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Ectophosphodiesterase/nucleotide phosphohydrolase (Enpp) nucleotidases: cloning, conservation and developmental restriction

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ABSTRACT Ectonucleotidase proteins occupy a central role in purine signalling regulation by sequentially hydrolysing ATP to ADP and to adenosine. The *ENPP (* or *PDNP)* gene family, which encodes ectophosphodiesterase/nucleotide phosphohydrolases, is a subfamily of these enzymes, which consists of 7 members in mammals. These proteins catalyse the generation of bioactive lipids, placing the ENPP enzymes as key regulators of major physiological signalling pathways and also important players in several pathological conditions. Here we report the cloning of all the members, except enpp5, of the enpp family in *Xenopus laevis* and *tropicalis*. Phylogenetic analyses demonstrate the high level of conservation of these proteins between amphibian and other vertebrate species. During development and in the adult frog, each gene displays a distinct specific expression pattern, suggesting potentially different functions for these proteins during amphibian embryogenesis. This is the first complete developmental analysis of gene expression of this gene family in vertebrates.

KEY WORDS: *ectonucleotidase, ectophosphodiesterase/nucleotide phosphohydrolases, purine metabolism, bioactive lipid metabolism, Xenopus laevis, embryogenesis, gene expression*

Introduction

Ecto-nucleotide pyrophosphates/phosphodiesterases (ENPP) form a group of widely and phylogenetically conserved proteins, from bacteria to plants. These enzymes, belonging to the superfamily of ectonucleotidases, catalytically interact with a wide range of nucleotides (purines and pyrimidines) and their derivatives (Zimmermann, 1999, 2001) They are able to hydrolyse the pyrophosphate and phosphodiester bonds of their substrates to nucleoside 5'monophosphates. ENPP2 displays a wider catalytic capacity by being able to hydrolyse AMP to adenosine. The ENPP family is, therefore, involved in the generation, breakdown and recycling of extracellular nucleotides (reviewed in Bollen *et al*., 2000; Stefan *et al*., 2006) and also in modulation of the nucleoside tri/diphosphates, known as P2 purinergic, receptor-mediated signalling (Grobben *et al*., 2000; Lazarowski *et al*., 2000). The recent discovery that ENPP2 is identical to lysophospholipase D (lysoPLD), which hydrolyses lysophosphatidylcholine (LPC) into lysophosphatidic acid (LPA; mono-acylglycerol-3-phosphate) or sphingosylphosphorylcholine (SPC) to sphingosine-1 phosphate (S1P) has widened the potential physiological functions of these enzymes (Tokomura *et al*., 2002; Umezu-Goto *et al*., 2002).

To date, seven members have been cloned in mammals and are numbered according to their order of discovery (reviewed in Zimmermann, 2001; Goding *et al*., 2003; Stefan *et al*., 2005). ENPP1 was initially discovered as a membrane glycoprotein on plasma cells, as Plasma-Cell differentiation antigen-1 or PC-1 (Takahashi *et al*., 1970; Rebbe *et al*., 1991). ENPP2α or autotaxin was first identified as a potent mobility-stimulating glycoprotein secreted by the human melanoma cell line A2058 (Stracke *et al*., 1992; Murata *et al*., 1994). Several splice variants have now been described, such as ENPP2β (PD-Iα), which lacks 52 amino acids

Abbreviations used in this paper: Enpp, ectophosphodiesterase/nucleotide phosphohydrolases.

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in the central domain of the protein and ENPP2γ which contains an additional region of 25 residues (Narita *et al*., 1994; Kawagoe *et al*., 1995). ENPP3 was first described as gp130^{RB13-6} a glycoprotein recognised by antibody RB13-6 but it is also known as B10, PD-1β or more recently as the antigen recognised by the monoclonal antibody 97A6 (Deissler *et al*., 1995; Scott *et al*., 1997; Jin-Hua *et al*., 1997; Bühring *et al*., 2001). These three family members display a very similar primary structure, with a short intracellular N-terminal region, a type II transmembrane domain and a large extracellular C-terminal domain. The latter consists of two somatomedin-B-like domains, involved in potential protein interactions, a catalytic domain with the conserved catalytic T210 and a nuclease-like domain with a putative "EF-hand" calcium binding motif (reviewed in Bollen *et al*., 2000). The "EF-hand" has been shown to be essential for ENPP1 and ENPP3 enzymatic activity (Andoh *et al*., 1999) however deletion of this motif has little or no effect on ENPP2 activity and properties (Lee *et al*., 2001). The single catalytic site mediating both nucleotide pyrophosphatase and phosphodiesterase activities (Gijsbers *et al*., 2001) is essential for the hydrolysis of lysophospholipids (Gijsbers *et al.,* 2003). Moreover, a single mutation in the mouse Enpp2 catalytic site leads to an inactive enzyme. Homozygous knock out mice bearing this mutation die at an early stage of development, suggesting that enzymatic activity of Enpp2 is critical to normal development (Ferry *et al*., 2007). However, domain-swapping experiments recently demonstrated that the lysophospholipase D activity of ENPP2 depends also on the N-terminal and C-terminal "nuclease like" domains (Cimpean *et al*., 2004). ENPP1 and 3 are ecto-enzymes whereas ENPP2 has now been shown to be synthesized as a pre-pro-enzyme and secreted (Jansen *et al*., 2005). ENPP4-7 are shorter proteins with a smaller catalytic extracellular domain and a type I transmembrane domain in their C terminus. However, truncated forms of ENPP6 and 7 can be secreted (Wu *et al*., 2004; Sakagami *et al*., 2005). ENPP4 and ENPP5 have been described as putative ENPP ectoenzymes based on their sequence homology with the previously identified members of the family (Gijsbers *et al*., 2001). However, little is known about their catalytic activity. ENPP6 is a choline-specific glycerophosphodiesterase, with lysophospholipase C activity towards lysophosphatidylcholine (LPC) (Sakagami *et al*., 2005) ENPP7 is better known as alkaline sphingomyelinase (alk-SMase) with no detectable nucleotidase activity (Duan *et al*., 2003).

Human ENPP1-5 proteins have been detected in many organs (Bollen *et al*., 2000) and despite their wide distribution, specific functions have been attributed to each member (reviewed in Goding *et al*., 2003). ENPP1, by producing pyrophosphate (PPi) from extracellular nucleotides, plays a central role in the regulation of calcification (reviewed in Terkeltaub, 2006). Tip-toe walking mice (Ttw), characterised by an ossification of the spinal ligaments and model of the human ossification of the posterior longitudinal ligament (OPLL), have a nonsense mutation in the *Enpp1* gene (Okawa *et al*., 1998). Mutations in human *ENPP1* cause several pathological calcification syndromes, such as Idiopathic Infantile Arterial Calcification (IIAC) (Rutsch *et al*., 2003) and Ossification of the Posterior Longitudinal Ligament of the spine (OPLL), a cause of myelopathy in Japan and Southeast Asia (Nakamura *et al*., 1999). ENPP1 has also been shown to directly interact with the insulin receptor (Maddux and Goldfine, 2000) and several studies have shown a correlation of the K121Q polymorphism with increase of insulin resistance in diabetes although the exact role of ENPP1 in insulin signalling remains controversial (see Goding *et al*., 2003). However, a recent study demonstrates the genetic link between *ENPP1* gene variants and childhood and adult obesity and type-2 diabetes (Meyre *et al*., 2005). ENPP3, which is specifically expressed in basophils and mast cells (Bühring *et al*., 1999) may be a marker for allergy diagnosis (Bühring *et al*., 2004). ENPP2, due to its ability to generate LPA, is involved in cell survival, proliferation and tumor cell motility (reviewed in Stefan *et al*., 2005; van Meeteren and Moolenaar*,* 2007). Creation of Enpp2 mutant mice revealed the importance of this protein for embryonic vasculature (Tanaka *et al*., 2006; van Meeteren *et al*., 2006) and it has been suggested that ENPP2 might play an important role during neurogenesis, since its transcripts are first detected in the developing nervous system in chick and mouse embryos (Bächner *et al*., 1999; Ohuchi *et al*., 2007). However, homozygous mutants are embryonic lethal at mid-gestation (E9.5-E10.5), preventing any further analysis. Human and mouse ENPP6 are predominantly found expressed in the kidney and might be involved in resorption of choline by degrading choline-containing compounds (Sakagami *et al*., 2005). ENPP7, specifically expressed in the intestine, is involved in the digestion of dietary sphingomyelin (SM) and may have a colonic anti-cancer activity (reviewed in Duan, 2006)

Although intensive work has demonstrated the physiological roles of ENPP in adults, little is known about the roles of the ENPP proteins during development. Using *Xenopus laevis* as a vertebrate model, we have analysed the expression pattern of each member of this family. We report the cloning and characterisation of 6 members of the enpp family in *X. laevis* and *X. tropicalis*. We compare their temporal and spatial expression during development and also their distribution in adult frog tissues. Their developmentally restricted and dynamic distributions strongly suggest that this family of proteins play important developmental roles and that these roles would be specific for each enpp member.

