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XIer2 is required for convergent extension movements during *Xenopus* development

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ABSTRACT Immediate early response 2 (Ier2) is a downstream target of fibroblast growth factor (FGF) signaling. In zebrafish, Ier2 is involved in left-right asymmetry establishment and in convergent extension movements. We isolated the *Xenopus ier2* gene based on sequence similarity searches using multiple vertebrate species. *Xenopus* Ier2 has high homology in the N-terminal region to other vertebrate Ier2 proteins, and *XIer2* transcripts were observed from oocytes through larval stages. Except for the maternal expression of *xier2*, the expression of this gene in the marginal region at gastrulation and in somites and the notochord at later stages is similar to the expression pattern of zebrafish *ier2*. XIer2 knockdown using antisense morpholinos resulted in defects of convergent extension leading to severe neural tube defects; overexpression of Ier2 showed similar, albeit milder phenotypes. Assays in animal cap explants likewise showed inhibition of elongation after blocking XIer2 expression. These results indicate that *Xenopus* Ier2 is essential for the execution of convergent extension movements during early *Xenopus* development.

KEY WORDS: *XIer2, Xenopus, convergent extension*

Introduction

Immediate early response 2 (Ier2) has been identified as a growth factor-inducible protein, and the signals involved in its expression have been described (Charles *et al.,* 1990; Latinkic and Lau, 1994), but the functions of Ier2 in the mouse embryo have not been reported to date. Recently we have isolated zebrafish Ier2 as a downstream target of FGF signaling and described its role in left-right asymmetry patterning in this animal (Hong and Dawid, 2009). In addition to affecting left-right asymmetry, knockdown of zebrafish Ier2 also results in defects in convergent extension movements from late gastrula through early segmentation stages. Convergent extension movements are critical in both *Xenopus* and zebrafish for the elongation of involuting tissue and for establishing the anterior-posterior body axis of the embryo (Keller *et al.,* 2000; Wallingford *et al.,* 2002; Heisenberg *et al.,* 2000; Sepich *et al.,* 2000).

To investigate whether the functions of Ier2 are conserved among different vertebrate species we examined the role of this protein in gastrulation and post-gastrulation development of a tetrapod. Here we report the isolation of the two *Xenopus ier2* genes and their expression during development. Using an antisense morpholino (MO) to attenuate Ier2 expression, we probed the role of Ier2 protein in the embryo and in animal explants. Our observations indicate that XIer2 has a critical role in convergent extension movements in the early development of this animal.

Results

Isolation of Xenopus laevis ier2

To examine the roles of *Xenopus* Ier2 during embryonic development we identified *Xenopus tropicalis* Ier2 by searching its genome using the highly conserved N-terminal region of other vertebrate Ier2 sequences. This search led to a contig, Scaffold_649, in the Ensembl genome browser, which provided us with full-length sequence information for *X. tropicalis ier2*. We designed PCR primers based on *X. tropicalis* sequence information, allowing us to isolate *X. laevis ier2* cDNA. As is common, *X. laevis* contains two pseu-

Abbreviations used in this paper: fgf, fibroblast growth factor; Ier, immediate early response gene; mo, morpholino.

