

The lysophosphatidic acid (LPA) and sphingosine-1-phosphate (S1P) receptor gene families: cloning and comparative expression analysis in *Xenopus laevis*

KARINE MASSÉ[#], JUN-ICHI KYUNO, SURINDER BHAMRA and ELIZABETH A. JONES*

Department of Biological Sciences, Warwick University, Coventry, U.K.

ABSTRACT Sphingosine-1-phosphate (S1P) and lysophosphatidic acid (LPA) are endogenous bioactive lipids which mediate a variety of biological cell responses such as cell proliferation, migration, differentiation and apoptosis. Their actions are mediated by binding to the G-protein-coupled endothelial differentiation gene (Edg) receptor subfamily, referred to as S1P1-5 and LPA1-5, and regulate a variety of signalling pathways involved in numerous physiological processes and pathological conditions. Their importance during embryogenesis has been demonstrated by the generation of knock-out mice and specific roles have been assigned to these receptors. However, potential functional redundancy and the lethality of some mutants have complicated functional analysis in these models. Here we report the cloning of the S1P and LPA receptors in *Xenopus laevis* and *tropicalis*. Phylogenetic analyses demonstrate the high level of conservation of these receptors between amphibian and other vertebrate species. We have conducted a comparative expression analysis of these receptors during development and in the adult frog, by both RT-PCR and whole mount *in situ* hybridisation. In particular, we show that S1P1, 2 and 5 display distinct embryonic specific expression patterns, suggesting potentially different developmental roles for these receptors, and therefore for their ligands, during amphibian embryogenesis.

KEY WORDS: *S1P receptors, LPA receptors, Xenopus, expression pattern, lipidic signalling*

Introduction

Besides their roles as structural components of cell membranes, sphingolipid and lipid phosphate molecules, such as sphingosine-1-phosphate (S1P) and lysophosphatidic acid (LPA), regulate diverse biological processes including cell proliferation, cell growth, cell survival, cell migration and cell morphogenesis (Spiegel and Milstien, 2003). Initially these molecules were identified as intracellular signalling components, but their roles as extracellular factors have now also been demonstrated with the discovery of specific cell membrane bound receptors and the evidence of their secretion by cells such as platelets. These discoveries have widened their potential range of physiological functions.

S1P and LPA can signal through a family of related orphan-G-protein-coupled receptors (GPCR), known as Endothelial Differentiation Genes (EDG) (Takuwa *et al.* 2002; Anliker and Chun,

2004). Lipid receptors are coupled to at least three major G protein families (G_i , G_q , G_{12}) and their interaction regulates the activity of numerous intracellular messengers through the activation or inhibition of the PLC/IP3/ Ca^{2+} , adenylyl cyclase, MAPK pathways and the small GTPase Rho, Rac and Ras proteins. At least 10 receptors have been identified in mammals, are grouped into two subfamilies depending on their ligands and have been renamed following NC-IUPHAR nomenclature guidelines (Lynch, 2002; Chun *et al.* 2002). The first subfamily, whose receptors mainly bind S1P as their high affinity ligand, includes S1P1/EDG1, S1P2/EDG5, S1P3/EDG3, S1P4/EDG6 and S1P5/EDG8

Abbreviations used in this paper: edg, endothelial differentiation gene; EST, expressed sequence tag; GPCR, G-protein coupled receptors; LPA, lysophosphatidic acid; RT-PCR, reverse transcription-polymerase chain reaction; S1P, sphingosine-1-phosphate; SPC, sphingosylphosphorylcholine.

***Address correspondence to:** Elizabeth A. Jones. Department of Biological Sciences, Warwick University, Coventry, CV4 7AL, U.K. Fax: +44-247-652-3701. e-mail: elizabeth.jones@warwick.ac.uk **#Present address:** Université Bordeaux, UMR-CNRS 5164, 33076 Bordeaux Cedex, France.

Supplementary Material for this paper is available at: <http://dx.doi.org/10.1387/ijdb.103068km>

Accepted: 11 March 2010. *Final author corrected PDF published online:* 2 July 2010. *Edited by:* Roberto Mayor.

ISSN: Online 1696-3547, Print 0214-6282

© 2010 UBC Press
Printed in Spain

(reviewed in Kluk and Hla, 2002; Sanchez and Hla, 2004). Sphingosylphosphorylcholine (SPC) can be a low affinity ligand for these receptors however LPA or ceramide do not compete with S1P for these receptors. The second subfamily includes LPA1/EDG2, LPA2/EDG4, LPA3/EDG7, LPA4/GPR23/P2Y9, LPA5/GPR92 and these receptors bind LPA with high affinity (Contos *et al.* 2000a). LPA1, 2 and 3 are closely related to the S1P receptors whereas the recently cloned LPA4 and 5 are evolutionary more distant and are referred to as non-Edg LPA receptors (Ishii *et al.* 2009). In adult mammals, LPA1-3 and S1P1-3 are ubiquitously expressed whereas the expression profile of S1P4-5 and LPA4-5 is more restricted (Anliker and Chun, 2004). For example, S1P4 is mostly detected in lymphoid and hematopoietic tissues and S1P5 in the central nervous system, spleen and skin (Sanchez and Hla, 2004). High level of expression of LPA4 is found in ovary whereas LPA5 is highly expressed in small intestine and the dorsal root ganglia (DRG) (Noguchi *et al.* 2003; Lee *et al.* 2006).

The broad tissue distribution of these receptors and their potential coupling to several G-protein subfamilies, suggest that S1P and LPA are involved in a multitude of physiological functions, such as angiogenesis, neurogenesis, myelination, wound healing and cholesterol metabolism (Kluk and Hla, 2002; Sengupta *et al.* 2004). Moreover, crosstalk between bioactive lipids and other signalling pathways has been demonstrated. For example, S1P can activate VEGF (vascular endothelial growth factor) receptors, acting in angiogenesis, cell growth and movement (reviewed in Spiegel and Milstien, 2003). The S1P3 receptor and PDGF (platelet-derived growth factor) receptors can interact and lead to Akt activation (Baudhuin *et al.* 2004). A variety of evidence identifies emerging roles for these receptors in pathologies, such as cancer, diabetes, atherosclerosis, Alzheimer's disease, Down's syndrome and autoimmune diseases such as Multiple Sclerosis (Mills and Moolenaar, 2003; Sengupta *et al.* 2004; Brinkmann, 2007). Recent *in vivo* studies have demonstrated the crucial roles of LPA receptors during breast and colon cancer progression (Liu *et al.* 2009; Lin *et al.* 2009). Therefore, LPA and S1P receptors have become targets for developing new drugs for these pathologies (reviewed in Brinkmann, 2007; Zhang *et al.* 2009).

The specific functions of each LPA and S1P receptor have been elucidated by the generation of knock-out mice. These have demonstrated the pivotal role of lipid pathways during cardiovascular and neuronal development (Osborne and Stainier, 2003; Saba, 2004). To date, single, double or triple null-mice have been reported for most of the receptor genes (with the exclusion of *LPA4*, *LPA5* and *S1PA*) and have demonstrated some specific functions for each receptor (reviewed in Brinkmann, 2007; Choi *et al.* 2008). LPA1-null mice show 50% perinatal lethality, due to suckling defects, and the survivors exhibited abnormal phenotypes such as craniofacial abnormalities (Contos *et al.* 2000b). Cortical development was affected in a spontaneous variant of these original knock-out mice (Estivill-Torrús *et al.* 2008). Analysis of the LPA3 mutant mice demonstrates the importance of this receptor in embryo implantation (Ye *et al.* 2005). Redundant functions for LPA receptors have been suggested since no obvious phenotype can be observed in LPA2-null mice, and LPA1/LPA2 double-null mice showed no additional phenotype to that of LPA1-null mice (Contos *et al.* 2002). S1P1 null-mice have the most severe phenotype, with death *in utero* caused by embryonic hemorrhage (Liu *et al.* 2000). Conditional S1P1 mutants demonstrated a role in maturation of

vascular endothelial cells and in lymphocyte egress (Allende *et al.* 2003, 2004; Matloubian *et al.* 2004; Kabashima *et al.* 2006). Despite roles of S1P2 in heart development suggested from the zebrafish mutant "miles apart" (Kupperman *et al.* 2000), no such phenotype is observed in the S1P2 null-mice (Ishii *et al.* 2002) even though loss of S1P2 induced vascular dysfunction in adult mice (Lorenz *et al.* 2007). However, the generation of three independent mutant mice strains demonstrated the fundamental role of S1P1 in the maintenance of auditory and vestibular systems since hearing deficiencies resulted in deafness (MacLennan *et al.* 2006; Kono *et al.* 2007; Herr *et al.* 2007). S1P3 null mice displayed no gross phenotype although some S1P-mediated responses were impaired (Ishii *et al.* 2001). Generation of double and triple mutant mice for these 3 S1P receptors confirmed their redundancy *in vivo* (Ishii *et al.* 2002; Kono *et al.* 2004). Fertile S1P5 knock-out mice confirmed the role of this receptor in the immune system but failed to display any evident neuronal defects despite its influence on oligodendrocyte development (Jaillard *et al.* 2005; Walzer *et al.* 2007).

Spatial-temporal concentrations of LPA and S1P are tightly regulated by several enzymes involved in either the anabolism or catabolism of these lysophospholipids (Spiegel and Milstien, 2003; Saba, 2004). The complexity of lipid signalling increases further due to intersections between LPA and S1P metabolism (Saba, 2004). Several members of the nucleotide pyrophosphatase/phosphodiesterase (ENPP) family, a subfamily of ectonucleotidases involved in the regulation of purinergic signalling, catalyse the generation of these bioactive lipids (reviewed in Stefan *et al.* 2005). ENPP2 or autotaxin is a lysophospholipase D (lysoPLD) which generates LPA from lysophosphatidylcholine (LPC) but also S1P from SPC (reviewed in Moolenaar *et al.* 2004; van Meeteren and Moolenaar, 2007). ENPP6 is a choline-specific glycerophosphodiesterase which produces phosphocholine by degrading choline-containing compounds such as LPC and SPC (Sakagami *et al.* 2005) and ENPP7 is the intestine-specific alkaline sphingomyelinase (alk-SMase) which hydrolyses sphingomyelin (SM) into ceramide and sphingosine (Duan, 2006).

