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The Notch-effector *HRT1* gene plays a role in glomerular development and patterning of the *Xenopus* pronephros anlagen

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Notch signaling has been shown to play a role in cell fate decisions in the *Xenopus* pronephros anlagen. Here, we show that the *Xenopus* Hairy-related transcription factor (*HRT*) gene *XHRT1*, and the Hairy/Enhancer of split (*HES*) genes *Xhairy1*, *Xhairy2b*, *esr9* and *esr10*, have distinct restricted dynamic expression patterns during pronephros development, and that their expression is regulated by Notch. *XHRT1*, which is the earliest and strongest gene expressed in the pronephric region, is initially transcribed predominantly in the forming glomus, where it is downregulated by antisense morpholino oligonucleotide inhibition of xWT1. Later, it is activated in the most dorsoanterior part of the pronephros anlagen that gives rise to the proximal tubules. In agreement with this dynamic expression profile, we found that early activation of Notch favors glomus, whereas only later activation promotes proximal tubule formation. We show that, among the bHLH-O factors tested, only XHRT1 efficiently inhibits distal tubule and duct formation, and that only its translational inhibition causes a reduction of the expression of proximal tubule and glomus markers. Using domain swap experiments, we found that the XHRT1 C-terminal region is crucial for its activity. Together, our results provide evidence that XHRT1 plays an important role in glomerular development and early proximodistal patterning that is distinct from those of the other pronephric bHLH repressors.

KEY WORDS: XHRT1, Notch, Pronephros

INTRODUCTION

The pronephros, which is the functional embryonic kidney of amphibian and fish embryos, is used as a model to study human kidney development and disease. In amphibians, the pronephros is a paired organ that consists of a single nephron composed of three basic components: (1) the glomus, which is the site of blood filtration; (2) the tubules, where filtrate resorption occurs; and (3) the duct, which carries the urine to the cloaca (Brändli, 1999; Vize et al., 2003; Ryffel, 2003; Jones, 2005).

All three components of the pronephros develop within the intermediate mesoderm right posterior to the head. In *Xenopus*, at late neurula (around stage 21), cells in the lateral layer of the intermediate mesoderm below somites 3-7 start to condense. Cells from the dorsoanterior region of the pronephric field will form the tubules, while those from the ventroposterior region migrate posteriorly out of the original kidney primordium to give rise to the majority of the duct. Concomitantly, cells in the adjacent medial layer undergo morphogenesis to form the glomus. The molecular mechanisms that control the early specification of the pronephros have been well studied in frog and chicken (Brennan et al., 1998; Brennan et al., 1999; Seufert et al., 1999; Obara-Ishihara et al., 1999; Carroll and Vize, 1999; Mauch et al., 2000; Chan et al., 2000; James and Schulteiss, 2005). By contrast, much less is known about the gene products that pattern the pronephric anlagen. The Wilms' tumor *xWT1* gene encoding a zinc finger transcription factor, which is expressed around the dorsal and anterior border of the future pronephros, is thought to have a role in the specification of the glomus by suppressing tubule and duct gene expression (Carroll and Vize, 1996; Wallingford et al., 1998). *Evi1* is another gene encoding a zinc finger transcription factor that may play a role in the partitioning of the pronephros; it is selectively expressed in the ventroposterior part of the pronephros anlagen, giving rise to the distal tubule and duct compartments (Van Campenhout et al., 2006). Notch signaling has also been shown to play an important role in the partitioning of the pronephros, inhibiting duct and distal tubule differentiation in the dorsoanterior region of the anlagen, where cells are normally fated to form proximal tubules and to increase the expression of the *xWT1* gene (McLaughlin et al., 2000; Van Campenhout et al., 2006). Studies in mice have demonstrated that Notch signaling is similarly required during metanephros development for glomerular podocyte and proximal tubule fates (McCright et al., 2001; Wang et al., 2003a; Cheng et al., 2003; Cheng and Kopan, 2005). However, the stages of nephron morphogenesis that are dependent upon the activation of Notch remain unidentified.

In *Xenopus*, the *XHRT1* gene (also named *Hey1*/*HERP2*/*Hesr-1*/*CHF2*), encoding a downstream basic helix-loop-helix Orange (bHLH-O) mediator of Notch signaling, has been shown to be expressed in numerous tissues during development, including the pronephros, and to be responsive to Notch signaling (Rones et al., 2002; Pichon et al., 2002). XHRT1 is a member of the HRT subfamily of bHLH-O proteins that forms heterodimers with hairy proteins through the bHLH-O and downstream sequences, and represses transcription in a groucho-independent manner (Iso et al., 2003; Taelman et al., 2004; Pichon et al., 2004). In the embryonic mouse metanephros, several intracellular Notch effectors have been found to be expressed in a segment-specific manner in early nephrons, but nothing is known as yet about their role in patterning cell fate decisions (Leimeister et al., 2003; Piscione et al., 2004; Chen and Al-Awqati, 2005).

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Here, we show that *XHRT1*, when compared with the other bHLH-O factors expressed in the developing kidney, plays a predominant role in the pronephros as a Notch effector, being required for glomus formation and for proximodistal patterning of the pronephric primordium. We show that this is due not only to its earlier temporal expression pattern, but also to intrinsic properties of the protein that the HES proteins lack.

MATERIALS AND METHODS

Plasmids

The *hGR/X-Su(H)/Ank*, *X-Su(H)DBM*, *Xhairy2b-MT-hGR*, *hGR-ESR9*, *hGR-ESR10* and *XHRT1-MT-hGR* expression plasmids have been described (Chitnis et al., 1995; Wettstein et al., 1997; Taelman et al., 2004). The following plasmids were constructed: *pCS2+MT XHes2-hGR*, encoding an inducible, Myc-tagged XHes2 protein; *pCS2+MT XHRT1-XHes2-hGR*, encoding an inducible, truncated form of XHRT1 (amino acids 1 to 160) to which has been attached the carboxy terminus of XHes2 (amino acids 129 to 191); and *pCS2+MT Hes2-XHRT1-hGR*, encoding an inducible, truncated form of XHes2 (amino acids 1 to 127) with the carboxy terminus of XHRT1 (amino acids 161 to 294). In addition, *pCS2+XHRT1a-mut-MT-hGR*, derived from *pCS2+XHRT1a-MT*-hGR, incorporates several mismatches (small letters) in the MO target sequence: 5′-ATGAAaaGaGGcCAtGAtTA (predicted start codon is underlined). *pCS2+XHRT1-eGFP* encodes the full-length XHRT1 protein fused with eGFP at its carboxy terminus. pCS2+xWT1-eGFP encodes a similar fusion with the N-terminal part of xWT1 (amino acids 1-91).

Morpholino oligonucleotides

Antisense morpholinos for xWT1, XHRT1, esr9, esr10 and Xhairy2b (GeneTools) consist of the following sequences (sequence complementary to the predicted start codon is underlined):
XHRT1 MO, 5′-TAGTCGTGTCCCCGCTTCATGGCTG-3′;
xWT1a MO, 5′-CATATCCCGGACATCAGACCCCATC-3′;
xWT1b MO, 5′-CATATCCCGCACATCAGATCCCATC-3′;
esr9 MO, 5′-CTGTCTGGTAATGGGATGTGATGGA-3′;
esr10 MO, 5′-CTGTTTAGTAAGTGGATATGATGGA-3′;
Xhairy2a MO, 5′-ATGGTATCTGCGGGCATGTTCAGTT-3′;
Xhairy2b MO, 5′-GGCATGTTCAGATGTTGTATCCGGA-3′.
Individual MOs, or a mixture of both MOs for xWT1, were injected at 15 ng/blastomere.

Embryo and injections

Xenopus eggs were obtained from hormone-induced (chorionic gonadotropin, Sigma) adult female frogs and fertilized using standard methods. Capped mRNAs were transcribed using the mMessage mMachine Kit (Ambion). For targeting the pronephros, synthetic RNA (500 pg) was injected into one blastomere in the lateral marginal zone of 8-cell stage embryos. *nuc-lacZ* mRNA (100-250 pg/blastomere) was used as a lineage tracer. Induction of hGR constructs in embryos was performed by addition of dexamethasone (Dex; 10 μM; Sigma). Injected embryos were fixed in MEMFA, stained for β-galactosidase activity with 5-bromo-4-chloro-3-indolyl-β-galactopyranoside (X-Gal, Bioline) or 6-chloro-3-indolyl-β-D-galactoside (Red-Gal, Research Organics) and stored in ethanol at –20°C. Only embryos that were phenotypically normal and show *lacZ* staining in the pronephic region were scored.

In situ hybridization

Whole-mount in situ hybridization was carried out as previously described (Harland et al., 1991). *X-Serrate-2* was identified through a search of the EST database (NIBB #XL054p21). The *X-Serrate-2* plasmid linearized with *Eco*R1 was transcribed with T7 polymerase. Plasmids used for generating the other in situ hybridization probes are: *XHRT1* (Pichon et al., 2002), *XHairy2b*, *Xhairy1* (Tsuji et al., 2003; Taelman et al., 2004), *Evi1*, *xSat1*, *xPDZK1* (Van Campenhout et al., 2006), *xCLC-K* (Vize, 2003), *ESR-4*, *ESR-5* (Jen et al., 1999), *ESR-6e* (Deblandre et al., 1999), *esr9*, *esr10* (Li et al., 2003), *XHes2* (M.S., unpublished), *Hes6* (Koyano-Nakagawa et al., 2000), *XSMP-30* (Sato et al., 2000), *xWT1* (Carroll and Vize, 1996), *Pax8* (Heller and Brandli, 1999), *X-Delta-1*, *N-tubulin* (Chitnis et al., 1995), *Ep. keratin* (Jonas et al., 1985), *X-Serrate-1* (Kiyota et al., 2001) and *Nephrin* (Gerth et al., 2005). For sections, embryos were gelatine embedded and vibratome sectioned.

