

Notch activates Wnt-4 signalling to control medio-lateral patterning of the pronephros

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Previous studies have highlighted a role for the Notch signalling pathway during pronephrogenesis in the amphibian *Xenopus laevis*, and in nephron development in the mammalian metanephros, yet a mechanism for this function remains elusive. Here, we further the understanding of how Notch signalling patterns the early *X. laevis* pronephros anlagen, a function that might be conserved in mammalian nephron segmentation. Our results indicate that early phase pronephric Notch signalling patterns the medio-lateral axis of the dorso-anterior pronephros anlagen, permitting the glomus and tubules to develop in isolation. We show that this novel function acts through the Notch effector gene *hrt1* by upregulating expression of *wnt4*. Wnt-4 then patterns the proximal pronephric anlagen to establish the specific compartments that span the medio-lateral axis. We also identified pronephric expression of *lunatic fringe* and *radical fringe* that is temporally and spatially appropriate for a role in regulating Notch signalling in the dorso-anterior region of the pronephros anlagen. On the basis of these results, along with data from previous publications, we propose a mechanism by which the Notch signalling pathway regulates a Wnt-4 function that patterns the proximal pronephric anlagen.

KEY WORDS: Notch, Wnt, Lunatic Fringe, Pronephros, *Xenopus*, Overexpression, Morpholino

INTRODUCTION

Recent research in the field of nephrology has determined the Notch signalling pathway as an important regulator of renal development (Dressler, 2008; Kopan et al., 2007; Liu et al., 2007; Taelman et al., 2006). Aberrant Notch signalling during development or in adulthood is a major cause of renal disease, specifically affecting glomerulus function (Barisoni, 2008; McCright et al., 2001; Mertens et al., 2008; Niranjana et al., 2008). Despite these studies, the mechanism by which the Notch signalling pathway regulates nephrogenesis remains unknown.

The Notch signalling pathway is a paracrine signalling pathway composed of the large transmembrane proteins Notch, Delta and Serrate (or Jagged in mammals and zebrafish). In the core pathway, Notch receptors bind Delta and Serrate ligands on opposing cells, which causes cleavage of the Notch receptor (Brou et al., 2000; Kopan and Goate, 2000), liberating its intracellular domain (Notch-ICD) (Schroeter et al., 1998; Taniguchi et al., 2002). Notch-ICD then translocates to the nucleus where it binds to the CSL transcription factor, switching it from a transcriptional repressor to a transcriptional activator (Jarriault et al., 1995). Consequently, altered gene expression directs the cell towards a specified cell fate. In addition to Notch-ICD canonical signalling, Notch-ICD independent and ligand-dependent signalling have also been reported to be important in the overall signal transduction of this pathway (Ascano et al., 2003; Ikeuchi and Sisodia, 2003; Kolev et al., 2005; LaVoie and Selkoe, 2003).

The pronephros, the embryonic kidney, develops from nephrogenic mesenchyme within the intermediate mesoderm lateral to the anterior somites (Brändli, 1999; Dressler, 2006; Jones, 2005). The pronephros is a paired organ, consisting of four distinct compartments; the glomus, coelomic cavity, nephrostomes and tubules (which are further characterized as proximal, intermediate,

distal and connecting tubules), together these components form a non-integrated nephron (Nieuwkoop and Faber, 1994; Reggiani et al., 2007; Saxén, 1987; Vize et al., 1995). The pronephros is a simple organ to study, showing both morphological and physiological similarities to more complex kidney forms, the meso- and metanephros, which make it an ideal model to study with reference to kidney development (Vize et al., 1995).

McLaughlin and co-workers (McLaughlin et al., 2000) were the first to highlight the role of the Notch signalling pathway in *Xenopus laevis* pronephrogenesis. This study showed that expression of components of the Notch signalling pathway in the pronephros appears in two phases. The early phase is characterized by *notch1*, *serrate1* and *deltal* expression in the dorso-anterior region of the pronephric anlagen between stages 21 and 32. This is then followed by late phase expression of *notch1* and *serrate1* more ventrally, in the developing proximal tubules. Knowledge of how the Notch signalling pathway regulates pronephrogenesis was further advanced by Taelman and colleagues (Taelman et al., 2006), who provided experimental evidence that early-phase pronephric Notch signalling promotes glomus formation, whereas late-phase pronephric Notch signalling promotes proximal tubule development.

In this study, we show that early-phase pronephric Notch signalling is required for medio-lateral patterning, a function that we suggest indirectly mediates proximal-distal patterning and permits the glomus and proximal tubules to develop in isolation. We provide evidence highlighting a novel role for Wnt-4 in establishing the glomus and nephrostomes, a function that is probably regulated by the Notch effector gene *hrt1* (also known as *hey1*). Furthermore, we report pronephric expression of *X. laevis* homologues of the *Drosophila fringe* gene, *lunatic fringe* and *radical fringe*, and propose a role for their gene products in proximal pronephrogenesis.

MATERIALS AND METHODS

Whole mount in situ hybridisation

Whole-mount in situ hybridisation was carried out as described elsewhere (Harland, 1991). The embryos were fixed in MEMFA (0.5 M MOPS, pH 7.4, 100 mM EGTA, 1 mM MgSO₄, 4% formaldehyde) and linearized

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plasmid from Na^+/K^+ -ATPase (*SmaI/T7*), *slc5a2* (*EcoRI/T7*), *odf3* (*EcoRI/T7*), *nephrin* (*SmaI/T7*), *Wnt-4* (*XhoI/T7*), *Notch-1* (*Clal/SP6*), *Delta-1* (*NdeI/T7*), *Serrate-1* (*HindIII/T7*), *lunatic fringe* (*NcoI/T7*), *radical fringe* (*BamHI/T7*), *CIC-K* (*NotI/T7*), *GATA-3* (*SmaI/T7*), *Xbrachyury* (*SacII/T3*), *MHC* (*NcoI/SP6*), *HRT-1* (*Not-I/T7*) and *Lim-1* (*XhoI/T7*) was used to generate digoxigenin-11-UTP-labelled (Boehringer Mannheim) antisense RNA probes from the polymerases indicated. Probes were visualised using anti-DIG-alkaline phosphatase secondary and NBT/BCIP for the colour reaction according to the manufacturer's recommendations (Boehringer Mannheim).

Embryo culture

Embryos were obtained by in vitro fertilisation of hormonally stimulated *Xenopus laevis* and staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1994). Standard embryological procedures and culturing conditions were used as described by Jones and Woodland (Jones and Woodland, 1986).

mRNA synthesis, morpholino antisense oligonucleotides and micro-injection

Capped RNAs were synthesised in vitro from Notch-ICD, $\Delta\text{Delta}^{\text{STU}}$, $\text{Su(H)}^{\text{DBM}}$ (Chitnis et al., 1995), *HRT-1-hGR* (Taelman et al., 2006), *Wnt-4*, *Lfng* and *Rfng* using the SP6 Message Machine Kit (Ambion). Typically 600 pg *notch-ICD* mRNA, 1 ng $\Delta\text{Delta}^{\text{STU}}$ mRNA, 1 ng $\text{su(H)}^{\text{DBM}}$ mRNA, 1 ng *hrt1* mRNA, 1 ng *wnt4* mRNA, 500 pg *lfng* mRNA, 2 ng *rfng* mRNA and 400 pg *beta-galactosidase* mRNA was injected. The *lfng*, *rfng*, *wnt4* and *hrt1* antisense morpholino oligonucleotides used were: *lfng* MO1 5'-gcttctccccagttcttcagcat-3'; *lfng* MO2 5'-gcgctacctctctctctctata-3'; *rfng* MO 5'-tgaggccaacgtaagtactctcat-3'; *wnt4* MO 5'-gtactctgggctcatctctgctgctg-3'; and *hrt1* MO 5'-tagctgtgtcccgccttcatggctg-3'; (Gene Tools LLC). The control morpholino is a random sequence of the same length. Typically 20 ng of *rfng* MO, *hrt1* MO and control MO, 10 ng of *wnt4* MO and 5 ng *lfng* MO1 or *lfng* MO2 was injected. Embryos were dejellied and injected with mRNA alone or in combination with MO (as specified in the text) into a V2 blastomere of an eight-cell-stage embryo to target the future pronephros (Dale and Slack, 1987; Moody and Kline, 1990), under 5% Ficoll in BarthX.

