The prepattern transcription factor *Irx3* directs nephron segment identity

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The nephron, the basic structural and functional unit of the vertebrate kidney, is organized into discrete segments, which are composed of distinct renal epithelial cell types. Each cell type carries out highly specific physiological functions to regulate fluid balance, osmolarity, and metabolic waste excretion. To date, the genetic basis of regionalization of the nephron has remained largely unknown. Here we show that *Irx3*, a member of the Iroquois (Irx) gene family, acts as a master regulator of intermediate tubule fate. Comparative studies in *Xenopus* and mouse have identified *Irx1*, *Irx2*, and *Irx3* as an evolutionary conserved subset of Irx genes, whose expression represents the earliest manifestation of intermediate compartment patterning in the developing vertebrate nephron discovered to date. Intermediate tubule progenitors will give rise to epithelia of Henle's loop in mammals. Loss-of-function studies indicate that *irx1* and *irx2* are dispensable, whereas *irx3* is necessary for intermediate tubule formation in *Xenopus*. Furthermore, we demonstrate that misexpression of *irx3* is sufficient to direct ectopic development of intermediate tubules in the *Xenopus* mesoderm. Taken together, *irx3* is the first gene known to be necessary and sufficient to specify nephron segment fate in vivo.

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Vertebrate kidneys are organs of complex structure and function. They are derived from the intermediate mesoderm in a process involving inductive interactions, mesenchyme-to-epithelium transitions, and branching morphogenesis that ultimately leads to the development of a fully mature excretory organ composed of thousands of nephrons, which are the functional units of the kidney (Saxén 1987). Along the proximodistal axis, each nephron is organized into proximal tubule, intermediate tubule, and distal tubule, which will connect with the collecting duct system. Each tubule can be further subdivided into separate segments based on histological criteria (Kriz and Bankir 1988). The segments are composed of specialized epithelial cell types with unique functional properties, such as solute transport, pH regulation, and water absorption. Importantly, the segments do not operate independently but rely on the correct spatial organization along the nephron to insure normal excretory functions. Absence of specific tubular segments, such as the proximal convoluted tubules, has been observed in man and usually causes stillbirth or neonatal lethality (Allanson et al. 1992). In recent years, major insights into transcription factors and signaling pathways that control the early stages of nephrogenesis have been gained (Vainio and Lin 2002; Dressler 2006). In contrast, little is known about the processes that underlie the subsequent patterning and regionalization of the nephron.

Studies in *Xenopus* and mice have suggested a need for Notch signaling activity and the Notch effector *HRT1/Hey1* in specifying proximal fates (McLaughlin et al. 2000; Cheng et al. 2003; Wang et al. 2003; Taelman et al. 2006). Recently, genetic analysis has revealed that *Notch2* is required for the differentiation of podocytes and proximal convoluted tubules (Cheng et al. 2007). Furthermore, specification of proximal lineages also requires *Lim1/Lhx1* gene function (Kobayashi et al. 2005), which may act as an upstream regulator of Notch signaling. Finally, survival of proximal tubular fates is dependent on *FGF8* (Grieshammer et al. 2005). Far less is known how the more distal nephron segments are specified. Mouse embryos that lack *Brn1* (Pou3f3), a POU domain transcription factor, initiate nephrogenesis but form truncated nephrons that lack the loop of Henle and distal tubules (Nakai et al. 2003). Whether *Brn1* is sufficient to specify these nephron segments is not known.

The analysis of mammalian kidney organogenesis by targeted gene disruptions is time-consuming and frequently requires the generation of conditional mutant alleles. Furthermore, in the case of redundant gene func-

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tions, the generation of compound mutant animals is necessary. We therefore turned to an amphibian animal model, the *Xenopus* embryo, to determine whether it could serve as a simple, cost-effective model to study the molecular basis of nephron segmentation. The *Xenopus* embryo generates within 2 d after fertilization a simple excretory organ, the pronephric kidney, which is essential for the survival of the larvae (Brändli 1999). Importantly, pronephric development is regulated by many of the same genes necessary for nephrogenesis in mammals, including *Wnt4* and *FGF8* (Saulnier et al. 2002b; Urban et al. 2006). First evidence for segmental organization of the pronephric nephron was provided by histological analysis (Mobjerg et al. 2000), and has recently been supported by studies demonstrating regionalized expression of transporter and ion channel genes along the proximodistal axis (Eid et al. 2002; Zhou and Vize 2004).

Results

The basic segmental organization of the nephron is conserved between pronephric and metanephric kidneys

We performed an extensive analysis of solute carrier (Slc) gene expression during *Xenopus* embryogenesis to identify novel segment-specific marker genes whose patterns may establish a comprehensive model of the pronephric nephron segmentation (Fig. 1). Database searches led to the identification of *Xenopus* cDNAs encoding 210 slc gene family members, which were used for whole-mount in situ hybridization studies. A total of 91 *slc* genes showed pronephric expression, usually in highly regionalized patterns. Selected examples of slc genes are shown in Figure 1A. In contrast, *pax2,* an early pronephric marker gene (Heller and Brändli 1997), was expressed