Western blots

Western blot analysis was performed as described (Taelman et al., 2004) using the 9E10 anti-Myc and anti-β-tubulin monoclonal antibodies (Sigma).

RESULTS

Distinct dynamic expression of *XHRT1*, *esr9*, *esr10*, *Xhairy1* and *Xhairy2b* during pronephros development

In a search for downstream effectors of Notch signaling in the early pronephros anlagen, we surveyed by whole-mount in situ hybridization the expression pattern of the previously identified *Xenopus* bHLH-O genes *XHRT1* (Pichon et al., 2002), *Xhairy1* (Taelman et al., 2004), *Xhairy2b* (Tsuji et al., 2003), *ESR-1* (Wettstein et al., 1997), *ESR-4*, *ESR-5* (Jen et al., 1999), *ESR-6e* (Deblandre et al., 1999), *esr9*, *esr10* (Li et al., 2003), *XHes2* (M.S., unpublished) and *Hes6* (Koyano-Nakagawa et al., 2000), and compared their expression with that of the ligands *X-Delta-1* (Chitnis et al., 1995), *X-Serrate-1* (Kiyota et al., 2001) and *X-Serrate-2*, identified by EST mining. *ESR-1*, *ESR-4*, *ESR-5*, *ESR-6e*, *XHes2* and *Hes6* mRNA were not detected in the pronephric anlagen between stages 20 and 36. *XHRT1*, *Xhairy1*, *Xhairy2b*, *esr9* and *esr10* are all expressed from the early tailbud stage in the dorsoanterior region of the developing pronephros, *XHRT1* expression being detectable slightly earlier than the others.

During early tailbud stages, *XHRT1*, *esr9* and *esr10* expression appears localized to the most dorsoanterior portion of the pronephric anlagen, whereas *Xhairy1* and *Xhairy2b* are more broadly expressed within the pronephric mesoderm (Fig. 1A,E,G,I,K,M). In transverse sections of stage 20-23 embryos, *XHRT1*, *esr9* and *esr10* transcripts are predominantly found, similarly to *xWT1* transcripts (Carroll and Vize, 1996), around the dorsoanterior border of the pronephros anlagen (Fig. 1B,D,F,H,J). By contrast, *Xhairy1* and *Xhairy2b* expression is detected both around and inside the developing pronephros, strong *Xhairy2b* staining being also observed in the sensorial layer of the ectoderm covering the pronephros anlagen (Fig. 1L,N). *X-Delta-1* is the only Notch ligand to be expressed in the developing pronephros at early tailbud stage. Its expression is detected in the lateral mesodermal layer in cells surrounded by the *XHRT1*-positive cells (Fig. 1C).

During late tailbud to early tadpole stages, *XHRT1* expression demarcates the most dorsoanterior portion of the pronephros, whereas *esr9*, *esr10*, *Xhairy1* and *Xhairy2b* occupy more ventral regions. Within this dorsoanterior portion of the pronephros, high levels of *XHRT1* expression progressively become restricted to the tip of the forming tubules, while expression of the other bHLH repressors remains broader (Fig. 2A,C,E,G,I,K,R). Sectioning of those embryos revealed that during this period, expression of *XHRT1*, *esr9* and *esr10* disappear in the medial layer and that they are now actively transcribed in the dorsoanterior portion of the pronephros anlagen itself. *Xhairy1* and *Xhairy2b*, which were initially transcribed in both layers, are now predominantly expressed in the lateral layer too (Fig. 2B,D,F,H,J,L). During this period, all Notch ligand genes are expressed in the dorsoanterior portion of the pronephros anlagen in a region slightly ventral to *XHRT1* expression. Whereas *X-Delta-1* expression is restricted to a band just below *XHRT1*, *Serrate2* expression is broader (Fig. 2M-Q). *X-Serrate-1* is expressed similarly to *X-Serrate2* at that stage (data not shown). Although pronephric expression of *XHRT1*, *esr9*, *esr10* and *Xhairy1* is no longer detectable at late

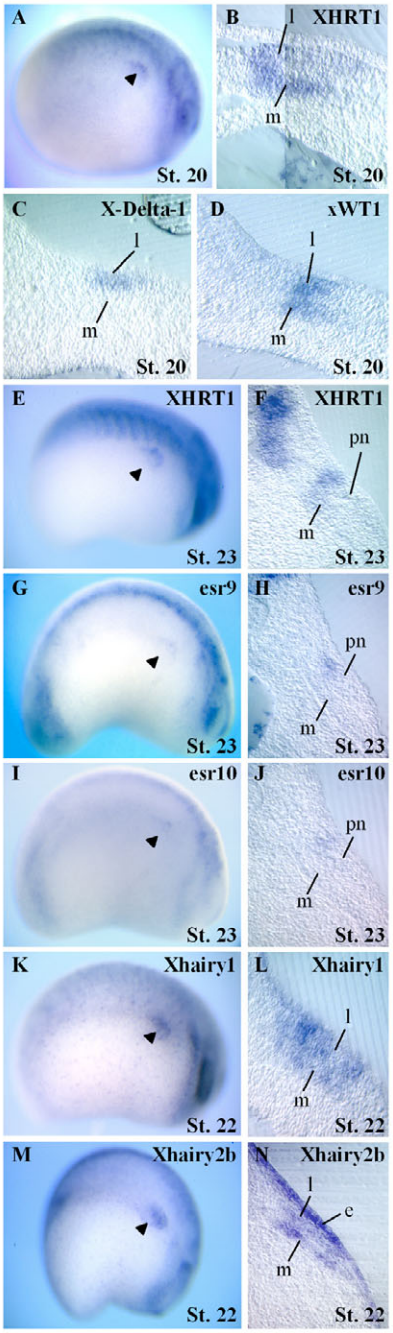

Fig. 1. Whole-mount in situ analysis of *XHRT1*, *esr9*, *esr10*, *Xhairy1* and *Xhairy2b* expression in comparison with *X-Delta-1* and *xWT1* in the pronephros region (arrowheads) of early tailbud stage embryos. Nieuwkoop-Faber stages are indicated (Nieuwkoop and Faber, 1967). (**A-D**) Comparison of the expression of *XHRT1*, *X-Delta-1* and *xWT1* in stage 20 embryos. (A) Whole embryo, lateral view, anterior right. (B-D) Transversal sections. *XHRT1* expression coincides with that of *xWT1* around the pronephros anlagen and surrounds that of *X-Delta-1*. (**E-N**) Comparison of the expression of *XHRT1*, *esr9*, *esr10*, *Xhairy1* and *Xhairy2b* in stage 22-23 embryos. (E,G,I,K,M) Whole embryos, lateral views, anterior right. (F,H,J,L,N) Transversal sections. Note that *esr9* and *esr10* are co-expressed with *XHRT1* around the dorsal border of the pronephros anlagen, and that *Xhairy1* and *Xhairy2b* expression is detected in both mesodermal layers and in the ectoderm. e, ectoderm; pn, pronephros; l, lateral intermediate mesodermal layer; m, medial intermediate mesodermal layer.

tadpole stages (stage 35), *Xhairy2b* expression is maintained in the proximal and distal tubules. *X-Serrate-1* is the only Notch ligand to remain expressed in the dorsoanterior portion of the pronephros at that time, and its expression appears to be similar to that of *Xhairy2b* (Fig. 2S,T). The spatially and temporally distinct expression patterns of those bHLH-O repressor-encoding genes suggest that they may have non-identical functions during pronephros development.

esr9, *esr10* and *Xhairy2b* are, like *XHRT1*, responsive to Notch signaling in the developing pronephros

The *XHRT1* gene has previously been shown to be responsive to Notch signaling in the pronephros (Rones et al., 2002). We investigated whether Notch signaling also affects the expression of the other bHLH-O genes. To study the consequence of activation of Notch signaling in the developing pronephros without affecting earlier developmental steps, we used an hormone-inducible form of the transcription factor Su(H) that mediates Notch signaling (Wettstein et al., 1997). Injected embryos were induced with dexamethasone at stage 18 and assayed for expression of the different bHLH-O genes between stages 25-30. We observed that, as in the case of *XHRT1*, activation of Notch signaling using an inducible Notch ICD-ankyrin fusion of Su(H) increased the pronephric expression of *esr*9 (n=24), *esr10* (n=35), *Xhairy1* (n=26) and *Xhairy2b* (n=25) in all injected embryos (Fig. 3A, parts a-j). In many embryos, expression of those bHLH-O genes expands within the posterior part of the intermediate mesoderm, the strongest staining being detected in the case of *esr9*, *esr10* and *XHRT1* in the lateral part of the intermediate mesoderm, while Xhairy1 and Xhairy2b expression is found in both layers (arrowheads). *Xhairy2b*, which is expressed in the ectoderm overlying the pronephros anlagen, was also strongly activated in the ectoderm (Fig. 3B, parts a-e). As reported in the case of *XHRT1*, suppression of Notch signaling using a dominant-negative form of Su(H) decreased the expression in the pronephros of *esr9*, *esr10*, *Xhairy1* and *Xhairy2b* (n=8 for *esr9*, 13 for *esr10*, 29 for *Xhairy1* and 31 for *Xhairy2b*; Fig. 3A, parts k-t). Thus, *esr9*, *esr10*, *Xhairy1* and *Xhairy2b* may function together with *XHRT1* in the developing pronephros as downstream mediators of Notch signaling.

