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Pax8 and Pax2 are specifically required at different steps of *Xenopus* pronephros development

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Abstract

The respective role of Pax2 and Pax8 in early kidney development in vertebrates is poorly understood. In this report, we have studied the roles of Pax8 and Pax2 in *Xenopus* pronephros development using a loss-of-function approach. Our results highlight a differential requirement of these two transcription factors for proper pronephros formation. Pax8 is necessary for the earliest steps of pronephric development and its depletion leads to a complete absence of pronephric tubule. Pax2 is required after the establishment of the tubule pronephric anlage, for the expression of several terminal differentiation markers of the pronephric tubule. Neither Pax2 nor Pax8 is essential to glomus development. We further show that Pax8 controls *hnf1b*, but not *lhx1* and *Osr2*, expression in the kidney field as soon as the mid-neurula stage. Pax8 is also required for cell proliferation of pronephric precursors in the kidney field. It may exert its action through the wnt/beta-catenin pathway since activation of this pathway can rescue MoPax8 induced proliferation defect and Pax8 regulates expression of the wnt pathway components, *dvl1* and *sfrp3*. Finally, we observed that loss of pronephros in Pax8 morphants correlates with an expanded vascular/blood gene expression domain indicating that Pax8 function is important to delimit the blood/endothelial genes expression domain in the anterior part of the dorso-lateral plate.

Keywords : pronephros, *Xenopus*, Pax2/8, *hnf1b*, wnt, proliferation

INTRODUCTION

The vertebrate kidney is an essential organ for water and salts homeostasis and to remove metabolic waste products from the body (Brandli, 1999; Saxen, 1987; Vize et al., 2003). It derives from the intermediate mesoderm and develops through a multi-step process where undifferentiated mesenchyme is converted into a highly complex organ. In mammals, three successive kidneys of increasing complexity, the pro-, meso- and metanephros, arise during development. Although the pronephros is rudimentary and non functional, it is required for the subsequent formation of the mesonephros and metanephros. Fish and amphibian do not form metanephros but generate a functional larval pronephros, followed by formation of a mesonephros in adult. All three kidneys share the same structural and functional unit, the nephron, but differ greatly in the number and spatial organization of nephrons. In *Xenopus*, the pronephros comprises a pair of single large non-integrated nephron, symmetrically localized on each side of the embryo (Brandli, 1999; Vize et al., 1997). It is composed of three distinct domains: the glomus, where the blood is filtrated into the coelomic cavity; the tubule where selective re-absorption and secretion is taking place, and the duct (also called connecting tubule) which transports the wastes to the cloaca. Fluids from the coelom are swept into the tubule through thin ciliated funnels called the nephrostomes. The pronephric tubule is segmented in distinct domains similar to those of the mammalian nephron, as evidenced by the differential expression of several membrane transporter genes (Raciti et al., 2008; Zhou and Vize, 2004). Due to its relatively simple organization and its experimental accessibility, the *Xenopus* pronephros has emerged as an attractive model to study the early events of kidney development. Many genes that have been shown to be necessary for the formation of *Xenopus* pronephros are also crucial for the formation of the more complex mammalian metanephros. Nevertheless, species differences exist and discovering such differences is essential to determine which processes and regulatory gene networks are evolutionary conserved

In *Xenopus* embryo, the kidney field (KF) emerges at the early neurula stage from the intermediate mesoderm that lies between the anterior paraxial mesoderm and the lateral plate. It is characterized by the co-expression of the transcription factor encoding genes *lhx1*, *pax8* and *osr1* and 2 (Carroll et al., 1999; Heller and Brandli, 1999; Tena et al., 2007). Renal precursors are already specified since KF explanted at the early neurula stage 14 grown *ex vivo* differentiate into glomus and tubule (Brennan et al., 1998, 1999). Pronephric morphogenesis is initiated at the early tailbud stage when cells from the somatic layer condense and segregate away from the lateral plate and somites to form the tubule anlage, which will later generate tubular epithelium. Cells in the adjacent splanchnic layer will undergo morphogenesis later on to form the glomus. They express *wt1*, a gene encoding a zinc finger transcription factor, thought to have a role in the specification of the glomus by suppressing tubule and duct gene expression (Carroll and Vize, 1996a; Wallingford et al., 1998). The tubule anlage is partitioned into two compartments: the antero-dorsal compartment giving rise to proximal tubule and the ventro-posterior compartment leading to distal tubule and duct. Renal precursors are proliferating under the control of a balance between canonical and non-canonical wnt signaling. When canonical signals are inhibited, cell proliferation rate decreases and the opposite occurs when non-canonical signals are downregulated (Mccoy et al., 2011).

Pax2/5/8 genes play crucial roles in embryonic development and organogenesis of the eye, inner ear, kidney and thyroid (Dressler et al., 1990; Nornes et al., 1990; Plachov et al., 1990). They encode transcription factors defined by the presence of a highly conserved 128-amino acid paired domain, a partial homeodomain, and a conserved octapeptide sequence with similarity to the engrailed homology domain (EH-1). *Pax2/5/8* can either activate or repress transcription of target genes by recruiting members of the groucho co-repressor family (Cai et al., 2003; Eberhard et al., 2000). They are frequently expressed at multiple stages of the developing organ and function with a certain degree of redundancy (Batista and Lewis, 2008; Bouchard et al., 2002). Using a gene replacement approach in the mouse, *Pax2/5/8* proteins have been shown to be interchangeable in inner ear indicating that their function is determined primarily by their spatio-temporal expression (Bouchard et al., 2000). *Pax2* and *Pax8* act redundantly in early mouse kidney development. Homozygous *pax2* *-/-* mouse embryos initially form the pro/mesonephros but the nephric duct degenerates before it is completely formed, resulting in kidney agenesis (Torres et al., 1995). *Pax8* inactivation alone does not result in any kidney malformation (Mansouri et al., 1998) while double mutant *pax2* *-/-pax8* *-/-* embryos fail to form the nephric duct, preventing the formation of all three embryonic kidneys (pro-, meso-, and meta- nephros) (Bouchard et al., 2002). The relative roles of *pax2* and *pax8* in kidney development in other vertebrates, in particular in *Xenopus*, remain still unclear. The zebrafish *pax2a* mutant *no isthmus* (Brand et al., 1996; Majumdar et al., 2000) shows defects in tubule epithelial differentiation and displays patterning defects that suggest a role in defining the boundary between glomerular and tubule progenitors. More recently, *pax2a/pax8* doubly deficient embryos were generated and shown to exhibit reduced expression of nephron segment markers (Naylor et al., 2013). In *Xenopus*, ectopic expression of *pax8* or *pax2* has a moderate effect on pronephric patterning, but co-expression with *lhx1* results in an enlargement of the pronephros and in some cases leads to the development of ectopic tubules (Carroll and Vize, 1999). However, data describing the specific effects of *pax2* and *pax8* knockdowns on pronephros development in *Xenopus* are currently lacking.

In this study, we investigate and compare the roles of *Pax8* and *Pax2* transcription factors on *Xenopus* pronephros development using a loss-of-function approach. We found that *Pax8* depletion leads to a complete absence of pronephric tubule marker gene expression associated with an expanded vascular/blood gene expression domain. *Pax2* is required after the establishment of the pronephric tubule anlage, for proximal tubule formation and distal tubule differentiation. We further show that, *Pax8* is required for *hnf1b*, but not *osr2* and *lhx1*, expression in the neurula KF. *Pax8* depletion also induces cell proliferation defects of pronephric precursors that are rescued by activation of the wnt/ β -catenin pathway. Moreover, expression of the wnt pathway components *dvl1* and *sfrp3* in the neurula KF is under the control of *Pax8*.

MATERIALS AND METHODS

Xenopus embryos and microinjections

Xenopus laevis were purchased from the CNRS *Xenopus* breeding Center (Rennes, France). Embryos were obtained after artificial fertilization, and were raised in modified Barth's solution (MBS). Stages were according to the normal table of *Xenopus laevis* (Nieuwkoop and Faber, 1967). Synthesis of capped RNA and microinjection of embryos were performed as described in (Umbhauer et al., 2000) using pCS2pax8 (Carroll and Vize, 1999), pCS2-TCF3-VP16GR (de Croze et al., 2011). For pSP64Tpax8-GR, we amplified *pax8* coding sequences using primers (forward: 5'-CGTAGATCTATGCCCAACAGCATCAGA-3'; reverse: 5'-TGCAGATCTACCATAAGGTCATAGGCTCC-3') and cloned the PCR fragment into pSP64T-XbraGRHA (Tada et al., 1997) digested with BglII. For pCS2pax8VP16, we amplified a PCR fragment corresponding to the first 1100 bp of the *pax8* coding sequence using (forward: 5'-GTCTGAATTCATGCCCAACAGCAGCATCAGA-3' reverse: 5'-TGAAGATCTTGGGTGTGAAAATGCGCTAA-3'). The PCR product was digested with EcoRI/BglII and introduced into pCS2Oct4λVP2 (Hammachi et al., 2012). For pCS2pax8ΔO, a *pax8* fragment of the first 573 bp was isolated from pCS2pax8 by ClaI/BamHI digestion. It was fused with a fragment amplified by PCR using primers (forward: 5'-AGTAGATCTACGATCACGCAGCCCAGCGCA-3' reverse: 5'-CATGCGCCGCCTACATAAGGTCATAGGCT-3'). The fused fragment was cloned into EcoRI site in pCS2. Antisense RNA was generated using SP6 RNA polymerase after enzyme digestion (Sall for pax8GR, NotI for pax8VP16, Asp 718 for pax8ΔO). A range of 140-280 pg of pax8, pax8ΔO, pax8-GR, pax8-VP16 RNAs were injected per blastomere. Morpholinos were purchased from Gene Tools. MoPax2 5'-GGTCTGCCTTGCAGTGCATATCCAT-3'. MoPax2 is 100% complementary to one pax2 pseudoallele sequence and displays one mismatch with the second pseudoallele. MoPax2.2 5'-TCTGCCTTGCAGTGCATATCCATG-3' is 100% complementary to both pseudoalleles. MoPax8 5'-CAGAGCAGCTCCTTGTAGCCAATGT-3', MoPax8.B 5'-CTAGTCCCTGGTAAATCCACAGTGC-3'. MoPax8 matches perfectly with one pseudoallele and displays 4 mispairs with the second one; MoPax8.B matches perfectly with the second pseudoallele and bears 3 mispairs with the first one (see Fig.S1B). MoC 5'-CACACCAGGTCGTTCTACCCAATCT-3'. Morpholinos (2-5 pmoles) were injected alone or mixed with 5 ng/nl of the lineage tracer rhodamine-lysinated dextran as previously described (Colas et al., 2008).

Explants dissections and incubation

Dissections were all performed in 1X MBS on 1% agar-coated dishes. Explants comprising the KF were dissected from mid-neurula embryos (stage 18). Using platinum loop and wire, pronephric field was isolated from somitic and lateral plate mesoderm and separated from the underlying endoderm. The overlying ectoderm was kept. Explants were immediately processed for RT-qPCR. The quality of the dissection was checked by analysing expression of renal, somitic, endodermal and epidermal markers (Fig. S5). Animal caps were isolated from late blastula (stage 9) embryos and immediately transferred into 1X MBS. Dexamethasone (DEX, Sigma D1756) was

prepared as a stock solution (10mM) in ethanol. It was used at a final concentration of 10 μ M. In control conditions, ethanol (diluted 1:1000) was added to the medium. For cycloheximide (CHX) treatments, animal cap explants were incubated 30 minutes at 21°C with 10 μ g/ml CHX prior to addition of DEX or carrier (i.e. ethanol) to the medium and incubation for 3h. Hydroxyurea and aphidicolin (HUA) treatment was done according to (Harris and Hartenstein, 1991).

Real-time quantitative PCR

RNA extraction, reverse transcription and PCR were carried out as described in (Le Bouffant et al., 2012). Sequences of the primers are given in Supplementary Table 1. The Comparative Ct method was used to determine the relative quantities of mRNA, using β -actin and ODC (not shown) mRNA as the endogenous reporter. Each RNA sample was analyzed in duplicate. Each data point represents the mean \pm SEM of at least three independent experiments. Data were analyzed using R Commander (R software) by paired Student's t-test. (* $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$).

