Conserved role of the urotensin II receptor 4 signalling pathway to control body straightness in a tetrapod

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Urp1 and Urp2 are two neuropeptides of the urotensin II family identified in teleost fish and mainly expressed in cerebrospinal fluid (CSF)-contacting neurons. It has been recently proposed that Urp1 and Urp2 are required for correct axis formation and maintenance. Their action is thought to be mediated by the receptor Uts2r3, which is specifically expressed in dorsal somites. In support of this view, it has been demonstrated that the loss of uts2r3 results in severe scoliosis in adult zebrafish. In the present study, we report for the first time the occurrence of urp2, but not of urp1, in two tetrapod species of the Xenopus genus. In X. laevis, we show that urp2 mRNA-containing cells are CSF-contacting neurons. Furthermore, we identified utr4, the X. laevis counterparts of zebrafish uts2r3, and we demonstrate that, as in zebrafish, it is expressed in the dorsal somatic musculature. Finally, we reveal that, in X. laevis, the disruption of utr4 results in an abnormal curvature of the antero-posterior axis of the tadpoles. Taken together, our results suggest that the role of the Utr4 signalling pathway in the control of body straightness is an ancestral feature of bony vertebrates and not just a peculiarity of ray-finned fishes.

1. Introduction

The urotensin II (UII) family is a multigenic family of neuropeptides, evolutionarily related to somatostatin [1], which consists of four paralogous genes called uts2, uts2-related peptide (urp, also called uts2b in mammals and uts2d in fishes), urp1 and urp2 [2,3]. While uts2 and urp exist both in fish and in tetrapods, so far, urp1 and urp2 have only been found in ray-finned fishes (actinopterygians) [2,3]. All peptides of this family act through a canonical family of G protein-coupled receptors called urotensin II receptors (Utr or Uts2r) [3]. The occurrence of multiple utr genes (utr1–utr5) has been reported in non-mammalian vertebrate genomes [3–5], which is in contrast with the single gene (utr1) present in mammals [3,6–9].

Since its discovery in 1980 [10], UII has prompted a large number of studies and it has been reported to regulate many physiological processes in the CNS and peripheral tissues, such as sleep, anxiety, depression, food intake, locomotion, neuroendocrine action, osmoregulation, cardiovascular functions and immunity (see [11] for review). In contrast with those of UII, the functions of Urps are much less understood. However, recent studies in zebrafish (Danio rerio) have shown that Urp1 and Urp2 play a critical role in spine morphogenesis [12–14].

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urp1 and urp2 genes have been first characterized from the Japanese eel (*Anguilla japonica*) [15] and zebrafish [16], respectively, and were subsequently detected in all teleost species investigated, as well as in the spotted gar (*Lepisosteus oculatus*), a ray-finned fish species that diverged from teleosts before the teleost-specific whole-genome duplication [2,3]. In zebrafish, urp1 and urp2 are primarily expressed in the spinal cord and the hindbrain. In the spinal cord, their transcripts mainly co-localize in a small population of sensory neurons called cerebrospinal fluid (CSF)-contacting neurons [16,17].

The view that Urp1 and Urp2 are required for correct axis formation and maintenance in zebrafish is supported by several lines of evidence: (i) urp2 and urp1 expression is strongly affected in various zebrafish mutants sharing a curved, instead of a straight, body axis feature [12–14]; (ii) in zebrafish embryos, knock-down of urp1 leads to a curled down axis, while its overexpression leads to the opposite curvature [12]; (iii) zebrafish mutant foruts2r3 (initially called uts2m), a member of the family of the urotensin II receptors specifically expressed in dorsal muscles, results in severe spine deformations in adult [12].

The morphogenetic defects caused by deregulation of the Urp1/2-Uts2r3 pathway in zebrafish are clearly reminiscent of some of the manifestations of idiopathic scoliosis (IS) in humans [12,13], a complex genetic disorder characterized by three-dimensional spinal curvatures [18,19]. Recently, zebrafish has emerged as a powerful system for studying IS, owing to well-developed genetic resources and a natural susceptibility to spinal curvature [20]. The studies cited above obviously belong within this context. However, the main question that arises is to what extent their results can be extrapolated to humans.

Today, it is well recognized that neither urp1 nor urp2 are present in mammals. By contrast, whether these peptides exist in other tetrapods has never been investigated to date. In the present study, we report for the first time the characterization of the urp2 (but not urp1) gene in two closely related tetrapod species, the western clawed frog (*Xenopus tropicalis*) and the African clawed frog (*X. laevis*). We demonstrate that *X. laevis* urp2 mRNA, as in zebrafish, mainly occurs in CSF-contacting neurons of the spinal cord and hindbrain. We also show that *X. laevis* utr4, the counterpart of zebrafish uts2r3, is primarily expressed in dorsal somites (see table 1 for the UII receptor nomenclature used in this article). Finally, we reveal that the gene knock-out of utr4 results in an abnormal curvature of the antero-posterior axis of the tadpoles that impacts their locomotion. Taken together, our results strongly suggest that the role of the Utr4 signalling pathway in the control of body straightness is an ancestral feature of bony vertebrates (osteichthyes) and not just a peculiarity of ray-finned fishes.

