Comparative expression analysis of cysteine-rich intestinal protein family members crip1, 2 and 3 during Xenopus laevis embryogenesis

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ABSTRACT Members of the cysteine-rich intestinal protein (Crip) family belong to the group 2 LIM proteins. Crip proteins are widely expressed in adult mammals but their expression profile and function during embryonic development are still mostly unknown. In this study, we have described for the first time the spatio-temporal expression pattern of the three family members crip1, crip2 and crip3 during Xenopus laevis embryogenesis by RT-PCR and whole mount in situ hybridization approaches. We observed that all three genes are expressed in the pronephros, branchial arches and the eye. Furthermore, crip1 transcripts could be visualized in the developing cranial ganglia and neural tube. In contrast, crip2 could be detected in the cardiovascular system, the brain and the neural tube while crip3 was expressed in the cranial ganglia and the heart. Based on these findings, we suggest that each crip family member may play an important role during embryonic development.

KEY WORDS: Xenopus laevis, cystein-rich protein, crip1-3, LIM

The first cysteine-rich intestinal protein (CRIP) was discovered as a marker for the suckling-to-weaning transition in the rodent intestine by Birkenmeier and Gordon (Birkenmeier and Gordon, 1986). Among the original names for the different Crip proteins was CRP2 (now Crip2) (Okano et al., 1993), which could easily be mistaken for a cysteine and glycine-rich protein (now classified as CSRP). Both subgroups, Crip and CSRP, belong to the group 2 LIM proteins and consist of three distinct representatives (Birkenmeier and Gordon, 1986, Hempe and Cousins, 1991, van Ham et al., 2003). While Crip1 contains only one single LIM domain, Crip2 and Crip3 carry two of them (Kirchner et al., 2001, Nalik et al., 1989, Wang et al., 1992). Such differences could be the result of DNA duplication events (Nalik et al., 1989). The LIM domain is a conserved cysteine-histidine-rich sequence with a double zinc-finger with the consensus sequence (CX,CX,N,CX,G)-(CX,CX,N,CX,G) (Bach, 2000, Liebhaber et al., 1990). LIM domains are postulated to promote protein-protein interactions exemplified by the interaction between the LIM domain of Crip2 and the PDZ domain IV of PTP-BL (protein tyrosine phosphatase PTP-BL five) (Bach, 2000, van Ham et al., 2003). Furthermore, the LIM domain is known to bind zinc that probably accounts for the zinc-binding properties of Crip1 reported by Hempe and Cousins (Hempe and Cousins, 1991, Hempe and Cousins, 1992). In addition, the zinc-dependent metalloprotease ADAM19 facilitate Crip2 secretion (Tanabe et al., 2010).

In adult mammals, all crip genes exhibit a wide tissue distribution, which might indicate essential roles in diverse cellular functions (Birkenmeier and Gordon, 1986, Casrouge et al., 2004, Chung et al., 2011, Hallquist et al., 1996, Karim et al., 1996, Kirchner et al., 2001, Levenson et al., 1993, Nalik et al., 1989, Okano et al., 1993, Tsui et al., 1994, van Ham et al., 2003, Wang et al., 1992, Yu et al., 2002). The different crip members have been linked to tissue differentiation and remodelling, immune response as well as suppression of angiogenesis and tumorigenesis (Chung et al., 2011, Davis et al., 1998, Hallquist et al., 1996, Khoo et al., 1996, Lanningham-Foster et al., 2002, Wei et al., 2011).

However, little is known about the expression profiles or functions of the crip family members during early embryogenesis in Xenopus laevis. Here, we describe the spatio-temporal expression pattern of crip1, 2 and 3 during Xenopus laevis embryogenesis by RT-PCR and whole mount in situ hybridization. We observed that all three genes are expressed in the pronephros, branchial arches and the eye. Furthermore, crip1 transcripts could be visualized in the developing cranial ganglia and neural tube. In contrast, crip2 could be detected in the cardiovascular system, the brain and the neural tube while crip3 was expressed in the cranial ganglia and the heart. Based on these findings, we suggest that each crip family member may play an important role during embryonic development.

Abbreviations used in this paper: crip, cysteine-rich intestinal protein; LIM, Lin1 1, Isl-1 and Mec-3; RT-PCR, reverse transcription polymerase chain reaction; WMISH, whole-mount in situ hybridization; X. laevis/tropicalis, Xenopus laevis/tropicalis.

