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Developmental Expression Pattern

The *Fox* gene family in *Xenopus laevis* : *FoxI2, FoxM1* and *FoxP1* in early development

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ABSTRACT We here describe the sequences and expression patterns of three new Fox (fork head box) transcription factors in *Xenopus laevis* embryos. *xlFoxI2,* another member of subclass I, is maternally transcribed. Zygotic transcripts are first detected during neurulation and become localised to the dorsal part of epibranchial placodes. *xlFoxM1* like *xlFoxP1* are the first members of subclasses M and P described in *Xenopus*. Both genes are maternally expressed and transcripts are found during early cleavage stages in the animal blastomeres. *xlFoxM1* is strongly upregulated during neurula stages and transcripts are localised in the neuroectoderm. Later, expression is found in the spinal cord, the rhombencephalon, the retina and in the branchial arches. *xlFoxP1* is activated during organogenesis and is mainly expressed in the brain, head mesenchyme and in the splanchnic layer of the lateral plate mesoderm.

KEY WORDS: *X. laevis, embryogenesis, fork head/winged helix factor, expression pattern*

Fox (fork head box) transcription factors are involved in various signalling pathways and cell fate decisions throughout development. According to conserved amino acid residues at distinct positions within the winged helix domain, these factors are categorised into the subclasses A to Q (Kaestner *et al.*, 2000). While for the mouse and human species, Fox family members from all subclasses have been reported, in the South African clawed frog, *Xenopus laevis*, currently only the subclasses A to L are known. Here we report the sequence and initial characterisation of two additional *Xenopus laevis* Fox gene family members, *xlFoxM1* and *xlFoxP1* and add *xlFoxI2* as a new member to the already existing subclass I. We describe the full length cDNAs encoding these proteins and investigate the temporal and spatial expression patterns of corresponding genes by RT-PCR and by whole mount *in situ* hybridisations.

Results and Discussion

xlFoxI2

The FoxI subclass is already known in different organisms to contain several members and has recently been analysed regarding the phylogenetic relationship inside the subclass based on the zebrafish members (Solomon *et al.*, 2003). By searching in EST data bases we have found an IMAGE-clone (4084049), which covers the complete translated region of a FoxI subclass member in *Xenopus laevis*. Referring to the suggested terminology regarding subclass I (Solomon *et al.*, 2003), this clone is preliminarily designated as xlFoxI2, even if the true orthologue relationship inside this subclass remains to be elucidated. The complete sequence of the IMAGE-clone has been determined and deposited under EMBL accession number AJ868112. Starting 78 bp in front of the start codon, the complete cDNA sequence comprises 1537 bp and a poly(A) tail. The translated region encompassing 1107 bp gives rise to a protein of 369 amino acids, with the fork head domain located between amino acids 115 and 224. The predicted amino acid sequence is aligned to the pseudo-allelelic variants xlFoxI1a and xlFoxI1b (Lef *et al.*, 1994) as well as to the close relative xlFoxI1c (Pohl *et al.*, 2002) (Fig. 1). While xlFoxI2 is 44% identical to xlFoxI1a and 46% to xlFoxI1b, it shows 48% identity to xlFoxI1c. However, conservation within the winged helix domain exhibits 84% and 86% identity between xlFoxI2 compared to xlFoxI1a and to xlFoxI1b, respectively and 88% to xlFox1c. Interestingly, all so far identified members of subclass I, *xlFoxI1a/b*, *xlFoxI1c* and *xlFoxI2*, show characteristic and different temporal expression patterns (Fig. 2) (Pohl *et al.*, 2002). Starting with high amounts of maternal transcripts, *xlFoxI2* expression decreases rapidly during early cleavage stages. Zygotic expression starts at neurulation and continues at low levels at stage 39. The *xlFoxI1a/b* genes, which exhibit identical patterns (Lef *et al.*, 1994), are strongly upregulated

Abbreviations used in this paper: Fox, fork head box.

