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Comprehensive analysis of Ephrin ligand and receptor expression reveals exclusive domains during nephrogenesis for epha4/epha7 and efna3

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ABSTRACT Interactions between ephrins and their Eph receptors regulate a broad range of cellular processes, including attraction, repulsion, adhesion and migration, all of which play crucial roles in tissue remodeling and homeostasis. While several ephrin ligands and Eph receptors are known to be expressed in the developing kidney, their specific roles, particularly during nephrogenesis, remain poorly understood. The development of the *Xenopus* pronephros provides an accessible and relatively simple model for studying vertebrate nephrogenesis. Through a comprehensive gene expression analysis of all ephrin ligands and Eph receptors present in *Xenopus* genomes, we have identified members of the Eph-ephrin signaling pathway that may contribute to pronephric development. Among them, *efna3*, which encodes an ephrin ligand, is strongly expressed in the ventral region of the pronephric anlage and later in the intermediate and distal segments of the developing tubule. This expression pattern is strikingly complementary to that of *epha4* and *epha7*, which are expressed in the region forming the proximal tubule. This suggests a potential role for efna3-epha4/epha7 signaling in establishing a boundary between these domains during pronephros development.

KEYWORDS: Nephron, Xenopus embryo, ephrins, eph receptors, pronephros

Introduction

The kidney plays a central role in fluid filtration, solutes absorption, excretion, and blood homeostasis. In vertebrates, kidney development proceeds through three stages: pronephros, mesonephros and metanephros (Saxén and Sariola, 1987). Despite their increasing complexity, they all share the nephron as a structural and functional basic unit. While the pronephros is rudimentary and non-functional in mammals, it serves as the functional kidney in amphibian tadpoles. It is made up of three components: the glomus that ensures blood filtration, the tubule where salts and macromolecules absorption take place, and the duct that connects to the cloaca. Similar to the mammalian metanephros, the tubule is divided into proximal, intermediate and distal segments, each performing specific physiological functions (Desgrange and Cereghini, 2015; Raciti *et al.*, 2008). Given the conservation of kidney development in vertebrates, the functional *Xenopus laevis* (African clawed frog) pronephros offers a simplified model for

nephrogenesis. The development of the *Xenopus* pronephros begins at the early neurula stage in the intermediate mesoderm, with the emergence of the kidney field characterized by the overlapping expression of the key transcription factors pax8 and lhx1 (Buisson *et al.*, 2015; Carroll and Vize, 1999; Cirio *et al.*, 2011). At the early tailbud stage, cells in the somatic layer begin to segregate from the remaining intermediate mesoderm and form the pronephric anlage, which can be visualized externally as a bulge positioned ventrally to the anterior somites. These cells will give rise to the segmented tubule whereas cells in the adjacent medial layer will form the glomus. Several transcription factors including *hnf1b*, *irx* genes and *evi1/mecom* have been implicated in tubule segmentation (Alarcón *et al.*, 2008; Buisson *et al.*, 2015; Desgrange and Cereghini, 2015; Heliot *et al.*, 2013; Reggiani *et al.*, 2007; Van Campenhout *et al.*, 2006). Tubule elongation involves convergent

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extension movements (Lienkamp *et al.*, 2010). Live imaging revealed that two opposing morphogenetic cell movements shape the primary loop which continues to form the intermediate segment (Lienkamp *et al.*, 2010). Inversin and Frizzled-8 control the dorsal to ventral movement of the proximal cells while signaling pathways for the posterior to anterior movement in the distal pronephros remain unclear (Lienkamp *et al.*, 2010). As the pronephric tubule extends toward the posterior, it meets the anterior migrating rectal diverticulum allowing connection to the cloaca. A functional pronephric kidney is complete at tadpole stage 35/36, two days after fertilization.

Eph-ephrin signaling is essential for short-distance cell-to-cell interactions, regulating cellular behaviors such as attraction, repulsion, adhesion or migration. It plays major roles in tissue remodeling and maintenance in both the embryo and adult (Kania and Klein, 2016). The signaling involves the binding of transmembrane Eph receptors to membrane-bound ephrin ligands (Taylor *et al.*, 2017). In addition to classical forward signaling through tyrosine kinase Eph receptors *via* oligomerization, autophosphorylation and phosphorylation of intracellular substrates, reverse signaling can also occur in cells expressing ephrin ligands (Weiss and Kispert, 2016). Two classes of ephrin ligands have been described. Class A ephrins have an extracellular globular domain tethered to the membrane with a glycosylphosphatidylinositol (GPI) anchor, while class B ephrins are transmembrane proteins with a short intracellular domain. EphA receptors generally interact with class A ephrins, while EphB receptors interact with class B ephrins, although some exceptions exist (e.g. ephrinB2 also binds to EphA4 and A7 (Weiss and Kispert, 2016)).

A total of fourteen human Eph receptors are encoded by *EPHA1-8*, *EPHA10*, *EPHB1-4* and *EPHB6* genes, while eight ephrin ligands are encoded by *EFNA1-5* and *EFNB1-3* (Taylor *et al.*, 2017*)*. Every human gene encoding ephrin ligands has an ortholog in *Xenopus laevis* and *Xenopus tropicalis* genomes, as do most of the Eph receptors, with the exception of *EPHA1* and *EPHB6* (https://www.xenbase.org/xenbase/, (Fisher *et al.*, 2023)). While *X. tropicalis* is a diploid species, *X. laevis* is allotetraploid. Nine sets of homeologous chromosomes have been identified in *X. laevis*, eight corresponding to the chromosomes 1-8 in *X. tropicalis*, while the last set corresponds to a fusion of chromosomes 9 and 10 of *X. tropicalis*. Every set of *X. laevis* homeologous chromosomes has a long (L) and a short (S) chromosome (Matsuda *et al.*, 2015). The homeologous .L subgenome generally appear to have retained the ancestral condition, while the .S subgenome evolved asymmetrically, being more disrupted by deletion and rearrangement (Session *et al.*, 2016). As a consequence, every *Xenopus* ephrin or eph receptor ortholog has a .L homeolog in *X. laevis*, while a .S homeolog can be missing as for example for *efnb1* for which only *efnb1.L* is present (https://www.xenbase.org/xenbase, (Fisher *et al.*, 2023)).