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any organism. In this study, we present the specific expression pattern of
\textit{crip1}, \textit{crip2} and \textit{crip3} throughout early \textit{Xenopus laevis}
embryogenesis, which are fundamental information for future
functional analyses. We thus provide in this paper the first com-
parative embryonic expression analyses of all three \textit{crip} genes
in a vertebrate organism.

\section*{Results and Discussion}

\subsection*{Xenopus cysteine-rich protein family members}

So far no expression or functional data have been reported about
the different members of the cysteine-rich protein family in
\textit{Xenopus}. For an initial analysis of \textit{crip} genes in \textit{Xenopus}, we
relied on publicly available \textit{Xenopus laevis/tropicalis} sequences.
The predicted protein sequences of each Crip family member
were highly conserved across species (Fig. 1 B-D). While Crip1
contained only one LIM-domain, both Crip2 and Crip3 contained
two LIM domains (LIM-1 and -2) (Fig. 1A), all of which were highly
conserved among different species (Fig. 1 B-D). Nevertheless,
all three Crip family members are closely related as phylogenetic
analysis showed (Fig. 1E). Furthermore, synteny analyses showed
that \textit{crip1} and \textit{crip2} are neighbouring genes. Their gene loci and
their neighbouring genes are highly conserved between \textit{Homo
sapiens, Mus musculus, Rattus norvegicus} and \textit{Xenopus tropi-
calis} (Fig. 2A). Surprisingly, in the \textit{Xenopus tropicalis} genome,
the genes \textit{pacs2, mta1} and \textit{crip1} had been inverted compared
to the genomes of human, mouse and rat for unknown reasons.
The gene loci of \textit{crip3} and its neighbouring genes are highly
conserved between \textit{Homo sapiens, Mus musculus} and \textit{Rattus
norvegicus} as well (Fig. 2B). The available \textit{Xenopus tropicalis}
genomic region covering \textit{crip3} (Gene ID 548528) is too short for
a detailed analysis and can therefore not be compared with
the gene loci of other organisms. The preserved protein structures
and gene localizations of the individual Crip family members
suggest a conserved expression as well as conserved function
of the \textit{crip} genes across species.

To investigate the temporal expression pattern of \textit{crip1-3} dur-
ing \textit{Xenopus laevis} embryogenesis, semi-quantitative reverse
transcription polymerase chain reaction (RT-PCR) experiments
using \textit{Xenopus laevis} specific \textit{crip1-3} primer pairs (Suppl. Figs.
1-3) were performed. Whereas \textit{crip1} and \textit{crip2} transcripts were
first detected during gastrulation as early as stage 10 and 12.5
respectively, *crip3* was maternally supplied and continuously expressed throughout early embryogenesis (Fig. 3).

We next analyzed the spatio-temporal expression of all three *crip* family members in *Xenopus laevis* embryogenesis by whole-mount in situ hybridization (WMISH). For further tissue-specific analysis, vibratome sections using stained embryos were performed. To examine the expression profiles of all three *crip* family members, we cloned *Xenopus laevis* specific cDNA fragments with the length of 550 bp (*crip1*), 539 bp (*crip2*) and 492 bp (*crip3*) and generated antisense DIG-labelled RNA probes (Suppl. Figs. 1-3). To sow the specificity of these probes, we performed a dot blot indicating the usability of the signed probes (Suppl. Figure 4).

**Expression of *crip1* during *Xenopus* embryogenesis**

The first tissue-specific expression of *crip1* was detected during gastrulation (data not shown) and became manifested at stage 13 in the anterior neural plate (Fig. 4B) being in line with the RT-PCR data. At stage 20, *crip1* was expressed in the developing neural tissue (Fig. 4C). At stage 25, *crip1* transcripts were detected at the dorsal side of the embryo and in the migrating cranial neural crest cells (Fig. 4D). In early tailbud stages, *crip1* was strongly expressed in the neural crest derived part of the second and third branchial arches and at the dorsal aorta and in the blood islands as well (Fig. 4 E-F, I; Fig. 5 G-J,T). Furthermore, *crip1* was clearly visible in the neural roof plate and the ventral region of the neural tube at stage 33 (Fig. 5K) and at the neural floor plate at stage 38.

