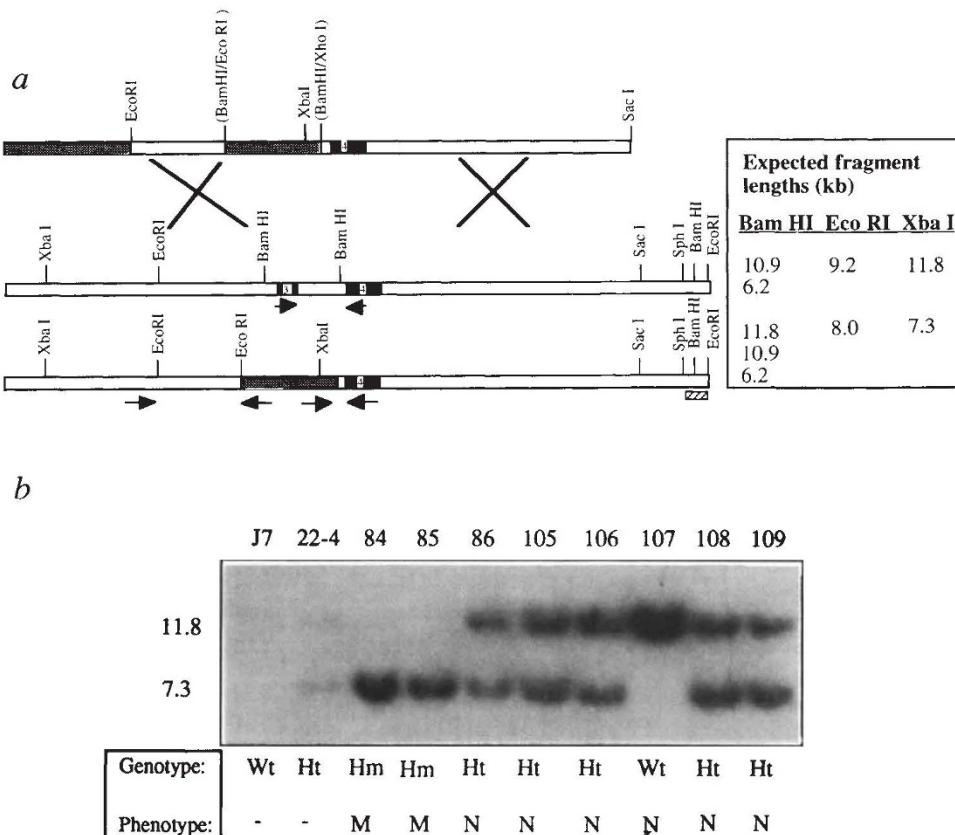
developing tubule⁹ (compare Fig. 4*o* and *s*). In mutant embryos, expression of both of these genes was never detected in kidneys scored as completely negative for tubule formation by histological analysis and was only detected in the rare case of a small and poorly organized aggregate (Fig. 4*m–t*). As normal *Wnt-4* expression was observed in several tissues that were not overtly affected in *Wnt-4*^{-/-} embryos (floorplate, spinal cord and mesonephric mesenchyme; Fig. 4*n* and data not shown), it is clear that the targeted allele was not transcribed. Thus, the failure to detect cells initiating *Wnt-4* expression suggests that *Wnt-4* may be required for full activation of its own expression in the kidney mesenchyme. Alternatively, cells lacking *Wnt-4* activity might rapidly adopt another fate. Further, *Wnt-4* is required,

directly or indirectly, for the activation and/or maintenance of *Pax8* expression. Activation of *Pax-8*, a paired domain containing transcription factor, may be critical for the regulation of other genes essential for tubule formation.