The LPA and S1P pathways have been conserved during evolution and their roles demonstrated from yeast to invertebrates and vertebrates even though the Edg receptors have not been identified in invertebrates (Spiegel and Milstien, 2003; Oskouian and Saba, 2004). Due to the complexity of the lipidic pathways and the potential redundancy *in vivo* of the functions of their diverse components, we opted to analyse their function during development using a simple but very well established vertebrate model, the amphibian *Xenopus laevis*. We have cloned all the amphibian *enpp* members and analysed their expression during *X. laevis* development (Massé *et al.* 2010). The distinct expression profile of *enpp2*, *enpp6* and *enpp7* suggests specific roles for these genes during embryogenesis and therefore for the lipids generated by their catalytic activity. Here, we report the cloning and characterisation of the 10 *edg* receptors of the LPA and S1P families in *X. laevis* and *X. tropicalis*. We compare their temporal and spatial expression during development and also their distribution in adult frog tissues. The developmentally restricted and dynamic distributions of these receptors strongly suggest that the LPA and S1P pathways might play important and diverse developmental roles, and together with our previously published work, provide the tools for the functional analysis of

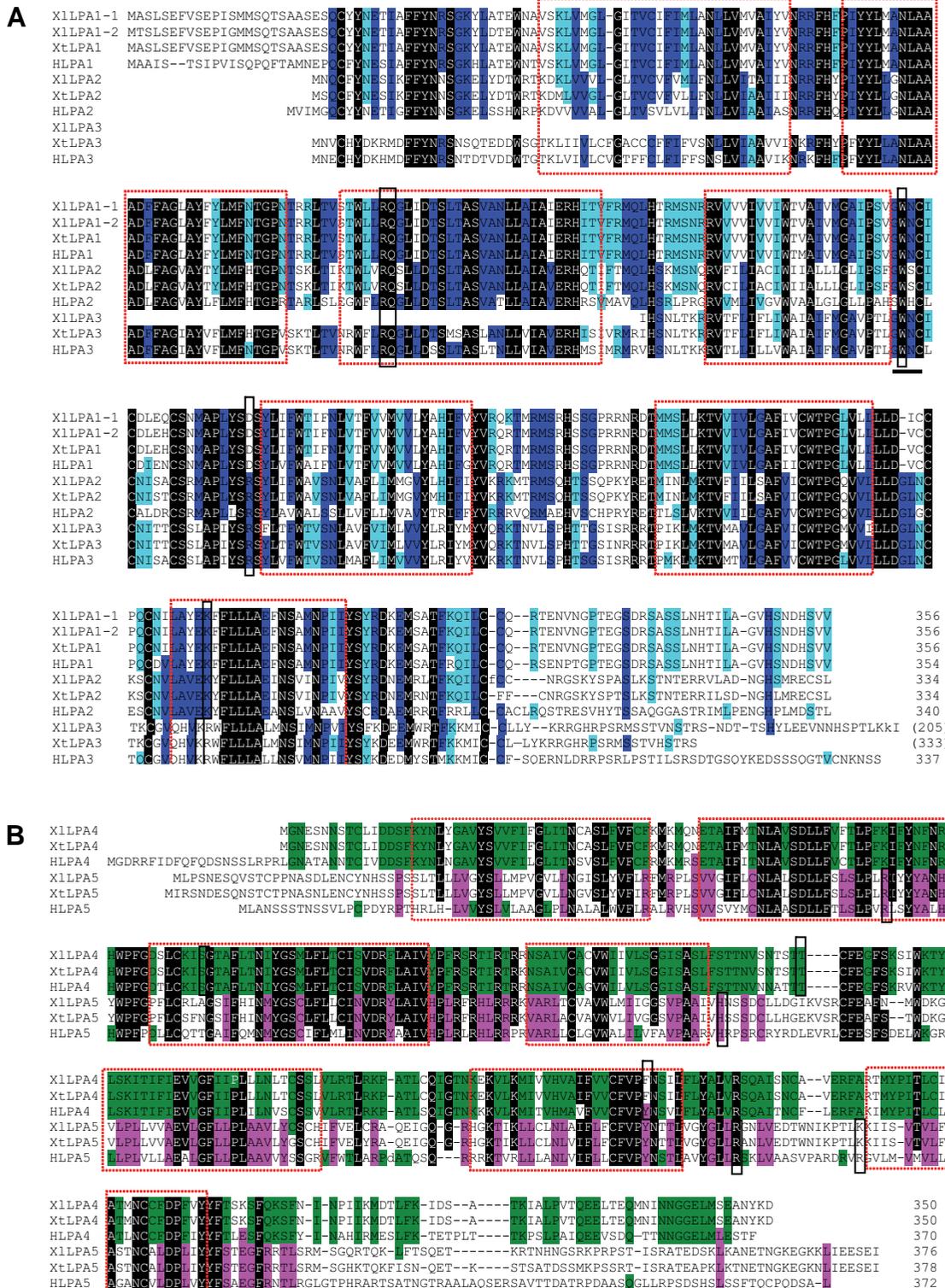


Fig. 1. Protein sequence alignment of *Xenopus* lysophosphatidic acid (LPA) receptors. Alignment of the LPA1-3 (A) or LPA4-5 (B) proteins was performed using CLUSTALW. The *X. laevis* (Xl) and *X. tropicalis* (Xt) proteins were aligned with their human orthologs (H). The Genbank accession numbers of the human LPA receptors are given in the legend of Fig. 3. The size of the proteins (AA numbers), complete or incomplete (in bracket) is also given. Spaces in the sequences are indicated by a dash. The 7 transmembrane domains are indicated by a red box. The amino acids involved in LPA binding are highlighted by the boxed regions. (A) Residues conserved in all sequences are indicated by a black background. Residues conserved in at least one group of orthologs are indicated by a light turquoise background. Residues conserved in at least two groups of orthologs are indicated by a dark blue background. The GWNC motif is underlined. (B) Residues conserved either in all sequences or in 5 out of the 6 proteins are indicated by a green background. Residues conserved in LPA4 sequences are indicated by a light grey background. Residues conserved in LPA5 sequences are indicated by a pink background.

these pathways during development (Massé *et al.* 2010).

Results

Cloning of the different receptor genes

A variety of strategies, as described in Supplementary Table

1A, were used in order to clone the different members of *LPA* and *S1P* gene families. TBLASTN search of the *X. laevis* and *tropicalis* databases on the NCBI website allowed the identification of full length I.M.A.G.E clones encoding several receptors (Accession numbers given in Supplementary Table 1B). *X. laevis* LPA1.1 and LPA1.2 and *X. tropicalis* LPA2 sequences have been

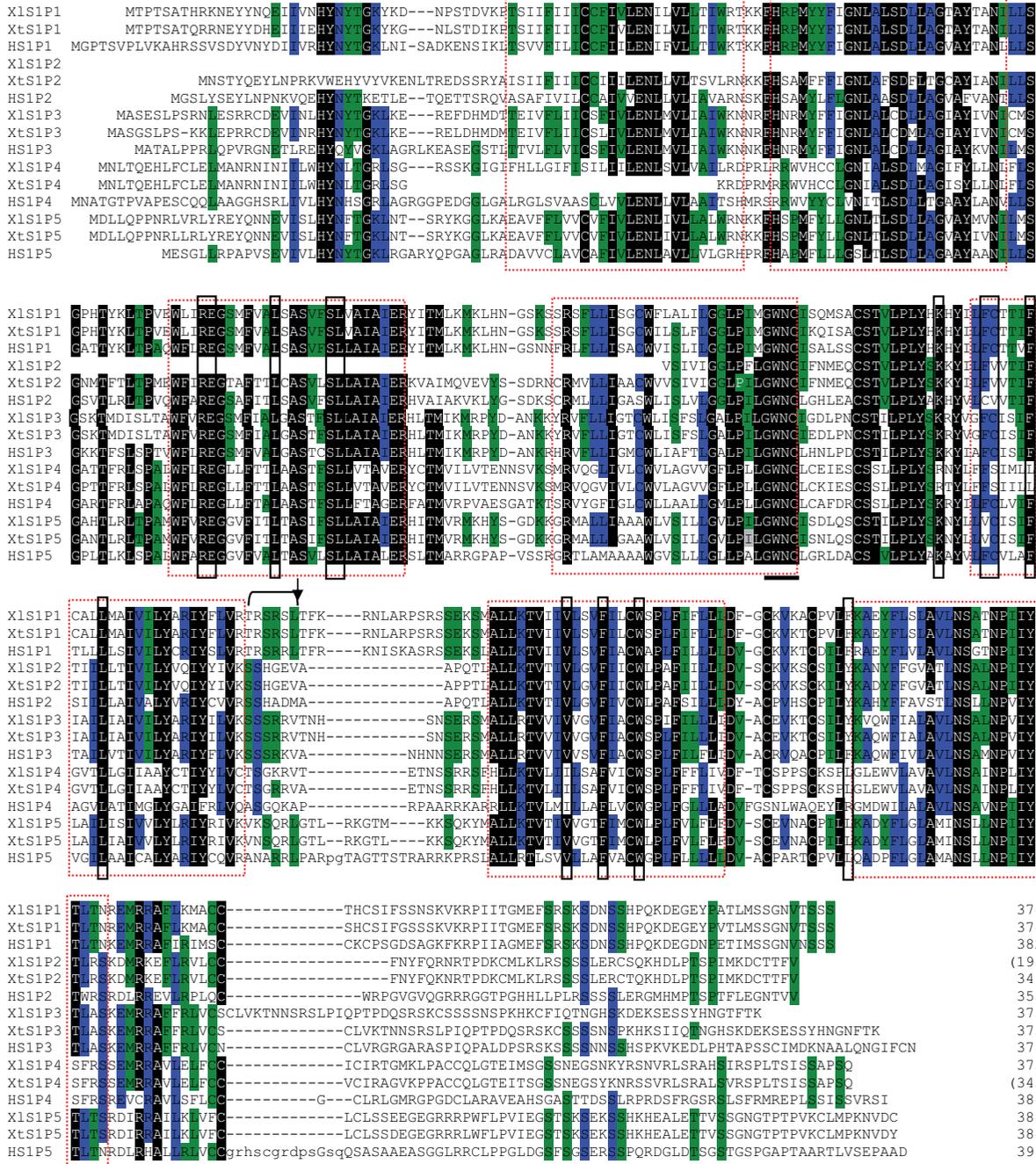


Fig. 2. Protein sequence alignment of *Xenopus* sphingosine-1-phosphate (S1P) receptors. The *X. laevis* (Xl) and *X. tropicalis* (Xt) proteins were aligned with their human orthologs (H) using CLUSTALW. Residues conserved either in all sequences or at least four of the five orthologs are indicated by a dark background. Residues conserved in at least three groups of orthologs are indicated by a blue background. Residues conserved in two groups of orthologs are indicated by a green background. The seven potential transmembrane domains (TMs) indicated by a red box. The S1P1 characteristic (RxRxT/S) motif is indicated by a bracket. The arrow indicates the phosphorylation site by the B/Akt protein kinase. The GWNC motif is underlined. The amino acids involved in the S1P binding are boxed. Spaces in the sequences are indicated by a dash. The Genbank accession numbers of the human S1P receptors are given in the legend of Fig. 3. The size of the proteins (AA numbers), complete or incomplete (in bracket) is also given.

previously published (Kimura *et al.* 2001; Lloyd *et al.* 2005). For the other receptors, *X. laevis* or *X. tropicalis* ESTs were identified and alignments of these sequences were performed to generate the consensus but incomplete sequence of their cDNA. Sequences were also 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 *X. tropicalis* embryonic and adult tissues to amplify the missing sequences. This work allowed us to obtain the complete protein sequences for all receptors except *X. laevis* and *X. tropicalis* LPA3, *X. laevis* S1P2 and *X. tropicalis* S1P4 (Figs. 1 and 2).

Protein and phylogenetic analysis of the LPA and S1P families

The *Xenopus* sequences of these receptors are highly similar (Figs. 1A,B and 2). The *X. laevis* and *X. tropicalis* orthologs share more than 95% identity and more than 97% similarity with the exception of LPA5 (88.4% identity and 92.1% similarity). The two *Xenopus* LPA4 orthologs share 99.4% identity and 100% similarity. However, the percentage of identity between the different members of these families is less than 60% in the same species. *X. laevis* LPA1.1 (or LPA1.2) shares 55% identity with LPA2 but only 20% identity with LPA4 or LPA5 sequences. *X. tropicalis* LPA3 shares approximately 50% identity with *X. tropicalis* LPA1 or LPA2 and only approximately 20% with *X. tropicalis* LPA4 or LPA5. The percentage of identity along the five S1P receptors in *X. laevis* or *X. tropicalis* is higher, between 30 to 50%, with S1P1,

3 and 5 sharing a higher percentage of identity. For example, *X. laevis* S1P1 share 49% and 49.7% identity with S1P3 and S1P5 respectively and only 35.3% with S1P4. *X. tropicalis* S1P1 and S1P2 share 44.7% identity. The lowest percentage of identity is between *X. laevis* S1P4 and S1P5 proteins (34.2%).

A phylogenetic analysis of these receptors in *Xenopus* and other vertebrates was carried out (Fig. 3, Supplementary Fig. 1). Clustering of these sequences demonstrates the clear separation of LPA from S1P proteins (Fig. 3). However, LPA1, 2, 3 are more related to S1P receptors than LPA4 and 5. Indeed, *Xenopus* LPA4 or LPA5 receptors only share approximately 20% identity with the other LPA and S1P receptors. By contrast, LPA1, 2 and 3 share 30% identity with the five S1P receptors in *Xenopus*. Among S1P receptors, S1P1, 3 and 5 are more related to each other than S1P2, with S1P4 appearing the most divergent member. *Xenopus* S1P4 shares only around 35% with the other four S1P receptors. 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. It is worth pointing out that the sequences of human S1P5 and zebrafish LPA2 in the database do not cluster with their orthologs, suggesting a potential divergence of the roles for these receptors during vertebrate evolution. However, a more complete phylogenetic analysis of the S1P5 receptors using the Ensembl database revealed that the *Xenopus tropicalis* sequence has been annotated as S1P5 protein, ruling out a potential mis-identification of the *Xenopus laevis* protein.