Immunohistochemistry

Whole-mount immunohistochemistry was performed using standard methods on MEMFA-fixed embryos. The primary antibodies used were monoclonal antibody 3G8, which detects the proximal tubules, and 4A6, which detects the intermediate and distal tubules (Vize et al., 1995). The secondary antibody was alkaline phosphatase-conjugated goat anti-mouse (Sigma). BCIP/NBT (Boehringer) or Fast Red TR/Napthol AS/MX (Sigma) was used for the colour reaction, according to the manufacturer's recommendations.

In vitro translations of *lfng* and *rfng* mRNA

Either mRNA alone, or mRNA with a MO, was translated in vitro in the Rabbit Reticulocyte Lysate System (Promega) with ^{35}S -Met according to the manufacturer's protocol. Reactions (25 μl) were denatured at 95°C in 2×SDS loading buffer (Harlow and Lane, 1988) and run on a 10% (w/v) SDS-PAGE resolving gel using a vertical mini-gel apparatus for 2 hours at 100 V. The gel was exposed to Kodak X-ray film overnight at room temperature, before being developed.

RESULTS

Early phase pronephric Notch signalling promotes formation of the nephrostomes

In order to mis-activate the Notch signalling pathway we injected *notch-ICD* mRNA, a truncated form of the Notch receptor that is constitutively active, or a dominant-negative form of the Delta ligand, $\Delta\text{Delta}^{\text{STU}}$ (Chitnis et al., 1995). Both constructs were targeted to the lateral mesoderm including the future pronephros by injection into a V2 blastomere of embryos at the eight-cell stage, with *beta-galactosidase* mRNA to act as a lineage tracer. Embryos were cultured to various stages of development and effects on different

markers of distinct regions of the pronephros were observed. Pronephric phenotypes were determined by comparing the injected and uninjected sides. All data presented here that produced pronephric phenotypes were analysed statistically, using the Chi-squared test, and were significantly different to embryos injected with control mRNA within 95% confidence limits. Experiments were repeated several times, yielding similar data.

Overexpression of *notch-ICD* mRNA in embryos harvested at stage 41 and whole-mount antibody stained with 3G8, which detects nephrostomes and proximal pronephric tubules, and 4A6, which detects intermediate and distal pronephric tubules (Vize et al., 1995), showed an increase in size of the proximal region of the pronephros at the expense of the distal pronephric region. A total of 76% of embryos had ectopic 3G8 staining, whereas 4A6 staining was reduced in 97% of embryos ($n=34$; Fig. 1A). Injection of $\Delta\text{Delta}^{\text{STU}}$ mRNA inhibited proximal pronephros formation but had no effect on distal pronephros development. 33% of embryos had reduced 3G8 staining, with only 5% having reduced 4A6 staining ($n=36$; Fig. 1B), suggesting that the Notch signalling pathway has no role in distal pronephros cell fate determination.

To investigate this phenotype in more detail, we injected *notch-ICD* mRNA and $\Delta\text{Delta}^{\text{STU}}$ mRNA and cultured embryos to stage 34. In situ hybridisations for two markers of pronephric tubules; Na^+/K^+ -ATPase, an ion transporter expressed in the proximal, intermediate and distal tubules, and *slc5a2*, a solute carrier expressed in the proximal tubules (Raciti et al., 2008; Reggiani et al., 2007), was then performed. Injection of *notch-ICD* mRNA reduced Na^+/K^+ -ATPase (also known as *atp1a1*) expression in 96% of embryos ($n=24$; Fig. 1C), and similarly reduced *slc5a2* expression in 95% of embryos analysed ($n=38$; Fig. 1E), thus tubulogenesis was completely inhibited. Injection of $\Delta\text{Delta}^{\text{STU}}$ mRNA reduced *slc5a2* expression in 76% of embryos ($n=34$; Fig. 1F), but had a localised effect on Na^+/K^+ -ATPase expression. In 23% of embryos Na^+/K^+ -ATPase expression was completely reduced, however the most prominent phenotype, observed in 47% of embryos, was a reduction in only the proximal tubule region of the Na^+/K^+ -ATPase expression pattern ($n=43$; Fig. 1D).

Since injection of *notch-ICD* mRNA inhibited proximal tubule formation (*slc5a2* reduction), but caused ectopic 3G8 staining, we concluded that this 3G8 staining must be completely nephrostomal, because 3G8 only stains nephrostome and proximal tubule domains. To confirm this, embryos were injected with *notch-ICD* mRNA and $\Delta\text{Delta}^{\text{STU}}$ mRNA as previously described, cultured to stage 34 and in situ hybridised for expression of *outer dense fibre 3* (*odf3*), a marker of multi-ciliated cells in the epidermis and nephrostomes. Overexpression of $\Delta\text{Delta}^{\text{STU}}$ inhibited nephrostomal *odf3* expression in 89% of embryos ($n=28$; Fig. 1H). However, as expected, we observed ectopic nephrostomal *odf3* expression in 74% of embryos injected with *notch-ICD* mRNA ($n=27$; Fig. 1G). As the percentage of embryos showing an increase in *odf3* expression was equivalent to those with ectopic 3G8 staining, we conclude that *notch-ICD* overexpression resulted in excess nephrostomal tissue.

McLaughlin and co-workers (McLaughlin et al., 2000) and Taelman and colleagues (Taelman et al., 2006) showed that early misactivation of the Notch signalling pathway induced ectopic glomus formation, a result we have confirmed; 81% of embryos injected with *notch-ICD* mRNA had increased staining of the glomus marker *nephrin*, as observed by in situ hybridisation ($n=27$) (Fig. 1I). $\Delta\text{Delta}^{\text{STU}}$ mRNA inhibited *nephrin* expression in 88% of embryos ($n=34$; Fig. 1J). In conclusion, early phase Notch signalling inhibits tubulogenesis and promotes glomus and nephrostome formation.