As our results indicate that *XHRT1* is expressed in a dynamic manner in the pronephros, we wanted to know whether this reflects a difference in the temporal responsiveness of the medial and lateral layers to activation of Notch signaling. Therefore, we analysed *XHRT1* expression in earlier embryos (stage 23). As observed in stage 25-30 embryos, activation of Notch induced *XHRT1* expression in both layers (Fig. 3B, part f). By contrast, *xWT1* is only activated in the medial layer at all stages analysed (Fig. 3B, part g). Thus, the successive expression of *XHRT1* in the medial and lateral mesodermal layers is not a consequence of a difference in the temporal competence of the two layers to respond to Notch activation.

XHRT1 early expression in the developing glomus is affected by translational inhibition of xWT1

The *xWT1* gene is thought to play an important role in the development of the pronephros by repressing lateral-specific gene expression in the portion of the pronephric mesoderm fated to form the glomus (Wallingford et al., 1998; Van Campenhout et al., 2006). To determine whether *xWT1*, which is activated at about the same time as *XHRT1* in the pronephros, is required for the expression of bHLH-O repressors in the forming glomus, we generated antisense MOs that block the translation of both *xWT1* pseudoalleles. Injection of those MOs specifically blocked the translation in vitro and in vivo

of its target mRNA (Fig. 4A; data not shown). Embryos injected with those xWT1 MOs were analysed by in situ hybridization with *XHRT1* and other pronephric markers. As expected, knockdown of xWT1 abolished the expression of *nephrin*, a marker of glomerular podocytes, which is directly activated in mice by WT1 (Wagner et al., 2004; Guo et al., 2004; Gerth et al., 2005) (100% inhibited, *n*=199; Fig. 4B,C). Expression of the *XSMP-30* proximal tubule (82% unaffected, *n*=72) and the *Pax8* (83% unaffected, *n*=24) and *Evi1* (90% unaffected, *n*=20) early pronephric markers was not affected (Fig. 4D-K). Interestingly, knockdown of xWT1 decreased the early glomus-specific expression of *XHRT1* (48% inhibited, *n*=125) but did not perturb its late expression in the pronephros anlagen (80% unaffected, *n*=35) (Fig. 4L-O). Together, these experiments are consistent with the idea that xWT1, which promotes glomus formation, may play a role in *XHRT1* early expression. They also suggest that there may be factors other than xWT1 that repress the expression of lateral-specific genes in the developing glomus.

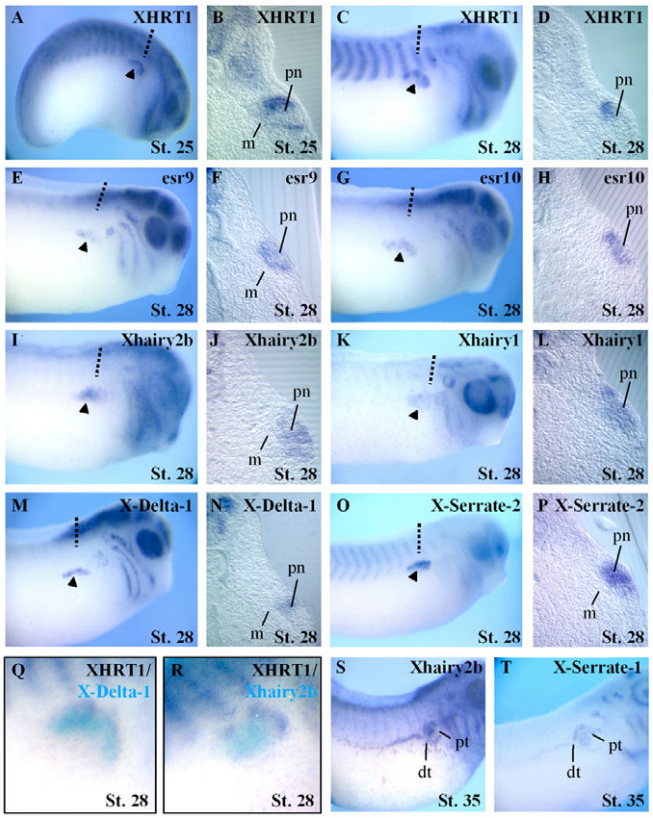

Fig. 2. Whole-mount in situ analysis of *XHRT1*, *esr9*, *esr10*, *Xhairy1* and *Xhairy2b* in comparison with *X-Delta-1* and *X-Serrate-2* in the pronephros region (arrowheads) of late tailbud to tadpole stage embryos. Nieuwkoop-Faber stages are indicated. (**A**) At stage 25, *XHRT1* is expressed in the most dorsoanterior portion of the pronephros anlagen. (**B**) Transversal section of the embryo shown in A at the level indicated. (**C-P**) Expression of *XHRT1*, *esr9*, *esr10*, *Xhairy1*, *Xhairy2b*, *X-Delta-1* and *X-Serrate-2* in stage 28 embryos. (A,C,E,G,I,K,M,O) Whole embryos, lateral views; (B,D,F,H,J,L,N,P) transversal sections of the corresponding embryos at the level indicated. Note that *esr9* and *esr10* staining appears slightly ventral compared with *XHRT1*. While *X-Delta-1* expression is restricted to a band just below *XHRT1*, *Serrate2* expression is broader. (**Q**,**R**) Double labeling of *XHRT1* and *X-Delta-1* or *Xhairy2b*. (**S**,**T**) At stage 35, *Xhairy2b* is co-expressed with *X-Serrate-1* in the proximal and distal tubules. dt, distal tubules; pn, pronephros; pt, proximal tubules; m, medial intermediate mesodermal layer.

XHRT1 overexpression inhibits pronephric distal tubule and duct formation

To determine if XHRT1, esr9, esr10 and Xhairy2b are functioning as mediators of Notch signaling in the pronephros anlagen, we used previously described hGR-inducible constructs (Taelman et al., 2004). Embryos were injected with the different *hGR* constructs mixed with β-galactosidase mRNA as a lineage tracer. In the

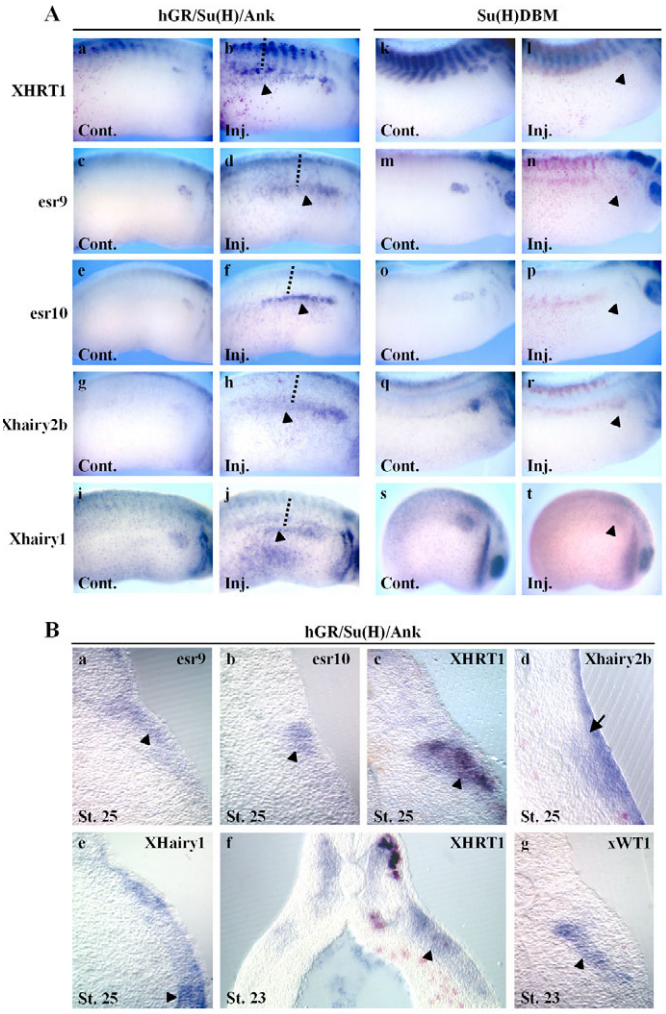

Fig. 3. *XHRT1*, *esr9*, *esr10*, *Xhairy1* and *Xhairy2b* are responsive to Notch signaling in the pronephric mesoderm (arrowheads). Whole-mount in situ analysis of stage 23-30 embryos injected with *hGR/Su(H)/Ank* or *Su(H)DBM* mRNA. The inducible construct was activated at stage 18. (**A**, parts a-t) Control and injected sides of embryos, anterior right. Note the posterior expansion of the expression of the different bHLH-O genes on the injected side of *hGR/Su(H)/Ank*-injected embryos (arrowheads). Note the inhibition of their expression in *Su(H)DBM*-injected embryos (arrowheads). Except in d,f,h and j, the injected side is revealed by β-gal staining (red). (**B**, parts a-e) Transversal sections of the embryos shown in b,d,f,h,j at the level indicated. Note that the strongest *esr9*, *esr10* and *XHRT1* ectopic staining is detected in the lateral part of the intermediate mesoderm, while Xhairy1 and Xhairy2b expression is found in both layers (arrowheads). *Xhairy2b* is also strongly activated in the ectoderm (arrow). (B, part f) Transversal sections in stage 23 embryos in the posterior portion of the pronephros showing that *XHRT1* ectopic expression in response to activation of Notch is already detected at that stage in both layers. (B, part g) Transversal section in the posterior portion of the pronephros of stage 23 embryos stained with *xWT1*. Ectopic staining of *xWT1* is restricted to the medial layer.