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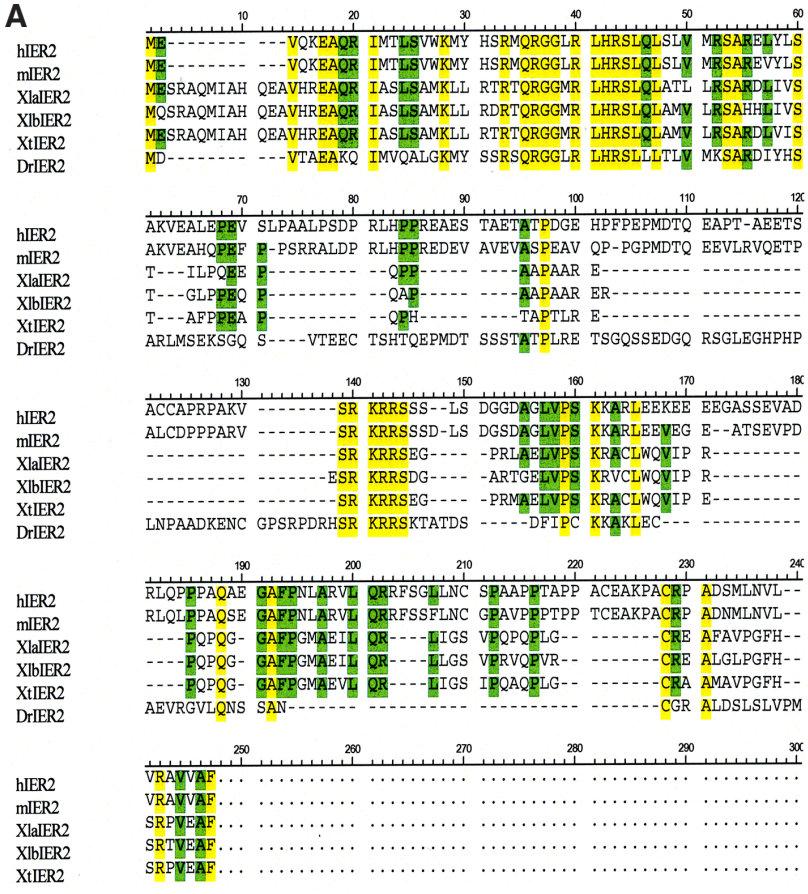


Fig. 1. Alignment of vertebrate ler2 sequences. (A) *Comparison of amino acid sequences of vertebrate ler2 proteins including human ler2 (h) (NM_004907), mouse ler2 (m) (NM_010499), X. laevis ler2a (Xla) (GQ120520), X. laevis ler2b (Xlb) (GQ120521), X. tropicalis (Xt) (predicted sequence based on Scaffold_649 of genomic sequence), and zebrafish ler2 (Dr) (NM_001142583). Conserved sequences are highlighted in yellow and green.* **(B)** *Phylogenetic tree of vertebrate ler2 proteins.*

doalleles of *ier2*, which share 87% amino acid sequence identity. The sequences have been deposited in the public data base as *xier2a* (GenBank accession # GQ120520) and *xier2b* (GenBank accession #GQ120521). Alignment of the corresponding protein sequences with ler2 from other vertebrate species shows that this protein displays substantial conservation among several vertebrates (Fig. 1A). The evolutionary relationship between vertebrate ler2 proteins is shown in Fig. 1B.

Expression of xier2 during development

To examine expression of *xier2*, we performed semi-quantitative RT-PCR analysis and whole mount *in situ* hybridization using both of *xier2a* and *xier2b*. *Xier2* transcripts of both genes were detected in maternal RNA and during embryogenesis through tadpole stages (Fig. 2A). The spatial expression of *xier2b* was visualized using whole mount *in situ* hybridization during development of *Xenopus*. Transcripts were observed before gastrulation in the animal region (Fig 2 B,C), and in the marginal region during gastrulation (Fig. 2 D,E). At st. 13 *xier2b* transcripts were detected in the hindbrain and paraxial mesoderm (Fig. 2F). These domains of expression are maintained during later stages, and in addition branchial arches, somites, and the notochord showed expression during tailbud to tadpole stages (Fig. 2 G-I). Sense strand controls showed no staining at any stage tested (Fig. 2 J-L). The *xier2a* expression pattern is similar to the pattern of *xier2b* shown in Fig. 2 (data not shown). Furthermore, in the marginal region, notochord and arch primordia, the expression of *xier2b* was similar to the pattern seen for zebrafish *ier2* (Hong and Dawid, 2009),