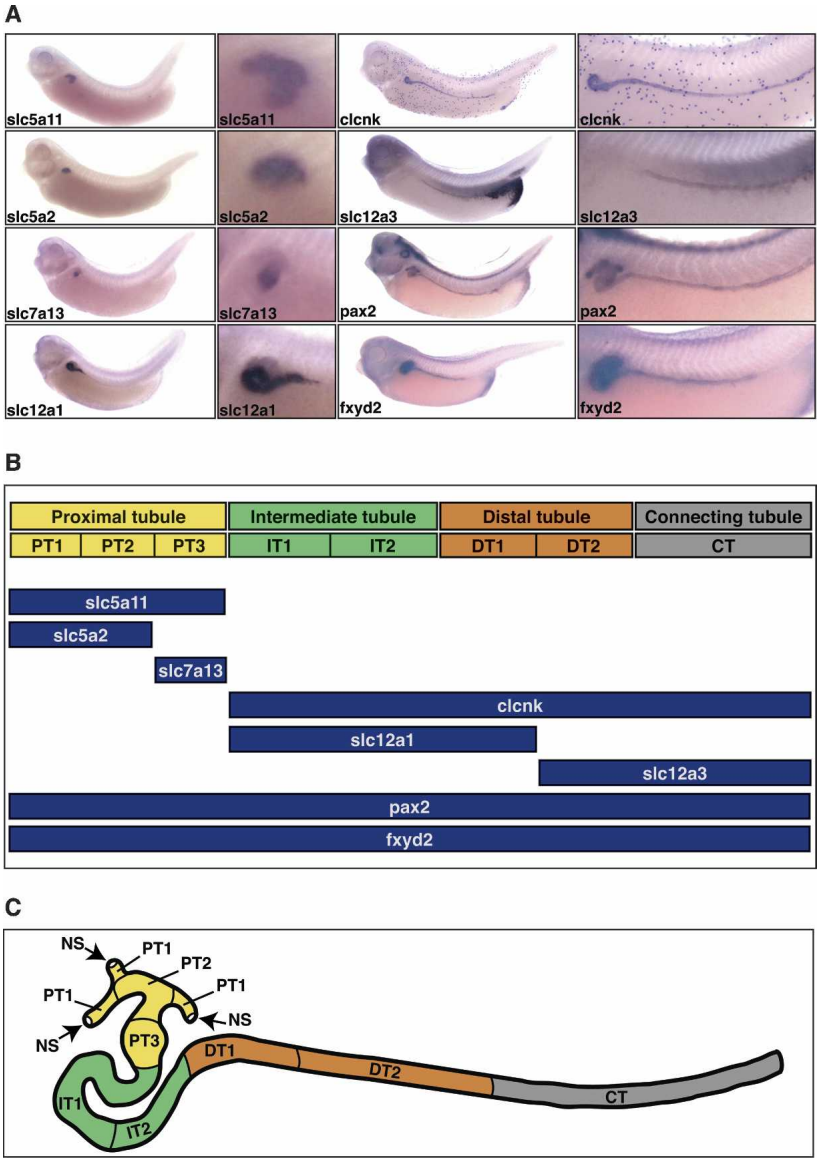

Figure 1. Segmental organization of the *Xenopus* pronephric nephron. (*A*) Spatial expression patterns of selected pronephric marker genes. *Xenopus* embryos (stage 35/36) were stained by whole-mount in situ hybridization for expression of *clcnk* (ClC-K), *fxyd2* (Na, K-ATPase γ subunit), *pax2, slc5a2* (SGLT2), *slc5a11* (SGLT1L), *slc7a13*, *slc12a1* (NKCC2), and *slc12a3* (NCC). Lateral views are shown with accompanying enlargements of the pronephric kidney region. (*B*) Summary of marker gene expression along the proximodistal axis of the pronephric nephron. The localization of the expression domains is shown *below* the corresponding segments. (*C*) Schematic representation of the tubular portion of the *Xenopus* pronephric kidney. A stage 35/36 pronephric kidney is shown with the four tubular compartments color-coded. Each tubule may be further subdivided into distinct segments: proximal tubule (yellow; PT1, PT2, PT3), intermediate tubule (green; IT1, IT2, IT3), distal tubule (orange; DT1, DT2), and connecting tubule (gray; CT). The nephrostomes (NS) are ciliated peritoneal funnels that connect the coelomic cavity to the nephron.

along the entire nephron. Slc gene expression patterns were carefully mapped along the mature pronephric nephron (Fig. 1B) and compared, where data was available, with the expression of the orthologous Slc genes in the adult mammalian kidney (Table 1). A full account of pronephric Slc gene expression, which will include marker genes for each nephron segment, will be presented elsewhere (D. Raciti, L. Reggiani, and A.W. Brändli, in prep.).

The segmental character of the pronephric nephron, which emerged from comparative gene expression studies, extends an older description (Zhou and Vize 2004) and demonstrates remarkable similarities with the organization of the mammalian metanephric nephron (Fig. 1B,C; Table 1). We therefore suggest, in line with the mammalian nomenclature (Kriz and Bankir 1988), that the pronephric nephron is composed of four basic domains: proximal tubule, intermediate tubule, distal tubule, and connecting tubule (CT, formerly known as pronephric duct). Each tubule may be further subdivided into distinct segments. For example, the proximal tubule is divided into three segments (PT1, PT2, and PT3), which share expression of many of the Slc genes characteristic for the S1, S2, and S3 segments of the mammalian proximal tubule (Fig. 1C). The CT has similarities at the gene expression level with the CT of the mammalian collecting duct system. A total of at least eight functionally distinct segments were defined. Taken together, the hallmarks of vertebrate nephron organization—the presence of distinct segmented tubular compartments—can be delineated already at the level of the *Xenopus* pronephric nephron.

Table 1. *Selected segment-specific marker genes of the pro- and metanephric nephron*

(A) Markers of the *Xenopus* pronephric nephron

Gene	GenBank acc. no.	Expression domains	References
slc5a2	CF520680	PT1, PT2	This study
slc5a11	AB008225	PT1, PT2, PT3	Eid et al. 2002; this study
slc7a13	BC060020	PT3	This study
slc12a1	BU904428	IT1, IT2, DT1	Zhou and Vize 2004; this study
clcnk	AJ011385	IT1, IT2, DT1, DT2, CT	Eid et al. 2002; this study
slc12a3	CA790325	DT2, CT	This study

(B) Markers of the mouse metanephric nephron

Gene	GenBank acc. no.	Expression domains	References
Slc5a2	NM_133254	S1, S2	Rubera et al. 2004; D. Raciti and A.W. Brändli, unpubl.
Slc5a11	NM_146198	n.d.	n.d.
Slc7a13	NM_028746	S3	Matsuo et al. 2002; D. Raciti and A.W. Brändli, unpubl.
Slc12a1	NM_183354	TAL	Mount et al. 1999
Clcnka	NM_24412	ATL	Kobayashi et al. 2001
Clcnkb	NM_019701	TAL, DCT, CNT, CD	Kobayashi et al. 2001
Slc12a3	NM_019415	DCT	Loffing et al. 2001

Note that only a single clcnk gene is known in *Xenopus*, whereas there are two mouse clcnk genes—*clcnka* and *clcnkb*. The expression of *Xenopus* clcnk in the intermediate, distal, and connecting tubules therefore has to be compared with the combined renal expression domains of mouse *Clcnka* and *Clcnkb*. See Figures 1 and 3 for abbreviations of *Xenopus* and mouse nephron segments, respectively.
(acc. no.) Accession number; (n.d.) not determined.

Expression of an evolutionary conserved subset of Iroquois (Irx) genes during nephron development

The genes involved in regionalization and proximodistal patterning of the nephron are still poorly understood. Irx genes encode homeodomain-containing transcription factors, which spatially prepattern the neural system in vertebrates and invertebrates (Gomez-Skarmeta and Modolell 2002). Moreover, Irx genes have been implicated in the specification of heart chambers (Bao et al. 1999). Interestingly, some Irx genes are also expressed during vertebrate kidney development (Bellefroid et al. 1998; Houweling et al. 2001). Given this data, we hypothesized that Irx genes play a role in patterning the vertebrate nephron. To support this idea, we first performed a detailed analysis of the spatio-temporal expression during nephrogenesis in *Xenopus* and mouse. The genomes of both species contain six members of the Irx gene family organized into two clusters: IrxA (containing *Irx1*, *Irx2*, and *Irx4*) and IrxB (containing *Irx3*, *Irx5*, and *Irx6*) (Peters et al. 2000; de la Calle-Mustienes et al. 2005).

In *Xenopus*, pronephric kidney organogenesis is initiated during late gastrulation with the specification of the pronephric anlagen, and is completed by stage 37/38 (Brändli 1999). Expression of *irx6* occurs late in *Xenopus* embryogenesis and is not associated with the developing pronephric kidney (de la Calle-Mustienes et al. 2005). Similarly, we failed to find any evidence for pronephric expression of *irx4* and *irx5* (L. Reggiani and A.W. Brändli, unpubl.; data not shown). In contrast, *irx1*, *irx2*, and *irx3* were expressed during pronephric kidney development in highly characteristic patterns (Fig. 2; Supplementary Fig. 1). *irx3* expression was initiated at stage 25 followed 8 h later at stage 29/30 by *irx1* and *irx2* (Fig. 2D; Supplementary Fig. 1). Interestingly, irx3 expression also preceded expression of segment-specific terminal differentiation markers such as *slc5a11* (formerly SGLT-1L) and *clnck* (ClC-K) at stages 29/30 and 31, respectively (Eid et al. 2002; Vize 2003). While *irx3* expression gradually ceased from stage 35/36 onward, *irx1* and *irx2* expression persisted in the pronephros (L. Reggiani and A.W. Brändli, unpubl.; data not shown). Most intriguingly, pronephric irx gene expression was highly regionalized and confined to a central region of the developing nephron (Fig. 2A–C). Mapping of the expression domains to distinct nephron segments of the stage 35/36 embryo