***In situ* hybridization**

Whole-mount *in situ* hybridization was carried out as described elsewhere (Harland, 1991). When double *in situ* hybridization was performed, embryos were hybridized with probes differentially labelled with fluorescein and digoxigenin. The first colour was developed using BCIP (Boehringer Mannheim) then BM Purple (Boehringer Mannheim) was used for the second color reaction. Embryos were sectioned at 60 μ m thickness with vibratome.

Immunodetection

Whole-mount immunofluorescence with the 3G8 and 4A6 antibodies (kindly provided by Dr. E.A. Jones, Warwick University, United Kingdom) was performed using standard methods on MEMFA fixed embryos. Immunostaining of frozen sections were performed as described in (Bello et al., 2008). The following primary antibodies were used : rabbit anti-Pax8 (ProteinTech, 10336-1-AP, diluted 1 :200), rabbit anti-Pax2 (Covance, PRB-276P, diluted 1 :200), rabbit anti-aPKC (Santa Cruz Biotechnology C-20, Sc-216, diluted 1:1000), rabbit anti-laminin-1 (Sigma L-9393, diluted 1:25), rabbit anti-collagen IV (Rockland, 66-401-106-0.5, diluted 1:50). Nuclear staining was carried out by a 1-min wash in PBS containing DAPI (Sigma, diluted 1:1000). Pictures were taken using either a Leica TDS SP5 or Nikon, Eclipse 80i, digital camera DXM 1200C. As secondary antibodies, we used Alexa Fluor 488 anti-mouse (Molecular probes, A11001, diluted 1:1000), Alexa Fluor 488 anti-rabbit (Molecular probes, A11008, diluted 1:1000), Alexa Fluor 568 anti-mouse (Molecular probes, A11004, diluted 1:1000), Alexa Fluor 568 anti rabbit (Molecular probes, A11036, diluted 1:1000).

BrdU incorporation

Cell proliferation was detected with 5-bromo-2'-deoxy-uridin (Hardcastle and Papalopulu, 2000). 10 nl BrdU (Roche) was injected bilaterally into the archenteron at stage 18. Embryos were fixed 1 hour later in MEMFA. Cryostat sections were first subjected to *in situ* hybridization for *pax8*, then to immunostaining for BrdU (Becton Dickinson, 347580, diluted 1:200) and Pax8. Numeration of BrdU-positive cells in the KF was performed by manual counting following delineation of the KF based on the *pax8* mRNA expression domain using Photoshop CS4. Within the KF area, we determined the BrdU labeling index (LI) which represents the proportion of BrdU-labeled cells over the total number of cells (numbered thanks to a DAPI staining). Statistical analysis was performed using Student's *t*-test (* $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$).

RESULTS

Pax8 and Pax2 tadpole morphants display distinct tubule phenotypes

In order to decipher the exact role of *pax8* and *pax2* on *Xenopus* pronephros formation *in vivo*, we have undertaken a loss of function approach using translational blocking morpholinos targeting either *pax8* (MoPax8) or *pax2* (MoPax2) transcripts. As multiple spliced isoforms have been described for *pax2* and *pax8* (Heller and Brandli, 1997; Short et al., 2012), we paid attention to design Mo that target all isoforms. We demonstrated their efficiency by showing their ability to abolish endogenous Pax8 or Pax2 expression in the embryo (fig.S1A). A Mo containing 7 mismatches relative to MoPax8 did not affect embryonic Pax8 expression and was used as a control (MoC) (fig. S1). In a first set of experiments, we performed injection of MoPax8, MoPax2 or MoC in embryos at the 4-cell stage in all four blastomeres. Embryos were cultured until tadpole stage 45 for phenotype analysis. Overall, 76 % (n=36) of MoPax8 and 85 % (n=68) of MoPax2 injected tadpoles display an edema in the antero-ventral region, a condition often caused by renal dysfunction impairing fluid homeostasis (Fig. 1A,a,b). Injection of MoC did not lead to any morphological phenotype defect (n=36) (Fig. 1A,c). To examine the effect of Pax8 and Pax2 loss of function on pronephric tissue, we used two antibodies, markers of pronephric terminal differentiation: the 3G8 antibody to identify the proximal tubule and the 4A6 antibody to visualize the intermediate and distal tubules (Vize et al., 1995). Embryos were injected at the 4-cell stage in the equatorial region of both left blastomeres with MoPax8, MoPax2 or MoC. They were cultured until tadpole stage 40 and processed for immunostaining. The right uninjected side was used as a control. Depletion of Pax8 resulted in a complete absence of 3G8 and 4A6 staining in 65 % (n=38) of the morphants (Fig. 1B,d,h). The remaining embryos showed a strong decrease of both 3G8 and 4A6 on the injected side (not shown). In MoPax2 injected tadpoles, reduced 3G8 staining revealed severe abnormalities of the proximal tubule (80%, n=21) (Fig. 1B,e,i). Immunodetection with 4A6 did not give any signal on the injected side in 42 % (n=21) of the MoPax2 morphants embryos; 48% showed a strong decrease of 4A6 staining (Fig. 1B,f,j). The absence of 4A6 staining induced by Pax2

knockdown in a significant proportion, led us to investigate whether it reflected a total absence of tubule or a defect in the tubular epithelial cells differentiation. Double immunostaining with 4A6 and antibodies against Laminin-1, Collagen IV or aPKC was carried out on tadpole transverse sections. On the control side, 4A6 stained the entire cell surface of pronephric tubular cells (Fig. 1C,o,p,q). The tubule was surrounded by laminin-1 and collagen IV, the main components of the kidney basement membranes (Fig. 1C,o,p). aPKC, an apical protein implicated in the control of epithelial polarity, was localized at the apical side of the tubular epithelium (Fig. 1C,q). On the injected side, even when 4A6 did not give any signal (Fig. 1C,l,m,n), a strong Laminin-1 and Collagen IV staining was delineating a tubular structure with a lumen (n=6) (Fig. 1C,l,m). aPKC was correctly localized at the apical side of the cells forming this tubule, suggesting that the apical basal cell polarity is maintained (n=4) (Fig. 1C,n). Similar analyses in MoPax8 morphants revealed a complete absence of tubule (data not shown).

For a more detailed description of the phenotype, we analyzed expression of several tubule pronephric marker genes by *in situ* hybridization at late tailbud stage: *pax2*, which is expressed in the three nephrostomes and the entire tubule; *smp30*, a marker of the proximal tubule (Sato et al., 2000); *outer dense fibre 3 (odf3)*, a marker of multiciliated cells in the epidermis and nephrostomes; *evi1* which is expressed in the distal tubule, the duct and part of the intermediate tubule (Van Campenhout et al., 2006). Comparison of the injected and uninjected sides showed that Pax8 knockdown strongly reduced or completely abolished expression of the tubule markers *pax2* (85%, n=34), *odf3* (90%, n=37), *smp30* (80%, n=30) and *evi1* (91%, n=32), indicating that tubulogenesis was completely inhibited (Fig. 2Aa-d). In Pax2 morphants, expression of *pax2* is severely affected in the proximal region but the expression domain corresponding to the intermediate and distal tubule was still present (62%, n=13) (Fig. 2Ae,e'). Expression of *odf3* was lost in the majority of the Pax2 depleted embryos (76%, n=29) (Fig. 2Ag,g') and *smp30* expression was strongly reduced (52%, n=29) (Fig. 2Af,f'). In the case of *evi1*, the expression domain was retained, except for the most proximal part (66%, n=29) (Fig. 2Ah,h'). Analysis of terminal differentiation markers encoding ion transporters and solute carriers (Raciti et al., 2008; Zhou and Vize, 2004) such as *slc4a4* (also called *NBC1*), *slc12a1* (also called *NKCC2*), *atp1b1* (ATPase Na⁺/K⁺ transporting beta1), *clcnkb1* and *claudin 19* in Pax2 morphants confirmed that the proximal part of the tubule, including the nephrostomes, was reduced (82%, n= 29, 64%, n= 28, 58%, n= 36, 77%, n= 36, 73%, n=38, respectively) (Fig.2B). In the intermediate and distal tubule, expression of *atp1b1* was not affected by the MoPax2 (100%, n=36) (Fig. 2Bk,k') whereas expression of *clcnkb1* was either completely abolished (41%, n=36) (Fig. 2Bl,l') or strongly reduced (36%, n=36). We also investigated expression of two glomus markers: *nephrin*, which is exclusively expressed in the glomus and *wt1* which is expressed strongly in the glomus and in the intermediate mesoderm surrounding the pronephric tubule and the heart (Carroll and Vize, 1996b; Gerth et al., 2005). We did not detect any significant effect of MoPax2 or MoPax8 on *wt1* and *nephrin* expression. In order to reveal a potential functional redundancy between *pax8* and *pax2*, we co-injected both morpholinos and analyzed the expression pattern of *nephrin* and *wt1* by *in situ* hybridization. For both genes, expression patterns on the injected and uninjected sides were similar (100%, n=21 for *wt1*; 100%, n=29 for *nephrin*) (Fig. 2C). These results were confirmed by RT-qPCR analysis performed on tailbud stage embryos injected at the 4-cell stage in all four blastomeres. Neither MoPax2 nor MoPax8 injected

alone or together has significant effect on *wt1* and *nephrin* expression levels (Fig. 2D). Since MoPax2 and MoPax8 are 100% complementary to one pseudoallele but display a few mismatches with the second pseudoallele (Fig.S1B), we repeated the experiments by injecting a MoPax2 that targets both *pax2* pseudoalleles and by co-injecting MoPax8 with a morpholino (MoPax8.B) that is 100% complementary to the second *pax8* pseudoallele. In both cases, the phenotypes were similar to the previous ones (Fig. S2, S3). These additional results support the idea that injection of MoPax2 and MoPax8 leads to a complete Pax2 and *pax8* knockdown, respectively. In all the experiments described, we used MoC injected embryos as controls, and did not detect any expression pattern modifications (data not shown).

Together, these data show that *pax8* and *pax2* are both required for proper pronephric tubule establishment but are dispensable for glomus formation. Moreover, Pax8 and Pax2 assume different roles in pronephric tubule development since their loss of function leads to strikingly distinct effects.

Pax8 but not Pax2 is required for tubule marker genes expression and anlage formation at the early tailbud stage

We next examined the effect of Pax8 and Pax2 loss of function upon earlier steps of kidney development, when the pronephric tubule anlage becomes morphologically distinguishable and regionalized. We used several marker genes known to be expressed in the pronephric region at tailbud stage 25: *lhx1*, *hnf1b*, *pax2*, *hrt1* and *evi1*. *Lhx1*, *hnf1b* and *pax2* are expressed in the entire pronephric region at this stage. *Hrt1* mRNA localization is restricted to the antero-dorsal part of the pronephric anlage (Taelman et al., 2006), while *evi1* mRNA is present in the posterior part of the pronephric region (Van Campenhout et al., 2006). Expression patterns were analyzed in injected embryos using *in situ* hybridization. Injection of MoPax8 led to either a drastic decrease or a complete inhibition of the expression of all the markers (76%, n=128 for *lhx1*; 86%, n=34 for *hnf1b*; 77%, n=30 for *pax2*; 90%, n=43 for *hrt1* and 95%, n=43 for *evi1*) (Fig. 3A,a-e). Co-injection with MoPax8.B led to similar phenotypes (Fig.S3). In contrast, injection of MoPax2 did not lead to any significant expression defect for *hrt1* (94%, n=31) and *lhx1* (100%, n=19). *Evi1* expression was identical to the control side in 71% (n=17) of the embryos and slightly diminished in the remaining embryos (Fig. 3A,f-j). Similar phenotypes are observed in response to MoPax2.2 (Fig.S2).

The first sign of pronephric morphogenesis is a change in cell shape in the somatic intermediate mesoderm at the early tailbud stage 21. Cells condense to form the pronephric tubule anlage that is obvious by stage 25 as a solid mass of cells surrounded by Laminin-1, below the anterior somites. We asked whether formation of the pronephric tubule anlage required Pax8 and/or Pax2 function. Embryos were injected on one side with MoPax8 or MoPax2, cultured until tailbud stage 25 and fixed. We then performed immunostaining with an anti-Laminin-1 antibody on transverse sections at the level of the pronephros area. On the control side, the pronephric tubule anlage is visualized as a group of somatic cells surrounded by Laminin-1 immunostaining (100%, n=5) (Fig. 3B,p',q',r',s'). No such structure is observed on the MoPax8 injected side (100%, n=5) (Fig. 3B,p). Laminin-1 is only detected in the extracellular matrix between

the epidermis and the somatic mesoderm as well as between the splanchnic mesoderm and the endoderm. In contrast, pronephric tubule anlage is fully segregated and delineated by Laminin-1 immunostaining on MoPax2 injected side (n=7) (Fig. 3B,q).