The percentage of identity between the members of an orthologous group of proteins is very variable; LPA1 is the most conserved receptor and LPA5 and S1P5 the least conserved

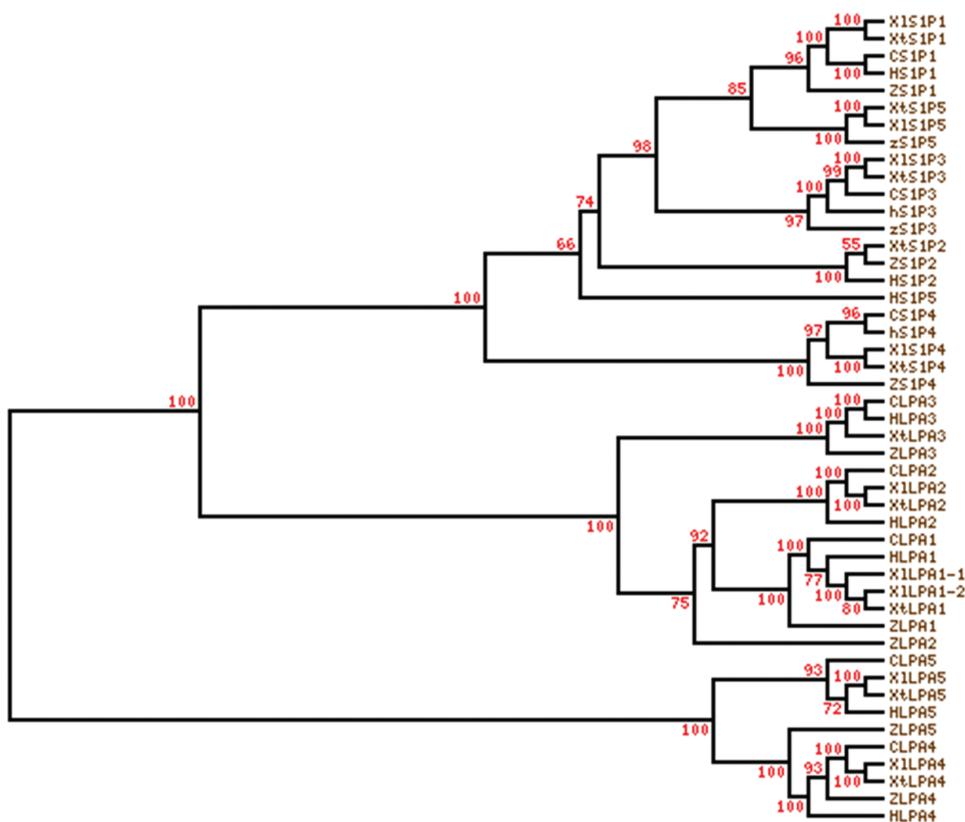


Fig. 3. Phylogenetic conservation of the lipidic receptors. A phylogenetic tree for the LPA and S1P receptors was constructed using the PHYLIP program. A cluster algorithm was used to build the tree. Bootstrap values are indicated at each node of the tree. The Genbank accession numbers of the different receptors are as follows: human LPA1: NP_001392; human LPA2: NP_004711; human LPA3: AAF91291; human LPA4: NP_005287; human LPA5: NP_065133; human S1P1: NP_001391; human S1P2: NP_004221; human S1P3: NP_005217; human S1P4: AAH14940; human S1P5: NP_110387; chick LPA1: ABY60418; chick LPA2: NP_001108553; chick LPA3: ABY60420; chick LPA4: XP_420150; chick LPA5: XP_416504; chick S1P1: XP_001231780; chick S1P3: XP_428818; chick S1P4: NP_001112363; zebrafish *lpa1*: NP_001004502; zebrafish *lpa2*: NP_001032783; zebrafish *lpa3*: XP_694990; zebrafish *lpa4*: XP8001334713; zebrafish *lpa5*: NP_001073524; zebrafish *s1p1*: NP_571766; zebrafish *s1p2*: XP_682912; zebrafish *s1p3*: XP_001345724; zebrafish *s1p4*: AAH66587; zebrafish *s1p5*: NP_001007317. The *X. laevis* LPA3 and S1P2 proteins were excluded since their sequence are incomplete. C: Chick; H: human; Xl: *X. laevis*; Xt: *X. tropicalis*; Z: Zebrafish.

members during vertebrate evolution. As shown in Supplementary Fig. 1, the mammalian, fish and amphibian S1P5 receptor sequences have diverged more during evolution than the other S1P members. *Xenopus* LPA1.1 and LPA1.2 share more than 90% identity with their mammal and chick orthologs and 85% with their zebrafish ortholog. *Xenopus* S1P5 and LPA5 only share 40 to 50% identity with their orthologs. The other receptors share more than 70% identity with their orthologs, except LPA2 and S1P2 for which the percentage of identity with the other vertebrate sequences is only around 60%. Except for S1P2 and S1P5, the zebrafish and *Xenopus* sequences share the lowest percentage of identity.

Temporal expression of LPA and S1P gene families during development of *Xenopus laevis*

The temporal expression of these genes during development was assessed using gene specific primers by RT-PCR as described in Supplementary Table 2 (Fig. 4). The S1P receptors are not expressed maternally, with the exception of S1P3 whose expression remains at a constant level during development at all the stages tested, except between stages 27 to 37. This apparent drop of expression was confirmed in another independent experiment, but its physiological significance remains unknown. If the numbers of cycles is increased, very weak maternal expression can be detected for S1P1 and S1P2. Zygotic expression of S1P1 and S1P2 is detectable weakly at stage 10.5 and their level of expression increases until stage 45. S1P5 expression is also detectable during gastrulation and remains expressed until stage 45 at a similar level. S1P4 is the only family member whose transcripts cannot be detected at any stage in this experiment.

All LPA genes, however, are expressed maternally with the exception of LPA3. The expression of LPA1.1, LPA1.2 and LPA4 remains at a similar level from the oocyte to stage 45, the last

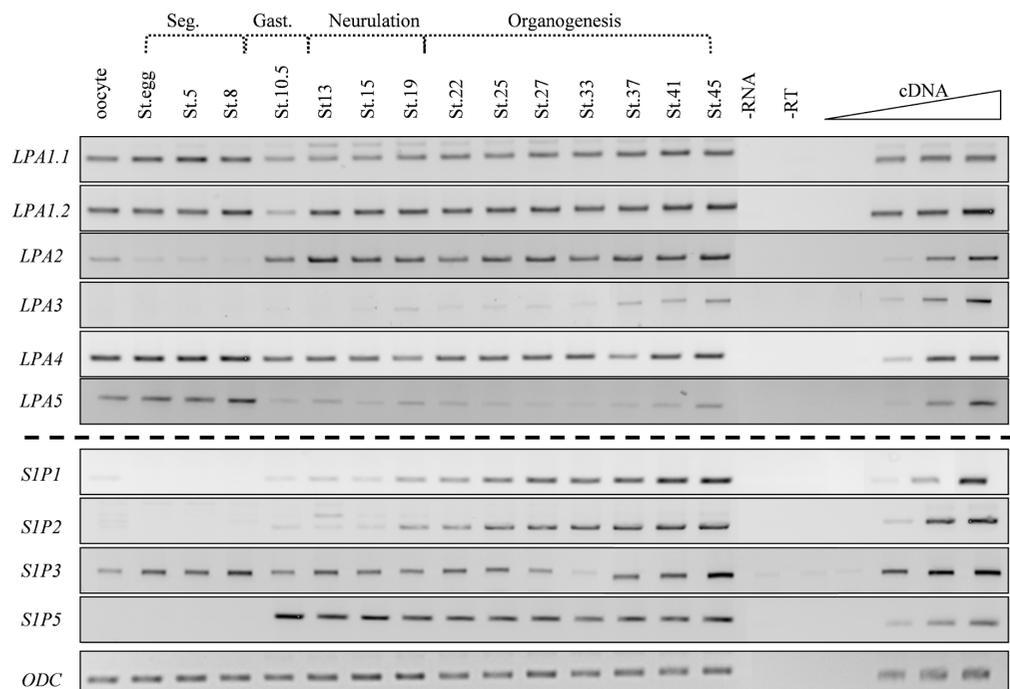
stage tested in this study. Maternal expression of LPA2 decreases quickly to an almost undetectable level at stage 8 and its zygotic expression is detected from gastrulation throughout development. LPA5 expression is the highest before MBT but its zygotic transcripts can be amplified until stage 45. Zygotic expression of LPA3 is switched on weakly during neurulation with an increase of expression at stage 37. However, its expression can be weakly detected during gastrulation with an increase of cycle numbers.

Spatial expression of LPA and S1P gene families during development of *Xenopus laevis*

The spatial expression of these genes in the embryo was assessed by two different complementary techniques. *In situ* hybridisation allows the identification of domains of high expression of genes; RT-PCR on the other hand identifies regions of both high and low expression domains in dissected embryos. *In situ* hybridisation was performed on embryos from stage 6 to stage 40/41 with specific antisense probes (see Supplementary Table 3). Sense probes were used as controls.

Zygotic expression of S1P1, S1P2 and S1P5 genes can be detected by *in situ* hybridisation and these receptors display distinct and different expression profiles during development (Fig. 5 A-C). S1P4 expression was not analysed by *in situ* hybridisation as its expression was only very weakly detected by RT-PCR. S1P1 expression is detected from stage 23 in the nervous system, in the brain and the neural tube as seen on cleared embryos (Fig. 5Ai,ii). This expression in the nervous system is more intense at stage 27 (Fig. 5-Aiii). Expression in the otic placode can also be observed from this stage. Neural expression can be more precisely observed in the forebrain, midbrain, hindbrain and spinal cord from stage 32 (Fig. 5-Av, vi). Strong expression can be detected in all the rhombomeres of the hindbrain from stage 37

Fig. 4. Temporal expression profile of LPA and S1P families genes. RT-PCR analysis showing expression pattern of the receptor transcripts in *Xenopus laevis* unfertilised eggs and embryos. Expression of the LPA1.1, LPA1.2, LPA4 and S1P3 genes is constant during development. Expression of S1P1, 2 and 5 is first detected at stage 10.5 and increases until stage 45, the latest stage tested whereas the expression of S1P5 remains at approximately the same level during development. LPA2 is a maternal gene; its zygotic expression is also switched on at stage 10.5 and expressed until stage 45. Expression of LPA3 can be weakly detected during neurulation and increases until stage 45 whereas LPA5 is expressed at its highest level during maternal stages. S1P4 transcripts cannot be detected in this experiment. ODC was used as a loading control. The linearity was performed with doubling dilutions of cDNA from stage 45 embryos except stage 5 for LPA5, stage 15 for S1P5 and stage 25 for LPA2. The major phases of embryogenesis are indicated. Seg: Segmentation; Gast: Gastrulation.



ODC was used as a loading control. The linearity was performed with doubling dilutions of cDNA from stage 45 embryos except stage 5 for LPA5, stage 15 for S1P5 and stage 25 for LPA2. The major phases of embryogenesis are indicated. Seg: Segmentation; Gast: Gastrulation.