Results

Cloning of the different enpp *genes*

All the members of the enpp family, except enpp5, were cloned using different strategies (Supplementary Table 1A). TBLASTN search of the *X. laevis* and *tropicalis* EST databases on the NCBI website allowed the identification of full length IMAGE clones or EST Unigenes coding for enpp2, enpp4, enpp6 and enpp7 (Accession numbers given in Supplementary Table 1B). When necessary, alignments of these sequences were performed to generate the consensus sequence of the cDNA. BLAST search on ESTs databases only allowed the cloning of partial sequences for *Xenopus enpp1* and *enpp3* cDNAs. The remaining sequences were identified by BLAST on the genomic *X. tropicalis* databases (JGI website) and the deduced cDNA sequences corrected by reference to the human sequence according to the Breathnach and Chambon law (Breathnach and Chambon, 1981). When needed, RT-PCRs were performed on *X. laevis* and *tropicalis* embryonic and adult tissues to amplify the missing sequences.

Protein and phylogenetic analysis of the enpp family

Consensus protein sequence deduced from conceptual translation of the cDNA sequences are given in Figs. 1 and 2 and each *Xenopus* enpp protein displays the characteristics features of this

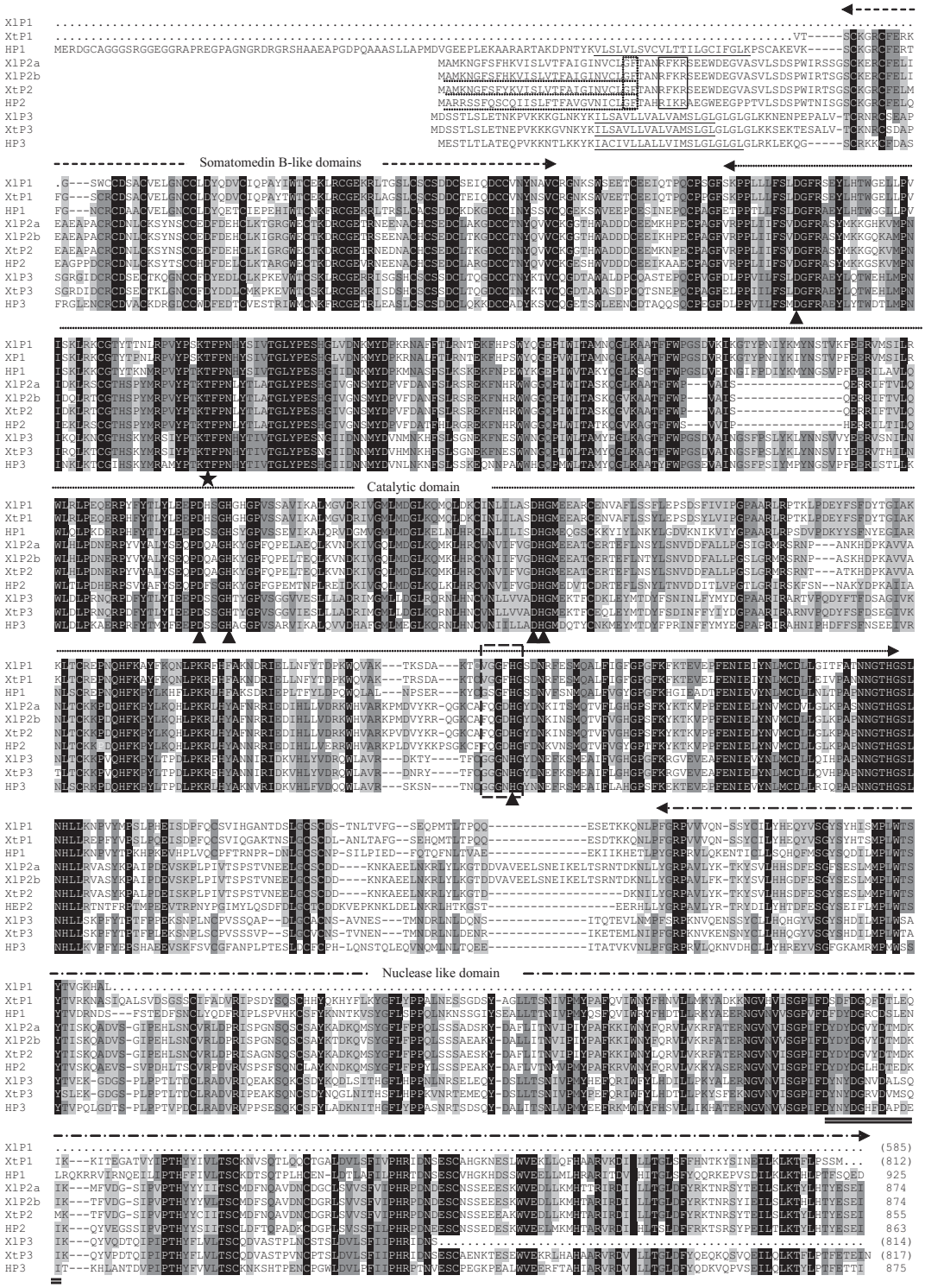

Fig. 1. Alignment of *Xenopus* enpp1, enpp2 and enpp3 proteins. *The* X. laevis *(Xl) and* X. tropicalis *(Xt) proteins were aligned with their human orthologs (H) using CLUSTALW. Residues conserved in all sequences are indicated by a black background. Residues conserved in two orthologous groups of enpp are indicated by a dark grey background. Similar or identical residues in at least 5 sequences are indicated by a light grey background. Missing sequences are indicated by dots. The different functional domains are indicated by the dashed and dotted arrows. The underlined sequences indicate the type 2 transmembrane domain in the N terminal region. In enpp2 sequences, the dotted underlined sequences indicate the signal peptide, the signal peptide cleavage site is marked by the dotted box and the consensus site for recognition by furin is marked by the black box. The GxGXXG motif, dinucleotide binding residues, is indicated by a dashed box. The double underlined sequences represent the EF hand motif. ★: Threonine catalytic-site; ▲: Putative metal binding residues. The Genbank accession numbers of the human ENPP proteins are given in the legend of Fig 3. The size of the proteins (AA numbers), complete or incomplete (in bracket) is also given.*

enzyme family. The N-terminal sequences (corresponding to the cytoplasmic tail and transmembrane domain) are missing from the *X. laevis* and *tropicalis* enpp1 proteins (Fig. 1). The C-terminal 60 amino acids are missing from *X.laevis* enpp3 sequence (Fig. 1). The *X. laevis* deduced enpp7 sequence is also incomplete, with the N-terminal 20 amino acids missing from our sequence (Fig. 2). *X. tropicalis* enpp7 sequences obtained by EST and genomic sequences analysis are identical following translation except in their C terminal domain; however the sequence derived from JGI genomic sequence is incomplete. The enpp3 sequence obtained by conceptual translation of the *X. tropicalis* genomic sequence differs in the C-terminus with our sequence based on EST analysis and is less conserved with the mammalian orthologs. Therefore, it is likely that our sequence provides the correct sequence for *X. tropicalis* enpp3 (Fig. 1).

A phylogenetic analysis of these enzymes in *Xenopus* and other vertebrates was carried out using the catalytic domains sequence of each protein. This analysis demonstrates the clear separation between enpp1-3 from enpp4-7 (Fig. 3). enpp1 proteins are more related to enpp3 proteins and enpp4 proteins are more related to enpp5 proteins. Moreover, each member is more related to its orthologs than to the other family members in the same species, suggesting that any function identified in *X. laevis* may well be conserved in other vertebrates. The percentage of identity between the catalytic domains of orthologous proteins is more than 60%, with enpp4 being the least conserved protein and enpp2 the most conserved member during vertebrate evolution (Table 1A). However, the percentage of identity between the catalytic domain of any *Xenopus* enpp1-3 and any *Xenopus* enpp 4-7 is only of 30% (Table 1B). As shown in Fig. 1, enpp1-3 proteins have a high percentage of identity over each different domain. For example, *X. tropicalis* enpp1 and enpp2 share 50.1%, 45.9% and 49.4% identity along their catalytic, nuclease-like and Somatomedin B-like domains respectively. The catalytic domain is the most conserved domain and *Xenopus* enpp1-3 share about 50% identity and more than 65% similarity with each other (Table 1B). A transmembrane type 2 domain is present in *Xenopus* enpp3 sequences at a similar position to that in human ENPP3, such a transmembrane domain could not be detected in *Xenopus* enpp1 proteins due to incomplete N-terminal sequences.