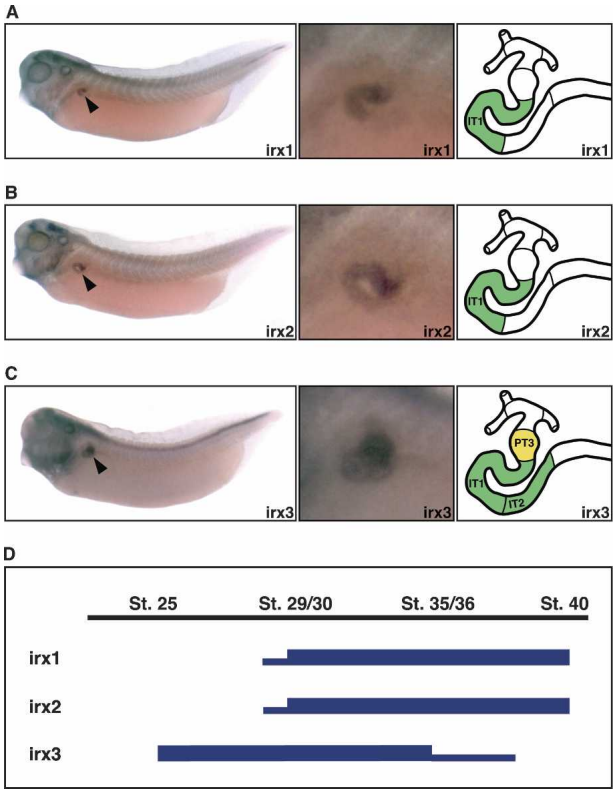

Figure 2. Expression of irx genes is highly regionalized in the developing pronephric kidney. (*A–C*) Expression patterns of *irx1* (*A*), *irx2* (*B*), and *irx3* (*C*) in the pronephric kidneys of stage 35/36 *Xenopus* embryos as determined by whole-mount in situ hybridization. Lateral views of whole embryos (*left* panels; arrowheads indicate pronephric expression), enlargements of the pronephric region (*middle* panels), and color-coded schematic representations of the segment-restricted expression domains (*right* panels) are shown. Note the sharp boundaries that limit the expression domains of irx genes in the developing nephron. (*D*) Summary of the temporal expression profiles of irx genes during pronephric kidney development. The embryonic stages of *X. laevis* development are indicated. High and low levels of irx gene expression are illustrated with thick and thin lines, respectively. The embryonic expression patterns in early embryos are shown in Supplementary Figure 1.

revealed that *irx1* and *irx2* expression was confined to the intermediate tubule segment IT1, whereas *irx3* was found in PT3, IT1, and IT2. Notably, sharp borders characterized the irx expression domains along the nephron. Taken together, we have identified a subset of irx genes whose expression patterns reveal subdivisions of the developing pronephric nephron. Most importantly, *irx3* is expressed well before the segment-restricted expression of terminal differentiation markers.

In the mouse, we examined expression of all six Irx genes in developing metanephric kidneys at embryonic day 18.5 (E18.5) and in adult kidneys. As in *Xenopus*, we detected renal expression of *Irx1*, *Irx2*, and *Irx3* but not for *Irx4*, *Irx5*, and *Irx6* (Figs. 3, 4; data not shown). During metanephric kidney development, signals from the ureteric bud initiate nephrogenesis by promoting condensation and epithelialization of the metanephric mesenchyme to form renal vesicles. Through a series of invaginations and elongations, renal vesicles are subsequently transformed first into comma-shaped and then into S-shaped bodies, which exhibit the first morphologic signs of nephron segmentation (Saxén 1987). Analysis of nephrogenesis in the E18.5 kidney revealed that Irx gene expression became first apparent in early comma-shaped bodies, where *Irx3* transcripts were detected at low levels (Fig. 3G). In S-shaped bodies, transcripts of all three Irx genes were confined to intermediate domains of the developing nephron with high level of expression for *Irx1*, intermediate for *Irx2*, and low for *Irx3* (Fig. 3E–G,I–K). In contrast, expression of *Brn1* was broader covering both intermediate and distal domains of the S-shaped body at this stage (Fig. 3H,L), which is consistent with its role in the development of intermediate and distal nephron derivatives (Nakai et al. 2003).

Regionalized Irx gene expression persisted in adult kidneys with expression domains being confined to specific nephron segments of the loop of Henle (Fig. 4). The loop of Henle is comprised of the S3 proximal tubule, the descending thin limb (DTL), the ascending thin limb (ATL), and the thick ascending limb (TAL) (Kriz and Bankir 1988). *Irx1* and *Irx2* transcripts were both present in S3 proximal tubule segments and in the TALs, whereas *Irx3* expression was confined solely to the S3 segment (Fig. 4). Expression was not detected in the thin limbs of Henle of the adult kidney. In summary, a subgroup of Irx genes—namely, *Irx1*, *Irx2*, and *Irx3*—is expressed in specific and overlapping patterns in the developing and adult mouse kidney. Most importantly, the developing nephron segments expressing Irx genes comprise the prospective loop of Henle (Fig. 3). Moreover, the comparison between *Xenopus* and mouse has revealed striking similarities with regard to the scope of Irx gene family member expression, developmental timing of Irx gene expression, and spatial expression domains during kidney development. This indicates that the mechanisms responsible for patterning the intermediate compartment of the vertebrate nephron have remained remarkably conserved during tetrapod evolution.

Irx3 gene function is required for patterning of the nephron

The expression studies in *Xenopus* and mouse suggest a function for Irx genes in patterning the nephron by specifying intermediate tubule and Henle's loop fates, respectively. Given the potential redundancy of Irx gene activities, we opted for a loss-of-function approach in *Xenopus* embryos to test this hypothesis. Morpholino (MO) antisense oligonucleotides were used to block irx gene functions by inhibiting mRNA translation either one by one or in combinations. Two independent MOs were designed for each *Xenopus* irx gene under investigation. Special care was taken to ensure that the selected MOs inhibit transcripts from both pseudoallelic irx genes typically present in the pseudotetraploid *Xenopus laevis*

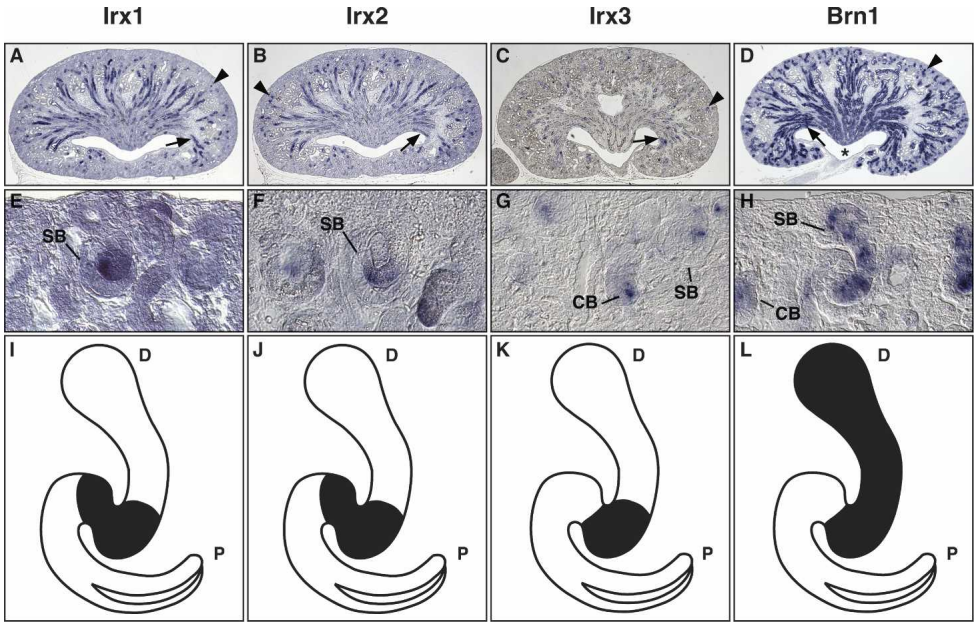

Figure 3. Irx gene expression marks an intermediate region of the S-shaped body. In situ hybridizations were performed on paraffin sections of E18.5 kidneys. Whole sagittal sections (*A–D*) and magnifications (*E–H*) of the renal cortex are shown to illustrate gene expression in developing nephrons. (*I–L*) The corresponding gene expression domains in the S-shaped body are indicated in the schematic drawings. (*A–C*) *Irx1* and *Irx2* are expressed in newly forming nephrons in the cortex (arrows) and the developing intermediate tubule epithelia (arrowheads). *Irx3* expression is similar to *Irx1* and *Irx2*, but fainter and more restricted. (*D*) *Brn1* expression in the newly forming nephrons (arrowhead) and developing distal tubule epithelia (arrow). Note that in contrast to Irx genes, *Brn1* is also highly expressed in epithelia of the renal papilla (asterisk). (*I–K*) Irx transcripts are confined to intermediate regions of S-shaped bodies. (*L*) *Brn1* transcripts are detected in intermediate and distal domains of the S-shaped body. (CB) Comma-shaped body; (SB) S-shaped body; (P) proximal pole of the nephron; (D) distal pole of the nephron.