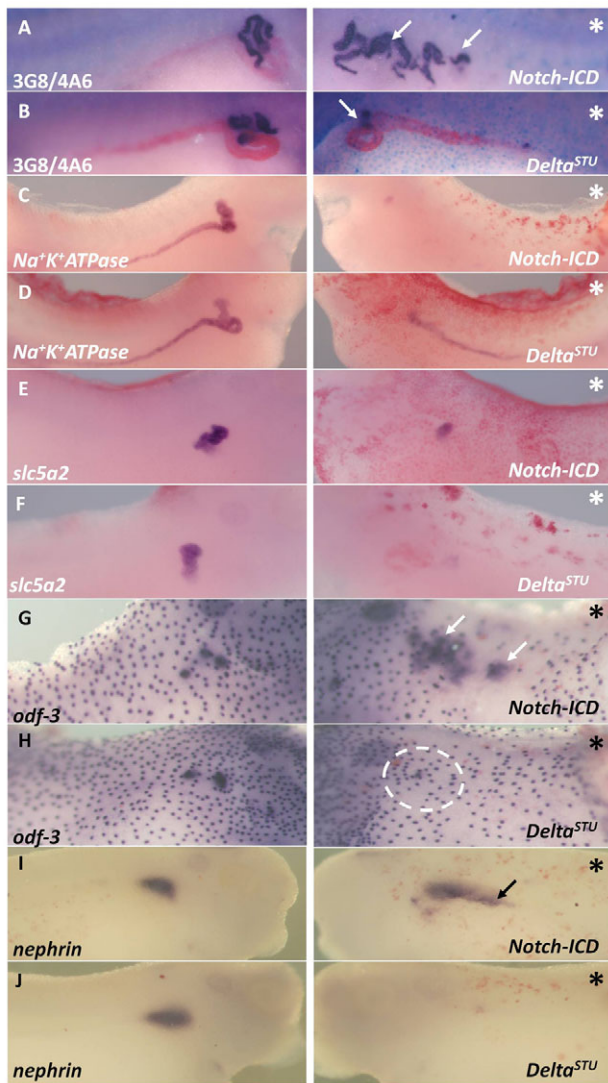


Fig. 1. Mis-activating early pronephric Notch signalling caused ectopic medial pronephrogenesis. (A–J) *X. laevis* embryos were injected at the eight-cell stage into a V2 blastomere to target tissues including the presumptive pronephric region. *notch-ICD* mRNA or *delta^{STU}* mRNA was co-injected with *beta-galactosidase* mRNA to act as a lineage tracer [blue (A,B); red (C–J) staining on the injected side]. At stage 41, 3G8 is ectopic (white arrows) and 4A6 is reduced in embryos injected with *notch-ICD* mRNA (A). *delta^{STU}* reduces 3G8 staining (white arrow) but has no effect on 4A6 staining (B). At stage 36, *Na⁺K⁺ATPase* expression is reduced after overexpression of *notch-ICD* (C), but only the proximal domain of *Na⁺K⁺ATPase* expression is reduced upon *delta^{STU}* overexpression (D). *slc5a2* expression is inhibited after overexpression of *notch-ICD* (E) and *delta^{STU}* (F). At stage 32, *odf3* (white arrows, G) and *nephrin* (black arrow, I) expression is ectopic upon *notch-ICD* mRNA injection. *odf3* (white circle, H) and *nephrin* (I) are inhibited by *delta^{STU}*. Asterisk denotes injected side.

Delta^{STU} is presumed to act as an anti-morph to inhibit Notch signalling, but its exact mechanism of action is unknown. To confirm that the phenotypes observed after *delta^{STU}* overexpression resulted from inhibited Notch-ICD signalling we targeted a dominant-negative Suppressor of Hairless construct, Su(H)^{DBM}, to the pronephros. Overexpressing *su(H)^{DBM}* produced exactly the same pronephric phenotypes as *delta^{STU}* overexpression (see Fig. S1 in the

supplementary material), confirming that the pronephric phenotypes were a consequence of reduced Notch-ICD signalling. To further control for the specificity of these results, we have shown identical phenotypes using hormone inducible Notch constructs (see Fig. S2 in the supplementary material), which are consistent with previous publications (McLaughlin et al., 2000; Taelman et al., 2006).

Overexpression of *wnt4* affects pronephrogenesis in an identical manner to overexpression of *notch-ICD*

During development, the Notch and Wnt signalling pathways frequently, if not always, act in an integrated manner to promote specific cell fates (Hayward et al., 2008). The Wnt gene *wnt4*, is expressed in the proximal tubules and nephrostomes during stages of development when the Notch signalling pathway is inducing nephrostome and glomus cell fate decisions (Saulnier et al., 2002). We hypothesised that these two pathways could be integrated and thus aid cell fate decisions in the dorso-anterior region of the pronephric anlagen. In order to investigate this possibility, *wnt4* mRNA and *beta-galactosidase* mRNA were co-injected as previously described, and embryos were cultured to various stages of development to determine the effect of *wnt4* overexpression on different markers of distinct regions of the pronephros.

wnt4 mRNA induced ectopic 3G8 staining in 69% of embryos, with 4A6 staining reduced in 77% of embryos ($n=26$; Fig. 2A). Expression of the tubule markers *Na⁺K⁺ATPase* and *slc5a2* was reduced in 83% ($n=23$; Fig. 2B) and 92% ($n=36$; Fig. 2C) of embryos, respectively. *wnt4* mRNA induced ectopic nephrostomal *odf3* expression in 76% of embryos ($n=34$; Fig. 2D) and increased the domain of *nephrin* expression in 75% of embryos ($n=32$; Fig. 2E). Thus, *wnt4* overexpression induced ectopic nephrostome and glomus formation at the expense of tubules, an identical phenotype to *notch-ICD* overexpression.

Notch-ICD signalling induces expression of *wnt4* in the pronephros

To observe whether Notch signalling affected *wnt4* expression in the pronephros, embryos were injected with *notch-ICD* mRNA or *delta^{STU}* mRNA into the V2 blastomere, cultured to stage 28, and analysed for *wnt4* expression by in situ hybridisation. Of embryos injected with *notch-ICD* mRNA, 64% had ectopic *wnt4* expression ($n=69$; Fig. 3A). Injection of *delta^{STU}* mRNA inhibited *wnt4* expression on the injected side in 70% of embryos ($n=71$; Fig. 3B). In conclusion, activation of the Notch-ICD dependent pathway induced *wnt4* expression. Conversely, suppression of Notch signalling inhibited *wnt4* expression.

To establish whether pronephric *wnt4* overexpression could upregulate components of the Notch signalling pathway, *wnt4* mRNA was injected into the V2 blastomere and embryos were cultured to stage 28 where whole mount in situ hybridisation for expression of *notch1*, *delta1* and *serrate1* was performed (Fig. 3C,D). *wnt4* overexpression had no significant effect on *notch1* (data not shown), *delta1* or *serrate1* expression levels. *delta1* expression was reduced in only 6% of embryos ($n=32$; Fig. 3C). Expression of *serrate1* was reduced in 14% of embryos ($n=36$; Fig. 3D). However, we did observe a change in the anterior-posterior register of gene expression of both *delta1* (84%) and *serrate1* (61%). Frequently, elements of the expression pattern were more distal (see Fig. 3B, marked with bars), and different from *delta1* or *serrate1* expression on the uninjected side. In summary, *wnt4* overexpression alters the expression pattern of *delta1* and *serrate1*, but does not seemingly inhibit or upregulate the expression levels of these genes.

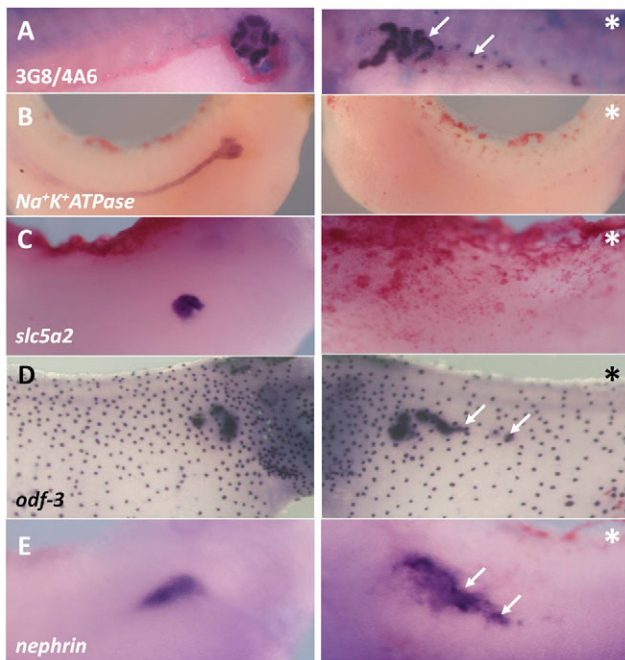


Fig. 2. *wnt4* overexpression induces the same phenotypes as injection of *notch-ICD* mRNA. (A–E) *X. laevis* embryos were injected as previously described. *wnt4* mRNA was co-injected with *beta-galactosidase* mRNA to act as a lineage tracer [blue (A); red (B–E) staining on the injected side]. At stage 41, 3G8 is ectopic (white arrows) and 4A6 is reduced on the injected side (A). At stage 36, *Na⁺K⁺ATPase* (B) and *slc5a2* (C) expression is reduced upon *wnt4* overexpression and at stage 32 *odf3* (D) and *nephrin* (E) expression is ectopic (white arrows). Asterisk denotes injected side.

Wnt-4 depletion prevents ectopic proximal pronephrogenesis caused by injection of *notch-ICD* mRNA

Given that *notch-ICD* overexpression induced *wnt4* expression, but *wnt4* overexpression had little effect on *delta1* and *serrate1* expression levels, we hypothesised that Wnt-4 could be acting downstream of the Notch signalling pathway. To investigate this possibility we simultaneously overexpressed *notch-ICD* and depleted endogenous *wnt4* translation using a morpholino oligonucleotide (MO) (Saulnier et al., 2002). Embryos injected with *notch-ICD* mRNA and *wnt4* MO were cultured to stage 32, where they were analysed for *nephrin* expression.