injected embryos, we examined how the expression of those bHLH-O genes affects pronephric gene markers. We first examined the ability of XHRT1, which is the earliest and strongest bHLH-O gene expressed in the pronephric primordium, to inhibit distal tubule and duct cell fates. Addition of dexamethasone at the end of neurulation (stage 18) resulted in a hormone-dependent inhibition at early tadpole stage of the formation of the distal tubule and duct as revealed by the *Evi1* marker (94% inhibited, *n*=32) (Van Campenhout et al., 2006). This phenotype is similar to that previously described upon activation of Notch signaling using a hGR/Su(H)/Ank construct (McLaughlin et al., 2000) (Fig. 5A-F). Similar results were observed using the *xClC-K* marker (data not shown). The same experiments were repeated for esr9, esr10 and Xhairy2b. However, unlike XHRT1, although effective in inhibiting primary neurogenesis, esr9, esr10 and Xhairy2b did not inhibit *Evi1* expression (unaffected: 72%, *n*=21; 76%, *n*=33; 88%, *n*=40; for esr9, esr10 and Xhairy2b, respectively; Fig. 5G-O). XHes2, another bHLH-O repressor, which is not found in the pronephros (M.S., unpublished), was also inefficient at repressing *Evi1* (80% unaffected, *n*=30) (Fig. 5P-R). It has been suggested that XHRT1 may participate in regulating aspects of gene expression that are linked to cell-cycle control and apoptosis (Wang et al., 2003b; Huang et al., 2004). The inhibition of distal tubule and duct formation could thus be explained by a decrease of proliferation or the apoptotic elimination of pronephric cells. However, immunostaining using an antibody recognizing phosphorylated histone H3, and TUNEL analysis of injected embryos, revealed no change in the pattern of mitotic and apoptotic cells (data not shown). We therefore conclude that activation of *XHRT1*, but not that of *esr9*, *esr10* or *Xhairy2b*, may play a crucial role during pronephros formation in the inhibition of distal tubule and duct cell fates.

Activation of Notch signaling in the pronephros anlagen has been shown to perturb the differentiation of the tubule network and to increase *xWT1* expression (McLaughlin et al., 2000). We therefore compared the ability of XHRT1 with that of Su(H)Ank to modulate the expression of the *XSMP-30* proximal tubule markers and the *xWT1* and *nephrin* glomus markers. We first observed that the responsiveness of these markers to activation of Notch signaling was temporally specific. Activation of Notch signaling before stage 25, when Notch bHLH-O effectors are expressed in the glomus, efficiently induced *xWT1* expression and inhibited *XSMP-30* expression. Later activation, when they are expressed in the dorsal part of the pronephros anlagen, had no effect on *xWT1* expression. In a few embryos activated between stage 22 and stage 27, *XSMP-30* expression was expanded (Fig. 6A). In contrast to the effects observed upon injection of hGR/Su(H)/Ank mRNA, early or late activation of *XHRT1* has no effect on *xWT1* (77% unaffected, *n*=39) and *nephrin* (85% unaffected, *n*=20). Although early activation decreases the expression of *XSMP-30* in some embryos (58% inhibited, *n*=33), late activation (stage 20-27) did not affect its expression (*n*=130; Fig. 6B-M). Similar results were obtained with other proximal tubule markers (*xSat1*, *xPDZK1*, 3G8; data not shown). We conclude that activation of Notch in the pronephric mesoderm is essential first for glomus and then for proximal tubule fates, and that XHRT1 only mediates part of its effects.

To further determine the importance of XHRT1 as a mediator of Notch signaling in the pronephros, we co-injected embryos with mRNA encoding *Su(H)DBM* together with *XHRT1-MT-hGR* mRNA. Injected embryos were induced at stage 22 and assayed for the expression of *Evi1* and *XSMP-30*. We observed that early inhibition of Notch signaling in untreated embryos reduces the expression of the proximal tubule marker *XSMP-30* and, as previously reported, elevates the expression of the distal tubule and duct marker *Evi1* (Fig. 7A-D,I). By contrast, in dexamethasone-treated embryos, we observed a reduction of both *XSMP-30* and *Evi1* expression (Fig. 7E-H,I). Thus, *XHRT1* could reverse the effect of *Su(H)DBM* on *Evi1* but is not sufficient to restore the expression of *XSMP-30*. Together, these results indicate that XHRT1 functions as an important downstream effector of Notch signaling. Compared with the other bHLH-O repressors, it appears to play a specific role in early pronephros development, contributing to the inhibition of distal tubule and duct cell fates in cells that form the glomus and the proximal tubules.

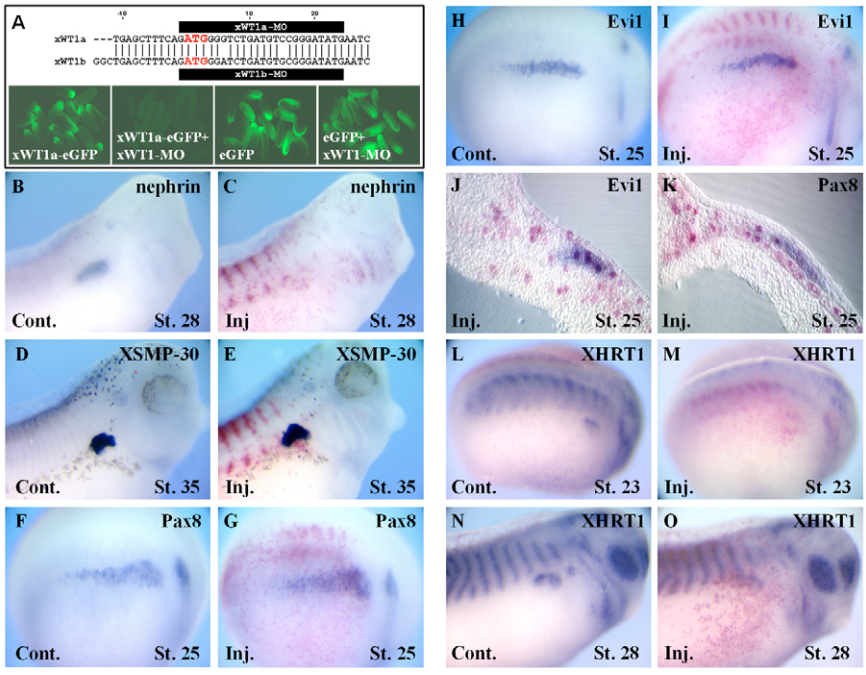

Fig. 4. *XHRT1* early expression in the developing glomus is affected by translational inhibition of xWT1. (**A**) Design of the xWT1 MOs that target both pseudoalleles. (Bottom) In vivo translation of 500 pg of *xWT1-eGFP* is specifically inhibited by 15 ng of xWT1 MOs. (**B-I**) Control and injected sides of xWT1-depleted embryos stained with the indicated probes. xWT1 MOs abolished the expression of *nephrin* but had no effect on *XSMP-30*, *Pax8* and *Evi1*. (**J**,**K**) Transversal sections of *Evi1*- and *Pax8*-stained xWT1-depleted embryos. Expression of both markers is unaffected by xWT1 knockdown. (**L-O**) Control and injected sides of xWT1-depleted embryos stained with *XHRT1*. Note that xWT1 knockdown decreases early but not late *XHRT1* pronephric expression.

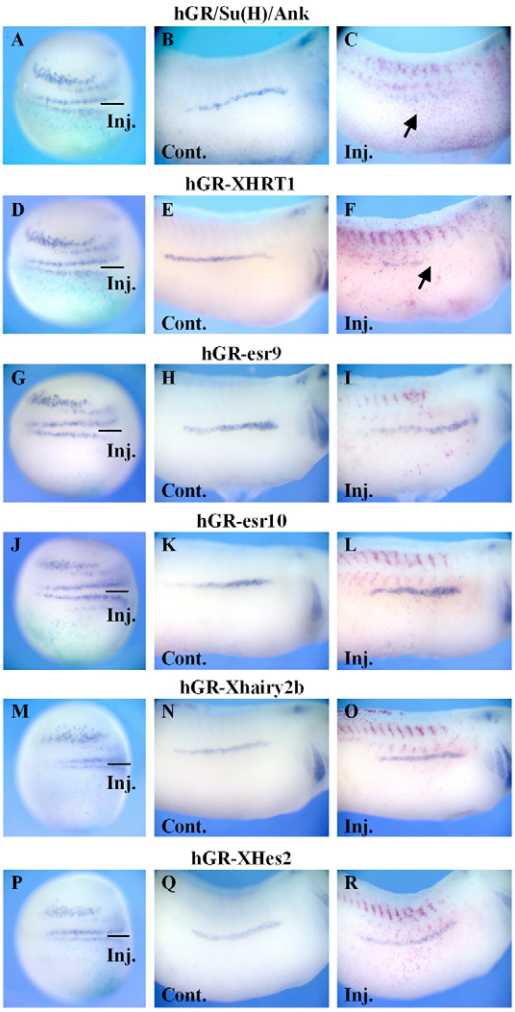

Fig. 5. XHRT1, but not esr9, esr10 or Xhairy2b inhibits pronephric distal tubule and duct formation. (**A-R**) Whole-mount in situ hybridization of embryos injected with 500 pg of mRNA encoding the indicated inducible constructs together with β-galactosidase mRNA analysed at stage 16 for *N-tubulin* (+Dex, stage 12; A,D,G,J,M,P) or stage 28 for *Evi1* expression (+Dex, stage 18; B,C,E,F,H,I,K,L,N,O,Q,R). Embryos at neurula stage are viewed from the dorsal side, injected side downwards. Lateral view of control and injected sides of stage 28 embryos are shown. (A-C) Embryos injected with *hGR/Su(H)/Ank* mRNA showed an inhibition of *N-tubulin* and *Evi1* expression (C, arrow). (D-R) Although overexpression of all bHLH-O genes inhibited *N-tubulin* in the neural plate, only *XHRT1* overexpression inhibited *Evi1* expression (F, arrow) in the pronephros. Lines in A,D,G,J,M,P indicate the injected and uninjected sides.