genome. All Irx MOs (Irx-MO) were able to inhibit in a concentration-dependent manner translation in cell-free coupled transcription–translation assays (Fig. 5). Importantly, Irx3-MO was unable to block translation of *irx1* and *irx2*. Different doses of Irx-MOs, typically 5 ng, were injected into single V2 blastomeres of eight-cell-stage embryos to target the prospective pronephric anlage (Saulnier et al. 2002b). The resulting embryos were ana-

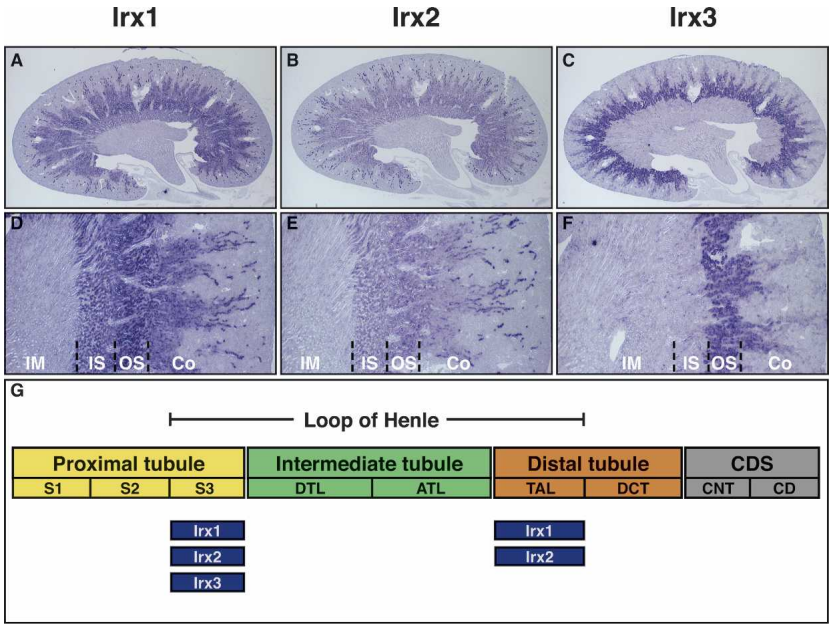

Figure 4. Irx gene expression is confined to distinct segments of Henle's loop in the adult metanephric kidney. (*A–F*) Expression of Irx genes in the adult kidney. In situ hybridizations were performed on paraffin sections. Whole sagittal sections (*A–C*) and magnifications (*D–F*) are shown to illustrate Irx gene expression in detail. (Co) Cortex; (OS) outer stripe of outer medulla; (IS) inner stripe of the outer medulla; (IM) inner medulla. (*A*,*B*,*D*,*E*) *Irx1* and *Irx2* transcripts are detected in S3 and TAL. (*C*,*F*) *Irx3* transcripts are confined to S3 only. (*G*) Summary of Irx gene expression in the adult metanephric kidney. The segmental organization of the adult metanephric nephron is shown schematically. The expression domains of each Irx gene are indicated *below* the scheme. (ATL) Ascending thin limb; (CD) collecting duct; (CDS) collecting duct system; (CNT) connecting tubule; (DCT) distal convoluted tubule; (DTL) descending thin limb; (S1, S2, S3) segments of the proximal tubule; (TAL) thick ascending limb.

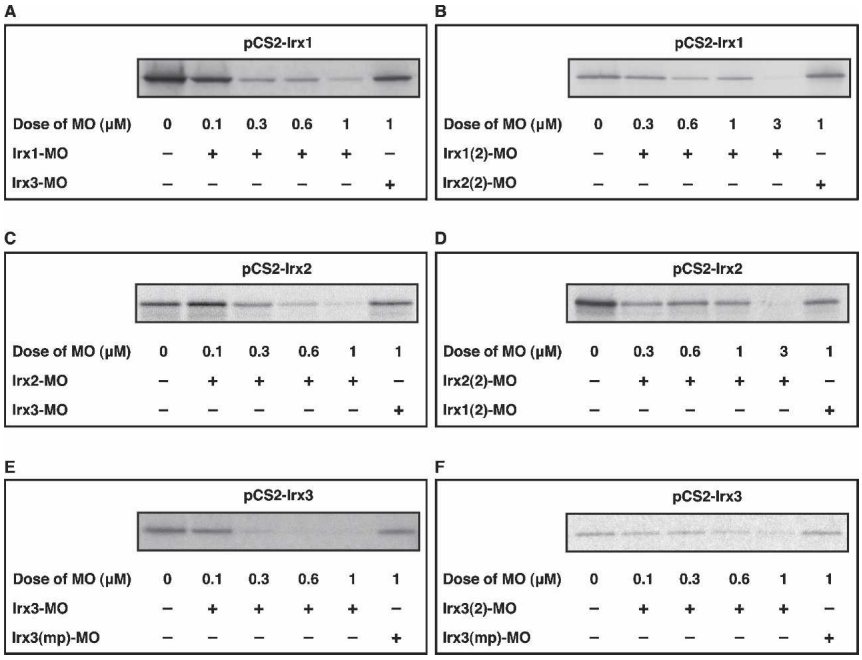

Figure 5. Inhibition of *irx1*, *irx2*, and *irx3* translation in vitro by antisense MOs. Plasmids (500 ng) encoding the ORF of *irx1*, *irx2*, or *irx3* were used as templates in cell-free coupled transcription–translation reactions. MOs were tested for inhibition of translation at the doses indicated. Cell-free transcription–translation reactions were performed in the presence of [^{35}S]methionine and analyzed by SDS-PAGE/autoradiography. (*A*,*B*) Dose-response analysis of inhibition of *irx1* translation by Irx1-MO (*A*) and Irx1(2)-MO (*B*). (*C*,*D*) Dose-response analysis of inhibition of *irx2* translation by Irx2-MO (*C*) and Irx2(2)-MO (*D*). (*E*,*F*) Dose-response analysis of inhibition of *irx3* translation by Irx3-MO (*E*) and Irx3(2)-MO (*F*).

lyzed by visual inspection for externally visible defects and by in situ hybridization for changes in pronephric marker gene expression.