We have identified two distinct genes coding for enpp2 in *X.laevis* (Fig. 1, Supplementary Fig. 1). These 2 *X. laevis* proteins are 95.8% identical with 37 amino acid differences along their length (Supplementary Fig. 1). The 3 amphibian enpp2 proteins display the same features, characteristic of the enpp family, and the nucleotide binding sequence, GXGXXG, is replaced by FXGXXG as in the other vertebrate ENPP2 proteins (Supplementary Fig. 1). Surprisingly, this sequence is also not conserved in *Xenopus* enpp1, suggesting that these amphibian proteins might display different enzymatic properties to their mammalian orthologs. The N terminal domain is less conserved between *Xenopus* and the other vertebrate proteins. However, as in the case of human ENPP2, the Signal-P-Program predicts that this region can act as a cleavable signal peptide with the cleavage site located between F28 and T29. Moreover, the consensus cleavage site RXK/RR for the endoprotease furin is present in all three frog enpp2 proteins (Fig. 1,

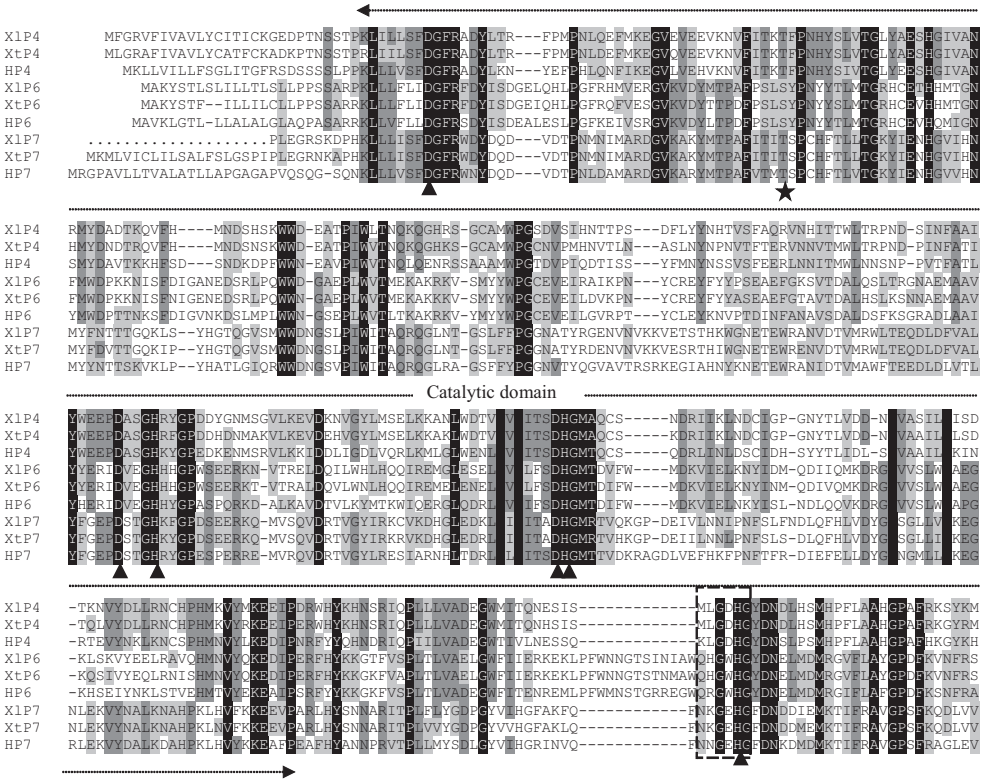

Fig. 2. Alignment of *Xenopus* enpp4, enpp6 and enpp7 proteins. *The* X. laevis *(Xl) and* X. tropicalis *(Xt) proteins were aligned with their human orthologs (H) using CLUSTALW. Residues conserved in all sequences are indicated by a black background. Residues conserved in two orthologous groups of enpp sequences are indicated by a dark grey background. Identical or similar residues in at least 4 sequences are indicated by a light grey background. Missing sequences are indicated by dots. The dotted arrow indicates the catalytic domain. The underlined sequences indicate the type 1 transmembrane domain in the C terminal region. The GxGXXG motif, dinucleotide binding residues, is indicated by a dashed box. ★: Catalytic-site residue (T or S); ▲: Putative metal binding residues. The Genbank accession numbers of the human ENPP proteins are given in the legend of figure 3. The size of the proteins (AA numbers), complete or incomplete (in bracket) is also given.*

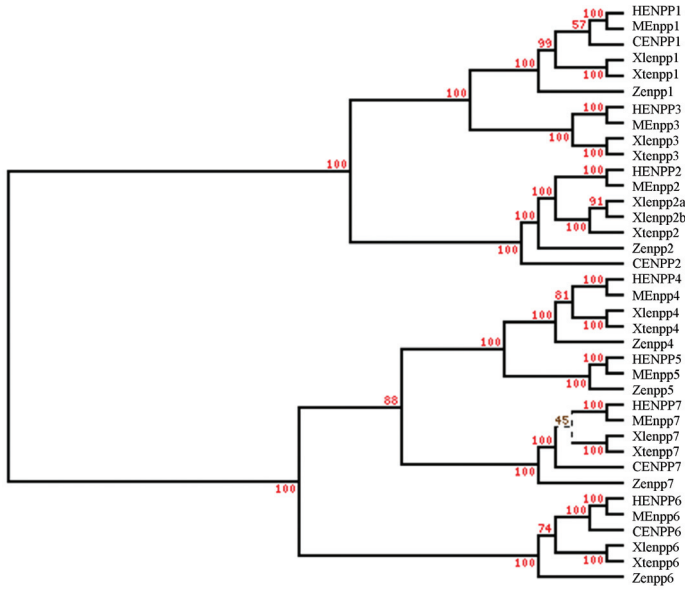

Fig. 3. Phylogenetic conservation of the catalytic domains of ENPP proteins. *A phylogenetic tree was constructed using the PHYLIP program. A cluster algorithm was used to build the tree. Bootstrap values are indicated at each node of the tree. The Genbank accession numbers of the ENPP proteins are as follows: human ENPP1: AAH59375; human ENPP2: AAH34961; human ENPP3: O14638; human ENPP4: EAX04292; human ENPP5: Q9UJA9; human ENPP6: NP_699174; human ENPP7: EAW89566; mouse Enpp1: NP_032839; mouse Enpp2: NP_056559; mouse Enpp3: NP_598766; mouse Enpp4: NP_950181; mouse Enpp5: Q9EQG7; mouse Enpp6: AAH96376; mouse Enpp7: NP_001025462; Chick ENPP1: XP_424539; chick ENPP2: XP_418466; chick ENPP6: XP_420512; chick ENPP7: XP_423912; zebrafish enpp1: CAI11601; zebrafish enpp2: NP_956897; zebrafish enpp4: AAH93443; zebrafish enpp5: NP_001025347; zebrafish enpp6: Q5BKW7; zebrafish enpp7: XP_685968. The chick ENPP4 protein was excluded since its sequence is incomplete. Xl:* X. laevis*; Xt:* X.tropicalis*; H: human; M: mouse; C: Chick; Z: Zebrafish.*

Supplementary Fig. 1). Furthermore, we have evidence that both *X. laevis enpp2* are secreted proteins (data not shown).Therefore we named this new gene *enpp2b* as opposed to *enpp2a* (the previously described *enpp2* on the NCBI database). Both *enpp2a* and *enpp2b* align with the same *X. tropicalis* genomic sequences (scaffold 330) and share similar percentage of identity to other vertebrate ENPP2 proteins (Table 1A).