To confirm the specificity of the MoPax8-induced phenotype, we decided to perform rescue experiments. In a first set of experiments, we tried to rescue the phenotype with an *in vitro* transcribed wild-type *pax8* RNA that did not contain the MoPax8-binding sequence. However, even at moderate doses (50-100 pg), injection of *pax8* RNA led to gastrulation defects, thus precluding study of pronephric development. To overcome this problem, we constructed an inducible form of Pax8 (*pax8-GR*) by fusing the *pax8* coding sequence to the ligand-binding domain of the human glucocorticoid receptor (Kolm and Sive, 1995). Embryos were first injected at the 4-cell stage in both left blastomeres with MoPax8. Then, approximately half of them were injected again with *pax8-GR* RNA. Dexamethasone was added to the culture medium at the end of gastrulation (stage13) to activate Pax8 function. In these conditions, gastrulation occurred normally and inhibition of pronephric *lhx1* expression caused by MoPax8 was partially rescued by inducing Pax8 function (Fig. 4).

Taken together, these findings show that interference with Pax8 but not Pax2 function prevents tubule anlage morphogenesis and expression of associated pronephric genes. This result indicates that the absence of pronephric tubule at tadpole stage in Pax8 morphants is consecutive to a defect in tubule anlage establishment.

***Hnf1b* expression in the kidney field (KF) is under the control of Pax8**

We sought to identify the earliest stage when the pronephric defects in Pax8 morphants were detectable. MoPax8 injected embryos were subjected to *in situ* hybridization with *lhx1*, *osr2* and *hnf1b* probes at neurula stages. *Lhx1* and *osr2* expression was identical on the injected and uninjected sides (100%, n=18 for *lhx1*; 100% for *osr2*, n=12) (Fig. 5A). In contrast, a clear and consistent downregulation of *hnf1b* was observed in the KF on the injected side at neurula stage 18 (100%, n=25) (Fig. 5A). Co-injection with MoPax8.B did not modify the phenotype (Fig. S4). Inhibition of *hnf1b* but not *osr2* and *lhx1* expression was confirmed by RT-qPCR analysis on KF explants (see materials and methods) (Fig. 5B). These observations demonstrate that Pax8 is required for the early *hnf1b* expression in the KF.

We then asked whether Pax8 gain of function was sufficient to induce *hnf1b* expression. To answer this question, we injected *in vitro* synthesized Pax8 RNA at the 4-cell stage, in the animal pole of the four blastomeres. Animal caps from injected and uninjected embryos were dissected at blastula stage 9, cultured for 3 hours and processed for *hnf1b* expression analysis by RT-qPCR. *Hnf1b* appeared to be significantly up-regulated in response to Pax8 ectopic expression (Fig. 6A). To address if activation of *hnf1b*

expression by Pax8 required protein synthesis, we used the inducible form Pax8-GR and cycloheximide (CHX), a protein synthesis inhibitor (Le Bouffant et al., 2012). Activation of Pax8 was triggered by incubating *Pax8-GR* injected animal caps in DEX. CHX was added to the culture medium 30 minutes before DEX treatment. We observed that CHX treatment by itself induced *hnf1b* expression in both injected and uninjected animal caps (Fig. 6B). This behavior was already noticed for many genes and may result from the release of gene repression, caused by synthesis inhibition of a repressor. Although CHX itself up-regulated *hnf1b*, DEX treatment still caused a threefold increase of *hnf1b* expression in a context of protein synthesis inhibition, suggesting that *hnf1b* might be a direct Pax8 target (Fig. 6B).

Pax8 knockdown leads to an increase of blood and endothelial genes expression domains in the dorso-lateral plate (DLP)

Several mechanisms can account for the lack of pronephric tubule anlage formation and defective pronephric tubule marker genes expression in morphants Pax8: a change in cell fate, a change in cell proliferation, or the induction of apoptosis. We first investigated a possible change of fate of the pronephric precursors by analyzing the expression pattern of somitic (*myoD*, *mlc*), lateral plate (*foxf1*, (El-Hodiri et al., 2001)), blood (*scl*, *lmo2*, (Mead et al., 2001; Walmsley et al., 2002) and endothelial (*msr*, *flk1*, (Cleaver et al., 1997; Inui et al., 2006)) marker genes. We did not detect any modification of the *myod* and *mlc* expression domains upon MoPax8 injection (Fig. 7A,a,a',c,c'). Since MoPax8 is a translational blocking morpholino, we reasoned that, cells that would normally express the Pax8 protein, should still contain *pax8* mRNA and thus should be revealed by *in situ* hybridization with a *pax8* probe. Therefore, we performed double *in situ* hybridization with *pax8* and either *foxf1* or *mlc*. The results clearly showed that, indeed, a population of cells expressing *pax8* mRNA was present at the tailbud stage 25 in the pronephric area of the injected side. These cells did not co-express *mlc* (100%, n=36) or *foxf1* (100%, n=29) (Fig. 7A,a,a',b,b'). We noticed that the *pax8* signal was stronger on the injected side compared to the uninjected side (Fig. 7Aa,a',b,b'). This could reflect an inhibitory effect of Pax8 on its own transcription or the bound MoPax8 might simply be stabilizing the transcript against degradation. Notably, the presence of a *pax8* mRNA expressing domain indicates that the absence of Pax8 did not lead to a massive apoptosis. This was further confirmed by TUNEL experiments (data not shown). Studies of blood and endothelial marker genes expression led to different outcomes. For all four tested markers (*msr*, *flk1*, *lmo2* and *scl*), comparison with the control uninjected side revealed a clear expansion of their expression domain in the anterior region where the tubule anlage normally forms (100% n=23 for *msr*; 86% n=22 for *flk1*; 81% n=37 for *lmo2* and 100%, n=24 for *scl*) (Fig. 7B). No such expansion was observed in MoC injected embryos (100%, n=15) (Fig. 7B). Anterior transverse sections of embryos subjected to double *in situ* hybridization with *pax8* and *lmo2* probes showed that *lmo2* staining was much stronger on the injected side in comparison to the uninjected side. The stained surface area is also larger (Fig. 7B,l,m). *Lmo2* and *pax8* staining did not overlap in the intermediate mesoderm, although we could not rule out a possible overlap in the most dorsal part of the *pax8* area. The increase of blood and endothelial gene expression in response to MoPax8 could be due to an increased proliferation of hematopoietic and

endothelial precursors. In order to test this hypothesis, we have blocked cell division in MoPax8 injected embryos and analysed *lmo2* expression. Embryos were treated with HUA (Harris and Hartenstein, 1991) from neurula stage 18 onward and *lmo2* expression was analysed by *in situ* hybridization. We observed that the enlargement of *lmo2* expression domain in the anterior region in response to the loss of Pax8 was maintained in absence of cell proliferation (70%, n=28) (Fig.7C).

Overall, these findings reveal a role of *pax8* in the delimitation of the blood/endothelial genes expression domain in the anterior part of the dorso-lateral plate.

Pax8 knockdown leads to cell proliferation defect that is rescued by activation of the wnt/ β -catenin pathway

Since the three mechanisms, i.e. change in cell fate, change in cell proliferation, or apoptosis, are not exclusive, we further decided to analyse the effect of Pax8 depletion on cell proliferation in the KF using a bromodeoxyuridine (BrdU) labelling assay. Neurula embryos (stage 18) were injected with BrdU into the archenteron. They were cultured one hour before fixation and cryostat sectioning. We combined BrdU staining with *pax8 in situ* hybridization and immunohistochemistry with anti-Pax8 antibodies. The first permitted to define the KF area, the second allowed to check that the Pax8 protein was indeed depleted by the MoPax8. We quantified the number of BrdU labeled cells in the KF area and determined the BrdU labeling index (LI) which represents the proportion of BrdU-labeled cells over the total number of cells in the KF area. We observed that depletion of Pax8 caused a significant decrease in the KF BrdU LI (Fig.8A,B). As expected, injection of the MoC has no effect on the BrdU LI (Fig. 8B).

The canonical wnt signaling has been shown to promote cell proliferation in the *Xenopus* pronephric area at the early tailbud stage (Mccoy et al., 2011). We therefore asked whether the effect of Pax8 deficiency on KF cell proliferation could be rescued by modulating the wnt/ β -catenin pathway. Canonical wnt signaling activation was achieved by overexpressing an inducible form of TCF3VP16 (TCF3VP16-GR, (de Croze et al., 2011)). TCF3VP16 is a modified version of the transcriptional effector of canonical wnt signaling in which the β -catenin-binding domain has been replaced with the VP16 transcriptional activator. We observed that activation of TCF3VP16-GR at neurula stage 15 rescued the effect of MoPax8 on BrdU incorporation (Fig. 8C).

These results indicate that Pax8 is required for proper cell proliferation in the KF, and that it might act through activation of the canonical wnt signaling.

Pax8 loss of function affects expression of several components of the wnt pathway

To explore the potential role of Pax8 on wnt/ β -catenin signaling in the KF, we studied the expression of several wnt signaling components. We focused our analysis on the wnt ligands known to be expressed in the KF (*wnt4*, *wnt5A*, *wnt8*, *wnt11b*, *wnt6*, *wnt9a*,

wnt9b, (Tetelin and Jones, 2010; Zhang et al., 2011)), the secreted wnt inhibitors (*crench*, *dkk1*, *dkk3*, *sfrp1*, *sfrp2*, *sfrp3*, *sfrp5*, *sizzled*) and the intracellular wnt components *dvl1*, *dvl2* and *dvl3*. We decided to assess expression levels of these genes by RT-qPCR on dissected KF explants at the neurula stage 18. Explants from MoPax8 injected embryos were compared to those from MoC injected as well as uninjected embryos. The dissection quality of the explants was controlled by showing an enrichment of the renal markers *lhx1* and *pax8* and an absence of significant expression of the endodermal and somitic markers, *sox17beta* and *myf5*, respectively (Fig. S2). Analysis of the wnt pathway components showed that although some variations were observed on average values, none of the tested genes was significantly affected by MoPax8 depletion with the exception of *dvl1* and *sfrp3*. Interestingly, *sfrp3* was upregulated in MoPax8 injected KF explants while *dvl1* was downregulated (Fig. 9A). In order to confirm these results, we performed *in situ* hybridization on transversely bisected embryos using *dvl1* and *sfrp3* probes. *Dvl1* staining was clearly observed in the pronephric area of the uninjected side whereas the equivalent region on the MoPax8 injected side did not show any staining (70%, n=12) (Fig. 9B). Injection of MoC did not have any effect on *dvl1* expression 100%, n=10) (Fig. 9B). *In situ* hybridization for *sfrp3* did not lead to any signal in the pronephric area even in presence of the MoPax8 (not shown). Since the *in situ* hybridization technique is far less sensitive than qPCR, it is therefore possible that the level of *sfrp3* expression induced by the loss of Pax8 was below the *in situ* limit of detection. We have then tested the ability of Pax8 to activate *dvl1* expression and repress *sfrp3* expression using the blastula animal cap as an *ex vivo* assay. Pax8 on its own was not sufficient to increase *dvl1* expression level. However, an activated form of Pax8 (pax8-VP16) in which the C-terminal domain has been replaced with the VP16 transcriptional activator, is able to increase *dvl1* expression level from three- to fourfold (Fig. 9C). Pax8 can repress transcription of target genes through its binding interaction with members of the groucho family. Since the octapeptide domain is an essential motif that mediates pax8-Groucho binding, we asked whether pax8 deleted from the octapeptide (pax8 Δ O) could induce *sfrp3* expression. We indeed observed that Pax8 Δ O RNA injection resulted in the upregulation of *sfrp3* in blastula animal caps. Pax8 ectopic expression did not lead to any significant modification of *sfrp3* expression (Fig. 9D).

All together, these results indicate that Pax8 regulates *dvl1* and *sfrp3* expression in the KF likely through transcriptional activator and repressor activities, respectively.