In a first set of experiments, we tested the effect of blocking *irx1*, *irx2*, and *irx3* gene functions simultaneously in the *Xenopus* embryo by unilateral injection of 5 ng of each Irx-MO into V2 blastomeres. The resulting embryos exhibited severe developmental defects, such as unilateral shortening of the body axes, which precluded the analysis of possible pronephric phenotypes (Supplementary Fig. 2). Thus, loss of *irx1*, *irx2*, and *irx3* gene functions appears to be incompatible with normal embryonic development in *Xenopus*. Next, we performed knockdowns of *irx1* and *irx2* gene functions either alone (5 ng and 10 ng MO) or in combination (5 ng MO each). In each case, we failed to observe any externally visible defects. Furthermore, the expression of pronephric marker genes (*pax2*, *clcnk*, *slc5a11*, and *slc12a1*) was overall normal (Supplementary Fig. 3A; data not shown). Similar results were obtained using alternate MOs directed against *irx1* and *irx2* (Supplementary Fig. 3B; data not shown). Taken together, our findings strongly suggest that both *irx1* and *irx2* are dispensable for morphogenesis and patterning of the *Xenopus* pronephric kidney. Interestingly, *Irx2* is neither required for kidney organogenesis in mice, since homozygous *Irx2* mutants are phenotypically normal (Lebel et al. 2003).

In contrast, injection of Irx3-MO led in the majority of the embryos to highly specific defects in pronephric kidney development, which are shown in Figure 5 and described in detail below. Irx3(2)-MO, a MO directed against the 5′ untranslated region (UTR) of *irx3*, caused similar pronephric phenotypes. With each Irx3-MO we observed in a smaller fraction, typically 25%, of the injected embryos malformations and absence of pronephric kidneys, indicating that *irx3* may have an early essential function during *Xenopus* development, which will be explored elsewhere. Embryos injected with Irx3(mp)-MO, a control nonblocking Irx3-MO-containing mutation in four positions, were phenotypically normal (Figs. 5, 6H; data not shown). *Irx3* knockdown embryos with normal overall appearance were selected and probed for evidence of defects in nephron organization. The transcription factor *pax2* is an early marker of the pronephric anlage, and its expression is subsequently found in all developing pronephric epithelia (Heller and Brändli 1997). Staining of Irx3-MO-injected embryos for *pax2* expression revealed the presence of pronephric kidneys, indicating that the specification of the pronephric fate had occurred during gastrulation. The pronephric kidneys were, however, frequently (72%, $n = 25$) characterized by abnormal morphology of the looped central domain, whereas the flanking proximal and distal domains appeared unaffected (Fig. 6A). As shown in Figure 6B, this phenotype was also observed in 65% ($n = 43$) of the Irx3-MO injected embryos stained for *fxyd2* (Na, K-ATPase γ subunit), which is expressed throughout the nephron (Eid and Brändli 2001). These findings indicate that loss of *irx3* gene function did not cause a general block in terminal differentiation of pronephric epithelia.

Next, we probed Irx3-MO-injected embryos for patterning defects along the proximodistal axis of the nephron using marker genes shown in Figure 1 and Table 1. Expression of *slc5a2*, a marker of PT1 and PT2, was retained, and there was no evidence indicating expansion of *slc5a2* expression into more distal territories of the nephron (Fig. 6C). In the case of *slc5a11* (Fig. 6D), the expression domains corresponding to PT1 and PT2 were again present, but expression in the distal PT3 domain was lost in the majority of the embryos (86%, $n = 37$). Analysis of *irx3* knockdown embryos with the PT3 marker *slc7a13* indicated that the loss of PT3 was less

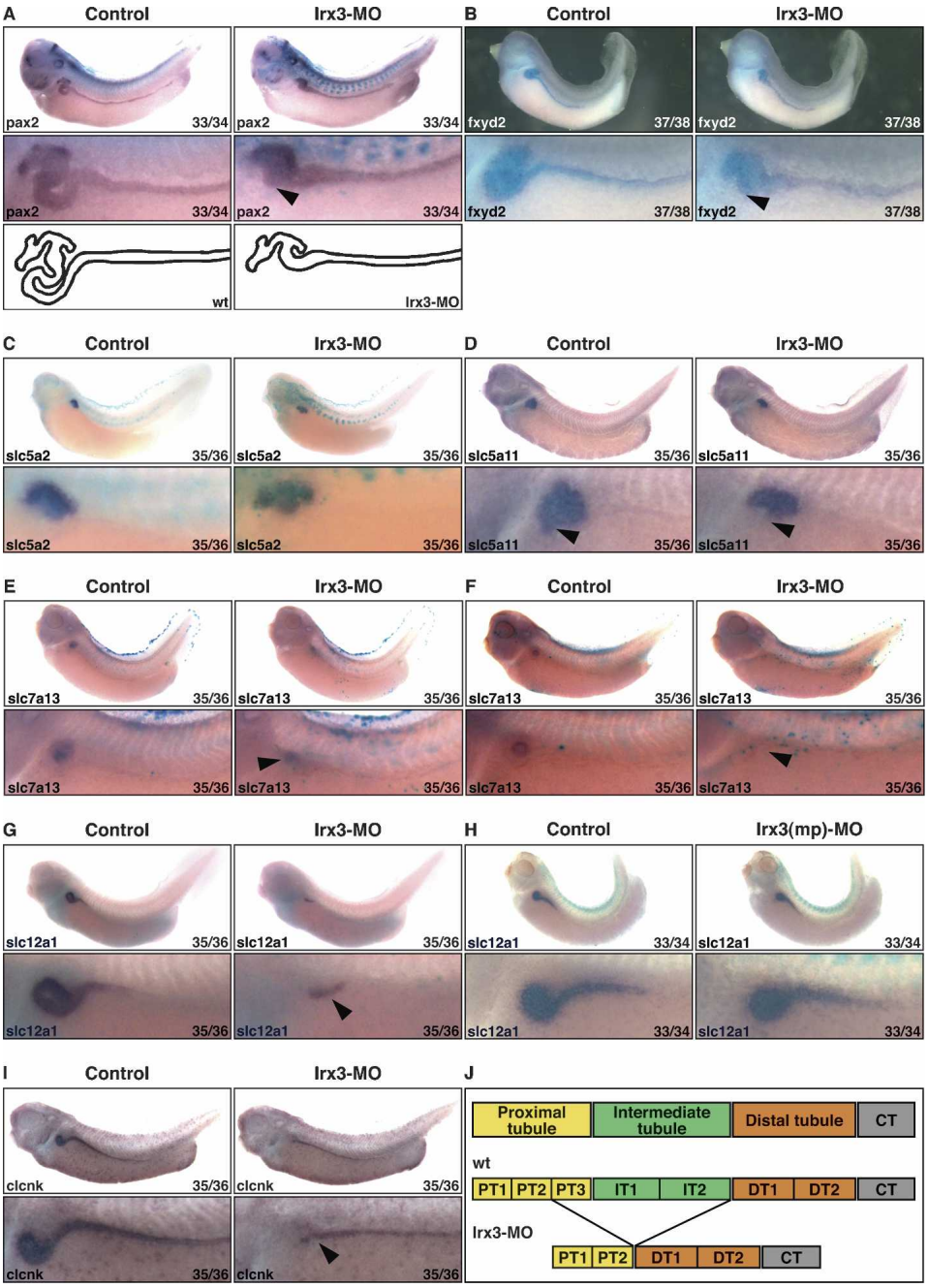

Figure 6. *Irx3* is required for intermediate tubule formation in the *Xenopus* pronephric kidney. (*A–I*) Irx3-MO (5 ng; *A–G*, *I*) or Irx3(mp)-MO (5 ng; *H*) and mRNA (0.25 ng) for the lineage tracer nuclear β-galactosidase were coinjected into single V2 blastomeres of eight-cell-stage embryos. Injected embryos were raised to the embryonic stage indicated, fixed, and processed for β-gal activity. Expression of marker genes was subsequently visualized by in situ hybridization. Control and injected sides are shown as lateral views with accompanying enlargements of the pronephric kidney region. (*A*,*B*) *Irx3* knockdown affects pronephric morphogenesis. Arrowheads indicate the central looped region that is abnormal. Schematic representations show the outline of the nephron in normal and Irx3-MO-injected embryos. (*C*) *Irx3* knockdown does not affect PT1 and PT2. (*D–F*) *Irx3* knockdown causes a loss of the proximal tubule segment PT3. The arrowhead indicates the position of the PT3 segment. Examples of strong reduction (*E*) and complete loss (*F*) of *slc7a13* expression are shown. (*G–I*) *Irx3* knockdown causes loss of IT1 and IT2 but not of DT1. Arrowheads indicate the location of DT1. Note that slc12a1 expression remains unaffected in the presence of the control Irx3(mp)-MO (*H*). (*J*) Summary illustrating the nephron segmentation defects seen in *irx3* knockdown embryos. See Figure 1C for the nomenclature of pronephric nephron segments and their abbreviations.