Eph-ephrin signaling is involved in key processes during *Xenopus* development including gastrulation, retinal progenitor migration in the eye field, hindbrain formation, angiogenesis, development of the pronephros and otic vesicle (Fagotto, 2020; Helbling *et al.*, 2000; Lee *et al.*, 2006; Sun *et al.*, 2018; Wang *et al.*, 2016; Wang *et al.*, 2020). This has greatly benefited from the experimental advantages offered by the *Xenopus* embryo for analyzing Eph–ephrin signaling at a mechanistic level (Hwang and Daar, 2017), as exemplified by studies on the formation of the ecto–meso-

dermal boundary at Brachet's cleft during gastrulation involving Epha4/Ephb4 receptors and Efnb2/Efnb3 ligands (Barua and Winklbauer, 2022; Fagotto, 2020).Two Eph receptors are known to be expressed in the developing pronephros. *EphA7*, specifically expressed in the proximal tubule at tadpole stages, regulates tubule cell adhesion by modulating the tight junction protein claudin6. In cultured cells, ephA7 binds and phosphorylates claudin6 (CLDN6), reducing its cell surface distribution (Sun *et al.*, 2018). Interestingly, a soluble form of ephA7 antagonizes ephA7 full length (EphA7-FL), stabilizing Claudin6 (CLDN6) at the membrane. Nicalin, a Nicastrin-like protein (Wang and Wang, 2021) binds the soluble form of epHA7, limiting its secretion and preventing the formation of EphA7 complex. *EphA4* is also expressed in the proximal part of the developing pronephric tubule, but its function has not been investigated (Smith *et al.*, 1997; Van Campenhout *et al.*, 2006). Whether these Eph receptors interact with an ephrin ligand in the developing pronephros, and if so, which ephrin, remains unknown.

Eph-ephrin signaling is also crucial in kidney and urinary tract development in mammals (Weiss and Kispert, 2016). In mice, EphA4 and EphA7 are essential for nephric duct insertion to the cloaca (Weiss *et al.*, 2014). EfnB1 is expressed during formation of the slit diaphragm of the glomerular podocyte (Hashimoto *et al.*, 2007) and Nephrin/EfnB1/NHERF2 complex can bind ezrin and actin leading to podocyte healing (Fukusumi *et al.*, 2021).

Given the complexity of the families of ephrin ligands and Eph receptors, it is important to have a comprehensive view of which of their members are expressed during renal development. As outlined above, the developing *Xenopus* pronephros is an accessible and relatively simple model of vertebrate nephrogenesis. We have therefore analyzed the expression of every gene encoding members of the ephrin/Eph families to identify those whose expression might prompt potential functions during the development of the nephron. *Efna3* encoding an ephrin ligand has a ventral expression domain in the developing pronephros that is complementary to a dorsal domain where genes encoding receptors epha4 and a7 are expressed, suggesting a role in the maintenance of a boundary between these domains.

Results and Discussion

Expression of ephrins and Eph receptors at the early tadpole stage (St35/36) in the pronephros

In an initial attempt to identify *eph* and *efn* family genes expressed in the pronephric nephron, we analyzed the expression of each member using whole-mount *in situ* hybridization (WISH) at the early tadpole stage (st35/36), when nephrogenesis is nearly completed (Fig. 1, Table 1). We therefore cloned cDNAs corresponding to all *eph* and *efn* family genes to synthesize riboprobes. Primer sequences used for RT-PCR were designed based on the .L homeologs (Table 2). Both .L and .S homeologs are present in the genomic data as gene models for each member of the *eph* and *efn* gene families, except for *efnb1* where only *efnb1.L* is present (www.xenbase.org, (Fisher *et al.*, 2023)). Some .S homeologs are expressed in oocytes or embryos at very low levels compared to their .L counterparts (*efna5*, *epha8*), or not at all (*efna3*, *epha10*) (Session *et al.*, 2016). For other *eph* and *efn* genes, the .L and .S homeologs show high sequence similarity, making it likely that the riboprobes detect the expression of both homeologs in these cases.

TABLE 1

EPHRIN AND EPH RECEPTOR-ENCODING GENE EXPRESSION IN THE PRONEPHROS IN EARLY TADPOLES (ST35/36)

Gene	Pronephric expression	Comments[a]	Reference[b]
efna1	NO	Vascular expression	
efna2	NO		
efna3	YES	IT+DT	
efna4	NO		
efna5	NO	vascular expression	
efnb1	NO	vascular expression	(Costa *et al.*, 2003; Giudetti *et al.*, 2014)
efnb2	YES	Glomus	(Helbling *et al.*, 2000; Kirmizitas *et al.*, 2017)
efnb3	NO		
epha2	NO	vascular expression	
epha3	NO	vascular expression	
epha4	YES	PT1+PT2+PT3	(Barnett *et al.*, 2012; Rothe *et al.*, 2017; Smith *et al.*, 1997; Winning and Sargent, 1994; Xu *et al.*, 1995)
epha5	NO	vascular expression	
epha6	NO	vascular expression	
epha7	YES	PT1 +PT2+PT3	(Wang *et al.*, 2016)
epha8	NO	vascular expression	
epha10	NO	Migrating hypaxial myoblasts	
ephb1	NO	vascular expression	(Helbling *et al.*, 2000; Smith *et al.*, 1997)
ephb2	YES	Prominent in IT1	(Helbling *et al.*, 2000)
ephb3	YES	Exclusively in PT3	(Helbling *et al.*, 2000)
ephb4	NO	vascular expression	(Cheong *et al.*, 2006; Helbling *et al.*, 2000)

Genes expressed in the pronephros appear in bold. [a]Additional comments about pronephric or mesodermal expression. [b]References about previously published gene expression data at early tadpole stage when available. IT1,2: intermediate tubule segments 1 or 2. PT1,2,3: proximal tubule segments 1, 2 or 3.

Many of *eph* and *efn* family genes are expressed at high levels in the head region, particularly in the developing brain, neurogenic placodes, visceral skeleton and eye. Since we needed to detect lower expression levels in the pronephric nephron, we had to overstain the head in several instances. Only one gene encoding class A ephrin ligands is expressed in the pronephros: *efna3* along with *efnb2* among class B ligands (Fig. 1). *Efna3* expression is remarkably prominent in the intermediate and distal segments of the pronephric tubule and has never been described previously. It will be studied in detail in the next paragraph (Figs. 2, 3). *Efnb2* is expressed at the level of the developing glomus in a region containing differentiating podocytes (Fig. S2). At stage 35/36, some genes encoding subfamily A of Eph receptors are detected in pronephros area: *epha4*, and *epha7*. As expected, *epha4* and *epha7* are expressed in the proximal part of the pronephric tubule. Two ephb genes are expressed at stage 35/36 in the pronephros: *ephb2* and *ephb3*. The former is detected in intermediate and distal segments and poorly expressed in proximal region, while the latter has a more restricted expression in the PT3 region of the proximal tubule (Figs. 1, 4).

Some genes encoding ephrin ligands or Eph receptors may be expressed only transiently in the developing pronephros, making them undetectable at stage 35/36. To address this possibility, we performed additional WISH analyses at earlier stages (Figs. S1−4) to complement the data shown in Fig. 1. Some members of the ephrin−Eph receptor family are expressed in the pronephric region but appear to be localized specifically to the developing nephron vasculature. This is the case of *efna1*, *efna5* (Fig. S1), *efnb1* (Fig. S2), *epha2, epha3, epha5, epha6* (Fig. S3) and *epha8* (Fig. S4). Vascular expression of *ephb4* has been previously documented (Helbling *et al.*, 2000). Additionally, while *epha10* is not detected in the pronephros at stage 35/36, instead being expressed in migrating hypaxial myoblasts and the eye, it is present at low levels in the developing pronephros at tailbud stages 25, 28, and 32 (Fig. S4).