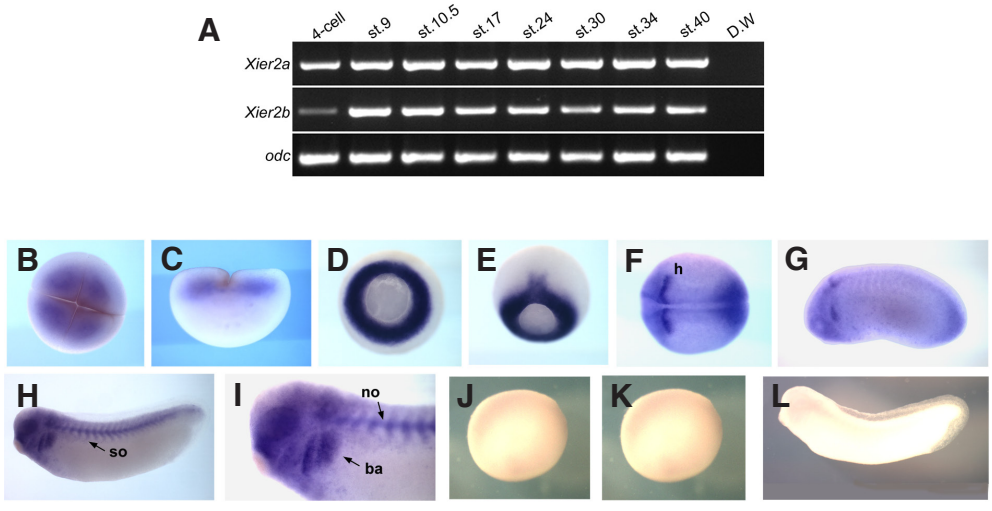


Fig. 2. Expression patterns of *xier2*. (A) *RT-PCR analysis of Xenopus* ier2a *and b genes from early cleavage to st 40. Odc was used as control.* **(B-I)** *Whole mount in situ hybridization of* xier2b**,** *using antisense strand probe;* **(J-L)** *sense strand used as control.* **(B,C)** *Maternal expression is seen in whole mount and transverse section of two-cell stage embryo* **(C)**. **(D,E)** *Restricted expression of* xier2b *in the marginal zone and involuting axial mesoderm during gastrulation at stage 11.* **(F,G)** *Hindbrain and somite expression of* xier2 *at st 13* **(F)** *and st 25* **(G)**. **(H,I)** *Expression of* xier2b *in branchial arches, notochord, and somites at stage 30;* **(I)** *is a magnified view of the head region. ba, branchial arches; h, hindbrain; no, notochord, so, somites.* **(J-L)** *Embryos at st 11* **(J,K)** *and st 29/30* **(L)** *hybridized with sense strand as controls.*

suggesting similar functions for Ier2 during embryonic development of *Xenopus* and zebrafish.