Both *Xenopus* enpp4, 6 and 7 are highly similar but are less conserved compared to enpp1-3 during evolution (Table 1A,B). Even though each member shares more than 50% identity with its orthologs, the percentage of identity between their catalytic domains is only 30% in *Xenopus* (Table 1A). However, their sequence analysis predicted a primary structure similar to their mammalian orthologs (Fig. 2): one transmembrane domain located at the C-terminal domain of the proteins and one N-terminal signal peptide with a cleavage site between residues AA19-20 for both enpp4, between AA 22-23 for *X.laevis* enpp6 and AA 20-21 for *X.tropicalis* enpp6 and between residues AA 19-20 for *X.tropicalis* enpp7 (this domain could not be identified in *X. laevis*

TABLE 1A

RELATEDNESS BETWEEN THE *X.LAEVIS* (XL) AND *X.TROPICALIS* (XT) NENPP CATALYTIC DOMAINS AND THEIR VERTEBRATE ORTHOLOGS

	X.laevis (pseudo)	*X.tropicalis*	Human	Mouse	Chick	Zebrafish
Xlenpp1		95.8 (97.9)	68.5(82.7)	66.9(81.6)	71.1(84.3)	62.2(77.4)
Xtenpp1			68.8(82.7)	67.5(81.9)	71.1(84.3)	62.5(77.2)
Xlenpp2a	95.9(98.6)	96.7(98.4)	82.0(91.0)	80.4(90.7)	72.1(79.3)	74.0(86.9)
Xlenpp2b		97.8(99.5)	82.3(91.6)	80.7(91.0)	72.6(80.0)	74.6(87.2)
Xtenpp2			83.7(91.8)	81.5(91.0)	73.8(80.5)	74.0(87.2)
Xlenpp3		93.7 (98.2)	72.6(86.8)	70.8(84.5)	nd	nd
Xtenpp3			72.6(86.8)	71.1(84.7)	nd	nd
Xlenpp4		85.3(92.8)	63.5(76.4)	62.1(78.0)	nd	59.6(76.2)
Xtenpp4			62.1(77.2)	62.6(78.8)	nd	59.6(77.0)
Xlenpp6		88.2(93.7)	67.0(80.4)	67.0(80.1)	68.6(84.0)	63.1(74.9)
Xtenpp6			66.8(80.4)	66.5(79.8)	67.5(83.2)	63.9(76.7)
Xlenpp7		93.3(97.1)	68.0(81.3)	67.7(81.6)	69.8(83.7)	66.8(79.8)
Xtenpp7			66.6(81.3)	66.6(82.1)	69.4(84.5)	66.2(80.6)

The percentage of amino acid identity and similarity (in bracket) between the ENPP catalytic domains was determined by pairwise alignment using the Needleman-Wunsch global alignment on the EMBL-EBI website. The Genbank accession numbers are given in the legends of Figure 3. nd, not determined.

TABLE 1B

RELATEDNESS OF THE CATALYTIC DOMAIN OF THE *X. LAEVIS* (XL) AND *X. TROPICALIS* (XT) ENPP (P) PROTEINS

	Xl enpp1	Xt enpp1	Xl enpp2a	Xl enpp2b	Xt enpp2	Xl enpp3	Xt enpp3	Xl enpp4	Xt enpp4	Xl enpp6	Xt enpp6	Xl enpp7
XlP1												
XtP1	95.8(97.9)											
XlP2a	50.1(66.9)	49.6(66.9)										
XlP2b	49.6(66.1)	49.1(66.1)	95.9(98.6)									
XtP2	50.1(66.4)	49.6(66.4)	96.7(98.4)	97.8 (99.5)								
XlP3	56(74.9)	55.8(75.4)	51.2(65.9)	51.7 (65.9)	51.4(66.1)							
XtP3	56.9(74.4)	57(74.7)	52.1(66)	52.6 (66.2)	52.3(66.5)	93.5(97.9)						
XlP4	36.2(52.2)	35.7(51.9)	34.7(48.5)	34.5 (49)	34.9 (49)	35.8(58)	36.2(56.8)					
XtP4	36.1(50.4)	35(50.4)	35.3(49.1)	35.1(49.4)	34.1(48.6)	34.7(55.6)	34.4(54.9)	85.3(92.8)				
XlP6	30.8(48.5)	30.5(47.8)	29.3(46.6)	28.3(46.5)	27.6(45.5)	28.9(46.8)	28.6(46.6)	34.4(53.7)	34.8(54.2)			
XtP6	30.2(46.9)	29.6(45)	28.4(48.1)	27.9(48.1)	26.7(46.8)	27.7(46.1)	27.1(45.8)	32.6(53.2)	33.9(53.1)	88(93.5)		
XlP7	31.3(46.5)	30.6(46.2)	31.1(45.8)	31.4(46.3)	31.2(45.2)	32.2(50.3)	31.8(50.3)	35.3(54.3)	35.2(54.5)	32.4(51.8)	29.6(51)	
XtP7	31.2(47.5)	30.9(47.3)	30.1(44.7)	30.4(44.5)	30.9(44.6)	32.1(50.1)	31.7(49.9)	35.2(55.9)	35.2(56.2)	32.8(53.1)	30.8(51.5)	93.4(97.1)

The percentage of amino acid identity and similarity (in bracket) between the catalytic domains of the different enpp proteins was determined by pairwise alignment using the Needleman-Wunsch global alignment on the EMBL-EBI website.

enpp7 due to incomplete N-terminal sequences). Finally, the nucleotide binding sequence GXGXXG is not conserved in any of these proteins, suggesting that as their mammalian orthologs, these amphibian proteins might not be involved in the purinergic signalling pathway but hydrolyse bioactive lipids. Moreover, the catalytic T residue is conserved in all sequences, and as in ENPP6 mammalian orthologs, is replaced by an S in the frog enpp6 proteins.

Spatial expression of the enpp *gene family in the adult frog*

The distribution of *enpp* gene expression in the adult frog was analysed by RT-PCR (Fig. 4). *enpp1* and *enpp4* are ubiquitously expressed genes, as their transcripts can be detected in each organ tested. *enpp3* also displays a wide expression pattern; however, the level of expression is higher in the intestinal tract, kidney, lung and eyes. *enpp6* is more expressed in ovary and kidney and no expression can be detected in the intestinal tract. *enpp2a*, *enpp2b* and *enpp7* display a more restricted expression pattern. *enpp7* is the most tissue specific gene as it is specifically highly expressed in the small intestine (duodenum and ileum). *enpp7* is the only gene of the *enpp* family not to be expressed in the nervous system. The pseudo-alleles of *enpp2* display a different expression profile: *enpp2a* expression can only be detected at low level in the nervous system and reproductive organs whereas *enpp2b* display a wider expression domain with a higher level of expression in the spinal cord, brain and ovary.

Temporal expression of the enpp *genes during* X. laevis *development*

The temporal embryonic expression of these genes was assessed by RT-PCR (Fig. 5). All genes are expressed maternally except *enpp7. enpp7* zygotic expression is only detected weakly at stage 41 but up-regulated by stage 45, the last stage tested in this experiment, ruling out a role of this gene during early development. *enpp1*, *enpp2b* and *enpp4* display a similar expression pattern. These genes are highly expressed at maternal stages. Their zygotic expression is weakly detected during gastrula (stage 10.5) but the level of expression increases until stage 45. Expression of *enpp2a* is more constant during development, with its level of expression being the lowest during gastrula. *enpp3* is expressed maternally at low levels. The zygotic expression of *enpp3* is strongly detected from stage 13 to stage 25 and then decreases from stages 27 to 37, but remains expressed until stage 45. *enpp6* is the highest maternally expressed member of the family. Its zygotic expression can be amplified after swimming tadpole stages; the weakly detected expression during gastrula and neurula stages may be as a consequence of non-degraded maternal transcripts.