Expression of *efna3* and *epha4/a7* at tailbud stage map complementary domains of the pronephric anlage

The Eph-ephrin signaling pathway plays a crucial role in tissue boundary formation. EfnA3 and EphA4 have been often observed to be distinctly expressed in certain tissues where they mediate

TABLE 2

SEQUENCE OF PRIMERS USED FOR CDNA CLONING OF THE DIFFERENT MEMBERS OF *EFN* AND *EPH* GENE MEMBERS WITH THE CORRESPONDING ACCESSION NUMBERS

Gene	Forward	Reverse	Accession
efna1.L	5'- ACGAGACCTGCAAGCCCAT-3'	5'- ACAGGGCTAGGTGGGTTAAAGTC-3'	NM_001095329
efna2.L	5'- TCTGTGGCACTGCCAGAAGT -3'	5'- TGGTGAAGATAGGCTCTGGAGACT-3'	XBmRNA2762
efna3.L	5'- GCCAACAGGCACTCGGTCTA-3'	5'- CTCTGCCATATCCCGAGGG -3'	XM_018231573
efna4.L	5'- CCTGAAGCTGCGGGTGTCT -3'	5'- GAGCCACTTGGGAATGAGCA -3'	NM_001127882
efna5.L	5'-CCACACCTTCTATTACATGCCTACA-3'	5'-ATGGGCGCAGGCACC-3'	XM_018264443
efnb1.L	5'- CGGCGCTGGGAAAGAACT-3'	5'- TGATGTCACTGGGTTCGCTG-3'	NM_001087479
efnb2.L	5'- TGGGATACAGTGCATGTGAGC-3'	5'- TGCGTTTTGGAGTGGCTAATG-3'	NM_001086485
efnb3.L	5'- TTAATCAGCATGTTTTCCCGG-3'	5'- AGTGAGGGCAAAAGGCACC-3'	NM_001085842
epha2.L	5'-GAGAACTTGGCTGGCTCACC-3'	5'-GGCCTCTGGCGCACACT-3'	NM_001087611
epha3.L	5'-ACGAGACTGCAACAGTATCCCTC-3'	5'-GAGCCGCCTGGTTCGTAGT-3'	XBmRNA11892
epha4.L	5'- TGCACTATAGCCCCCAGCA-3'	5'- CCGACCATCATTCTTCCGAA-3'	NM_001085992.1
epha5.L	5'- GGGAAGAAATTGGAGAGGTAGATG -3'	5'- AGCCATTCACCTTCTGCACTG - 3'	XM_031903379.1
epha6.L	5'- AGCTGTCCATGAGTTTGCCAA -3'	5'- CCACGTCCAGCGTTGTGA – 3'	XBmRNA11875
epha7.L	5'-TCAAGTCTTGGAGCCCAACC-3'	5'-GGGCAACAAGGTCCACAATG-3'	XM_031902278
epha8.L	5'- CCCCCAACAGATGAGCCTAG-3'	5'- TGTTAAACTGCCCATCCCGA - 3'	XM_018241840
epha10.L	5'- TCCACCTAATGGATGGGAGG -3'	5'- TGACCTGACTGGCCCTGAA – 3'	XM_018246715
ephb1.L	5'-CACGGCAGTTGGGTTTGACT-3'	5'-CCCATCGTTTTGCCGTAGAA-3'	XM_018265884
ephb2.L	5'-GGGTACACGGAGAAACAGCG-3'	5'-GGAAGCGCCAATCAAACTTC-3'	XM.0182241849
ephb3.L	5'- TGGCGGTAGTTCGGTCTGTT-3'	5' ACGTGGAGCTGAGGGCAC-3'	XM_018262082
ephb4.L	5'- CTCTTCGCATAGGGCCCC-3'	5'- CTCAAGCAGATGGGCCTCAT-3'	NM_001136172

Note that due to sequence conservation between the two homeologs, probes generated from the amplified sequences may detect expression of both.
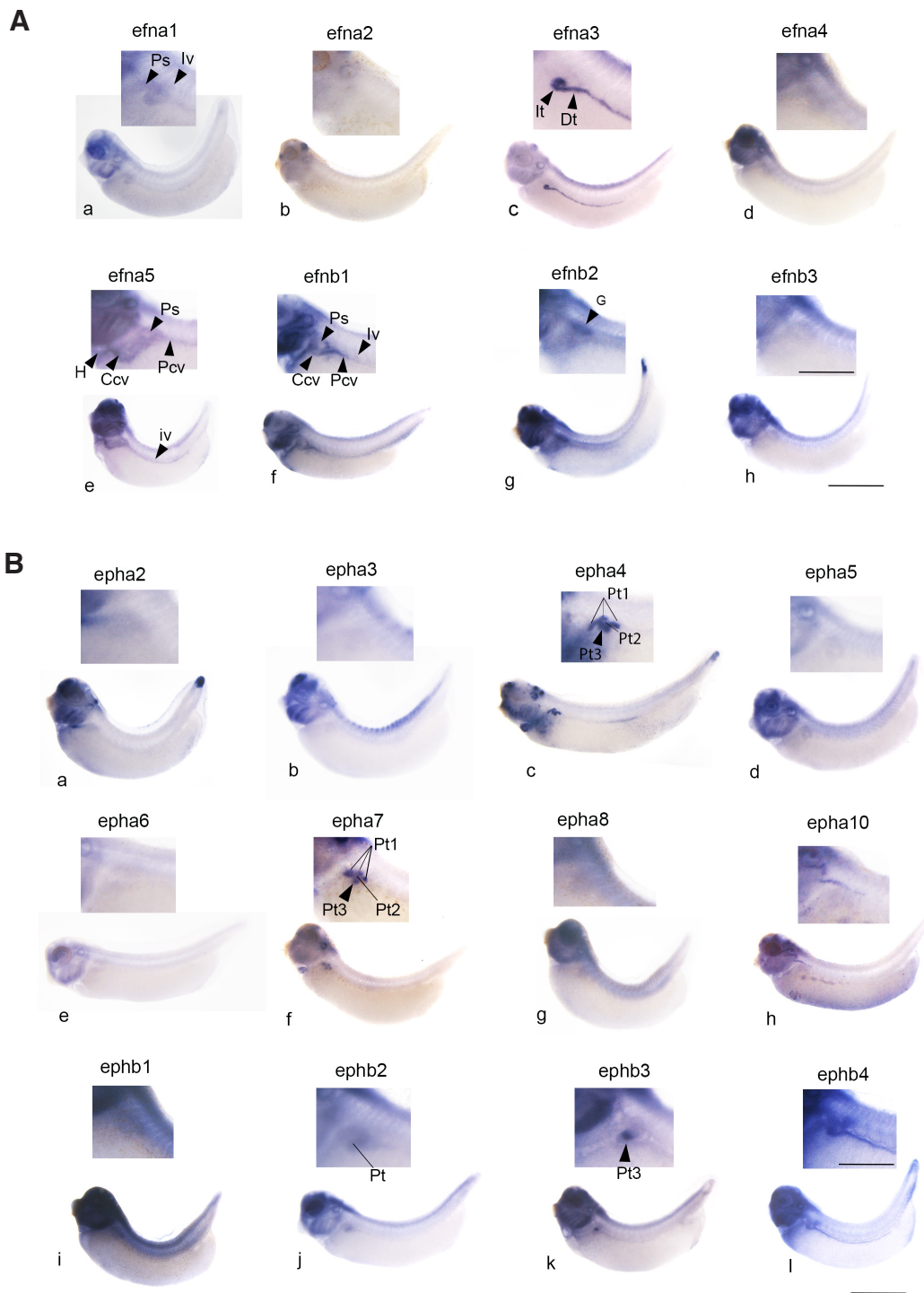
Fig. 1. Expression of genes encoding the members of the Eph-ephrin signaling components in the pronephros at early tadpole stage 35/36. The enlarged region corresponding to the pronephric region is shown with a general view of the tadpole. **(A)** Genes encoding ephrin ligands. Expression of *efna3* and *b2* is detected in the developing pronephros; *efna3* is expressed in the intermediate and distal segments of the tubule (It, Dt). Expression of *efnb2* is observed in the glomus region (G). Expression of *efna5* and *b1* is detected at the level of the vessels associated with the pronephros: in the pronephric sinus (Ps), the posterior cardinal vein (Pcv), the common cardinal vein (Ccv) and the intersomitic veins (Iv). *Efna5* expression is also observed in the heart (H). *Efna1* seems to be weakly expressed in the pronephric sinus and intersomitic vein. Scale bars: 0.8 mm for whole embryo and 0.3 mm for embryo magnification. **(B)** Genes encoding Eph receptors. Expression of *epha4*, *a7*, *b3 and b2* are clearly detected in the pronephros. *Epha4 and EphA7* are expressed in the PT1, PT2 and PT3 segments of the proximal tubule (Pt1, three lines, Pt2, line and Pt3 arrowhead) while *ephb3* is detected only in PT3 (arrowhead). *Ephb2* is detected in the proximal, intermediate and the first distal segment of the tubule. No pronephric expression of *epha2*, *a3*, *a5*, *a6*, *a8*, *a10* and *b1* is observed. Expression of *ephb4* is detected in the vessels associated with the pronephros. Pt, It, Dt: proximal, intermediate and distal tubule. Scale bars: 1.7 mm for whole embryo and 0.5 mm for embryo magnification.