Single injections of *notch-ICD* mRNA induced ectopic expression of *nephrin* in 94% of embryos ($n=47$, data not shown). 74% of embryos injected with the *wnt4* MO had reduced *nephrin* expression ($n=27$; Fig. 4A). Co-injection of *notch-ICD* mRNA and the *wnt4* MO reduced expression of *nephrin* in 50% of embryos, with no embryos showing ectopic *nephrin* expression ($n=28$; Fig. 4B). We also repeated this experiment to determine the effect on 3G8 and 4A6 staining, and observed a 71% reduction in both 3G8 and 4A6 staining when *notch-ICD* mRNA and the *wnt4* MO are co-injected ($n=24$, data not shown). Again, none of these embryos had ectopic 3G8 staining. Consequently, ectopic proximal pronephrogenesis normally caused by overexpression of *notch-ICD* mRNA was completely prevented by the *wnt4* MO. In conclusion, this result suggests that Wnt-4 acts downstream of the Notch signalling pathway in the proximal pronephros to influence medio-lateral patterning.

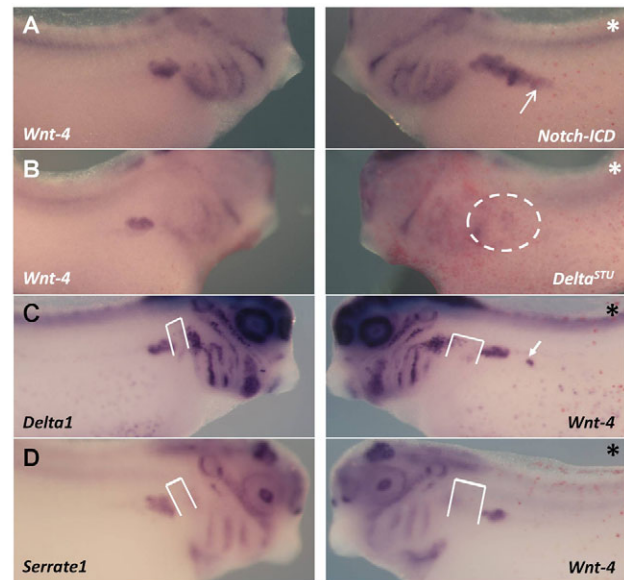


Fig. 3. Notch signalling regulates pronephric *wnt4* expression.

(A–D) *X. laevis* embryos were injected as previously described, with mRNA co-injected with *beta-galactosidase* mRNA to act as a lineage tracer (red staining on the injected side). Embryos were cultured to stage 28 when *wnt4* (A, B), *delta1* (C) and *serrate1* (D) expression was detected by in situ hybridisation. Mis-activation of Notch signalling by injection of *notch-ICD* mRNA causes ectopic *wnt4* expression (white arrow, A), whereas *delta^{STU}* mRNA inhibits *wnt4* expression (B). *wnt4* overexpression does not have an effect on *delta1* or *serrate1* expression levels (B). Expression of both these genes is altered in the anteroposterior register; frequently, expression is more distal (white brackets) and rarely ectopic (white arrow, C). Asterisk denotes injected side.

We next attempted the reciprocal experiment; overexpressing *wnt4* with concomitant inhibition of Notch-ICD signalling. Co-injection of *wnt4* mRNA with either *su(H)^{DBM}* mRNA (57% increased, $n=21$; Fig. 4C) or *delta^{STU}* mRNA (67% increased, $n=36$; see Fig. S3A in the supplementary material) caused ectopic *nephrin* expression, again suggesting Wnt-4 functions downstream of Notch signalling. We also observed the effects these co-injections had on proximal tubulogenesis. Co-injections of *wnt4* mRNA and *su(H)^{DBM}* mRNA caused ectopic *slc5a2* expression in 43% of embryos (21% of embryos had reduced *slc5a2* expression; $n=28$; Fig. 4D). This result is surprising because single injections of either *wnt4* mRNA or *su(H)^{DBM}* mRNA reduced *slc5a2* expression (Fig. 2A; see Fig. S2C in the supplementary material). Similarly, co-injection of *wnt4* mRNA and *delta^{STU}* caused ectopic proximal tubulogenesis in 15% of embryos, with only 8% of embryos having reduced *slc5a2* expression ($n=26$; see Fig. S3B in the supplementary material).

hrt1 acts upstream of *wnt4* in the pronephros

The Hairy-related transcription factor gene *hrt1*, encodes a downstream mediator of Notch signalling that has been shown to be responsive to Notch signalling in numerous tissues (Pichon et al., 2002; Ronés et al., 2002). HRT-1 has been shown to regulate glomus formation and patterning of the pronephros anlagen (Taelman et al., 2006). We aimed to determine whether *hrt1* overexpression and MO knockdown affected *wnt4* expression in the pronephros in order to position the activity of HRT-1 upstream or downstream of Wnt-4 during proximal pronephrogenesis.

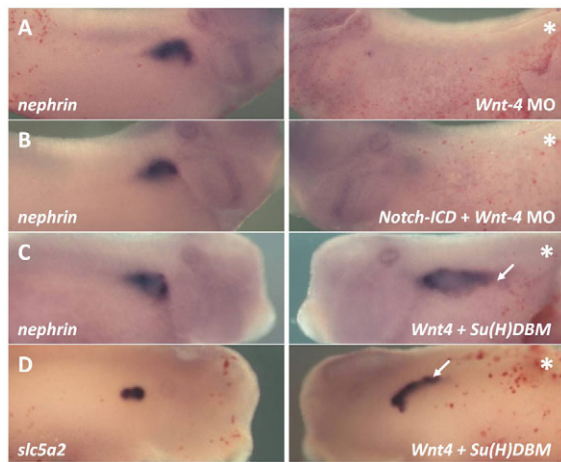


Fig. 4. *wnt4* acts downstream of the Notch pathway in the pronephros. (A–D) *X. laevis* embryos were injected as previously described and left to develop to stage 32, when in situ hybridisation detecting expression of *nephrin* and *slc5a2* was performed. (A) Knockdown of *wnt4* translation using a MO-inhibited *nephrin* expression. (B) Co-injection of *notch-ICD* mRNA and the *wnt4* MO also reduces *nephrin* expression. Co-injection of *su(H)^{DBM}* mRNA with *wnt4* mRNA, causes ectopic glomus formation (white arrow, C) and proximal tubulogenesis (white arrow, D). Asterisk denotes injected side.

We targeted mRNA encoding a hormone-inducible *hrt1-hGR* construct to the future pronephros as described previously. Embryos were left to develop to stage 18, where half of the injected embryos were switched into a dexamethasone containing culture medium to activate the message. Embryos were fixed at stage 28, stained for lineage label and analysed for *wnt4* expression. Embryos injected with *hrt1-hGR* mRNA alone displayed no phenotype (3% reduced, $n=38$; Fig. 5A). Of embryos incubated in dexamethasone, 29% displayed ectopic *wnt4* expression (Fig. 5B), 10% had reduced *wnt4* expression, and the remainder had no phenotype ($n=41$). We also knocked down *hrt1* translation using a MO previously shown to specifically deplete endogenous HRT-1 (Taelman et al., 2006). Injection of this *hrt1* MO to the pronephros caused a reduction in *wnt4* expression in 69% of embryos scored ($n=26$; Fig. 5C).

To place HRT-1 in the genetic hierarchy, we examined the effect of *notch-ICD* and *wnt4* overexpression on *hrt1* expression. Mis-activation of the Notch signalling pathway induces *hrt1* expression (Taelman et al., 2006). We reproduced this phenotype; 80% of embryos injected with *notch-ICD* mRNA had ectopic *hrt1* expression ($n=70$; Fig. 5D). However, following *wnt4* overexpression, no embryos showed regions of ectopic *hrt1* expression. A total of 92% of embryos showed a slight phenotype, of which 73% had disrupted *hrt1* expression, with the remaining embryos having reduced *hrt1* expression ($n=28$; Fig. 5E). Thus, overexpression of *wnt4* did not significantly affect *hrt1* expression. We suggest the disrupted pronephric expression profile of *hrt1* after *wnt4* overexpression is a product of the overall effect Wnt-4 has on pronephrogenesis (as observed in Fig. 2A). Thus HRT-1 acts upstream of Wnt-4 and is required for *wnt4* expression.