DISCUSSION

We report a detailed study of Pax2 and Pax8 functions in *Xenopus* pronephric development. We show that, unlike in mouse, Pax2 and Pax8 are differentially required for proper pronephros formation. Pax8 is necessary for the earliest steps of pronephric development and its depletion leads to a complete absence of pronephric tubule. Pax2 is required after the establishment of the tubule pronephric anlage, for proximal tubule formation and distal tubule differentiation. We further show that Pax8 assumes different roles in early pronephric development: 1/ it activates *hnf1b* expression in the neurula

KF 2/ it controls cell proliferation of pronephric precursors likely through activation of the wnt/ β -catenin 3/ it is implicated in the delimitation of the blood/endothelial genes expression domain in the anterior part of the dorso-lateral plate.

Our results highlight the differential requirement for Pax2 and Pax8 during *Xenopus* pronephric development. The developmental defects upon loss of Pax8 function can be traced to as early as the mid-neurula stage while alterations in response to Pax2 depletion are first observed at the late tailbud stage. Moreover, Pax8 depletion leads to a much stronger kidney phenotype than Pax2 depletion and Pax8 only, is required for pronephric tubule fate specification. Pax2 knockdown leads to defects in a subset of tubule differentiation markers. The expression domain of several markers in the proximal tubule is reduced (*smp30*, *slc4a4*, *atp1b1*, 3G8) and the expression of two nephrostomal markers (*odf3* and *claudin19*) are absent. Interestingly, markers genes of the distal tubule are not similarly affected by Pax2 depletion. *Clnkb1* is expressed normally in the distal tubule while *atp1b1* expression is lacking as well as the 4A6 antigen. This suggest that *pax2* is required for tubule differentiation through the regulation of the expression of specific terminal differentiation genes. The Pax8 and Pax2 knockdown pronephric phenotypes are in agreement with their expression patterns. *Pax8* mRNAs are detected by *in situ* hybridization as soon as the late gastrula stage 12.5 in the KF. Low levels of *pax2* transcripts have been detected by semi-quantitative RT-PCR in dissected KF explants at the early neurula stage 13, but strong pronephric expression of *pax2* starts at the early tailbud stage (Haldin et al., 2008; Heller and Brandli, 1999) where it overlaps with that of *pax8*. Notably, a *pax8* mutant called *ruby* has been recently identified in a forward genetic screen in *Xenopus tropicalis*. Mutant embryos have a reduced expression of *pax8* and tadpoles develop edema around the heart as a consequence of renal dysfunction (del Viso et al., 2012). The predominant role of *pax8* upon *pax2* in the control of kidney cell fate specification revealed by our study is not conserved in vertebrates. Kidney organogenesis is normal in *pax8*^{-/-} mutant mice that die postnatally of a defect in thyroid gland development (Mansouri et al., 1998). Analysis of double mutant *pax2*^{-/-}*pax8*^{-/-} embryos demonstrate that *pax2* and *pax8* have redundant functions and are both required for specifying the nephric lineage (Bouchard et al., 2002). Functional redundancy of Pax2a and Pax8 seems also to occur in zebrafish pronephros development. It has been recently shown that expression of *hnf1ba/b* in the nephron in singly deficient embryos for either Pax2a or Pax8 is not affected, while doubly deficient animals show a severe reduction of *hnf1ba/b* transcripts (Naylor et al., 2013). Our results point out *hnf1b* as a major target of Pax8 in the *Xenopus* KF. Whether it is a direct target is not known but cycloheximide experiments are in favor of this hypothesis. The loss of *hnf1b* expression is unlikely to account for all the Pax8 morphant defects since expression of a dominant negative form of Hnf1b affects the proximal tubule only (Bohn et al., 2003; Heliot et al., 2013). Although one should bear in mind that expression of a dominant negative form may not lead to a complete loss of function, this phenotype differs from the Pax8 knockdown.

Pax2 and *Pax8* are not required for the establishment of the glomus compartment in *Xenopus*. This finding was quite unexpected since data obtained in zebrafish and in mouse revealed *pax2/wt1* regulatory interactions during renal development (Dressler et al., 1993; Ryan et al., 1995; Schedl and Hastie, 2000; Yang et al., 1999). In mouse, Pax2 activation causes repression of the podocyte regulator *wt1* and consequently a dramatic reduction of *nephrin* expression (Wagner et al., 2006). In zebrafish, restriction of the *wt1* expression domain to medial cells requires *pax2a* expression in lateral cells of the

pronephric primordium. In absence of Pax2a, presumptive tubule cells express podocyte-specific markers (Majumdar et al., 2000). The absence of a significant effect of *pax8* and *pax2* loss of function on *wt1* expression in *Xenopus* may be the consequence of fundamental differences between the developmental processes involved in the establishment of the filtration unit. The zebrafish pronephros and the mammalian nephron of the metanephros are integrated nephrons (lacking nephrostomes) where the tubule and glomerulus differentiate in intimate contact. In *Xenopus*, primordia of the glomus and tubule are more distinctly separated (Drummond et al., 1998; Vize et al., 2003).

Our results show that one role of Pax8 is to maintain proper cell proliferation in the KF at neurula stage. Several observations suggest that Pax8 and Pax2 could directly or indirectly influence cell proliferation during embryonic development (Burton et al., 2004; Freter et al., 2012; Li et al., 2004). When aberrantly expressed in adult tissues, Pax2 and Pax8 may exhibit oncogenic properties and have emerged as potential diagnostic markers for several cancers including renal cell carcinomas and Wilm's tumor (Dressler and Douglass, 1992; Eccles et al., 1992; Ordonez, 2012a, b; Tong et al., 2009). The mechanisms by which Pax8 and Pax2 mediate cell cycle control are poorly characterized. In human cancer cell lines, Pax8 regulates the G1/S transition by regulating the expression of the transcription factor E2F1 and stabilizing the retinoblastoma protein (Li et al., 2011). Pax2 promotes proliferation of colon cancer cells by inducing cyclin D1 expression through the AP-1 protein (Zhang et al., 2012). We show that the proliferation defect induced by the loss of Pax8 is rescued by an activated form of Tcf-3. We therefore propose that Pax8 controls pronephric precursors proliferation through activation of the wnt/ β -catenin pathway. This view gains further support given the regulation of *dvl1* and *sfrp3* expression by Pax8 in the KF. It is noteworthy that blocking proliferation at neurula stage with cell division inhibitors Hydroxyurea and Aphidicolin (HUA) does not interfere with pronephros anlage formation (Naylor and Jones, 2009). Therefore, cell proliferation defects on their own cannot explain all aspects of the MoPax8 phenotype, such as the absence of early pronephric marker genes expression.

Our data reveal a potential role for Pax8 in the control of the wnt pathway through the regulation of genes encoding components of this pathway. Several publications report a direct transcriptional control of wnt signaling components by Pax2. Pax2 directly activates *wnt4* in JTC12 cell line derived from the proximal renal tubule (Torban et al., 2006), *sfrp2* in immortalized mouse metanephric mesenchyme cells (Brophy et al., 2003), and *wnt5A* in the human embryonic kidney derived cell line HEK293 (Tamimi et al., 2008). Interestingly, in the *Xenopus* KF, Pax8 controls the wnt transducer *dvl1* and the secreted wnt antagonist *sfrp3* in an opposite manner. Upregulation of *sfrp3* expression in animal caps, in response to Pax8 deleted of the octapeptide, suggests that Pax8 functions as a transcriptional repressor to regulate *sfrp3* expression by recruiting Groucho proteins. *Grg4* and *Grg5* are expressed in the pronephric anlage at the early

tailbud stage (Molenaar et al., 2000), overlapping with *pax8* expression. Pax8 ectopic expression in animal caps is, however, not sufficient to significantly inhibit *sfrp3* expression. It is possible that low levels of endogenous *sfrp3* expression in animal caps preclude from detecting a repression, or that groucho proteins are missing. Notably, we showed that *dvl1* expression in mesoderm is restricted to the kidney field at the neurula stage 18 (Fig.9B). Its downregulation in response to MoPax8 is concomitant to *hnf1b* inhibition and therefore could be either a cause or a consequence of *hnf1b* downregulation. Whether modulation of *dvl1* and *sfrp3* expression leads to activation of the wnt/ β -catenin pathway is still an open question. Although nuclear localization of β -catenin is considered to be a hallmark of wnt activation (Anderson et al., 2002), we were unable to detect any change in β -catenin nuclear localization in KF cells when Pax8 was depleted. Our attempt to analyze by western blot the activated form of β -catenin did not reveal any effect either (data not shown). However, we cannot rule out the possibility that, using immunocytochemistry or western blotting, we missed subtle differences in either the level or the kinetic of β -catenin nuclear localization. While our data are consistent with Pax8 activating the wnt/ β -catenin pathway, a wnt-dependent control of *pax8* expression occurs in KF cells. Inhibition of wnt/ β -catenin signaling within the pronephric region at the neurula stage 16 results in downregulation of early pronephric markers among which *pax8* and the loss of pronephric tubules and glomus at later stages. The developmental period during which this control occurs is restricted to the first half of neurulation, since inhibition at stage 19 does not alter tubulogenesis (Lyons et al., 2009). These data highlight the complex regulatory interactions taking place between Pax8 and the wnt pathway. They would imply that a positive regulatory loop might exist between *pax8* and the wnt pathway, at least before stage 19.

The kidney and the blood/vasculature originate from adjacent regions of the fish and frog embryos (Brown et al., 2000; Ciau-Uitz et al., 2010; Gering et al., 1998; Kimmel et al., 1990; Walmsley et al., 2002). Careful analysis of gene expression patterns in *Xenopus* has demonstrated overlapping expression of vascular and blood genes (*fli1*, *msr*, *gata2*, *scl*) with the kidney markers *lhx1* and *Gata3* in the dorsolateral plate (DLP) at tailbud stage (Walmsley et al., 2002). Later on, distinct stripes of cells can be distinguished that express either pronephric markers or more dorsally hematovascular markers. It is still unclear whether blood and vessels develop from the same multi-potent cells as kidney, or whether mosaicism exists within the KF. In Pax8 morphants, we observed a reproducible enlargement of the expression domain of blood and endothelial marker genes (*lmo2*, *gata2*, *flk1*, *msr*) in the anterior part of the embryo. This enlargement is restricted to the most dorsal part of the dorso-lateral plate and can be attributed neither to a complete switch of cell fate from renal towards blood/endothelial fate (Fig.7B,l-m) nor to an overproliferation of hematopoietic precursors (Fig.7C). One possibility would be that the pronephric anlage sends a signal that restricts blood and endothelial genes expression to the most dorsal part of the dorso-lateral plate. In absence of Pax8, this signal is missing, leading to an increase of blood and endothelial genes expression. In zebrafish, *Osr1* knockdown results in a complete loss of anterior kidney progenitors and a compensatory increase in the number of angioblast cells. (Mudumana et al., 2008). This phenotype is associated with the decreased expression of *pax2a* in the anterior intermediate mesoderm. The authors however rule out a predominant role of Pax2a loss

in the expansion of the blood/vascular lineages since Pax2a or Pax2a/Pax8 knockdown does not cause *scl* upregulation. In any case, further work is needed to understand the function of Pax8 in delimiting the blood/endothelial genes expression domain in the anterior part of the dorso-lateral plate.

In summary, our findings reveal that the specific functions of *pax2* and *pax8* in early kidney development are not strictly conserved in vertebrates. Most of the differences may reflect species-specific variations of the Pax2 and Pax8 expression domains or divergent developmental processes. We demonstrate that in *Xenopus*, Pax8 is a major regulator of the first steps of pronephric development, through the control of cell proliferation and *hnf1b* expression in the KF. Moreover, our work reveals that Pax8 function is necessary to delimit the blood/endothelial genes expression domain in the anterior part of the dorso-lateral plate.