XHRT1 depletion reduces the expression of proximal tubule and glomus markers

To determine whether XHRT1 is required for glomus and proximal tubule development, we generated a MO that targets a 100% conserved 25 bp stretch, including the AUG initiation codon, in the two *XHRT1* pseudoalleles. In vivo and in vitro, the XHRT1-MO specifically and efficiently blocks the translation of the corresponding mRNA (Fig. 8A). XHRT1-MO-injected embryos displayed a decrease in the expression of all proximal tubule-specific markers tested [including *XSMP-30* (72%, *n*=59), *xPDZK1* (88%, *n*=17) and *xSat1* (68%, *n*=15)] and downregulated the expression of *xWT1* (77%, *n*=40) and *nephrin* (88% inhibited, *n*=17; Fig. 8B-I; data not shown). Transverse sections revealed that the glomus was present and that in many injected embryos, the proximal tubules were reduced in size (Fig. 8J,K). These effects are not due to a change in the pattern of mitotic and apoptotic cells, as revealed by analysis of the injected embryos by phosphorylated histone H3 immunostaining and TUNEL (data not shown). Expression of the distal tubule and duct markers *Evi1* and *xClC-K* appears unaffected (*Evi1* 64%, *n*=37; *xClC-K* 72%, *n*=11; Fig. 8L,M; data not shown). Expression of other markers, such as *Ep. keratin*, and *N-tubulin*, was also unaltered (*Ep. Keratin*, none inhibited, *n*=27; *N-tubulin*, none inhibited, *n*=25; Fig. 8N,O). Injection of an MO designated against *esr9*, *esr10* or *xHairy2b* that efficiently inhibits their target mRNA did not affect the expression of any of the pronephric genes tested (see Fig. S1 and S2 in the supplementary material), which further supports the idea that XHRT1 has a specific function in the partitioning of the pronephros anlagen.

In order to determine whether the phenotype caused by the injection of XHRT1-MO can be rescued by co-injection of *XHRT1* mRNA, we generated an inducible *XHRT1a-mut* construct (*XHRT1a-mut-MT-hGR*). Co-injection with *XHRT1a-mut-MT-hGR* was sufficient to restore normal expression of *XSMP-30* in dexamethasone-treated XHRT1-MO-injected embryos, indicating that the XHRT1-MO knockdown phenotype is specific (Fig. 8P). We next investigated whether blocking *XHRT1* activity could block the effect of *hGR/Su(H)/Ank*. As shown in Fig. 8Q, we observed that activation of Notch signaling at stage 22-25 has no effect, or in some embryos, increases the expression of XSMP-30. A reduction of *XSMP-30* expression was observed in embryos co-injected with the XHRT1-MO. Thus, injection of XHRT1-MO is sufficient to impede the effect of overexpression of *hGR/Su(H)/Ank* on *XSMP-30* further supporting the idea that XHRT1 is an important component of the Notch signaling pathway that leads to glomus and proximal tubule formation.

The specific activity of XHRT1 is conferred by its C-terminal region

Our results support the idea that, compared with the other bHLH-O repressors tested, XHRT1 has a specific function in the developing pronephros. To identify the region(s) that are required for its activity, we performed domain-swapping experiments between XHRT1 and one of the bHLH-O repressors inactive in the pronephros. We chose the novel bHLH-O gene *XHes2* because both genes are expressed in the retina where XHes2 but not XHRT1 promotes Müller glial development (Satow et al., 2001) (M.S., unpublished), which may provide another assay to identify the regions required for their distinct regulatory functions. As we and others have previously shown that the bHLH and the Orange domains of XHRT1 are required for efficient DNA-binding and homo- and heterodimerization (Taelman et al., 2004), we decided to keep the bHLH-O regions of both proteins intact and swap their C-terminal sequences (Fig. 9A). Both chimeric molecules were linked to glucocorticoid receptor ligand-binding domain sequences to generate inducible constructs. Injected embryos were induced at stage 18 and assayed for the expression of *Evi1*. All constructs were effectively translated when expressed in embryos (Fig. 9B). As shown in Fig. 9C, only the hybrid containing the XHRT1 C-terminal domain fused to the XHes2 bHLH-O domain reduced *xEvi1* expression to a similar extent as the wild-type XHRT1 protein. Thus, the specific properties of XHRT1 in the pronephros appear to be linked to its C-terminal region.

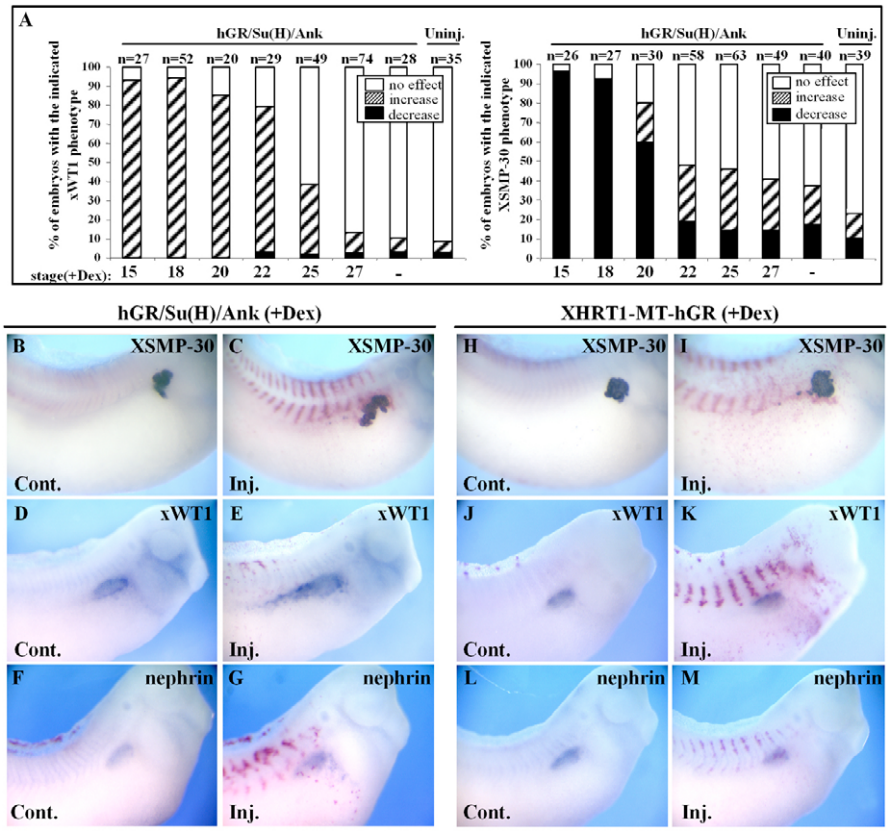

Fig. 6. Comparison of the ability of *hGR/Su(H)/Ank* and *XHRT1-MT-hGR* to affect the expression of proximal tubule and glomus markers. (**A**) Activation of Notch signaling until stage 25 expands *xWT1* efficiently, while later activation had no effect. *XSMP-30* expression was increased in some embryos activated between stages 22 and 27. Embryos were co-injected with *hGR/Su(H)/Ank* and β-galactosidase mRNA. Injected embryos were treated, or not, with Dex at the indicated times and analysed at stage 32-35. Changes in the expression of *xWT1* and *XSMP-30* at each stage were scored in individual embryos by comparing the injected and injected sides in at least two different injections. Embryos were classified into three phenotypes (no changes, increase or decrease). *n*, number of cases analysed. (**B-M**) Control and injected sides of embryos injected with the indicated mRNA together with β-galactosidase mRNA, treated with Dex at stage 18 (*xWT1*, *nephrin*) or 25 (*XSMP-30*) and analysed with the indicated probes. Note that *hGR/Su(H)/Ank* expands *xWT1*, *nephrin* and, in a few cases, *XSMP-30*, while *XHRT1-MT-hGR* has no effect.

DISCUSSION

In this work, we analysed the expression and function of several potential downstream effectors of Notch signaling during pronephric development. Our results indicate that *XHRT1*, compared with the other bHLH-O factors expressed in the pronephros, plays a predominant role in the patterning of the kidney, favoring first glomus and later proximal tubule formation. We also provide evidence that its distinct specific function in the developing kidney is not only due to its earlier temporal expression pattern but also to the intrinsic properties of the protein.

Restricted dynamic expression of *XHRT1*, *Xhairy1*, *Xhairy2b*, *esr9 and esr10* in *Xenopus* compared with higher vertebrates

Notch-1, *X-Delta-1* and *X-Serrate-1* have distinct expression patterns in the developing pronephros, suggesting that spatiotemporal control of Notch activity is an important determinant of the patterning of the early kidney anlage (McLaughlin et al., 2000). In agreement with those observations, our results indicate that five bHLH-O genes, namely *XHRT1*, *Xhairy1* and *Xhairy2b* (related to murine *HES1*), *esr9* and *esr10* (related to *HES5*) have distinct