**Fig. 2. Synteny analyses of the *crip* family members in *Homo sapiens*, *Mus musculus*, *Rattus norvegicus*, *Xenopus tropicalis* and *Danio rerio*.**

(A) The schematic overview shows the comparison of the *crip1* and *crip2* genes and their neighbouring gene loci in *H. sapiens* (chromosome 14), *M. musculus* (chromosome 12), *R. norvegicus* (chromosome 6) and *X. tropicalis* (unplaced scaffold). The gene length and distances between them are not drawn to scale. Conserved genes are indicated by the same color code and non-conserved genes by white boxes. The orientation of the genes open reading frames are depicted by black arrows. Abcc10 ATP-binding cassette sub-family C (CFTR/MRP) member 10, asxl2 additional sex combs like 2 (*Drosophila*), ATP5G1P1 ATP synthase, H+ transporting, mitochondrial Fo complex, subunit G (subunit 9) pseudogene, Brf1 BRF1 RNA polymerase III transcription initiation factor 90 kDa subunit, CUL9 cullin 9, Dlk2 delta-like 2 homolog (*Drosophila*), Dnph1 2’-deoxynucleoside 5’-phosphate N-hydrolase 1, dtmb dystrobrevin beta b, ELK2BP ELK2B member of ETS oncogene family pseudogene, hadhab hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase (trifunctional protein) alpha subunit b, IGH immunoglobulin heavy locus/immunoglobulin heavy chain complex, Igha immunoglobulin heavy chain alpha, Ighg immunoglobulin heavy chain (gamma polypeptide), Kif3cb kinesin family member 3Cbeta, Mta1 metastasis associated 1, Pacs2 phosphofurin acidic cluster sorting protein 2, Ptgr2 prostaglandin reductase 2, rnf8 ring finger protein 8, rtf1 Rtf1 Paf1/RNA polymerase II complex component homolog, Slc22a7 solute carrier family 22 (organic anion transporter) member 7, Slc30a1b solute carrier family 30 (zinc transporter) member 1b, Slc39a1b zinc finger protein 36 C3H type-like 1a, Zfp318 zinc finger protein 318.
At stage 38 and 41, *crip1* was expressed in the anlagen of the developing cartilage structures including the quadrate cartilage, Meckel’s cartilage, ceratobranchial cartilage and ceratohyal cartilage as well as the branchial arches (Fig. 4 G-H; Fig. 5 M-O). Additionally, *crip1* was found in the sensory layer of the epidermis and the ganglion cell layer of the retina (Fig. 5 N,S). Moreover, a specific *crip1* expression could be detected in the cardiac arteries, the second heart field and the ventral aorta (Fig. 4 J-K; Fig. 5 M-P-R). These results are in line with data by others demonstrating *CRIP1* mRNA expression in fetal rat and human hearts (Tsui et al., 1994). *Crip1* transcripts were also detected in the tubules of the developing pronephros (Fig. 5U) and lung (Fig 5V). Not much is known about *crip1* expression during early embryogenesis in other vertebrate species. Further studies will be required to determine whether *crip1* expression is conserved across species.

Expression of *crip2* during Xenopus embryogenesis

The tissue-specific expression of *crip2* started during gastrulation (data not shown) what is in agreement with RT-PCR results. At stage 13, *crip2* was strongly expressed in the anterior neural plate (Fig. 6A). At stage 23, *crip2* was expressed in the anterior neural tissue (Fig. 6B) and at stage 25 at the dorsal side of the embryo and the migrating neural crest cells (Fig. 6C). In tailbud stages,

Fig. 3. Temporal expression patterns of *crip1-3* during *Xenopus laevis* embryogenesis analysed by semi-quantitative RT-PCR approaches with cDNA templates of the indicated stages. As loading control gapdh (glycerinaldehyde-3-phosphate dehydrogenase) and as negative controls –RT (reverse transcriptase) reactions lacking the enzyme reverse transcriptase were used. (A) *Crip1* and *crip2* expression were first detected during gastrulation at stage 10 and 12.5 respectively and continuously expressed till stage 38. (B) In contrast *crip3* transcripts were maternally supplied and continuously expressed till stage 40.