### 2. Material and methods

#### 2.1. Animals

Animal husbandry *X. laevis* (outbred, wild type) were obtained from the National Biological Resource Center in Rennes (France). Animals were maintained at 23°C until stage NF52-55 then were sacrificed. Embryos were obtained by *in vitro* fertilization using wild-type *X. laevis* according to [21].

#### Table 1. Nomenclature of urotensin II receptors.

<table>
<thead>
<tr>
<th>Species</th>
<th>Ensembl nomenclature</th>
<th>NCBI ID</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>X. tropicalis</em></td>
<td>urotensin 2 receptor- like 1 (uts2rl1)</td>
<td>XP_001334493.1</td>
</tr>
<tr>
<td><em>X. laevis</em></td>
<td>urotensin 2 receptor (uts2)</td>
<td>XP_005179851.1</td>
</tr>
</tbody>
</table>

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2.2. Molecular cloning of *Xenopus* Urp2 cDNA

A genomic sequence from *X. tropicalis* potentially encoding a urp2-like sequence (ACFWKYCIRQWK) was recently reported [22]. Based on this nucleotide sequence, first primers and nested primers were designed to amplify the 5′-end of an *X. laevis* urp2 cDNA using the Advantage 2 PCR kit (Clontech). 5′RACE-ready cDNAs were constructed from 1 µg of poly(A⁺) RNA using the SMARTer RACE cDNA Amplification kit (Clontech), as previously described [23]. PCR was carried out in a MyCycler thermal cycler (Bio-Rad) under the following conditions: initial denaturation at 95°C/2 min, 5 cycles of 94°C/30 s and 72°C/2 min; 5 cycles of 94°C/30 s, 70°C/1 min and 72°C/1 min; 25 cycles of 94°C/30 s 68°C/1 min and 72°C/1 min; and the final extension at 72°C/7 min. The primers used were Xla_urp2 Rev x Universal Primer A Mix (UPM) then Xla_urp2 Rev Nest x Nested Universal Primer A Mix (NUP) (see electronic supplementary material, table S1 for primer sequences). PCR products were subcloned into the pGEM-T vector (Promega, Charbonnières-les-Bains, France) and Sanger sequenced (eurofinsgenomics.eu). A Blast search of the *X. laevis* genome database (available in Xenbase, http://www.xenbase.org) was performed using the 5′RACE *X. laevis* urp2 sequence as a query. This search allowed us to detect two *X. laevis* urp2 genes, called urp2S and urp2 L. Based on the genomic DNA sequences, four pairs of primers (Xla_urp2F, Xla_ urp2S Rev, Xla_urp2 L F and Xla_ urp2 L Rev; see electronic supplementary material, table S1 for primer sequences) were designed to amplify the corresponding cDNA sequences. The basic cycling conditions of the PCRs were set as follows: initial denaturation at 94°C/2 min, 35 cycles of 94°C/30 s, gene-specific annealing for 45 s and 72°C/60 s and the final extension at 72°C/6 min. PCR products were sequenced as described above. The coding sequence of the two *X. laevis* urp2 cDNAs has been deposited in the GenBank database under the accession numbers MZ054702 and MZ054703 for urp2L and urp2S, respectively. Data from *X. laevis* were used to identify the full-length *X. tropicalis* urp2 cDNA and to decipher organization of the corresponding gene.

2.3. Identification of urotensin II receptors

*Xenopus laevis* utr gene predictions were identified in the Xenbase browser and the National Center for Biotechnology Information (NCBI) genome resource (https://www.ncbi.nlm.nih.gov/genome/) by using the *X. tropicalis* utr gene sequences [4] as queries.

In the present study, we used the phylogenetic nomenclature initially proposed by Tostivint et al. [3] and more recently taken up by Konno et al. [4] and Cui et al. [5]. According to this nomenclature, the only mammalian Utr is Utr1, while Utr4 is the counterpart of zebrafish Uts2r3 and Utr2 is the Utr subtype missing in *X. tropicalis* (table 1).

2.4. Phylogeny and synteny analyses

A set of 47 vertebrate UII and Urp precursor sequences was collected from NCBI and Ensembl databases and supplemented by the sequences characterized in the present study. All sequences were aligned using the Clustal algorithm [24] then manually adjusted. Molecular phylogenetic relationships were analysed using the neighbour-joining (NJ) algorithm [25] with MEGA6 software [26]. The reliability of phylogenetic trees was assessed with bootstrapping (1000 iterations). The accession numbers of the sequences used in the phylogenetic analysis are shown in the electronic supplementary material, figure S1. Ensembl database (http://www.ensembl.org/index.html) and Genomicus [27] (version 100.1, https://www.genomicus.biologie.ens.fr/genomicus-100.01/cgi-bin/search.pl) were used to determine the conserved syntenic pattern of urp2 gene in the western clawed frog (*X. tropicalis*), chicken (*Gallus gallus*), human (*Homo sapiens*), spotted gar (*L. oculatus*) and zebrafish (*D. rerio*).