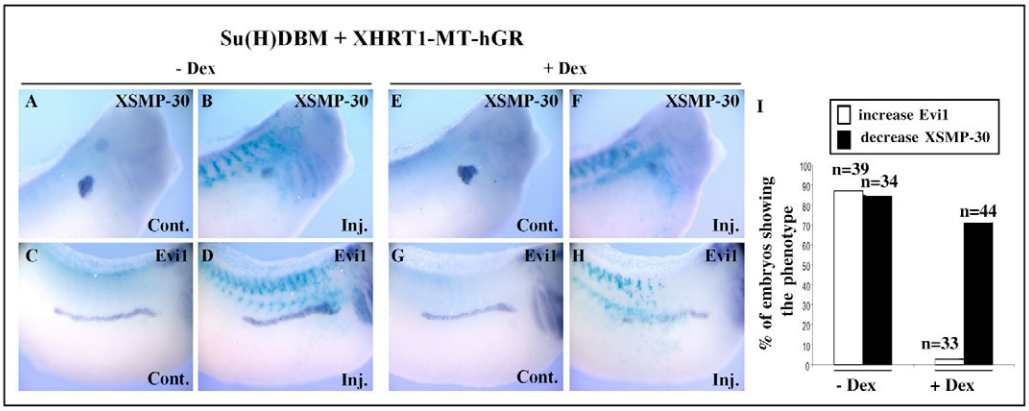

Fig. 7. *XHRT1* reverses the effect of *Su(H)DBM* on *Evi1* but is not sufficient to restore *XSMP-30* expression. (**A-H**) Late tailbud/early tadpole stage embryos injected with 500 pg of *Su(H)DBM* mRNA together with 500 pg of *XHRT1a-mut-MT-hGR* mRNA, untreated (A-D) or dexamethasone treated (E-H) at stage 22. β-galactosidase RNA was co-injected to identify the injected side. Note the decrease of *XSMP-30* and the increase of *Evi1* expression in untreated embryos, and the reduction of both *XSMP-30* and *Evi1* expression in treated embryos. (**I**) Quantification of the results. Embryos were classified into two phenotypes (increase *Evi1* and decrease *XSMP-30*). *n*, number of cases analysed.

dynamic restricted expression patterns within the developing pronephros and are regulated by Notch. In most cases, their expression pattern resembles that of their murine orthologs. In mouse, *HRT1* mRNA is detected during the earliest stage of development of the nephron. At the comma and S-shaped body stages, *HRT1* transcripts are localized to the more proximal regions of the developing nephron that will form the loop of Henle to the developing podocytes (Leimeister et al., 2003; Chen and Al-Awqati, 2005). We found that in *Xenopus*, *XHRT1* is expressed early in the developing pronephros region in the pronephric mesoderm that will give rise to the glomus. *X-Delta-1* is the only Notch ligand to be expressed at those early stages in the pronephric region. It is expressed in cells of the lateral layer in close contact with *XHRT1*-expressing cells, suggesting that it may trigger *XHRT1* activation in the surrounding cells. *xWT1* is another potential regulator of *XHRT1*, as both genes are activated at about the same time in the forming glomus and its translational inhibition decreased *XHRT1* expression. Interestingly, we observed that the expression of lateral specific genes is not expanded in the xWT1 morphants, suggesting that xWT1 is not the only factor that controls mediolateral patterning of the pronephros. Later on, expression of *XHRT1* in the medial layer is downregulated and strong staining is detected transiently in the dorsoanterior proximal-tubule forming part of the pronephros anlagen itself. Thus, both in mouse and in *Xenopus*, *HRT1* is early and selectively expressed in proximal compartments of the developing nephron, suggesting an early role in glomus and proximal tubule formation. At early tadpole stage, we observed that *XHRT1* expression is restricted to the tip of the forming tubules, suggesting another later role in the subcompartmentalization of the tubules.

HES1 in the mouse is expressed in most cells during early stages of kidney development. Its expression becomes more restricted later during development (Leimeister et al., 2003; Chen and Al-Awqati, 2005). Similarly in *Xenopus*, *Xhairy1* and *Xhairy2b* are initially expressed in both pronephric mesodermal layers, suggesting that they may have early a broader role than *XHRT1*. In contrast to the other bHLH-O genes, *Xhairy2b* expression is maintained in late tadpoles. Its expression at that stage resembles that of the Notch ligand *X-Serrate-1*. As recent investigations have demonstrated that the Notch ligands not only deliver their signal by binding to Notch

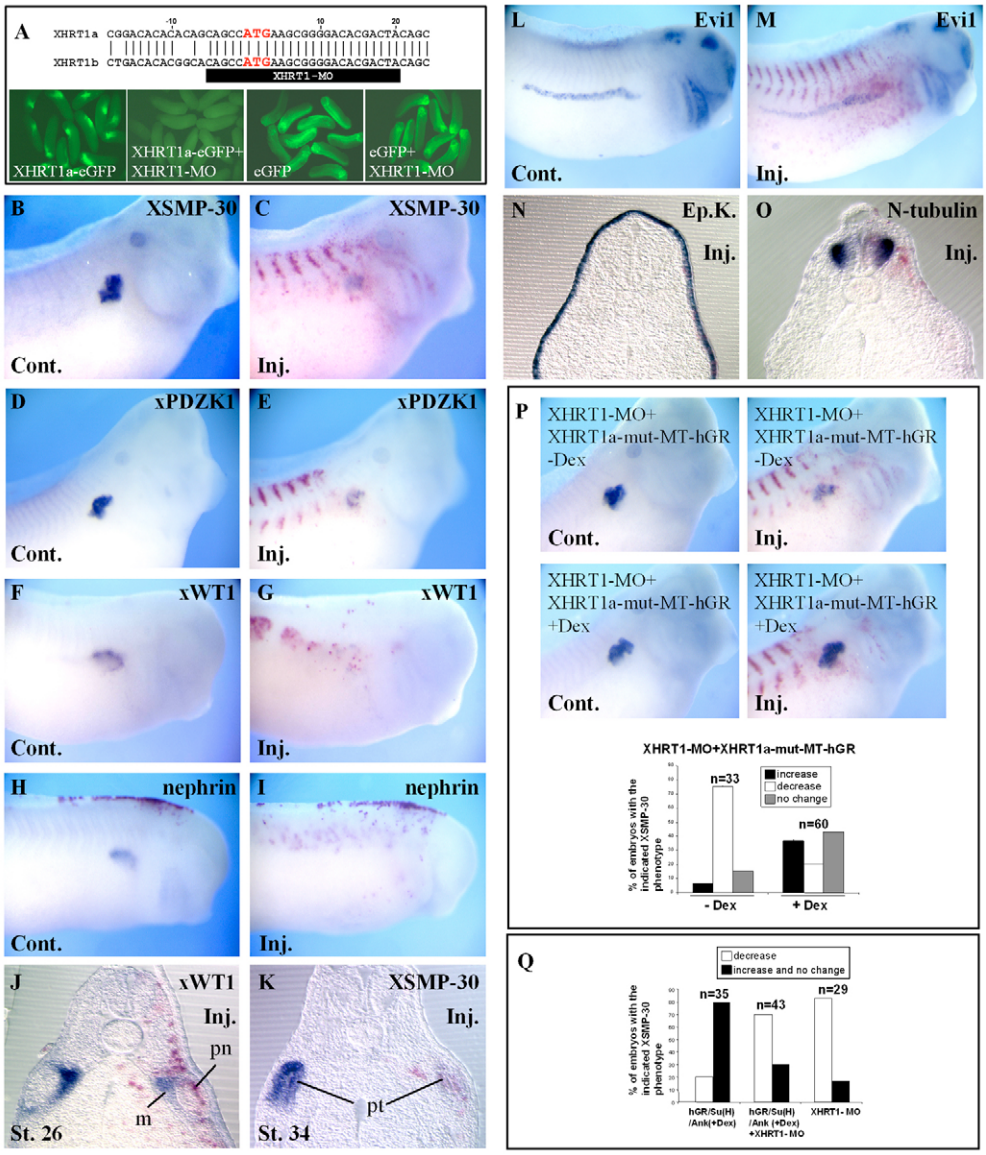

Fig. 8. Antisense morpholinos against XHRT1 reduces the expression of glomus and proximal tubule markers. (**A**) Design of the XHRT1-MO that targets both pseudoalleles. (Bottom) In vivo translation of *XHRT1a-eGFP* is specifically inhibited by XHRT1-MOs. Embryos were injected with 500 pg of *XHRT1-eGFP* or *eGFP* mRNA, alone or in combination with 15 ng of the XHRT1-MO, as indicated. (**B-O**) Embryos injected with 15 ng XHRT1-MO and β-galactosidase mRNA analysed with the indicated markers. (B-I) Control and injected sides of XHRT1-MO-injected embryos with decreased *XSMP-30*, *xPDZK1*, *xWT1* and *nephrin* expression. (J,K) Transversal sections of XHRT1-MO-injected embryos. (L,M) XHRT1 knockdown has no effect on *Evi1* expression. (N,O) Transversal sections of XHRT1-MO-injected embryos. *Ep. keratin* and *N-tubulin* expression is unaffected by XHRT1 knockdown. (**P**) Co-injection of the XHRT1-MO with 500 pg of *XHRT1a-mut-MT-hGR* mRNA rescues *XSMP-30* expression in stage 22 dexamethasone-treated XHRT1-MO-injected embryos. Changes in the expression of *XSMP-30* were scored and classified as in Fig. 6A. (**Q**) Co-injection of 15 ng XHRT1-MO inhibits the effect of overexpression of *hGR/Su(H)/Ank* (500 pg) on *XSMP-30* expression. Injected embryos were dexamethasone treated at stage 22. Changes in the expression of *XSMP-30* were classified into two groups (no change or increase, decrease). *n*, number of embryos analysed; m, medial intermediate mesodermal layer; pn, pronephros; pt, pronephric tubules.