Spatial expression of the enpp *gene family during development of* X. laevis

The spatial expression of the *enpp* genes was detected by whole-mount *in situ* hybridisation (WISH). WISH was carried out on embryos from stage 5 to stage 45, based on the temporal expression of each gene established by RT-PCR. Specific antisense probes were used to detect each gene and sense probes were used to control the specificity of the staining. In some cases, non-specific staining in the notochord was detected with the sense probes. As shown in Figs. 6 and 7, the expression of all *enpp* members can be detected throughout *X. laevis* development except *enpp3*. Moreover, *enpp* genes display distinct expression domains suggesting specific roles for each member

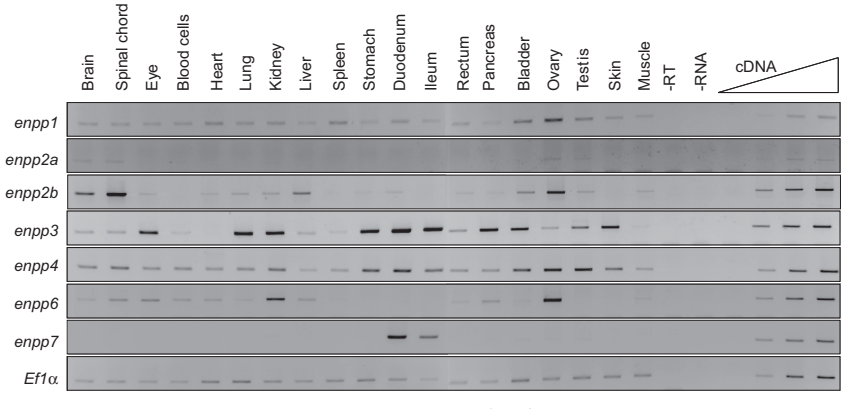

Fig. 4 (Left). Spatial expression profiles of *enpp* genes in the adult frog. *RT-PCR analysis showing the expression pattern of the* enpp *genes in* X. laevis *adult tissues.* enpp1 *and* enpp4 *are expressed in all tissues tested.* enpp2b, enpp3 *and* enpp6 *display a wide expression profile whereas* enpp2a *and* enpp7 *are more tissue specific.* EF1α *was used as a loading control. The linearity was performed with doubling dilutions of cDNA from the following tissues: heart for* enpp1, *brain for* enpp2a *and* enpp2b, *skin for* enpp3, *ovary for* enpp4, *kidney for* enpp6 *and ileum for* enpp7.

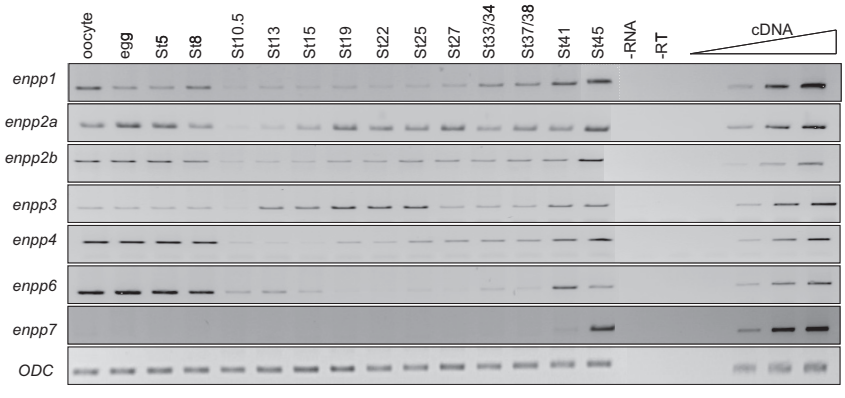

Fig. 5 (Left). Temporal expression profiles of *enpp* genes during embryogenesis. *RT-PCR analysis showing the temporal expression profile of the* enpp *genes in* X. laevis *unfertilised eggs and embryos.* enpp1, enpp2a, enpp2b, enpp3 *and* enpp4 *are expressed at almost all stages tested. The level of* enpp6 *expression is higher during maternal stages whereas* enpp7 *expression is only detected from stage 41.* ODC *was used as loading control. The linearity was performed with doubling dilutions of cDNA from stage 5 embryos for* enpp2b, enpp4 *and* enpp6 *and from stage 45 embryos for* enpp1, enpp2a, enpp3 *and* enpp7.

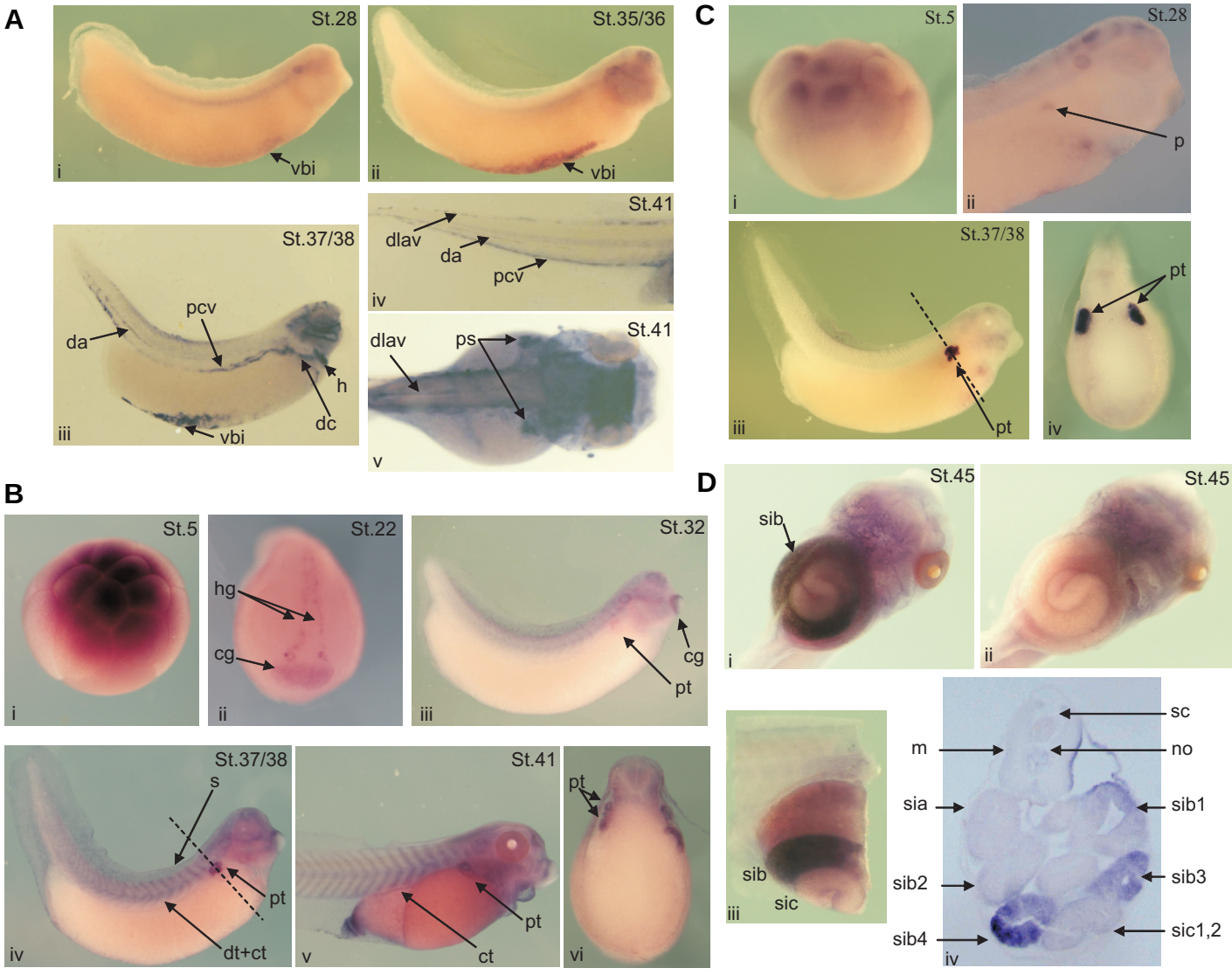

Fig. 6. Spatial expression profile of *enpp1, 4, 6* and *7* during development. *Whole mount* in situ *hybridisation with DIG-labelled antisense RNA probes was performed on embryos from stages 5-41 for* enpp1, 4, 6 *and stages 32-45 for* enpp7. *All embryos were analysed from the same experimental set. The differences in colours result from clearing the embryos and photographing them against a white background to visualise staining.* **(A)** *Whole-mount* in situ *hybridisation analysis of* enpp1 *expression. Lateral view at stages 28 (i), 35/36 (ii) and 37/38 (iii). Detail of the fin at stage 41 (iv) and dorsal view of a stage 41 embryo (v). Anterior is right and dorsal is up, except in (v). da, dorsal aorta; dc, duct of cuvier; dlav, dorsal longitudinal anastomosing vessel; h, heart; pcv, posterior cardinal vein; ps, pronephric sinus; vbi, ventral blood island.* **(B)** *Whole-mount* in situ *hybridisation analysis of* enpp4 *expression. Animal view at stage 5 (i), anterior view at stage 22 (ii), lateral view at stages 32 (iii), 37/38 (iv) and 41 (v). For the lateral views, dorsal is up and anterior is right. Transverse sections at stage 37/38 through the proximal tubules (vi). Dorsal is up. The dotted line through the pronephric tubules (iii) correspond to planes of section in (vi). cg: cement gland; ct, collecting tubules; dt, distal tubules; hg, hatching gland; pt, proximal tubules; s, somites.* **(C)** *Whole-mount* in situ *hybridisation analysis of* enpp6 *expression. Lateral view at stage 5 (i), lateral view at stage 28 (ii) and 37/38 (iii). Dorsal is up and anterior is right. Transverse sections in the proximal tubules level at 37/38 (iv). The dotted line through the pronephric tubules correspond to planes of section in (iv). p, pronephros; pt, proximal tubules.* **(D)** *Whole-mount* in situ *hybridisation analysis of* enpp7 *expression. Ventral view of stage 45 embryos hybridized with anti-sense (i) and sense (ii) probe, lateral view of the gut region of a stage 45 embryo (iii), cryostat section (iv) at 28µm of a stage 45 embryo through the gut. no, notochord; m, muscles; sc, spinal cord; sia, first sections of the small intestine; sib, external coil of small intestine; sic, internal coil of small intestine (from Chalmers and Slack, 1998).*

during *X. laevis* development.