2.5. Gene expression analysis

2.5.1. RNA extraction and cDNA synthesis from embryos and tadpoles

*Xenopus laevis* embryos and tadpoles at various developmental stages (NFI, 10, 21, 24, 37, 41 and 50, according to [28]) were anaesthetized with 0.01% MS-222, rinsed in sterile water then transferred in Sorenson tubes containing 100 µl of lysis solution (provided in RNAqueous micro kit—see below). Three biological replicates were collected for each developmental stage. Tubes were flash-frozen in liquid nitrogen and stored at −80°C prior to RNA extraction. RNA was extracted using RNAqueous-Micro Total RNA Isolation Kit (Ambion, Thermofisher) following the manufacturer’s instructions. The concentration of RNA was determined using a spectrophotometer (Nanodrop Thermofisher). Next, an Agilent bioanalyzer was employed to verify the quality of the collected RNA. Only samples with RNA Integrity Numer (RIN) above 7.5 were included for further processes. After, reverse transcription was conducted on 500 ng of RNA using a high capacity cDNA RT kit (Applied BioSystem) following the manufacturer’s instructions (in a total 20 µl reaction). cDNA was a 20-times diluted (5 µl of cDNA in 95 µl of nuclease-free water) then stored at −20°C until use.

2.5.2. RNA extraction and cDNA synthesis from tissues of juvenile frogs

Total RNA was extracted from various tissues (brain, spinal cord, eye, skin, skeletal muscles, lung, heart, liver, spleen, stomach, intestine, kidney, ovary and testis) of six juvenile frogs and purified by using RNABLE (Eurobio). Samples were treated with DNase I (Roche) to remove potential contamination by genomic DNA and then purified with phenol/chloroform extraction. The integrity of RNA was assayed by electrophoresis on a 1% agarose gel. For each tissue, 0.5 µg of total RNA was reverse transcribed using the GoScript reverse transcriptase (Promega) with random primers.

2.5.3. Real-time quantitative PCR

Real-time quantitative PCR (RT-qPCR) was carried out using QuantStudio 6 flex (Life Technologies) on 384 well-plates, with a standard reaction per well containing 1/20 diluted cDNA as template (1 µl per well) plus 5 µl of mix (Power SYBR Green PCR Master Mix, Applied BioSystem). For each sample, the RT-qPCR reaction was conducted in duplicates. Water and no-template controls were used as negative controls for each primer set. The following cycling conditions were used: 1 cycle of 95°C for 2 min, followed by 45 cycles of 95°C for 15 s and 60°C for 30 s. RT-qPCR data
were analysed using the QuantStudio 6 and 7 Flex RT PCR System (Life Technologies). Cycle threshold (Ct) values were obtained using auto baseline and applied to all amplicons of the same primer set. Reference genes used to normalize measurements were as follows: SUB1 homolog (sub1-L) and solute carrier family 35 member B1 (slc35b1-L) for developmental analysis and ribosomal protein S13 (rps13) for tissue analysis in accordance with previous studies performed by [29] and [4], respectively. Primer sequences are listed in the electronic supplementary material, table S1. Plot was made using R software [30] (available in https://www.R-project.org/) and ggplot2 package [31].

2.6. In situ hybridization

2.6.1. Riboprobe synthesis

To generate the \textit{X. laevis} urp2-L RNA probe, the 5’RACE urp2-L cDNA previously cloned into pGEM-T was used as a template. To generate the four \textit{X. laevis} utr-L receptor RNA probes, partial sequences of their cDNAs were cloned into PGEM-T after RT-PCR amplification of the corresponding transcripts then used as templates. The same procedure was followed to produce the \textit{pdk2II} probe (see electronic supplementary material, table S1 for primer sequences). Sense and antisense digoxigenin (Dig)-labelled probes were synthesized from the linearized plasmids with T7 or Sp6 RNA polymerases and RNA Labelling mix (Roche).

2.6.2. Tissue preparation and hybridization procedures

Embryos (at stages NF29–30) and juvenile frogs were killed in 1% MS-222. Whole-mount embryos and dissected brains and spinal cords were fixed overnight at 4°C in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) and stored in 100% methanol at −20°C. Fixed embryos were rehydrated through a descending series of methanol solutions then bleached in 16.25% \textit{H}_2\textit{O}_2, 8.33% formamide and 0.83 × saline sodium citrate (SSC) buffer for 10 min. Fixed tissues were cryopreserved in 30% sucrose/PBS at 4°C. After, they were rehydrated, mounted in Eukitt (Sigma), then photographed with DM5500 light microscope (Leica).

2.7. CRISPR/Cas9-mediated genome editing

2.7.1. Small guide RNA design and injection

Knock-out frogs were generated by CRISPR/Cas9 genome editing and thereafter are called as crispants (CRISPR-mediated mutants). CRISPR target sequences (20-nucleotide sequence followed by a protospacer adjacent motif (PAM) or ‘NGG’ were designed by using an online CRISPR design tool from Integrated DNA Technologies (IDT, https://eu.idtdna.com/pages) and crRNA ordered from IDT (electronic supplementary material, table S1). To prepare sgRNA, crRNA was duplexed with tracrRNA according to the manufacturer instructions. For the \textit{utr4} knock-out, two sgRNAs were designed between transmembrane domains 2 and 4 in order to frame the ligand-binding domain of the receptor [11]. Given the occurrence of two \textit{utr4} homoeologues in \textit{X. laevis} [32], located on chromosomes 7L and 7S, respectively, the sgRNAs were designed so that there was not more than one mismatch between the sgRNA target site and the sgRNA sequence on either of the homoeologues (see electronic supplementary material, figure S2). The two sgRNAs were used simultaneously. To screen knock-out animals, an additional sgRNA was designed to disrupt \textit{tyrosinase} (\textit{tyr}), a gene required for pigmentation. Knock-out of this gene using either CRISPR/Cas9, ZFNs or TALENs results in albinism in \textit{Xenopus} [33–35], making the detection of knock-out animals apparent. Before injection, sgRNA (20 µM) were added to Cas9 protein (30 µM) to obtain a Cas9:sgRNA ratio of 1:2 and incubated for 10 min at 37°C.