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during the early blastula stage reaching a maximum number of transcripts at the gastrula stage. Thereafter, the amount of transcripts declines during neurulation, with low levels even detected at stage 40. Finally, *xlFoxI1c* starts expression at the late gastrula stage and transcripts constantly accumulate during later embryogenesis (Pohl *et al.*, 2002). Thus it is evident that the genes belonging to subclass I are subject to different regulatory mechanisms and that different developmental stages are associated with the expression of distinct xlFoxI proteins.

Results obtained by whole mount *in situ* hybridisation confirm the temporal patterns as determined by RT-PCR. High amounts of *xlFoxI2* transcripts are present at early cleavage stages in the animal half of the blastomeres (Fig. 3A, B). Zygotic expression is exclusively found in the placodes of the head (Fig. 3C). In contrast to the placodal expression of *xlFoxI1a* (Fig. 3F) and *xlFoxI1c* (Fig. 3G), *xlFoxI2* is only expressed in the dorsal part of the epibranchial placodes (for an overview see Schlosser and Northcutt, 2000). As shown by a transverse section (Fig. 3D), stained xlFoxI2 positive cells are located between the mesodermal foregut tissue and the more dorsal head mesenchyme. A horizontal section demonstrates that *xlFoxI2* is expressed within a restricted region located near the tip of the first, second and third visceral pouch (Fig. 3E).

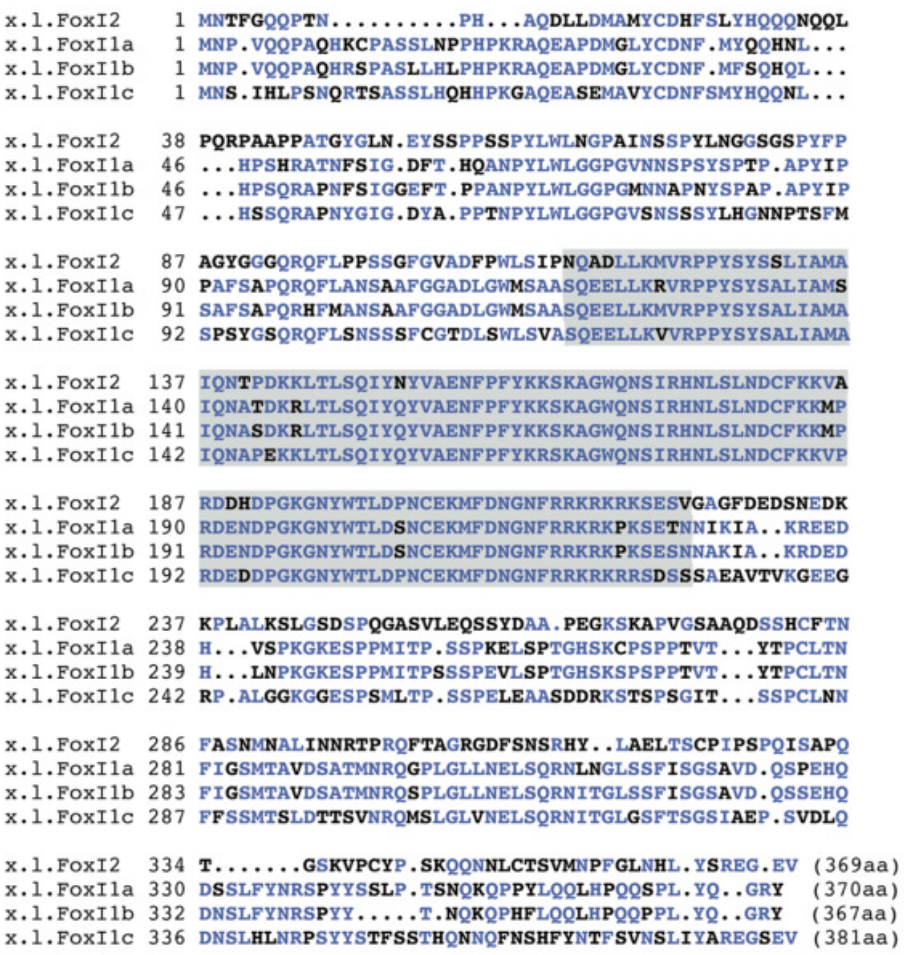

Fig. 1. FoxI2 sequences. *Alignment of the predicted amino acid sequence of* Xenopus laevis *(x.l.) FoxI2 to FoxI1a, FoxI1b (Lef* et al.*, 1994) and FoxI1c (Pohl* et al.*, 2002). Identical residues are shown in blue. The fork head domain is shaded in gray.*