enpp1 maternal expression is detected in the animal pole of the embryo at stage 3 and 8 (data not shown). Zygotically, *enpp1* is weakly detected at stage 28 in the ventral blood island (Fig. 6Ai). At this stage, the staining observed in the notochord and otic vesicle may be non-specific since it is observed with the sense control probe (data not shown). At stage 35, the staining in the blood compartment is more intense but no staining can be observed in the dorsal lateral plate, the other blood compartment (Fig. 6Aii). At later stages, *enpp1* is still expressed in the ventral blood island and its transcripts can be detected in the vasculature of the embryo, such as the posterior cardinal vein, the dorsal aorta

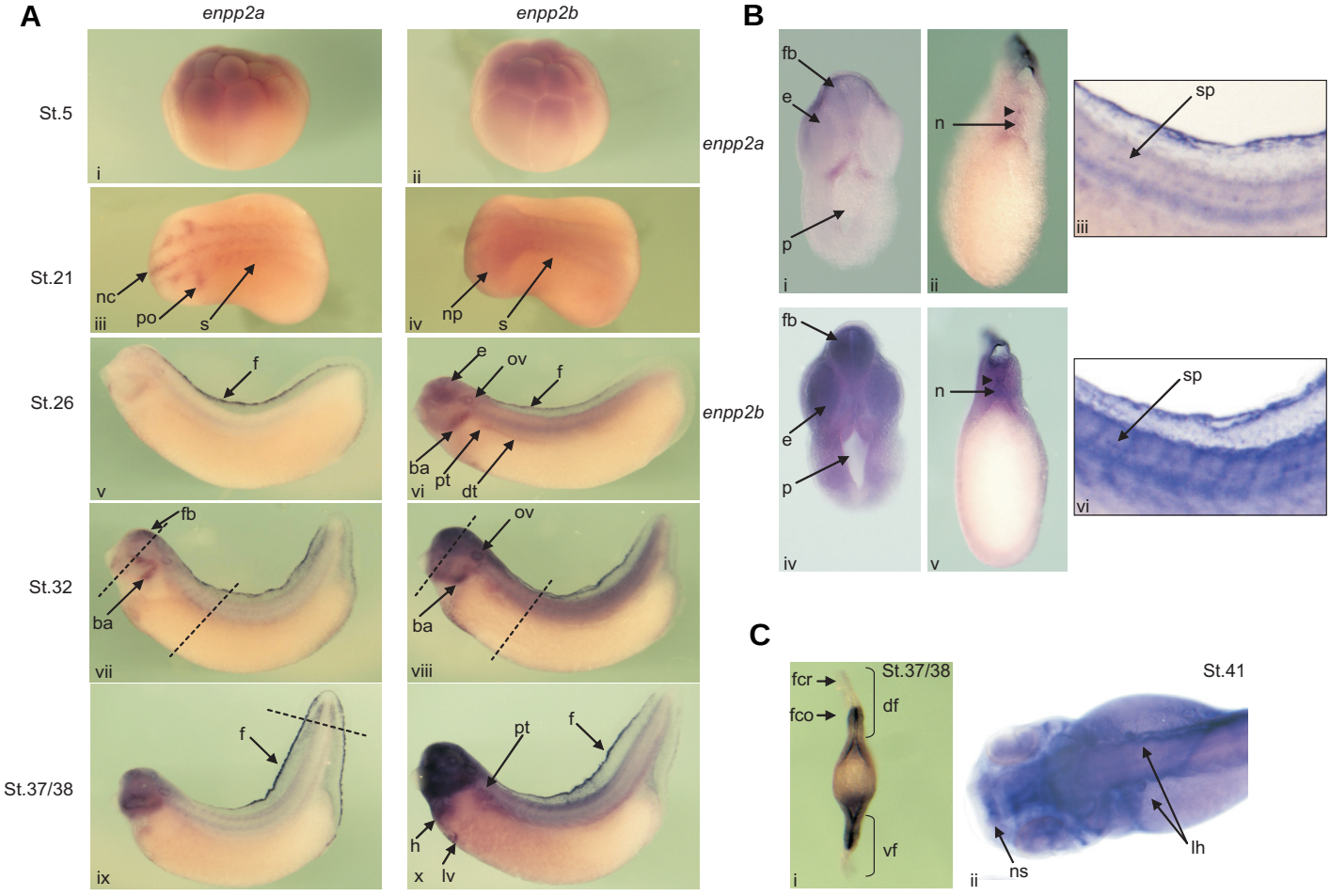

Fig. 7. Comparison of the spatial expression profile of *enpp2a* and *enpp2b* genes during development. (A) *Whole-mount* in situ *hybridisation with an* enpp2a *and* enpp2b *DIG labelled anti-sense RNA probe was performed on embryos from stages 5-41. Animal view at stage 5 (i, ii). Lateral view at stage 21 (iii, iv), stage 26 (v, vi), stage 32 (vii, viii) and stage 37/38 (ix, x). Dorsal is up and anterior is left. The dotted line through the fins (ix) corresponds to plane of section in Ci.* **(B)** *Whole-mount* in situ *hybridisation with an* enpp2a *and* enpp2b *DIG labelled antisense RNA probe on stage 32 embryos. Transverse section through the head (i, iv) and trunk (ii, v) levels. Lateral view of the trunk region (iii, vi). Arrowheads indicate the staining in the spinal cord.* **(C)** *Whole-mount* in situ *hybridisation with an* enpp2a *DIG labelled antisense RNA probe. Transverse section through the fin at stage 37/38 (i). Dorsal view of a stage 41 cleared embryo (ii). ba, branchial arches; df, dorsal fin; dt, distal tubules; e, eye; f, fin; fb, forebrain; fcr, fin crest; fco, fin core; h, heart; lh, lymphatic heart; lv, liver diverticulum; n, notochord; nc, neural crest; np, neural plate; ns, nasal placode; ov, otic vesicle; p, pharynx; pt, pronephric tubules; s, somites; sp, spinal cord; vf, ventral fin.*

and the duct of Cuvier and also in the heart (Fig. 6Aiii). At stage 41, *enpp1* remains strongly expressed in the vasculature of the embryos, as seen in the tail (Fig. 6Aiv) and also its transcripts can be detected in the pronephric sinus (Fig. 6Av).

enpp4 is strongly expressed before MBT in the blastomeres of the animal pole of the embryos (Fig. 6Bi). Its zygotic expression is first detected in the cement gland from stage 16. The expression in this tissue is maintained until stage 38 (Fig. 6Bii-iv). From stage 22 until stage 28, staining can be observed in the hatching gland, as shown on Fig. 6Bii. From stage 32, *enpp4* is expressed in the developing pronephros and becomes strongly expressed in the proximal tubules (Fig. 6Biii,iv, section 6Bvi) although weaker expression can also be detected in the intermediate, distal and collecting tubules (Fig. 6Biv). Expression in the kidney remains detectable until stage 41, the last stage tested in this experiment (Fig. 6Bv). From tailbud stages, *enpp4* is also expressed in the somites (Fig. 6Biii-v).

