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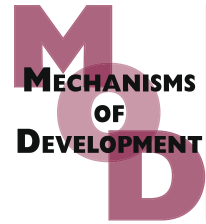

Requirement of Wnt/β-catenin signaling in pronephric kidney development

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ABSTRACT

The pronephric kidney controls water and electrolyte balance during early fish and amphibian embryogenesis. Many Wnt signaling components have been implicated in kidney development. Specifically, in *Xenopus* pronephric development as well as the murine metanephroi, the secreted glycoprotein Wnt-4 has been shown to be essential for renal tubule formation. Despite the importance of Wnt signals in kidney organogenesis, little is known of the definitive downstream signaling pathway(s) that mediate their effects. Here we report that inhibition of Wnt/β-catenin signaling within the pronephric field of *Xenopus* results in significant losses to kidney epithelial tubulogenesis with little or no effect on adjoining axis or somite development. We find that the requirement for Wnt/β-catenin signaling extends throughout the pronephric primordium and is essential for the development of proximal and distal tubules of the pronephros as well as for the development of the duct and glomus. Although less pronounced than effects upon later pronephric tubule differentiation, inhibition of the Wnt/β-catenin pathway decreased expression of early pronephric mesenchymal markers indicating it is also needed in early pronephric patterning. We find that upstream inhibition of Wnt/β-catenin signals in zebrafish likewise reduces pronephric epithelial tubulogenesis. We also find that exogenous activation of Wnt/β-catenin signaling within the *Xenopus* pronephric field results in significant tubulogenic losses. Together, we propose Wnt/β-catenin signaling is required for pronephric tubule, duct and glomus formation in *Xenopus laevis*, and this requirement is conserved in zebrafish pronephric tubule formation.

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1. Introduction

Tubule formation occurs in many developmental contexts, yet many of the underlying signaling processes driving differentiation and morphogenesis are incompletely understood. A classic system for studies of inductive interactions and mesenchyme-to-epithelial transitions, inclusive of tubule formation, has been the vertebrate kidney (Saxén, 1987).

Mammals progress through three developmental kidney stages: the pronephros, the mesonephros and the metanephros (Vize et al., 1997). Amphibian and fish embryos do not form the third stage but generate an embryonic pronephros, followed by formation of a mesonephros in adults (Vize et al., 1997). These kidney forms have similar architecture: they have the same basic unit of filtration, the nephron. Additionally, similar inductive events, signaling cascades and gene products drive their differentiation and morphogenesis (Brandli, 1999; Hensey et al., 2002; Vize et al., 1997). The pronephros, which is as functionally complex as later kidney forms, is experimentally attractive because of its simpler structure. It is composed of a single nephron, inclusive of tubules, a duct and a glomus (Carroll et al., 1999a; Vize et al., 1997, 2003). Various constructs, including mRNAs coding for native or mutant proteins of interest, can be targeted to the pronephros by injecting them into blastomeres that will ultimately give rise to this structure. Thus, development can be altered by activating or interfering with chosen developmental pathways. Early markers of nephron formation, such as *lhx-1* and *pax-8*, have been shown to be active in the nephrogenic mesenchyme prior to epithelialization, morphogenesis, and tubule maturation in both the pronephros and metanephros (Vize et al., 1997, 2003). Later, the conversion of nephrogenic mesenchyme to polarized nephric epithelia requires the action of Wnt signaling components. Many Wnt signaling components have been implicated in kidney development including Wnt-2b, Wnt-4, Wnt-6, Wnt-9b, Wnt-11, Frz4, Frz8, LRP6, Dkk1, CK2, GSK3, Tcf2, Pygo1 and Pygo2 (Carroll et al., 2005; Dressler, 2006; Itaranta et al., 2002; Kuure et al., 2007; Kyuno et al., 2008; Lin et al., 2001; Majumdar et al., 2003; Satow et al., 2004; Saulnier et al., 2002; Schedl, 2007; Schwab et al., 2007; Stark et al., 1994b, 2000; Valerius et al., 2002). Wnt-4 in particular has been shown to be important in both the *Xenopus* pronephros and murine metanephros (Saulnier et al., 2002; Stark et al., 1994b). In both systems, loss of Wnt-4 inhibits mesenchyme condensation and thus the epithelialization of nephric tubules (Saulnier et al., 2002; Stark et al., 1994a). In *Xenopus*, expression of Wnt-9a, Wnt-9b and Wnt-11b have also been observed in the pronephric kidney (E.A. Jones, unpublished results).

In mice, several Wnts participate in kidney formation, with Wnt-9b also contributing to metanephric tubulogenesis (Carroll et al., 2005). Unlike Wnt-4, which is expressed in the metanephric mesenchyme, Wnt-9b is expressed in the stalks of the invading ureteric bud (Carroll et al., 2005). Wnt-9b knockouts do not undergo metanephric mesenchymal to epithelial transition, and markers of nephron formation, such as *pax-8*, *lhx-1*, and *wnt-4* are not expressed (Carroll et al., 2005). Wnt-6, which is also expressed in the murine ureteric bud, induces tubulogenesis from mesenchyme *in vitro* (Itaranta et al., 2002). Other Wnts include Wnt-11, expressed in the ureteric epithelium and needed for ureteric branching (Majumdar et al., 2003), as well as Wnt-2b, expressed in the perinephric mesenchyme and likewise is involved in ureter formation (Lin et al., 2001).

Wnts may affect kidney development via canonical and/or non-canonical signaling trajectories. Wnts activating the canonical pathway bind both Frizzled (Fz) and LRP receptors resulting in activation of the transcription factor β-catenin. In the absence of Wnt signals, β-catenin is marked for degradation by a multiprotein complex that includes GSK-3β, APC, and Axin (Huang and He, 2008; Sokol and Wharton, 2007; Widelitz, 2005; Willert and Jones, 2006). In the presence of canonical Wnt signals, Dishevelled and LRP sequester the degradation complex, allowing β-catenin to accumulate (Schwarz-Romond et al., 2007; Zeng et al., 2005). β-Catenin then enters the nucleus to associate with and relieve Lef/Tcf-mediated transcriptional repression, resulting in target gene activation (Widelitz, 2005). Wnt signaling that does not act through the canonical β-catenin signaling trajectory is by definition considered non-canonical Wnt signaling. The two primary non-canonical Wnt signaling pathways are the planar cell polarity (PCP) pathway, involving Rho GTPases and JNK, and the calcium pathway, involving PKC and CAMKII (Wallingford and Habas, 2005; Widelitz, 2005).

Growing evidence suggests that canonical Wnt/β-catenin signals act in kidney development. Mice bearing a β-catenin-responsiveTcf/βGal reporter transgene reveal canonical Wnt activity in the nephrogenic mesenchyme during tubulogenesis (Iglesias et al., 2007), removal of β-catenin from metanephric progenitors reduces nephron number and organization (Park et al., 2007), and β-catenin deficiencies in the ureteric bud produce abnormal ureteric branching (Bridgewater et al., 2008). In organ culture, constitutive β-catenin signaling in epithelial progenitors induces Tcf/Lef-dependent epithelial transcripts (Schmidt-Ott et al., 2007), and in cultured Madin–Darby Canine Kidney (MDCK) epithelial cells, Wnt-4 activates canonical Wnt/β-catenin signals (Lyons et al., 2004). While suggestive, each of these studies has either been conducted *in vitro* or *ex vivo*, or has reduced β-catenin function using an approach that could have perturbed cell–cell adhesion in addition to canonical Wnt target gene expression. This latter concern arises from the fact that in addition to its signaling roles, β-catenin is an essential component of cadherin complexes present at cell–cell adhesive junctions, where it contributes to the indirect dynamic association of cadherins with the underlying cortical actin cytoskeleton (Brembeck et al., 2006; Nelson, 2008).

Here, using amphibian and zebrafish pronephric model systems, we test the hypothesis that Wnt/β-catenin signaling, specifically, is required for kidney development. We show that inhibiting this pathway inhibits formation of pronephric epithelial tubules, duct and glomus, reducing expression of both early and late pronephric markers. We thus demonstrate the requirement of canonical Wnt/β-catenin signals in multiple components and stages in pronephric development.

2. Experimental procedures

2.1. *Xenopus laevis embryos, in vitro transcription, and microinjections*

Embryo preparation, *in vitro* transcription and microinjections were performed using standard methods (Montross et al., 2000). Microinjections were targeted to the V2 blastomere at the eight-cell stage and the V2.2 blastomere at the sixteen-cell stage. The V2.2 blastomere in the sixteen-cell stage provides major contributions to the development of the pronephros, somites, trunk crest and hindgut, while the V2 blastomere in the eight-cell stage provides major contributions to the development of the fin epidermis, the lateral plate mesoderm and the proctodeum in addition (Moody, 1987; Moody and Kline, 1990). To verify proper delivery to the pronephric field, dextran rhodamine (Molecular Probes) was co-injected with mRNAs. After injections, embryos were placed in a solution of 5% Ficoll in 1× MMR for 3 h before transfer to 0.1× MMR. For those embryos injected with mRNAs encoding inducible protein chimeras, dexamethasone (10 μM) was added to the 0.1× MMR at the times indicated and refreshed every 2 days. Embryos were then reared at 16–18 °C in 0.1× MMR with 0.5% gentamycin until ready for fixation and processing. For detection of proper lineage delivery, a fluorescent stereomicroscope was utilized (Leica MZFLIII).

2.2. *Inhibiting/activating constructs*

The β-Engrailed chimera was created as previously described by replacing the C-terminal transactivation domain of β-catenin with the transcriptional repression domain of *Drosophila* Engrailed (Montross et al., 2000). The dominant negative (N-terminal deletion) XTcf3 cDNA construct was obtained as a kind gift from the laboratory of Hans Clevers (Molenaar et al., 1996). The inhibitory EnR-LefΔN-GR755A hormone-inducible GR chimera was generated as previously described (Deroo et al., 2004). The previously uncharacterized LefΔN-βCTA-GR755A construct was generated by PCR amplification of the C-terminal transactivation domain (CTA) of β-catenin from LefΔN-βCTA (Vleminckx et al., 1999). This PCR fragment was then cut with BamHI and MluI (a MluI site was generated during PCR at the 3′ end of the amplicon). The LefΔN-βCTA fragment was then ligated into the GR-containing vector fragment of p958 digested with BamHI and MluI. The p958 vector is a pCS2+ based plasmid from Andreas Hecht and Daniel Kessler that contains LefΔN-GR in which the GR domain is preceded by a MluI site (Kessler, 1997).

2.3. *Heat-shock inducible Dkk1GFP transgenic zebrafish*

A detailed description of the hsDkk1GFP transgenic zebrafish lines has been published previously (Stoick-Cooper et al., 2007). Briefly, mmGPF5 (Siemering et al., 1996) was fused to the C-terminus of zebrafish *dkk1* (Genbank Accession # AB023488). The fusion protein was cloned downstream of a 1.5 kb fragment of the zebrafish hsp70-4 promoter (Halloran et al., 2000) and upstream of the SV40 polyadenylation signal of the vector pCS2+. Transgenic lines were established via plasmid injection of 1-cell stage embryos.