Overexpression of *notch-ICD* inhibits formation of the lateral pronephric mesoderm

Overexpression of *notch-ICD* caused highly significant pronephric phenotypes, increasing the size of the glomus and nephrostomes at the expense of the proximal tubules. To establish

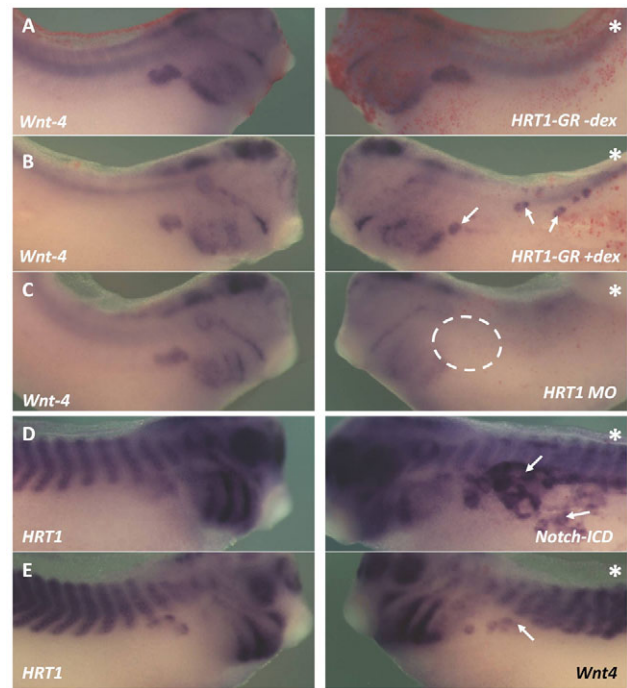


Fig. 5. *hrt1* is induced by *notch-ICD* mRNA injection and promotes *wnt4* expression. (A–E) *X. laevis* embryos were injected as previously described and left to develop to stage 28, when in situ hybridisation detecting expression of either *wnt4* (A–C) or *hrt1* (D,E) was performed. Embryos injected with hormone-inducible *hrt1-hGR* mRNA, but not incubated in dexamethasone to activate the message, have normal pronephric *wnt4* expression (A). Embryos injected with *hrt1-hGR* mRNA but incubated in dexamethasone from stage 18 onwards, display ectopic *wnt4* expression (B). MO depletion of HRT-1 inhibited *wnt4* expression (C). Overexpression of *notch-ICD* causes a large increase in pronephric *hrt1* expression (D), whereas *hrt1* expression is largely unaffected by *wnt4* mRNA injection (E). Asterisk denotes injected side.

the exact spatial relationships between these two differentiated tissues and other internal structures, the injected embryos were analysed in section.

Embryos injected with *notch-ICD* mRNA and stained for *nephrin* expression by in situ hybridisation at stage 32, marking the medial pronephric region, were sectioned (Fig. 6A). In these embryos, the medial pronephric mesoderm extended into the normal region of lateral pronephric mesoderm after *notch-ICD* mRNA injection. The only other gene whose expression is increased after overexpression of *notch-ICD* is *odf3*, a marker specific for the nephrostomes in the pronephros. Despite their position on the lateral side of the coelom, nephrostomes form from medial pronephric mesoderm (Howland, 1916). Thus it is perhaps unsurprising ectopic glomus formation is accompanied by ectopic nephrostome formation. In conclusion, we suggest that lateral pronephric mesoderm does not form in embryos overexpressing *notch-ICD*, the entire anlagen switches to medial pronephric fates.

Embryos, injected with *notch-ICD* mRNA and co-injected with *gfp* mRNA lineage label, were sorted for left- or right-injected sides at stage 26. At stage 41, embryos were fixed, wax-embedded, sectioned and analysed histologically with haematoxylin and eosin. Sections through the proximal pronephric region showed an abnormal mass of cells without tubular structure on the injected side

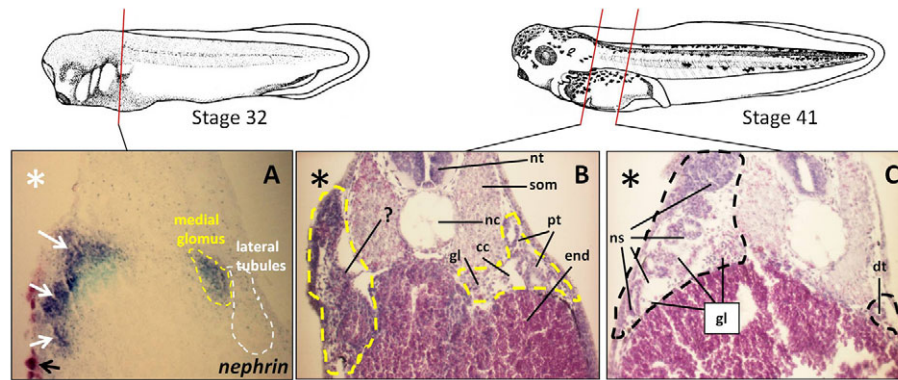


Fig. 6. Early *notch-ICD* and *wnt4* overexpression promotes medial pronephrogenesis at the expense of lateral pronephric cell fates.

(A-C) *X. laevis* embryos injected with *notch-ICD* mRNA, cultured to stage 32, and in situ hybridised for *nephrin* expression, were paraplast sectioned (A). *notch-ICD* overexpression causes the domain of *nephrin* expression to extend laterally. Paraplast wax sectioning of stage 41 embryos injected with *notch-ICD* mRNA highlights the severity of this phenotype (B,C). Normal pronephric layout in section can be observed on the uninjected side of B. On the injected side of this embryo, a mass of non-distinct cells can be observed proximally (B), and in the distal region ectopic nephrostomal tubules and podocytes can be detected (C). Regions of interest are outlined (dashed lines). Asterisk denotes injected side. gl, glomus; cc, coelomic cavity; pt, proximal tubules; end, endoderm; nc, notochord; som, somites; nt, neural tube; ns, nephrostomes; dt, distal tubules.

(Fig. 6B). Normal pronephric development of the proximal pronephric region could be clearly seen on the uninjected side of this embryo. The glomus, indicated by dispersed podocytes, the coelomic cavity, and the proximal tubules all formed in their correct positions. In sections through the distal pronephric region ectopic glomus and nephrostomal tubule formation was clearly visible on the injected side (Fig. 6C), whereas a single distal tubule was visible on the uninjected side.

***lunatic fringe* and *radical fringe* are expressed in the dorso-anterior region of the proximal pronephros at tail bud stages of development**

Fringe proteins are glycosyl transferases that extend carbohydrate chains on the EGF repeats of the extracellular domain of the Notch receptor. In *Drosophila*, such post-translational modifications promote Notch interactions with its Delta ligand (Bruckner et al., 2000; Moloney et al., 2000). Previous studies have highlighted roles for downstream Notch effector genes during proximal pronephrogenesis (Rones et al., 2002; Taelman et al., 2006), yet an understanding for how Notch signalling is regulated, and how this regulation affects pronephros development, has not been investigated. If *X. laevis* homologues of the *Drosophila* *fringe* gene, *lunatic fringe* (*lfng*) and *radical fringe* (*rfng*), were temporally and spatially expressed appropriately in the pronephros, this finding would further understanding of the mechanism by which Notch signalling is regulated in the proximal pronephros.