The *enpp6* gene displays a very specific expression domain. Prior to MBT, *enpp6* is expressed in the animal pole (Fig. 6Ci). After MBT, no staining can be observed until late tailbud stages. At stage 28, *enpp6* is expressed in the brain and at a lower level in the developing pronephros (Fig. 6Cii). The level of expression in the kidney increases and at stage 37/38, *enpp6* is highly and specifically expressed in the pronephric proximal tubules (Fig. 6Ciii,iv) where it remains expressed until stage 41, the last stage tested.

enpp7 displays the most tissue restricted expression domain. As expected from its temporal expression profile, *enpp7* is only detected from stage 43 by *in situ* hybridisation. At stage 45, *enpp7* is highly and specifically expressed in the gut (Fig. 6Di,iii), in the external coil 3 and 4 of the small intestine (Fig. 6Div) whereas no expression of *enpp7* can be observed in the anterior (sia) and the internal coil of the small intestine (sic) (Fig. 6Div). No staining in the gut region can be seen with the sense probe (Fig. 6Dii)

The pseudoalleles *enpp2a* and *enpp2b* display different staining expression patterns. Both genes are expressed strongly in the animal pole of the embryo at stage 5 and 8 (Fig. 7Ai,ii). *enpp2b* is expressed in the developing neural plate from stage 15, whereas expression of *enpp2a* can not be detected before the end of neurulation. However, at stage 21, *enpp2a* is expressed in the somites, in sensory placodes and neural crest (Fig. 7Aiii). *enpp2b* is also expressed in the somites and in the anterior nervous system (Fig. 7Aiv). Expression can also be observed in the developing eyes after clearing the embryos (data not shown). At tailbud stages, *enpp2a* expression is mainly detected in developing dorsal fin whereas *enpp2b* displays a broader expression pattern with strong staining detected in the eye, otic vesicle, branchial arches, somites, in the dorsal fin and in the pronephric tubules (Fig. 7Av,vi). Weaker expression of *enpp2a* can also be detected in the nervous system, pronephros and cement gland (Fig. 7Av). At stage 32, staining in the nervous system can be detected for both genes (Fig. 7B): *enpp2a* and *enpp2b* are both expressed in small populations of cells in the ventral part of the spinal cord (Fig. 7B ii,iii,v,vi) whereas *enpp2b* seems more expressed in the central nervous system than *enpp2a* (Fig. 7Bi,iv). From stage 32 to stage 41, *enpp2a* remains strongly expressed in the dorsal and ventral fins (Fig. 7Avii, ix), in the fin core, composed of extracellular matrix and neural crest cells, but not in the fin crest which is a single epithelial layer as shown by transversal section (Tucker and Slack, 2004)(Fig. 7Ci). Expression in otic vesicle, branchial arches and somites can also be observed from these stages. At tadpole stages, *enpp2b* remains expressed in all tissues detected at earlier stages. Moreover, expression in the heart and in the liver diverticulum can be observed at stage 37/38 (Fig. 7Aviii). At stage 41, both *enpp2a* and *enpp2b* are expressed in several placodes, including the olfactory placodes (data not shown). *enpp2a*, but not *enpp2b*, can be detected in the lymphatic hearts (Fig. 7Cii).

Discussion

This paper reports the cloning of 6 members of the *enpp* gene family in *Xenopus* and their expression during development and in adult frog tissues. Although this family has been intensively studied during the last few years, there is little data regarding the expression of these genes during development, except for ENPP2, autotaxin (Bächner *et al*., 1999; Ohuchi *et al*., 2007). Our work is the first to comprehensively compare the expression pattern of all *enpp* genes during the development of any vertebrate and also to compare embryonic expression patterns with those in adult tissues.

Evolutionary conserved members of the enpp family are present in Xenopus

Our study reveals that all members of the enpp family, except *enpp5*, are present in *X. laevis* and *tropicalis*. We failed to amplify any *enpp5* sequences, suggesting that there may be no *enpp5* ortholog in either *Xenopus* species. Phylogenetic analysis demonstrates that the enpp family is highly conserved between frog species and also during vertebrate evolution both at the protein level and at the level of genomic organisation. Indeed, BLAST analyses demonstrate that *enpp1* and *enpp3* genomic sequences are located on the same scaffold (scaffold 200) in *X. tropicalis*. This linkage (or synteny) is conserved in other vertebrate species such as human, mouse, chimpanzee and cows, suggesting that *enpp1* and *enpp3* might originate by duplication of an ancestral gene, this duplication having taken place before the emergence of amphibia. This is consistent with the fact that in protein sequence, enpp1 is more related to enpp3 than to its enpp2 homolog.

Two enpp2 *genes in* Xenopus laevis

During this work, we isolated two genes encoding enpp2 in *X. laevis*, named *enpp2a* and *enpp2b*. These two sequences must correspond to the two pseudo-allelic *enpp2* sequences since they both identify by BLAST the same *X. tropicalis* genomic sequences. Moreover, these two genes display different expression patterns during development which suggests that these two sequences have been retained in *X. laevis* due to spatial sub-functionalization. Studies based on full length cDNA clone analysis have demonstrated that approximately 14% of paralogous pairs in *X. laevis* show differential expression patterns indicative of sub-functionalization (Morin *et al*., 2006). Recently, we demonstrated by *in situ* hybridisation that this was indeed the case for the 2 pseudo-alleles coding for Annexin 4 (Massé *et al*., 2007). In mammalian species, several transcripts encoding ENPP2, which result from alternative splicing, have been identified. They display different expression patterns (van Meeteren and Moolenaar, 2007; Giganti *et al*., 2008), although the physiological relevance for these different isoforms is still unknown.

Based on sequence analysis, it seems that *enpp2a* and *enpp2b* have duplicated from an ancestral gene and have evolved independently at an equal rate. However, based on expression patterns, we suggest that *enpp2b* is closer to the ancestral gene since the mouse ortholog of *enpp2b* has a similar expression pattern in the neural plate, somites and kidney (Bächner *et al*., 1999). It is worth noting that the *enpp2* gene, described in Xenbase, corresponds to our *enpp2b* sequence whereas the sequence referred to as *enpp2* on the NCBI website encodes the enpp2a protein.

EST analysis for the other *enpp* members indicates pseudo-allelic sequences are present in *X. laevis*, but we have not investigated this any further. It might be interesting to analyse if their expression patterns diverge as *enpp2a* and *enpp2b*.

The expression patterns of enpp *genes are temporally and spatially regulated*

Although enpp1, 2a, 2b and 3 are highly related in sequence, their expression patterns differ during development and in the adult frog, in both a temporal and spatial manner. The expression pattern of the *enpp3* gene suggests a unique role for this family member during the early phase of development. The level of expression of the other *enpp* genes increases until stage 45, the last stage tested in this study. *enpp4*, *enpp6* and *enpp7* also display unique embryonic expression profiles. In the adult frog all tissues tested express more than one member of the enpp family. As in mammalian species, the *enpp* genes display distinct but overlapping expression profiles (Bollen *et al*., 2000; Sakagami *et al*., 2005; Duan *et al*., 2003). *enpp7* displays the most tissue restricted expression pattern, being only detected in the adult intestine. All these genes are expressed maternally, except *enpp7*. Hydrolysis of lipids is critical for fertilization and LPC has

been suggested to induce the membrane fusion needed for the sperm acrosomal reaction and sphingomeylin (SM) may play a role during membrane fusion and egg-sperm interactions by stabilizing rafts. SM and LPC levels decrease during fertilization, reflecting the activation of sphingomyelinase and lysoPLD enzymes such as the enpp proteins (Petcoff *et al.,* 2008).

Distinct expression patterns suggest specific roles during development

We have shown that all *enpp* genes, except *enpp5*, are expressed during *X. laevis* development. The zygotic expression of *enpp1*, *2a*, *2b*, *3* is up-regulated during gastrula stages suggesting potential roles during early phases of development. However, their spatial expression profile seems to indicate distinct functions. During *Xenopus* embryogenesis, *enpp1* is highly expressed in blood compartments, and in the adult its ubiquitous expression may be due to expression in adult blood cells. In mammals, ENPP1 was first cloned as PC-1, a glycoprotein of mouse B-cells (Takahashi *et al*., 1970). The embryonic distribution of *enpp1* in the ventral blood island in *Xenopus*—possibly reflects that production of B-cell precursors which occurs in this region (Du Pasquier *et al*., 2000).