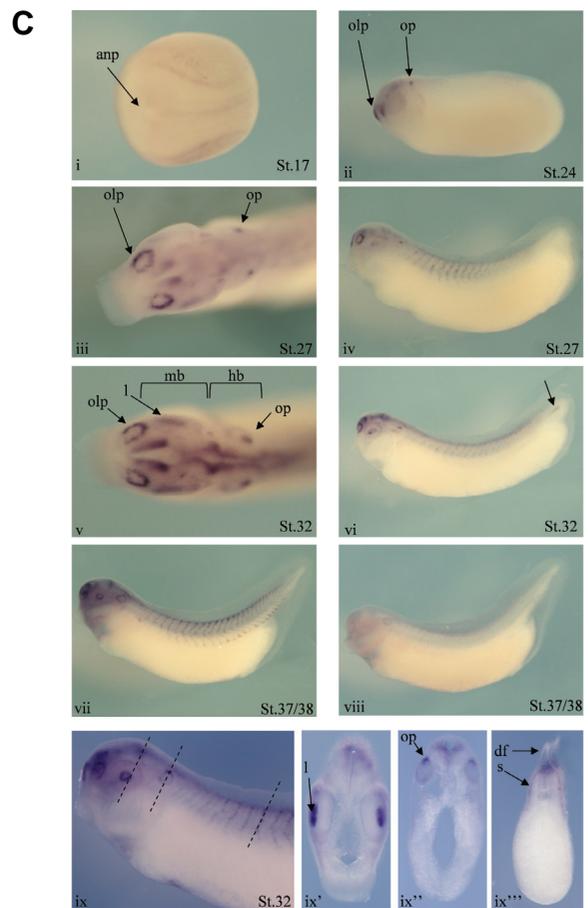
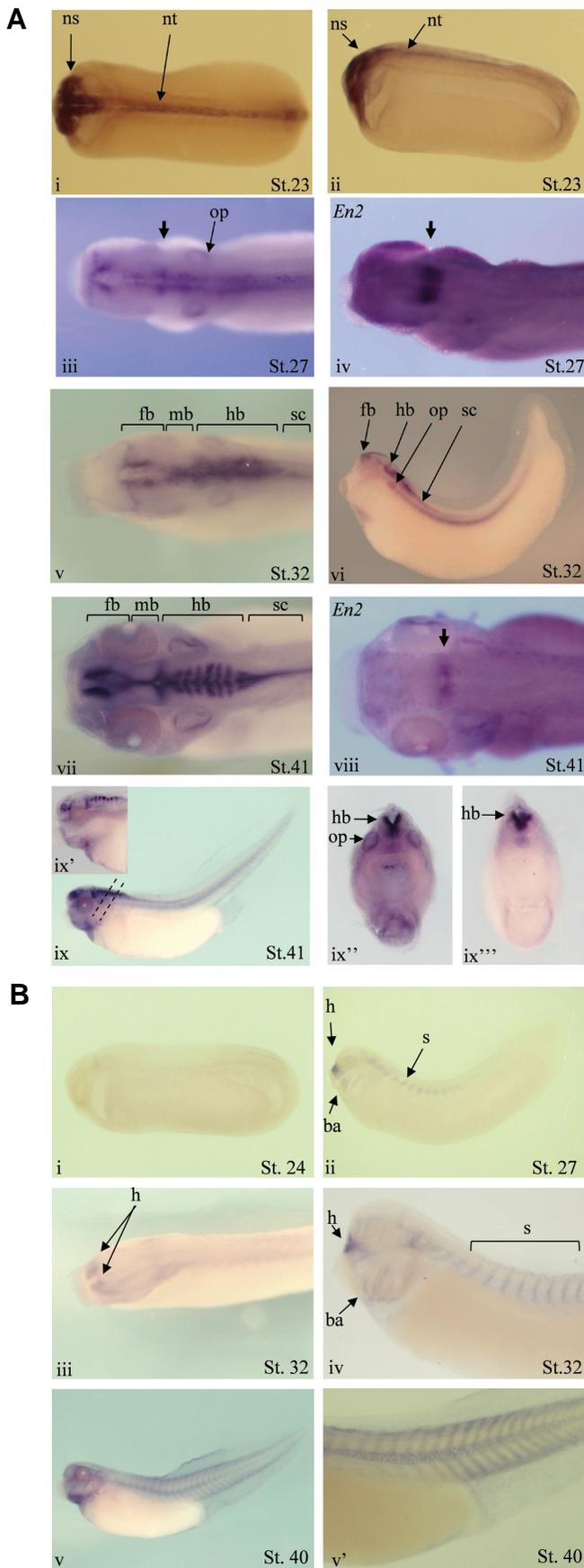


Fig. 5. Embryonic spatial expression profile of S1P genes. Whole mount in situ hybridisation with DIG-labelled RNA probe was performed on embryos from stages 6-41 for S1P genes, except for S1P4. **(A)** Whole mount in situ hybridisation analysis of S1P1 expression. Dorsal and lateral views of a cleared embryo at stage 23 (i, ii), of uncleared embryos at stage 27 (iii), at stage 32 (v, vi), at stage 41 (vii, ix). Sagittal section (ix') of the head at stage 41. Transverse sections (ix'', ix''') at stage 41. The dotted lines through the embryo (ix) correspond to planes of the transverse sections. Dorsal view of a stage 27 (iv) and stage 41 (viii) embryo stained with En2 antisense probe. The arrow indicates the midbrain/hindbrain boundary marked by En2 expression. For the lateral views, dorsal is up and anterior is left. fb: forebrain; hb: hindbrain; mb: midbrain; ns: neural system; nt: neural tube; op: otic placode; sc: spinal cord. **(B)** Whole mount in situ hybridisation analysis of S1P2 expression. Lateral view of cleared embryos at stage 24 (i), stage 27 (ii). Dorso-lateral view of an uncleared embryo (iii) and lateral view of a cleared embryo (iv) at stage 32. Lateral view of a uncleared embryo at stage 40 (v). Detail of the stained somites of a cleared stage 40 embryo (v'). For the lateral views, dorsal is up and anterior is left. ba: branchial arches; h: hypophyseal anlagen; s: somites. **(C)** Whole mount in situ hybridisation analysis of S1P5 expression. Anterior view of an embryo at stage 17 (i), lateral view at stage 24 (ii). Details of the head (iii) and lateral view (iv) of a stage 27 embryo. Details of the head (v) and lateral view (vi) of a stage 32 embryo. Lateral views at stage 37/38 of an embryo hybridized with the antisense probe (vii) or with the sense probe (viii). Lateral view of the stained stage 32 embryo (ix) used for the transverse sections (ix', ix'', ix'''). The dotted line through the embryo corresponds to plane of the sections. The arrow indicates the expression in the posterior notochord in the tail tip. For lateral views, dorsal is up and anterior is left. anp: anterior neural plate; df: dorsal fin; hb: hindbrain; l: lens; mb: midbrain; olp: olfactory placode; op: otic placode; s: somites.

until stage 41 (Fig. 5Avii,ix'). Sectioning of a stage 41 stained embryo showed that expression of *S1P1* is limited to the dorsal region of the rhombomeres (Fig. 5Aix'-ix'''). Transcripts are still detected at this late stage in the forebrain, midbrain, otic vesicle and spinal cord (Fig. 5Avii,ix-ix'''). No expression is detected with the sense probe in these tissues. Comparative *En2* *in situ* hybridisation analysis showed that *S1P1* is intensively expressed in the midbrain/hindbrain boundary from stage 27 to 41 (compare Fig. 5Aiii,iv and Fig. 5Avii,viii). However, whereas *En2* is more expressed at early stages, the level of expression of *S1P1* is higher at late stages.

S1P2 expression is first detected in tadpole embryos, since no staining can be observed before stage 27 (compare Fig. 5Bi,ii). At stage 27, expression is seen in the hypophyseal anlagen (Fig. 5Bii). This staining remains until stage 32 (Fig. 5Biii,iv). Expression of *S1P2* in the somites and in the branchial arches can also be detected from stage 27 in cleared embryos (Fig. 5Bii). *S1P2* remains expressed in these tissues until stage 40 (Fig. 5Biv-v'). *S1P2* is detected in vertical stripes along the somites but its expression is weaker in the presomitic mesoderm at the caudal end of the tadpole (Fig. 5Bv). Low levels of apparent expression in the notochord are non-specific as this level of staining was also observed with the sense probe (data not shown).

S1P5 transcripts can be detected from stage 17 during neurulation (Fig. 5Ci). At this stage, *S1P5* is expressed in a horse-shoe shaped domain around the neural plate, characteristic of cranial placode markers such as *Six1* (Schlosser, 2006). From tailbud stages, *S1P5* displays a restricted expression pattern with heavy staining in olfactory, optic and otic placodes (Fig. 5Cii-vii). Expression in the midbrain and hindbrain can also be detected from stage 32 (Fig. 5Cv-vii). Transient specific expression in the posterior part of the notochord in the tail tip can also be seen at stage 32 (arrow, Fig. 5Cvi). *S1P5* is also found in the forming somites from stage 28 to stage 37 (Fig. 5Ciii-vii). This expression profile is specific as no expression in the nervous system, placodes or in the somites can be detected with the sense probe (Fig.

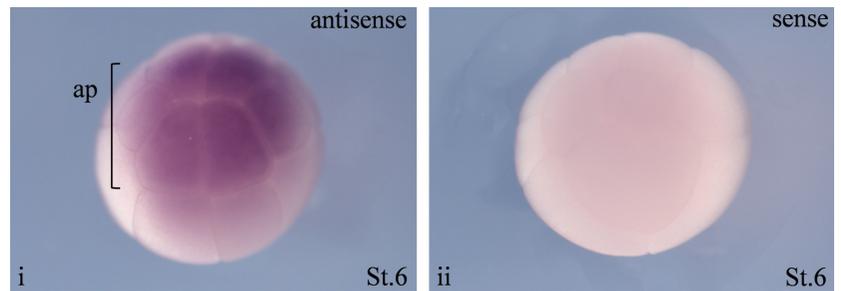


Fig. 6. Embryonic spatial expression profile of the *LPA5* gene. Whole mount *in situ* hybridisation with DIG-labelled antisense (i) and sense (ii) *LPA5* probes was performed. Expression was only detected at stage 6 in the animal pole (ap) of the embryos. Animal pole is up.

5Cviii). Sectioning of a stage 32 stained embryo (Fig. 5Cix) showed *S1P5* expression is limited to the lens (Fig. 5Cix'), to the dorsal region of the neural tube and the otic vesicle (Fig. 5-Cix''), to the nuclear region of the somites and the mesenchymal cells contributing to the dorsal fin (Fig. 5-Cix''').

Specific expression of the *LPA* genes was only detected for *LPA5* by *in situ* hybridisation, suggesting that these genes are expressed at low levels during development, below the level of detection of this technique. Maternal *LPA5* transcripts were detected in the animal pole of stage 6 embryos (Fig. 6Ai). No staining was detected with the sense probe (Fig. 6Aii). Zygotic *LPA5* transcripts were however not detected, as expected from the RT-PCR analysis.

RT-PCR was also performed on a series of embryo dissections in order to confirm the expression pattern of these genes (Fig. 7). This analysis is however not quantitative as the volumes of the input cDNAs in dissected samples were equalised with respect to *ODC* gene expression and thus the dissected samples do not represent the total expression of each gene in one embryo. However, this analysis completes and extends the *in situ* hybridisation analysis, especially for the *LPA* genes, whose expression is clearly at low levels in all tissues and was not detected by *in situ* hybridisation. *S1P1* and *2* transcripts are very weakly detected at stage 9 in the marginal zone of the embryo.