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FIGURE LEGENDS

Figure 1: Tadpoles depleted for Pax 2 or Pax8 display pronephric defects

(A) Morphology of stage 45 tadpoles injected at the 4-cell stage in all four blastomeres with MoPax8 (a), MoPax2 (b) or MoC (c). A large edema is developed in the antero-ventral region of the MoPax8 and MoPax2 injected tadpoles. **(B)** Whole-mount immunofluorescence with 3G8 and 4A6 antibodies. Embryos were injected with MoPax8 (d,h), MoPax2 (e,i,f,j) or MoC (g,k) at the 4-cell stage in the equatorial region of both left blastomeres. Embryos were cultured to the tadpole stage 40 and processed for immunostaining. The right uninjected side is used as control (h,i,j,k). Pax8 knockdown leads to the absence of 3G8 and 4A6 reactivity. In MoPax2 injected tadpoles, 3G8 staining is strongly reduced on the injected side compared to the control side; 4A6 signal is absent. **(C)** Double immunofluorescence on tadpole (stage 40) transverse sections with 4A6 (l-q, revealed in green) and laminin-1 (l,o, revealed in red), collagen IV (m,p, revealed in red) or aPKC (n,q, revealed in red) antibodies. DAPI staining is shown in blue. Embryos were injected with MoPax2 on the left side. The right uninjected side is used as control. A tubular structure, lacking 4A6 staining, but surrounded by laminin-1 and collagen IV is present on the MoPax2 injected side. aPKC is also correctly localized at the apical side of the cells forming this tubule. Insets show higher magnification of the tubule (one focal plane). Scale bar in B: 500 μ m, Scale bar in C: 40 μ m

Figure 2: Pax 8 and Pax2 loss of function differentially affects tubule but not glomus marker genes expression at late tailbud stage

(A,B) Whole mount *in situ* hybridization for tubule marker genes. Embryos were injected with MoPax8 (a-d) or MoPax2 (e-m) at the 4-cell stage, in the equatorial region of both left blastomeres. They were cultured until the tailbud stage 33-35, fixed and analyzed by *in situ* hybridization with specific probes for the indicated genes. For each embryo, injected (a-m) and uninjected (a'-m') sides are shown. Pax8 depletion prevents expression of marker genes all along the entire tubule expression domain. In Pax2 morphants, gene expression in the proximal part of the tubule, including nephrostomes, is affected. Expression in the distal tubule is not modified by the MoPax2 except for the terminal differentiation marker *clcnkb1* whose expression is completely inhibited. Insets show higher magnification of the anterior pronephric territory **(C)** Whole mount *in situ* hybridization for the glomus marker genes *wt1* and *nephrin*. Embryos were injected with a mixture of MoPax8 and MoPax2 in the equatorial region of both left blastomeres at the 4-cell stage and cultured until the tailbud stage 33. Injected sides are shown in (n,o), uninjected sides in (n',o'). Insets show higher magnification of the anterior pronephric territory **(D)** RT-qPCR analysis of *wt1* and *nephrin*. Embryos were injected with the indicated Mo at the 4-cell stage in all four blastomeres. They were cultured until the late tailbud stage 33 and processed for RT-qPCR analysis. Average values from three independent experiments. Neither MoPax2 nor MoPax8 injected alone or together significantly affects *wt1* and *nephrin* expression compared to the control (MoC).

Figure 3: Pax8 but not Pax2 loss of function inhibits tubule marker genes expression and pronephric tubule anlage formation at the early tailbud stage

(A) Whole mount *in situ* hybridization for tubule marker genes. Embryos were injected with MoPax8 (a-e), MoPax2 (f-j) or MoC (k-o) at the 4-cell stage, in the equatorial region of both left blastomeres. They were cultured until tailbud stage 25, fixed and analyzed by *in situ* hybridization with the indicated probes. For each embryo, injected (a-o) and uninjected (a',o') sides are shown. Pax8 but not Pax2 depletion strongly reduces expression of *lhx1*, *hnf1b*, *pax2*, *hrt1*, and *evi1* in the pronephric area (B) Immunofluorescence with an anti-laminin-1 antibody on transverse sections of stage 25 embryos injected with MoPax8 (p,q) or MoPax2 (r,s) on one side. For each embryo, injected (p-s) and uninjected (p'-s') sides are shown. DAPI staining is shown in blue (q,q',s,s'). On uninjected sides and MoPax2 injected side, the pronephric tubule anlage is visualized as a group of somatic cells radially organized, surrounded by laminin-1 immunostaining (p',q',r,s,r',s'). No such cell arrangement is observed on the MoPax8 injected side. (p-q). Laminin-1 is only detected in the extracellular matrix between the epidermis and the somatic mesoderm, as well as between the splanchnic mesoderm and the endoderm. Scale bar: 140 μ m

Figure 4: Inhibition of *lhx1* expression in response to MoPax8 is rescued by expressing Pax8

Embryos were first injected at the 4-cell stage in both left blastomeres with MoPax8. They were split into two groups. Embryos from one group were injected again with RNA Pax8-GR. Dexamethasone was added to the culture medium at the end of gastrulation (stage13). Phenotype was assessed by studying *lhx1* expression by *in situ* hybridization at tailbud stage 25. Phenotypes were classified into three categories: strong inhibition (a), weak inhibition (b) and normal expression (c). Inhibition of pronephric *lhx1* expression caused by MoPax8 was partially rescued by inducing Pax8 function just after completion of gastrulation. Histogram shows quantitative results from three independent experiments (d). n= number of analyzed embryos in total.

Figure 5: *Hnf1b* but not *osr2* and *lhx1* expression is inhibited in the KF of Pax8 morphant at neurula stage

(A) *In situ* hybridization for *lhx1*, *osr2* and *hnf1b* genes. Embryos were injected with MoPax8 at the 4-cell stage, in the equatorial region of both left blastomeres. At neurula stage 18, they were fixed, transversely bisected at the level of the pronephric area and analyzed by *in situ* hybridization with the indicated probes. For each embryo, the dotted line indicates the frontier between the injected side (on the left) and the uninjected side (on the right). *Hnf1b* expression is inhibited in the pronephric area (arrow) on the injected side. (B) RT-qPCR analysis of *lhx1*, *osr2* and *hnf1b* gene expression in KF explants dissected at neurula stage 18 from MoC and MoPax8 injected embryos. Unlike *lhx1* and *osr2* expression which is not modified, *hnf1b* expression is downregulated in

response to Pax8 depletion. Average values from four independent experiments. * $P < 0.05$

Figure 6: Pax8 ectopic expression is sufficient to up-regulate *hnf1b* in ectoderm animal caps in absence of protein synthesis

(A and B) RT-qPCR analysis of *hnf1b* expression in blastula animal caps. (A) Embryos were injected with *pax8* RNA in the animal pole of all four blastomeres at the 4-cell stage. Animal caps from injected and uninjected embryos were dissected at blastula stage 9, cultured for 3 hours and processed for RT-qPCR. A significant increase of *Hnf1b* expression is observed in response to Pax8. Average values from five independent experiments (B) Embryos were injected with *pax8-GR* RNA in the animal pole of all four blastomeres at the 4-cell stage. Animal caps from injected and uninjected embryos were dissected at blastula stage 9. They were pre-incubated with CHX for 30 minutes, or with 1× MBS alone. CHX pre-incubated explants were then cultured for 3 hours in CHX alone or in CHX+DEX; the others were cultured without CHX for 3 hours in presence or in absence of DEX. CHX alone causes an approximately fivefold increase of *hnf1b* expression. DEX treatment still causes a threefold increase of *hnf1b* expression relatively to CHX alone. Average values from three independent experiments. * $P < 0.05$

Figure 7: Expression of somitic, lateral plate, blood and endothelial marker genes in Pax8 morphants

(A) Expression of somitic (*myoD* and *mlc*) and lateral plate markers (*foxf1*). Embryos were injected with MoPax8 at the 4-cell stage, in the equatorial region of both left blastomeres. They were cultured until tailbud stage 25 (a,a',b,b') or neurula stage 18 (c,c'), fixed and analyzed by whole-mount *in situ* hybridization with the indicated probes. For each embryo, injected (a,b,c) and uninjected (a',b',c') sides are shown. Interference with Pax8 function does not modify the expression pattern of neither *myoD*, *mlc* and *foxf1*. (B) Expression of blood (*scl* and *lmo2*) and endothelial markers (*msr* and *flk1*). Embryos were injected with MoPax8 (d-g) or MoC (h-k) at the 4-cell stage, in the equatorial region of both left blastomeres. They were cultured until tailbud stage 27 and processed for whole-mount *in situ* hybridization with the indicated probes. For each embryo, injected (d-j,h-k) and uninjected (d'-g',h'-k') sides are shown. Double *in situ* with *pax8* and *lmo2* probes are shown in whole mount (d, d') as well as in section performed at the level of the anterior (l) and posterior (m) part of the pronephros anlage and in the posterior region of the embryo (n). The dotted line delineates injected (left) and uninjected (right) halves. In Pax8 morphants, the expression domain of *scl*, *lmo2*, *msr* and *flk1* is enlarged in the anterior region where the tubule anlage normally forms. Double *in situ* hybridization with *pax8* and *lmo2* probes shows a much stronger *lmo2* signal dorsal to *pax8* expression domain on the injected side at the level of the pronephros anlage (l,m). In A and B, *pax8* is revealed in light blue while the other genes are revealed in dark blue. Scale bar: 200 μm , (C) Expression of *lmo2* in Pax8 morphants in absence of cell division. Embryos were injected with MoPax8 at the 4-cell stage, in the equatorial region of both left blastomeres. Half of them were cultivated in the presence of HUA from neurula stage 18 onwards. They were processed for whole-mount *in situ* hybridization at stage 27. The enlargement of the *lmo2* expression domain

in the anterior region in response to the loss of Pax8 (o) is maintained in HUA treated embryos (p).

Figure 8: MoPax8 knockdown results in cell proliferation defect in the KF that is rescued by activation of the canonical wnt pathway

(A) Embryos were injected at the 4-cell stage in the equatorial region of both left blastomeres and cultured until stage 18. They were then injected with BrdU into the archenteron, cultured for an additional one hour and fixed for *in situ* hybridization and immunostaining. On transverse sections, we combined *in situ* hybridization for *pax8* (a,a') with immunolocalization for BrdU (b,b') and Pax8 (d,d'), and DAPI staining (c,c'). MoPax8 injected side (a-d), uninjected side (a'-d'). The outlined area in a and a' corresponds to the KF determined by the expression of *pax8* mRNA. The number of BrdU stained nuclei was counted in this region Scale bar: 80 μ m (B) Histogram showing the BrdU labeling index (LI) in MoC and MoPax8 injected embryos. For each embryo, uninjected side is compared with the injected side. The LI represents the proportion of BrdU-labeled cells over the total number of cells in the KF area. BrdU LI is significantly decreased by Pax8 depletion. (C) Histogram showing the BrdU LI in embryos co-injected with MoPax8 and TCF-VP16-GR. Dexamethasone was added (+DEX) or not at stage 15. Activation of TCF-VP16-GR rescues the effect of MoPax8 on the BrdU LI. n= number of analyzed embryos in total. For each embryo, 4 sections were analyzed. Average values from three independent experiments. ** $P < 0.005$, *** $P < 0.001$

Figure 9: Pax8 regulates the expression of components of the wnt signaling pathway

(A) Expression of genes of the wnt signaling pathway analyzed by RT-qPCR in KF explants dissected at neurula stage 18 from uninjected or injected embryos with MoC or MoPax8. We considered the difference as significant (*) when the p value between MoPax8 and MoC as well as between MoPax8 and uninjected was < 0.05 when both *b-actine* and *ODC* are used as endogenous reporter. *dlv1* is downregulated and *sfrp3* is upregulated in response to Pax8 depletion. In contrast, the expression of the secreted wnt inhibitors *crescent*, *dkk1*, *dkk3*, *sfrp1*, *sfrp2*, *sfrp5*, *sizzled*, of the wnt ligands *wnt4*, *wnt5A*, *wnt8*, *wnt11b*, *wnt6*, *wnt9a*, *wnt9b* as well as *dlv2* and *dlv3* is not modified. Average values from five independent experiments. (B) *In situ* hybridization with a *dlv1* probe. Embryos were injected with MoPax8 or MoC at the 4-cell stage, in the equatorial region of both left blastomeres. At neurula stage 18, they were fixed, transversely bisected at the level of the pronephric area and analyzed by *in situ* hybridization. For each embryo, the dotted line delineates injected (left) and uninjected halves (right). *Dlv1* is expressed in the intermediate mesoderm and its expression is inhibited in response to MoPax8 (arrow). (C, D) RT-qPCR analysis of *dlv1* (C) and *sfrp3* (D) expression in blastula animal caps. Embryos were injected with the indicated RNA (*pax8*, *pax8-VP16*, *pax8dO*) in the animal pole of all four blastomeres at the 4-cell stage. Animal caps from injected and uninjected embryos were dissected at blastula stage 9, cultured for 3 hours and processed for RT-qPCR. A significant increased of *dlv1* expression is observed in response to Pax8-VP16. *Sfrp3* is upregulated in response to Pax8 Δ O. Average values from four independent experiments. * $P < 0.05$

Supplementary data

Figure S1: MoPax8 and MoPax2 block endogenous embryonic Pax8 and Pax2 expression, respectively

(A) Immunofluorescence with anti-Pax8 (a-b) and anti-Pax2 (c, d) antibodies performed on transverse sections of embryos injected with MoPax8.2 (a, a'), MoC (b, b') or MoPax2 (c, d) in both left blastomeres at the 4-cell. The KF region of injected (a, b) and uninjected side (b, b') is shown at the late neurula stage 18. The dorsal part of a stage 25 embryo containing the neural tube (NT) and the medial part containing the pronephros (arrow) are shown in c and d, respectively. The dotted line delineates injected (left) and the uninjected (right) halves. Pax8 is detected both in the splanchnic and somatic layers of the KF on the uninjected side, while it is absent on the MoPax8 injected side (a,a'). MoC has no effect on the endogenous Pax8 expression (b'). Pax2 expression in the neural tube and in the pronephros is inhibited on the MoPax2 injected side (c,d). Scale bar in a and b: 100 μ m, scale bar in c and d: 400 μ m **(B)** Location of the Mo target sequences used in this study on *pax2* and *pax8* pseudoalleles. Nucleotides that differ between the two pseudoalleles are in blue. The start codon is in red.