xlFoxM1

FoxM1 was originally isolated in a search for proteins that are phosphorylated during M-phase (Westendorf *et al.*, 1994) without having been identified as a winged helix factor. After the isolation of the complete cDNA from mouse (Genbank accession number: NM_008021, Korver *et al.*, 1997a) and the human orthologue (Genbank accession number: NM_202002, Ye *et al.*, 1997; Korver *et al.*, 1997 b; Yao *et al.*, 1997) it became clear, that the expression of *FoxM1* (also named MPP2, Trident, FKHL16, HFH-11 and WIN) is strictly correlated to proliferating cells.

xlFoxM1 was originally identified in form of the incomplete EST-clones BJ618321 and XL058d22. Screening of a stage 30 cDNA library led to the isolation of an incomplete cDNA overlapping with XL058d22. With 5´-primers derived from BJ618321 and 3´-primers derived from our cDNA, the complete coding sequence could finally be amplified from a reverse transcribed stage 30 RNA. The cDNA contains 2762 bp and a poly(A) tail (deposited under EMBL accession number AJ853462). The protein is encoded by 2277 bp, thus comprising 759 amino acids. The fork head domain is located at the N-terminal half in between amino acids 251 and 360. Figure 4A shows an alignment of the *Xenopus* sequence to the human and mouse orthologues. While the human and mouse proteins share 79% identity, the identities between xlFoxM1 and human FOXM1 (36%) and mouse Foxm1 (37%) are rather low. However, this low degree is mainly due to a stretch of deviating amino acids following the fork head domain. In this context it should be noted that several mammalian splice variants are described (Yao *et al.*, 1997). Adjacent to this region, the rate of identity is significantly higher and that of the winged helix domains is even 84% (see Fig. 4A, calculated by DIALIGN, Morgenstern *et al.*, 1998). To determine the temporal expression pattern of *xlFoxM1* in *Xenopus* embryos, RNAs of different developmental stages were analysed by RT-PCR. As shown in Fig. 2, maternal gene transcription yields high amounts of RNA in early cleavage stages, but transcripts are rapidly degraded until the blastula stage. Zygotic expression of *xlFoxM1* starts during neurulation and transcripts persist and accumulate until the end of organogenesis.

The spatial expression was determined by *in situ* hybridisation (Fig. 5). Transcripts are found in the animal half at early cleavage stages (Fig. 5A), but are absent from gastrula stage embryos. During neurulation, expression is observed in the neural folds and, later, in the spinal cord as well as in the eye field (Fig. 5B, C). This localisation becomes even more prominent at stage 22, when transcripts demarcate the region of the eye (Fig. 5D). Thus, it can

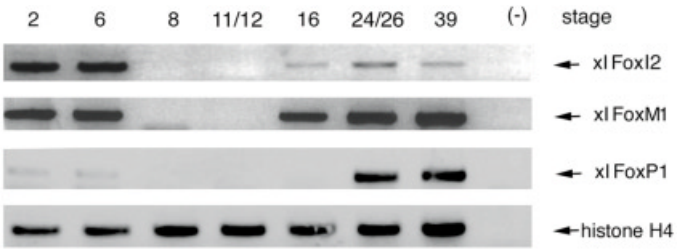

Fig. 2. Temporal expression of *xlFoxI2, xlFoxM1* and *xlFoxP1*. *RT-PCR for* xlFoxI2, xlFoxM1 *and* xlFoxP1 *was performed with RNAs of different developmental stages. (-) indicates a negative control in the absence of RNA. Histone H4 was used as internal control.*