Convergent extension defects result from knockdown and overexpression of XIer2

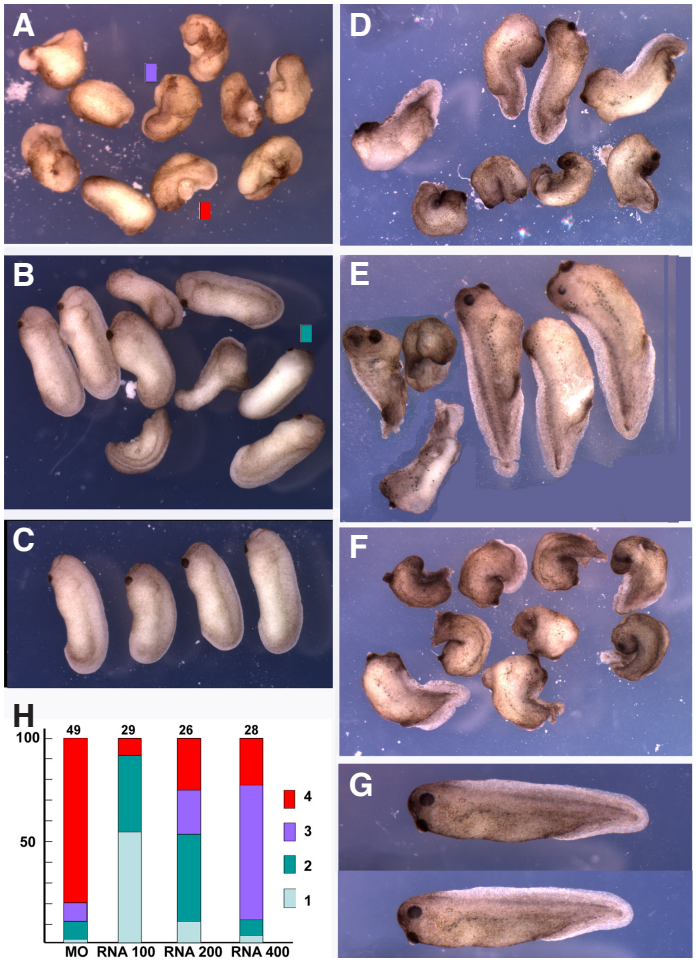
To investigate the function of XIer2 in *Xenopus* development, anti-sense XIer2 MOs were designed to deplete endogenous XIer2 by targeting the translation start site. We injected Xier2 MO into 4-cell stage embryos into each of the two dorsal blastomeres and analyzed the resulting phenotype. Injection of XIer2b MO at 40 ng per embryo resulted in a severe phenotype indicating inhibition of convergent extension movements, leading to a shortened axis and frequently an open neural tube, whereas injection of 60ng control MO had no effect on development (Fig. 3 A,D,H). Similar levels of an MO targeting XIer2a had a much weaker effect on development, and therefore we restricted our attention on the XIer2b MO. Overexpression of Ier2 by injection of synthetic mRNA also led to phenotypes that were less severe than those of MO injection, and were dosage dependent (Fig. 3 B,E,F,H). Rescue of whole-embryo knock-down phenotype was not achieved, possibly because both MO and mRNA generate a phenotype and achieving a true balance proved impractical. However, the specificity of the MO is indicated by the rescue that could be achieved in animal explant experiments, as shown below.

To further study the effect of this MO we used animal caps that are induced to elongate by treatment with activin; induced animal caps undergo cell movements that are appropriate model systems for convergent extension in the embryo (Tada and Smith, 2000; Wallingford *et al.*, 2000). Untreated animal caps fail to elongate (Fig. 4A), while activin-treated explants elongate (Fig. 4B). This elongation was strongly inhibited by knockdown of XIer2b (Fig. 4C) and importantly, was rescued to a large extent by the co-injection of 100pg of *xier2b* mRNA (Fig. 4D). The respective phenotypes were 100% penetrant in these treatments (Fig. 4). These data support the view that Ier2 function is required for convergent extension movements in the *Xenopus* gastrula embryo.

The phenotypes observed after Xier2 knock-down were not due to inhibition of mesoderm induction. Several markers of mesoderm differentiation including *xbra*, *bmp4*, and *fzb*, as well as the organizer marker *gsc* and the neural marker *sox2* were unaffected in animal caps by injection of Xier2 MO (Supplementary Fig. S1A). Likewise, the paraxial mesoderm marker *myoD* was expressed in MO as well as RNA injected embryos, albeit in distorted shape (Supplementary Fig. S1 B-D). These observations support the view that Xier2 has a role in convergence and extension movements in the embryo.