Figure S2: Pronephric phenotype induced by injection of MoPax2.2

Whole mount *in situ* hybridization for tubule and glomus marker genes. Embryos were injected with MoPax2.2 at the 4-cell stage, in the equatorial region of both left blastomeres. They were cultured until the tailbud stages 33-35 **(A)** or 25 **(B)** and analyzed by *in situ* hybridization with specific probes for the indicated genes. For each embryo, injected (a-q) and uninjected (a'-q') sides are shown. Pax2 depletion does not lead to any change in the expression of pronephric marker genes at stage 25. At the late tailbud stage, gene expression in the proximal part of the tubule, including nephrostomes, is affected by MoPax2.2 while expression in the distal tubule is not modified except for the terminal differentiation marker *clcnkb1* whose expression is completely inhibited. Expression of the glomus marker genes *wt1* and *nephrin* is not significantly affected in MoPax2.2 injected embryos. Insets show higher magnification of the anterior pronephric territory. Numbers indicate how many embryos showed the phenotype among the total number of injected embryos.

Figure S3: Pronephric phenotype induced by co-injection of MoPax8 and MoPax8.B

Whole mount *in situ* hybridization for tubule and glomus marker genes. Embryos were injected with MoPax8.B alone or in co-injection with MoPax8 at the 4-cell stage, in the equatorial region of both left blastomeres. They were cultured until the tailbud stages

33-35 **(A)**, early tailbud stage 23-25 **(B)** and analyzed by *in situ* hybridization with specific probes for the indicated genes. For each embryo, injected (a-v) and uninjected (a'-v') sides are shown. A strong reduction of the tubule marker genes (*pax2*, *odf3*, *smp30*, *lhx1*, *hnf1b*, *pax2*, *hrt1*, and *evi1*) expression is observed in response to the injection while the glomus markers *wt1* and *nephrin* are still expressed. Insets show higher magnification of the anterior pronephric territory.

Figure S4: *Hnf1b* but not *osr2* and *lhx1* expression is inhibited in the neurula KF in response to the co-injection of MoPax8 and MoPax8.B

In situ hybridization for *lhx1*, *osr2* and *hnf1b* genes. Embryos were injected with MoPax8.B alone or in co-injection with MoPax8 at the 4-cell stage, in the equatorial region of both left blastomeres. At neurula stage 18, they were fixed, transversely bisected at the level of the pronephric area and analyzed by *in situ* hybridization with the indicated probes. For each embryo, the dotted line indicates the frontier between the injected side (on the left) and the uninjected side (on the right). *Hnf1b* expression is inhibited in the pronephric area (arrow) on the injected side.

Figure S5: Characterization of the kidney field explants.

RT-qPCR analysis of *lhx1*, *pax8*, *myf5*, *sox17beta* and *cytokeratin* expression in KF explants and whole embryos. KF explants were dissected at neurula stage 18 from uninjected or injected embryos with MoC or MoPax8. The mRNAs renal markers *Pax8* and *lhx1* were enriched in the explants in contrast to the endodermal *sox17beta* and the somitic *myf5* mRNAs. Explants including the kidney field and the overlying ectoderm, the epidermal gene *cytokeratin* is also expressed. Average values from four independent experiments. * $P < 0.05$ ** $P < 0.005$, *** $P < 0.001$.

Highlights

- Pax8 is a major regulator of the first steps of pronephric development
- Pax2 is required for the expression of *clckn*, a tubule differentiation marker gene
- Pax8 is required for the expression of *hnf1b* in the kidney field
- Pax8 controls expression of the wnt pathway genes *dvl1* and *sfrp3* in the kidney field
- Pax8 is required for cell proliferation of pronephric precursors

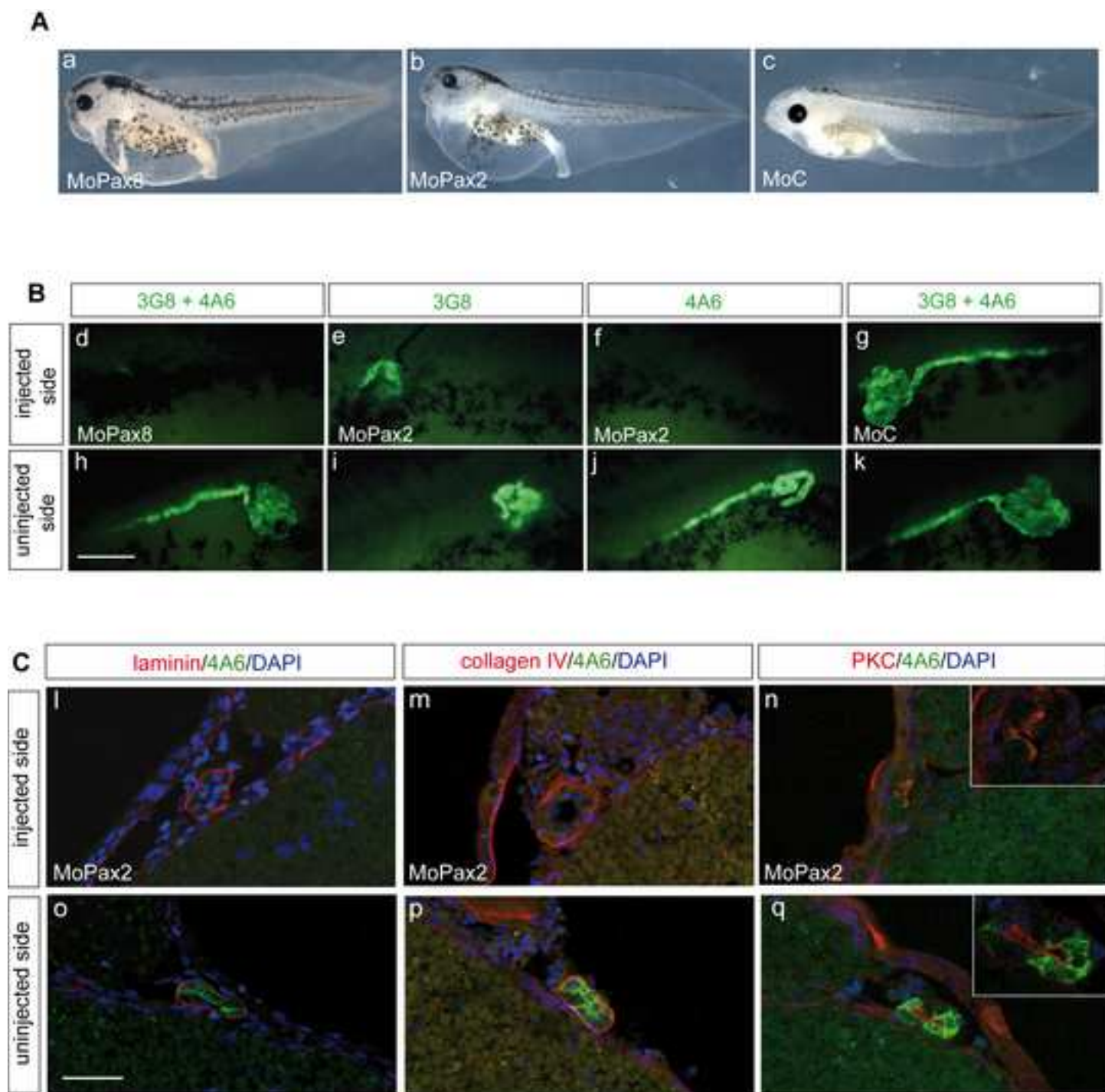


Figure 1
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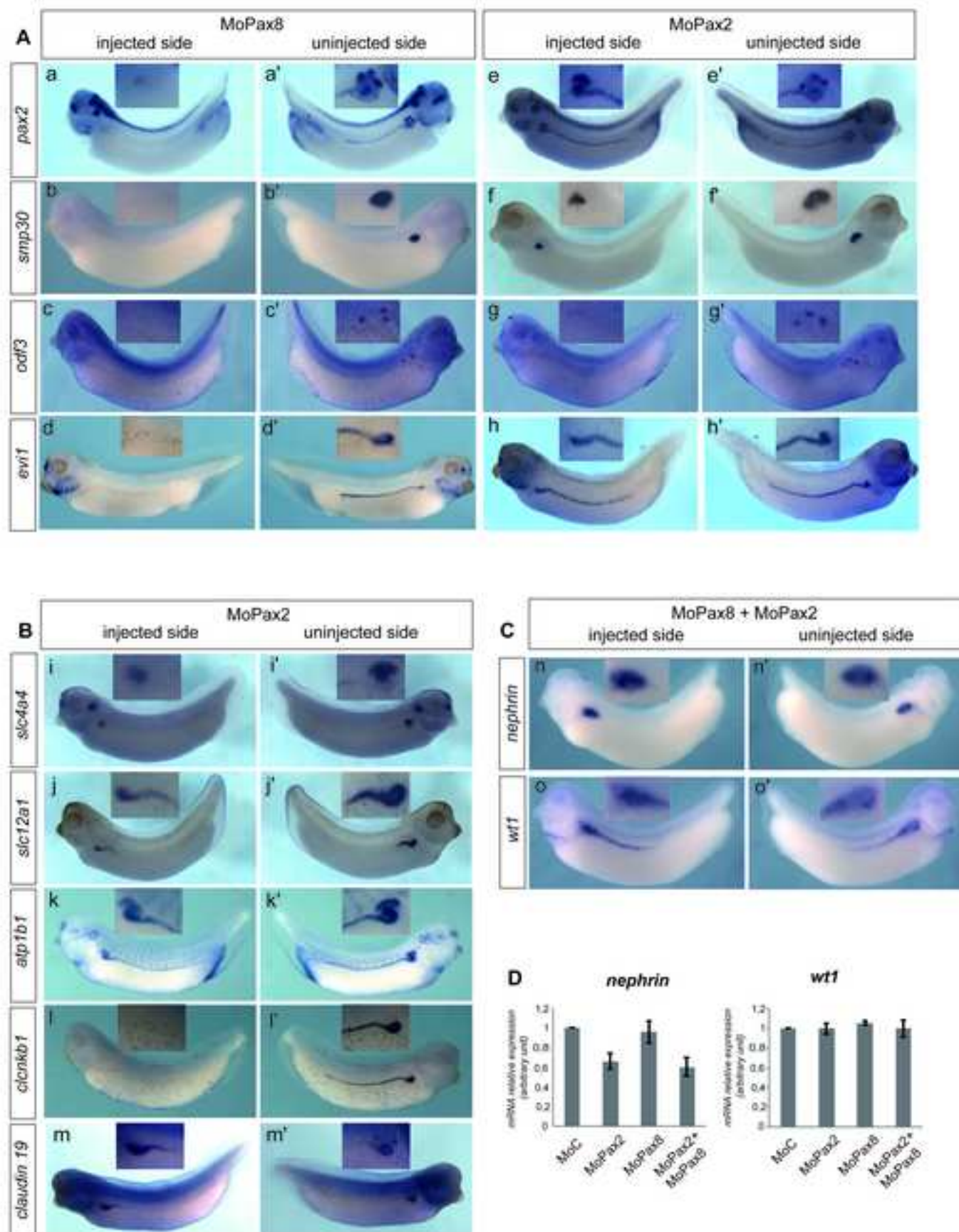