Kidney development is critically dependent on the ureter. For example, mice lacking *c-ret* activity, a receptor protein tyrosine kinase normally expressed in the ureteric bud¹⁰, die because of complete or partial failure of kidney development¹¹. Expression of *c-ret* and *c-ros*, a second receptor tyrosine kinase transcribed in the ureteric epithelium^{12,13}, was activated and maintained in the tips of the ureteric bud in mutant kidneys (Fig. 4*u–y*; data not shown). This result, together with the observed branching of the ureter, supports the conclusion that development of the

FIG. 2 Gene targeting at the *Wnt-4* locus. *a*, Schematic representation of the *Wnt-4* targeting vector (upper), *Wnt-4* locus (middle) and expected recombination event (lower). Successful gene replacement by the targeting vector results in the deletion of roughly 2.2 kb of *Wnt-4* genomic sequence, including all of the coding sequence derived from exon 3 (amino acids 106 to 196 in the predicted *Wnt-4* protein sequence²⁵), and insertion of a *PGK-neo* selection cassette²⁶, in the same transcriptional orientation as that of the *Wnt-4* gene. The arrows indicate PCR primers, and the hatched box a DNA probe used to identify putative targeted ES cell clones. *b*, Southern blot analysis of gene targeting at the *Wnt-4* locus and transmission of the targeted allele. An 11.8 kb *Xba*I fragment, indicative of the wild type *Wnt-4* allele, is detected in the parental CJ7 ES cell line²⁷. An additional fragment of 7.3 kb, corresponding to the targeted allele, is detected in DNA from clone 22-4. Additional Southern analysis with *Bam*H and *Eco*RI digests, and with a 5' flanking probe, confirmed that the predicted targeting event occurred in clone 22-4 (data not shown). Lanes 84 to 109 represent selected DNA samples from 18.5 d.p.c. progeny derived from intercrosses between mice heterozygous for the targeted *Wnt-4* allele. Agenesis of the kidney was apparent in all homozygous (Hm) embryos at this stage. No phenotype was observed in heterozygous (Ht) or wild-type (Wt) littermates.

METHODS. The gene targeting construct, containing 1.6 kb of 5' and 4.5 kb of 3' homology, was transfected into the CJ7 ES cell line²⁷ and clones selected using positive (*PGK-neo* cassette²⁶) and negative (*MC1TK* cassette²⁸) selection with G418 and FIAU, respectively, as described previously²⁹. Surviving clones were screened for homologous recombination by PCR using a 5' oligonucleotide homologous to sequences in intron 2 of *Wnt-4* (5'-GCGTTCTGCCTCCCTCTGCCGGGG-3'), which lies just upstream of the 5' homology region in the targeting construct, and a 3' oligonucleotide (5'-GGGAGCCGGTGGC-GCTACCGGTGG-3') homologous to PGK promoter sequences in the *PGK-neo* cassette (arrows in *a*). Sample preparation and PCR amplification was as described previously²⁹, except that the initial PCR was performed on pools of 5 samples. Gene replacement at the *Wnt-4* locus leads to the amplification of a 1.6 kb 5' homology region. Because a band of the expected size was not observed with ethidium bromide staining, the PCR products were transferred to nylon membranes and hybridized with a ³²P-labelled oligonucleotide homologous to sequences within the amplified region (5'-GCTGAGTGGCTAGAGCCCCAG-3'). One positive pool



(22) was identified; on further analysis, one sample of the five clones in this pool was PCR positive. The overall targeting frequency was ~1 in 200 clones which survived double selection. DNA was prepared from this clone (22-4) and analysed for correct targeting by Southern blot analysis using both 5' and 3' single copy probes and multiple diagnostic restriction enzyme digests. Having verified that homologous recombination had occurred as expected, 22-4 cells were used to generate chimeras and germ-line transmission of the targeted allele was monitored by Southern analysis using the indicated probe (hatched box in *a*). All subsequent genotyping was done by Southern blot or by PCR. The wild-type *Wnt-4* allele was detected by PCR using a 5' oligonucleotide (5'-CTTCACAAACGAGGGCTGGCAGG-3') and a 3' oligonucleotide (5'-CACCCGCAATGTTGTCAGATGG-3') homologous to nucleotides 655–679 in exon 3 and 683–706 in exon 4 of the *Wnt-4* cDNA²⁵. The targeted *Wnt-4* allele was identified by using a 3' primer homologous to sequences within the *PGK-neo* cassette (5'-GCATTGTCTGAGT-AGGTGTCATTC-3') and the *Wnt-4* exon 4 primer described above. PCR amplifies fragments of about 400 bp (targeted allele) and 700 bp (wild-type allele). PCR was done for 45 cycles of: 1 min at 93 °C, 65 °C for 30 s, 72 °C for 30 s, followed by 5 min at 72 °C.

ureteric epithelium, at these stages, is independent of both *Wnt-4* expression and tubule formation.

