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Research Paper

Anxa4 Genes are Expressed in Distinct Organ Systems in *Xenopus laevis* and *tropicalis* But are Functionally Conserved

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KEY WORDS

annexin 4, kidney, liver, evolution, paralog and ortholog, *Xenopus laevis*, *Xenopus tropicalis*, morpholino, organogenesis

ABBREVIATIONS

Anx	Annexin
Anx4	Annexin4
anxa4a	*X. laevis* Annexin4
Xt anx4	*X. tropicalis* anxa4
anxa4b	*X. laevis* annexin4 pseudoallele
sd	standard deviation

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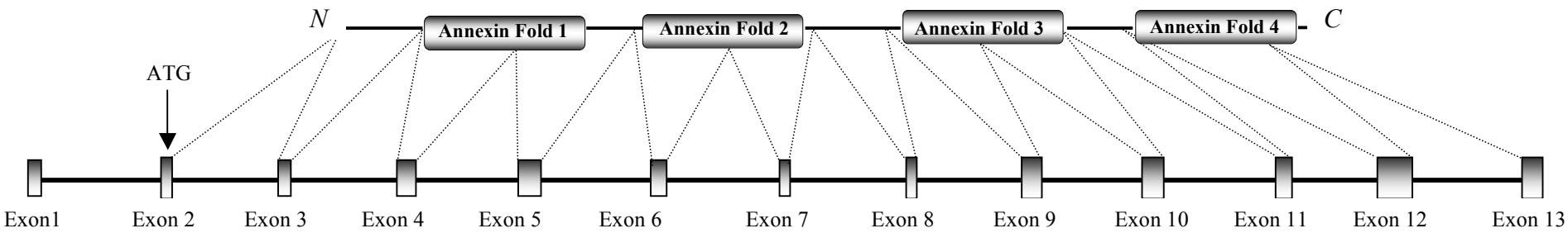
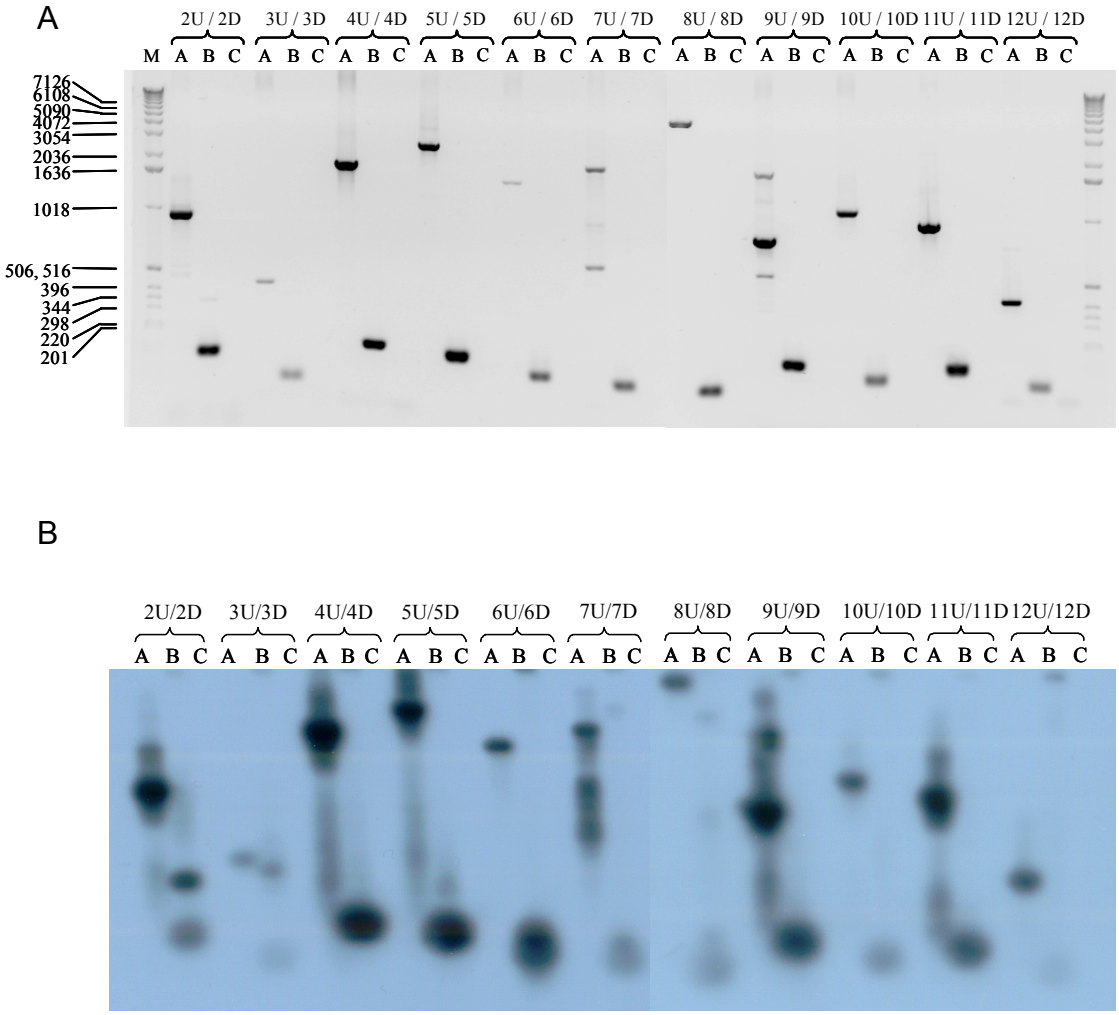
SUPPLEMENTARY MATERIALS