A

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CLUSTAL 2.1 multiple sequence alignment


ephrin-A3b_[Danio_rerio]          ------MALYALCLFLLT-LTCTNFALVTAARHAVHWNSSNILLRKEGYT
ephrin-A3_[Oryzias_latipes]       ------MALAAPSLWVLTSLTWVTVLQVSANRHSVYWNSSNIHLRREGFT
ephrin-A3a_[Danio_rerio]          ------MALAAVSLYLLALAWTNLRPVHGNNRHAVYWNSSNIHLRREGYT
ephrin-A3_[Homo_sapiens]          MAAAPLLLLLLLVPVPLLPLLAQGPGGALGNRHAVYWNSSNQHLRREGYT
ephrin-A3_[Macaca_mulatta]        MAAAPLLLLLLLVPVPLLPLLAQGPGGALGNRHAVYWNSSNQHLRREGYT
ephrin-A3_[Mus_musculus]          MAAAPLLLLLLLVPVPLLPLLAQGPGGALGNRHAVYWNSSNQHLRREGYT
ephrin-A3L_[Xenopus_laevis]       ---------MAVVFVLLFPLLFLFPR-SGANRHSVYWNSSNYHLRRDGYT
ephrin-A3_[Xenopus_tropicalis]    ---------MAGVFVLLFPLLSLFPR-SGANRHSVYWNSSNYHLRRDGYT
                                           *            **:*:***** **::*:*


ephrin-A3b_[Danio_rerio]          LQVNVNDYLDIYCPHYNSSQ---------RGIAEQYVLYMVSYRGYRTCD
ephrin-A3_[Oryzias_latipes]       MQVNVNDYLDIYCPHYNDSQR-------MVGTGEQYVLYMVSYRGYRNCD
ephrin-A3a_[Danio_rerio]          VQVSVNDYLDIYCPHYNQSQRGS----LERGVAEQYVLYMVSYRGYRTCD
ephrin-A3_[Homo_sapiens]          VQVNVNDYLDIYCPHYNSSGVGPGAGPGPGGGAEQYVLYMVSRNGYRTCN
ephrin-A3_[Macaca_mulatta]        VQVNVNDYLDIYCPHYNSSGVGPGAGPGPGGGAEQYVLYMVSRNGYRTCN
ephrin-A3_[Mus_musculus]          VQVNVNDYLDIYCPHYNSS-------GPGGGAEQYVLYMVNLSGYRTCN
ephrin-A3L_[Xenopus_laevis]       VQVDVNDYLDIYCPHYNES-------VVEHKMEQYILYMVSYEGYRTCN
ephrin-A3_[Xenopus_tropicalis]    VQVNVNDYLDIYCPHYNET-------PGEHKMEQYILYMVSYEGYRTCN
                                  :**.************** .:          ***:****. ***.*:


ephrin-A3b_[Danio_rerio]          PQLGFKRWECNRPHAPHAPIKFSEKFQRYSAFSLGYEFHVGQEYYYISTP
ephrin-A3_[Oryzias_latipes]       PQMGFKRWECNRPHAPHAPIKFSEKFQRYSAFSLGYEFHVGHEYYYISTP
ephrin-A3a_[Danio_rerio]          PQMGFKRWECNRPHAPHAPIKFSEKFQRYSAFSLGYEFNVGHEYYYISTP
ephrin-A3_[Homo_sapiens]          ASQGFKRWECNRPHAPHSPIKFSEKFQRYSAFSLGYEFHAGHEYYYISTP
ephrin-A3_[Macaca_mulatta]        ASQGFKRWECNRPHAPHSPIKFSEKFQRYSAFSLGYEFHAGHEYYYISTP
ephrin-A3_[Mus_musculus]          ASQGSKRWECNRPHAPHSPIKFSEKFQRYSAFSLGYEFHAGQEYYYISTP
ephrin-A3L_[Xenopus_laevis]       ISQGFKRWECNRPHVPHSPIKFSEKFQRYSAFSLGYEFHAGHEYYYISTP
ephrin-A3_[Xenopus_tropicalis]    ISQGFKRWECNRPHAPHSPIKFSEKFQRYSAFSLGYEFHAGHEYYYISTP
                                   . * ******* *..*:****************:.*:********