**Fig. 4. Crip1 spatial expression pattern during X. laevis embryogenesis.** Embryonic stages are indicated in each panel. The white dashed circles depict the cement gland. (A) At stage 3, embryos were negative for *crip1* expression (lateral view). (B) Anterior view of a stage 13 embryo. *Crip1* was expressed in the anterior neural plate (black arrowhead). (C) The anterior view of a stage 20 embryo shows *crip1* expression in the anterior neural tissue (white arrowhead). (D-H) Lateral views with anterior to the left. (I-K) Ventral views with anterior to the top. (D) At stage 25 *crip1* transcripts were detected in the migrating cranial neural crest cells (orange arrowhead) and at the dorsal side of the embryo (green arrowhead). (E-F) *Crip1* was expressed in the hyoid arch (ha), in the dorsal aorta (da) with a stronger expression in the posterior part (green arrowhead) and in the ventral blood islands (violet arrowhead). (G-H) In later stages *crip1* transcripts were strongly detected in the head mesenchyme, namely the quadrate cartilage (QC), ceratobranchial cartilage (CBC), and ceratohyal cartilage (CHC) as well as. *Crip1* transcripts were in the dorsal aorta (da) detected. Furthermore, *crip1* was expressed in the pronephric tubule convolute (black arrow) and in the posterior cardinal vein (pvc). The stomadeum including the region where the cloaca will form (blue arrowhead) as well as the tip of the tail (yellow arrowhead) showed a strong *crip1* expression, too. (J-K) Also the second heart field (red arrowhead) and ventral aorta (va) were positive for *crip1*. 
Expression pattern of crip1-3 in Xenopus laevis

Fig. 5. Tissue specific expression of crip1 in Xenopus embryos stage 33, 38 and 41. (A,C,E) Lateral views with anterior to the left. (B,D,E) Ventral views with anterior to the top. Black lines indicate level of sections shown in G-V. (G,I,K,M-V) Transversal sections with the dorsal side to the top. (H,L) Horizontal sections with anterior to the top. The notochord (nc) and pronephric tubules (pt) are depicted by dashed circles, respectively. (M,O,T,U) At stage 33 crip1 transcripts were detected in the neural crest derived part of the second and third branchial arches namely the hyoid arch (ha) and the anterior branchial arch (aba) but not in the posterior third branchial arch (pba) (black arrowheads). (I-L) Furthermore, crip1 was strongly expressed in the blood islands (I-J) and neural roof plate (nrp) and a specific ventral region of the neural tube (green arrowhead). (K-L) At stage 33 crip1 was expressed in the ventral aorta (va), in the sensory layer of the epidermis (sle) and the cranial cartilage structures as indicated: quadrate cartilages (QC), Meckel’s cartilages (MC), ceratohyal cartilages (CHC) as well as in the branchial arches (ba). (N,S) Additionally crip1 was found in the ganglion cell layer (GCL) of the eye. (O,T) Similar to stage 33, at stage 38 crip1 was expressed ventral part of the neural tube (green arrowhead), the neural roof plate (nrp) and in the neural floor plate (nfp). (T) Furthermore, crip1 transcripts were detected in the surrounding layer of the dorsal aorta (da). (P-R) Sections through the heart region revealed crip1 expression in the arteries (a), at stage 38 weakly in the endocardium (white arrowhead) and at stage 41 in the second heart field (red arrowheads). (U-V) At stage 41 crip1 was expressed in the pronephric tubules (pt) and in the lung tubes (lt). le, lens; INL and ONL, inner and outer nuclear layer; RPE, retinal pigmented epithelium.
Fig. 6 (Left). Spatial expression pattern of crip2 during *X. laevis* embryonic development. Embryonic stages are indicated in each panel. The cement gland is depicted as white dashed circles. (A) Animal view of a stage 13 embryo. Crip2 expression was detected in the anterior neural plate (black arrowhead). (B) The anterior view of a stage 23 embryo showed a crip2 expression in the anterior neural tube (white arrowhead). (C) Lateral views with anterior to the left. (E,G,I) Ventral views with anterior to the top. (F) At stage 25 crip2 transcripts were detected in the migrating cranial neural crest cells (orange arrowhead) and the dorsal side of the embryo (yellow arrow). (D) Crip2 was expressed in the cardiac tissue especially of the first heart field (red arrowheads), the endocardium (ec) and the cardiac vascular nerves (yellow arrowhead). (D) At stage 29 crip2 transcripts were strongly detected at the dorsal side (yellow arrow), the posterior cardinal vein (green arrowhead) and at the tip of the tail (blue arrowhead). Furthermore, crip2 was expressed in the fore-, mid- and hindbrain (fb, mb, hb) as well as in the profundal placode (pPr) and the trigeminal placode (pV). (F,H) Later during organogenesis crip2 transcripts were additionally detected in the anterior part of pronephros (black arrow) and the connecting tubule of the pronephros (ctp). (F) At stage 34 the profundal ganglion (gPr), the trigeminal ganglion (gV), the facial epibranchial ganglia egVII, egIX and egXI and the cells that contribute to the vagal and posterior lateral line ganglion (gVPL) were positive for crip2. (H) At stage 37/38 crip2 was also expressed the quadrate cartilages (QC), branchial arches (ba) and dorsal aorta (da).