2.4. *Immunohistochemistry*

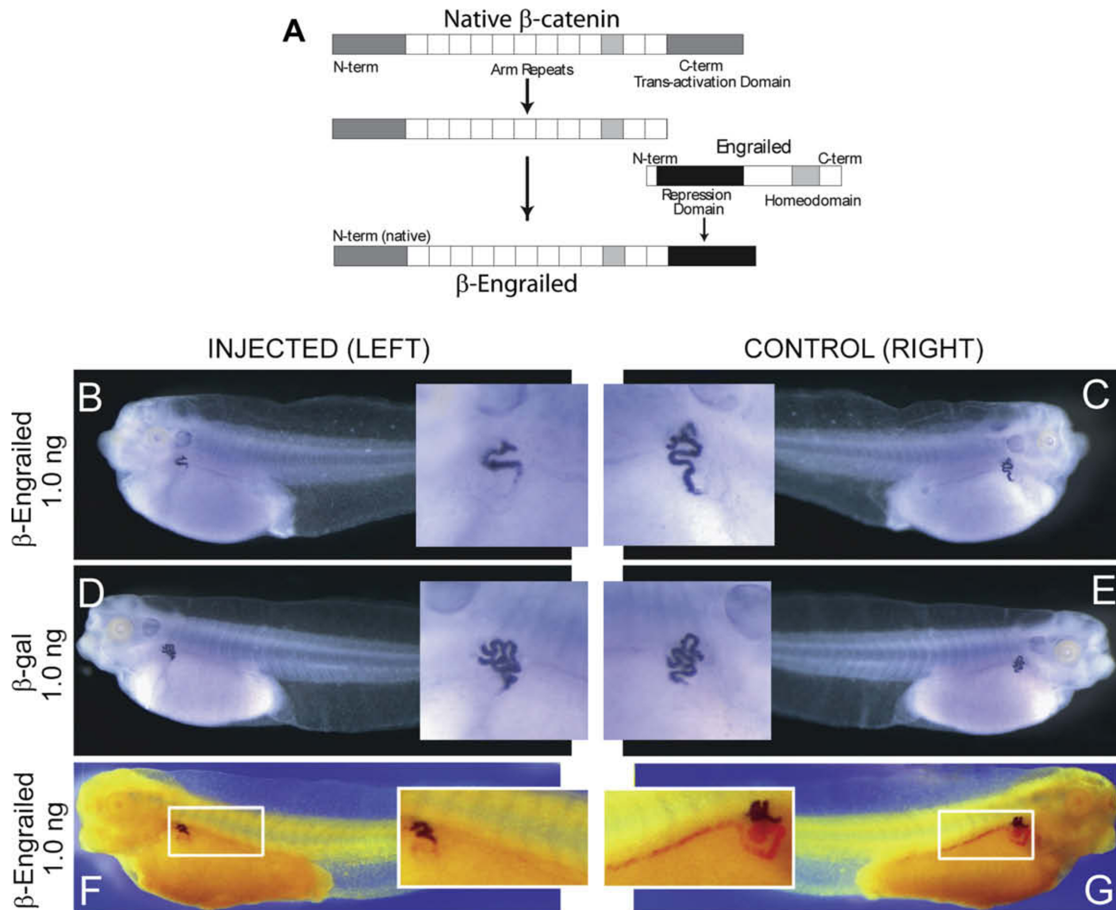
Xenopus embryos were fixed in MEMFA (0.1 M MOPS (pH 7.4), 2 mM EGTA, 1 mM $MgSO_4$, and 4% formaldehyde) for 1 h at room temperature and then washed 2× in 100% methanol, dehydrated at −20 °C overnight in fresh 100% methanol, rehydrated with 1× PBT (1× PBS, 2 mg/mL BSA, 0.1% Triton X-100), and blocked with 20% goat serum in 1× PBT for 1–3 h. Zebrafish embryos were fixed in Dent's fixative (80% ethanol, 20% DMSO) overnight at −20 °C, dehydrated in 100% methanol overnight at −20 °C, and rehydrated and inhibited as above. Primary antibodies were diluted in 1× PBT with 10% goat serum to optimal concentration, incubated with embryos overnight at 4 °C, and washed 5× 1 h in 1× PBT. Primary antibodies used in *Xenopus* were monoclonal 3G8 to detect pronephric epithelial tubules, monoclonal 4A6 to detect pronephric duct (Vize et al., 1995), and monoclonal 12/101 to detect somites (Kintner and Brookes, 1984; Seufert et al., 1999). In zebrafish, the monoclonal α6F (α-1 subunit of Na/K ATPase) was used to detect pronephric epithelial tubules and ducts (Drummond et al., 1998; Takeyasu et al., 1988). Embryos were blocked again, then incubated overnight at 4 °C in goat anti-mouse secondary antibody (1:2000) conjugated to alkaline phosphatase (BioSource), washed 5× 1 h in 1× PBT, and stained for alkaline phosphatase using NBT/BCIP ready-to-use tablets (Roche). When double staining, the above process was repeated and Fast Red (Roche) employed to stain for alkaline phosphatase. To bleach epithelial pigment, embryos were placed in two parts methanol and one part 30% H_2O_2 under

Fig. 1 – β-Engrailed or dnXTcf3 inhibit pronephric tubule and duct development. (A) Schematic overview of β-Engrailed chimera showing replacement of native β-catenin's carboxy-terminal transactivation region with the *Drosophila* Engrailed repression domain. Left V2 blastomeres of eight-cell stage embryos were co-injected with various doses of β-Engrailed (versus nuclear-directed β-galactosidase as RNA loading control) and rhodamine dextran lineage tracer. Embryos were fixed at stages 37–41, analyzed for correct lineage, and processed for tubule immunostaining using the 3G8 monoclonal antibody (blue) or for duct immunostaining using the 4A6 monoclonal antibody (red). (B) β-Engrailed generates loss of 3G8 kidney tubule expression as shown in the corresponding enlarged inset. (F) β-Engrailed also generates loss of 4A6 kidney duct expression as shown in the corresponding enlarged inset (tubule = blue, duct = red). (D) β-Gal injected embryos, or non-injected control sides of embryos (C, E, and G), show normal 3G8 pronephric tubule and 4A6 pronephric duct expression. (H) The Pronephric Index (PNI) scoring system was utilized to quantify reductions in 3G8 tubule expression, with scores of 2.41 and 2.74 for β-Engrailed and dnXTcf3, respectively, indicating a significant loss (greater than PNI of 2.0). Uninjected and nuclear β-gal injection controls indicated no significant loss.

bright fluorescent lighting for 6–12 h, with several changes of methanol/H_2O_2. In some cases, embryos were cleared in Murray's clearing medium (two volumes of benzyl benzoate, one volume of benzyl alcohol). All images were collected using ImagePro Plus software and an Optronics camera mounted directly to a Leica MZFLIII brightfield stereomicroscope.

2.5. *In situ hybridization, fluorescent whole-mount in situ (FISH), and fluorescent & colorimetric in situ (FCIS)*

Embryos were fixed in MEMFA and stored in 100% methanol at −20 °C for no more than 10 days prior to DIG-labelled RNA probe hybridization. Probes used include: Na Glu-



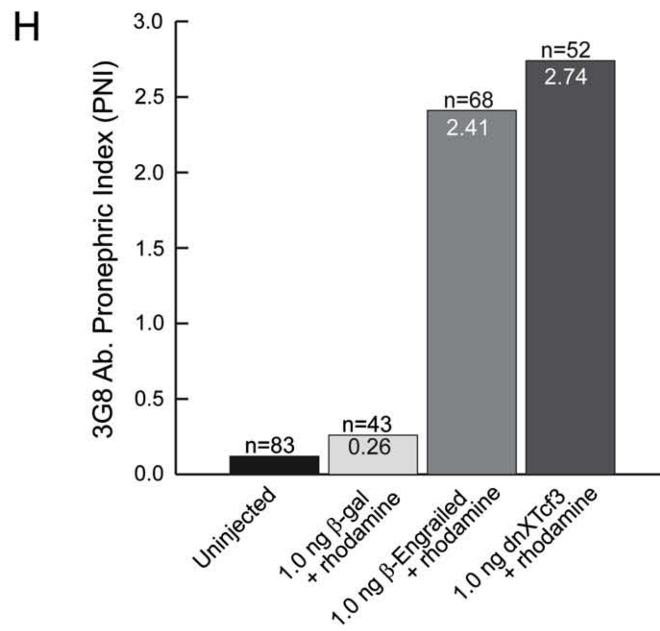


Table 1 – Analysis of pronephric marker protein expression in β-Engrailed injected Xenopus embryos.

Markers	Frequency of reduced or absent expression	Total analyzed
3G8 (tubule)	60%	68
4A6 (duct)	86%	28

cose = *slc5a1*, NKCC2 = *slc12a1*, VHNF = *hnf1β*, XLIM1 = *lhx1*, Pax8 = *pax8*, XNBC1 = *slc4a4*, NaKATPase = *atp1a1*, CRESCENT = *frzb2*, and NEPHRIN = *nhps1* (Carroll and Vize, 1999; Eid and Brandli, 2001; Gerth et al., 2005; Shibata et al., 2000; Wild et al., 2000; Zhou and Vize, 2004). Standard probe synthesis, embryo hybridization, and colorimetric processing methods were utilized (Harland, 1991). Fluorescent (FISH) and combined fluorescent and colorimetric (FCIS) methods were performed as described (Davidson and Keller, 1999; Zhou and Vize, 2004).

2.6. Histological sections

Histological sections were prepared by embedding previously stained embryos in paraplast. Sections were then cut at a thickness of 10 μm, counter stained with eosin and mounted.

2.7. Western blot analysis

We employed antibodies directed against HA (12CA5) for temporal expression analysis of the HA-tagged hormone-inducible GR chimeras. Anti-actin antibodies (Sigma) were employed as a control for protein loading. In brief, embryos were collected at the time and conditions indicated, and solubilized in 20 μl per embryo of 0.5% Triton X-100 buffer (0.5% Triton X-100, 10 mM HEPES pH 7.4, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA) with proteinase inhibitor cocktail (Sigma). After centrifugation (14,000 rpm, 5 min), supernatants were collected and samples denatured upon addition of an equal volume of 4× SDS sample buffer (200 mM Tris–Cl, pH 6.8, 40% glycerol, 8% SDS, 200 mM DTT, 0.08% bromophenol blue), with boiling at 95 °C for 5 min. Half-embryo equivalents were loaded per lane on a SDS–polyacrylamide gel and subsequently transferred onto nitrocellulose membrane. Blocking and antibody incubation occurred in 2% bovine serum albumin-TBST (25 mM Tris–HCl, pH 8.0, 125 mM NaCl, 0.5% Tween-20) and ultimately SuperSignal WestPico (Pierce Biotechnology, Inc.) was used to detect HRP (horseradish peroxidase)-conjugated secondary antibodies.

2.8. Xenopus transgenesis

TOPTK-iGFP transgenic tadpoles were generated as previously described (Denayer et al., 2006; Deroo et al., 2004). Dexamethasone (10 μM) was added at stage 14, stage 23, or stage 32, and embryos analyzed at stages 19/20, stages 28–30 and stage 36.

3. Results

3.1. β-Catenin/Engrailed (β-Engrailed) fusion protein inhibits pronephric epithelial tubule and duct development in Xenopus laevis

To determine if Wnt/β-catenin signaling is required for pronephric development, we first tested the impact of reducing Wnt/β-catenin signaling in *Xenopus* embryos. In previous work, we and others found that a β-catenin/Engrailed fusion protein, β-Engrailed, specifically inhibits Wnt/β-catenin signals in *Xenopus* embryos, mammalian cell lines, and mouse embryo systems (Lyons et al., 2004; Montross et al., 2000; Tepera et al., 2003). β-Engrailed is a chimera consisting of the native N-terminal and Armadillo domains of *Xenopus* β-catenin (lacking the C-terminal transactivation domain) joined to the repressor domain of Drosophila Engrailed (Fig. 1A). Here, β-Engrailed was introduced into the pronephric field (single V2 blastomere) of eight-cell stage embryos in conjunction with dextran rhodamine as a lineage tracer. β-Engrailed reproducibly inhibits formation of pronephric tubules in a dose-dependent manner (Supplemental Fig. 1) on injected (Fig. 1B) as opposed to uninjected sides (Fig. 1C) of the same embryos. This was visualized by rearing embryos to tadpole stages (stages 37–41), whereupon they were fixed and analyzed via

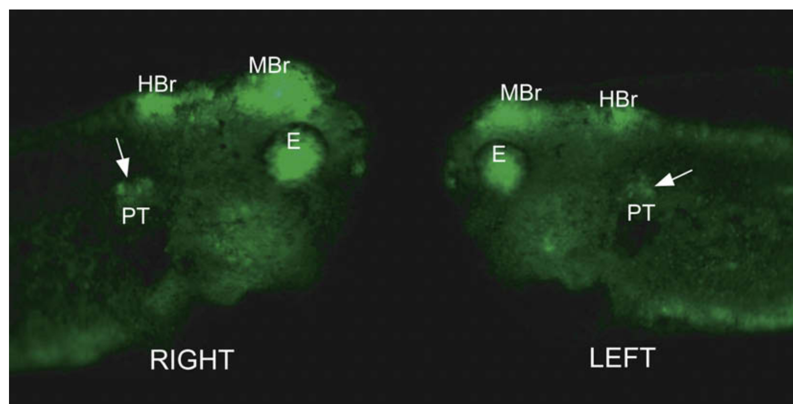


Fig. 2 – Wnt/β-catenin signaling activity in pronephric tubules of *Xenopus* transgenic TOPTK-iGFP reporter tadpoles. Lateral views of anterior portion of left and right sides of a representative transgenic tadpole (fixed stage 36) treated with dexamethasone (10 μM) at stage 32 for 16 h at 15 °C. Wnt/β-catenin signaling is observed in the eyes (E), mid-brain (MBr), hind-brain (HBr) and pronephric tubules (PT, arrows).

whole-mount immunohistochemistry using the pronephric epithelial tubule-specific 3G8 monoclonal antibody. Control embryos co-injected with dextran rhodamine and β-galactosidase mRNA show no effect on either the injected (Fig. 1D) or the uninjected sides (Fig. 1E). Consistent with our β-Engrailed results, dominant negative XTcf3 also produced a dose-dependent inhibition of tubule formation (not shown). Expression of β-Engrailed also reduced pronephric duct formation, as visualized using monoclonal antibody 4A6 (compare Fig. 1F and G, respectively injected versus uninjected sides of the same embryos). Basic quantitation indicated that reduced or absent tubule staining occurred in 60% of embryo sides injected with