pronounced, but could still be detected in 36% (n = 55) of the injected embryos (Fig. 6E,F). Effects of *irx3* knockdown on the formation of the intermediate tubule were analyzed by staining for *slc12a1* and *clcnk* expression (Fig. 6G–I). The marker gene *slc12a1* is expressed distal to *slc5a11* and *slc7a13* in the intermediate tubule and DT1. Interestingly, *slc12a1* expression in the intermediate tubule segments IT1 and IT2 was no longer detectable, but residual staining remained associated with DT1 in the majority of Irx3-MO-injected embryos (72%, n = 138). Similarly, expression of clcnk was lost in IT1 and IT2 but retained in DT1 and the more distal nephron segments (82%, n = 33). In summary, our findings indicate that, consistent with its expression pattern (Fig. 2C), *irx3* gene function is required for the formation of PT3 and intermediate tubules during nephron development (Fig. 6J).

Next we asked whether the expression of pronephric irx genes is dependent on *irx3* gene function. For this purpose, irx3 knockdown embryos were raised until they reached stage 29/30, when expression of all three irx genes could be detected (Fig. 2D; Supplementary Fig. 1). The analysis revealed that *irx3* knockdown embryos failed to initiate expression of *irx1* as well as of *irx2* in 71% of the injected embryos (n = 14 for each gene), indicating that expression of these genes requires *irx3* gene function (Fig. 7A,B). In contrast, regionalized pronephric *irx3* transcripts were readily detected in the majority of the injected embryos (63%, n = 16) (Fig. 7C). Hence, the block of *irx3* translation does not impair *irx3* transcription, indicating that the *irx3* gene is not subjected to autoregulation during early pronephric kidney development. Importantly, the regionalized pronephric expression pattern of *irx3* is retained in the *irx3* knockdown embryos. This suggests that nephron patterning is initiated normally but subsequent steps of intermediate tubule development are disrupted, resulting in the loss of the PT3, IT1, and IT2 segments. Notably, since the expression of *irx1* and *irx2* requires *irx3* gene function, they are not able to compensate for the loss of *irx3*.

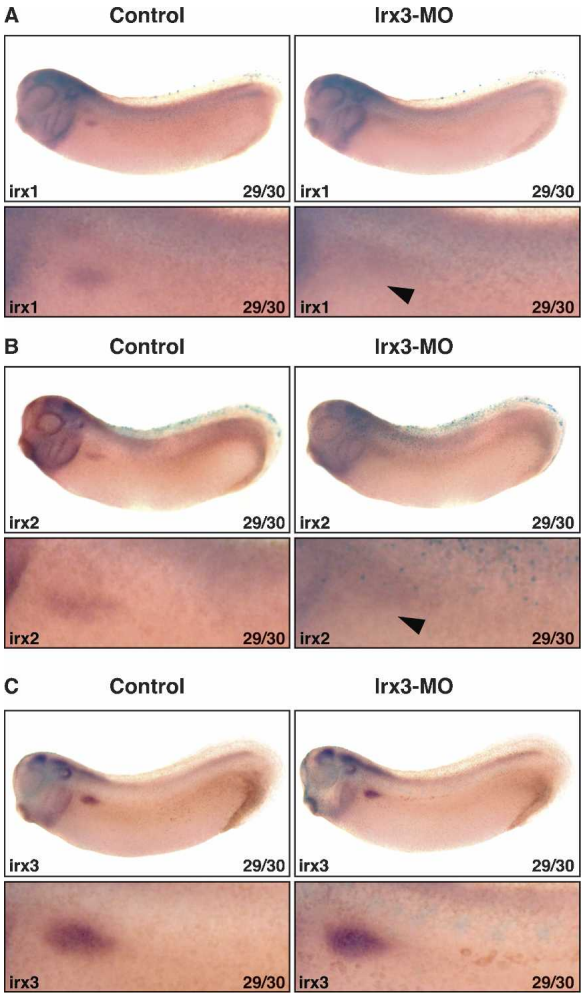

Figure 7. Pronephric expression of *irx1* and *irx2* but not *irx3* requires *irx3* gene function. Irx3-MO (5 ng) and mRNA (0.25 ng) for the lineage tracer nuclear β-galactosidase were coinjected into single V2 blastomeres of eight-cell-stage embryos. Injected embryos were raised to the embryonic stage indicated, fixed, and processed for β-gal activity. Expression of marker genes was subsequently visualized by in situ hybridization. Control and injected sides are shown as lateral views with accompanying enlargements of the pronephric kidney region. (*A*,*B*) *Irx3* knockdown disrupts *irx1* and *irx2* expression in the developing pronephric kidney. Arrowheads indicate the pronephric area devoid of *irx1* (*A*) and *irx2* (*B*) expression. (*C*) *Irx3* knockdown does not affect *irx3* expression.

Irx3 is sufficient to specify intermediate tubule fate

To assess whether *irx3* might specify intermediate tubule fate, ectopic expression experiments were carried out. Injections of *irx3* mRNA (0.15 ng or 0.25 ng) into one blastomere of two-cell-stage embryos or the V2 blastomere of eight-cell-stage embryos were performed. Embryos where then raised, fixed, and processed by in situ hybridization for expression of the intermediate tubule marker *slc12a1*. Of the injected embryos, 40%–60% failed to gastrulate normally resulting in severe developmental abnormalities, such as defects in neural tube closure. Irx3-injected embryos devoid of obvious developmental defects were subjected to marker gene analysis. Remarkably, *irx3* misexpression resulted in the formation of ectopic *slc12a1*-expressing intermediate tubule tissues (Fig. 8). This phenotype occurred in 5%–14% of the embryos (n = 190) in three independent experiments. Interestingly, ectopic *slc12a1*-expressing tissues were always located in the intermediate mesoderm posterior to the pronephric kidney. This suggests that only the posterior intermediate mesoderm is competent to undergo cell fate change to form ectopic intermediate tubule tissue in response to *irx3* misexpression. Taken together, these results demonstrate that ectopic expression of *irx3* is sufficient to specify intermediate tubule fate.