ephrin-A3b_[Danio_rerio]          THHHGRSCLRLRVYVCCSTASDSDDEPQPTEPDYTLRPNIKIDDLDDYDN
ephrin-A3_[Oryzias_latipes]       THHHGRSCLRLRVYVCCSTASDTDDEPEPTEPDYTLRPGLKIDDIDEFN-
ephrin-A3a_[Danio_rerio]          IHHHGHSCLRLRVYVCCSTVSNSDDDSSP--PDYTVRP--KLHDIDEFN-
ephrin-A3_[Homo_sapiens]          THNLHWKCLRMKVFVCCASTSHSGEKPVPTLPQFTMGPNVKINVLEDFEG
ephrin-A3_[Macaca_mulatta]        THNLHWKCLRMKVFVCCASTSHSGEKPVPTLPQFTMGPNVKINVLEDFEG
ephrin-A3_[Mus_musculus]          THNLHWKCLRMKVFVCCAS------------------------KDFEG
ephrin-A3L_[Xenopus_laevis]       THNHRRSCLKMKVFVCCASTSHSGEKHSPTLPQFTIGPEVN---IEDLDN
ephrin-A3_[Xenopus_tropicalis]    THNHRRSCLKMKVFVCCASTAHSGEKHSPTLPQFTIGPEVK---IEDLDH
                                  *:  .**:::*:***::                      .: :


ephrin-A3b_[Danio_rerio]          --PEVPKLEKSISGSSPSRDRLLLTVASLLLIALSVS-
ephrin-A3_[Oryzias_latipes]       --PFIPKLEKSVSGSSPSRDRLLLTVTMLFLGALFIS-
ephrin-A3a_[Danio_rerio]          --PEIPKLEKSVSGSSPSGDRLLITVATLLLAALLVS-
ephrin-A3_[Homo_sapiens]          ENPQVPKLEKSISGTSPKREHLPLAVGIAFFLMTFLAS
ephrin-A3_[Macaca_mulatta]        ENPQVPKLEKSISGTSPKREHLPLAVGIAFFLMTFLAS
ephrin-A3_[Mus_musculus]          ENPQVPKLEKSISGTSPKREHLPLAVGIAFFLMTLLAS
ephrin-A3L_[Xenopus_laevis]       FNPEIPKLEKSVSGSSPKRKHLHLTVAVCLLIMTLLAS
ephrin-A3_[Xenopus_tropicalis]    FNPEIPKLEKSVSGSSPKREHLHLTVAFCLLIMALLAS
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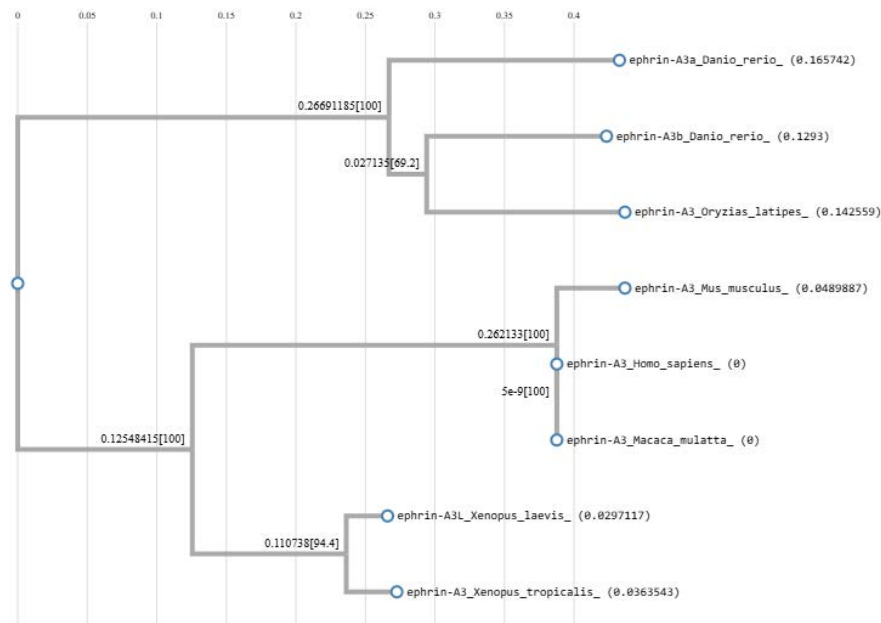
B