Discussion

In this study we report the isolation of *Xenopus laevis ier2* cDNA, and a comparison of the amino acid sequences of Ier2 proteins



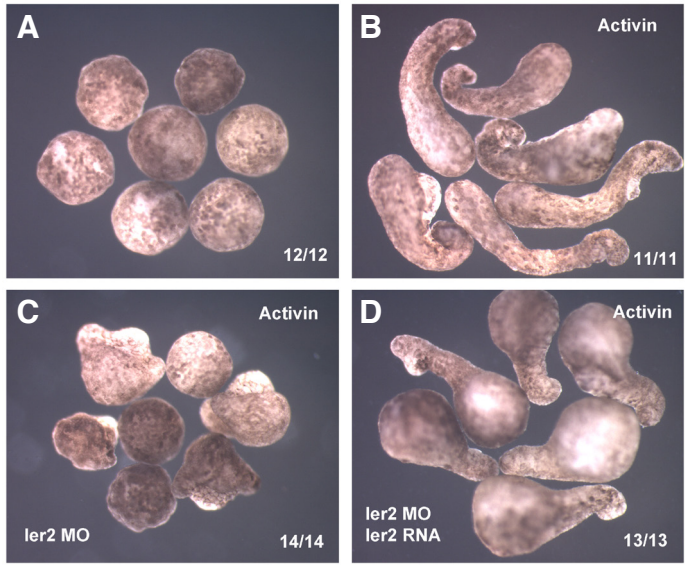


Fig. 3 (Left). Phenotypes generated by Ier2 knock-down and overexpression. (A-C) *stages 25-27;* **(D-G)** *stage 35/36.* **(A,D)** *Ier2 MO, 40 ng;* **(B)** ier2 *RNA, 100 pg;* **(E)** ier2 *RNA, 200 pg;* **(F)** ier2 *RNA, 400 pg;* **(C,G)** *uninjected.* **(H)** *quantification of phenotypes: 1, normal; 2, elongated but abnormal; 3, very short axis with closed blastopore; 4, very short axis with open blastopore. Colored rectangles are placed next to embryos illustrating each phenotype in (A,B). Total number of embryos scored for each injection is shown on top of the histogram, based on two independent experiments.*

Fig. 4 (Right). Convergent extension defects in animal caps caused by knockdown of XIer2. *Control animal caps round up* **(A)**, *but elongate after addition of activin* **(B)**. *Elongation is inhibited by Ier2 knockdown* **(C)**, *and is rescued by co-injection of* xier2 mRNA **(D)**. *The number of animal caps showing the phenotype portrayed and the number of caps tested is listed in each panel. The data are based on two independent experiments.*

in several vertebrates. As observed previously, vertebrate Ier2 proteins have highly conserved N-terminal sequences while the remaining region shows considerable sequence variation (Hong and Dawid, 2009), and this conclusion holds true for the homologs in *X. laevis* and *X. tropicalis* (Fig. 1). The expression pattern of the *ier2* gene in *Xenopus* again shows considerable similarity to the pattern previously observed in zebrafish (Hong and Dawid, 2009). In zebrafish, zygotic expression of *ier2* is restricted to the marginal region and developing notochord during gastrulation. At later stages the expression pattern is more dynamic, including the brain, portions of the branchial arches and blood vessels. Many of the expression domains of *Xenopus ier2* were the same as those in the zebrafish during early development, except that maternal expression was observed for *Xenopus ier2* but not for zebrafish. The similar expression in the marginal zone of both species during gastrulation is of interest because of the defects in convergent extension movements that result in both zebrafish (Hong and Dawid, 2009) and *Xenopus* embryos as a consequence of the knockdown of Ier2. Convergent extension prominently involves the marginal region and the developing notochord, and dorsal marginal explants spontaneously undergo convergent extension movements. These movements lead to the elaboration of the anterior-posterior axis in the embryo.