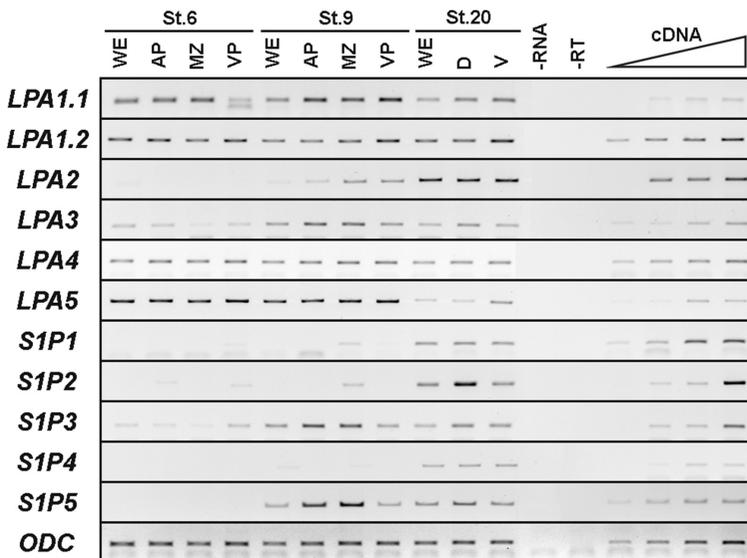


Fig. 7. Embryonic spatial expression profile of *LPA* and *S1P* families genes. RT-PCR analysis showing the spatial expression of the *LPA* and *S1P* genes during *Xenopus laevis* development. All the *LPA* genes appear to be expressed at the same level in the different parts of the embryos, regardless of the stage tested. Differences in the expression level can be observed for the *S1P* genes, with *S1P1* and *S1P2* only expressed in the marginal zone at stage 9, *S1P3* and *4* more expressed in the animal pole and marginal zone at this stage and *S1P2* more expressed in the dorsal region at stage 20. At stages 6 and 9, animal pole, marginal zone and vegetal pole containing respectively the presumptive ectoderm, mesoderm and endoderm were dissected from the embryos. At stage 20, the dorsal region of the embryo was separated from the ventral region of the embryo. *ODC* was used as a loading control. More cycles were added during the amplification in order to amplify genes found being expressed weakly at these early stages in Fig. 4. The linearity was performed with doubling dilutions of cDNA from stage 20 whole embryos. AP: Animal pole; D: Dorsal; MZ: Marginal zone; V: Ventral; VP: Vegetal pole; WE: Whole embryo.

Later on, only *S1P2* displays a differential expression between the dorsal and ventral region of the embryo, reflecting the staining in the forming somites observed by *in situ* hybridisation. *S1P3* appears less expressed in the vegetal pole at stage 9 although at stage 6, its transcripts can only be detected in this part of the embryo. *S1P4* expression can be weakly detected at stage 20, due to an increase of cycle number of the PCR in this experiment. Finally, *S1P5* is more expressed in the animal pole and marginal zone at stage 9 and later on, in the dorsal region, corresponding to the placodes, neural and somitic tissues.

All *LPA* genes are expressed ubiquitously in these dissected embryos, confirming the lack of any major sites of expression seen by *in situ* hybridisation. *LPA5* expression is the highest during early stages of development, confirming the previous results.

Spatial expression of LPA and S1P gene families in tissues of the adult frog

The spatial expression of the members of *LPA* and *S1P* families in the adult frog was analysed by RT-PCR. As shown in Fig. 8, the members of the *S1P* family display broad expression patterns. *S1P5* is ubiquitously expressed and *S1P2* is expressed in all tissues except blood cells. *S1P1* and *3* are also expressed in a variety of tissues with the exception of the reproductive organs for *S1P1* and stomach, pancreas and bladder for *S1P3*. *S1P4* is preferentially expressed in the spleen and bladder although transcripts can be weakly amplified from other tissues except eyes, blood cells, stomach, and ileum.

LPA receptors also display different expression patterns. The expression profile of *LPA1.1* and *LPA1.2* genes is quite similar being expressed in all tissues tested; however, the respective levels of their expression differs in some tissues. For example, *LPA1.1* is more expressed than *LPA1.2* in the blood cells and liver whereas the expression level of *LPA1.2* is higher

than *LPA1.1* in the eyes and bladder. *LPA2*, *4* and *5* appear to be expressed in a wide range of organs. However, their transcripts cannot be or are only very weakly detected in some tissues. *LPA2* is absent in blood cells and muscle, *LPA4* transcripts are not found in spinal cord, blood cells and ileum and *LPA5* cannot be detected in blood cells. *LPA3* displays a more restricted expression profile. Its transcripts can only be amplified in the eyes, bladder, and skin and very weakly in the brain and muscle.

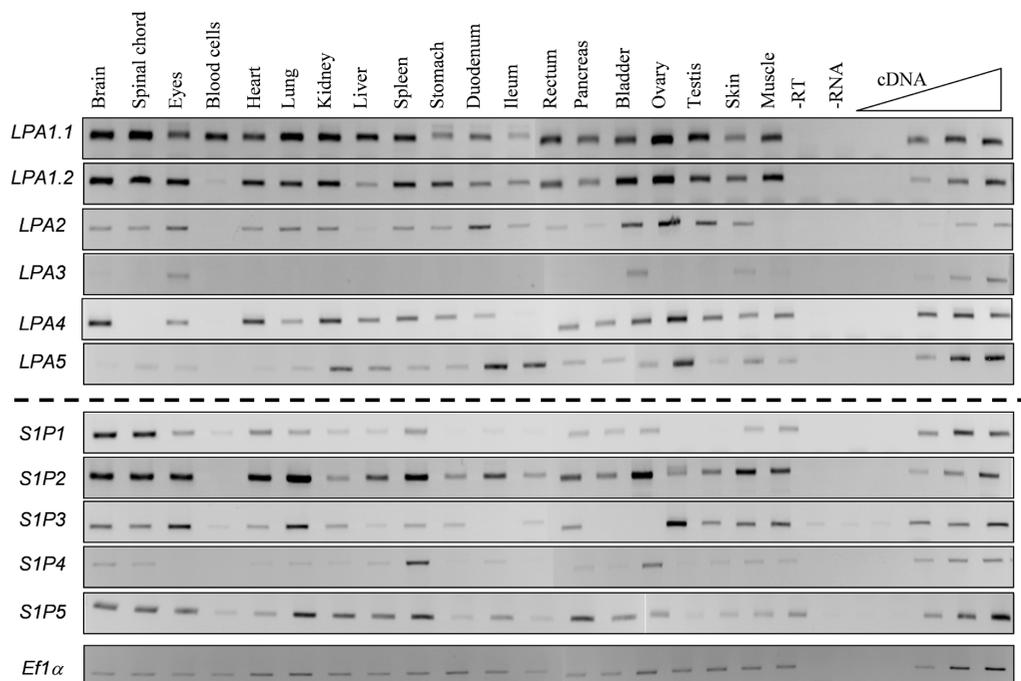
Our study demonstrates that the 10 S1P/LPA receptors display overlapping expression patterns, with several tissues, such as nervous system, spleen, lung, kidney, bladder expressing several lipidic receptors at high levels. However, in the blood cells, only *LPA1.1* transcripts can be detected.

Discussion

This paper reports the cloning of 10 members of the *edg* gene family encoding the LPA and S1P receptors in *Xenopus* and the analysis of their expression profiles during development and in adult frog tissues. During this study, a complementary, systematic study of these receptors during mouse development was published (Ohuchi *et al.* 2008). Although some similarities can be drawn between these two studies, several differences in the expression profiles indicate potentially distinct roles that these receptors might have acquired during vertebrate evolution. The adult expression of the 10 LPA/S1P receptors have been characterised in mammals allowing comparison with their expression patterns in the adult frog tissues (reviewed in Anliker and Chun, 2004; Meyer zu Heringdorf and Jakobs, 2007). Our analysis indicates that the expression profile of these genes has been conserved during evolution and as in mammals, with the exception of *LPA3* and *S1P4*, these receptors are widely expressed. Our study is the first to describe the comparative expression profile of these 10 receptors

Fig. 8. Spatial expression profile of LPA and S1P families genes in the adult frog.

RT-PCR was performed with total RNA extracted from *Xenopus laevis* adult tissues. Each gene of the two families shows a distinct expression pattern. *LPA1.1* and *LPA1.2* present a similar ubiquitous expression profile. *LPA2*, *4* and *5* were found expressed in almost all tissues studied whereas the expression of *LPA3* is very specific being expressed in only 3 tissues. The S1P family members are also expressed in almost all tissues studied, with the exception of *S1P4* which has high levels of expression in spleen and bladder. *EF1α* was used as a loading control. The linearity was performed with doubling dilutions of cDNA from the following tissues: bladder for *LPA3*; brain for *S1P1*; kidney for *LPA1.1*, *LPA1.2* and *LPA2*; lung for *S1P5*; muscle for *S1P2*; ovary for *S1P3*, *LPA4* and *LPA5*; spleen for *S1P4*.



in adult tissues and to compare these patterns with those observed during embryogenesis.

Evolutionary conserved members of the LPA and S1P families are present in *Xenopus*

Our study reveals that all the identified members of these bioactive lipidic receptors are present in *X. laevis* and *X. tropicalis*. *Xenopus laevis* LPA1.1 and LPA1.2 and *Xenopus tropicalis* LPA2 receptors have already been cloned (Kimura *et al.* 2001; Lloyd *et al.* 2005). Although the sequences for several LPA/S1P receptors were available in databases, we cloned cDNAs for 8 of these receptors by *in silico* analysis, due to the increased coverage of ESTs and the *X. tropicalis* genomic DNA sequencing projects. In particular we identified sequences for *X. tropicalis* LPA3 and S1P3 that previous studies failed to identify (Lloyd *et al.* 2005; Ohuchi *et al.* 2008). Bioinformatics analysis indicated that all the sequences display the characteristic extracellular N terminus and the 7 transmembrane domains (TM) of these receptors which are located at the same position to the human orthologous sequences. Moreover, the G-protein coupled receptor family 1 signature and secondary modification sites for potential N-glycosylation, phosphorylation (by protein kinase C, casein kinase II), N-myristoylation and amidation were identified by ScanProsite analysis of the *Xenopus* protein sequences. The GWNC motif at the C-terminus of the TM4 is conserved in all *Xenopus* S1P sequences. This sequence is also found in *Xenopus* LPA1 and 3, but as in its mammalian orthologs, it is not conserved in *Xenopus* LPA2 (GWSC in *Xenopus* proteins, SWHC in human sequence), LPA4 and 5. The consensus sequence RxRxxT/S, characteristic of human S1P1, is conserved in the *Xenopus* receptors, suggesting that phosphorylation of the T residue of this receptor by protein kinase B/Akt, necessary for Rac activation might also occur in *Xenopus* (Lee *et al.* 2001). The amino acid residues R124, Q125, W186, R/D204 and K294, (based on human LPA1 sequence) which have been shown to be critical for interaction of LPA 1-3 with the polar head group of the LPA molecule are conserved in *Xenopus* proteins (Fujiwara *et al.* 2005; Valentine *et al.* 2008). The residues S114 and T187, but not Y265, of LPA4 protein (based on human sequence) which hydrogen bond with the polar head group of LPA, are conserved in *Xenopus* proteins (Li *et al.* 2009). The residues R78, H160, R261, but not R276, of LPA5 (based on human sequence) which are involved in ligand recognition, are conserved in *Xenopus* proteins (Williams *et al.* 2009). Several residues which have been implicated in the binding of S1P to its human receptor S1P1 are conserved in the other mammal orthologs (Parrill *et al.* 2000; Fujiwara *et al.* 2007). Seven of these 10 are also present in all *Xenopus* receptors, especially R124 and E125 (based on the human sequence) which are essential for S1P binding (Parrill *et al.* 2000). Moreover, E125 determines the ligand specificity for S1P *versus* LPA (Wang *et al.* 2001). Surprisingly, K204 which is essential for S1P binding to mouse S1P4 and receptor activation is not conserved in the *Xenopus* proteins (Inagaki *et al.* 2005). As in other mammalian S1P receptors, a basic residue in the *Xenopus* orthologs replaces the R292 involved in the interaction with the phosphate group of S1P (Parrill *et al.* 2000). Therefore we suggest that the *Xenopus* LPA and S1P receptors might bind their respective ligands in a similar way to the mammalian ones.

Three new receptors have been recently shown to bind bioactive

lipids. The orphan GPCR GPR87 receptor can bind LPA (Tabata *et al.* 2007). The P2Y5, identified as LPA6, and LPA8/P2Y10 receptors, belonging to the nucleotide P2Y receptors family, are activated by LPA and LPA/S1P respectively (Murakami *et al.* 2008; Yanagida *et al.* 2009). BLAST search identified *X. laevis* and *tropicalis* clones similar to these mammalian sequences.