be concluded that *xlFoxM1* is involved in early stages of eye formation. During tailbud stages, *xlFoxM1* expression is still restricted to the neuroectoderm, predominantly to the hindbrain, the eye and the spinal cord (Fig. 5E). With ongoing development, expression is also found at lower levels in the branchial arches (Fig. 5F). Sections of embryos at stage 35 reveal distinct expression of *xlFoxM1* in the rhombencephalon, in the eye retina but not the in forming lens and in the branchial arches (Fig. 5 G-I). While in mouse development Foxm1 was found to be ubiquitously localised in all cell types during proliferation, its expression is downregulated when cells enter terminal differentiation (Korver *et al.*, 1997a; Ye *et al.*, 1997). In adult human and mouse tissues, FoxM1 is predominantly found within the thymus and testis and, at a lower extent, in intestine, liver and lung. Interestingly, a *Foxm1* knock out mouse was found to be post-natally lethal due to a failure in development of the myocardium. Both for hepatocytes and myocytes, polyploidy was observed in combination with a dramatic increase in DNA content (Korver *et al.*, 1998). Human *FOXM1* is localised on chromosome 12p13. Chromosomal abnormalities of this region are known from several tumors which were initially explained by the cyclin dependent kinase inhibitor $p27^{kip1}$ localised in close proximity (Korver *et al.*, 1997b). However, it has recently been found that FoxM1 itself is an important part of the precise machinery ensuring the correct progression through the cell cycle. This is achieved via a direct regulation of cyclin B1 and D promoters by FoxM1, in association with a diminished expression of $p27^{Kip1}$ (reviewed in Costa *et al.*, 2003).

xlFoxP1

The subclass P of Fox transcription factors gained special interest, because *FOXP2* was found to be associated with language disorders in humans (Lai *et al.*, 2001). By sequence comparisons with great apes, *FOXP2* was suggested to be an important target of selection during recent evolution in humans (Enard *et al.*, 2002). FOXP1 was isolated in search for a protein known to be involved in several human tumors (Banham *et al.*, 2001). Members of the FoxP subclass are also of interest, because these proteins contain a zinc-finger and a leucine-zipper located N-terminal to their fork head domain. For the first time in case of Fox proteins, FoxP factors are not only able to bind as heterodimers to DNA, but were shown to interact with each other due to these motifs (Wang *et al.*, 2003; Li *et al.*, 2004). *xlFoxP1* was originally identified by the IMAGE-clone 3747208. However, this clone encodes only the C-terminal part of the protein. Further database searches for sequences encoding the N-terminal part led to additional EST clones (e.g. BE679963) which were used to elongate the *Xenopus FoxP1* sequence. Finally, the complete coding sequence could be amplified by RT-PCR from stage 30 RNA. The *xlFoxP1* cDNA sequence encompasses 1968 bp starting 54 bp in front to the start codon. It contains 180 bp of the 3´-UTR and, additionally, a poly(A) tail (deposited under EMBL accession number AJ853463). Since the EST-clone contains a stop codon in front of the translation start site, the derived amino acid sequence is shorter than those listed for mammals (*homo sapiens* Genbank accession number: NM_032682; *mus musculus* Genbank accession number: NM_053202). However, it should be mentioned that different isoforms with varying start sites are also reported for mice (Wang *et al.*, 2003). Moreover, translation start site of the zebrafish *FoxP1* (www.ensemble.org/ ENSDART00000023619) and, interestingly, also of the closely related FoxP2 gene in human (NM_014491) were uniformly determined to the respective start codon as we have found for *xlFoxP1*. The open reading frame of *xlFoxP1* contains 1734 bp encoding 578 amino acids. The fork head domain is located within the C-terminal half, a rather unusual feature for a Fox protein. The alignment of FoxP1 protein sequences of *X. laevis*, human, mouse and zebrafish is shown in Fig. 4B. Both on DNA and amino acid level, FoxP1 shows the highest homology