How does Ier2 function in its role in convergent extension? We have previously shown in zebrafish that *ier2* is a target gene of Fgf signaling, and is critical in the transmission of the Fgf signal during formation of Kupffer's vesicle, and ultimately in the establishment of left-right asymmetry (Hong and Dawid, 2009). It is possible that Ier2 is a more general downstream effector of the FGF pathway, affecting processes beyond left-right asymmetry. Such a hypothesis might well account for the requirement for Ier2 in convergent extension as Fgf signaling is well known as an important regulator of this process. The role of Fgf in this context appears to be mediated by different molecules that do not appear to constitute a linear pathway. Brachyury, a well-known Fgf target gene, is required for gastrulation movements in addition to its various roles in mesoderm differentiation and determination of caudal tissues (Conlon and Smith, 1999). However, we find that Ier2 knock-down does not inhibit *xbra* expression in activin treated animal caps (Supplementary Fig. S1). Some of the effects of Fgf in convergent extension are regulated by the inhibitor Sprouty2 which appears to affect movements differentially from mesoderm differentiation and gene expression (Nutt *et al.*, 2001). More surprising is the observation that Xnr3, related in sequence to the Tgf-β family, affects convergent extension through the Fgf receptor 1 (Yokota *et al.*, 2003), and finally an additional Fgf target involved in gastrulation movements has been found more recently (Chung *et al.*, 2005). We suggest that Ier2 is a novel example of this growing array of Fgf target genes that have, among other functions, a role in the control of convergent extension, a key process in the establishment of the vertebrate body plan during gastrulation.

Materials and Methods

Isolation of Xenopus ier2 cDNAs and RT-PCR assay

Isolation of X. laevis ier2 cDNAs was based on homology searches using the conserved N-terminal region of other vertebrate ier2 genes. Scaffold_649 of the X. tropicalis data base from Ensembl (http://www.ensembl.org/Xenopus_tropicalis) provides full information ier2 genomic sequences. The full-length ORFs of X. laevis ier2 was amplified by PCR and subcloned into BamHI-EcoRI sites of the pCS2+ vector. The following primer set was used:
F:5'-AAGATGCAGAGCCGAGCCCAGATG-3',
R:5'-CCCCGGTAGCGCTTAGAAAGCCTC-3'. RT-PCR was performed as previously described (Tanegashima et al., 2004). Total RNA was isolated from X. laevis embryos using TRIzol reagent (Invitrogen), and first-strand cDNA synthesis was performed using SuperScript III (Invitrogen). The primers used are:

xier2a F5'- CAAACATTAGCAGGCGAGGGTTC-3',
R5'- CATCTTATGTTGTTTCCCTAGTC-3';
xier2b F5'-CAGAGCCGAGCCCAGATG -3',
R5'-GGTAGCGCTTAGAAAGCCTC -3'.

Ornithine decarboxylase (ODC) was used as internal control in RT-PCR assays.

Sequence alignment and phylogenetic tree

Multiple amino acid sequence comparisons and phylogenic tree analysis were carried out with DNASIS MAX version 2.0 (MiraiBio, Hitachi software). Accession numbers are listed in the legend to Fig. 1.

Whole mount in situ hybridization

Whole mount *in situ* hybridization was carried out based on Harland (1991). Anti-sense probe was generated by BamHI linearization and transcription with T7 polymerase from pCS2$^+$-XIer2a and b constructs. BM-Purple (Roche) was used for substrates for color reaction.

Injection of mRNA and morpholino

Capped synthetic *xier2* RNA was generated using mMessage mMachine Kit (Ambion) and was injected at levels indicated in the text. XIer2 anti-sense oligonucleotide (MO) targeting the translation initiation sites of *xier2a, xier2b* and standard control MO were purchased from Gene Tools LLC. The sequences are as follows:

XIer2a MO: 5'- CCATCTCACTTTCCAATGCTGAACC-3';
XIer2b MO: 5'- GCATCTTGTGCTGCTTCTTCCGCGC-3';
Ctrl MO 5'-CCTCTTACCTCAGTTACAATTTATA-3'.

The bold letters in XIer2 MO indicate the translation start sequences. Forty ng of XIer2 MO or 60ng of Control MO were injected into the dorsal blastomeres at the four-cell stage.

Animal cap assay

Animal cap assay were carried out as previously described (Tanegashima *et al.*, 2008).

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