Fig. 7 (Bottom). Crip2 tissue specific expression in Xenopus embryos stage 34, 37/38 and 41. (A,C,E) Lateral views with anterior to the left. (B,D) Ventral views with anterior to the top. Black lines show level of sections demonstrated in F-L. (F-I,K,M-U) Transversal sections with the dorsal side to the top. (J, L) Horizontal sections with anterior to the top. The notochord (nc) and pronephric tubules (pt) are depicted by dashed circles, respectively (L-R-U). (F-H) During Xenopus embryogenesis crip2 was expressed in the developing eye, more precisely the ganglion cell layer (GCL). In contrast, no expression was detected in the lens (le), the inner and outer nuclear layer (INL, ONL) or the retinal pigmented epithelium (RPE). (I,J) At stage 34 crip2 was found in the neural tube except at the most dorsal and ventral parts (orange arrowheads). (K,L) Furthermore, crip2 expression was detected in the glomerulus (g) and the specific pronephric tubules (ptU). (M-P) Cardiac sections at stage 34 and 37/38 revealed crip2 expression in the outflow tract (oft), the pericardial roof (r) as well as the myo- and endocardium (m, e) of the developing heart, but not the pericardium (p). (Q-U) Crip2 was expressed in various tissue of the brain, predominantly in the mid- and hindbrain (mb, hb). The ganglia and placodes of the profundal (gPr, gPv) and trigeminal (gV, gV) show distinct crip2 expression as well as the cornea epithelium (CE/JO). (R-I) Consecutive transversal sections at the level of the otic vesicle (otv) showed crip2 transcripts in the sensory layer of epithemis (sle), neural crest cells (ncc), the fused ganglia of the glossopharyngeal and middle lateral line nerves (gX>M) and the branchial arches (ba).
crip2 transcripts were detected in various parts of the brain (Fig. 7 Q-U) as well as in the posterior neural tube (Fig. 7I). Note, that in the neural tube, differentiated neurons were crip2 positive whereas neural stem cells located in the centre of the neural tube were negative for crip2. The facial epibranchial ganglia egVII, egX and egXI, the cells that contribute to the vagal and posterior lateral line ganglion as well as the placodes and ganglia of the profundal and trigeminus nerve also showed distinct crip2 expression at sages 34 (Fig. 6 D,F). Additionally, crip2 was expressed in the sensory layer of the epidermis, neural crest cells, the fused ganglia of the glossopharyngeal and middle lateral line nerves (Fig. 6F; Fig. 7Q-S) and the branchial arches (Fig. 6H; Fig. 7 T-U). In the ganglion cell layer of the eye, crip2 transcripts were detected as well (Fig. 7 F-H). Furthermore, crip2 expression was visualized in the developing pronephros (Fig. 6 F,H; Fig. 7 K-L) and in the pericardial roof, the myo- and endocardium of the developing heart (Fig. 6 D-I; Fig. 7 M-P).