	E1	I1	E2	I2	E3	I3	E4	I4	E5	I5	E6	I6	E7	I7	E8	I8	E9	I9	E10	I10	E11	I11	E12	I12	E13
X. laevis	37	>1400	55(9)	833	88	379	95	*~1700*	114	*~2350*	91	*~1350*	80	*~1950*	57	*~4100 (5500)*	94	*~700*	96	*~1100*	59	*~900*	123	338	112(60)
X. tropicalis	45	8373	57(9)	744	88	375	95	1414	114	1273	91	784	80	396	57	2757	94	558	96	1328	59	2840	123	398	114(60)
H. sapiens	26	ND	59(9)	6479	88	16391	95	1757	114	1417	91	2597	80	1979	57	3391	94	2411	96	583	59	1359	123	4633	943(60)
M. musculus	47	27640	49(3)	5004	88	2760	95	2850	114	959	91	1570	80	973	57	2080	94	4126	96	1447	59	1158	123	4011	911(60)
D.rerio	257	10490	58(9)	2941	88	2869	95	5479	114	3480	91	99	80	446	57	1645	94	2203	96	80	59	76	123	2360	219(60)

Supplementary Figure 1. Exon / intron organization of *anxa4* genes. Exons are represented by shaded boxes and introns by a horizontal line. The total size in base pairs is given in the corresponding column. Coding sequences are underlined. Exon 2 contains the start codon (atg) and exon 13 contains the stop codon (taa). Sizes of exons 1 and 13 were determined by reference to the cDNA sequences (See Table 1 for Accession numbers). Each exon is positioned relative to the anxa4 protein domains. Figures in *italics* are generated from size estimates obtained by reference to PCR amplified bands to size markers; these introns have not been fully sequenced. *X.laevis* intron 1 was never amplified by PCR but was located in the 5'UTR using the Universal Genome Walker kit (CLONTECH laboratories, Inc). Its exact size could not be determined. Genomic sequences for the human, mouse and zebrafish *anxa4* genes were identified by BLAST on the NCBI website. The Genbank accession numbers for these sequences are: *H.sapiens*, NT_022184; *M.musculus*, NT_039353 and *D.rerio*, NW_001513125. *X.tropicalis anxa4* genomic sequences, located on the scaffold 143, were identified by BLAST on the JGI *Xenopus* website (http://genome.jgi-psf.org/Xentr4/Xentr4.home.html). ND, not determined.



Supplementary Figure 2. Identification of the *X. laevis anxa4* introns.

(A) PCR amplification of introns 2 – 12 of *anxa4* gene.