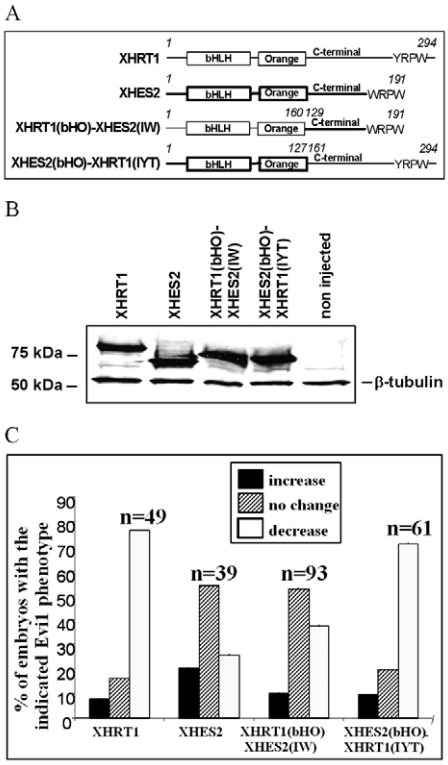

Fig. 9. XHRT1 specific function in the pronephros is dependent on its C-terminal region. (**A**) Schematic representation of the XHRT1, XHes2 and chimeric proteins. All constructs encode Myc tag and hGR fusion proteins (not represented). The numbers correspond to the amino acids of the protein domains. (**B**) Western blot analysis of the expression level of XHRT1, XHes2 and chimeric proteins. Extracts prepared from animal caps derived from embryos injected with 250 pg of each construct were immunoblotted with anti-Myc and anti-β-tubulin antibodies. (**C**) Comparison of the activity of XHRT1, XHes2 and chimeric proteins. Embryos were injected with 500 pg mRNA of each construct. The embryos were treated with dexamethasone at stage 18 and fixed at stage 26. Changes in *Evi1* expression were scored as in Fig. 6A.

receptors, but also by playing a functional role in the cells in which they are expressed (Ascano et al., 2003; LaVoie and Selkoe, 2003; Ikeuchi and Sisodia, 2003), it is tempting to speculate that X-Serrate-1 may play a role in *Xhairy2b* activation.

Hes5 expression in the mouse is restricted to the middle segment of S-shaped bodies that gives rise to the loop of Henle (Piscione et al., 2004; Chen and Al-Awqati, 2005). Similarly, in *Xenopus*, *esr9* and *esr10* expression are detected in the pronephros anlagen of late tailbud embryos in a region slightly more ventral to *XHRT1* expression. At that stage, expression of *esr9* and *esr10* resembles that of the Notch ligands *X-Serrate-1* and *X-Serrate-2*, suggesting that they may be involved in their regulation. Further experiments are needed to analyse the contribution of the distinct Notch ligands to the spatiotemporal regulation of bHLH-O genes in the pronephros.

Role of Notch signaling and downstream bHLH-O targets in the specification of glomus and proximal tubules within the pronephros anlagen

In the *Xenopus* pronephros and the mouse metanephroi, Notch activation has been shown to be essential for proximal tubule and glomus formation (McLaughlin et al., 2000; McCright et al., 2001; Wang et al., 2003a; Cheng et al., 2003; Cheng and Kopan, 2005; Van Campenhout et al., 2006). Here, we show that activation of Notch in the pronephric primordium favors first glomus and later proximal tubule fates, which correlates with the temporal expression pattern of XHRT1, esr9 and esr10. To the best of our knowledge, this is the first time that the precise temporal dependence on Notch activation of those two processes has been investigated. We observed that overexpression and the morpholino knockdown of XHRT1, but not that of esr9, esr10 or Xhairy2b, phenocopy the defects observed upon activation and inhibition of Notch signaling. These results indicate that XHRT1 may act to repress distal tubule and duct cell fates in the portion of pronephric mesoderm fated to form the glomus and the proximal tubules. They also suggest that XHRT1 has a role distinct to that of the other bHLH-O repressors in the earliest stage of pronephros development. However, we cannot exclude that these finding may arise from a reduced efficiency of the corresponding MOs. Kidneys from mice where the *Hes1* or *Hes5* genes were deleted show no defects, whereas compound homozygotes for both *Hes1* and *Hes5* die before kidney development (Chen and Al-Awqati, 2005). Further evaluation of the targeted disruption of these genes in conditional knockout mice is required to determine their contribution in nephron patterning.

In contrast to Notch activation using an inducible form of an activated *Su(H)* construct, *XHRT1* overexpression does not increase proximal tubule/glomus formation. We also observed that XHRT1 does not restore the expression of proximal tubule markers in embryos where Notch has been inhibited by injection of a *Su(H)DBM* construct, suggesting that it mediates only part of the effects executed by Notch. xWT1 is another transcriptional repressor that has also been suggested to have a role in the repression of tubule and duct specific genes in the forming glomus (Wallingford et al., 1998; Van Campenhout et al., 2006). Further investigations are required to elucidate the hierarchical relationship that links XHRT1 and xWT1, and to identify other factors that may contribute to glomus/proximal tubule cell fate decisions.

In *HRT1* single mutant or in *HRT1*/*HRT2* double mutant mice, no kidney defects have been reported (Fisher et al., 2004; Kokubo et al., 2005). The difference in phenotype between *Xenopus* and mouse may be due to differential evolution or expression of the HRT genes in both species. Differential evolution of the *HRT2* gene has been recently reported in fish (Winkler et al., 2003). In the mouse, *HRT1* and *HRT3*, but not *HRT2*, have overlapping expression in the developing nephrons (Leimeister et al., 2003). The identification of the functional role of *HRT1* in mouse nephrogenesis will require the analysis of the phenotype through nephrogenesis of *HRT1*/*HRT3* double knockout mice.

XHRT1 specific function in the pronephros is dependent on its C-terminal sequences

Swapping experiments between XHRT1 and the related bHLH-O XHes2 that is inactive in the pronephros have shown that the specific properties of XHRT1 are dependent on their divergent C-terminal sequences. Whereas the XHes2 protein has a C-terminal domain of 62 amino acids terminated by a WRPW motif, the XHRT1 C-terminal domain is much longer (133 amino acids) and does not contain the WRPW motif; this is replaced by a related sequence (YRPW) near its C terminus. In E(spl) in *Drosophila*, this region has also been shown to be important, as mutants that lack the sequences C terminal to the Orange domain act as dominant-negative variants (Giebel and Campos-Ortega, 1997). In zebrafish Her4, the Orange

domain-WRPW interval is also essential for its ability to block neurogenesis (Takke et al., 1999). At present, the functional role of the bHLH-O C-terminal sequences is unclear. In XHRT1, those sequences are involved, together with the bHLH and Orange domains, in dimerization and selection of the bHLH partners, and they possess intrinsic repression activity (Taelman et al., 2004). In HES1, the C-terminal domain allows interaction with the Runt-related protein CbFa1 (McLarren et al., 2000). Further studies are needed to clarify the role of the C-terminal sequences in XHRT1-specific function.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/133/15/2961/DC1

References

Ascano, J. M., Beverly, L. J. and Capobianco, A. J. (2003). The C-terminal PDZ-ligand of Jagged1 is esential for cellular transformation. *J. Biol. Chem.* **278**, 8771-8779.

Brändli, A. W. (1999). Towards a molecular anatomy of the Xenopus pronephric kidney. *Int. J. Dev. Biol.* **43**, 381-395.

Brennan, H. C., Nijjar, S. and Jones, E. A. (1998). The specification of the pronephric tubules and duct in Xenopus laevis. *Mech. Dev.* **75**, 127-137.

Brennan, H. C., Nijjar, S. and Jones, E. A. (1999). The specification and growth factor inducibility of the pronephric glomus in Xenopus laevis. *Development* **126**, 5847-5856.

Carroll, T. and Vize, P. D. (1996). Wilm's tumor suppressor gene is involved in the development of disparate kidney forms: evidence from expression in the Xenopus pronephros. *Dev. Dyn.* **206**, 131-138.

Carroll, T. and Vize, P. D. (1999). Synergism between Pax8 and lim-1 in embryonic kidney development. *Dev. Biol.* **214**, 46-59.

Chan, T.-C., Takahashi, S. and Asashima, M. (2000). A role for Xlim1 in pronephros development in Xenopus laevis. *Dev. Biol.* **228**, 256-269.

Chen, L. and Al-Awqati, Q. (2005). Segmental expression of Notch and Hairy genes in nephrogenesis. *Am. J. Physiol.* **288**, 939-952.

Cheng, H. T. and Kopan, R. (2005). The role of Notch signaling in specification of podocyte and proximal tubules within the developing mouse kidney. *Kidney Int.* **68**, 1951-1952.

Cheng, H. T., Miner, J. H., Lin, M., Tansey, M. G., Roth, K. and Kopan, R. (2003). γ-Secretase activity is dispensable for mesenchyme-to-epithelium transition but required for podocyte and proximal tubule formation in developing mouse kidney. *Development* **130**, 5031-5042.

Chitnis, A., Henrique, D., Lewis, J., Ish-Horowicz, D. and Kintner, C. (1995). Primary neurogenesis in Xenopus embryos regulated by a homologue of the Drosophila neurogenic gene Delta. *Nature* **375**, 761-766.

Deblandre, G. A., Wettstein, D. A., Koyano-Nakagawa, N. and Kintner, C. (1999). A two step mechanism generates the spacing pattern of the ciliated cells in the skin of Xenopus embryos. *Development* **126**, 4715-4728.

Fisher, A., Schumacher, N., Maier, M., Sentner, M. and Gessler, M. (2004). The Notch target genes Hey1 and Hey2 are required for embryonic vascular development. *Genes Dev.* **18**, 901-911.

Gerth, V. E., Zhou, X. and Vize, P. D. (2005). Nephrin expression and three-dimensional morphogenesis of the Xenopus pronephric glomus. *Dev. Dyn*. **233**, 1131-1139.

Giebel, B. and Campos-Ortega, J. A. (1997). Functional dissection of the Drosophila Enhancer of split protein, a suppressor of neurogenesis. *Proc. Natl. Acad. Sci. USA* **94**, 6250-6254.

Guo, G., Morrison, D. J., Licht, J. D. and Quaggin, S. E. (2004). WT1 activates a glomerular-specific enhancer identified from the human nephrin gene. *J. Am. Soc. Nephrol.* **15**, 2851-2856.