Up to the 15th day of pregnancy, there is no visible decrease in size or increase in cell death in mutant kidneys. This period is normally marked by a general decrease in cell proliferation in developing tubules². Thus, it seems unlikely that *Wnt-4* regulates cell growth or survival. Together, our evidence is most consistent with a model in which *Wnt-4* acts as an autoactivator of the mesenchymal to epithelial transition. Thus, initiation of *Wnt-4* expression in a cluster of induced cells leads to these same cells forming the pretubular aggregate. Continued expression of *Wnt-4* suggests an additional role in later stages of tubule morphogenesis which cannot be addressed at present.

Clearly, some mechanism must account for the discrete localization of *Wnt-4* to the aggregates. One possibility is that induction of mesenchyme by the ureter leads to both the activation of *Wnt-4* expression and the competence to respond to this signal. Thus, only those cells in the distal mesenchyme that were previously in contact with the ureter are induced to aggregate by a *Wnt-4* signal. Indeed, aggregating cells are unable to elicit tubule formation in uninduced nephrogenic mesenchyme¹⁴. Alternatively, aggregating mesenchymal cells may inhibit neighbouring cells from adopting the same fates.

One likely step in the mesenchyme to epithelial transition is regulation of cell adhesion. Interestingly, ectopic expression of *Wnt-4* (R. Moon, personal communication) and *Wnt-5a*, in the *Xenopus* embryo¹⁵, may increase cell adhesiveness. Moreover, *armadillo*, which is a *Drosophila* homologue of beta-catenin, a cytoplasmic protein essential for E-cadherin-mediated adhesion^{16,17}, is required for *wingless* (the *Drosophila* orthologue of *Wnt-1*) signalling^{18,19}. *Wnt-1* has been shown to modulate cell adhesion *in vitro*^{20,21}, and the expression of the cell adhesion molecule, E-cadherin *in vivo*²². Interestingly, E-cadherin is expressed in the early kidney tubule, after activation of *Wnt-4* and *Pax-8* expression²³. Hence, it is tempting to speculate that

FIG. 4 Expression of mesenchymal and epithelial marker genes in kidneys of *Wnt-4* mutants. *WT-1* is expressed in the undifferentiated nephrogenic mesenchyme (nm) at 12.5 (a) and 14.5 (c) d.p.c. in *Wnt-4*^{+/+} or ^{+/-} embryos. Expression is also seen in early and late stages of tubule morphogenesis at 14.5 d.p.c. (arrowheads in c). *WT-1* expression in the kidneys of *Wnt-4*^{-/-} embryos is restricted to morphologically undifferentiated mesenchyme (b, d). *N-Myc* expression at 12.5 d.p.c. localizes to a subset of induced mesenchyme in kidneys from wild-type (e) and homozygous mutant embryos (f). Expression is also seen in more advanced stages of tubule development in the normal kidney (g), but not in kidneys from *Wnt-4*^{-/-} embryos (h). *Pax2* shows complex expression, first in the invading ureteric epithelium and condensed mesenchyme (i), then also in comma- and S-shaped bodies (arrowheads), and proximal and distal collecting duct (cd) epithelium, at 14.5 d.p.c. (k). Expression is unchanged in the ureter and kidney mesenchyme of *Wnt-4* mutants at 12.5 d.p.c. (l), and extends into the collecting duct epithelium at 14.5 d.p.c. (l); however, epithelial expression is limited to ureteric derivatives. *Wnt-4* (m-p) and *Pax-8* (q-t) are both expressed in pre- and early tubular aggregates (m, q), as well as in comma- and S-shaped bodies (o, s). In *Wnt-4* mutants only occasional expression of either gene is seen in the kidney and expression is restricted to poorly developed aggregates (arrowed in p and t). o and s are adjacent sections. *Wnt-4* expression in the central nervous system and mesonephric mesenchyme (ms in n) is unaltered in *Wnt-4*^{-/-} embryos. Expression of *c-ret* localizes to the tips of the ureter at 12.5 (u) and 14.5 (x) d.p.c. In *Wnt-4* mutants, expression persists at the tips of the newly branched ureter at 12.5 (v) and until at least 14.5 d.p.c. (y), suggesting that development of the ureteric bud is not dependent on *Wnt-4*. Scale bar, 10 µm.