One-cell stage \textit{Xenopus} embryos were injected with approximately 10 nanolitres of a solution containing three different Cas9-sgRNA complexes; 2 sgRNAs targeting both homoeologues of \textit{utr4} together with one sgRNA targeting the \textit{tyrosinase} gene. During subsequent development, abnormal and dead embryos (mainly due to gastrulation defects) were removed in order to conduct morphological phenotyping.

2.7.2. Detection and sequencing of mutations in injected tadpoles

In a first test designed to check the sgRNAs, genomic DNA was extracted from tail clips of 10 tadpoles in 50 mM NaOH for 15 min at 95°C then neutralized with 1/10 volume of Tris 10 mM pH 8. All the samples were then pooled. To evaluate the efficiency of targeted deletion guided by the sgRNA pair, PCR analyses were carried out from this pooled DNA using primers flanking the targeted regions. Wild-type and truncated genomic fragments were resolved by gel electrophoresis. In order to search for mutations at the target sites of each sgRNA, non-truncated fragments were purified, sequenced on both strands then analysed by tracking indels by Inference of CRISPR Edits (ICE; Synthego, https://www.synthego.com/products/bioinformatics/crispr-analysis). After validation of the sgRNAs, the same test was performed on genomic DNA from 10 new tadpoles, but this time dealt separately, to
determine the relationship between their genotype and phenotype.

2.8. Phenotype analysis of utr4 crispsants

2.8.1. Body curvature analysis

Xenopus laevis utr4 crispant and control tadpoles (from stages NF46 to 52) were euthanized, fixed in 4% PFA, stored in 100% methanol and then re-hydrated in PBS. Tadpoles were imaged in a bright field with a Lumenera Infinity 3-6UR microscope camera attached to an Olympus Microscope operated with Infinity Analyze software in an agarose plate submerged in PBS laterally with the head pointing to the left. To define the body curvature, the images were analysed with the Fiji Angle tool. The line was drawn starting from the posterior half of the tail mid line to the head (figure 10), and the value of the angle formed in between the two lines was defined by the Angle tool for each tadpole.

To evaluate the statistical difference in body curvature in utr4 crispant and control tadpoles, the measured angles were applied to Student’s t-test (function t.test, two-tailed, unequal variance) using Microsoft Excel 2020 (function t.test, two-tailed, unequal variance). Tadpoles were considered as curled down when the head-to-tail angle was less than 160°.

Figure 1. Primary structure of frog Urp2 precursors. (a) Nucleotide sequence of the X. tropicalis urp2 cDNA and deduced amino acid sequence. (b) Alignment of the aa sequences of X. tropicalis and X. laevis prepro-Urp2. An asterisk denotes conserved residues. Precursor sizes are indicated. Xtr, X. tropicalis; Xla, X. laevis. (c) Comparison of Urp2 and Urp1 primary sequences in Xenopus and zebrafish. Initiation (ATG) and stop codons are boxed. Signal peptides are in grey. Putative bioactive peptides are highlighted in red. Cleavage sites are in bold. The sequences of the two X. laevis Urp2 cDNAs have been deposited in the GenBank database under the accession number MZ054702 and MZ054703, respectively.
2.8.2. Quantification of locomotor activity

To record the locomotion of tadpoles, we used a Phantom Miro M110 camera (Vision Research, Elancourt, France) set at 700 or 1000 frames per second (fps). Stage NF53 tadpoles were placed in a large Petri dish (30 cm diameter with 3 cm of water depth), stimulated to swim and recorded for 3 s. We recorded five utr4 crispant and three control tadpoles. Three videos per individual were recorded, saved and analysed using ProAnalyst (Xcitex, Cambridge, MA, USA) software. For each frame, the eye was digitized either manually or using the auto-tracking routine implemented in ProAnalyst. Raw coordinates were exported to Excel and smoothed using a zero phase-shift low-pass Butterworth filter with a cut-off frequency set at 50 Hz. Next, we calculated the cumulative distance swum along the path. Instantaneous velocities and accelerations were calculated by numerical differentiation of the smoothed cumulative displacement profile. We extracted peak velocity, acceleration and deceleration from the kinematic data and further calculated the straight-line distance between the beginning and the end of the video recording. We calculated the sinuosity of the trajectory by dividing the cumulative distance by the straight-line distance. All variables were log10-transformed and analysed using IBM SPSS statistics (v. 26). We used univariate analyses of variance to test for differences in swimming kinematics between utr4 crispant and control tadpoles for each variable tested.