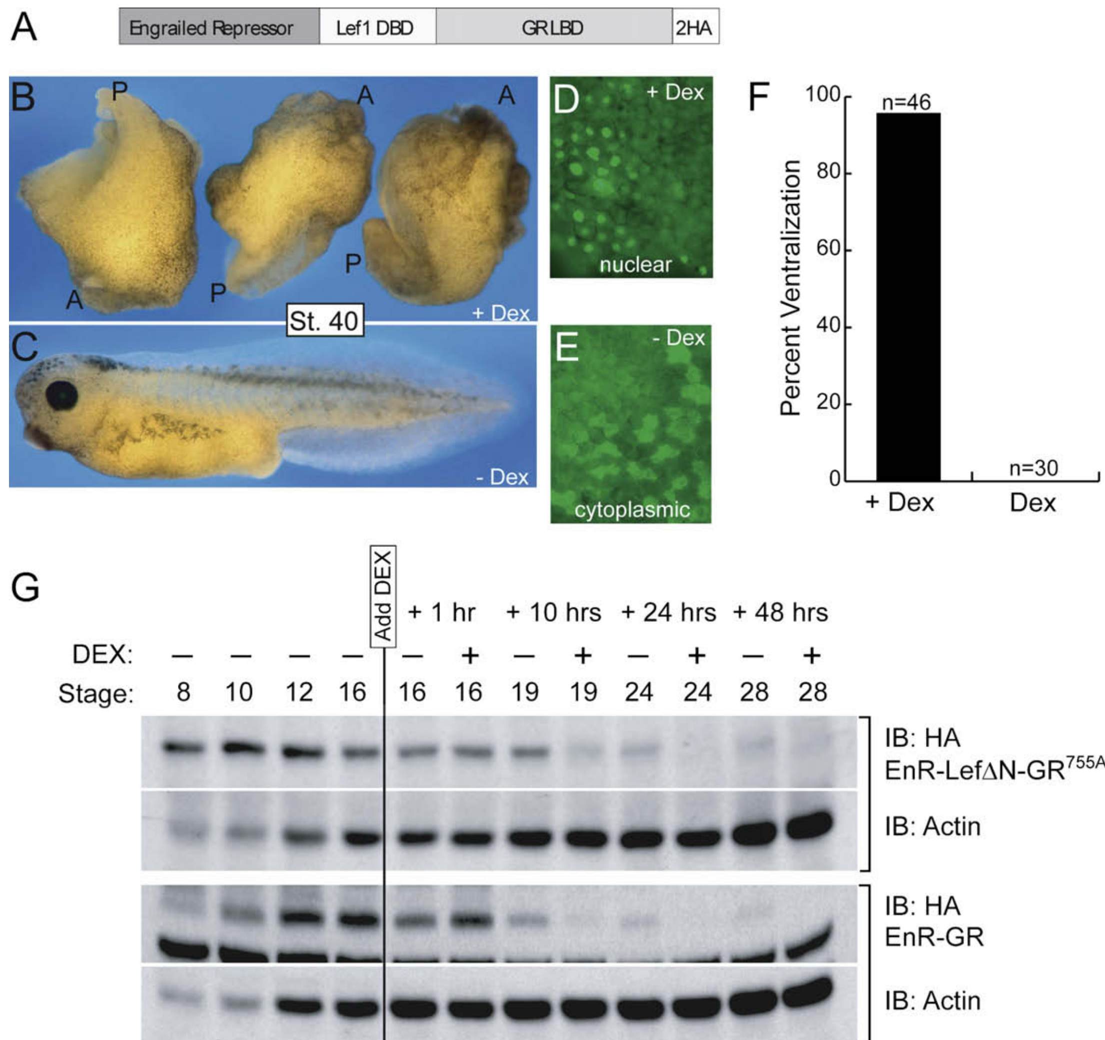


Fig. 3 – Characterization of EnR-LefΔN-GR755A, an inducible fusion construct inhibiting Wnt/β-catenin signaling. (A) EnR-LefΔN-GR755A schematic indicating the Drosophila Engrailed repressor domain (aa 1–299), mouse Lef1 DNA-binding HMG box (aa 265–391), human Glucocorticoid receptor hormone-binding domain (aa 512–777), and double HA epitope-tag. To abrogate potential recruitment of transcriptional coactivators, the TAF-2 motif in the GR domain was mutated (glutamine to alanine substitution at position 755). *Xenopus* embryos were injected with 0.5 ng EnR-LefΔN-GR755A into both animal-dorsal blastomeres at the four-cell stage and grown to stage 40 with or without dexamethasone. In the presence of dexamethasone, embryos exhibited strong ventralization phenotypes (B), while embryos not treated with dexamethasone developed normally (C). Fluorescent (FITC) immunohistochemistry of animal caps excised from stage 9/10 embryos show nuclear localization of EnR-LefΔN-GR755A in the presence of dexamethasone (D) and cytoplasmic localization in the absence of dexamethasone (E) (1.0 ng EnR-LefΔN-GR755A injected into single animal-dorsal blastomere at the 2-cell stage). (F) Embryos from panels B and C were scored using the Dorso-Anterior Index (DAI) system with embryos scoring between 0 and 4 designated positive for ventralization while embryos scoring 5 were normal. A high proportion of embryos were ventralized in the presence of dexamethasone, while none were ventralized in dexamethasone absence. (G) To assess EnR-LefΔN-GR755A protein stability following dexamethasone addition at later stages, a time course was undertaken of embryos injected with 0.5 ng EnR-LefΔN-GR755A (or control EnR-GR) into a single vegetal-ventral (V2) blastomere at the eight-cell stage. HA epitope-tag Western blotting (IB = immunoblot) shows that each chimera is stably present until addition of dexamethasone at stage 16, at which time each experiences increased metabolic turnover with dexamethasone.

β-Engrailed ($n = 68$), whereas reduced or absent duct staining was 86% ($n = 28$) (Table 1).

To better quantify pronephros formation, an established pronephric index (PNI) scoring system was utilized that compares the extent of tubule development (visualized through 3G8 antibody staining of embryos at stages 37–38), on experimental (injected) versus control sides of embryos (left versus right) (Wallingford et al., 1998). At stage 37/38, a normal *Xenopus* pronephros is composed of five tubule components each being assigned one point. Fully formed tubules would thus score five, whereas complete loss of tubules would score zero. The PNI is expressed as the difference between control and experimental sides, where scores greater than two are considered significant (not attributable to normal variation) (Vize et al., 1995; Wallingford et al., 1998). PNI analysis indicated that expression of β-Engrailed (PNI = 2.41, $n = 68$), or of dnXTcf3 (PNI = 2.74, $n = 52$), produced substantial tubule loss (Fig. 1H). Overall, these results indicate that inhibiting Wnt/β-catenin signaling in the pronephros hinders development of the tubules and duct.

3.2. *Wnt/β-catenin signaling is active in the pronephros*

As blockage of Wnt/β-catenin signaling inhibited development in the pronephros, we examined if canonical Wnt signaling is active there. Indeed, previously characterized TOPTK-iGFP reporter tadpoles harboring a hormone-inducible GFP reporter (Denayer et al., 2006). Induction with 10 μM dexamethasone at stage 32, followed by fixation/processing st stage 36 reproducibly generated an eGFP signal within forming tubules, as well as within eyes, mid-brain and hind-brain (positive controls) (Fig. 2). Recently, another more sensitive *Xenopus* transgenic reporter system that employs multimerized Lef/Tcf binding sites upstream of GFP, has revealed canonical Wnt/β-catenin activity as early as stage 25 (Vleminckx, unpublished results). These data indicate Wnt/β-catenin signaling is evident in the pronephric anlage.

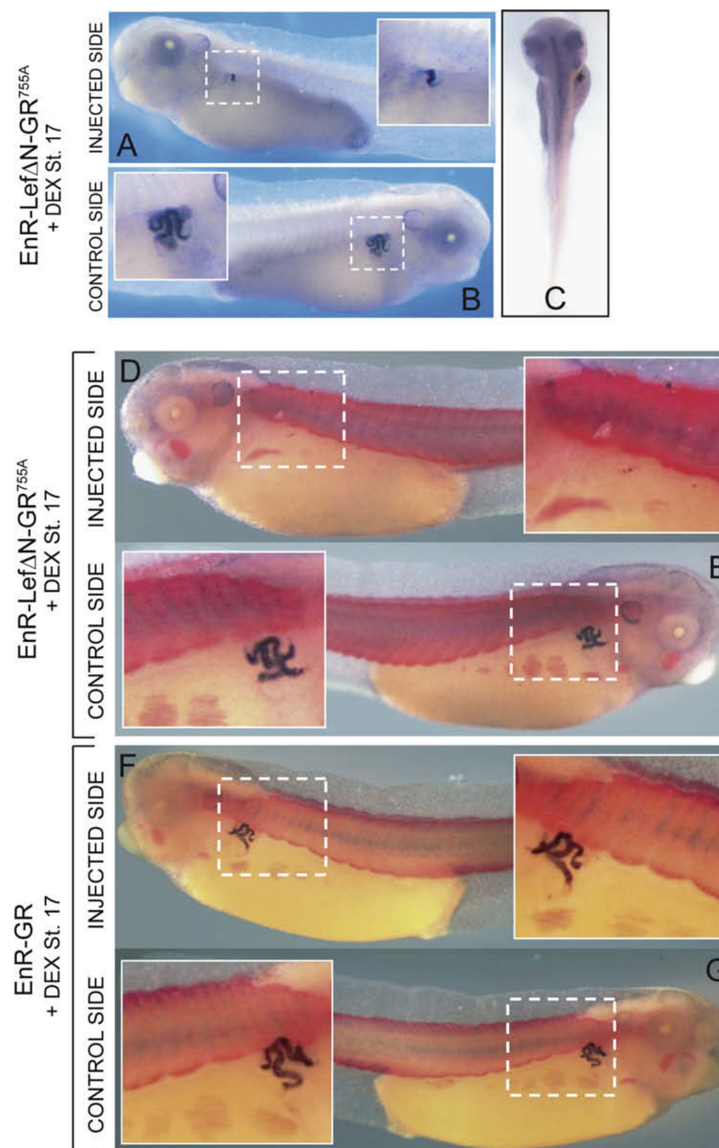
3.3. *Inducible inhibition of Wnt/β-catenin signaling inhibits pronephric tubule formation with minimal effects on axis or somite development*

Since axis bending followed high-dose inhibition of Wnt/β-catenin signaling using β-Engrailed in *Xenopus* (Supplemental Fig. 1), we wished to rule out concerns that pronephric kidney tubule loss might be a secondary consequence of earlier developmental effects upon surrounding tissues. Previous work has shown a conserved role for Wnt/β-catenin signaling in vertebrate somitogenesis (Aulehla and Herrmann, 2004), while in *Xenopus* and chick embryos the overlying anterior somites contribute to establishing intermediate mesoderm and the competence of pronephric mesenchyme (Mauch et al., 2000; Seufert et al., 1999). To obtain temporal control of pathway inhibition, we employed a chimeric hormone-inducible construct, EnR-LefΔN-GR755A (Deroo et al., 2004), to inhibit expression of Wnt/β-catenin target genes at times prior to the onset of Wnt-4 transcription at stage 19 in the presumptive pronephros (Saulnier et al., 2002). The EnR-LefΔN-GR755A fusion consists of the *Drosophila* Engrailed repression domain (amino acids 1–299), the mouse Lef1 DNA-binding domain (HMG box amino acids 265–391), the human glucocorticoid receptor hormone-binding domain (amino acids 512–777 with E to A substitution at 755), and two HA tags (Fig. 3A). To verify the inhibitory activity of EnR-LefΔN-GR755A in *Xenopus* embryos, we injected 0.5 ng of the fusion mRNA into both dorsal-animal blastomeres at the four-cell stage and kept embryos in culture with or without dexamethasone. As reported when first characterized (Deroo et al., 2004), robust ventralization resulted in the embryos exposed to dexamethasone (dorso-anterior index scores of 1–2) (Fig. 3B), while normal embryo phenotypes prevailed without dexamethasone (Fig. 3C). Further, nuclear translocation of EnR-LefΔN-GR755A (HA-tagged) upon dexamethasone treatment was verified using immunofluorescence of isolated blastula-stage ectoderm (Fig. 3D), while it remained cytoplasmic in untreated cultures (Fig. 3E). Most EnR-Lef1ΔN-GR755A injected embryos (>95%), were ventralized upon dexamethasone incubation, whereas no untreated embryos were ventralized (Fig. 3F).