We performed whole-mount in situ hybridisations on uninjected embryos using 3'UTR-specific DIG-labelled antisense RNA probes to detect the temporal and spatial expression of *lfng* and *rfng* in *X. laevis* (Fig. 7). A previous study investigated the expression pattern of *lfng* in *X. laevis* (Wu et al., 1996); however, pronephric expression was not detected. Using an improved in situ hybridisation protocol, we observed pronephric expression of both *lfng* and *rfng*. *lfng* is expressed in the dorso-anterior region of the proximal pronephros between stages 22 and 32. Pronephric *rfng* expression was detected in the same region between stages 26 and 32. The temporal and spatial expression of *lfng* and *rfng* therefore suggests they might have a role in regulation of the Notch signalling pathway because *notch1*, *serrate1*, *serrate2*, *delta1* and

wnt4 are all expressed in this pronephric region at a similar time during development (McLaughlin et al., 2000; Rones et al., 2002; Saulnier et al., 2002; Taelman et al., 2006).

Overexpression of *Radical fringe* causes ectopic formation of the entire proximal pronephros

In order to observe whether mis-expressing *lfng* and *rfng* caused pronephric phenotypes, morpholino oligonucleotides (MOs) were designed to specifically knockdown translation of endogenous *lfng* and *rfng* transcripts (for MO sequences see Fig. S4A-C in the supplementary material), and overexpression experiments were performed. In vitro translation of *lfng* and *rfng* mRNA, in a rabbit reticulocyte lysate system using ^{35}S -methionine, could be knocked down by each specific MO (see Fig. S4D-F in the supplementary material).

We observed gross developmental defects upon mis-expression of *lfng* (see Figs S5-S7 in the supplementary material). Consequently, we were unable to perform functional analysis of *Lfng* in the pronephros. Furthermore, targeted injection of the *rfng* MO to knockdown endogenous *rfng* expression had no statistically significant effect on development of any region of the pronephros. *Na⁺K⁺ATPase*, *slc5a2* and *nephrin* expression were normal in these embryos (data not shown). Antibody staining of the mature pronephros with 3G8 and 4A6 caused a statistically insignificant 6% decrease in 3G8 staining and 2% decrease in 4A6 staining ($n=47$; see Fig. S5F in the supplementary material). Normal levels of *rfng* are therefore not required for normal development of the pronephros during embryogenesis.

By contrast, overexpression of *rfng* had a dramatic effect on pronephros development: *rfng* mRNA overexpression caused ectopic expression of the early pronephric marker *lim1* in 94% of embryos at stage 26 ($n=17$; Fig. 8A). Expression of *hrt1* was also increased by *rfng* overexpression in 77% of embryos ($n=35$; Fig. 8B). Unlike injection of *notch-ICD* mRNA, *rfng* overexpression caused ectopic 4A6 staining (63%, $n=41$), in addition to ectopic 3G8 staining (93%, $n=41$; Fig. 8C). Frequently, the proximal pronephric domain appeared to be completely duplicated in more distal regions (white arrow in Fig. 8C). Expression of tubule markers *Na⁺K⁺ATPase* ($n=23$; Fig. 8D) and *slc5a2* ($n=22$; Fig. 8E) was

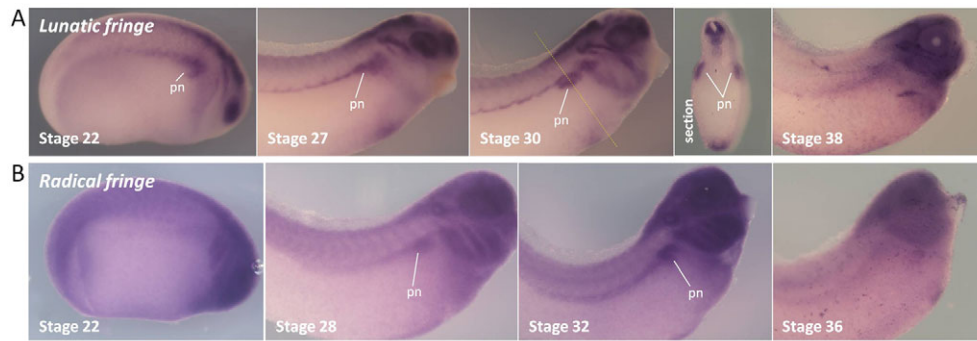


Fig. 7. *lfng* and *rfng* are expressed in appropriate temporal and spatial regions for a role in regulation of pronephric Notch signalling. (A,B) Whole-mount in situ hybridisation was carried out with a DIG-labelled anti-sense RNA probe for *lfng* (A) and *rfng* (B). *lfng* expression is detected in the dorso-anterior pronephros anlagen between stages 22 and 32. Transverse section of a stage 30 embryo clearly identifies this pronephric expression. At stage 38, pronephric *lfng* expression is lost. *rfng* expression in the pronephros is detected slightly later, at stage 26, and persists to stage 32/33. By stage 36, no pronephric expression of *rfng* could be detected. pn, pronephros.

increased in 67% and 59% of embryos, respectively. Ectopic formation of the glomus was observed in 81% of embryos examined by in situ hybridisation for *nephrin* expression ($n=21$; Fig. 8F). Ectopic nephrostome formation was also observed, with the domain of *odf3* expression increased in 62% of embryos ($n=21$, data not shown). In situ hybridisation detecting expression of *gata3*, a marker of the distal tubules, was also observed. *gata3* expression was reduced in 84% of embryos ($n=112$; Fig. 8G). In conclusion, Rfng promotes formation of the proximal pronephros and suppresses distal pronephrogenesis.

In vertebrates, Fringe proteins promote Notch-ligand interactions (Haines and Irvine, 2003; Hicks et al., 2000; Tsukumo et al., 2006). We have shown that Wnt-4 acts downstream of Notch signalling (see above), thus we wished to confirm that Rfng also acted upstream of Wnt-4. Co-injection of *rfng* mRNA with the *wnt4* MO reduced *nephrin* expression in 65% of embryos ($n=23$; Fig. 8H) and also inhibited *slc5a2* expression in 82% of embryos ($n=22$; Fig. 8I). This result indicates Rfng acts upstream of Wnt-4, most likely by mediating Notch-ligand interactions in the proximal pronephros.

Overexpression of *rfng* induces expression of components of the Notch signalling pathway and *wnt4*

Fringe proteins are enzymes whose activity is dependent on available substrate (Haines and Irvine, 2003). To understand the mechanism by which Rfng induces ectopic pronephrogenesis, we injected *rfng* mRNA as previously described and performed in situ hybridisation for expression of *wnt4*, *delta1* and *serrate1* at stage 32 (Fig. 9). Injection of *rfng* mRNA caused ectopic *wnt4* expression in 73% of embryos ($n=62$; Fig. 8A). Expression of *delta1* and *serrate1* was ectopic in 84% and 80% of embryos, respectively, when *rfng* was overexpressed ($n=51$, Fig. 9B; $n=65$, Fig. 9C). We suggest that *rfng* overexpression caused ectopic pronephrogenesis by promoting Notch signalling, as has been described in other systems (Hicks et al., 2000; Tsukumo et al., 2006). Promotion of pronephric Notch signalling activates *wnt4* expression, the protein product can be secreted in more distal positions than those found normally, thus producing ectopic proximal pronephros cell fates in distal regions.

DISCUSSION

We have established three novel findings that extend our knowledge of the mechanism by which the Notch signalling pathway regulates proximal pronephrogenesis in *X. laevis*. We

show that early-phase Notch signalling patterns the medio-lateral axis of the dorso-anterior region of the pronephros anlagen. This activity is mediated by regulation of a downstream Wnt-4 function. We also identify *lfng* and *rfng* expression in this pronephric region, which most likely act as regulators of Notch signalling in the proximal pronephros. These findings further the mechanistic understanding for how the Notch signalling pathway regulates proximal pronephrogenesis.

Misactivation of the Notch signalling pathway induces ectopic nephrostome and glomus development, but inhibits tubulogenesis

Early mis-activation of the Notch signalling pathway in the pronephros, by injection of *notch-ICD* mRNA, caused increased nephrostomal and glomus formation but inhibited tubulogenesis completely. This novel identification of a nephrostomal phenotype has not been observed previously because pronephric markers used to indicate the effects of mis-expressing components of the Notch signalling pathway failed to distinguish between nephrostomes and proximal tubules. McLaughlin and co-workers (McLaughlin et al., 2000) reasoned that the increase in 3G8 staining and *lim1* and *pax2* expression they observed after mis-activation of the Notch signalling pathway was due to an increase in proximal pronephric development (glomus, nephrostomes and proximal tubules). However, complete inhibition of tubulogenesis, indicated by loss of $Na^+K^+ATPase$ expression, suggested that the ectopic 3G8 staining observed after *notch-ICD* overexpression was completely nephrostomal. This theory was confirmed by *notch-ICD* mRNA injection inducing ectopic *odf3* expression. We therefore extend the results currently in the literature to show mis-activation of early phase pronephric Notch signalling induces ectopic glomus and nephrostome development, at the expense of tubules. Indeed, mis-activation of early phase pronephric Notch signalling caused the entire anlagen to acquire a medial pronephric fate.