Expression of S1P genes suggests specific roles in *Xenopus*

The embryonic gene expression profile of the *S1P* genes has been reported in other vertebrate species, especially in the mouse in which a comparative analysis of their expression during development has been published allowing direct comparison with our study (Ohuchi *et al.* 2008; Meng and Lee, 2009). Three members of S1P receptor family display specific and distinct expression profiles during frog embryogenesis. *S1P3* expression is not regulated during embryogenesis and we failed to detect its expression by *in situ* hybridisation. In mouse and chick embryos, this receptor is expressed widely in non neuronal cells but null-S1P3 mice display no gross phenotype suggesting that this receptor is not required for normal development and function (Ishii *et al.* 2001; Ohuchi *et al.* 2008; Meng and Lee, 2009). Our result shows that *S1P4* is only weakly expressed during embryogenesis. In the mouse, *S1P4* is first expressed ubiquitously, but its expression is not detected or is only at low levels after E12.5, suggesting that like in the frog, this gene might not play a major role during embryogenesis although expression in specific neural tissues might suggest a potential role during neural formation (Ohuchi *et al.* 2008; Meng and Lee, 2009). No knock-out mice are available to confirm this hypothesis. However, *S1P4* adult expression is mostly highly detected in the frog spleen, as in both human and mouse, suggesting potential roles of this receptor in lymphoid cell functions (Gräler *et al.* 1998).

The zygotic expression of *S1P1*, *S1P2* and *S1P5* is switched on at the same stage, during early gastrulation. However, their distinct spatial expression profiles suggest specific roles during embryogenesis. *S1P2* is majorly expressed in the developing somites and at stage 9, its expression is detected in the marginal zone. The mouse ortholog is expressed in somites and also in the mesenchyme of several organs (Ohuchi *et al.* 2008; Meng and Lee, 2009). Moreover, the zebrafish *S1P2* or *mil* gene is expressed in the paraxial mesodermal cells which give rise to the somites (Kupperman *et al.* 2000). This mesodermal expression pattern has been conserved during evolution and *Xenopus* S1P2 might play a role during mesoderm formation. However, due to the disparate phenotypes between mouse and zebrafish mutants, it is difficult to speculate as to the potential phenotype following mis-expression in *Xenopus*. *S1P2* is not expressed in the mouse and frog developing nervous system but found in the developing branchial arches, emphasizing the potential conservation of the function of this receptor in mammals and non-mammalian vertebrates (Ohuchi *et al.* 2008; Meng and Lee, 2009).

Xenopus S1P1 transcripts are only localised in the developing nervous system and this exclusive expression of *S1P1* in the brain is in agreement with the expression profile of its ortholog in zebrafish (Im *et al.* 2000). This exclusive neuronal expression contrasts with the expression profile of mammalian *S1P1* receptors. In mouse and rat, this receptor is more widely expressed, although strong expression can be detected in the nervous system from E14 (Lado *et al.* 1994; Liu and Hla, 1997; McGiffert

et al. 2002). Earlier expression in the forebrain can be detected from E10.5, although the major domain of expression at this stage is the developing heart and blood vessels (Ohuchi *et al.* 2008). A similar expression profile has been recently published in chick embryos (Meng and Lee, 2009). However, the phenotypic analysis of the null-S1P1 mice demonstrated the role of this receptor during neural development, consistent with its expression in the neuroepithelium and ventricular zone of the brain and neural tube (Mizugishi *et al.* 2005; Meng and Lee, 2009). We did not detect zygotic expression of *S1P1* outside the nervous system. Moreover, the high level of its expression in the adult brain and spinal cord suggests that, as in mammals, S1P1 must be the receptor mediating the effects of S1P in the nervous system.

The expression profile of *S1P5* is quite different between *Xenopus* and mammals. In the adult, *S1P5* is ubiquitously expressed in the adult frog whereas its expression is more restricted in mammalian tissues, the exact profile depending on the species analysed (Im *et al.* 2001; Niedernberg *et al.* 2002). During embryogenesis, *S1P5* is expressed in the somites, lens, otic vesicle and in the mesoderm in the caudal region of mouse and *Xenopus* embryos but mouse *S1P5* is majorly expressed in the presomitic mesoderm whereas *Xenopus S1P5* is a marker for cranial placodes (Ohuchi *et al.* 2008; Meng and Lee, 2009). Moreover, no specific signals can be detected after E12.5 whereas its expression can still be detected at stage 41 in the tadpole. These data suggest that S1P5 might play distinct and different roles in mouse and *Xenopus*. This is in agreement with the *in vitro* observation that rS1P5 display anti-proliferative effects in contrast to hS1P5 (Niedernberg *et al.* 2002). Moreover, a recent study revealed strong expression in the mouse nervous system from E.14.5, suggesting that the expression profile of this receptor in mammals is still uncertain (Meng and Lee, 2009). Our protein sequence analysis showed that S1P5 is the least Edg conserved member, emphasizing potential different functional roles this receptor might have acquired during evolution. However, despite lack of major obvious phenotype, S1P5 null mice display defects in oligodendroglial cellular functions and natural killer cell trafficking (Jaillard *et al.* 2005; Walzer *et al.* 2007). *Xenopus S1P5* is expressed in developing cranial placodes. S1P has been involved in cell migration during development and we suggest that S1P5 might play a crucial role during placode cell migration as it has been suggested for S1P1 and vascular smooth muscle cells migration or S1P2 and cardiac precursor cells migration (Kupperman *et al.* 2000; Liu *et al.* 2000).

LPA expression suggests housekeeping functions in *Xenopus*

LPA1 and LPA2 have already been cloned in *Xenopus* (Kimura *et al.* 2001; Lloyd *et al.* 2005). Our results indicate a wider distribution for *LPA1.1* and *LPA1.2* in adult frog than previously published, but confirm that these transcripts are strongly expressed in the nervous system and ovary (oocytes) (Kimura *et al.* 2001). Therefore, these differences probably reflect the increased sensitivity of RT-PCR analysis over the previously used Northern-Blot analysis. The expression profiles of *Xenopus LPA1* and *2* have been published previously but no *in situ* hybridisation data are available for these genes (Lloyd *et al.* 2005). Our RT-PCR analysis is consistent with published temporal expression profile for these genes and is included for completeness in our analysis.

As in mammals, *LPA1.1*, *LPA1.2*, *LPA2* are ubiquitous whereas *LPA4* and *LPA5* are expressed at low level in many tissues but at higher level in ovary and small intestine respectively (Noguchi *et al.* 2003; Lee *et al.* 2006). However, *LPA3* is ubiquitously expressed in mammals whereas its expression is only detected in the amphibian bladder, eyes and skin (Bandoh *et al.* 1999; Im *et al.* 2000). In embryos, we could only detect *LPA5* by *in situ* hybridisation, the other LPA receptors being expressed ubiquitously at low level by RT-PCR, certainly below the level of detection of the *in situ* hybridisation technique. In mouse, the expression of all LPA family members display specific but overlapping domains but the intensity of the signals is weak, except for LPA1, suggesting a low level of expression for these genes (Ohuchi *et al.* 2008).

Our RT-PCR results suggest that the 5 LPA receptors might display a similar ubiquitous embryonic expression pattern, pointing towards housekeeping cellular functions for these receptors during frog development. This is in agreement with the phenotype obtained following miss-expression of the LPA1 and 2 receptors in *Xenopus*, which demonstrate the role of LPA signalling in cortical actin assembly and cytoarchitecture (Lloyd *et al.* 2005). However, their temporal expression is regulated during *Xenopus* embryogenesis suggesting some developmental role. The high level of *LPA5* expression during maternal stages suggests it might be the major receptor to mediate LPA signalling during the early phases of development. Hydrolysis of lipids is critical for fertilization and LPC has been suggested to induce the membrane fusion needed for the sperm acrosomal reaction and sphingomyelin (SM) may play a role during membrane fusion and egg-sperm interactions by stabilizing rafts (Petcoff *et al.* 2008). As *S1P3* is the only *S1P* receptor member to be maternally expressed, it may be also involved in mediating the actions of S1P during these early phases of embryogenesis.

We have previously published the expression profile of *enpp2a* and *enpp2b* genes encoding for *Xenopus laevis* autotaxin proteins, the major lysoPLD enzyme producing extracellular LPA (Massé *et al.* 2010). Surprisingly, no correlations between the expression profiles obtained by *in situ* hybridisation of these enzymes and the *LPA1-5* receptors can be made, except that *enpp2a* and *enpp2b* display a similar temporal expression to *LPA1.1* and *LPA1.2*. One possibility is that the different receptors have redundant functions as it has been suggested in rodents (see Choi *et al.* 2008) and therefore a low level of expression of each receptor is sufficient to mediate the actions of LPA, especially since these 5 receptors seem to be ubiquitously expressed. It is also possible that the three new LPA receptors might display a more similar expression profile to the *enpp2* genes, although *LPA6* seems to be highly expressed in the skin/ectoderm from EST analysis. This is in agreement with the involvement of the human protein in the regulation of hair follicle development and hair growth (Pasternack *et al.* 2008; Shimomura *et al.* 2009). The analysis of their expression is currently in progress in the laboratory. Furthermore, it is more than possible that not all the LPA receptors have yet been identified and new orphan GPCR receptors might be shown to bind LPA.

In conclusion, our study demonstrates that at least 10 of the lipidic receptors are present in the frogs. Their wide adult distribution suggests that the S1P and LPA molecules might regulate diverse cellular functions in all amphibian tissues, but especially

in the nervous system, spleen and kidney as it has been shown in mammals (reviewed in Fukushima, 2004; Jo *et al.* 2008). However, the embryonic expression profiles of these receptors suggest potential distinct and novel roles for S1P1, S1P2 and S1P5 and LPA5 and therefore for their ligands during development. S1P and LPA signalling pathways have been conserved during evolution however, their receptors have only been identified in vertebrates (Oskouian and Saba, 2004). Therefore, studying their functions in frogs should bring new evidence of the roles they have acquired during vertebrate evolution and also to their involvement in human pathologies. Since we have previously published the cloning and the developmental expression patterns of the *enpp* family genes whose members can generate LPA and S1P (Massé *et al.* 2010), as a consequence of this work we can now misexpress both enzymes and receptors to identify their combinatorial roles in development. *Xenopus* therefore provides an excellent model in which to dissect these signalling pathways and establish their roles during embryogenesis.

Materials 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, receptor 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 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. Dissections of appropriately staged embryos were performed in Barth X using forceps and an eyebrow hair knife.

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* (Bassez *et al.* 1990) or *Ef1 α* (Mohun *et al.* 1989). 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 (Massé *et al.* 2010). In order to minimise the cross-reactions between homologous genes, specific antisense probes were made of each gene, preferentially designed in the 3'UTR of each receptor sequence. In each case, sense probes were used to control the specificity of the staining. Details of the probes are documented in Supplementary Table 3.

Acknowledgments

We thank P. Jarrett for the maintenance of the breeding frogs. This work was supported by the Wellcome Trust, Grant no 082071.