Next, we characterized the temporal protein expression profile and turnover of EnR-LefΔN-GR755A, while also testing the control chimera EnR-GR, which lacks the Lef1 HMG box DNA-binding domain. Following the corresponding mRNA injections into V2 blastomeres of eight-cell stage embryos, each GR-protein exhibited a similar temporal expression profile (Fig. 3G). All were stable through early tailbud stages in the absence of dexamethasone (stages

Fig. 4 – EnR-LefΔN-GR755A inhibits pronephric epithelial tubulogenesis. (A) Injection of 0.5 ng of EnR-LefΔN-GR755A into a single V2 blastomere at the eight-cell stage and dexamethasone addition at stage 17 results in a significant or complete loss of pronephric tubule epithelium on the injected side as assessed using the 3G8 antibody. All embryos were fixed at stages 37–41. Inset shows enlargement of the 3G8-stained pronephric region. (B) The control side of the embryo shows normal 3G8 pronephric tubule staining in the presence of dexamethasone. Tubulogenesis is normal in injected or non-injected sides of non-dexamethasone treated embryos (not shown). (C) Embryo dorsal views indicate normal axis lengths with minimal axis bending. (D) Double immunostaining with 3G8 (blue) and 12/101 somite marker (red) shows EnR-Lef1ΔN-GR755A-injected embryo sides completely lack pronephric tubules while exhibiting largely normal somitogenesis (somite number unaffected and minimal effects upon segmentation). (E) Non-injected side of EnR-Lef1ΔN-GR755A-injected embryos show normal 3G8 tubule and 12/101 somite staining. (F and G) Injected and non-injected sides of EnR-GR (negative-control construct) injected embryos display normal tubule and somite development. (H) The Pronephric Index (PNI) scoring system was used to quantify the 3G8 tubule staining at stage 37/38 of EnR-LefΔN-GR755A versus control EnR-GR-injected embryos. In the plus dexamethasone condition, EnR-LefΔN-GR755A-injected embryos show a significant loss of pronephric tubulogenesis (PNI score of 2.64), while in the absence of dexamethasone no significant loss was observed. The control chimera EnR-GR (with or without dexamethasone) showed no significant loss of tubulogenesis.

19–20), but underwent more rapid turnover upon dexamethasone addition at neurula stages. Ten hours after dexamethasone addition, little to no GR-protein is detected, although residual low level activity of EnR-LefΔN-GR755A may conceivably reduce Wnt target gene expression into tadpole stages.



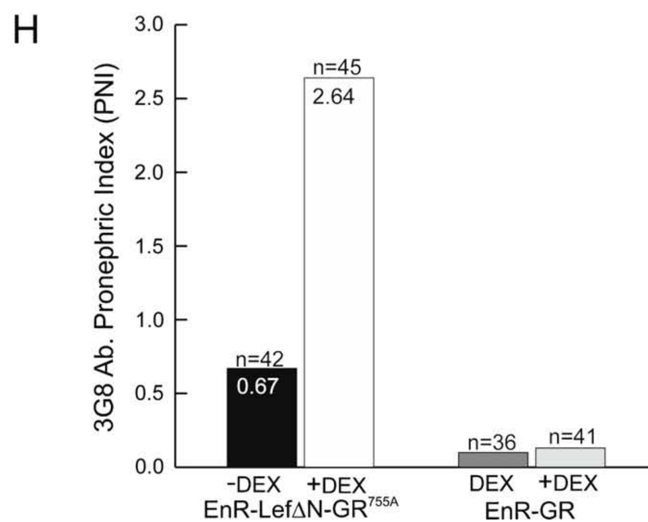


Table 2 – Analysis of pronephric marker protein expression in EnR-LefΔN-GR755A injected Xenopus embryos.

Markers	Frequency of reduced or absent expression	Total analyzed
3G8 (tubule)	78%	45

Having established such parameters, we asked if EnR-LefΔN-GR755A induced at neurula stage could inhibit kidney tubule development. EnR-LefΔN-GR755A or EnR-GR (0.5 ng) was microinjected with dextran rhodamine (lineage tracer) into single V2 blastomeres at the eight-cell stage, and dexamethasone-induced at stage 17. Embryos were then fixed at stages 37–41, selected for the intended V2 blastomere lineage and stained for the pronephric tubule-specific 3G8 marker. Only minimal axis bending occurred when dexamethasone was added to EnR-LefΔN-GR755A injected embryos at stage 17 (Fig. 4C), yet robust tubule inhibition was evident on injected sides (Fig. 4A). 3G8 staining was reduced or absent in 78% of tadpoles analyzed ($n = 45$) (Table 2), and their pronephric index (PNI) score was 2.64 (Fig. 4H). No axis bending (not shown) or 3G8 tubule phenotypic effects were detected in control EnR-GR-injected embryos (Fig. 4H). Further, little to no somitogenesis defects (normal somite number and segmentation) were evident in embryos co-stained with the 12/101 somite marker when pronephric tubule formation was inhibited (Fig. 4D). Altogether, these findings indicate that pronephric tubule loss following EnR-LefΔN-GR755A induction is directly related to the inhibition of Wnt/β-catenin signaling in the presumptive pronephric field.

3.4. Inhibition of Wnt/β-catenin signaling results in global, not subdomain-specific, loss of pronephric tubules, duct and glomus

We next asked if inhibiting the Wnt/β-catenin pathway alters expression of differentiated pronephric tubule, duct or glomus markers (Fig. 5A) (Eid and Brandli, 2001; Gerth et al., 2005; Shibata et al., 2000; Zhou and Vize, 2004). Following lineage trace verification of EnR-LefΔN-GR755A delivery to the pronephric region, proximal (VHNF = *hnf1*β, Na Glucose = *slc5a1*) and distal (NKCC2 = *slc12a1*, XNBC1 = *slc4a4*), as well as general ($Na^{+}K^{+}$ ATPase = *atp1a1*) tubule and duct markers were analyzed at various stages by *in situ* hybridization and colorimetric and/or fluorescent detection. Two glomerular markers, nephrin (*nhps1*) and Xcrescent (*frzb2*), were also examined. Down-regulation of subdomain-specific tubule markers was observed (Table 3), including *slc4a4* and *slc12a1* (Fig. 5B–G), as well as the more general tubule and duct marker *atp1a1* (Table 3 and Fig. 5H–J). Of the two glomus markers, *nhps1* expression was reduced by 60% (Table 3 and Fig. 5K–M), whereas *frzb2* was reduced 13% relative to the control side (Table 3). Overall, these results are consistent with for the role of Wnt/β-catenin signals in pronephric tubule, duct and glomar epithelialization.

To assist in analyzing β-catenin's role in pronephric tubulogenesis, we examined cross sections of embryos expressing EnR-LefΔN-GR755A (injected left V2 blastomere at eight-cell stage, dexamethasone-induced at stage 16, fixed and stained at stages 35–36). Fluorescent *in situ* hybridization of the NaK ATPase (*atp1a1*) indicated failures in pronephric tubule and duct epithelialization (mesenchyme-to-epithelial transition/MET) (Fig. 6A, Table 3). Additionally, the absence in injected sides of the marker nephrin (*nhps1*) suggested that MET had also failed within glomar structures (Fig. 6B, Table 3).

3.5. Inhibition of Wnt/β-catenin signaling results in decreased expression of early pronephric mesenchyme markers

To address if Wnt/β-catenin signaling is required to maintain earlier pronephric marker expression, such as intermediate mesenchyme specification or aggregation, we examined VHNF (*hnf1*β), XLim1 (*lhx1*), Pax8 (*pax8*) (Carroll and Vize, 1999; Wild et al., 2000). EnR-LefΔN-GR755A inductions were executed at stage 15/16, followed by fixation at stages 23, 25, and 27. *In situ* staining reveals significant reductions in *lhx1* and *hnf1*β expression on injected sides (Fig. 7A,E) in 34% and 46%, respectively, of embryos examined (Table 4). While a moderate reduction of *pax8* expression (Fig. 7C) was observed in 63% of embryos examined (Table 4). It is noteworthy, however, that in contrast to the complete inhibition of late epithelial tubule or domain-specific markers (Fig. 5), the response of mesenchyme markers was more subtle and never eliminated (Fig. 7).

3.6. Dkk1 inhibits pronephric epithelial tubule development in zebrafish

To assess if Wnt/β-catenin signals are required for pronephric epithelial tubule formation in another vertebrate species, we analyzed transgenic zebrafish embryos expressing Dkk1. Dkk1 is an extracellular inhibitory ligand of the Wnt co-receptor LRP5/6 (Glinka et al., 1998; Kawano and Kypta, 2003; Semenov et al., 2001; Yamamoto et al., 2008; Zorn, 2001) that is thought to primarily inhibit canonical Wnt/β-catenin rather than non-canonical signaling trajectories (Kawano and Kypta, 2003). The Dkk1 transgene employed is heat-shock inducible, and was activated at the five somite stage (12 h post fertilization) when the teleost pronephros is being specified (Drummond, 2003, 2000; Drummond et al., 1998). This stage is the point at which expression of *lhx1*, an early pronephric marker, becomes noticeable (Kimmel et al., 1995; Toyama and Dawid, 1997). Subsequent heat-shocks (18, 24, 36, 48 h post fertilization) were performed to maintain or enhance the expression of Dkk1, and embryo wholemounts analyzed using monoclonal antibody α6F, which marks epithelial tubules and ducts expressing the Na^{+}/K^{+} ATPase (Drummond et al., 1998; Takeyasu et al., 1988). Heterozygote Dkk1 embryos heat-shocked from the five somite stage onwards (Fig. 8B and C, two representative embryos) showed weak (25%), moderate (8%) or complete (14%) loss of the Na^{+}/K^{+} ATPase marker (Fig. 8D), while wild-type heat-shocked embryos show robust staining (Fig. 8A). These data indicate that reducing Wnt/β-catenin signaling via Dkk1 overexpression results in a significant loss of pronephric tubule epithelium in a large proportion of embryos.