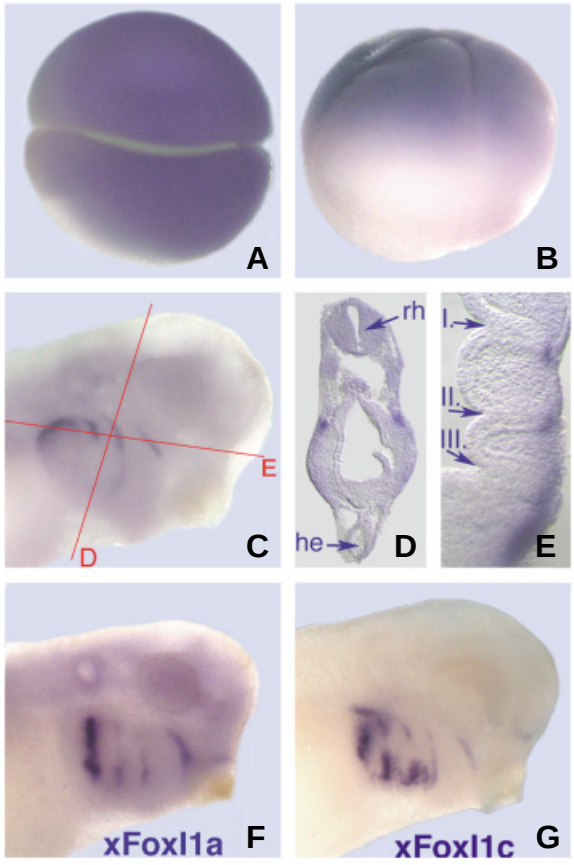

Fig. 3. Whole mount *in situ* hybridisation of *xlFoxI2*. **(A)** *2-cell stage, animal view;* **(B)** *4-cell stage, lateral view;* **(C)** *stage 35, the red lines denote sections shown in* **(D,E)** *which are transverse and horizontal sections respectively, through the dorsal epibranchial placodes;* **(F)** *stage 35 stained for* xlFoxI1a*;* **(G)** *stage 34 stained for* xlFoxI1c*. he, heart anlage; rh, rhombencephalon; I., II., III., first, second and third visceral pouches.*