Overexpression of *wnt4* reproduces the phenotypes observed after *notch-ICD* overexpression

Overexpression of *wnt4* highlighted a novel role for the Wnt signalling pathway in pronephric anlagen patterning. Consistent with this, *wnt4* is expressed in the dorso-anterior pronephros during early tail-bud stages of development (Saulnier et al., 2002). We

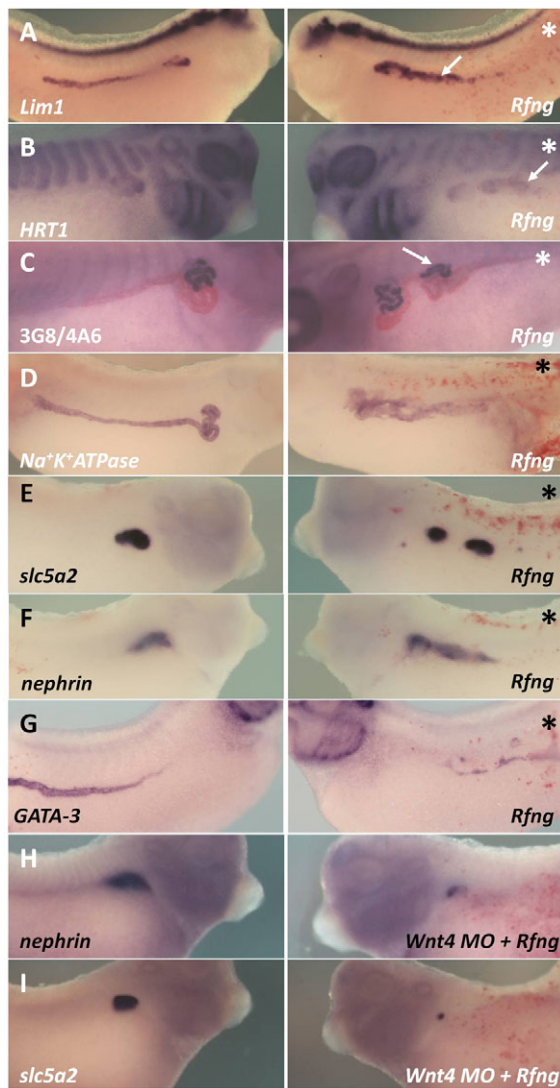


Fig. 8. *rfng* overexpression caused ectopic proximal pronephrogenesis. (A–I) *X. laevis* embryos were injected with *rfng* mRNA as previously described. *rfng* overexpression causes ectopic *Lim1* (A) and *hrt1* expression (B) at stage 28. At stage 41, 3G8 and 4A6 immunostaining is ectopic; frequently the entire proximal pronephros is duplicated in the distal region (white arrow, C). Similarly, *Na⁺K⁺ATPase* (D), *slc5a2* (E) and *nephrin* (F) expression is also ectopic. *GATA-3*, which is expressed solely in the distal tubule, is completely absent (G). Co-injection of *rfng* mRNA with the *wnt4* MO abrogates these effects; *nephrin* (H) and *slc5a2* (I) expression is reduced in these embryos. Asterisk denotes injected side.

overexpressed *wnt4* and showed that it caused identical phenotypes to *notch-ICD* overexpression: ectopic nephrostome and glomus formation, accompanied with inhibited tubulogenesis. Intriguingly, Saulnier and colleagues showed that overexpression of *wnt4* disrupted nephrostome formation and proximal tubulogenesis without affecting more distal tubule development (Saulnier et al., 2002). These findings are similar to our own, although they failed to observe ectopic nephrostome formation or inhibition of distal tubule development. We suggest that this difference is due to the different amounts of exogenous message injected in these two experimental series. We injected 1 ng of *wnt4* mRNA compared with 0.25 ng *wnt4* mRNA injected by the other researchers (Saulnier et al., 2002);

hence, the phenotypes they observed were less pronounced. They also knocked down translation of endogenous *wnt4* transcripts using a MO and showed this inhibited proximal tubule development, but left distal tubulogenesis largely unaffected (Saulnier et al., 2002). We have confirmed this result and extended these observations to show a reduction in glomus size. Thus, *wnt4* MO knockdown produces an identical phenotype to *delta^{STU}* and *su(H)^{DBM}* overexpression. The Wnt signalling pathway is therefore required for proximal pronephrogenesis and the correlation between the phenotypes observed following overexpression of both *wnt4* and *notch-ICD*, and inhibition of these pathways using *delta^{STU}* mRNA and *wnt4* MO injection, suggest these two pathways might be integrated.

The Notch-ICD dependent pathway is required for *wnt4* expression and Wnt-4 acts downstream of pronephric Notch signalling

We aimed to see whether the Notch and Wnt signalling pathways were integrated by observing the effect mis-activation of either pathway had on expression of components of the other pathway. We found that injection of *notch-ICD* mRNA caused ectopic *wnt4* expression on the injected side. Suppression of the Notch signalling pathway by injection of *delta^{STU}* mRNA inhibited *wnt4* expression on the injected side, a phenotype that correlates well with the inhibitory effects caused by overexpressing *delta^{STU}* on proximal pronephrogenesis.

Overexpression of *wnt4* did not have an obvious effect on overall levels of *notch1*, *delta1* or *serrate1* expression, indicating that Wnt-4 does not either induce or suppress expression of components of the Notch signalling pathway. This result is informative because it suggests there is no positive-feedback loop between the Notch and Wnt signalling pathways in the proximal pronephros and the Notch-ICD-dependent pathway is required upstream for *wnt4* expression. We have provided evidence that Wnt-4 acts downstream of pronephric Notch signalling since co-injection of a *wnt4* MO with *notch-ICD* mRNA prevented the ectopic glomus and nephrostome phenotype observed when *notch-ICD* overexpression is performed alone. Additionally, overexpression of *wnt4* and concomitant suppression of Notch signalling still caused ectopic glomus formation. This result suggests that Wnt-4 acts downstream of Notch signalling, and also illustrates that the major role of Notch signalling in the proximal pronephros is to regulate Wnt-4 function, therefore suggesting that Wnt-4 is the integral protein that patterns the proximal pronephric anlagen.

We also investigated the role of HRT-1 in regulation of *wnt4* expression. HRT-1 has previously been shown to regulate glomus formation and patterning of the proximal pronephros (Taelman et al., 2006). We show that HRT-1 is capable of inducing *wnt4* expression, and MO depletion of HRT-1 prevented *wnt4* expression, although we do not know whether these effects are either direct or indirect. By contrast, we observed a very slight disruption of *hrt1* expression following *wnt4* overexpression, which we believe is due to the overall effect of *wnt4* overexpression on pronephrogenesis. Thus, we conclude that Notch-ligand interactions promote *hrt1* expression, which then promotes *wnt4* expression, and Wnt-4 functions to pattern the proximal pronephros.