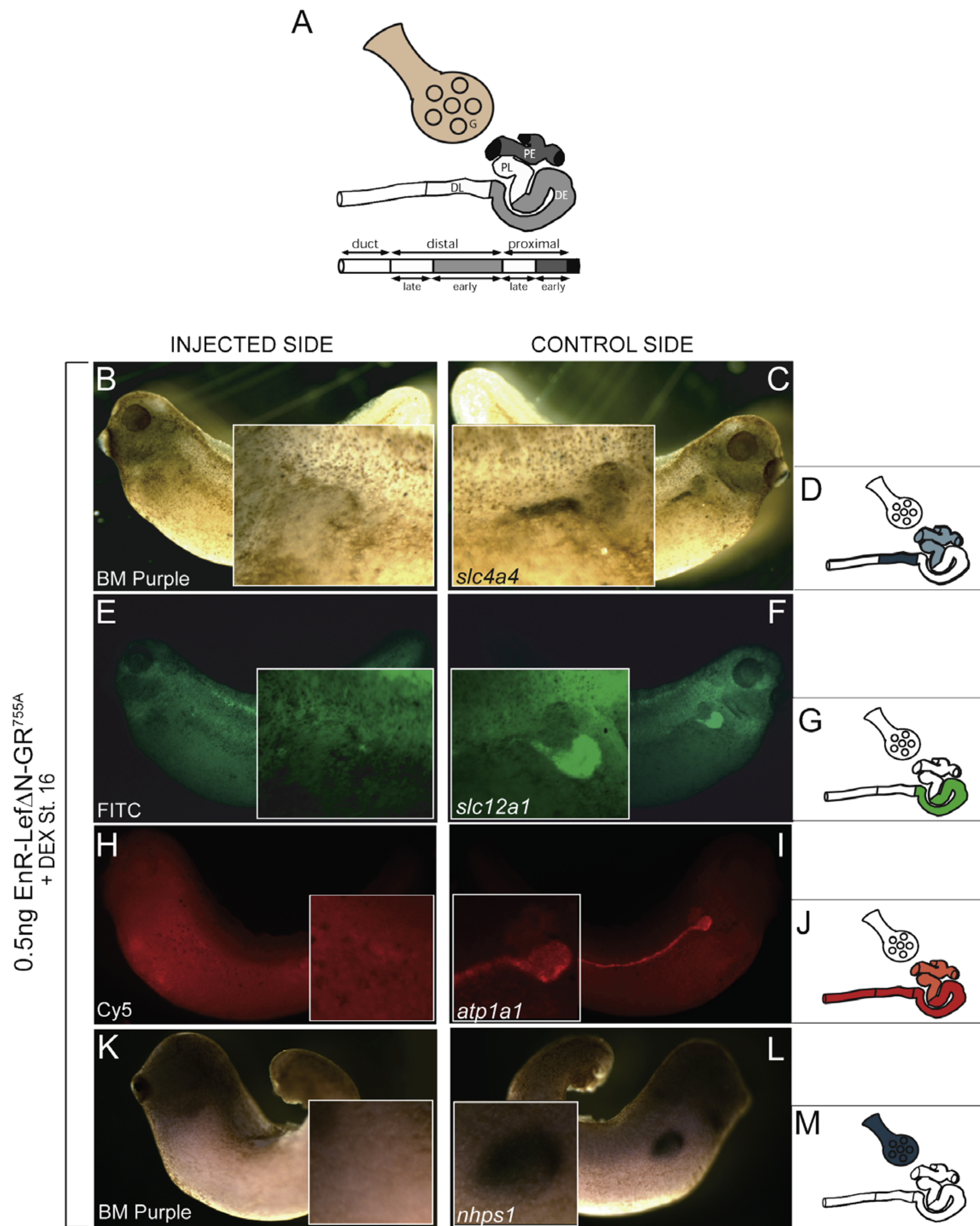


Fig. 5 – EnR-LefΔN-GR755A inhibits expression of multiple pronephric glomus, epithelial tubule, and duct-specific genes. (A) Schematic showing pronephric glomus, tubule and duct segment nomenclature at stage 35. The glomus, labeled G, is shown in beige. The early and late segments within both the proximal and distal domains of tubules are shown in dark and light gray, respectively. The duct is shown in white. Injection of EnR-LefΔN-GR755A (0.5 ng) with rhodamine dextran tracer was performed into single V2 blastomeres at the eight-cell stage and dexamethasone was added at stage 16. Embryos were fixed at stages 35/36 (B–C, E–F, H–I, K–L) and expression of several pronephric marker genes analyzed by whole-mount colorimetric or fluorescent *in situ*. Schematics represent the expression domains of four later stage pronephric markers (D, G, J, M). Shown are injected and control sides of embryos, each including insets of enlarged pronephric regions. Upon the inhibition of Wnt/β-catenin signaling, the strong late distal expression of the sodium bicarbonate cotransporter XNBC1 (*slc4a4*) (D) is lost on the injected (B) versus control side (C). Earlier weaker expression of this same marker is also lost (not shown). The early distal Na-K-2Cl cotransporter NKCC2 (*slc12a1*) (G) shows complete loss of expression on injected (E) versus control side (F). Expression of the NaK ATPase (*atp1a1*) in both the pronephric tubules and duct (J) is lost on the experimental side (H) as compared to the control side (I) when Wnt/β-catenin signaling is inhibited. Additionally, inhibition of Wnt/β-catenin signaling causes the normal expression of nephrin (*nhps1*) in the glomus (M) to be reduced in the injected side (K) as compared with the control side (L).

Table 3 – Analysis of pronephric epithelial tubule marker gene expression in EnR-LefΔN-GR755A injected Xenopus embryos.

Markers	Frequency of reduced or absent expression	Total analyzed
Na Glucose (*slc5a1*)	27%	11
NaK ATPase (*atp1a1*)	73%	22
NBC1 (*slc4a4*)	64%	11
NKCC2 (*slc12a1*)	58%	31
VHNF1 (*hnf1β*)	72%	52
Crescent (*frz2b*)	13%	16
Nephrin (*nhps1*)	60%	20

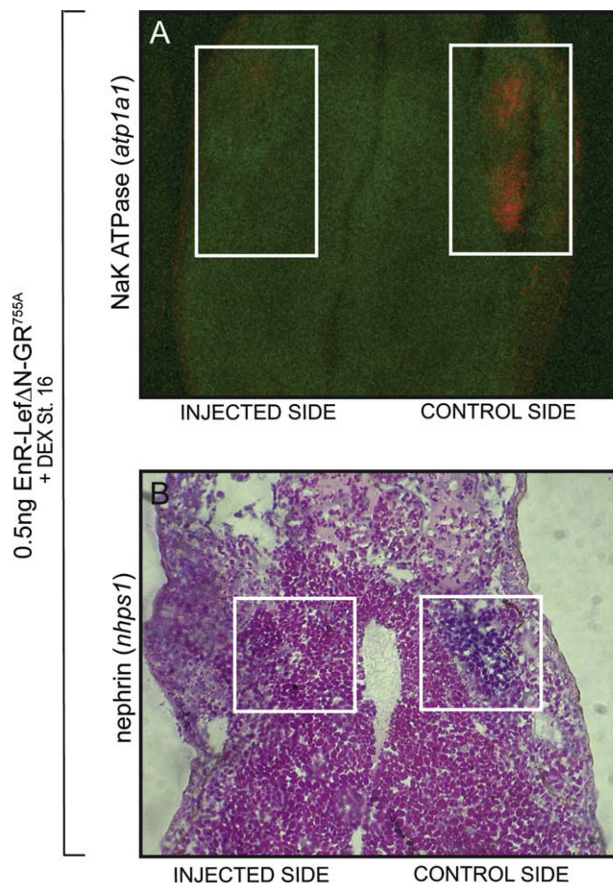


Fig. 6 – EnR-LefΔN-GR755A reduces expression of NaK ATPase and nephrin in the pronephric region. Cross sections of embryos injected with EnR-LefΔN-GR755A (left V2 blastomere at eight-cell stage), induced at stage 16, fixed at stage 35/36, and stained by *in situ* hybridization. (A) Fluorescent *in situ* hybridization of cross sections of stage 35/36 embryos shows that EnR-LefΔN-GR755A inhibits NaKATPase (*atp1a1*) expression within the injected side (left) versus the control side (right). (B) *In situ* hybridization shows that expression of nephrin (*nhps1*), a glomus marker, is inhibited by EnR-LefΔN-GR755A in injected (left) sides of stage 35/36 embryos as compared to control (right) sides.

3.7. Hyperactivation of Wnt/β-catenin signaling inhibits pronephric epithelial tubule development in *Xenopus laevis*

To test if increased Wnt/β-catenin signaling alters pronephric tubulogenesis in *Xenopus*, we employed a hormone-inducible fusion, LefΔN-βCTA-GR755A, activated prior to endogenous Wnt-4 transcriptional onset. The previously uncharacterized LefΔN-βCTA-GR755A fusion consists of the DNA-binding region of mouse Lef1 (HMB box amino acids 265–394), two HA tags, the C-terminal transactivation domain (CTA) of β-catenin, and the human glucocorticoid receptor hormone-binding domain (amino acids 512–777 with E to A substitution at 755) (Fig. 9A). Inducible activation of LefΔN-βCTA-GR755A was confirmed in *Xenopus* embryos by expressing it in ventral-vegetal blastomeres of four-cell embryos incubated with or without dexamethasone. As expected, ectopic (duplicate) axes only arose from ventral tissues following dexamethasone treatment (Fig. 9B and C). Likewise, nuclear translocation of LefΔN-βCTA-GR755A (HA-tagged) was solely evident in isolated ectoderm from embryos incubated in the presence of dexamethasone (Fig. 9D and E). Induced LefΔN-βCTA-GR755A embryos displayed a 97% frequency of ectopic axes, while few secondary axes were observed in the absence of dexamethasone (Fig. 9F).

To assess LefΔN-βCTA-GR755A protein stability (HA-tagged), a temporal expression profile was obtained. Following mRNA injection (V2 blastomeres of eight-cell stage embryos), resulting LefΔN-βCTA-GR755A protein was quite stable through tailbud (stages 24–29) in the absence of dexamethasone (Fig. 9G), while dexamethasone addition at neurula stages increased LefΔN-βCTA-GR755A turnover (stages 19–24) (Fig. 9G). Ten hours after dexamethasone addition, little to no protein was detected.

Very low levels (0.1 ng) of the inducible LefΔN-βCTA-GR755A chimera, which is a potent activator of the Wnt/β-catenin pathway (Fig. 9A–F), caused inhibition of tubule formation (Fig. 9H). When fixed and stained for 3G8 antibody at stages 33–36, some of these embryos displayed a single tubule (~20%). Thus, either inhibiting or over-activating Wnt/β-catenin signaling perturbs pronephric development (tubulogenesis), suggesting that a balanced endogenous level of this signaling pathway must be maintained.

4. Discussion

For some time, Wnt pathways have been known to be required in vertebrate kidney development (Carroll and McMahon, 2000; Vainio, 2003; Vainio and Uusitalo, 2000; Yoshino et al., 2001). In *Xenopus* and murine model systems, Wnt-4 is essential for the mesenchyme-to-epithelial cellular transition that generates tubular epithelium (Saulnier et al., 2002; Stark et al., 1994b). More recently in mice, Wnt-9b has been found to be required for the earliest inductive responses in the metanephric mesenchyme (Carroll et al., 2005), and β-catenin loss resulted in failed metanephric mesenchyme condensation/epithelialization (Park et al., 2007). Using accepted methods to target β-catenin's signaling roles as opposed to those in cell–cell adhesion (cadherin complex), and using multiple molecular tools and model systems, we

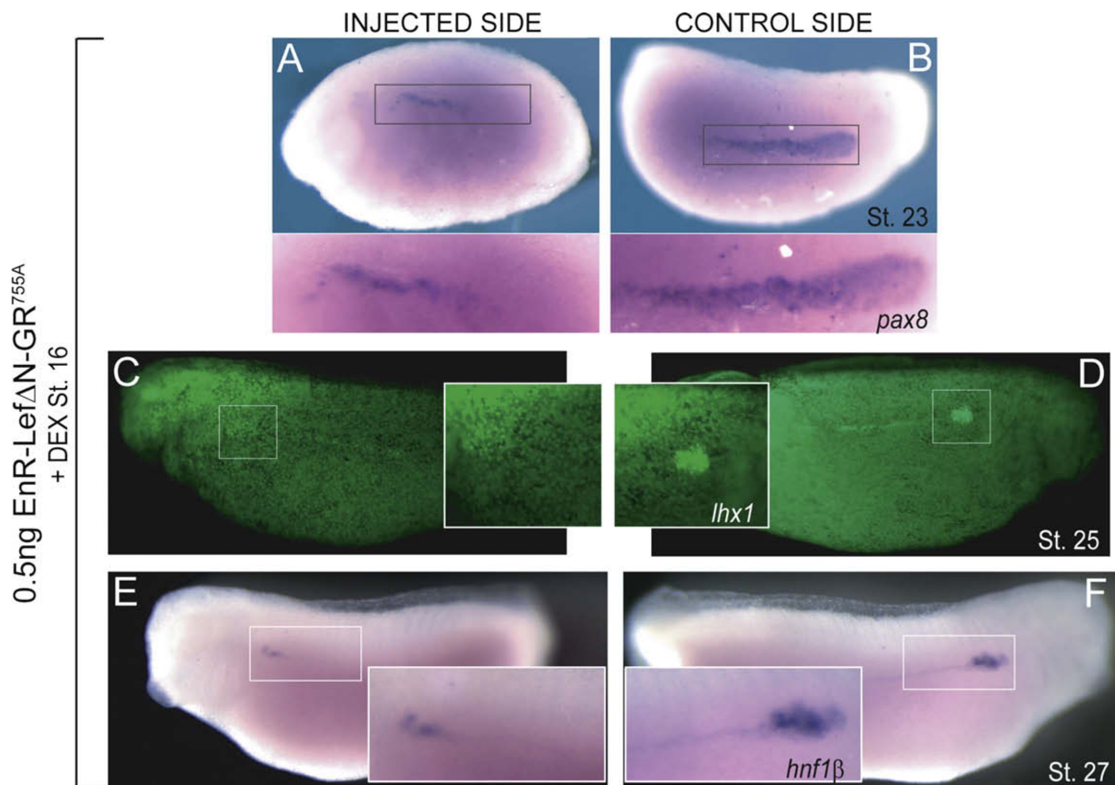


Fig. 7 – EnR-LefΔN-GR755A generates reduced expression of pronephric mesenchyme markers. EnR-LefΔN-GR755A (0.5 ng) with rhodamine dextran tracer was injected into single V2 blastomeres at the eight-cell stage and dexamethasone was added at stage 16. Embryos were fixed at stage 23 (A and B), stage 25 (C and D), and stage 27 (E and F). The injected side of embryos shows reduced expression of pronephric mesenchyme markers XPax8 (*pax8*) (A), XLim1 (*lhx1*) (C), and vHNF1 (*hnf1β*) (E), shown via whole-mount colorimetric or fluorescent *in situ*. The control side of embryos shows expected normal distribution of pronephric mesenchyme markers (B, D, F). Injected and control side images shown are from the same embryo and insets are enlargements of the pronephric region.

provide direct evidence for the requirement of Wnt/β-catenin signaling in the genesis of vertebrate kidney tubules, duct and glomus.

Our data show that Wnt/β-catenin signaling is active and required for formation of tubules, duct and glomus in the *Xenopus* pronephros. Inhibition of Wnt/β-catenin signaling within the pronephric region results in the inhibition of early and late marker expression within the tubules, duct and glomus of *Xenopus*. The requirement of Wnt/β-catenin signaling for the development of vertebrate pronephric tubules is substantiated by inhibition studies of tubule development in zebrafish. Additionally, we have found that hyperactivation of Wnt/β-catenin signaling results in inhibition of pronephric tubulogenesis in *Xenopus*.