The putative intron boundaries of the *Xenopus laevis anxa4* gene were predicted by reference to the organization of mammalian *anxa4* genes. Primers were designed complementary to exon sequence adjacent to the putative intron boundaries (Supplementary Table 1). Each intron was amplified by PCR from *X. laevis* genomic DNA extracted from adult blood. Each amplification was optimised by gradient PCR by modifying the $MgCl_2$ concentration and the annealing temperature. The optimized conditions used for each intronic amplification, are indicated in Supplementary Table 1. PCR was carried out using the Taq polymerase from Invitrogen and Expand Long Template PCR polymerase (intron 7). For each primer pair, lane A shows the amplified genomic fragment, lane B shows the amplified cDNA fragment and lane C shows the no DNA input control. M, 1Kb DNA marker (Invitrogen).

(B) Southern blot analysis of the PCR amplified intronic sequences using *anxa4a* cDNA labelled with α^{32}P-dGTP. For each primer pair, lane A shows the amplified genomic fragment, lane B shows the amplified cDNA fragment and lane C shows the no DNA input control.

Intron		Sequence at exon-intron junction				Amino acid interrupted
		5' splice donor			3' splice acceptor	
	Xl	ATG GCA GCA	**gt**aagtataaacct……ttcctgttctat	**ag**	CTC GGA ACT AAG	
	Xt	ATG GCA ACA	**gt**aagtatgaacct……tttctgttctct	**ag**	CTA GGA ACT AAG	
2	*Hs*	ATG GCC ATG	**gt**aagttgtgaaat……tcttgtgtgctc	**ag**	GCA ACC AAA GGA	
	Mm	ATG	**gt**gagtgcctcgcc……ttctgtgccttc	**ag**	GAA GCC AAA GGA	
	Dr	ATG GCA GCG	**gt**aagtgaaataac……tttttttactgt	**ag**	TTG GGA AAC CGT	
	Xl	ATG AAA GGA GCA G	**gt**gagtactagtac……cccttgttttct	**ag**	GC ACC GAT GAA	Gly-33
	Xt	ATG AAA GGA GCA G	**gt**gagtactgatat……gtgcccgtttct	**ag**	GC ACC GAT GAA	Gly-33
3	*Hs*	ATG AAA GGG CTC G	**gt**atgtgtcctgct……ccttcttccccc	**ag**	GC ACC GAT GAA	Gly-33
	Mm	ATG AAA GGC CTC G	**gt**atgtacctttct……catcttgtctcc	**ag**	GT ACT GAT GAA	Gly-30
	Dr	ATG AAA GGA GCA G	**gt**gagagagaccat……aagctacttttc	**ag**	GT ACC AAC GAG	Gly-33
	Xl	ACT GTT GGA AAA	**gt**aagtcaagccaa……attatcatttttt	**ag**	GAT CTT GAT GAT	
	Xt	ACT ATT GGA AAA	**gt**aagtcaaaccaa……gttcttatttttt	**ag**	GAT CTG GAG GAT	
4	*Hs*	ACC ATC GGC AGG	**gt**aggccacagtct……ttcttgcattgc	**ag**	GAC TTG ATA GAC	
	Mm	ACC ATT GGC AGG	**gt**agggcccagtct……ctcgtttgttgc	**ag**	GAC CTG ATT GAG	
	Dr	AGC GTG GGA AAG	**Gt**aaaacgtaacac……aaacgctctttc	**ag**	GAA TTG ATG GAC	
	Xl	AAA GCC ATG AAG	**gt**gggtgttctgag……gctatggttttt	**ag**	GGT GCA GGG ACA	
	Xt	AAA GCC ATG AAG	**gt**gggtgttctgaa……gtttgttttttc	**ag**	GGT GCA GGG ACA	
5	*Hs*	AGG GCC ATG AAG	**gt**ctgtgctcttcc……ggtttcttgttt	**ag**	GGA GCC GGC ACT	
	Mm	AGG GCC ATG AAG	**gt**ctgtggtcccct……gccctcacatttt	**ag**	GGA GCT GGC ACG	
	Dr	AAC GCC ATC AAG	**gt**tagtgaggatag……tattttgtgctc	**ag**	GGA GCA GGG ACG	