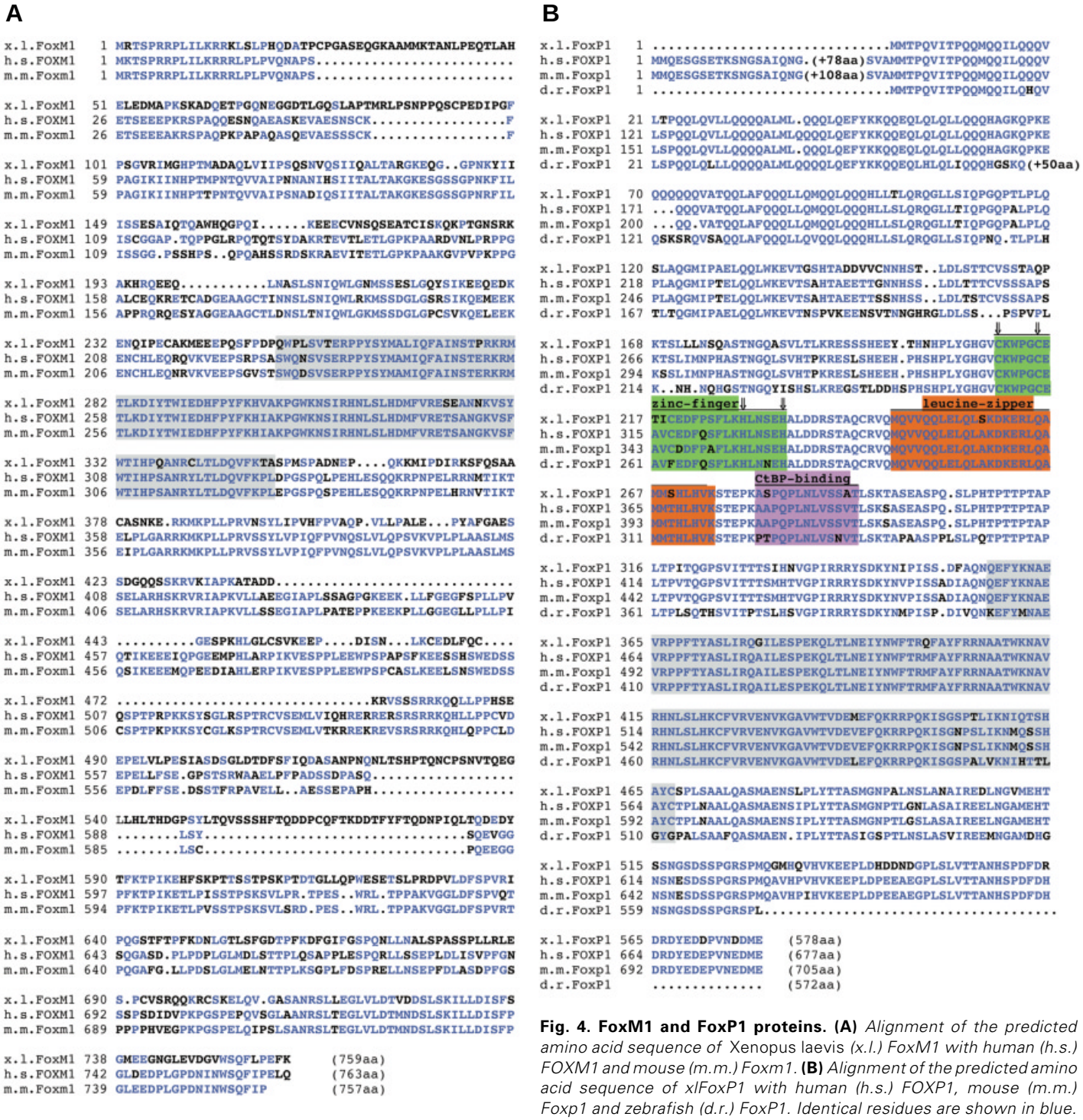

Fig. 4. FoxM1 and FoxP1 proteins. (A) *Alignment of the predicted amino acid sequence of* Xenopus laevis *(x.l.) FoxM1 with human (h.s.) FOXM1 and mouse (m.m.) Foxm1.* **(B)** *Alignment of the predicted amino acid sequence of xlFoxP1 with human (h.s.) FOXP1, mouse (m.m.) Foxp1 and zebrafish (d.r.) FoxP1. Identical residues are shown in blue. The fork head domain is shaded gray. The green box shows the position of the Gli-like zinc-finger with the relevant cysteine and histidine residues denoted by arrows, the red box marks the leucine-zipper, the magenta box represents the CtBP-binding side.*

between different species, that to our knowledge was ever reported for Fox genes. xlFoxP1 protein is 86% identical to its mouse and human orthologues and 70% identical to zebrafish FoxP1. Both the fork head domain (grey) and the zinc-finger motif (green) are highly conserved. The latter was found to be closely related to the first zinc-finger found in Gli-proteins (Shu *et al.*, 2001). Furthermore, the leucine-zipper that is localised directly adjacent to the zinc-finger and a binding site for the transcriptional co-repressor CtBP-1 (Li *et al.*, 2004) are also found to be conserved. Fig. 2 shows the temporal expression pattern of *xlFoxP1*. Low levels of maternal transcripts are present, but disappear before gastrulation. Zygotic transcription starts at stage 26 and continues throughout embryo-