The spatio-temporal expression of Crip2 during embryogenesis was also described in more detail in mice and zebrafish (Sun et al., 2008, Wei et al., 2011, Yu et al., 2002). Crip2 is detected in pre-streak embryo, up-regulated with the onset of gastrulation and shows an abundant expression throughout the developing heart which is consistent with our expression study. Later during murine embryogenesis, Crip2 is expressed in the heart primordial (E7.5), heart tube (E8.5) and strongly in the myo- and endocardium as well as the coronary vascular endothelial cells in the atrium and ventricle of embryonic (E9.5-15.5) and adult mouse hearts. Again, this is in agreement with crip2 expression in Xenopus. Additionally, murine Crip2 was detected in lymphatic endothelial cells, in dorsal root ganglia and around the spinal cord, the neural tube including the brain, lungs, pelvic ganglia and root ganglia and around the spinal cord, the neural tube as well (Fig. 7F-H). Furthermore, during murine embryogenesis, crip2 was strongly expressed in the branchial arches (egVII, egIX and egXI, the cells that contribute to the vagal and posterior lateral line ganglion as well as the placodes and ganglia of the profundal and trigeminus nerve also showed distinct crip2 expression at sages 34 (Fig. 6 D,F). Additionally, crip2 was expressed in the sensory layer of the epidermis, neural crest cells, the fused ganglia of the glossopharyngeal and middle lateral line nerves (Fig. 6F; Fig. 7Q-S) and the branchial arches (Fig. 6H; Fig. 7 T-U). In the ganglion cell layer of the eye, crip2 transcripts were detected as well (Fig. 7 F-H). Furthermore, crip2 expression was visualized in the developing pronephros (Fig. 6 F,H; Fig. 7 K-L) and in the pericardial roof, the myo- and endocardium of the developing heart (Fig. 6 D-I; Fig. 7 M-P).

Expression of crip3 during Xenopus embryogenesis

Like in the RT-PCR experiments, crip3 transcripts could be detected early on by WMISH approaches. At stage 3, crip3 was visualized in the animal half of the embryo (Fig. 8A). At stage 13, crip3 was detected in the heart tube, ventricular cardiomyocytes and aortic vessels in pharyngeal arches 3-6 (24-36 hpf) (Sun et al., 2008). Hence, crip2 was recently used as cardiac neural crest cell marker (Wang et al., 2013). Collectively, these data indicate a conserved expression of crip2 across species.

Fig. 8. Spatial expression of crip3 during X. laevis embryogenesis. Embryonic stages are indicated in each panel. The white dashed circles depict the cement gland. (A) Animal view. Crip3 expression was detected at the animal half at 4-cell stage (black arrowhead). (B) Crip3 is expressed in the anterior neural plate. (C) The anterior view of a stage 20 embryo showed crip3 expression in the anterior neural tube (an). (D-H) Lateral views with anterior to the left. (I-K) Ventral views with anterior to the top. (D-H) At stage 25 and 29 crip3 was expressed in the migrating neural crest cells (orange arrowhead in (D,E) and at later stages in the branchial arches (ba, G,H). (F,G) During organogenesis a very weak crip3 signal was seen in the dorsal side of the embryo (green arrowheads). (E-K) Furthermore, during Xenopus embryogenesis crip3 was detected in the developing eye (green arrow) and cardiac tissue (red arrowheads).
in the lens and the retina, in particular the ganglion cell layer, at stages 37/38 and 41 (green arrows in Fig. 8 E-H; Fig. 9 B-J-L).

Little is known about the embryonic crip expression in other organisms. Human CRIP3 could be detected in the fetal thymus, spleen, brain, heart, kidney, liver and lung partially in line with our observations (Casrouge et al., 2004). These limited findings make a comparison of the expression across species difficult.

As all three crip family members show a distinct expression pattern during early Xenopus development (Table 1), it would be interesting to examine their function during embryonic development by using knock-down approaches in future studies.

**Material and Methods**

**Xenopus laevis embryos**

Xenopus embryos were obtained, cultured according to standard protocols (Sive et al., 2000) and staged according to (Nieuwkoop and Faber, 1994).

**Cloning of Xenopus laevis crip1-3**

X. laevis cDNA fragments of 550 bp (crip1; Acc. No. KP036486), 539 bp (crip2; Acc. No. KP036487) and 492 bp (crip3; Acc. No. KP036488) were cloned into the pSC-B vector (Stratagene, La Jolla, Ca) by using cDNAs from stages 28 (crip1), 33 (crip3) or 42 (crip2) of X. laevis embryos. The primers were designed according to conserved sequence regions (X. laevis crip1-a: NM_001093834.1; X. tropicalis crip2: NM_001079267.1; X. tropicalis crip3: NM_001015811.1). Following cloning primers were used: crip1a_forw: 5'-CACCAAGCAACATGCCAAGTGT-3'; crip1a_rev: 5'-GTAACATATAACACCTGTTGAATGCTAT-3'; crip2_forw: 5'-ATGGCTTCAAGTGATGCTAT-3'; crip2_rev: 5'-CCATAAGCAAGCTTGTGCGATG-3'; crip3_forw: 5'-CTTCGCAAGAAATGAGCTCTCT-3'; crip3_rev: 5'-CCGTAATGGAATTCGAG-3'. The proof reading Phusion DNA polymerase (Agilent Tech., Santa Clara, CA) was used for all PCR reactions. Amplification accuracies and inserts orientation were confirmed by sequencing. Sequences were deposited at NCBI with the accession numbers KP036486 (crip1), KP036487 (crip2) and KP036488 (crip3).