Table 4 – Analysis of early pronephric marker gene expression in EnR-LefΔN-GR755A injected Xenopus embryos.

Markers	Frequency of reduced or absent expression	Total analyzed
pax8 (St. 23)	63%	61
lhx1 (St. 25)	34%	29
hnf1β (St. 26/27)	46%	58

In vivo Wnt pathway reporter studies in *Xenopus* have primarily focused on early developmental events, such as those occurring at cleavage, blastula or neurula stages. A Wnt/β-catenin signaling mapping system, harboring a hormone-inducible GFP reporter, has provided useful spatial information at later developmental stages (Denayer et al., 2006). For example, stage 46 transgenic tadpoles exhibit robust eGFP signal in pronephric tubules. Closer examination of the same reporter system resolved Wnt/β-catenin activity at stages 35/36 (Fig. 2). Another more sensitive reporter system in *Xenopus*, has revealed activity as early as stage 25 (Vleminkcx, unpublished results), a time-frame between the onset of *Xwnt-4* transcript expression (stages 19/20) and the first signs of epithelial tubule formation (stages 30/31) in *Xenopus*. Earlier Wnt/β-catenin activity may be present *in vivo* and contributory to intermediate mesoderm specification and/or survival. However, our data suggest it must be below the resolution of current reporter systems in *Xenopus*. While our own immunofluorescence studies in *Xenopus* did not reveal nuclear β-catenin in kidney mesenchyme during tubulogenesis (stages 30–39, not shown), immunohistochemical experiments in human fetuses at 10–18 weeks of development (much later than tubule development) evidence distinct nuclear β-catenin within kidney mesenchyme surrounding renal tubules (Eberhart and Argani, 2001).

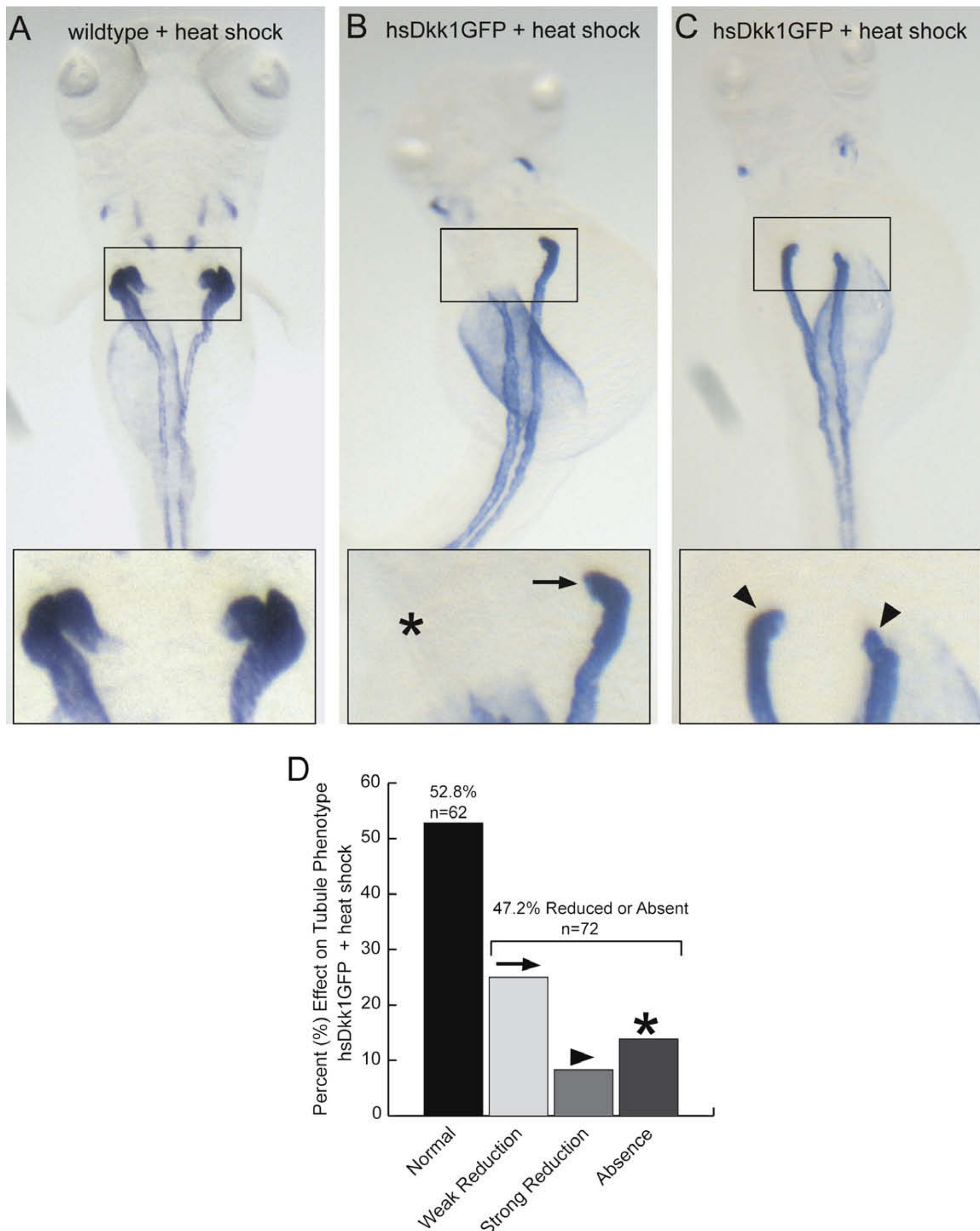


Fig. 8 – Loss of pronephric tubule tissue in zebrafish tadpoles following transgenic Dkk1 misexpression. Dorsal views of dkk1 wild-type (A) and heterozygous (B and C) transgenic embryos heat-shocked several times starting at 12 h post fertilization (5 somite stage), followed by fixation and staining at 72 h post fertilization with the epithelial pronephric tubule and duct-specific antibody α6F. Insets show enlargements of the pronephric tubule region, with an arrow indicating weak tubule reduction (B, right side), arrowheads indicating strong reduction (C, left and right sides), and an asterisk indicating complete absence (B, left side) of α6F tubule expression. Wild-type embryos exhibit normal α6F staining of pronephric tubules (A). α6F Expression in hsDkk1GFP transgenic embryos was tabulated, with 47.2% of embryos displaying a reduction or complete absence of expression (D).

First using non-inducible transcriptional-level inhibition of Wnt/β-catenin signaling within the *Xenopus* pronephros, we observed inhibition of tubule and duct formation (Fig. 1 and Supplemental Fig. 1). Although targeting the pronephric region by injecting constructs into the V2 blastomere is widely accepted, it has limitations given that V2 blastomeres

contribute to other tissues including somites (Moody, 1987; Moody and Kline, 1990). To limit secondary effects, we next employed an inducible system to inhibit Wnt/β-catenin signaling. Induction of inhibition at the transcriptional-level (stage 16/17), resulted in reduced pronephric tubule formation with little effect on somites (Fig. 4), and also reduced expression of differentiated tubule, duct and glomus markers (Fig. 5).

Upon inhibiting Wnt/β-catenin signaling in zebrafish using a heat-shock inducible Dkk1 construct, which inhibits upstream at the level of the cell membrane, pronephric development was likewise impaired (Fig. 8). However, unlike *Xenopus* (Figs. 1 and 5), duct formation appeared to proceed. This difference may be due to distinctions in upstream Dkk1, versus more downstream inhibition of canonical Wnt/β-catenin signaling used in *Xenopus*. Because Dkk1 is an antagonistic ligand of LRP 5/6 co-receptors, it prevents transduction of canonical signals (Semenov et al., 2001), whereas inhibitory constructs used in *Xenopus* are expected to inhibit transcription of canonical signaling target genes.

Interestingly, inhibition of Wnt/β-catenin at stage 19 or later in *Xenopus* does not alter epithelial tubulogenesis (not shown). The loss of tubules observed when inhibition was initiated at stage 16/17 is consistent with *wnt-4* expression beginning in the presumptive pronephric region (intermediate mesoderm) at stage 18 (Carroll et al., 1999b). Induction of inhibition at stage 19 or following produces no effect, perhaps because prior *wnt-4* expression, or that of an unidentified *Xenopus* Wnt, already initiated the tubulogenic program. It is also possible that the amount of inhibiting protein drops below a level capable of efficient inhibition by stage 19, though stability controls indicate that some protein remains until at least stage 28 (Fig. 3).

Mouse studies suggest that early expression of *wnt-4* and *fgf8* together induce *lhx1*, maintaining kidney mesenchyme survival prior to directing the mesenchymal to epithelial transition (Perantoni et al., 2005). Although it remains possible that cell death is a contributing factor influencing tubule loss following inhibition of Wnt/β-catenin signaling, TUNEL assays performed at early stages showed no increase in cell death in injected pronephric fields (not shown). Overall, our data show partially decreased expression of *pax8*, *lhx1* and *hnf1*β, suggesting that Wnt/β-catenin signals contribute to mesenchyme maintenance prior to epithelialization (Fig. 7). The subsequent reduction in tubule marker expression indicates that *pax8*, *lhx1* and *hnf1*β expression, and/or maintained Wnt/β-catenin signaling itself, are needed during the epithelial transition as well (Figs. 5 and 6).

Although our data are consistent with β-catenin transducing Wnt-4 signals, an alternative explanation is that inhibiting Wnt/β-catenin signaling prior to stage 18 prevents *wnt-4* expression itself. In fact, others have indicated that *wnt-4* expression is regulated by Wnt signaling (Carroll et al., 2005; Itaranta et al., 2002; Park et al., 2007). *wnt-9b*, which is expressed in the tips of the invading ureteric bud, is epistatic to *wnt-4* expression in the metanephric mesenchyme (Carroll et al., 2005). Additional evidence suggests that this signal may be transduced via the canonical pathway, as the Wnt-9b phenotype can be rescued using exogenous Wnt-1 or stabilized β-catenin (Carroll et al., 2005; Park et al., 2007). Additionally, it appears that Wnt-4 is capable of transducing either canonical or non-canonical Wnt signals (Chang et al., 2007; Lyons et al., 2004; Maurus et al., 2005; Ungar et al., 1995). It is thus presently unclear whether the signals downstream of Wnt-4 in kidney are canonical, non-canonical or both.

As in inhibiting Wnt/β-catenin signaling, hyperactivation of the pathway results in tubule loss (Fig. 9), with a proportion of these embryos (~20%) displaying only one as opposed to three nephrostomal segments. This observation is consistent with misexpression studies of *wnt-4* in *Xenopus*, which generated a single fused tubule appearing by stages 33/34 as opposed to enhanced tubulogenesis (Saulnier et al., 2002). It is unclear if the single tubule phenotype we observe following β-catenin pathway hyperactivation reflects a coalescence of nephrostomes or simply the elimination or delay of tubule development. As our phenotype resembles that of *wnt-4* overexpression (or depletion), our results are consistent with the possibility that Wnt-4 employs β-catenin to execute at least some of its tubulogenic roles. In any case, our findings support the view that kidney tubule formation requires an intermediate balance of Wnt/β-catenin signaling, with either too little or too much being detrimental.

This view gains further support given the failure of murine metanephric tubules to epithelialize when rescue of *wnt-4* or *wnt-9b* null tissue is attempted using β-catenin, suggesting that Wnt/β-catenin signals must be attenuated during tubulogenesis (Park et al., 2007). Further relevant findings have arisen from the ICAT null mouse, which in addition to having forebrain and craniofacial abnormalities, lacks kidneys (Satoh et al., 2004). Since ICAT disrupts complex formation between β-catenin and Tcf, its loss would be expected to heighten expression of Wnt/β-catenin target genes in the prospective kidney region. Activated β-catenin has also been found to be associated with renal tumors and cystic kidney diseases (Benzing et al., 2007; Tycko et al., 2007). Additionally, certain ciliary proteins found to be functionally compromised in cystic kidney disorders have been implicated in reduction of canonical (β-catenin mediated) in favor of non-canonical Wnt signaling (Kishimoto et al., 2008; Simons et al., 2005). Other evidence has likewise supported a relationship between non-canonical Wnt signaling and ciliagenesis (Wallingford, 2006). Thus, our data in combination with other recent data suggest that canonical Wnt signals necessary for pronephric mesenchymal to epithelial transition are attenuated followed by the intensification of non-canonical Wnt signaling.

In summary, our studies indicate that Wnt/β-catenin signaling is required for pronephric kidney development in *Xenopus laevis* and zebrafish. We expect that Wnt-4 and/or other Wnts employ β-catenin mediated signals in specifying or maintaining prospective pronephric mesenchyme that will subsequently undergo mesenchyme conversion into epithelial tubules. In overview, Wnts are further likely to activate β-catenin-independent pathways involved in pronephric cytoskeletal organization, morphogenesis and cell polarity, although this remains to be clearly demonstrated. Our work supports a direct requirement for β-catenin signaling in vertebrate renal development.