Fig. 2. Conservation of *Xenopus efna3* in vertebrates. (A) Multiple alignment of vertebrate *efna3* protein sequences. **(B)** Phylogenetic tree. List of accession numbers used for sequences analysis is shown in the Materials and Methods section.
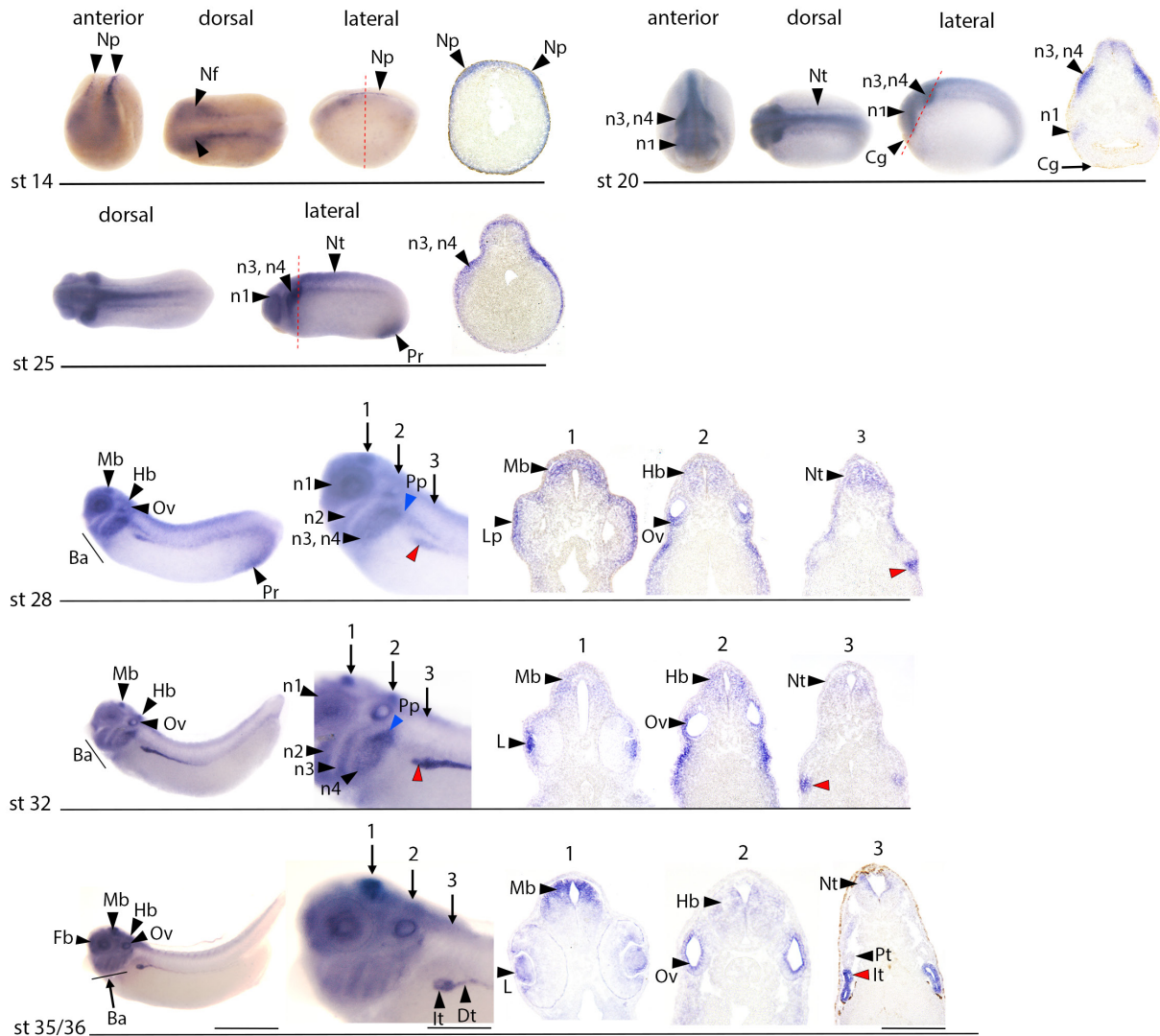
Fig. 3. Expression of *efna3* at stages of pronephric development. WISH analysis at stages ranging from early neurula stage 14 (appearance of the kidney field) to early tadpole stage 35/36 (functional pronephros). Anterior, dorsal and lateral views are shown at neurula stages (st14 and 20). Lateral and dorsal views are shown at tailbud stage 25. Only lateral views are shown for tailbud stages 28 and 32, and early tadpole (st35/36). At stage 14, 20 and 25, images of sectioned embryos are shown with red dotted lines, indicating the corresponding levels on whole mounts. For convenience, these levels are just indicated with arrows 1, 2, and 3 at stage 28, 32 and 35/36 (note that the sections were not made from the whole mount specimen shown). *Efna3* starts to be detected in the developing tubule from the stage 28. It is detected prior to stage 28 in the developing nervous system and migrating cephalic neural crest cells. *Efna3* is expressed at stage 14 in the primary neuronal precursors (Np), and weakly in the anterior neural folds (Nf). Neural expression expands at st20 to the closing neural tube (Nt). It is strong in the emerging first (n1) and third/fourth (n3, n4) branchial arches neural crest streams. Cg: cement gland. At stage 25, *efna3* expression is strong in branchial neural crest streams populating the first arch around the eye rudiment (n1), as well as the third and fourth arches (n3, n4). It is also detected in the dorsal neural tube (Nt). *Efna3* is also expressed in the proctodeal region (Pr). At stage 28, *efna3* is now expressed in the ventral region of the pronephric anlage (red arrowhead). It is detected in the dorsal region of the midbrain (Mb) and in the hindbrain (Hb), as well as in the developing branchial arches (Ba). In addition to the first branchial arch around the eye (n1), and the third and fourth branchial arches (n3, n4), *efna3* is now also detected in the second branchial arch (n2). Expression of *efna3* is observed in developing neural placodes, in the epidermis overlying the optic vesicle that contains the lens placode (Lp), in the ventral region of the otic vesicle (Ov) and in the developing posterior lateral line placode (Pp, blue arrowhead). *Efna3* is also detected in the proctodeal region (Pr). Pronephric expression at stage 32 is very strong in a region of the tubule at the origin of the intermediate and distal segments (red arrowhead). *Efna3* continues to be detected in the anterior midbrain (Mb) and in a hindbrain (Hb) region likely corresponding to rhombomere 4. It continues to be detected in the four branchial arches (n1, n2, n3 and n4), in the posterior lateral line placode and in the otic vesicle (Ov). It is now expressed in the developing lens (L). Expression at stage 35/36 is similar to stage 32 in the intermediate and distal segments of the developing tubule (lt, Dt). Expression is diminishing in the branchial arches (Ba) and the hindbrain (Hb) but is very strong in dorsal midbrain (Mb), otic vesicle (Ov), and lens (L). Scale bar: 1.7 mm for whole embryo, 0.5 mm for embryonic magnification and 280 µm for section.
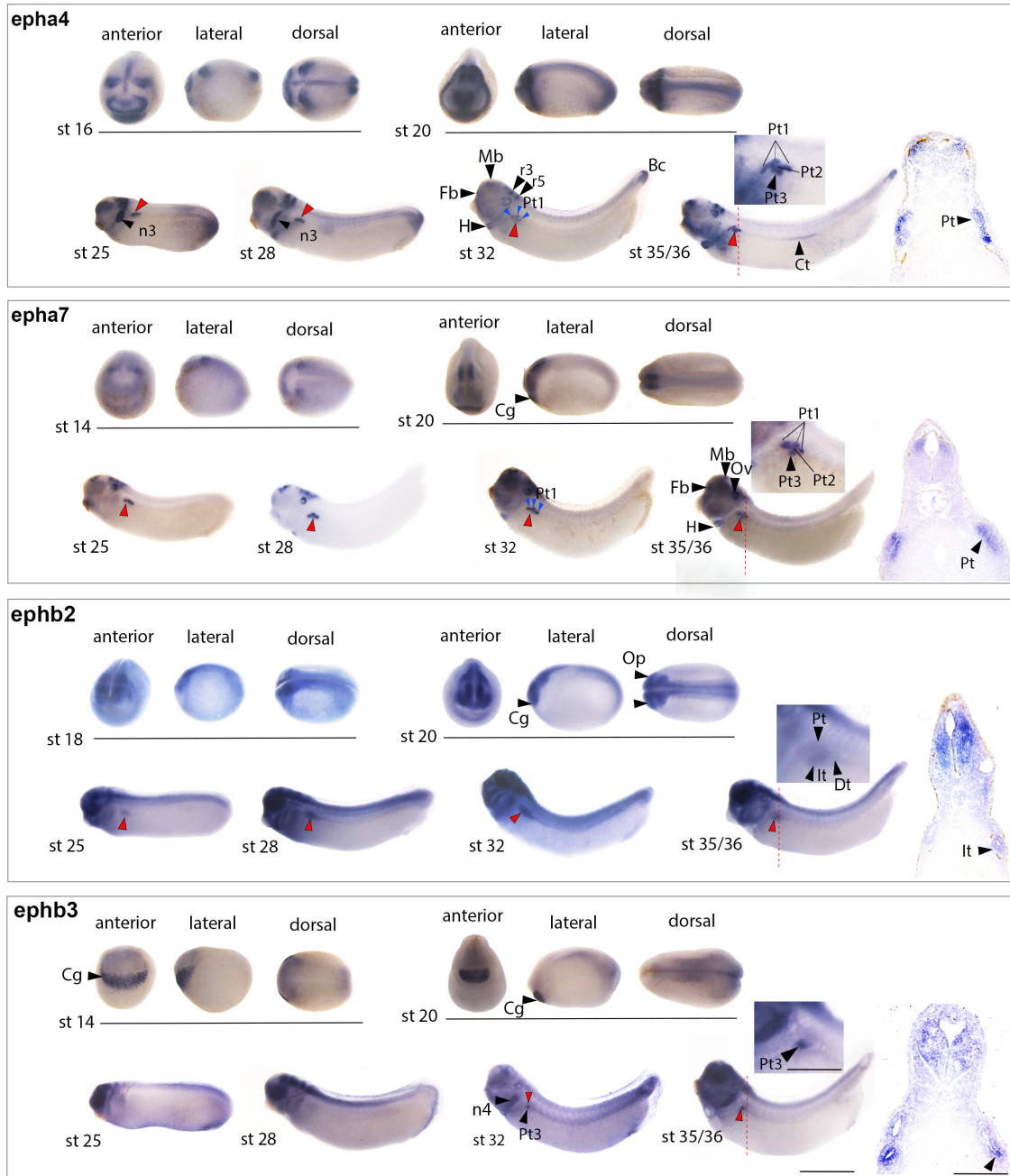
Fig. 4. Expression of Eph receptors *epha4*, *a7*, *b2* and *b3* at stages of pronephric development. WISH analysis at stages ranging from neurula stage 14-18 (kidney field) to early tadpole stage 35/36 (functional pronephros). Anterior, dorsal and lateral views are shown at neurula stages (st14-18 and 20). Only lateral views are shown for tailbud stages 25, 28 and 32, and early tadpole (st35/36). Images of sectioned embryos at stage 35/36 are shown, with red dotted lines, indicating the corresponding levels on whole mounts (note that the sections were not made from the whole mount specimen shown). Pronephric expression of *epha4*, *a7* and *b2* is detected from stage 25 onward, while *ephb3* is only detected in the pronephros at stage 32. *Epha4* and *a7* are mostly co-expressed in the dorsal region of the pronephric anlage at stages 25 and 28. Their expression is maintained in the emerging PT1 segments at stage 32 (blue arrowheads) and expands at stage 35/36 in PT2 (line) and PT3 (arrowhead). EphA4 is also expressed in connecting tubule (Ct). Expression of e*phb2* is observed in the whole anlage at stages 25 and 28, and later in the developing tubule especially in intermediate region (It), while *ephb3* transcripts are detected in the PT3 segment at stages 32 and 35/36. Expression of *epha4* in the developing central nervous system (Fb, Mb, r3, 5: rhombomeres 3 and 5; cephalic neural crest n3: neural crest destined for third arch), developing heart (h) and tailbud (Bc) confirms previously published observations (Barnett *et al.*, 2012; Rothe *et al.*, 2017; Smith *et al.*, 1997; Winning and Sargent, 1994; Xu *et al.*, 1995), as well as *epha7* expression in forebrain (Fb) and hindbrain (Hb), Cement gland (Cg), otic vesicle (Ov) and developing heart (H) (Wang *et al.*, 2016). *Ephb2* expression at neurula stage is observed in the anterior neural plate. At stage 20, it is observed in the whole neural tube and in the optic placode (Op), and at lower levels in the cement gland (Cg). It is detected at tailbud stage in the developing eye and migrating neural crest cells populating every branchial arch. Expression of *ephb3* at neurula stage is principally observed in the cement gland (Cg) and also at low levels of expression in the neural plate. At tailbud stages 25 and 28, it is detected in the developing eye, midbrain and with a segmented pattern in the hindbrain, and at lower levels in migrating cephalic neural crest. Strong *ephb3* expression is detected in the fourth branchial arch at stages 32 and 35/36 (n4). The red arrowheads indicate the pronephric anlage at stages 25 and 28, and the pronephros at stage 32 and 35/36. Scale bar: 1.7 mm for whole embryo, 0.5 mm for embryonic magnification and 280 μm for section.