Discussion

Within the nephron, the loop of Henle has a unique structure that enables the generation of hypertonic urine

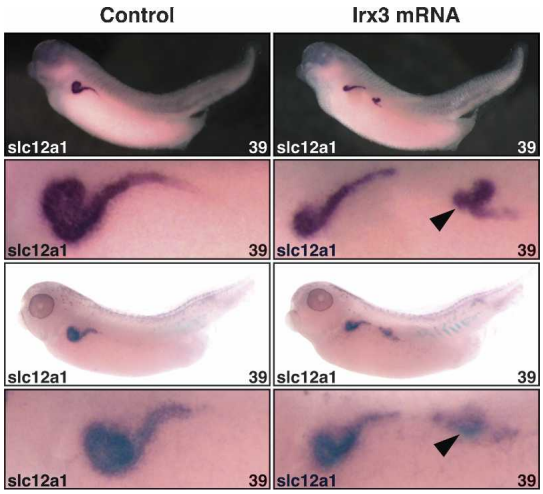

Figure 8. *Irx3* is sufficient for intermediate tubule formation in the *Xenopus* pronephric kidney. Single V2 blastomeres of eight-cell-stage embryos were injected with *irx3* mRNA (0.15 ng). Injected embryos were raised to the embryonic stage indicated, fixed, and processed for β-gal activity. Expression of the *slc12a1* marker gene was subsequently visualized by in situ hybridization. Lateral views of control and injected sides of two representative embryos displaying the gain-of-function phenotype are shown with accompanying enlargements of the pronephric kidney region. The arrowheads indicate the ectopic intermediate tubule tissues expressing *slc12a1*.

and the maintenance of water homeostasis. It is composed of four nephron segments (S3, DTL, ATL, and TAL) (Kriz and Bankir 1988) that arise from the intermediate region of the developing nephron. Four distinct stages of Henle's loop development can be distinguished: the anlage, the primitive loop, the immature loop, and the mature loop (Neiss 1982; Nakai et al. 2003). To date, Brn1 is the only gene known to be essential for development of the loop of Henle, which is arrested at the primitive loop stage in *Brn1* mutants (Nakai et al. 2003).

In the present study, we have identified Irx genes as critical factors for patterning the intermediate region of the developing vertebrate nephron. In the *Xenopus* and mouse, a conserved subset of Irx genes was expressed in overlapping domains, constituting an intermediate region of the developing nephron that will give rise to PT3 and the intermediate tubule in *Xenopus* and Henle's loop in the mouse. Hence, Irx gene expression prefigures the future segmental organization of the nephron. Unlike many transcription factors regulating early development, Irx gene expression in the developing nephron is not transient but remains segmentally restricted into adulthood. In the mouse, expression persists in the thick limbs (S3 and TAL) but not in the thin limbs of Henle (DTL and ATL). Interestingly, direct evidence for a late function of Irx genes was recently provided by studies of *Irx5*-deficient mice, where *Irx5* maintains asymmetric potassium channel expression (Costantini et al. 2005). Thus, Irx genes may serve as determining factors in the developing nephron and subsequently as maintenance factors ensuring persistent segment-specific gene expression in the mature nephron. Notably, expression of *Hes5,* a hairy-related basic helix–loop–helix (bHLH) transcription factor and target of Notch signaling, is also restricted to an intermediate segment of the S-shaped body (Piscione et al. 2004; Chen and Al-Awqati 2005). *Hes5* mutant mice, however, fail to display any defects in nephrogenesis (Chen and Al-Awqati 2005).

The gain- and loss-of-function studies reported here directly address the functional significance of Irx genes in specifying nephron segment identity. The overlapping gene expression patterns of Irx genes suggest redundant roles in the developing nephron. This notion was confirmed as blocking of *irx1* and *irx2* translation either alone or together did not affect nephron patterning and differentiation in *Xenopus*. Redundant roles for Irx genes have also been reported in the mouse, where the loss of function of Irx genes does not significantly affect embryonic development (Bruneau et al. 2001; Lebel et al. 2003; Costantini et al. 2005). In contrast, knockdown of *Xenopus irx3* function caused a profound patterning defect in the developing pronephric nephron manifesting in the deletion of segments spanning from PT3 to IT2. Interestingly, the affected region did not adopt the fate of adjacent nephron segments, as we failed to observe any marker gene expansion. This indicates that *irx3* may not function to repress proximal and/or distal fates in the developing intermediate tubule segments. It is therefore also unlikely that there is a default program establishing initially either a proximal or distal fate in the prospective central region of the developing nephron, which will subsequently adopt intermediate tubule fate upon *irx3* gene activation. Rather, our findings indicate that intermediate tubule fate is established together with proximal and distal tubule fates. We conclude that *irx3* is required to specify PT3 and the entire intermediate tubule. Given that the pronephric nephron shares a segmental complexity that is remarkably comparable with that of the metanephric nephron, we infer that Irx genes may play an equally important role in nephron patterning and segmentation in mammals. More specifically, we propose that Irx genes are required to specify nephron segments that will give rise to the loop of Henle.

In *Drosophila*, the Iroquois complex (Iro-C) encodes a cluster of three related homeodomain genes—named *araucan, caupolican,* and *mirror*—that are homologs of the vertebrate Irx genes (Cavodeassi et al. 2001). Overexpression of Iro-C genes imposes notum differentiation fate on cells of the imaginal wing disc (Wang et al. 2000; Aldaz et al. 2003). Interestingly, the gain-of-function experiments reported here demonstrate unequivocally that *Xenopus irx3* acts as a master regulator of intermediate tubule development that is sufficient to specify intermediate tubule fate in vivo. It therefore appears that the ability of irx-related genes to specify cell fate has been conserved between *Drosophila* and vertebrates. Remarkably, the ectopic intermediate tubules induced by *irx3* undergo terminal differentiation and were detected at frequencies of 5%–14%, which are comparable with the induction of ectopic eyes in 5.8% of the *Xenopus* embryos injected with the master regulator *pax6* (Chow et

al. 1999). The *irx3* gain-of-function phenotype appears at first sight surprising, since Irx genes are thought to act mainly as transcriptional repressors (Bilioni et al. 2005). However, *IRX4* was shown previously to act as a transcriptional activator required for heart development in chicken (Bao et al. 1999). Moreover, FGF signaling can stimulate phosphorylation of Irx proteins that will convert them from transcriptional repressors to activators (Matsumoto et al. 2004). Thus, the transcriptional activity of Irx proteins is regulated in a context-dependent manner. Ectopic intermediate tubules are only detected in the intermediate mesoderm and not elsewhere in the developing embryo. Moreover, only the posterior intermediate mesoderm is competent to respond to ectopic *irx3* expression. Interestingly, this portion of the intermediate mesoderm has nephrogenic potential, as it will later give rise to the mesonephric kidney. Whether the ectopic intermediate tubules are of pronephric or mesonephric origin cannot presently be determined due to the lack of specific markers.

The loop of Henle is a structure exclusively found in birds and mammals, which are the only vertebrate groups that retain body water by producing urine osmotically more concentrated than the plasma from which it is derived (Casotti et al. 2000). The kidneys of larval and adult amphibians do not develop loops of Henle. Their urine concentration is hypoosmotic to plasma, and they produce very dilute urine in freshwater (Vize et al. 2003). Despite the inability to generate loops of Henle, our analysis of irx gene expression and function in *Xenopus* embryos clearly demonstrates the existence of a patterning mechanism to specify an intermediate compartment in the pronephric nephron. The physiological roles of the intermediate tubule in the *Xenopus* pronephros are currently not known, but are likely to involve the reabsorption of salt ions as evidenced by the prominent expression of *slc12a1* (Na-K-Cl symporter). The molecular mechanism for specifying intermediate tubule fate therefore predates the establishment of Henle's loop in higher vertebrates. Moreover, it indicates that the ability to generate an intermediate tubule compartment is evolutionary ancient, and may have been established at the onset of tetrapod evolution.

Besides Irx genes, *Brn1,* a POU transcription factor, represents the other early patterning gene required for the specification of the intermediate nephron and essential for the function of Henle's loop (Nakai et al. 2003). Polarized Brn1 expression is evident already at the renal vesicle stage and covers a broad intermediate and distal domain of the developing nephron, while Irx gene expression is detectable in early comma-shaped bodies and confined solely to the intermediate region in S-shaped bodies. Whether *Brn1* and *Irx* genes act in concert in the same pathway or represent separate parallel pathways that insure intermediate tubule development is currently not known. Preliminary studies indicate that the *Xenopus* embryo expresses several class III POU transcription factors including brn1 during pronephric kidney organogenesis (L. Reggiani and A.W. Brändli, unpubl.). The *Xenopus* pronephros may therefore be a useful model to study the epistatic relationship of these genes.