References

- ALLENDE ML, YAMASHITA T, PROIA RL (2003) G-protein-coupled receptor S1P1 acts within endothelial cells to regulate vascular maturation. *Blood* 102: 3665-3667.
- ALLENDE ML, DREIER JL, MANDALA S, PROIA RL (2004) Expression of the sphingosine 1-phosphate receptor, S1P1, on T-cells controls thymic emigration. *J Biol Chem* 279: 15396-15401.
- ALTSCHUL SF, GISH W., MILLER W, MYERS EW, LIPMAN DJ (1990) Basic local alignment search tool. *J Mol Biol* 215: 403-410.
- ANLIKER B, CHUN J (2004) Cell surface receptors in lysophospholipid signalling. *Semin Cell Dev Biol* 15: 457-465.
- BANDOH K, AOKI J, HOSONO H, KOBAYASHI S, KOBAYASHI T, MURAKAMI-MUROFUSHI K, TSUJIMOTO M, ARAI H, INOUE K (1999) Molecular cloning and characterization of a novel human G-protein-coupled receptor, EDG7, for lysophosphatidic acid. *J Biol Chem* 274: 27776-27785.
- BARNETT MW, OLD RW, JONES EA (1998) Neural induction and patterning by fibroblast growth factor, notochord and somite tissue in *Xenopus*. *Dev Growth Differ* 40: 47-57.
- BASSEZ T, PARIS J, OMILLI F, DOREL C, OSBORNE HB (1990) Post-transcriptional regulation of ornithine decarboxylase in *Xenopus laevis* oocytes. *Development* 110: 955-962.
- BAUDHUIN LM, JIANG Y, ZASLAVSKY A, ISHII I, CHUN J, XU Y (2004) S1P3-mediated Akt activation and cross-talk with platelet-derived growth factor receptor (PDGFR). *FASEB J* 18: 341-343.
- BREATHNACH R, CHAMBON P (1981) Organization and expression of eucaryotic split genes coding for proteins. *Annu Rev Biochem* 50: 349-383.
- BRINKMANN V (2007) Sphingosine 1-phosphate receptors in health and disease: mechanistic insights from gene deletion studies and reverse pharmacology. *Pharmacol Ther* 115: 84-105.
- BRODSKY L.I., VASILIEV A.V., KALAIKIDZIS YA.L., OSIPOV YU.S., TATUZOV R.L., FERANCHUK S.I. (1992) GeneBee: the Program Package for Biopolymer Structure Analysis. *Dimacs* 8: 127-139.
- CHOI JW, LEE CW, CHUN J (2008) Biological roles of lysophospholipid receptors revealed by genetic null mice: an update. *Biochim Biophys Acta* 1781: 531-539.
- CHUN J, GOETZL EJ, HLA T, IGARASHI Y, LYNCH KR, MOOLENAAR W, PYNE S, TIGYIG (2002) International Union of Pharmacology. XXXIV. Lysophospholipid receptor nomenclature. *Pharmacol Rev* 54: 265-269.
- CONTOS JJ, ISHII I, CHUN J (2000a) Lysophosphatidic acid receptors. *Mol Pharmacol* 58: 1188-1196.
- CONTOS JJ, FUKUSHIMA N, WEINER JA, KAUSHAL D, CHUN J (2000b) Requirement for the lpa1 lysophosphatidic acid receptor gene in normal suckling behavior. *Proc Natl Acad Sci USA* 97: 13384-13389.
- CONTOS JJ, ISHII I, FUKUSHIMA N, KINGSBURY MA, YE X, KAWAMURA S, BROWN JH, CHUN J (2002) Characterization of lpa(2) (Edg4) and lpa(1)/lpa(2) (Edg2/Edg4) lysophosphatidic acid receptor knockout mice: signaling deficits without obvious phenotypic abnormality attributable to lpa(2). *Mol Cell Bio* 22: 6921-6929.
- DUAN RD (2006) Alkaline sphingomyelinase: An old enzyme with novel implications. *Biochim Biophys Acta* 1761: 281-291.

- ESTIVILL-TORRÚS G, LLEBREZ-ZAYAS P, MATAS-RICO E, SANTÍN L, PEDRAZA C, DE DIEGO I, DEL ARCO I, FERNÁNDEZ-LLEBREZ P, CHUN J, DE FONSECA FR (2008) Absence of LPA1 signaling results in defective cortical development. *Cereb Cortex* 18: 938-950.
- FUJIWARA Y, SARDAR V, TOKUMURA A, BAKER D, MURAKAMI-MUROFUSHI K, PARRILL A, TIGYI G (2005) Identification of residues responsible for ligand recognition and regioisomeric selectivity of lysophosphatidic acid receptors expressed in mammalian cells. *J Biol Chem* 280: 35038-35050.
- FUJIWARA Y, OSBORNE DA, WALKER MD, WANG DA, BAUTISTA DA, LILIO M K, VAN BROCKLYN JR, PARRILL AL, TIGYI G (2007) Identification of the hydrophobic ligand binding pocket of the S1P1 receptor. *J Biol Chem* 282: 2374-2385.
- FUKUSHIMA N (2004) LPA in neural cell development. *J Cell Biochem* 92: 993-1003.
- GRÄLER MH, BERNHARDT G, LIPP M (1998) EDG6, a novel G-protein-coupled receptor related to receptors for bioactive lysophospholipids, is specifically expressed in lymphoid tissue. *Genomics* 53: 164-169.
- HARLAND RM (1991) *In situ* hybridization: an improved whole-mount method for *Xenopus* embryos. *Methods Cell Biol* 36: 685-695.
- HERR DR, GRILLET N, SCHWANDER M, RIVERA R, MÜLLER U, CHUN J (2007) Sphingosine 1-phosphate (S1P) signaling is required for maintenance of hair cells mainly via activation of S1P2. *J Neurosci* 27: 1474-1478.
- IM DS, UNGAR AR, LYNCH KR. (2000) Characterization of a zebrafish (*Danio rerio*) sphingosine 1-phosphate receptor expressed in the embryonic brain. *Biochem Biophys Res Commun* 279: 139-143.
- IM DS, CLEMENS J, MACDONALD TL, LYNCH KR (2001) Characterization of the human and mouse sphingosine 1-phosphate receptor, S1P5 (Edg-8): structure-activity relationship of sphingosine 1-phosphate receptors. *Biochemistry* 40: 14053-14060.
- INAGAKI Y, PHAM TT, FUJIWARA Y, KOHNO T, OSBORNE DA, IGARASHI Y, TIGYI G, PARRILL AL (2005) Sphingosine 1-phosphate analogue recognition and selectivity at S1P4 within the endothelial differentiation gene family of receptors. *Biochem J* 389: 187-195.
- ISHII I, FRIEDMAN B, YE X, KAWAMURA S, MCGIFFERT C, CONTOS JJ, KINGSBURY MA, ZHANG G, BROWN JH, CHUN J (2001) Selective loss of sphingosine 1-phosphate signaling with no obvious phenotypic abnormality in mice lacking its G protein-coupled receptor, LP(B3)/EDG-3. *J Biol Chem* 276: 33697-33704.
- ISHII I, YE X, FRIEDMAN B, KAWAMURA S, CONTOS JJ, KINGSBURY MA, YANG AH, ZHANG G, BROWN JH, CHUN J (2002) Marked perinatal lethality and cellular signaling deficits in mice null for the two sphingosine 1-phosphate (S1P) receptors, S1P(2)/LP(B2)/EDG-5 and S1P(3)/LP(B3)/EDG-3. *J Biol Chem* 277: 25152-25159.
- ISHII S, NOGUCHI K, YANAGIDA K (2009) Non-Edg family lysophosphatidic acid (LPA) receptors *Prostaglandins and Other Lipid Mediators* 89: 57-65.
- JAILLARD C, HARRISON S, STANKOFF B, AIGROT MS, CALVER AR, DUDDY G, WALSH FS, PANGALOS MN, ARIMURA N, KAIBUCHI K, ZALC B, LUBETZKI C (2005) Edg8/S1P5: an oligodendroglial receptor with dual function on process retraction and cell survival. *J Neurosci* 25: 1459-1469.
- JO S-K, BAJWA A, AWAD AS, LYNCH KR, OKUSA MD (2008) Sphingosine-1-phosphate receptors: biology and therapeutic potential in kidney disease. *Kidney Int* 73: 1220-1230.
- KABASHIMA K, HAYNES NM, XU Y, NUTT SL, ALLENDE ML, PROIA RL, CYSTER JG (2006) Plasma cell S1P1 expression determines secondary lymphoid organ retention versus bone marrow tropism. *J Exp Med* 203: 2683-2690.
- KIMURA Y, SCHMITT A, FUKUSHIMA N, ISHII I, KIMURA H, NEBREDA AR, CHUN J (2001) Two novel *Xenopus* homologs of mammalian LP(A1)/EDG-2 function as lysophosphatidic acid receptors in *Xenopus* oocytes and mammalian cells. *J Biol Chem* 276: 15208-15215.
- KLUK MJ, HLA T (2002) Signaling of sphingosine-1-phosphate via the S1P/EDG-family of G-protein-coupled receptors. *Biochim Biophys Acta* 1582: 72-80.
- KONO M, MI Y, LIU Y, SASAKI T, ALLENDE ML, WU YP, YAMASHITA T, PROIA RL (2004) The sphingosine-1-phosphate receptors S1P1, S1P2, and S1P3 function coordinately during embryonic angiogenesis. *J Biol Chem* 279: 29367-29373.
- KONO M, BELYANTSEVA IA, SKOURA A, FROLENKOV GI, STAROST MF, DREIER JL, LIDINGTON D, BOLZ SS, FRIEDMAN TB, HLA T, PROIA RL (2007) Deafness and stria vascularis defects in S1P2 receptor-null mice. *J Biol Chem* 282: 10690-10696.
- KUPPERMAN E, AN S, OSBORNE N, WALDRON S, STAINIER DY (2000) A sphingosine-1-phosphate receptor regulates cell migration during vertebrate heart development. *Nature* 406: 192-195.
- LADO DC, BROWE CS, GASKIN AA, BORDEN JM, MACLENNAN AJ (1994) Cloning of the rat edg-1 immediate-early gene: expression pattern suggests diverse functions. *Gene* 149: 331-336.
- LEE MJ, THANGADA S, PAIK JH, SAPKOTA GP, ANCELLIN N, CHAE SS, WU M, MORALES-RUIZ M, SESSA WC, ALESSI DR, HLA T. (2001) Akt-mediated phosphorylation of the G protein-coupled receptor EDG-1 is required for endothelial cell chemotaxis. *Mol Cell* 8: 693-704.
- LEE CW, RIVERA R, GARDELL S, DUBIN AE, CHUN J. (2006) GPR92 as a new G12/13- and Gq-coupled lysophosphatidic acid receptor that increases cAMP, LPA5 *J Biol Chem* 281: 23589-23597.
- LI G, MOSIER PD, FANG X, ZHANG Y (2009) Toward the three-dimensional structure and lysophosphatidic acid binding characteristics of the LPA(4)/p2y(9)/GPR23 receptor: A homology modeling study. *J Mol Graph Model* 28: 70-79
- LIN S, WANG D, IYER S, GHALEB AM, SHIM H, YANG VW, CHUN J, YUN CC (2009). The absence of LPA2 attenuates tumor formation in an experimental model of colitis-associated cancer. *Gastroenterology* 136: 1711-1720.
- LIU CH, HLA T (1997) The mouse gene for the inducible G-protein-coupled receptor edg-1. *Genomics* 43: 15-24.
- LIU Y, WADA R, YAMASHITA T, MI Y, DENG CX, HOBSON JP, ROSENFELDT HM, NAVA VE, CHAE SS, LEE MJ, LIU CH, HLA T, SPIEGEL S, PROIA RL. (2000) Edg-1, the G protein-coupled receptor for sphingosine-1-phosphate, is essential for vascular maturation. *J Clin Invest* 106: 951-961.
- LIU S, UMEZU-GOTO M, MURPH M, LU Y, LIU W, ZHANG F, YU S, STEPHENS LC, CUI X, MURROW G, COOMBES K, MULLER W, HUNG MC, PEROU CM, LEE AV, FANG X, MILLS GB (2009) Expression of Autotaxin and Lysophosphatidic Acid Receptors Increases Mammary Tumorigenesis, Invasion, and Metastases *Cancer Cell* 15: 539-550.
- LLOYD B, TAO Q, LANG S, WYLIE C (2005) Lysophosphatidic acid signaling controls cortical actin assembly and cytoarchitecture in *Xenopus* embryos. *Development* 132: 805-816.
- LORENZ JN, AREND LJ, ROBITZ R, PAUL RJ, MACLENNAN AJ (2007) Vascular dysfunction in S1P2 sphingosine 1-phosphate receptor knockout mice. *Am J Physiol Regul Integr Comp Physiol* 292: 440-446.
- LYNCH KR (2002) Lysophospholipid receptor nomenclature. *Biochim Biophys Acta* 1582: 70-71.
- MCGIFFERT C, CONTOS JJ, FRIEDMAN B, CHUN J (2002) Embryonic brain expression analysis of lysophospholipid receptor genes suggests roles for s1p(1) in neurogenesis and s1p(1-3) in angiogenesis. *FEBS Lett* 531: 103-108.
- MACLENNAN AJ, BENNER SJ, ANDRINGA A, CHAVES AH, ROSING JL, VESEY R, KARPMAN AM, CRONIER SA, LEE N, ERWAY LC, MILLER ML (2006) The S1P2 sphingosine 1-phosphate receptor is essential for auditory and vestibular function. *Hear Res* 220: 38-48.
- MASSÉ K, BALDWIN R, BARNETT MW, JONES EA (2004) X-epilectin: a novel epidermal fuclectins regulated by BMP signalling. *Int J Dev Biol* 48: 1119-1129.
- MASSÉ K, BHAMRA S, ALLSOP G, DALE N, JONES EA (2010) enpp nucleotidases: cloning, conservation and developmental restriction. *Int J Dev Biol* 54: 181-193
- MATLOUBIAN M, LO CG, CINAMON G, LESNESKI MJ, XU Y, BRINKMANN V, ALLENDE ML, PROIA RL, CYSTER JG (2004) Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. *Nature* 427: 355-360.
- MENG H, LEE VM (2009) Differential expression of Sphingosine-1-Phosphate receptors 1-5 in the developing nervous system. *Dev Dyn* 238: 487-500.
- MEYER ZU HERINGDORF D, JAKOBS KH (2007) Lysophospholipid receptors: signalling, pharmacology and regulation by lysophospholipid metabolism. *Biochim Biophys Acta* 1768: 923-940.
- MILLS GB, MOOLENAAR WH (2003) The emerging role of lysophosphatidic acid in cancer. *Nat Rev Cancer* 3: 582-591.