repulsive interactions. In mammals for example, such interactions are essential for the migration of cortical interneurons in the basal telencephalon (Rudolph *et al.*, 2010) and for hippocampal dendritic spine formation (Bourgin *et al.*, 2007; Murai *et al.*, 2003). EfnA3 has also been implicated in axonal guidance through its interaction with the EphA4 receptor (Steinecke *et al.*, 2014).

As outlined above, *efna3* is only poorly characterized in *Xenopus*. According to Xenbase data (Fisher *et al.*, 2023), only the homeologue L is expressed in *Xenopus laevis* (Session *et al.*, 2016*)*. The deduced amino sequence is strongly conserved in vertebrates (Fig. 2A), particularly in tetrapods (Fig. 2B), sharing 68.2% and 68.4% identity with the human and mouse sequences, respectively.

We further studied how *efna3* is expressed during the development of the pronephros, from the early neurula stage (st14), when the pronephric field is established, to the early tadpole stage (st35/36) (Fig. 3), and have compared these observations with the expression patterns of eph receptors-encoding genes expressed in the pronephric tubule, not only *epha4*, but also *epha7*, *ephb2* and *ephb3* (Fig. 4).

Efna3 is detected in the developing tubule from the tailbud stage 28 (Fig. 3), but is expressed earlier in other tissues. At early neurula stage 14, *efna3* expression is observed in the primary neuronal precursors and weaker in the anterior neural plate. Its neural expression expands at neurula stage 20 to a large part of the neural tube, with strongest expression in the emerging first and third branchial neural crest streams (Fig. 3). No Eph receptor is detected in the developing pronephros at these stages.

At the tailbud stage 25, *efna3* is highly expressed in migrating neural crest populating the third and fourth branchial arches. It is also detected around the eye vesicle in migrating neural crest cells of the first branchial arch, and in the developing midbrain, posterior hindbrain and spinal cord. Expression is also observed in the proctodeal region. At this stage, *efna3* is not expressed in the developing pronephros, but receptors *epha4* and *a7* are strongly expressed in the dorsal region of the pronephric anlage. *Ephb2* expression is also detected, although at lower levels. No detectable expression of *ephb3* can be observed (Fig. 4).

At tailbud stage 28, *efna3* is clearly expressed in the ventral region of the pronephric anlage (Fig. 3). Its expression persists in the proctodeal region, the developing nervous system and branchial arches. In addition to being detected in the first and third/fourth arches, *efna3* is now expressed in the second arch. High levels of expression are observed in the ventral region of the otic vesicle and in the epidermis overlying the optic vesicle, including the lens placode. *Efna3* is detected ventrally to the otic vesicle in the developing posterior lateral line placode (Schlosser, 2003; Schlosser and Northcutt, 2000). Strong expression is also observed in the anterior midbrain. *Epha4* and *a7* are co-expressed in the dorsal region of the pronephric anlage with highest intensities in the three nephrostomes-forming regions (Fig. 4). *Ephb2* expression is detected in a wider area comprising most of the developing pronephros. *Ephb3* is not detected in the pronephros (Fig. 4).

Efna3 expression at late tailbud stage 32 is similar to st28, with the exception of the proctodeal region where it is no more detected. *Efna3* is strongly expressed in the ventral region of the pronephric anlage at the origin of the intermediate and distal segments of the tubule. Expression in developing branchial arches and anterior midbrain persists. It is strong in a region of the hindbrain immediately above the otic vesicle that likely corresponds to the rhombomere 4, as well as in the developing posterior lateral line placode. It is also strongly detected in the developing lens. Eph receptors *epha4*, *a7*, *b2* and *b3* are all expressed in the developing pronephros. *Epha4* and *a7* are now expressed as three dots in the PT1 segments of the tubule, while *ephb2* is detected in a wider domain encompassing the proximal, intermediate and distal segments. *Ephb3* expression is restricted to the PT3 segment (Fig. 4).