Figure 2
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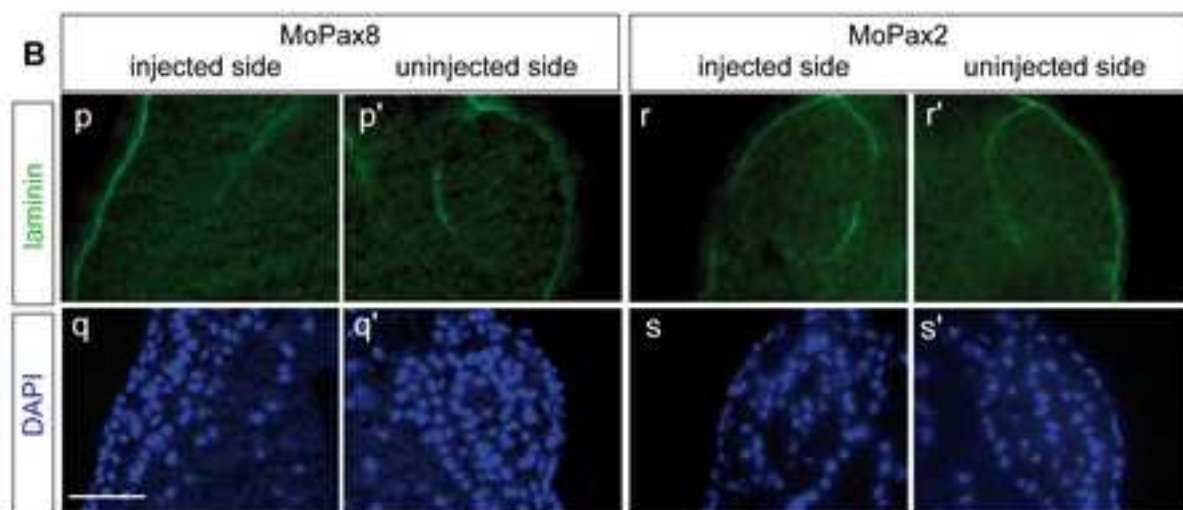
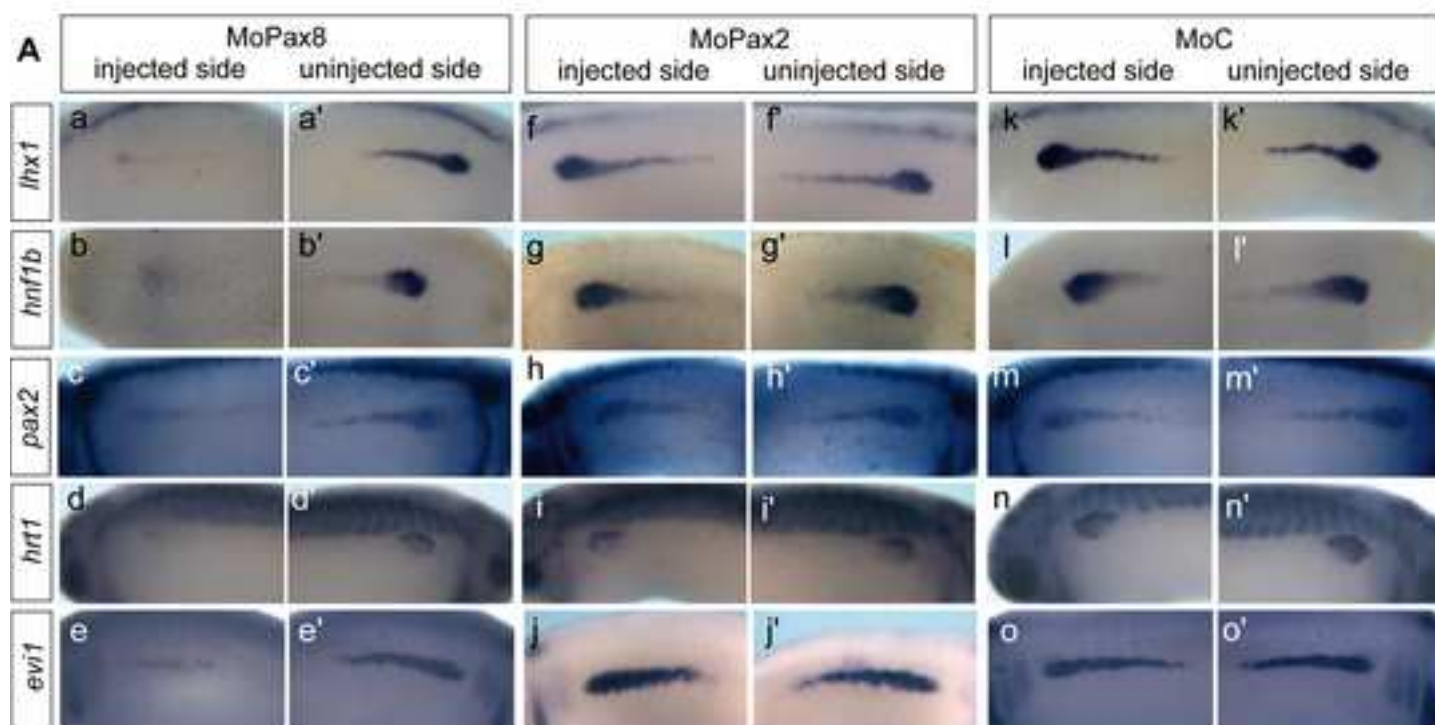


Figure 3
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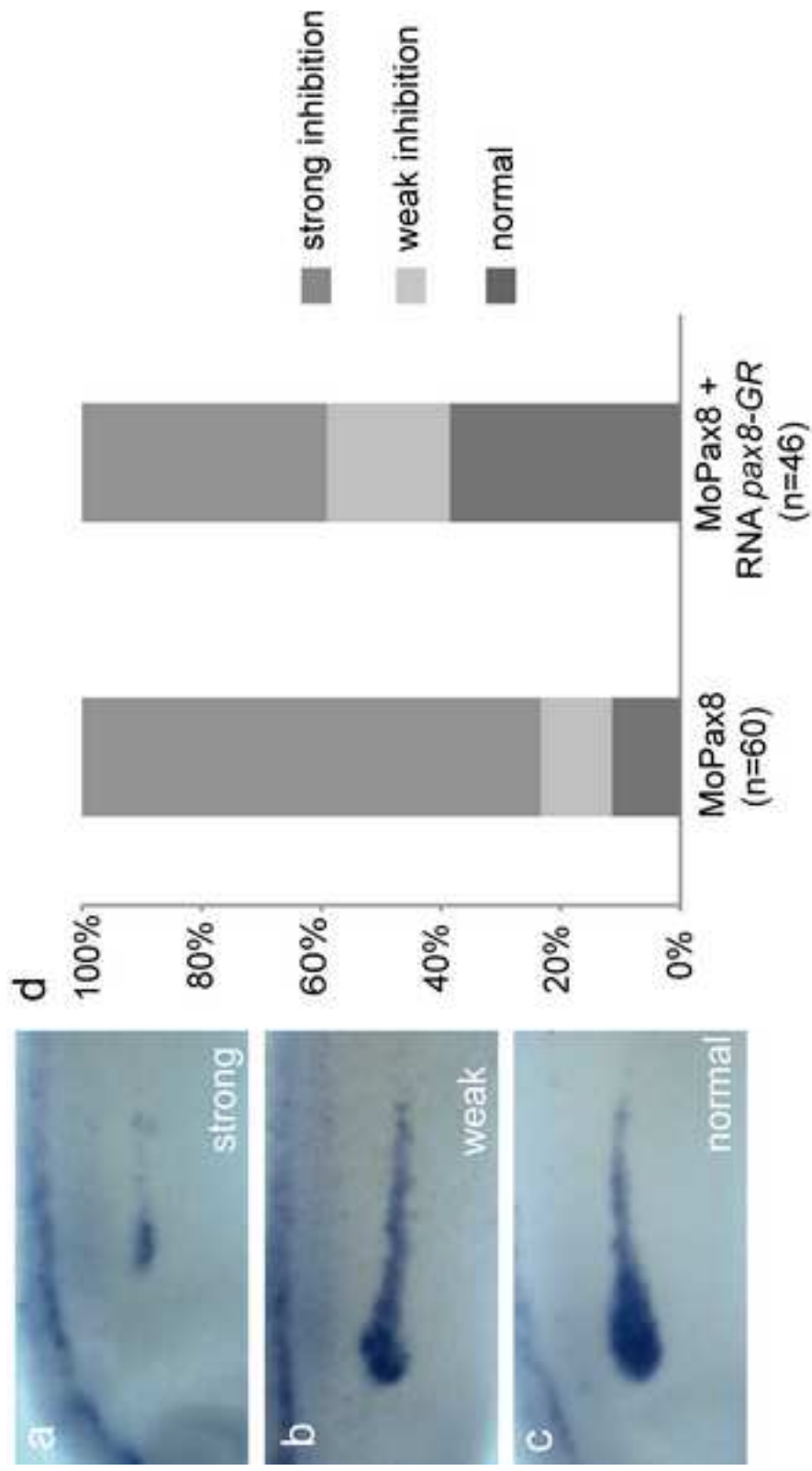


Figure 4
(color, 1.5 - column)