3. Results

3.1. Structure of Urp2 precursor cDNAs and genes in Xenopus tropicalis and Xenopus laevis

The nucleotide and deduced amino acid sequences of the X. tropicalis urp2 cDNA are shown in figure 1a. The coding region of this cDNA consists of 456 base pairs (bp) that encode a 152 amino acid (aa) protein. The primary structure of the protein, called prepro-Urp2, encompasses a 23-aa putative signal peptide [36], a 115-aa cryptic sequence, a 3-aa proteolytic site of the protein, called prepro-Urp2, encompasses a 23-aa putative signal peptide [36], a 115-aa cryptic sequence, a 3-aa proteolytic cleavage site KKR and the Urp2 peptide sequence ACFWK-YCQKNK. Comparison of the cDNA with genomic sequence revealed that the X. tropicalis urp2 gene is composed of 5 exons and 4 introns (electronic supplementary material, figure S3). The fifth exon fully encodes the putative mature peptide Urp2, as in zebrafish [16]. In X. laevis, two distinct urp2 cDNAs were found (electronic supplementary material, figure S4). Both cDNAs were called urp2-L and urp2-S on the basis of the chromosome location of the corresponding genes (chromosome 2L and 2S, respectively) [32]. X. laevis prepro-Urp2-L and Urp2-L exhibit a high level of sequence identity with X. tropicalis prepro-Urp2 (approx. 86–91% sequence identity for each; figure 1b; electronic supplementary material, table S2). The primary sequence of Urp2 is the same for both precursors. As X. tropicalis urp2 gene, X. laevis urp2-L and -S genes are composed of 5 exons and 4 introns (electronic supplementary material, figures S5 and S6).

3.2. Comparison of prepro-Urp2 sequences from other species

Frog and fish Urp2 precursors display only low-sequence identity (from 21.7 to 29.0%, depending on the species, electronic supplementary material, table S2). They do not exhibit appreciable sequence similarities outside the Urp2 domain (electronic supplementary material, figure S7). Frog Urp2 contains one more aa residue than fish Urp2 and it differs at two additional positions (1 and 8) (figure 1c). Frog Urp2 differs from fish Urp1, which is one residue shorter [2], at positions 8 and 9 (figure 1c).

3.3. Identification of Xenopus laevis urotensin II receptors

As in X. tropicalis [41], four Utr subtypes were identified in X. laevis, namely Utr1, Utr3, Utr4 and Utr5, each of them consisting of two homoeologues (L and S) (electronic supplementary material, figures S8 and S9). It is noteworthy that Utr3-S and Utr4-S (but not Utr3-L and Utr4-L) lack multiple transmembrane domains (electronic supplementary material, figure S9).

3.4. Phylogenetic analysis of the UII and Urp precursors

Based on an amino acid alignment of 47 selected UII and Urp precursor sequences (electronic supplementary material, figure S10), a phylogenetic tree was constructed using the NJ distance-based method [25]. As depicted in figure 2, the phylogenetic tree segregated the UII and Urp sequences into four main clades which correspond to the four paralogues UII, Urp, Urp1 and Urp2. Bootstrap support values for these groups were 90, 79, 97 and 50%, respectively.

3.5. Synteny analysis of the urp2 genes in vertebrates

To further resolve the orthologue relationship between fish and frog urp2 genes, a synteny analysis was performed. For this purpose, the genomic environment of urp2 genes was determined in X. tropicalis and compared to that of various representative ostechthyian species, namely human, chicken, spotted gar and zebrafish. As shown in figure 3, the three closest genes to urp2 in X. tropicalis, rcan1, clic6 and gart are also located in the vicinity of urp2 in ray-finned fishes (see also electronic supplementary material, table S3 for more details).

3.6. Urp2 gene expression

3.6.1. Urp2 gene expression during Xenopus laevis development

Gene expression profiles of urp2-L and urp2-S genes were examined by RT-qPCR during X. laevis development from fertilization (NF1) to pre-metamorphosis (NF50). mRNA level of urp2-L was found to strongly increase from NF21 to NF37 and became roughly stable thereafter (figure 4). Almost no urp2-S mRNA was detected at the different stages examined.

3.6.2. Tissue expression of urp2 in Xenopus laevis frog

RT-qPCR was used to determine the distribution of urp2-L and urp2-S mRNAs in different tissues of juvenile frogs (figure 5). The highest amount of urp2-L mRNA was measured in the spinal cord. urp2-L mRNA was also detected in the brain, eye, skin, muscles, lung, heart, kidney and testis but at a much lower level. In all other tissues, urp2-L expression was very low or undetectable. Almost no urp2-S mRNA could be detected in the tissues examined.
3.6.3. Localization of urp2 mRNA in *Xenopus laevis* embryos

The localization of *urp2*-L mRNA was studied in *X. laevis* embryos (NF29-30) by *in situ* hybridization. As depicted in figure 6a, the *urp2*-L staining was mainly detected along the ventral part of the spinal cord. At higher magnification, *urp2*-L-positive cells were observed ventrally to the central canal (figure 6a'). No staining was detected in the spinal cord with the sense *urp2*-L riboprobe (figure 6b).