Efna3 expression at stage 35/36 is almost identical to stage 32, although it appears to diminish in the branchial arches. In the pronephros, it is detected in the intermediate and distal segments of the tubule (Fig. 3). Expression of Eph receptors *epha7* expands to the PT2 and PT3 segment, while *epha4* is now detected in the entire proximal segments and posteriorly in the connecting tubule. Interestingly, pronephric expression of *epha4* in the collecting duct is reminiscent of the Epha4 expression in the developing mouse kidney that has been shown to be essential for nephric duct insertion to the cloaca (Weiss *et al.*, 2014). Expression of *ephb2 and b3* is identical to stage 32 (Fig. 4). A summary of gene expression domains for Ephrin ligands and Eph receptors in the tubule segments at stage 35/36 is provided in Fig. 5.
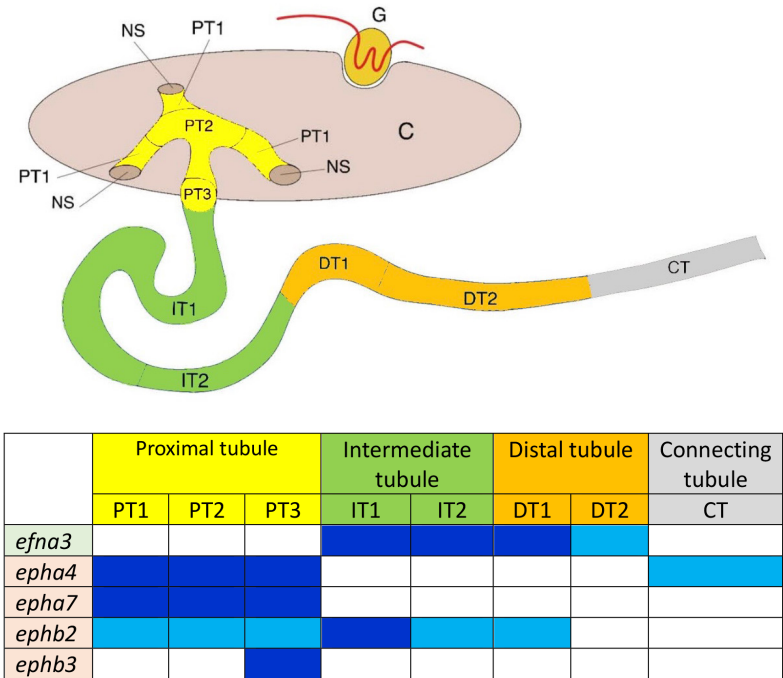

	Proximal tubule			Intermediate tubule		Distal tubule		Connecting tubule
	PT1	PT2	PT3	IT1	IT2	DT1	DT2	CT
efna3				dark blue	dark blue	dark blue	turquoise	
epha4	dark blue	dark blue	dark blue					turquoise
epha7	dark blue	turquoise	turquoise					
ephb2	turquoise	turquoise	turquoise	dark blue	turquoise	turquoise		
ephb3			dark blue					

Fig. 5. Summary of gene expression domains for Ephrin ligands and Eph receptors in pronephros segments at stage 35/36. Schematic representation of the pronephros and table (inspired by Raciti *et al.*, 2008); Ephrin ligands are outlined in green and Eph receptors in light orange. Dark blue: strong expression. Turquoise blue: middle expression. *Efnb2* expression is detected in the glomus at stage 35/36 and not in the tubule and is therefore not included in the table.

The expression domains of *efna3* and those of *epha4* and *epha7* are strikingly complementary at stages 28, 32 and 35 suggesting that they may play a role in the patterning of the pronephric anlage. Dorso-ventral patterning of the pronephric anlage establishes a dorsal domain, which gives rise to the glomus and proximal tubule, and a ventral domain, which forms the intermediate and distal segments of the tubule. This process is largely dependent on Notch signaling, which is active in the dorsal domain (McLaughlin *et al.*, 2000), whereas evi1/mecom may counteract Notch in the ventral domain (Van Campenhout *et al.*, 2006). Efna3 and epha4 and/or epha7 may thus play a role in maintaining the boundary between these two domains. Although this has not been demonstrated for Efna3-Epha4/Epha7 signaling, crosstalk between the Notch axis and ephrin-Eph signaling is well documented (Ventrella *et al.*, 2017). Such crosstalk plays a central role during vascular development, as demonstrated in the mouse retina where Efnb2-dependent arterial development involves an increase of *Efnb2* expression downstream of active Notch (Iso *et al.*, 2006), as well as ephrin acting upstream of Notch signaling (Stewen *et al.*, 2024). *Efnb2* appears to be expressed in the dorsal region of the pronephric anlage from st28 onward, and later in the glomus (Fig. S2). It is therefore possible that Efnb2 may also be involved in the dorso-ventral segregation process of the pronephric anlage dependent on Notch.

Materials and Methods

Xenopus embryos

Xenopus laevis were purchased from TEFOR Paris-Saclay (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, 1975). All animal experiments were carried out according to approved guidelines validated by the Ethics Committee on Animal Experiments "Charles Darwin" (C2EA-05) with the "Autorisation de projet" number 02164.02.

In situ hybridization and embryo sectioning

To generate plasmids leading to synthesis of cRNA probes, PCR products were generated from cDNA library using specific primers listed in Table 2. PCR products were cloned in pCRII vector according to manufacturer instructions (Invitrogen) and digoxigenin-labeled cRNA probes were transcribed from linearized plasmids using SP6 or T7 polymerase. WISH was carried out as previously described (Le Bouffant *et al.*, 2014) after embryo fixation in MEMFA for one hour and dehydration in ethanol (Harland, 1991).

Embryos selected for sectioning after WISH were further washed 3 times in PBS and embedded in gelatin for cryostat sectioning as described in (Bello *et al.*, 2008). Sixteen μm sections were made on a Leica CM 3050S. Observation and images acquisition were performed on a Zeiss Macroapotome Axiozoom V16. Each magnification is indicated in figure legends. For each probe, 15-20 embryos of each stage were analyzed and 5 representative embryos were used for sections.

Alignment and tree

Proteins sequences were collected on Uniprot platform and Clustalw 2.1 free software was used for alignment and tree. Accession numbers of these sequences are the following:

EfnA3 *Danio rerio* (3a: XP_009292344.1; 3b: NP_571929.1), EfnA3 Medaka (XP_011483598.1), EfnA3 *Xenopus laevis* (NP_001080496.1), EfnA3 *Xenopus Tropicalis* (NP_001011360.1), EfnA3 *Macaka mulatta* (NP_001252612.1), EfnA3 *Mus musculus* (NP_001364045.1), EfnA3 *Homo sapiens* (NP_004943.1).

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