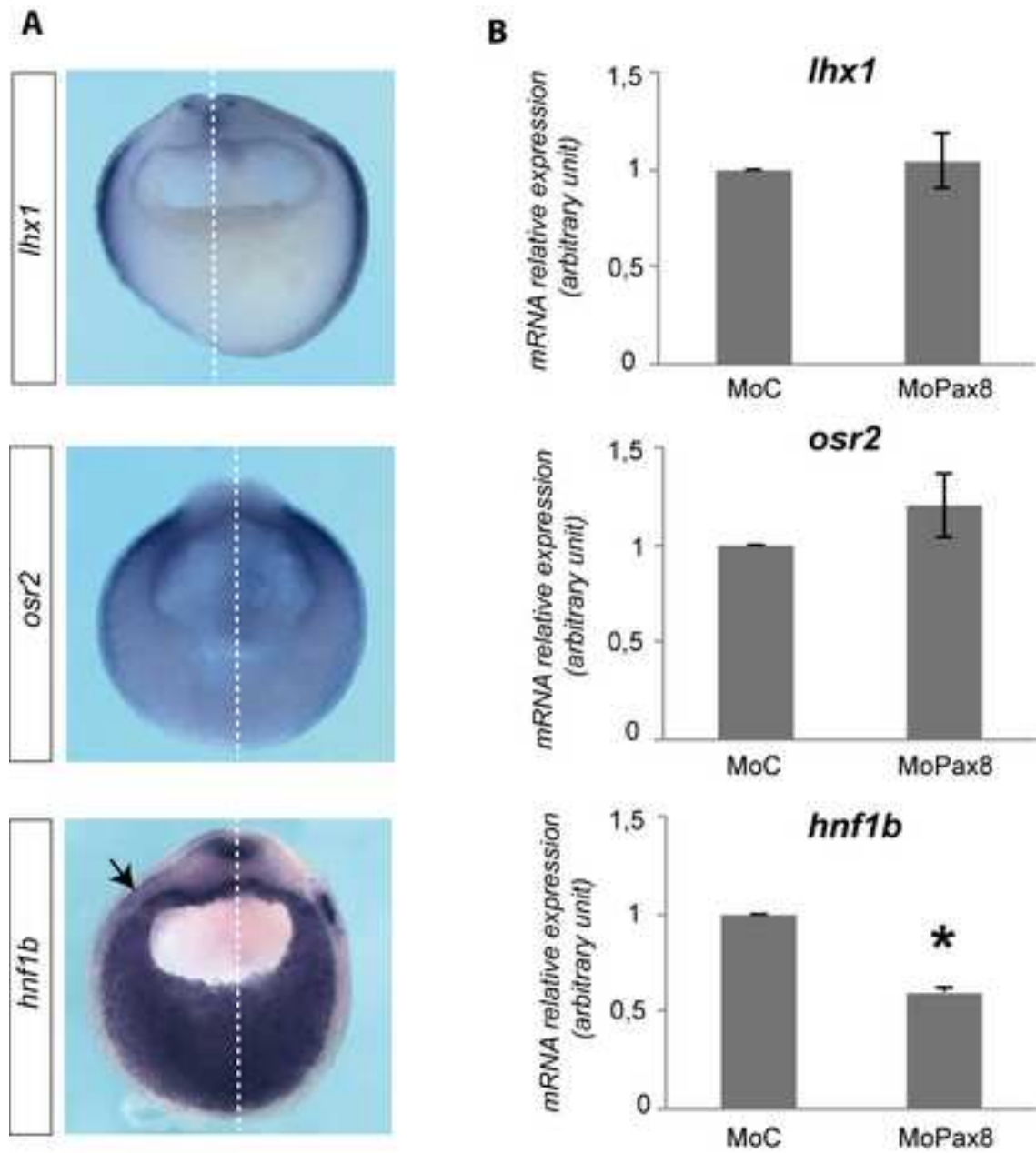


Figure 5
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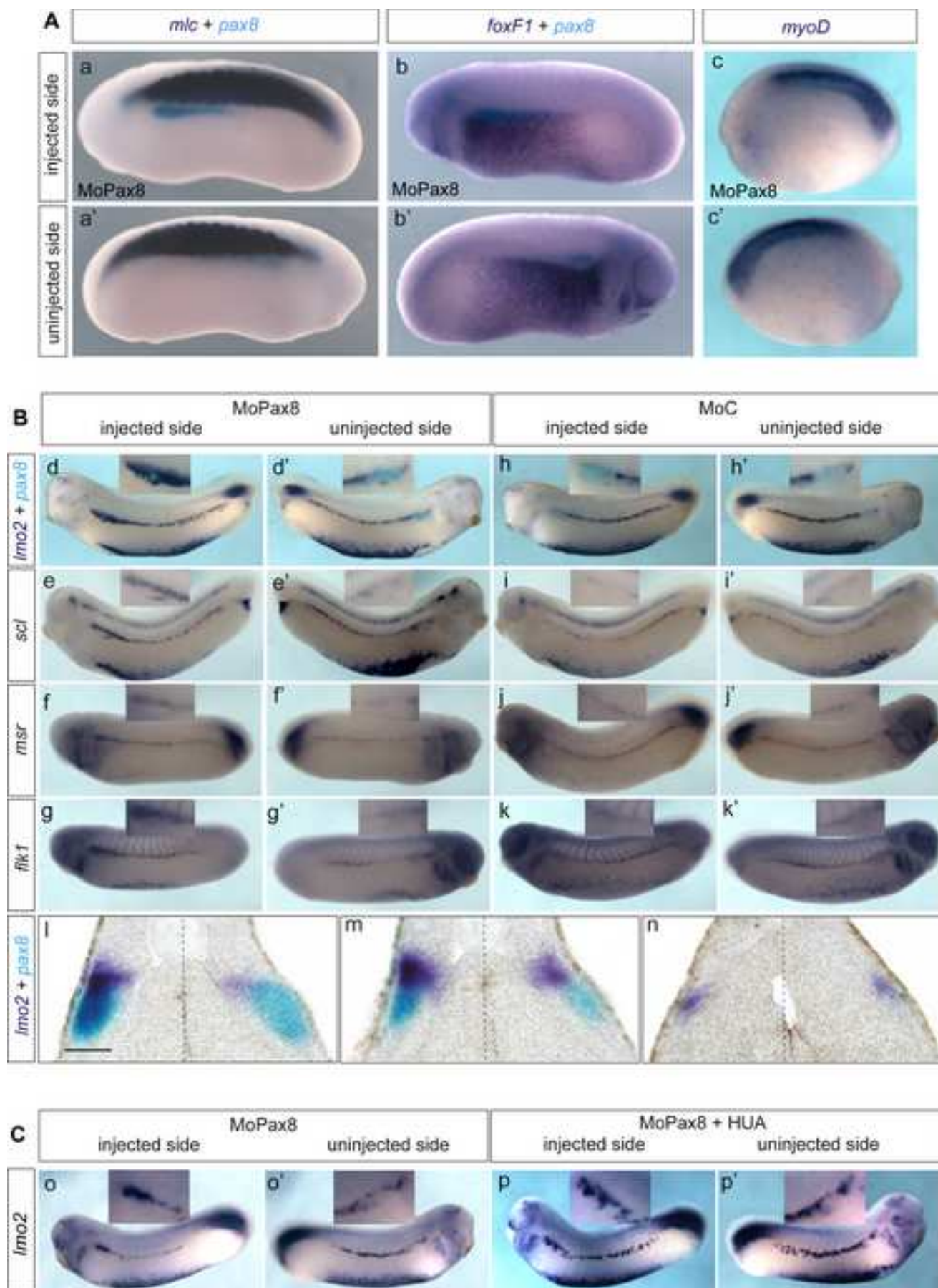


Figure 7
(color, 2-column)

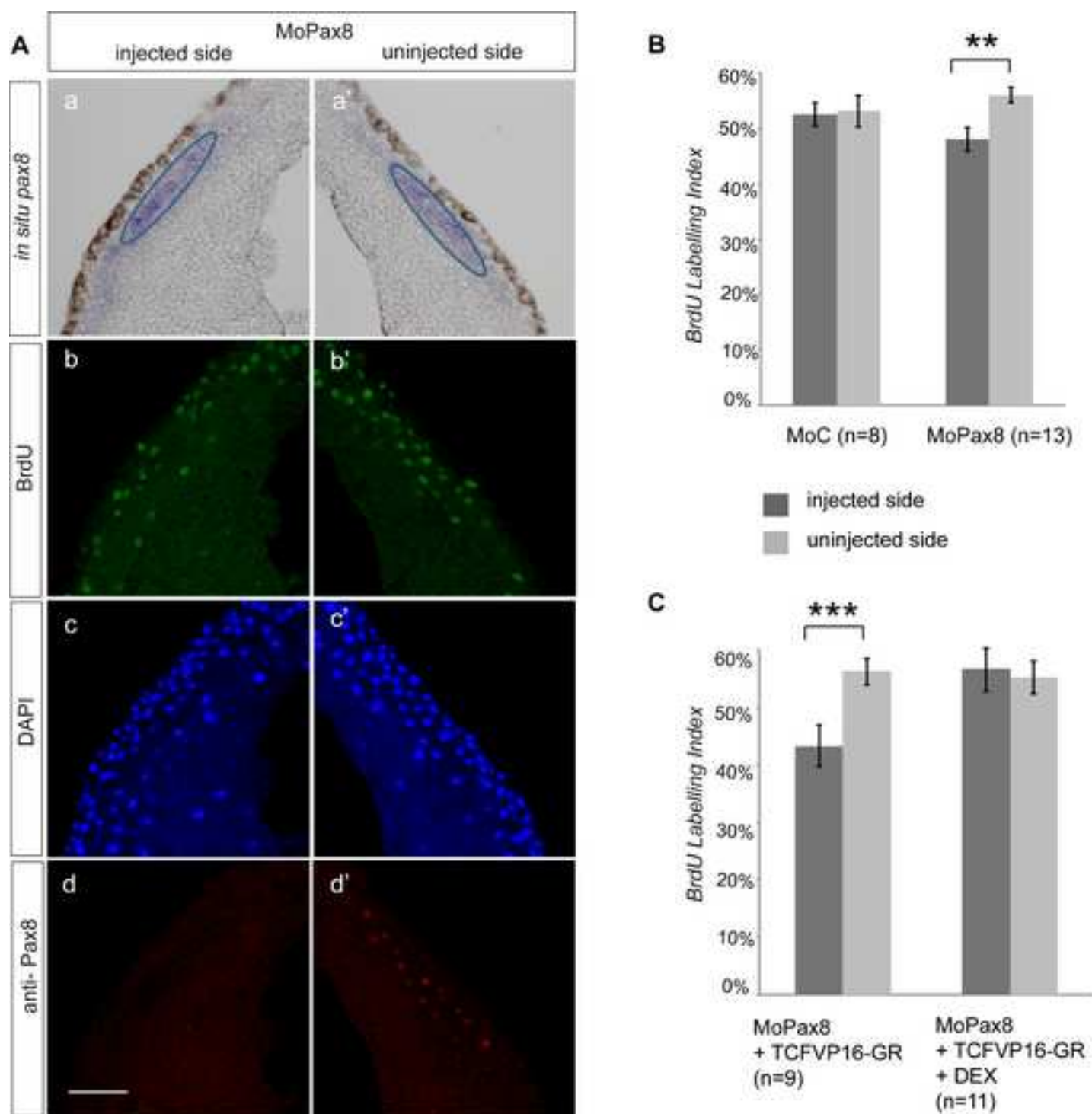


Figure 8
(color, 2-column)

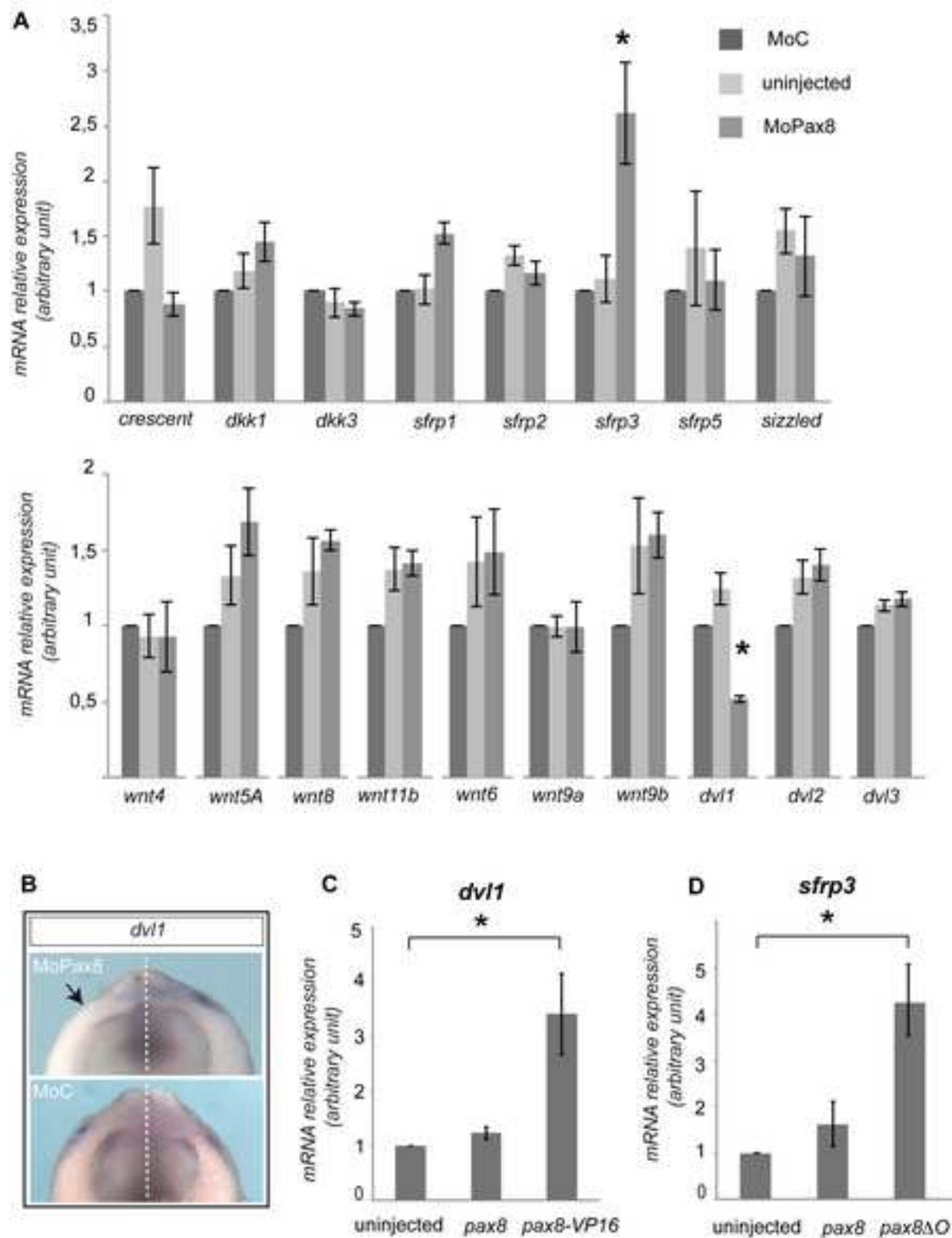


Figure 9
(color, 2-column)