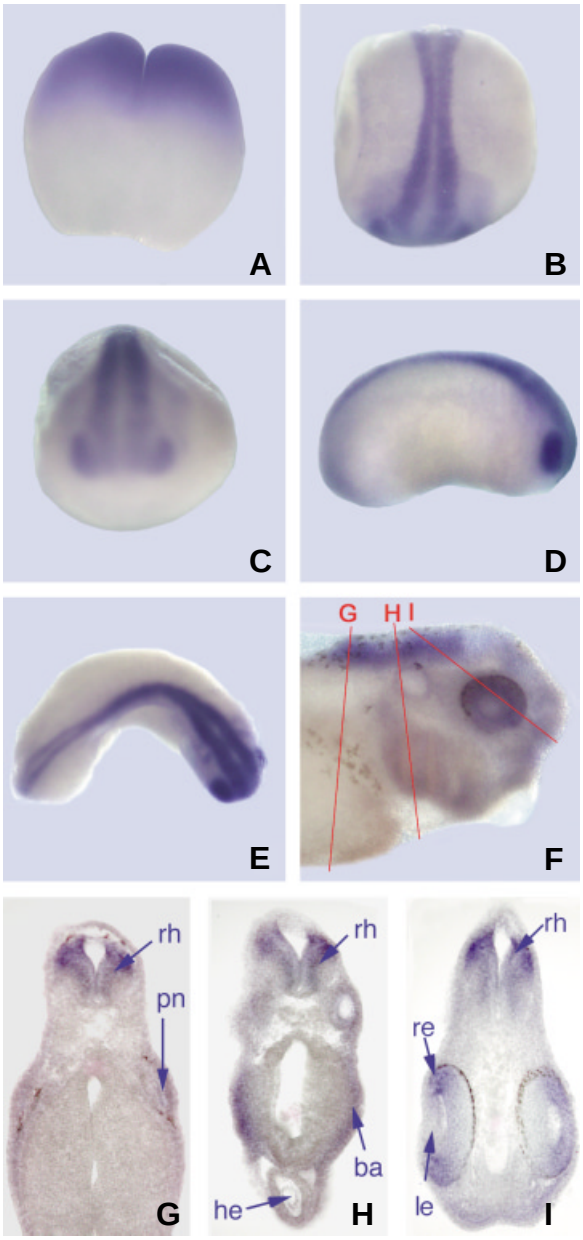

Fig. 5. Whole mount *in situ* hybridisation of *xlFoxM1*. (A) *2-cell stage, lateral view;* **(B)** *stage 16, dorsal view, anterior to the bottom;* **(C)** *stage 19, anterior view;* **(D)** *stage 22, lateral view;* **(E)** *stage 26, dorsal view;* **(F)** *stage 35; in (D-F), anterior is to the right. Red lines in (F) denote sections shown in (G-I).* **(G)** *Transverse section revealing* xlFoxM1 *expression in the rhombencephalon;* **(H)** *transverse section demonstrating additional staining of the branchial arches;* **(I)** *horizontal section of the head with staining of the retina, anterior is to the bottom. ba, branchial arches; le, lens; he, heart; pn, pronephros; rh, rhombencephalon.*

genesis. *In situ* hybridisations reveal the presence of *xlFoxP1* transcripts in the animal blastomeres of early cleavage stages (Fig. 6A). At tailbud stages, *xlFoxP1* expression is visible in regions of the brain, eyes and the splanchnic mesodermal layer of the lateral plate mesoderm surrounding the gut (Fig. 6B,G). At stage 35, xlFoxP1 is expressed within the lens of the eye, in distinct regions of the head mesenchyme and the area anterior to the gut (Fig. 6C,F). In the brain the anterior most staining is restricted to the outer region of the mesencephalon (Fig. 6E). With ongoing development additional expression is found in the curling gut (Fig. 6D). This corresponds to the *in situ* analyses performed in mice, where Foxp1, besides its expression in the lung, is also described in the developing central nervous system and in the intestine (Shu *et al.*, 2001; Tamura *et al.*, 2003). Thus, the relationship between the mammalian and *Xenopus FoxP1* genes is not only reflected by sequence homology but also by similar expression patterns.

