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 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/Her2-1/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).
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 glomerus 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-S(+)-DAB, 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+MTXhes2-hGR, encoding an inducible, Myc-tagged Xhes2 protein; pCS2+MTXhRT1-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+MTXhes1-XhRT1-hGR, encoding an inducible, truncated form of XHRT1 (amino acids 1 to 127) with the carboxy terminus of XHRT1 (amino acids 161 to 294). In addition, pCS2+XHRT1-a-mut-MT-hGR, derived from pCS2+XHRT1-a-MT-hGR, incorporates several mismatches (small letters) in the MO target sequence: 5'-ATGAAGaGCGCAGATGAAGAATGAAAACTG-3' derived from pCS2+XHRT1a-MT-hGR; and pCS2+XHRT1-MT-hGR, encoding an inducible, truncated form of XHRT1 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 aMO, 5'-TATGCGTCCGGCCGGTCTGCGGCTGTCG-3'; xWT1 aMO, 5'-CATATCCCGGACATCCGACCCTCAT-3'; xWT1 bMO, 5'-CATATCCCGGACATCCGACCCTCAT-3';
- esr9 MO, 5'-CTGTCTGTTGAACTGGATGTGAAGA-3';
- esr10 MO, 5'-TGTGTTGAACTGGATGTGAAGA-3';
- Xhairy2a MO, 5'-ATGATGATGACGGCCGCGATTTGCTT-3';
- Xhairy2b MO, 5'-GATCATTGGATGACGGCCGCGATTTGCTT-3';

Individual Mors, or a mixture of both Mors 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-d-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 β-galactosidase activity in the pronephric region were scored.

**In situ hybridization**

Whole-mount in situ hybridization was carried out as previously described (Harland et al., 1991). X-Serratel-1 was identified through a search of the EST database (NIBB, xKLOUQp21). The X-Serratel-2 plasmid linearized with EcoRI was transcribed with T7 polymerase. Plasmids used for generating the other in situ hybridization probes are:

- XHRT1 (Pichon et al., 2002), Xhairy1 and Xhairy2b
- X-Serrate-1 (Harland et al., 1991).

Whole-mount in situ hybridization was carried out as previously described (Taelman et al., 2004) using the Xhairy1 (Taelman et al., 2004), Xhairy2b (Tsuij et al., 2003), X-ESR1 (Wettstein et al., 1997), X-ESR5 (Jen et al., 1999), X-ESR6 (Deblandre et al., 1999), Xhairy1 (Taelman et al., 2004), Xhairy2b (Tsuij et al., 2003), ESR-1 (Wettstein et al., 1997), ESR-4, ESR-5 (Jen et al., 1999), ESR-6e (Deblandre et al., 1999), 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-6, ESR-6e, Xhes2 and Hes6 mRNA were not detected in the pronephric anlagen between stages 20 and 36. XHRT1, Xhaiy1, 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 surrounding 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-Serratel-1 is expressed similarly to X-Serratel2 at that stage (data not shown). Although pronephric expression of XHRT1, esr9, esr10 and Xhairy1 is no longer detectable at late
**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 esr9 (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 X SMP-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.

**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. 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 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.

![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 glomerular 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 maker Evi1 (Fig. 7A-D). By contrast, in dexamethasone-treated embryos, we observed a reduction of both XSMP-30 and Evi1 expression (Fig. 7E-H). 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.
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**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,J,K,L,N,O,Q,R). Embryos at neurula stage are viewed from the dorsal side, injected side shown. 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 (R, 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 xCIC-K appears unaffected (Evi1 64%, n=37; xCIC-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. As shown in Fig. 9A, 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 xEvil 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.
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.

**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

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**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.

**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. 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 XHRT1-a-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 XHRT1-a-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 hGRSu(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 EvII 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 metanephros, 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 YRPW motif, the XHRT1 C-terminal domain is much longer (133 amino acids) and does not contain the YRPW 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 XenH1, 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 (McLaren et al., 2000). Further studies are needed to clarify the role of the C-terminal sequences in XenH1-specific function.

We thank Makoto Asahima, Andre Brändli, Elizabeth Jones, Jonathan Licht, Chris Kintner, Peter Vize and David Wilkinson for providing us with various constructs and antibodies, and Sadia Kricha for technical assistance. This work was supported by the Belgian program Interuniversity Attraction Poles (grant P35/35 to E.J.B.), by the Communauté Française de Belgique (grant number 3.4555.01 and 3.4556.01), and by a grant from the DFG in the context of the CMBP to T.P.

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

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