The functions of ENPP2 in cell proliferation, migration and survival are certainly linked to LPA production and to its lysoPLD activity (van Meeteren and Moolenaar, 2007) and not to the purine pathways, despite the fact that ATP is an inhibitor of tumour cell proliferation and adenosine a stimulator of angiogenesis (Stefan *et al*., 2006). *Xenopus enpp2* genes are both expressed in embryonic and adult nervous systems and neural crest derivatives such as the fin, reflecting its importance during cell mobility. The expression in neural tissue is conserved during vertebrate evolution (Bächner *et al*., 1999; Ohuchi *et al*., 2007) suggesting that enpp2 is critical to both normal development and function of the nervous system. Human ENPP2 is involved in the secretion of cerebrospinal fluid, differentiation of oligodendrocytes but also in pathological conditions such as multiple sclerosis, neuropathic pain and Alzheimer-type dementia (ATD) (reviewed in Yuelling and Fuss, 2008; Dennis *et al*., 2005) Despite the importance of purine signalling in the nervous system, it seems that these neuronal functions are likely to be mediated by a novel domain located in the ENPP2 C-terminal region, the MORFO domain (Yuelling and Fuss, 2008). Mouse knock-outs for Enpp2 are embryonic lethal and therefore uninformative (Tanaka *et al*., 2006; van Meeteren *et al*., 2006)

In *Xenopus* embryogenesis, *enpp3* is strongly expressed during neurulation. Moreover, in the adult, *enpp3* transcripts can be detected in almost every tissue tested but are at a higher level in lung, kidney, intestine tract and skin. This expression profile reflects the importance of purinergic signalling in the extracellular environment of epithelial cells (reviewed in Schwieber and Zsembery, 2003) mammalian enpp3 having been shown to be involved in airway, liver and intestinal epithelial functions (Stefan *et al*., 2006)

The *enpp4-7* members are all expressed later in development, during organogenesis. *enpp4* expression level increases at late neurulation and the zygotic expression of *enpp6* and *7* can only be detected at late swimming tadpole stages, ruling out any role for these 2 proteins during early developmental processes. The pronephric expression of *enpp6* is upregulated when the pronephros becomes fully formed and functional (Vize, 2003) suggesting a role in the differentiated function of this organ. *enpp7* is highly expressed in the gut at the start of independent feeding, when the tadpole gut has acquired its coiled structure and is fully functional (Chalmers and Slack, 1998). Therefore, we could speculate that these two *enpp* gene family members might be more involved in the physiological function of these organs as has been suggested in humans. ENPP7 hydrolyses sphingomyelin (SM) in the intestinal tract and therefore plays anti-proliferative and anti-inflammatory roles by generating ceramide and also can affect cholesterol uptake (reviewed in Duan, 2006). ENPP6 might be involved in the resorption of choline by degrading choline-containing compounds such as LPC and SPC but also may have a role in the kidney by producing bioactive lipids such as arachidonoylglycerol and sphingosine (Sakagami *et al*., 2008).

Several enpp family members play a conserved role in vertebrate kidney development/function

Several *enpp* family genes are highly expressed in the pronephros but also in the adult kidney, the mesonephros. *enpp2b* is transiently expressed in the tubules during tailbud stages. In the mouse, Enpp2 is also expressed in the embryonic kidney (Bächner *et al*., 1999). Our work is the first study to describe a strong and specific expression during pronephric development of *enpp4.* In mammals, no data are available concerning the expression of this gene. In the adult frog, *enpp4* is also expressed in the kidney, but as in human, its expression in the adult tissues appears to be ubiquitous (data from Unigene Hs709868). *enpp6* displays the strongest expression in the kidney. Unlike *enpp2b* and *enpp4*, this member is only expressed in the proximal tubules. This is in agreement with the expression of its human ortholog, found only in the proximal tubules and thin descending limbs of Henle (Sakagami *et al*., 2005). This specific and strong expression in the kidney is also seen in the adult frog, emphasising the important roles that enpp6 could play in kidney physiology in vertebrates. *enpp3* is also expressed strongly in the adult kidney and *enpp1* and *enpp2b* transcripts can also be detected at low levels in this organ. Therefore, the kidney appears to be a major site of expression for the *enpp* gene family. In human, each *enpp* gene, except enpp7, is expressed in the adult metanephros (Bollen *et al*., 2000; Sakagami *et al*., 2005). This is consistent with the expression in that organ of LPA2, LPA4 and S1P1-4, bioactive lipid receptors (Contos *et al*., 2000; Awad *et al*., 2006) and also with the importance of the purinoreceptors (reviewed in Unwin *et al*., 2003), whose misexpression has been implicated in polycystic kidney disease (Turner *et al*., 2004). Purinergic signalling plays essential roles in both normal renal physiology and pathological renal cases (reviewed in Hovater *et al*., 2008).

Xenopus *is an appropriate model to study* enpp *gene function*

There is a high degree of conservation of expression profile of this family between frogs and higher vertebrates. For example, *enpp7* is only expressed in the frog small intestine, which is consistent with the mammalian gene expression (Duan *et al*., 2003) and *enpp6* transcripts are preferentially detected in the kidney, as its human ortholog (Sakagami *et al*., 2005). Therefore, we suggest that their functions will also be similar. We have recently shown that ectonucleotidases of the E-NTPDase family

are expressed in a developmentally regulated manner, and one member of this family, E-NTPDase2, is responsible for triggering eye development via its effects on purinergic signalling (Massé *et al*., 2006, 2007). This previously published work illustrates that the power of the amphibian *X. laevis* to establish previously unknown developmental signalling pathways during development. Functional analysis of the *enpp* genes family during kidney formation, one of the major sites of their expression, is now in process in the laboratory, using a combination of morpholino knock-down and over-expression strategies.

Material and Methods

Bioinformatics

X. laevis and *X. tropicalis* EST clones were identified by a TBLASTN (Basic Local Alignment Search Tool) search on the NCBI EST others databases using the sequence from the orthologous human proteins (Altschul *et al*., 1990). Genomic *X. tropicalis* sequences were identified by the same search on the Joint Genome Institute (JGI) website (http://genome.jgi-psf.org/Xentr4/Xentr4.home.html). Accession numbers of all sequences used in this study are given in Supplementary Table 1B. Pairwise alignments were performed using the Needle program based on Needleman-Wunsch global alignment algorithm (European Bioinformatics Institute website) (Needleman and Wunsch, 1970). Multiple alignment was performed using the software CLUSTALW (Thompson *et al*., 1994). For phylogeny studies, catalytic domain sequences were aligned using the GeneBee algorithm and a phylogenetic tree produced using the PHYLIP program (Brodsky *et al*., 1992) Conceptual translation of cDNA and protein sequence analysis was performed on the ExPaSy website http://www.expasy.org/tools/#ptm).

DNA

The identified I.M.A.G.E clones were ordered from the Geneservice Ltd Cambridge (Supplementary Table 1B). DNA was extracted from several individual colonies, verified by restriction analysis and sequenced from both strands. When required, primers were designed to complete the sequencing of each clone.

Embryo culture and dissections

Culture of the embryos was performed as described previously (Massé *et al.,* 2004). The embryos were staged according to Nieuwkoop and Faber, 1994.

RT-PCR

RNA extraction from embryonic and adult tissues and cDNA synthesis were performed as described by Barnett *et al*., 1998. For each gene, specific primers were designed based on the *X. laevis* sequences (Supplementary Table 2). Each PCR was optimised and verified by sequencing as described previously. PCR was then carried out with non-radioactive nucleotides according to details in Supplementary Table 2. For each experiment, the quantity of input cDNA was determined by equalisation of the samples with a constant gene, either *ODC* or *Ef1α*. Linearity of signal was controlled by carrying out PCR reactions on doubling dilutions of cDNA, illustrated by the triangle. Negative controls were performed as indicated.

Whole-mount in situ *hybridisation*

Whole-mount *in situ* hybridisation was performed essentially as described in Harland, 1991 with several modifications. In order to minimise the cross-reactions between homologs, specific probes were designed in the 3'UTR of each gene. However, since *X. laevis enpp1*, *enpp3* and *enpp7* UTR sequences are not available, templates from the coding region of these genes were used to generate the antisense probes. In each case, sense probes were used to control the specificity of the staining. Details of the probes are documented in Supplementary Table 3**.** Each probe was made using a DIG RNA labelling kit (Roche) and the original DNA template was carefully removed after incubation with DNaseI. Several washes in MAB buffer (0.1M Maleic Acid, 0.15M NaCl) were performed following hybridisation of the probe. Hybridised probes were detected with a pre-absorbed anti DIG alkaline phosphatase-labelled antibody. The coloration reaction was performed with BM-Purple (Roche).

Embedding and sectioning

After whole-mount *in situ* hybridisation, enpp7 stained embryos were embedded in acrylamide as described by Seville *et al*., 2002. The blocks were sectioned on a microtome at 28 µm thickness. Transverse sections were also performed with a razor blade on fixed embryos.

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