	Xl	ACC TAC AAA ATA A	**gt**aagtattataaa……tttctgctttgc	**ag**	AA TAT GGT AAA	Lys-133
	Xt	**ACC TAC AGA ATA A**	**gt**aagtagaaaaag……ttttgcattac	**ag**	AA TAT GGT AAA	Lys-133
6	*Hs*	ACC TAC CAG CAG C	**gt**acgtgacatccg……cactctcttgct	**ag**	AA TAT GGA CGG	Gln-133
	Mm	ACA TAC CAG CAG C	**gt**aagtagcagccc……caatttttggt	**ag**	AA TAT GGA AGG	Gln-131
	Dr	**GCT TAC AAG AAA G**	**gt**gcatgctagtta……cctcattatctc	**ag**	AA CAT GAC AAG	Glu-133
	Xl	TCT TTG GCT GCT	**gt**aagtaccacaac……ttgttcatttgc	**ag**	GGT GGG AGA GAC	
	Xt	TCT TTG GCT GCT	**gt**aagtaccacaac……gcattcctttgc	**ag**	GGT GGG AGA GAC	
7	*Hs*	TCT CTG TCA GCT	**gt**gagtgactgctt……tttctcatcctc	**ag**	GGT GGG AGG GAT	
	Mm	TTC CTG TCG GCG	**gt**gcgtggcttcct……ccatcttcccac	**ag**	GCT GGC AGG GAT	
	Dr	TCA TTG CTC ACG	**gt**aaataaacaaat……tgctttgtttac	**ag**	GCT GGG CGT GAC	
	Xl	CAA GAT GCC AAT	**gt**aagtcagtctat……cactttcttctc	**ag**	GAC CTA TAT GAA	
	Xt	CAG GAT GCC AAT	**gt**aagtcagtctat……tactttcttccc	**ag**	GAA CTA TAT GAA	
8	*Hs*	CAG GAT GCC CAG	**gt**tggtagaactgc……ttgtttggcttc	**ag**	GAC CTG TAT GAG	
	Mm	CAG GAC GCC CAG	**gt**ttgtggaccgca……ttgtttgtcatc	**ag**	GAA CTG TAT GAG	
	Dr	CAG GAT GCA AAG	**gt**gaggattttgac……ttatgctttatc	**ag**	GAC ATC TAT GAG	
	Xl	CAC TTA CTG AAA G	**gt**gagcatgtgaga……gctttttttctc	**ag**	TT TTT GAA GAG	Val-210
	Xt	CAC TTG CTG AAG G	**gt**gagcttatctcc……ggttttattctc	**ag**	TT TTT GAT GAA	Val-210
9	*Hs*	CAC CTG TTG CAT G	**gt**aaggcacttact……gttattgtctgc	**ag**	TG TTT GAT GAA	Val-210
	Mm	CAT CTG CTC CAC G	**gt**gggtagccacct……gttattggtcat	**ag**	TG TTT GAT GAA	Val-208
	Dr	GCC CTT CTG CGA G	**gt**aaacacagtaaa……ctgtttttcccc	**ag**	TG TTT CAG GAG	Val-210
	Xl	CTT TTG GCA ATC G	**Gt**aagtatgtcagg……tctttgccttgc	**ag**	TG AAA TGC ATA	Val-242
	Xt	CTT TTG GCA GTC G	**gt**gagtacgttagg……tctttgccttgc	**ag**	TG AAA TGC TTG	Val-242
10	*Hs*	CTG CTG GCT ATA G	**gt**aagctggtaggg……ttttctttgac	**ag**	TA AAG TGC ATG	Val-242
	Mm	CTG TTG GCT ATA G	**gt**aagctggtgagc……cgttttctccac	**ag**	TG AAG TGC ATG	Val-240
	Dr	TTT CTG GCA ATA G	**gt**cagcgttagtgc……gtttgccttttc	**ag**	TG AAA GTC ATA	Val-242
	Xl	AAA TCT ATG AAG	**gt**gagtgtttttgt……cttctgtgttgc	**ag**	GGT CTG GGG ACA	
	Xt	AAA TCT ATG AAG	**gt**gagccctttttt……**cttctgttttgc**	**ag**	**GGT CTG GGG ACA**	
11	*Hs*	AAA TCG ATG AAG	**gt**aaatggccttat……gttttcttcctt	**ag**	GGC TTG GGC ACC	
	Mm	AAA TCC ATG AAG	**gt**aagcagctgtgc……actcccttcctc	**ag**	GGC TTA GGC ACT	
	Dr	AAG TCA ATG AAG	**gt**acacaaaatcac……atatcatttctt	**ag**	**GGT TTA GGG ACT**	
	Xl	TCA TTT ATT AAG	**gt**gagagaagaatt……tttgttttccgc	**ag**	GGT GAC TGC TCG	
	Xt	**TCC TTT ATT AAG**	**gt**aagagaggaatt……ttttgtttctgc	**ag**	GGT GAC TGC TCT	
12	*Hs*	TCG TTC ATC AAG	**gt**aggtcacagcag……tccctatcgaac	**ag**	GGT GAC ACA TCT	
	Mm	TCT TTC ATC AAG	**gt**aggaacatcatc……ctcctttctccc	**ag**	GGT GAC ACT TCC	
	Dr	**TCC TTT ATA AAG**	**gt**gagacattacac……ttgtctttttct	**ag**	GGC GAC ACG TCG	