METHODS. *In situ* hybridization to embryo sections was as described²⁴. The probes used were as described: *WT-1*⁵, *N-myc*³⁰, *Pax-2*⁷, *Wnt-4*³, *Pax-8*⁹, *c-Ret*¹⁰.

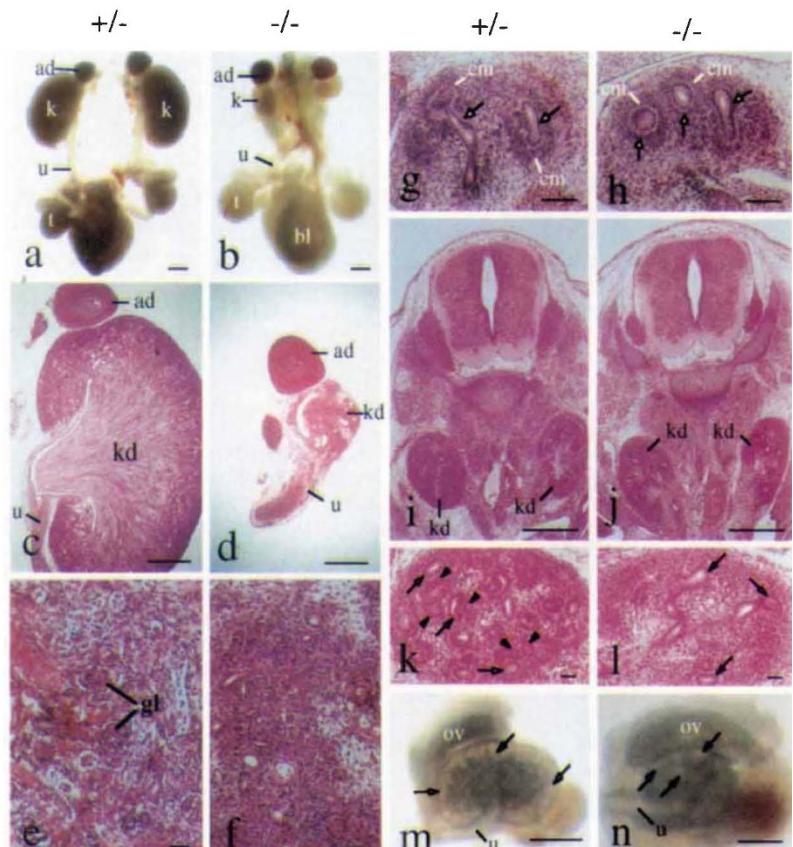
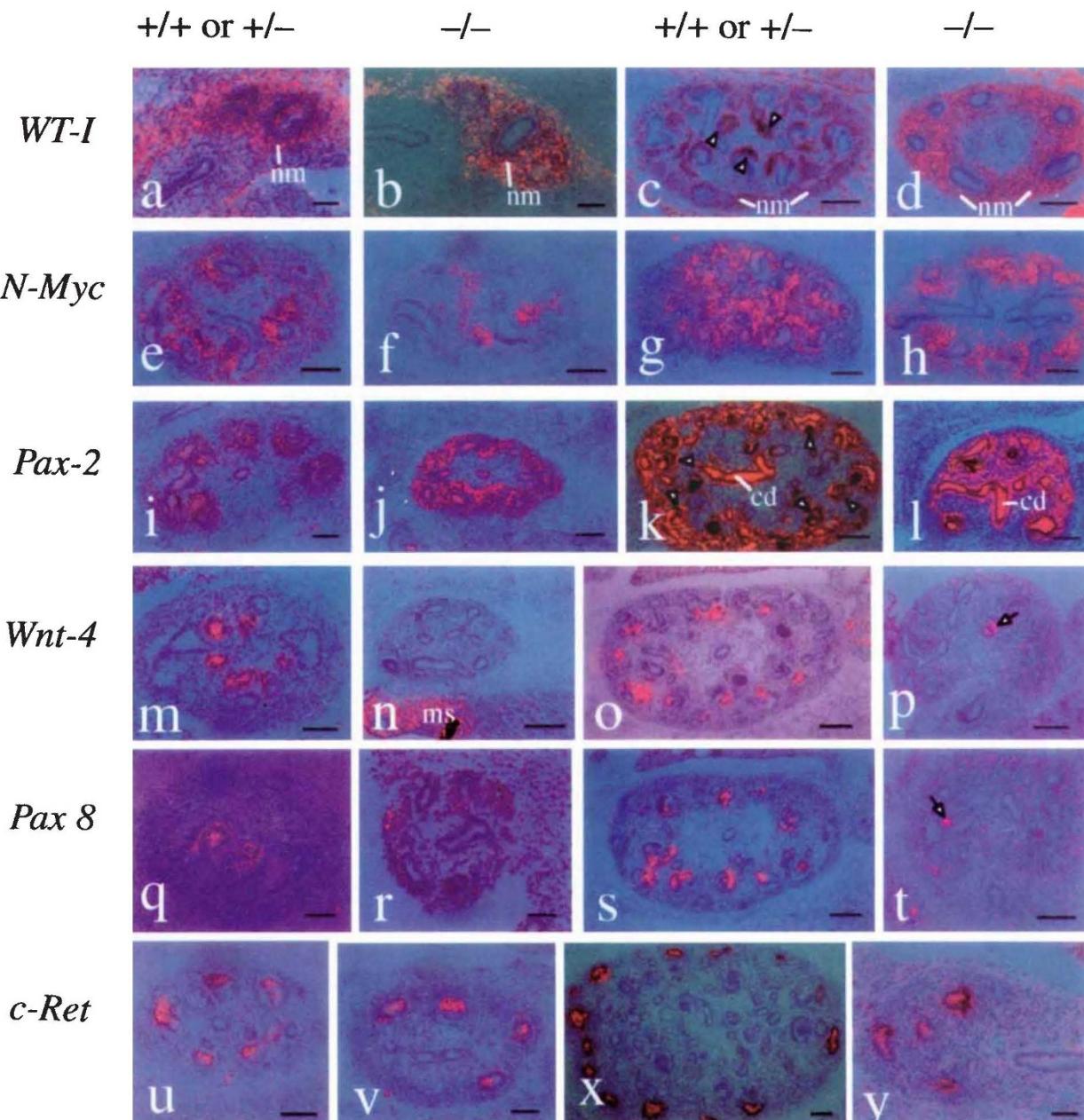


FIG. 3 Histological analysis of kidney development in *Wnt-4* mutant embryos. Whole-mount views (a, b, m, n) and histological sections (c–l) of kidneys from embryos taken at 18.5 d.p.c. (a–f), 12.5 d.p.c. (g, h), and early (i–l) and late (m, n) on the 15th day of pregnancy. e, f, High-power views of c and d, respectively. k, l, High-power views of i and j, respectively. For description, see text. Arrows indicate the invading and branching ureteric epithelium; arrowheads, epithelial tubular aggregates. ad, Adrenal gland; bl, bladder; cm, condensed mesenchyme; gl, glomerulus; kd, kidney; ov, ovary; t, testis; u, ureter. Scale bars, e, f, g, h, k, l, 10 µm; a–d, i, j, m, n, 100 µm. METHODS. Embryos were collected into PBS, a sample of the yolk sac or liver was removed for genotyping and the remaining embryo, or isolated kidneys, were fixed in Bouin's fixative. Samples were dehydrated, embedded in wax and sectioned at 3 to 6 µm. Sections were dewaxed, rehydrated and stained with haematoxylin and eosin.



Wnt-4 may regulate mesenchymal aggregation and tubule formation through modulation of cell adhesion factors. □

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