**Protein alignment and synteny analyses, phylogenetic tree**

The ClustalW2 program from the EMBL-EBI hompage was used for amino acid sequence alignment, homology calculation and generation of the phylogenetic tree. Following sequences were used: human CRIP1: NP_001302.1, mouse Crip1 NP_031789.1, rat Crip1: NP_001231796.1, X. tropicalis Crip1: NP_001165119.1, X. laevis Crip1-a: NP_001087303.1, zebrafish Crip1: NP_001153291.1, human Crip2: NP_001303.1, mouse Crip2: NP_077185.1, rat Crip2: NP_071946.1, X. tropicalis Crip2: NP_001072735.1, zebrafish Crip2: NP_998662.2, human Crip3: NP_996805.2, mouse Crip3 isoform TLP-A: NP_858050.1 (homology) and isoform TLP-B: NP_444460.1 (phylogeny), rat Crip3: NP_001102773.1, X. tropicalis Crip3: NP_001015811.1, NCBI GeneBank and Xenbase Genome Browser v7.1 were used for synteny analyses, genomic structure and chromosomal organisation. Human, mouse, rat, X. tropicalis and zebrafish crip1, crip2 and crip3 were compared.

**RNA isolation and RT-PCR assays**

To examine the temporal expression pattern of crip1-3, total RNA of Xenopus embryos at different developmental stages (stages 1 to 41) was isolated using thepeq-GOLD RNApure kit (Peqlab, Erlangen, D). cDNA was generated using random primers and the SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). RT-PCRs were performed using the Phire Hot Start II DNA polymerase (Thermo Scientific, Waltham, MA) and following primers: GAPDH_RT_forw: 5'-GGGTATCCTCCCTGGGAAAAG-3'; GAPDH_RT_rev: 5'-ATTTGCTGATGCTTCGAG-3'; Crip1_RT_forw: 5'-GGATATCCTCCTGGGAAAAG-3'; Crip1_RT_rev: 5'-ATTGGGACT-
Expression pattern of crip1-3 in Xenopus laevis

TACTCGGCCAC-3'; Crip2_RT_for: 5'-GCCCGAAGTGACAAAGACT-3'; Crip2_RT_rev: 5'-GCTGGAAAGCTTGGTGAGAC-3'; Crip3_RT_forwr: 5'-CTTCCGACGAGAGTGACCTCTT-3'; Crip3_RT_rev: 5'-CCGTAGCATGACAGTGCCAG-3'. Note that crip1 primers were designed using the previously published Xenopus laevis sequence (Acc. No. NM_06195324.1) and therefore do not completely match with the sequence shown in Suppl. Fig. 1. Annealing temperatures were: gapdh: 55°C; crip1: 52°C; crip2: 51°C; crip3: 54°C and the product lengths were: gapdh: 230 bp; crip1: 366 bp; crip2: 332bp; crip3: 488 bp.

Dot blot

Dot blotting using Hybond-N+ nucleic acid transfer membranes (GE Healthcare, Cleveland, Ohio) was done to test RNA probes for their specificity. 1 μg of plasmid DNA of crip1-3 was diluted in 2x SSC buffer, denatured, dot blotted and hybridized with crip1-3 digoxigenin-labeled antisense RNA probes according to the manufactures instruction (GE Healthcare, 9.5 protocol for dot blotting and 10.2 hybridization protocol). Blocking and staining of the blots with BM-Purple (Roche, Basel) was done according the WMISH protocol.

Whole mount in situ hybridization (WMISH) and sectioning

To investigate the spatio-temporal expression of crip1-3 digoxigenin-labeled antisense RNA probes were generated by in vitro transcription with either T7 or T3 RNA polymerase (Roche, Basel). WMISH analyses were performed according to established protocols (Hemmati-Brivanlou et al., 1990) using fixed Xenopus embryos at different developmental stages, which were subsequently stained with BM-Purple (Roche, Basel). For more detailed analyses of the gene expressions vibratome sections of 25μm thickness were performed as previously described (Guo et al., 2011).

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

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