Harland, R. M. (1991). In situ hybridization: an improved whole-mount method for Xenopus embryos. *Methods Cell Biol.* **36**, 685-695.

Heller, N. and Brandli, A. W. (1999). Xenopus Pax-2/5/8 orthologues: novel insights into Pax gene evolution and identification of Pax-8 as the earliest marker for otic and pronephric cell lineages. *Dev. Genet.* **24**, 208-219.

Huang, Q., Raya, A., DeJesus, P., Chao, S.-H., Quon, K. C., Caldwell, J. S., Chanda, S. K., Izpisua-Belmonte, J. C. and Schultz, P. (2004). Identification of p53 regulators by genome-wide functional analysis. *Proc. Natl. Acad. Sci. USA* **101**, 3456-3461.

Ikeuchi, T. and Sisodia, S. (2003). The Notch ligands, Delta1 and Jagged2, are substrates for presenilin-dependent "γ-secretase" cleavage. *J. Biol. Chem.* **278**, 7751-7754.

Iso, T., Kedes, L. and Hamamori, Y. (2003). HES and HERP families: multiple effectors of the Notch signaling pathway. *J. Cell. Physiol.* **194**, 237-255.

James, R. G. and Schultheiss, T. M. (2005). BMP signaling promotes intermediate mesoderm gene expresssion in a dose-dependent, cell-autonomous and translation-dependent manner. *Dev. Biol.* **288**, 113-125.

Jen, W. C., Gawantka, V., Pollet, N., Niehrs, C. and Kintner, C. (1999). Periodic repression of Notch pathway governs the segmentation of Xenopus embryos. *Genes Dev.* **13**, 486-499.

Jonas, E., Sargent, T. D. and Dawid, I. B. (1985). Epidermal keratin gene expressed in embryos of Xenopus laevis. *Proc. Natl. Acad. Sci. USA* **82**, 5413-5417.

Jones, E. A. (2005). Xenopus: a prince among models for pronephric kidney development. *J. Am. Soc. Nephrol.* **16**, 313-321.

Kiyota, T., Jono, H., Kuriyama, S., Hasegawa, K., Miyatani, S. and Kinoshita, T. (2001). X-Serrate-1 is involved in primary neurogenesis in Xenopus laevis in a complementary manner with X-Delta-1. *Dev. Genes Evol.* **211**, 367-376.

Kokubo, H., Miyagawa-Tomita, S., Nakazawa, M., Saga, Y. and Johnson, R. L. (2005). Mouse hers1 and hesr2 genes are redundantly required to mediate Notch signaling in the developing cardiovascular system. *Dev. Biol.* **278**, 301-309.

Koyano-Nakagawa, N., Kim, J., Anderson, D. and Kintner, C. (2000). Hes6 acts in a positive feedback loop with the neurogenins to promote neuronal differentiation. *Development* **127**, 4203-4216.

LaVoie, M. J. and Selkoe, D. J. (2003). The Notch ligands, Jagged and Delta, are sequentially processed by α-secretase and presenilin/γ-secretase and release signaling fragments. *J. Biol. Chem.* **278**, 34427-34437.

Leimeister, C., Schumacher, N. and Gessler, M. (2003). Expression of Notch pathway genes in the embryonic mouse metanephros suggests a role in proximal tubule development. *Gene Expr. Patterns* **3**, 595-598.

Li, Y., Fenger, U., Niehrs, C. and Pollet, N. (2003). Cyclic expression of esr9 gene in Xenopus presomitic mesoderm. *Differentiation* **71**, 83-89.

Mauch, T. J., Yang, G., Wright, M., Smith, D. and Schoenwolf, G. C. (2000). Signals from the trunk paraxial mesoderm induce pronephros formation in chick intermediate mesoderm. *Dev. Biol.* **220**, 62-75.

McCright, B., Gao, X., Shen, L. Y., Lozier, J., Lan, Y., Maguire, M., Herzlinger, D., Weinmaster, G., Jiang, R. L. and Gridley, T. (2001). Defects in development of the kidney, heart and eye vasculature in mice homozygous for a hypomorphic Notch2 mutation. *Development* **128**, 491-502.

McLarren, K. W., Lo, R., Grbavec, D., Thirunavukkarasu, K., Karsenty, G. and Stifani, S. (2000). The mammalian basic helix-loop-helix protein HES-1 binds to and modulates the transactivating function of the runt-related factor Cbfa1. *J. Biol. Chem.* **275**, 530-538.

McLaughin, K. A., Rones, M. S. and Mercola, M. (2000). Notch regulates cell fate in the developing pronephros. *Dev. Biol.* **227**, 567-580.

Nieuwkoop, P. D. and Faber, J. (1967). *Normal Table of Xenopus laevis (Daudin)*. Amsterdam: North Holland.

Obara-Ishihara, T., Kuhlman, T., Niswander, L. and Herzlinger, D. (1999). The surface ectoderm is essential for nephric duct formation in intermediate mesoderm. *Development* **126**, 1103-1108.

Pichon, B., Taelman, V., Kricha, S., Christophe, D. and Bellefroid, E. J. (2002). XHRT-1, a hairy and Enhancer of split related gene with expression in floor plate and hypochord during early Xenopus embryogenesis. *Dev. Genes Evol.* **212**, 491-495.

Pichon, B., Taelman, V., Bellefroid, E. J. and Christophe, D. (2004). Transcriptional repression by the bHLH-Orange factor XHRT1 does not involve the C-terminal YRPW motif. *Biochim. Biophys. Acta* **1680**, 46-52.

Piscione, T. D., Wu, M. Y. J. and Quaggin, S. E. (2004). Expression of Hairy/Enhancer of Split genes, Hes1 and Hes5, during murine nephron morphogenesis. *Gene Expr. Patterns* **4**, 707-711.

Rones, M. S., Woda, J., Mercola, M. and McLaughlin, K. A. (2002). Isolation and characterization of Xenopus Hey-1: a downstream mediator of Notch signaling. *Dev. Dyn.* **225**, 554-560.

Ryffel, G. U. (2003). What can a frog tell us about kidney development. *Nephron Exp. Nephrol.* **94**, 35-43.

Sato, A., Asashima, M., Yokota, T. and Nishinakamura, R. (2000). Cloning and expression pattern of a Xenopus pronephros-specific gene, XSMP-30. *Mech. Dev.* **92**, 273-275.

Satow, T., Bae, S.-K., Inoue, T., Miyoshi, G., Tomita, K., Bessho, Y., Hashimoto, N. and Kageyama, R. (2001). The basic helix-loop-helix gene hesr2 promotes gliogenesis in mouse retina. *J. Neurosci.* **21**, 1265-1273.

Seufert, D. W., Brennan, H. C., DeGuire, J., Jones, E. A. and Vize, P. D. (1999). Developmental basis of pronephric defects in xenopus body plan phenotypes. *Dev. Biol.* **215**, 233-242.

Taelman, V., Van Wayenbergh, R., Sölter, M., Pichon, B., Pieler, T., Christophe, D. and Bellefroid, E. J. (2004). Sequences downstream of the

bHLH domain of the Xenopus hairy-related transcription factor-1 act as an extended dimerization domain that contributes to the selection of the partners. *Dev. Biol.* **276**, 47-63.

Takke, C., Dornseifer, P., Weizcäcker, E. V. and Campos-Ortega, J. A. (1999). her4, a zebrafish homologue of the Drosophila neurogenic gene E(spl), is a target of Notch signaling. *Development* **126**, 1811-1821.

Tsuji, S., Cho, K. W. and Hashimoto, C. (2003). Expression of a basic helix-loop-helix transcription factor Xhairy2b during Xenopus laevis development. *Dev. Genes Evol.* **213**, 407-411.

Van Campenhout, C., Nichane, M., Antoniou, A., Pendeville, H., Bronchain, O. J., Marine, J. C., Mazabraud, A., Voz, M. L. and Bellefroid, E. J. (2006). Evi1 is expressed specifically in the distal tubule and duct of the Xenopus pronephros and plays a role in its formation. *Dev. Biol.* **294**, 203-219.

Vize, P. D. (2003). The chloride conductance channel ClC-K is a specific marker for the Xenopus pronephric distal tubule and duct. *Gene Expr. Patterns* **3**, 347-350.

Vize, P. D., Woolf, A. S. and Bard, J. B. L. (2003). *The Kidney. From Normal Development to Congenital Disease*. Amsterdam: Academic Press.

Wagner, N., Wagner, K. D., Xing, Y., Scholz, H. and Schedl, A. (2004). The major podocyte protein nephrin is transcriptionally activated by the Wilms' tumor suppressor WT1. *J. Am. Soc. Nephrol.* **15**, 3044-3051.

Wallingford, J. B., Carroll, T. J. and Vize, P. D. (1998). Precocious expression of the Wilm's tumor gene xWT1 inhibits embryonic kidney development in Xenopus laevis. *Dev. Biol.* **202**, 103-112.

Wang, P., Pereira, F. A., Beasley, D. and Zheng, H. (2003a). Preselinins are required for the formation of comma- and S-shaped bodies during nephrogenesis. *Development* **130**, 5019-5029.

Wang, W., Prince, C. Z., Hu, X. and Pollman, M. J. (2003b). HRT1 modulates vascular smooth cell proliferation and apoptosis. *Biochem. Biophys. Res. Commun.* **308**, 596-601.

Wettstein, D. A., Turner, D. L. and Kintner, C. (1997). The Xenopus homolog od Drosophila Suppressor of Hairless mediates Notch signaling during primary neurogenesis. *Development* **124**, 693-702.

Winkler, C., Elmasri, H., Klamt, B., Nicolas, J. and Gessler, M. (2003). Characterization of hey bHLH genes in teleost fish. *Dev. Genes Evol.* **213**, 541-553.