3.6.4. Localization of *urp2* mRNA in the central nervous system of *Xenopus laevis* frog

The expression of the *urp2* gene in the brain and spinal cord was studied in juvenile frogs by *in situ* hybridization. In the brain, the *urp2*-L staining was observed in the ventral midline of the medulla oblongata, ventrally to the fourth ventricle (figure 7a). In the spinal cord, *urp2*-L-expressing cells were seen ventrally to the central canal (figure 7b). As depicted...
3.7. Utr gene expression

3.7.1. Utr gene expression during Xenopus laevis development

Gene expression profile of utr genes (utr1-L, utr3-L, utr4-L and utr5-L) was examined by RT-qPCR during X. laevis development from NF1 to NF50 (figure 8). Utr1-L mRNA level strongly increased from NF10 to NF21 and appeared a bit lower between NF37 and NF50. Utr3-L displayed a similar expression profile to that of utr1-L, but with a slightly earlier phase of increase. utr4-L mRNA level increased from NF10 to NF 24 and became roughly stable thereafter. utr5-L mRNA level stayed very low until NF41 then slightly increased between NF41 and NF51. utr-S mRNA levels were very low at all stages examined (data not shown).

3.7.2. Localization of urotensin II receptors mRNAs in Xenopus laevis embryos

The localization of urotensin II receptor mRNAs was studied in X. laevis embryos (NF29-30) by in situ hybridization (figure 9). The utr1-L staining was mainly detected in the notochord (figure 9a,a’), while the utr4-L staining was primarily present in dorsal somites (figure 9b,b’). It is noteworthy that in juvenile frogs, utr4-L expression was very low in skeletal muscles (electronic supplementary material, figure S11). No apparent staining was observed with utr2-L (figure 9c) and utr5-L (figure 9d) antisense riboprobes (table 1).

3.8. Utr4 crispsants

3.8.1. Generation and phenotype analysis

In zebrafish, mutants for uts2r3 (utr4 counterpart) lead to spine deformation in larvae and adults [12]. To test whether the function of the Utr4 signalling pathway is conserved in X. laevis, we produced CRISPR/Cas9-mediated mutant (i.e. crispsants). We designed two RNA guides, each targeting both utr4-L and utr4-S, and injected them into fertilized eggs together with a guide RNA against the tyrosinase (tyr) gene as a control for injection efficiency (see Material and methods; electronic supplementary material, figure S2). The efficiency of utr4 disruption was assayed by PCR using primers flanking the targeted region. As shown in the electronic supplementary material, figure S12, expected deleted fragments were detected from both utr4-L and utr4-S at 320 and 560 bp, respectively. Moreover, the analysis of Sanger trace data from non-deleted PCR products showed that the knock-out score (percentage of frameshifting indels) at the two targeted sites were 39% and 71% for utr4-L and utr4-S (see electronic supplementary material, table S4). Thus, on average, 82% of non-deleted utr4-L copies were disrupted (85% for utr4-S). Altogether these results show an efficient disruption of utr4.

Embryos knocked-out for both utr4 and tyr (utr4 crispsants) developed normally until stage NF40-42.
NF42, an abnormal curvature of the antero-posterior axis was observed (figure 10a, b). While in control tadpoles (invalidated for only tyr), the average angle between the head and the body was 164° (±4.3° standard deviation, s.d., n = 41), this angle was only 153° (s.d. 7.7, p < 0.001, n = 29) in utr4 crispants (figure 10c).

Electronic supplementary material, figure S13 depicts the relationship between genotype and phenotype of 10 tadpoles randomly selected among the 29 utr4 crispants mentioned above. The results show that (i) unmutated or slightly mutated tadpoles exhibit a wild-type phenotype and (ii) all tadpoles that exhibit a curved phenotype are mutated with
either medium or strong mutation level. Taken together, these results strongly suggest that the curved phenotype is caused by utr4 disruption. The occurrence of some mutated crispants without abnormal curvature could be due to mosaicism.

3.8.2. Quantification of locomotor activity

A summary of the kinematic variables is provided in table 2. Swimming in utr4 crispant tadpoles was erratic and showed regular long-axis rotations (figure 11). This resulted in a greater cumulative displacement along the path in crispants ($F_{1,18} = 10.22; p = 0.005$; table 2) while swimming a similar straight-line distance ($F_{1,18} = 1.19; p = 0.29$). Although crispants had a greater sinuosity (table 2), the difference was not statistically significant ($F_{1,18} = 2.62; p = 0.12$). Swimming in crispants was further characterized by higher mean ($F_{1,18} = 4.60; p = 0.046$) and peak velocity ($F_{1,18} = 8.42; p = 0.01$) and higher peak accelerations ($F_{1,18} = 4.45; p = 0.049$) and decelerations ($F_{1,18} = 4.91; p = 0.04$).

Table 2. Mean kinematic variables of utr4 crispants. Table entries are means ± s.d. Significant differences ($p < 0.05$) are in italics.

<table>
<thead>
<tr>
<th></th>
<th>wild type</th>
<th>knock-out</th>
</tr>
</thead>
<tbody>
<tr>
<td>straight-line distance (mm)</td>
<td>55.04 ± 46.46</td>
<td>70.53 ± 39.83</td>
</tr>
<tr>
<td>cumulative distance (mm)</td>
<td>69.80 ± 54.18</td>
<td>231.15 ± 204.94</td>
</tr>
<tr>
<td>sinuosity</td>
<td>1.43 ± 0.42</td>
<td>6.02 ± 7.97</td>
</tr>
<tr>
<td>peak velocity ($mms^{-1}$)</td>
<td>120.56 ± 138.65</td>
<td>3856.03 ± 9785.67</td>
</tr>
<tr>
<td>mean velocity ($mms^{-1}$)</td>
<td>22.10 ± 17.74</td>
<td>61.58 ± 58.32</td>
</tr>
<tr>
<td>peak acceleration ($ms^{-2}$)</td>
<td>14.14 ± 16.95</td>
<td>568.07 ± 1451.47</td>
</tr>
<tr>
<td>peak deceleration ($ms^{-2}$)</td>
<td>−12.86 ± 17.95</td>
<td>−569.74 ± 1460.51</td>
</tr>
</tbody>
</table>