Experimental Procedures

RT-PCR, *in situ* hybridisation and handling of *Xenopus* embryos was done according to standard procedures (for more details see: Pohl and Knöchel, 2001). Developmental stages were determined according to Nieuwkoop and Faber, 1967.

The IMAGE-clone 4084049 was commercially obtained by RZPD (Deutsches Resourcenzentrum für Genomforschung GmbH, Berlin). Primers used for amplification of complete coding regions for xlFoxP1 and xlFoxM1 are as follows:

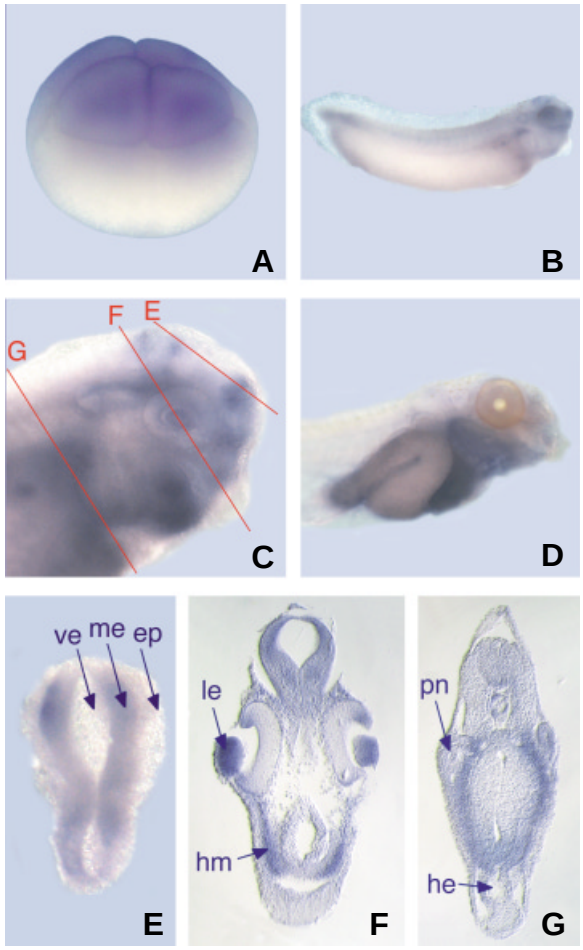

Fig. 6. Whole mount *in situ* hybridisation of *xlFoxP1*. (A) *8-cell stage, lateral view;* **(B)** *stage 31;* **(C)** *stage 35, red lines in (C) indicate the plane of sections shown in (E-G);* **(D)** *stage 41, (B-D) are lateral views;* **(E)** *horizontal section, anterior is to the bottom;* **(F,G)** *transverse sections, dorsal is on top. ep, epidermis; he, heart; hm, head mesenchyme; le, lens; me, mesencephalon; pn, pronephros; ve, brain ventricle.*

xlFoxP1-for: 5´-CCC ACA AGA GGA ATG ACA AAC-3´;
xlFoxP1-rev: 5´-TTA CTC CAT GTC GTC ATT TAC-3´;
xlFoxM1-for: 5´-ATG CAT TTT GAG CTC TCA ATG-3´;
xlFoxM1-rev: 5´-AGT TAA GAA TCT ACA GAA CAC TTG-3´.

Primers used in RT-PCR for temporal expression were
xlFoxI2-RT-for: 5´-GAC AGC AGT CAC TGT TTC AC-3´;
xlFoxI2-RT-rev: 5´-GGC CGA AGG GAT TCA TGA CAG-3´;
xlFoxP1-RT-for: 5´-CAT GAT TCC AGC TGA ACT GC-3´;
xlFoxP1-RT-rev: 5´-GCA CTC GAT ACT AGG TTC AG-3´,
xlFoxM1-RT-for: 5´-CCC AGA GTG TGC AAA GAT GG-3;
xlFoxM1-RT-rev: 5´-TTC ACA CGC TTG CTG CTT T-3´.

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