A role for Fringe proteins during pronephrogenesis

lfng and *rfng* are expressed in the dorso-anterior pronephros, suggesting that Fringe proteins are active in regulation of the Notch signalling pathway in the pronephros. Interestingly, *lfng*, *rfng* and *manic fringe* are expressed in the proximal compartment of the mammalian nephron, although their functional role is unknown

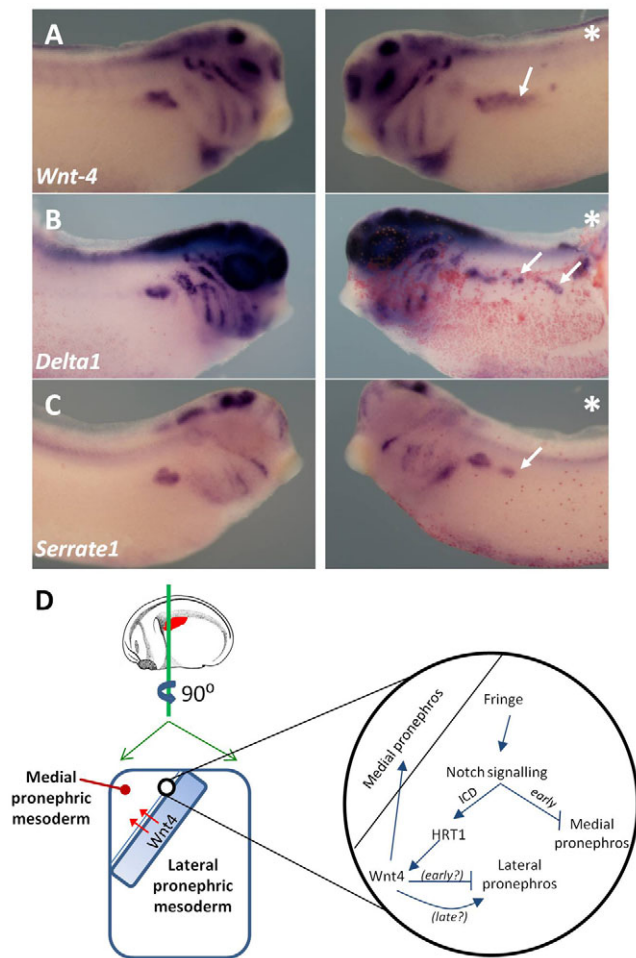


Fig. 9. *rfng* promotes expression of *wnt4* and components of the Notch pathway. (A–C) *X. laevis* embryos were injected as previously described and cultured to stage 28, with *beta-galactosidase* mRNA co-injected to act as a lineage tracer (red staining on injected side). Ectopic *wnt4* (A), *delta1* (B) and *serrate1* (C) expression was detected after *rfng* mRNA injection. Asterisk denotes injected side. (D) A model for early stage proximal pronephrogenesis regulated by the Notch signalling pathway. We propose a pool of cells undergoing Notch signalling and located on the lateral side of the dorso-anterior pronephros anlagen at tail bud stages of development, mediate medio-lateral patterning, permitting the glomus and tubules to develop independently. Blue arrows indicate proposed genetic hierarchy; red arrows indicate directional secretion of Wnt4. See text for full description.

(Leimeister et al., 2003). Misexpression of *lfng* caused gross developmental defects, thus we were unable to confirm a role for Lfng in pronephrogenesis. However, *rfng* mRNA was able to rescue the effects of *lfng* depletion (see Fig. S8 in the supplementary material), supporting previous studies that suggest it has functional homology with Lfng (Rampal et al., 2005). Overexpression of *rfng* increased only the size of the proximal pronephros region at the expense of the distal pronephros, a result that correlates with the predicted proximal pronephric phenotypes we expected to observe given the expression profile of this gene in the pronephros. *rfng* overexpression also caused ectopic expression of *delta1*, *serrate1* and *wnt4*, suggesting that Fringe proteins mediate Notch signalling in this pronephric region, which then regulates *wnt4* expression and ultimately proximal pronephrogenesis.

Does early phase pronephric Notch signalling establish a developmental boundary between the medial and lateral pronephric mesoderms?

We initially expected an asymmetrical Notch-ligand interaction mechanism for Notch-mediated boundary formation – as has been described in the *Drosophila* imaginal wing disc (Johnston et al., 1997) – to be conserved in the pronephros anlagen. This hypothesis was strengthened by our novel observations that early phase pronephric Notch signalling regulates the medio-lateral patterning of the pronephros anlagen and that both *lfng* and *rfng* are expressed in this dorso-anterior pronephric compartment. However, discrete spatial expression of Delta and Serrate genes across the medio-lateral axis of the proximal pronephros anlagen is not observed. Previous studies have outlined expression profiles for *wnt4* (Saulnier et al., 2002), *delta1*, *serrate1*, *notch1* (McLaughlin et al., 2000), *hrt1* and *serrate2* (Taelman et al., 2006). All these genes are expressed in the dorso-anterior region of the pronephros anlagen during the stages of lateral and medial pronephric mesoderm separation. Surprisingly, all these genes (apart from *hrt1*, which is initially expressed in both the lateral and medial pronephric mesoderms, before localising to the lateral pronephric mesoderm around stage 25) are expressed on the lateral side of the dorso-anterior pronephric mesoderm. None of these genes is expressed in the medial pronephric mesoderm where the future glomus develops. This lack of medial expression of genes involved in Notch signalling suggests that differential Notch-ligand interactions do not mediate separation of the lateral and medial pronephric mesoderms in *X. laevis*. Therefore, medio-lateral patterning of the dorso-anterior pronephros must occur by a novel Notch-mediated mechanism.

Taelman and co-workers (Taelman et al., 2006) showed that early phase pronephric Notch signalling promoted glomus development and later proximal tubulogenesis. Overexpression of *rfng* seemingly overcame these temporal effects and promoted formation of the entire proximal pronephros. We suggest one explanation for this phenotype could be the new-found complexity of the Notch signalling pathway (Bray, 2006; Fiuza and Arias, 2007; Kadesch, 2004). Misactivation of Notch signalling by overexpression of *notch-ICD* will only affect the Notch-ICD-dependent pathway; theoretically all cells in the anlagen will undergo gene expression activated by the CSL transcription complex. However, Notch-ICD-independent pathways are known to exist and are being realised as an important part of the overall signal transmitted by Notch-ligand interactions (Brennan and Gardner, 2002; Bush et al., 2001; Karsan, 2008). Unlike injection of *notch-ICD* mRNA, *rfng* overexpression would probably have a more global effect on Notch signalling because vertebrate Fringe proteins promote Notch-ligand interactions rather than one specific aspect of Notch signal transduction (Hicks et al., 2000; Tsukumo et al., 2006). Thus, we suggest that Notch-ICD-independent pathways have a role in development of the proximal pronephros. In addition, upon inhibition of Notch-ICD dependent signalling by overexpression of *su(H)^{DBM}*, we have shown that Wnt-4 was able to promote proximal tubulogenesis. This surprising result is important because it indicates that Wnt-4 is capable of promoting formation of all proximal pronephric domains. Since we have shown that Wnt-4 is the integral gene regulated by Notch signalling that controls proximal pronephrogenesis, it is likely that the temporal effects of Notch signalling identified by Taelman and colleagues (Taelman et al., 2006) mediate Wnt-4 function such that Wnt-4 promotes glomus formation early, and proximal tubulogenesis later.

Collating the data we have presented here with published results, we propose the following model for Notch-mediated pronephric patterning (Fig. 9D). Notch signalling begins in a pool of cells on the lateral side of the dorso-anterior pronephros anlagen around stage 22, with Notch-ligand interactions promoted by fringe proteins. The Notch-ICD signal induces expression of *wnt4*, which is then secreted by these cells, perhaps acting as a morphogen, generating glomus cell fates in the medial pronephros. This signal is maintained throughout tail-bud stages of development until the lateral and medial pronephric mesoderms physically separate, around stage 28, after which medio-lateral patterning is not required and proximal pronephric Notch signalling then promotes proximal tubulogenesis.

In summary, the mechanism for Notch-mediated boundary formation observed in the *Drosophila* imaginal wing disc does not separate the medial and lateral pronephric mesoderms in *X. laevis*. Instead, we propose a pool of cells, under the control of the Notch signalling pathway regulates medio-lateral patterning in a temporal fashion. We suggest that future experimentation should focus on the function of Fringe proteins, whether Wnt-4 acts temporally, and the effect Notch-ICD-independent pathways could have on renal patterning. Given the apparent conservation of mechanisms associated with renal development, such investigations are likely to uncover novel aspects of nephrogenesis that could explain the causes of nephropathy associated with aberrant Notch signalling in humans.

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Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/136/21/3585/DC1>

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