Supplementary Figure 3: **Exon / intron splice junctions of *anxa4* gene sequences.**
Each intronic amplification was ligated into pGEMt-easy as recommended by the manufacturer (Promega, UK) and confirmed as *anxa4* genomic sequences by in-house sequencing. Intronic boundaries were identified by alignment between the genomic and cDNA sequences and according to the intron/exon boundary consensus sequences (Breathnach and Chambon, 1981). Sequences at the exon/intron boundaries from different species were compared by alignment. Shading represents conservation between species at this base in two or more of the presented intronic sequences. Intronic boundaries **gt**…**ag** are shown in bold. Xl, *Xenopus laevis*; Xt, *Xenopus tropicalis*; Hs, *Homo sapiens*; Mm, *Mus musculus*; Dr. *Danio rerio*.

Breathnach, R. and Chambon, P. Organization and expression of eucaryotic split genes coding for proteins. *Annu Rev Biochem* 1981; 50:349-83.

Supplementary Table 1. Primers designed to amplify up *X. laevis anxa4* introns

Intron	Sequence	Optimum [$MgCl_2$]	Optimum Temperature (^oC)	Cycles	
2	U-CAGCCGACATCTGCTTAGAA D-CCTTTCATGGCGTTCCTCAG	3mM	60	30	This work
3	U-CTGAGGAACGCCATGAAAGG D-GCAATGACGTCAATGAC	7. 5mM	60	30	This work
4	U-GTCATTGACGTCATTGC D-GTTCCTCCACATCATAGAGA	5mM	60	30	This work
5	U-CCGAACTGACAGGGAACTTT D-TCTCCTCTGCGCTGCGAGAT	3mM	60	30	This work
6	U-TCTCGCAGCGCAGAGGAGAT D-CTCTCTGGAACATGAAAGAC	5mM	60	30	This work
7	U-GGAGGACGATATTTGCTCAG D-TCATTCACGGTGCTGCTCTG	1. 5mM	60	30	This work Seville et al. (2002)
9	U-CAGAGCAGCACCGTGAATGA D-CCACTTCTTCTCACCGGCTT	4mM	60	30	This work
10	U-AAGCCGGTGAGAAGAAGTGG D-GTGTCCCGACATTTCAGATT	3mM	60	30	This work
11	U-AGATCTTGAGGCCAGTATAA D-CAATCGTTCTGCAAAGTAGG	5mM	60	30	This work
12	U-AAGAGCAGGCCAGCCTACTT D-TCTTGAACTCGCAGCGGATT	1. 5mM	60	30	This work
13	U-TCCGCTGCGAGTTCAAGAAG D-GAGCACCTTCCTGTAATCTC	3mM	60	30	This work