The kidney is a common target of systemic diseases, developmental syndromes, and drug toxicity. As a consequence, selective damage or loss of epithelial cell types may incur and impair normal renal functions. Given its instructive properties, *Irx3* may be exploited to drive differentiation of renal progenitors derived from embryonic stem cells (Kim and Dressler 2005) toward intermediate tubule fate. Hence, the present work provides not only a novel framework for understanding how transcription factors direct formation of nephron segments but also suggests new approaches for renal tissue engineering and possible treatment of renal diseases by cell replacement therapy.

Materials and methods

Gene nomenclature

The standard gene nomenclature suggested by Xenbase (http://www.xenbase.org) and adopted by the NCBI for *Xenopus* genes is utilized rather than the original gene names to maximize compatibility with data available from other model systems. Where possible, *Xenopus* gene names are the same as the human orthologs.

Cloning of cDNAs, sequencing, and sequence analysis

Expressed sequence tag (EST) databases were screened to identify the cDNAs encoding slc transporters from *X. laevis* (D. Raciti, unpubl.). Among them, *slc5a2* (GenBank accession no. CF520680), *slc7a13* (BC060020), *slc12a1* (BU904428), and *slc12a3* (CA790325) were employed in this study as pronephric marker genes. Double-stranded DNA sequencing was performed in-house. Assembly of nucleotide sequence traces and analysis of nucleotides and protein sequences was performed using the DNAStar Lasergene software package (version 6.0).

Plasmid constructs

The following plasmids containing the complete ORF of *Xenopus irx1* (Gomez-Skarmeta et al. 1998), *irx2* (Gomez-Skarmeta et al. 1998), and *irx3* (Bellefroid et al. 1998) were constructed for in vitro coupled transcription–translation reaction and in vitro RNA synthesis: pCS2-Irx1, pCS2-Irx2, and pCS2-Irx3. The cDNAs were amplified by PCR (Expand High-Fidelity PCR System, Roche Diagnostics) and subcloned into the $pCS2^+$ vector (Turner and Weintraub 1994) using T4 DNA Ligase (Fermentas). All constructs were confirmed by DNA sequencing.

Xenopus *embryo manipulation and in situ hybridization*

In vitro fertilization, culture, and staging of *Xenopus* embryos were performed as previously described (Brändli and Kirschner 1995; Helbling et al. 1998). Probe synthesis, whole-mount in situ hybridization, β-galactosidase staining, and bleaching of embryos were carried out as described (Helbling et al. 1998, 1999; Saulnier et al. 2002b). Digoxigenin probes were generated from linearized plasmids encoding *irx1* (Xiro) and *irx2* (Xiro2) (Gomez-Skarmeta et al. 1998), *irx3* (Xiro3) (Bellefroid et al. 1998), *irx4* and *irx5* (Garriock et al. 2001), *pax2* (Heller and Brändli 1997), *clcnk* (ClC-K) (Maulet et al. 1999), *fxyd2* (Na, K-ATPase γ-subunit) (Eid and Brändli 2001), *slc5a11* (SGLT1-L)

(Eid et al. 2002), *slc7a13* (GenBank accession no. BC060020), *slc12a1* (BU904428), *slc12a3* (CA790325), *slc5a2* (CF520680), and *wnt4* (Saulnier et al. 2002a). Sense strand controls were prepared from all plasmids and then tested negative by in situ hybridization.

Contour model of the pronephric nephron and marker gene mapping

The pronephric expression patterns of 91 slc genes, along with a full account of how the segmental organization of the *Xenopus* pronephric kidney was determined and how the accompanying nomenclature was established, will be presented elsewhere (D. Raciti, L. Raggiani, and A.W. Brändli, in prep.). In brief, a first schematic representation of the contour of the stage 35/36 nephron was developed from *Xenopus* embryos stained by whole-mount in situ hybridization with a combination of probes for the pronephric marker genes *fxyd2* (Eid and Brändli 2001), *pax2* (Heller and Brändli 1997), and *wnt4* (Saulnier et al. 2002a). Two dozen stained embryos were inspected to generate a two-dimensional contour drawing of the nephron on paper. Refinements to the initial contour model were made after inspection of hundreds of embryos stained with other pronephric marker genes. The final contour model of the nephron shown in Figure 1C was made with Illustrator CS2 (Adobe).

The pronephric expression patterns of 91 slc genes were projected onto the contour model to define the segments of the nephron. Unambiguous morphological features, such as the nephrostomes, a characteristically broad proximal tubule domain known as common tubule (Fox 1963) (subsequently named PT3), and the looped part of the pronephric nephron (IT1, IT2, and DT1), were used as landmarks to identify the relative location of the boundaries of the expression domains. The final borders between the nephron segments are defined by the boundaries of multiple marker genes.

Microinjection of Xenopus *embryos*

The following antisense MO oligonucleotides were ordered from GeneTools to inhibit translation of *Xenopus* mRNAs (sequence complementary to the predicted start codon is underlined; mispaired nucleotides are indicated with small letters): Irx1-MO, 5′-CATGTCTCTCCGGCAGGGAAATCGC-3′; Irx1(2)-MO, 5′-CCCAGCTGCGGGAAGGACATGTCTC-3′; Irx2-MO, 5′-GGTAACCCTGAGGATAGGACATGGT-3′; Irx2(2)-MO, 5′-GCAGAAGCACAGAATCGCCGGGGCT-3′; Irx3-MO, 5′-AGCTGTGGGAAGGACATGGTGCAGC-3′; Irx3(mp)-MO, 5′-ACgTGTGGGtAGGACAgGGTGaAGC-3′; Irx3(2)-MO, 5′-GAATCCCCTTTTATGACCTGACTTT-3′.

In vitro coupled transcription translation assays were carried out as described previously (Saulnier et al. 2002b). When not indicated differently, 5 ng of individual MOs were injected in V2 blastomere of eight-cell-stage embryos (Moody and Kline 1990). RNA synthesis and microinjection were performed as described (Helbling et al. 1998) except that RNA purification was done by phenol-chloroform extraction. RNA encoding the lineage tracer nuclear β-galactosidase (nucβgal) was usually coinjected at 0.25 ng per blastomere.

In situ hybridization of mouse kidneys and kidney sections

Mouse embryos were obtained from matings of NMRI wild-type animals. For timed pregnancies, plugs were checked in the morning after mating, noon was taken as 0.5 d post-coitum (dpc). Microdissected metanephric kidneys were fixed in 4% PFA and stored in 100% methanol at –20°C prior to in situ hybridization analysis. In situ hybridization analysis of 10-µm paraffin sections of embryonic kidneys at E18.5 and 6-wk-old adult kidneys was performed according to an established protocol (Soufan et al. 2004). Digoxigenin-labeled probes were transcribed from linearized plasmids encoding the following mouse cDNAs: *Irx1* (GenBank accession no. BU703212), *Irx2* (BI111057), *Irx3* (BI525434), *Irx4* (BE367799), *Irx5* (Bosse et al. 2000), and *Irx6* (Peters et al. 2000). For probe synthesis, the mouse *Brn1* cDNA (Hara et al. 1992) was subcloned by PCR into pGEM-TEasy (Promega).

Photography and computer graphics

Mouse kidney sections were embedded in moviol and photographed on Leica Axioplan with a ProgResC14 digital camera. All images of mouse specimens were processed in Adobe Photoshop 8.0. Digital photographs of whole-mount *Xenopus* embryos were taken with an AxioCam Color camera mounted on a Zeiss SteREO Lumar.V12 stereomicroscope. Composite figures were assembled and labeled with Adobe Photoshop CS2 and Adobe InDesign CS2. Schematic figures were drawn using Adobe Illustrator CS2.

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