- MIZUGISHI K, YAMASHITA T, OLIVERA A, MILLER GF, SPIEGEL S, PROIA RL (2005) Essential role for sphingosine kinases in neural and vascular development. *Mol Cell Biol* 25: 11113-11121.
- MOHUN TJ, TAYLOR MV, GARRETT N, GURDON JB (1989) The CArG promoter sequence is necessary for muscle-specific transcription of the cardiac actin gene in *Xenopus* embryos. *EMBO J* 8: 1153-1161.
- MOOLENAAR WH, VAN MEETEREN LA, GIEPMANS BN (2004) The ins and outs of lysophosphatidic acid signaling. *Bioessays* 26: 870-881.
- MURAKAMI M, SHIRAIISHI A, TABATA K, FUJITA N (2008) Identification of the orphan GPCR, P2Y(10) receptor as the sphingosine-1-phosphate and lysophosphatidic acid receptor. *Biochem Biophys Res Commun* 371: 707-712.
- NEEDLEMAN SB, WUNSCH CD (1970) A general method applicable to the search for similarities in the amino acid sequence of two proteins. *J Mol Biol* 48: 443-453.
- NIEDERNBERG A, SCHERER CR, BUSCH AE, KOSTENIS E. (2002) Comparative analysis of human and rat S1P(5) (edg8): differential expression profiles and sensitivities to antagonists. *Biochem Pharmacol* 64: 1243-1250.
- NIEUWKOOP PD, FABER J (1994) Normal table of *Xenopus laevis* (Daudin). Garland Publishing, Inc. New York.
- NOGUCHI K, ISHII S, SHIMIZU T (2003) Identification of p2y9/GPR23 as a novel G protein-coupled receptor for lysophosphatidic acid, structurally distant from the Edg family. *J Biol Chem* 278: 25600-25606.
- OHUCHI H, HAMADA A, MATSUDA H, TAKAGI A, TANAKA M, AOKI J, ARAI H, NOJI S (2008) Expression patterns of the lysophospholipid receptor genes during mouse early development. *Dev Dyn* 237: 3280-3294.
- OSBORNE N, STAINIER DY (2003) Lipid receptors in cardiovascular development. *Annu Rev Physiol* 65: 23-43.
- OSKOUIAN B, SABA JD (2004) Death and taxis: what non-mammalian models tell us about sphingosine-1-phosphate. *Semin Cell Dev Biol* 15: 529-540.
- PARRILL AL, WANG D, BAUTISTA DL, VAN BROCKLYN JR, LORINCZ Z, FISCHER DJ, BAKER DL, LILIOM K, SPIEGEL S, TIGYI G (2000) Identification of Edg1 receptor residues that recognize sphingosine 1-phosphate. *J Biol Chem* 275: 39379-39384.
- PASTERNAK SM, VON KÜGELGEN I, ABOUD KA, LEE YA, RÜSCHENDORF F, VOSSK, HILLMER AM, MOLDERINGS GJ, FRANZT, RAMIREZA, NÜRNBERG P, NÖTHEN MM, BETZ RC (2008) G protein-coupled receptor P2Y5 and its ligand LPA are involved in maintenance of human hair growth. *Nat Genet* 40: 329-334.
- PETCOFF DW, HOLLAND WL, STITH BJ (2008) Lipid levels in sperm, eggs, and during fertilization in *Xenopus laevis*. *J Lipid Res* 49: 2365-2378.
- SABA JD (2004) Lysophospholipids in development: Miles apart and edging in. *J Cell Biochem* 92: 967-992.
- SAKAGAMI H, AOKI J, NATORI Y, NISHIKAWA K, KAKEHI Y, NATORI Y, ARAI H (2005) Biochemical and molecular characterization of a novel choline-specific glycerophosphodiester phosphodiesterase belonging to the nucleotide pyrophosphatase/phosphodiesterase family. *J Biol Chem* 280: 23084-23093.
- SANCHEZ T, HLA T (2004) Structural and functional characteristics of S1P receptors. *J Cell Biochem* 92: 913-922.
- SCHLOSSER G (2006) Induction and specification of cranial placodes. *Dev Biol* 294: 303-351.
- SENGUPTA S, WANG Z, TIPPS R, XU Y (2004) Biology of LPA in health and disease. *Semin Cell Dev Biol* 15: 503-512.
- SHIMOMURA Y, GARZON MC, KRISTAL L, SHAPIRO L, CHRISTIANO AM (2009) Autosomal recessive woolly hair with hypotrichosis caused by a novel homozygous mutation in the P2RY5 gene. *Exp Dermatol* 18: 218-221.
- SPIEGEL S, MILSTIEN S (2003) Sphingosine-1-phosphate: an enigmatic signaling lipid. *Nat Rev Mol Cell Biol* 4: 397-407.
- STEFAN C, JANSEN S, BOLLEN M (2005) NPP-type ectophosphodiesterases: unity in diversity. *Trends Biochem Sci* 30: 542-550.
- TABATA K, BABA K, SHIRAIISHI A, ITO M, FUJITA N (2007) The orphan GPCR GPR87 was deorphanized and shown to be a lysophosphatidic acid receptor. *Biochem Biophys Res Commun* 363: 861-866.
- TAKUWA Y, TAKUWA N, SUGIMOTO N (2002) The Edg family G protein-coupled receptors for lysophospholipids: their signaling properties and biological activities. *J Biochem* 131: 767-771.
- THOMPSON JD, HIGGINS DG, GIBSON TJ (1994) CLUSTALW: improving the sensitivity of progressive multiple sequence alignment through sequences weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* 22: 4673-4680.
- VALENTINE WJ, FELLS JI, PERYGIN DH, MUJAHID S, YOKOYAMA K, FUJIWARA Y, TSUKAHARA R, VAN BROCKLYN JR, PARRILL AL, TIGYI G (2008) Subtype-specific residues involved in ligand activation of the endothelial differentiation gene family lysophosphatidic acid receptors. *J Biol Chem* 283: 12175-12187.
- VAN MEETEREN LA, MOOLENAAR WH (2007) Regulation and biological activities of the autotaxin-LPA axis. *Prog Lipid Res* 46: 145-160.
- WALZER T, CHIOSSONE L, CHAIX J, CALVER A, CAROZZO C, GARRIGUE-ANTAR L, JACQUES Y, BARATIN M, TOMASELLO E, VIVIER E (2007) Natural killer cell trafficking *in vivo* requires a dedicated sphingosine 1-phosphate receptor. *Nat Immunol* 8: 1337-1344.
- WANG DA, LORINCZ Z, BAUTISTA DL, LILIOM K, TIGYI G, PARRILL AL (2001) A single amino acid determines lysophospholipid specificity of the S1P1 (EDG1) and LPA1 (EDG2) phospholipid growth factor receptors. *J Biol Chem* 276: 49213-49220.
- WILLIAMS JR, KHANDOGA AL, GOYAL P, FELLS JI, PERYGIN DH, SIESS W, PARRILL AL, TIGYI G, FUJIWARA Y (2009) Unique Ligand Selectivity of the GPR92/LPA5 Lysophosphatidate Receptor Indicates Role in Human Platelet Activation. *J Biol Chem* 284: 17304-17319.
- YANAGIDA K, MASAGO K, NAKANISHI H, KIHARA Y, HAMANO F, TAJIMA Y, TAGUCHI R, SHIMIZU T, ISHII S (2009) Identification and characterization of a novel lysophosphatidic acid receptor, p2y5/LPA6. *J Biol Chem* 284: 17731-17741.
- YE X, HAMA K, CONTOS JJ, ANLIKER B, INOUE A, SKINNER MK, SUZUKI H, AMANO T, KENNEDY G, ARAI H, AOKI J, CHUN J (2005) LPA3-mediated lysophosphatidic acid signalling in embryo implantation and spacing. *Nature* 435: 104-108.
- ZHANG H, XU X, GAJEWIAK J, TSUKAHARA R, FUJIWARA Y, LIU J, FELLS JI, PERYGIN D, PARRILL AL, TIGYI G, PRESTWICH GD (2009) Dual activity lysophosphatidic acid receptor pan-antagonist/autotaxin inhibitor reduces breast cancer cell migration *in vitro* and causes tumor regression *in vivo*. *Cancer Res* 69: 5441-5449.

Further Related Reading, published previously in the *Int. J. Dev. Biol.*

See our recent Special Issue **Placenta** edited by Joan S. Hunt and Kent L. Thornburg at:
<http://www.ijdb.ehu.es/web/contents.php?vol=54&issue=2-3>

A novel role for Glucocorticoid-Induced TNF Receptor Ligand (Gitr1) in early embryonic zebrafish development

Lynn D. Poulton, Kathleen F. Nolan, Corina Anastasaki, Herman Waldmann and E. Elizabeth Patton
Int. J. Dev. Biol. 54: 815-825 (10.1387/ijdb.082841p)

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

Karine Massé, Surinder Bhamra, Gavin Allsop, Nicholas Dale and Elizabeth A. Jones
Int. J. Dev. Biol. (2010) 54: 181-193

Receptor-mediated uptake and transport of macromolecules in the human placenta

Henning Schneider and Richard K. Miller
Int. J. Dev. Biol. (2010) 54: 367-375

Targeted disruption of fibroblast growth factor receptor-1 blocks maturation of visceral endoderm and cavitation in mouse embryoid bodies

Milan Esner, Jiri Pachernik, Ales Hampl and Petr Dvorak
Int. J. Dev. Biol. (2002) 46: 817-825

Evolution and developmental expression of nuclear receptor genes in the ascidian *Herdmania*.

Christine Devine, Veronica F Hinman and Bernard M Degnan
Int. J. Dev. Biol. (2002) 46: 687-692

Sequence and translation initiation properties of the xenopus TGFbeta5, PDGF-A, and PDGF-alpha receptor 5' untranslated regions

A W van der Velden, A Los, H O Voorma and A A Thomas
Int. J. Dev. Biol. (2000) 44: 851-859

Retinoic acid receptors and nuclear orphan receptors in the development of *Xenopus laevis*

C Dreyer and H Ellinger-Ziegelbauer
Int. J. Dev. Biol. (1996) 40: 255-262

5 yr ISI Impact Factor (2009) = 3.253