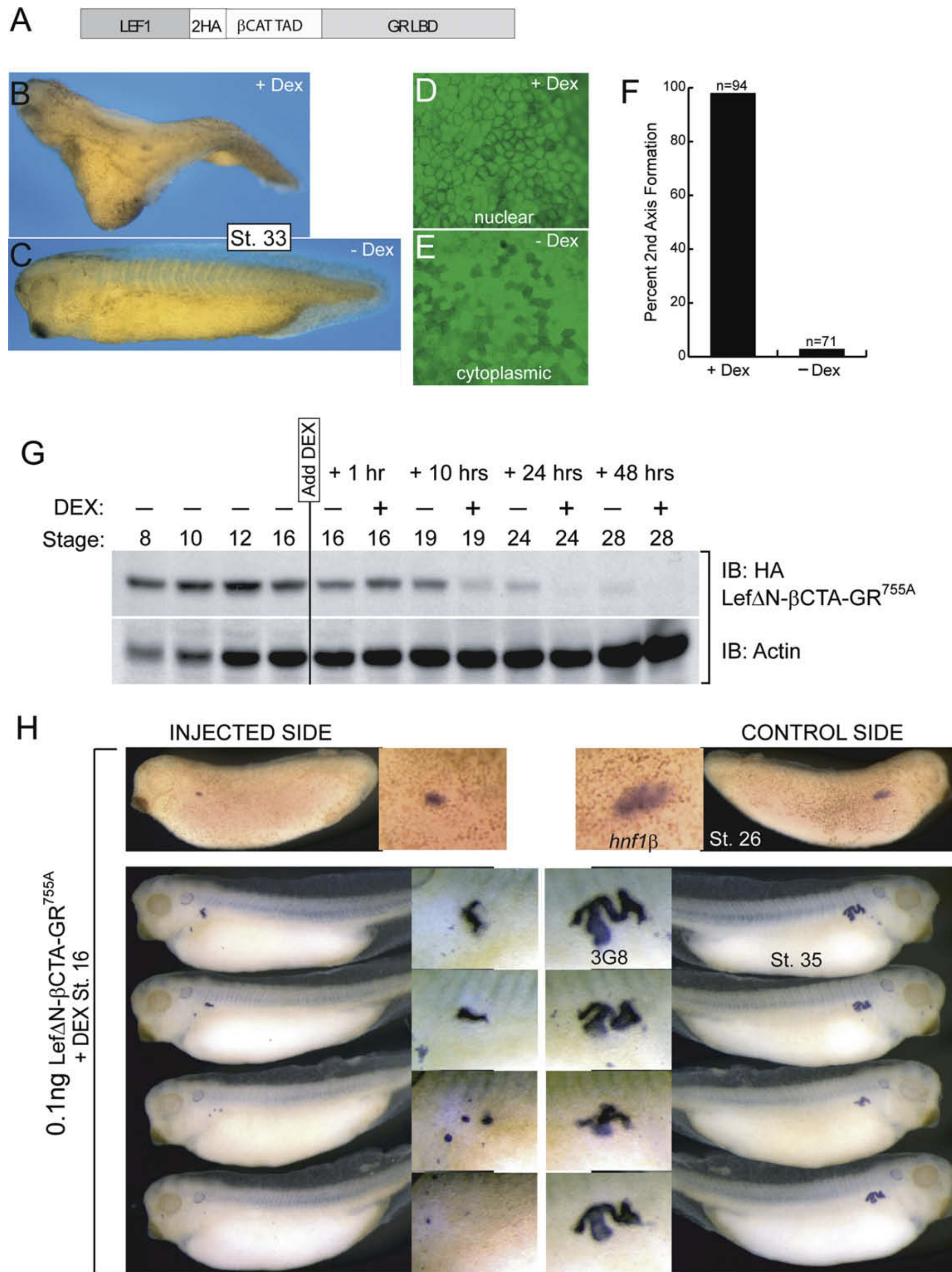


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Fig. 9 – Characterization and pronephric phenotypic analysis of LefΔN-βCTA-GR755A, an inducible fusion construct promoting Wnt/β-catenin signaling. (A) LefΔN-βCTA-GR755A schematic including the mouse Lef1 DNA-binding HMG box (aa 265–391), a double HA epitope-tag, the mouse β-catenin transactivation domain, and the human Glucocorticoid receptor hormone-binding domain (aa 512–777). *Xenopus* embryos were injected with 0.1 ng LefΔN-βCTA-GR755A into a single ventral-vegetal blastomere at the four-cell stage and grown to stages 33–35 with or without dexamethasone. In the presence of dexamethasone, embryos showed a robust level of secondary axis phenotypes (B), while embryos not treated with dexamethasone developed normally (C). Fluorescent (FITC) immunohistochemistry of animal caps excised from stage 9/10 embryos show nuclear localization of LefΔN-βCTA-GR755A in the presence of dexamethasone (D) and cytoplasmic localization in the absence of dexamethasone (E) (prior to animal cap excision, embryos were injected with 1.0 ng LefΔN-βCTA-GR755A into single animal-dorsal blastomeres at the 2-cell stage). (F) Most all embryos noted in B (presence of dexamethasone) exhibited one or more features reflecting an ectopic dorsal axis, while none of those noted in C (absence of dexamethasone) exhibited duplicate axes. (G) To assess LefΔN-βCTA-GR755A protein stability following dexamethasone addition, a time course was undertaken of embryos injected with 0.5 ng LefΔN-βCTA-GR755A into single vegetal-ventral (V2) blastomeres at the eight-cell stage. HA epitope-tag Western blotting shows the inducible chimera (like that of EnR-LefΔN-GR755A and EnR-GR) is stably present until addition of dexamethasone at stage 16, at which time each experiences increased metabolic turnover. (H) *Xenopus* embryos injected with 0.1 ng LefΔN-βCTA-GR755A into single vegetal-ventral (V2) blastomeres at the eight-cell stage (+dexamethasone at stage 16) showed decreased expression of an early pronephric tubule epithelial marker *hnf1β* (in situ at stage 26), and variable decreased expression of a more mature tubule epithelial marker 3G8 (immunostaining at stage 35). The left panels show whole tadpoles along with enlargements of observed phenotypes on injected sides, while right panels depict uninjected/control sides with corresponding enlargements.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mod.2008.11.007.

REFERENCES

Aulehla, A., Herrmann, B.G., 2004. Segmentation in vertebrates: clock and gradient finally joined. Genes Dev. 18, 2060–2067.
Benzing, T., Simons, M., Walz, G., 2007. Wnt signaling in polycystic kidney disease. J. Am. Soc. Nephrol. 18, 1389–1398.
Brandli, A.W., 1999. Towards a molecular anatomy of the *Xenopus* pronephric kidney. Int. J. Dev. Biol. 43, 381–395.
Brembeck, F.H., Rosario, M., Birchmeier, W., 2006. Balancing cell adhesion and Wnt signaling, the key role of beta-catenin. Curr. Opin. Genet. Dev. 16, 51–59.
Bridgewater, D., Cox, B., Cain, J., Lau, A., Athaide, V., Gill, P.S., Kuure, S., Sainio, K., Rosenblum, N.D., 2008. Canonical WNT/beta-catenin signaling is required for ureteric branching. Dev. Biol. 317, 83–94.
Carroll, T., Wallingford, J., Seufert, D., Vize, P.D., 1999a. Molecular regulation of pronephric development. Curr. Top. Dev. Biol. 44, 67–100.
Carroll, T.J., McMahon, A.P., 2000. Secreted molecules in metanephric induction. J. Am. Soc. Nephrol. 11 (Suppl. 16), S116–S119.
Carroll, T.J., Park, J.S., Hayashi, S., Majumdar, A., McMahon, A.P., 2005. Wnt9b plays a central role in the regulation of mesenchymal to epithelial transitions underlying organogenesis of the mammalian urogenital system. Dev. Cell 9, 283–292.
Carroll, T.J., Vize, P.D., 1999. Synergism between Pax-8 and lim-1 in embryonic kidney development. Dev. Biol. 214, 46–59.
Carroll, T.J., Wallingford, J.B., Vize, P.D., 1999b. Dynamic patterns of gene expression in the developing pronephros of *Xenopus laevis*. Dev. Genet. 24, 199–207.
Chang, J., Sonoyama, W., Wang, Z., Jin, Q., Zhang, C., Krebsbach, P.H., Giannobile, W., Shi, S., Wang, C.Y., 2007. Noncanonical Wnt-4 signaling enhances bone regeneration of mesenchymal stem cells in craniofacial defects through activation of p38 MAPK. J. Biol. Chem. 282, 30938–30948.
Davidson, L.A., Keller, R.E., 1999. Neural tube closure in *Xenopus laevis* involves medial migration, directed protrusive activity, cell intercalation and convergent extension. Development 126, 4547–4556.
Denayer, T., Van Roy, F., Vleminckx, K., 2006. In vivo tracing of canonical Wnt signaling in *Xenopus* tadpoles by means of an inducible transgenic reporter tool. FEBS Lett. 580, 393–398.
Deroo, T., Denayer, T., Van Roy, F., Vleminckx, K., 2004. Global inhibition of Lef1/Tcf-dependent Wnt signaling at its nuclear endpoint abrogates development in transgenic *Xenopus* embryos. J. Biol. Chem. 279, 50670–50675.
Dressler, G.R., 2006. The cellular basis of kidney development. Annu. Rev. Cell Dev. Biol. 22, 509–529.
Drummond, I., 2003. Making a zebrafish kidney: a tale of two tubes. Trends Cell Biol. 13, 357–365.
Drummond, I.A., 2000. The zebrafish pronephros: a genetic system for studies of kidney development. Pediatr. Nephrol. 14, 428–435.
Drummond, I.A., Majumdar, A., Hentschel, H., Elger, M., Solnica-Krezel, L., Schier, A.F., Neuhauss, S.C., Stemple, D.L., Zwartkruis, F., Rangini, Z., Driever, W., Fishman, M.C., 1998. Early development of the zebrafish pronephros and analysis of mutations affecting pronephric function. Development 125, 4655–4667.
Eberhart, C.G., Argani, P., 2001. Wnt signaling in human development: beta-catenin nuclear translocation in fetal lung, kidney, placenta, capillaries, adrenal, and cartilage. Pediatr. Dev. Pathol. 4, 351–357.
Eid, S.R., Brandli, A.W., 2001. *Xenopus* Na, K-ATPase: primary sequence of the beta2 subunit and in situ localization of alpha1, beta1, and gamma expression during pronephric kidney development. Differentiation 68, 115–125.