4. Discussion

The present study reports the characterization of urp2 genes in the *Xenopus* genus. *X. tropicalis* possesses a single urp2 gene, while two homeologues are present in *X. laevis*, in agreement with the pseudotetraploid status of this species.
So far, urp2, first identified in the Japanese eel [15] and zebrafish [16] was only known in ray-finned fishes [2]. Therefore, as far as we are aware of, our study provides the first conclusive evidence for the presence of urp2 in tetrapods.

The molecular organization of the Urp2 precursor is the same in all species examined, with a signal peptide followed by a long central segment, a conserved pair of basic residues and, finally, the Urp2 sequence at their C-terminus. In fish, the Urp2 sequence encompasses ten aa with a fully conserved cyclic region at position 2–7 followed by three residues SQN [15,16]. In frogs, the Urp2 contains one more residue at its C-terminus which exhibits the original IQNK extension. Despite these differences, the orthology between fish and frog urp2 genes is clearly supported by phylogenetic and synteny analyses.

It has been previously shown that urp2 arose through the two whole-genome duplication events (1R and 2R) that took place early during vertebrate evolution [2,16]. The occurrence of urp2 in both fish and amphibians is in full agreement with this model. Moreover, its absence in both chicken and mammals suggests that it has been subsequently lost in the amniote lineage. It is noteworthy that the urp1 gene, which is thought to have appeared at the same time as urp2 in 2R [2,16] could not be found in the frog’s genome.

In zebrafish, urp2 is primarily expressed in the hindbrain and spinal cord, both in the embryo and the adult. In the spinal cord, urp2 mRNA was located in a small population of sensory neurons called CSF-contacting neurons while in the brainstem, urp2-expressing cells were found at the ventral edge of the fourth ventricle [16,17]. The present study reveals that this expression pattern is well conserved in X. laevis, since urp2 mRNA-containing cells also expressed pkl2L1, a specific marker for CSF-contacting neurons [38]. urp2 expression was apparent in spinal CSF-contacting neurons well before hatching and remained visible in the same cell type beyond metamorphosis when tadpoles turn into frogs. It should be noted that only the urp2-L transcript could be significantly detected in X. laevis tissues suggesting that the urp2-S copy is very little expressed and therefore may become pseudogenized either in the near or more distant future [39].

Urp1 and Urp2 have been recently shown to be required for correct axis formation and maintenance in zebrafish. The knock-down of urp1 leads to a curled down axis in zebrafish embryos, while its overexpression leads to the opposite curvature [12]. Although the knock-down of urp2 has no effect in wild-type embryos, its overexpression, as that of urp1, can rescue body axis defects in ciliary mutants, suggesting that Urp1 and Urp2 actions are largely redundant [12]. These actions are thought to be mediated by Uts2r3, a member of the Utr family specifically expressed in muscles, since the uts2r3 knock-down also produced body curvature in zebrafish embryos [12]. Based on these data, it has been hypothesized that uts2r3-mediated contractile activity of dorsal muscle brings about proper axial morphogenesis [12].

Recently, Konno et al. [4] reported the occurrence of four Utr subtypes in X. tropicalis (Utr1, Utr3, Utr4 and Utr5), instead of five in zebrafish [5]. In agreement with these findings, we showed that four Utr homoeologues pairs are also present in X. laevis. Utr3-S and Utr4-S were found to lack multiple transmembrane domains, suggesting that they are not functional. Generally, utr-S genes are all little expressed suggesting that, as urp2-S, they may become pseudogenized.

We identified X. laevis Utr4-L as the frog counterpart of zebrafish Uts2r3, evolutionarily and functionally. Thus, as in zebrafish, utr4-L is specifically expressed in the dorsal somitic musculature of the X. laevis embryo. Moreover, the gene knock-out of utr4 resulted in a severe axial curvature of the tadpoles. The first signs of the spinal axis deformation in utr4 crispants could not be detected until stage NF42, which is consistent with the fact that utr4 expression peaks shortly before hatching. Overall, the phenotype of Xenopus utr4 crispants is reminiscent to that of zebrafish uts2r3 mutants.

The curvature of the body axis in the vertical plane in utr4 crispant tadpoles significantly impacted their locomotion. Swimming in crispants was erratic and included significant long-axis rotation leading to a somewhat higher sinuosity and a significantly higher cumulative displacement along the path. This resulted in higher swimming speeds [40], accelerations and decelerations. However, the ultimate straight-line distance swim by the crispant tadpoles in the same time frame was not different. Consequently, utr4 crispant tadpoles shall compensate for their off-trajectory movements by swimming faster. The increased curvature in the sagittal plane in utr4 crispant tadpoles combined with the oscillatory movements of the tail resulted in a torque being generated causing the long-axis rotation of the tadpole and the greater cumulative distance swim by the tadpole to achieve the same straight-line displacement.