Gerth, V.E., Zhou, X., Vize, P.D., 2005. Nephrin expression and three-dimensional morphogenesis of the *Xenopus* pronephric glomus. Dev. Dyn. 233, 1131–1139.
Glinka, A., Wu, W., Delius, H., Monaghan, A.P., Blumenstock, C., Niehrs, C., 1998. Dickkopf-1 is a member of a new family of secreted proteins and functions in head induction. Nature 391, 357–362.
Halloran, M.C., Sato-Maeda, M., Warren, J.T., Su, F., Lele, Z., Krone, P.H., Kuwada, J.Y., Shoji, W., 2000. Laser-induced gene expression in specific cells of transgenic zebrafish. Development 127, 1953–1960.
Harland, R.M., 1991. In Situ Hybridization: An Improved Whole Mount Method for *Xenopusk* Embryos*Xenopus laevis*: Practical Uses in Cell and Molecular Biology. Academic Press, San Diego. pp. 685–696.
Hensey, C., Dolan, V., Brady, H.R., 2002. The *Xenopus* pronephros as a model system for the study of kidney development and pathophysiology. Nephrol. Dial. Transplant. 17, 73–74.
Huang, H., He, X., 2008. Wnt/beta-catenin signaling: new (and old) players and new insights. Curr. Opin. Cell Biol. 20, 119–125.
Iglesias, D.M., Hueber, P.A., Chu, L., Campbell, R., Patenaude, A.M., Dziarmaga, A.J., Quinlan, J., Mohamed, O., Dufort, D., Goodyer, P.R., 2007. Canonical WNT signaling during kidney development. Am. J. Physiol. Renal Physiol. 293, F494–F500.
Itaranta, P., Lin, Y., Perasaari, J., Roel, G., Destree, O., Vainio, S., 2002. Wnt-6 is expressed in the ureter bud and induces kidney tubule development in vitro. Genesis 32, 259–268.
Kawano, Y., Kypta, R., 2003. Secreted antagonists of the Wnt signalling pathway. J. Cell Sci. 116, 2627–2634.
Kessler, D.S., 1997. Siamois is required for formation of Spemann's organizer. Proc. Natl. Acad. Sci. USA 94, 13017–13022.
Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B., Schilling, T.F., 1995. Stages of embryonic development of the zebrafish. Dev. Dyn. 203, 253–310.
Kintner, C.R., Brookes, J.P., 1984. Monoclonal antibodies identify blastemal cells derived from dedifferentiating muscle in newt limb regeneration. Nature 308, 67–69.
Kishimoto, N., Cao, Y., Park, A., Sun, Z., 2008. Cystic kidney gene seahorse regulates cilia-mediated processes and Wnt pathways. Dev. Cell 14, 954–961.
Kuure, S., Popsueva, A., Jakobson, M., Sainio, K., Sariola, H., 2007. Glycogen synthase kinase-3 inactivation and stabilization of beta-catenin induce nephron differentiation in isolated mouse and rat kidney mesenchymes. J. Am. Soc. Nephrol. 18, 1130–1139.
Kyuno, J., Masse, K., Jones, E.A., 2008. A functional screen for genes involved in *Xenopus* pronephros development. Mech. Dev. 125, 571–586.
Lin, Y., Liu, A., Zhang, S., Ruusunen, T., Kreidberg, J.A., Peltoketo, H., Drummond, I., Vainio, S., 2001. Induction of ureter branching as a response to Wnt-2b signaling during early kidney organogenesis. Dev. Dyn. 222, 26–39.
Lyons, J.P., Mueller, U.W., Ji, H., Everett, C., Fang, X., Hsieh, J.C., Barth, A.M., McCrea, P.D., 2004. Wnt-4 activates the canonical beta-catenin-mediated Wnt pathway and binds Frizzled-6 CRD: functional implications of Wnt/beta-catenin activity in kidney epithelial cells. Exp. Cell Res. 298, 369–387.
Majumdar, A., Vainio, S., Kispert, A., McMahon, J., McMahon, A.P., 2003. Wnt11 and Ret/Gdnf pathways cooperate in regulating ureteric branching during metanephric kidney development. Development 130, 3175–3185.
Mauch, T.J., Yang, G., Wright, M., Smith, D., Schoenwolf, G.C., 2000. Signals from trunk paraxial mesoderm induce pronephros formation in chick intermediate mesoderm. Dev. Biol. 220, 62–75.
Maurus, D., Heligon, C., Burger-Schwarzler, A., Brandli, A.W., Kuhl, M., 2005. Noncanonical Wnt-4 signaling and EAF2 are required for eye development in *Xenopus laevis*. EMBO J. 24, 1181–1191.
Molenaar, M., van de Wetering, M., Oosterwegel, M., Peterson-Maduro, J., Godsave, S., Korinek, V., Roose, J., Destree, O., Clevers, H., 1996. XTcf-3 transcription factor mediates beta-catenin-induced axis formation in *Xenopus* embryos. Cell 86, 391–399.
Montross, W.T., Ji, H., McCrea, P.D., 2000. A beta-catenin/engrailed chimera selectively suppresses Wnt signaling. J. Cell Sci. 113 (Pt. 10), 1759–1770.
Moody, S.A., 1987. Fates of the blastomeres of the 16-cell stage *Xenopus* embryo. Dev. Biol. 119, 560–578.
Moody, S.A., Kline, M.J., 1990. Segregation of fate during cleavage of frog (*Xenopus laevis*) blastomeres. Anat. Embryol. (Berl) 182, 347–362.
Nelson, W.J., 2008. Regulation of cell–cell adhesion by the cadherin–catenin complex. Biochem. Soc. Trans. 36, 149–155.
Park, J.S., Valerius, M.T., McMahon, A.P., 2007. Wnt/beta-catenin signaling regulates nephron induction during mouse kidney development. Development 134, 2533–2539.
Perantoni, A.O., Timofeeva, O., Naillat, F., Richman, C., Pajni-Underwood, S., Wilson, C., Vainio, S., Dove, L.F., Lewandoski, M., 2005. Inactivation of FGF8 in early mesoderm reveals an essential role in kidney development. Development 132, 3859–3871.
Satoh, K., Kasai, M., Ishidao, T., Tago, K., Ohwada, S., Hasegawa, Y., Senda, T., Takada, S., Nada, S., Nakamura, T., Akiyama, T., 2004. Anteriorization of neural fate by inhibitor of beta-catenin and T cell factor (ICAT), a negative regulator of Wnt signaling. Proc. Natl. Acad. Sci. USA 101, 8017–8021.
Satow, R., Chan, T.C., Asashima, M., 2004. The role of *Xenopus* frizzled-8 in pronephric development. Biochem. Biophys. Res. Commun. 321, 487–494.
Saulnier, D.M., Ghanbari, H., Brandli, A.W., 2002. Essential function of Wnt-4 for tubulogenesis in the *Xenopus* pronephric kidney. Dev. Biol. 248, 13–28.
Saxén, L., 1987. Organogenesis of the KidneyDevelopmental and Cell Biology Series, vol. 19. Cambridge University Press, Cambridge; New York.
Schedl, A., 2007. Renal abnormalities and their developmental origin. Nat. Rev. Genet. 8, 791–802.
Schmidt-Ott, K.M., Masckauchan, T.N., Chen, X., Hirsh, B.J., Sarkar, A., Yang, J., Paragas, N., Wallace, V.A., Dufort, D., Pavlidis, P., Jagla, B., Kitajewski, J., Barasch, J., 2007. Beta-catenin/TCF/Lef controls a differentiation-associated transcriptional program in renal epithelial progenitors. Development 134, 3177–3190.
Schwab, K.R., Patterson, L.T., Hartman, H.A., Song, N., Lang, R.A., Lin, X., Potter, S.S., 2007. Pygo1 and Pygo2 roles in Wnt signaling in mammalian kidney development. BMC Biol. 5, 15.
Schwarz-Romond, T., Metcalfe, C., Bienz, M., 2007. Dynamic recruitment of axin by Dishevelled protein assemblies. J. Cell Sci. 120, 2402–2412.
Semenov, M.V., Tamai, K., Brott, B.K., Kuhl, M., Sokol, S., He, X., 2001. Head inducer Dickkopf-1 is a ligand for Wnt coreceptor LRP6. Curr. Biol. 11, 951–961.
Seufert, D.W., Brennan, H.C., DeGuire, J., Jones, E.A., Vize, P.D., 1999. Developmental basis of pronephric defects in *Xenopus* body plan phenotypes. Dev. Biol. 215, 233–242.
Shibata, M., Ono, H., Hikasa, H., Shinga, J., Taira, M., 2000. *Xenopus* crescent encoding a Frizzled-like domain is expressed in the Spemann organizer and pronephros. Mech. Dev. 96, 243–246.
Siemering, K.R., Golbik, R., Sever, R., Haseloff, J., 1996. Mutations that suppress the thermosensitivity of green fluorescent protein. Curr. Biol. 6, 1653–1663.

Simons, M., Gloy, J., Ganner, A., Bullerkotte, A., Bashkurov, M., Kronig, C., Schermer, B., Benzing, T., Cabello, O.A., Jenny, A., Mlodzik, M., Polok, B., Driever, W., Obara, T., Walz, G., 2005. Inversin, the gene product mutated in nephronophthisis type II, functions as a molecular switch between Wnt signaling pathways. Nat. Genet. 37, 537–543.
Sokol, S.Y., Wharton Jr., K.A., 2007. WNTers in La Jolla. Development 134, 3393–3399.
Stark, K., Vainio, S., Vassileva, G., McMahon, A.P., 1994a. Epithelial transformation of metanephric mesenchyme in the developing. Nature 372, 679–683.
Stark, K., Vainio, S., Vassileva, G., McMahon, A.P., 1994b. Epithelial transformation of metanephric mesenchyme in the developing kidney regulated by Wnt-4. Nature 372, 679–683.
Stark, M.R., Rao, M.S., Schoenwolf, G.C., Yang, G., Smith, D., Mauch, T.J., 2000. Frizzled-4 expression during chick kidney development. Mech. Dev. 98, 121–125.
Stoick-Cooper, C.L., Weidinger, G., Riehle, K.J., Hubbert, C., Major, M.B., Fausto, N., Moon, R.T., 2007. Distinct Wnt signaling pathways have opposing roles in appendage regeneration. Development 134, 479–489.
Takeyasu, K., Tamkun, M.M., Renaud, K.J., Fambrough, D.M., 1988. Ouabain-sensitive (Na+ + K+)-ATPase activity expressed in mouse L cells by transfection with DNA encoding the alpha-subunit of an avian sodium pump. J. Biol. Chem. 263, 4347–4354.
Tepera, S.B., McCrea, P.D., Rosen, J.M., 2003. A beta-catenin survival signal is required for normal lobular development in the mammary gland. J. Cell Sci. 116, 1137–1149.
Toyama, R., Dawid, I.B., 1997. Lim6, a novel LIM homeobox gene in the zebrafish: comparison of its expression pattern with lim1. Dev. Dyn. 209, 406–417.
Tycko, B., Li, C.M., Buttyan, R., 2007. The Wnt/beta-catenin pathway in Wilms tumors and prostate cancers. Curr. Mol. Med. 7, 479–489.
Ungar, A.R., Kelly, G.M., Moon, R.T., 1995. Wnt4 affects morphogenesis when misexpressed in the zebrafish embryo. Mech. Dev. 52, 153–164.
Vainio, S.J., 2003. Nephrogenesis regulated by Wnt signaling. J. Nephrol. 16, 279–285.
Vainio, S.J., Uusitalo, M.S., 2000. A road to kidney tubules via the Wnt pathway. Pediatr. Nephrol. 15, 151–156.
Valerius, M.T., Patterson, L.T., Witte, D.P., Potter, S.S., 2002. Microarray analysis of novel cell lines representing two stages of metanephric mesenchyme differentiation. Mech. Dev. 112, 219–232.
Vize, P.D., Jones, E.A., Pfister, R., 1995. Development of the *Xenopus* pronephric system. Dev. Biol. 171, 531–540.
Vize, P.D., Seufert, D.W., Carroll, T.J., Wallingford, J.B., 1997. Model systems for the study of kidney development: use of the pronephros in the analysis of organ induction and patterning. Dev. Biol. 188, 189–204.
Vize, P.D., Woolf, A.S., Bard, J., 2003. The kidney: from normal development to congenital abnormalities. Academic Press, Amsterdam; Boston. pp. xiii, 522 p.
Vleminckx, K., Kemler, R., Hecht, A., 1999. The C-terminal transactivation domain of beta-catenin is necessary and sufficient for signaling by the LEF-1/beta-catenin complex in *Xenopus laevis*. Mech. Dev. 81, 65–74.
Wallingford, J.B., 2006. Planar cell polarity, ciliogenesis and neural tube defects. Hum. Mol. Genet. 15 (Spec. No. 2), R227–R234.
Wallingford, J.B., Carroll, T.J., Vize, P.D., 1998. Precocious expression of the Wilms' tumor gene xWT1 inhibits embryonic kidney development in *Xenopus laevis*. Dev. Biol. 202, 103–112.
Wallingford, J.B., Habas, R., 2005. The developmental biology of dishevelled: an enigmatic protein governing cell fate and cell polarity. Development 132, 4421–4436.
Widelitz, R., 2005. Wnt signaling through canonical and non-canonical pathways: recent progress. Growth Factors 23, 111–116.
Wild, W., Pogge von Strandmann, E., Nastos, A., Senkel, S., Lingott-Frieg, A., Bulman, M., Bingham, C., Ellard, S., Hattersley, A.T., Ryffel, G.U., 2000. The mutated human gene encoding hepatocyte nuclear factor 1beta inhibits kidney formation in developing *Xenopus* embryos. Proc. Natl. Acad. Sci. USA 97, 4695–4700.
Willert, K., Jones, K.A., 2006. Wnt signaling: is the party in the nucleus? Genes Dev. 20, 1394–1404.
Yamamoto, H., Sakane, H., Michiue, T., Kikuchi, A., 2008. Wnt3a and Dkk1 regulate distinct internalization pathways of LRP6 to tune the activation of beta-catenin signaling. Dev. Cell 15, 37–48.
Yoshino, K., Rubin, J.S., Higinbotham, K.G., Uren, A., Anest, V., Plisov, S.Y., Perantoni, A.O., 2001. Secreted Frizzled-related proteins can regulate metanephric development. Mech. Dev. 102, 45–55.
Zeng, X., Tamai, K., Doble, B., Li, S., Huang, H., Habas, R., Okamura, H., Woodgett, J., He, X., 2005. A dual-kinase mechanism for Wnt co-receptor phosphorylation and activation. Nature 438, 873–877.
Zhou, X., Vize, P.D., 2004. Proximo-distal specialization of epithelial transport processes within the *Xenopus* pronephric kidney tubules. Dev. Biol. 271, 322–338.
Zorn, A.M., 2001. Wnt signalling: antagonistic Dickkopfs. Curr. Biol. 11, R592–R595.