It is noteworthy that the developmental expression dynamics of utr4 is very similar to that of urp2, which agrees with the idea that Urp2 is a natural ligand of Utr4 in the somatic musculature. Taken together, our results show that the role of
the Utr4 signalling pathway in the control of body straightness, first reported in zebrafish, is conserved in X. laevis. Hence, it is likely that this pathway was already operant in the osteichthyan ancestor, more than 400 million years ago [41].

In zebrafish, the view that Utr4 can be activated by other ligands than Urp2 cannot be ruled out, while in frog, it is well supported by a recent study [4]. With this respect, the occurrence of UII and Urp in motoneurons of the X. laevis spinal cord [23,42] makes both peptides additional plausible candidates due to the close functional relationships between muscles and motoneurons. In this regard, it is noteworthy that in zebrafish, Urp (but not UII) is also expressed in spinal motoneurons [17,43,44]. Further studies would be needed to test the roles of Urp and/or UII in both Xenopus and zebrafish in the control of body straightness.

In zebrafish, the Reissner fibre has also been found to play an essential role for axis morphogenesis in the zebrafish embryo [45–47]. The Reissner fibre is an acellular thread formed by the aggregation of the Scl-spondin glycoprotein which extends caudally through the central canal of the spinal cord [48]. It has been found that scsospondin mutant embryos lacking the Reissner fibre fail to extend a straight body axis during embryonic development [45,46]. Interestingly, the Reissner fibre appears to be required for the expression of urp2 in CSF-contacting neurons [13,14], which is in agreement with recent findings showing that it is functionally coupled to the mechanosensory function of CSF-contacting neurons [49]. The Reissner fibre is highly conserved and present in the central canal of almost all vertebrate species [48] including X. laevis [50]. The role of the Utr4 pathway is therefore probably part of a strongly conserved mechanism of control of the body axis morphology in vertebrates which links the Reissner fibre, the CSF-contacting neurons and the somitic musculature.

It is likely that the Utr4 signalling pathway has been lost several times in the amniote lineage since Utr4 lacks both in the chicken and mammals but still present in the lizard Anolis carolinensis [3,5]. Whether other molecular players now ensure the functions previously exerted by Utr4 (plus Urp1 and/or Urp2) is an important issue in mammals. Some of these players could also belong to the urotensin II-ergic and/or Urp2) is an important issue in mammals. Some of these players could also belong to the urotensin II-ergic and/or Urp2) is an important issue in mammals. Some of these players could also belong to the urotensin II-ergic pathway in the control of body straightness, is an ancestral feature of bony vertebrates and not just a peculiarity of ray-finned fishes. Hence, it is likely that this pathway was already operant in the osteichthyan ancestor, more than 400 million years ago [41].

In the present study, we characterized for the first time the urp2 gene in a tetrapod. We showed that X. laevis urp2 is mainly expressed in CSF-contacting neurons of the spinal cord and hindbrain. We also showed that its putative receptor utr4 is primarily expressed in dorsal somites. Finally, we revealed that the utr4 knock-out results in a severe axial curvature of the tadpoles. Taken together, our results strongly suggest that the role of the Utr4 signalling pathway in the control of body straightness is an ancestral feature of bony vertebrates and not just a peculiarity of ray-finned fishes. Understanding why this pathway has been lost in amniotes and determining how it has been replaced is an essential challenge for the future. In particular, answers to these questions should have critical applications in human pathology, notably to identify novel pathogenic mechanisms of IS [53].

5. Conclusion

In the present study, we characterized for the first time the urp2 gene in a tetrapod. We showed that X. laevis urp2 is mainly expressed in CSF-contacting neurons of the spinal cord and hindbrain. We also showed that its putative receptor utr4 is primarily expressed in dorsal somites. Finally, we revealed that the utr4 knock-out results in a severe axial curvature of the tadpoles. Taken together, our results strongly suggest that the role of the Utr4 signalling pathway in the control of body straightness is an ancestral feature of bony vertebrates and not just a peculiarity of ray-finned fishes. Understanding why this pathway has been lost in amniotes and determining how it has been replaced is an essential challenge for the future. In particular, answers to these questions should have critical applications in human pathology, notably to identify novel pathogenic mechanisms of IS [53].

6. Ethics

All animal experimental procedures were approved by the Institutional Ethics Committee at the Museum National d’Histoire Naturelle and the European Communities Council Directive (2010/63/EU) under the animal protocol APAFIS#26986–200082717256159 v12 for the housing and experimentation on Xenopus laevis.

7. Data accessibility

Novel nucleotide sequences reported in this paper have been deposited in the GenBank database under accession nos. M2054702 and M2054703.

8. Authors’ contributions

F.A. performed phenotype analysis of tadpole crispsants; M.L. produced tadpole crispsants with the help of S.L.M.; A.-L.G. performed genotype analysis of tadpole crispsants and gene expression analyses with the help of D.L.; A.H. performed the analysis of locomotor activity of tadpole crispsants; J.-B.F. supervised generation of tadpole crispsants; G.P. designed sgRNAs for Crispr experiments and supervised phenotype analysis of tadpole crispsants; H.T. conceived, designed and coordinated the study. He performed cDNA cloning with the contribution of C.d.F. and M.B. and phylogeny analyses. He wrote the manuscript with the help of F.A., M.L., A.H., J.-B.F. and G.P. All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

9. Competing interests

The authors declare that they have no competing interests.

10. Funding

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References


