

The developmental expression of *foxl2* in the dogfish *Scyliorhinus canicula*

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Abstract

The *FoxL2* genes are a subfamily of the Fox (forkhead box) gene family. *FOXL2* is mutated in the disorder Blepharophimosis, Ptosis, and Epicanthus Inversus Syndrome (BPES), which is characterized by eyelid malformations, and Premature Ovarian Failure (POF). In the mouse expression is seen in the perioptic mesenchyme, developing eyelids, ovary and pituitary. We have isolated a *foxl2* cDNA from the dogfish *Scyliorhinus canicula* (also known as the lesser spotted catshark), allowing the characterisation of this gene's sequence and expression from a lineage that diverged early in the evolution of gnathostomes. Molecular phylogenetic analysis strongly grouped this sequence with the gnathostomes within the FoxL2 subfamily. We demonstrate the early expression of *Scyliorhinus canicula foxl2* in the mandibular head mesoderm and later in continuous populations of mandibular arch cells and mandibular head mesenchyme cells around the developing pituitary. As development proceeds expression decreases in the mesenchyme of the head but is seen in the mesenchyme around the eye and later in the developing eyelids. Additionally expression is seen in regions of pharyngeal arch mesoderm and in ectoderm from which gill buds will form. This expression is maintained in the developing and elongating gill buds. Thus, *S.canicula foxl2* is a marker for the mandibular mesoderm and gill buds and its expression is conserved in the perioptic mesenchyme, developing eyelids and pituitary.

1. Results and discussion

The Fox genes are a family of transcription factors characterised by a 110 amino acid DNA binding domain. They are involved in a wide range of biological processes in the adult and during development and have been implicated in a number of diseases

(Carlsson and Mahlapuu, 2002). *FOXL2* is mutated in the disorder Blepharophimosis, Ptosis, and Epicanthus Inversus Syndrome (BPES), which is characterized by eyelid malformations, and Premature Ovarian Failure (POF) (Crisponi *et al.*, 2001). Consistent with this phenotype, mouse *Foxl2* expression is seen in the perioptic mesenchyme, developing eyelids and ovary, and also in the pituitary (Crisponi *et al.*, 2001; Treier *et al.*, 1998). *Foxl2* is one of the earliest markers of ovary development and its expression in this tissue has been reported from the mouse, chicken, goat, turtle, and the teleost fishes medaka (*Oryzias latipes*), rainbow trout (*Oncorhynchus mykiss*) and the Nile tilapia (*Oreochromis niloticus*) (Baron *et al.*, 2004; Crisponi *et al.*, 2001; Loffler *et al.*, 2003; Nakamoto *et al.*, 2006; Pailhoux *et al.*, 2001; Wang *et al.*, 2004). Additionally in the Nile tilapia RT-PCR has revealed expression in the brain, pituitary, and gills (Wang *et al.*, 2004). Sequence analysis has shown high conservation in mammals, including a polyalanine tract, the expansion of which is associated with eyelid malformations without POF (type II BPES). Analysis of non-mammalian *foxl2* sequences has shown an absence of a polyalanine tract (Cocquet *et al.*, 2002; Wang *et al.*, 2004). Amongst the invertebrates, *foxl2* genes have been identified from various genomes (Mazet *et al.*, 2003) but have only been studied in the sponge, *Suberites domuncula* (Adell and Muller, 2004)

1.1 Isolation of *S. canicula foxl2* and sequence analysis

Foxl2 appears in a single copy gene in the genomes of mammals, *Gallus gallus* and *Xenopus tropicalis* while a second diverged duplicate has been identified in the genomes of some teleosts (Baron *et al.*, 2004). The *S. canicula foxl2* cDNA is 1726bp long and encodes a 198 amino acid protein (Fig.1). It shows the highest protein

sequence identity to *X. tropicalis* foxl2, 77% over the whole length and 96% in the forkhead domain. Like sequences from other non-mammalian species the polyalanine tract is absent, however a polyalanine tract was found in foxl2 from the Opossum (*Monodelphis domestica*) suggesting this feature arose in the mammals before the separation of the placental and marsupial lineages. Phylogenetic analysis places *S. canicula* foxl2 with the vertebrate FoxL2 sequences with a high bootstrap value.

1.2 Developmental expression of *S.canicula* foxl2

S. canicula embryos were staged according to Ballard *et al.* (1993) and the developmental expression of foxl2 investigated between stages 17 and 31. *S. canicula* foxl2 expression is detected at stage 17 (Ballard *et al.*, 1993) in the mandibular head mesoderm (Fig.2A). Sectioning shows expression is restricted to the medial wall of the head cavity (Fig.2B). As development proceeds through stages 18 and 19 expression is seen in the mandibular mesenchyme of the first pharyngeal arch and under the mesencephalon (Fig.2C). Serial sections through the head show expression in the mesenchyme around Rathke's pouch (Fig.2F), the mandibular head cavities (Fig.2F) and in the aortic blood vessels of the mandibular arches (Fig.2G). At stage 21 expression is still seen in the mesenchyme under the mesencephalon and can be detected here up to stage 24 (Fig.2H, I, J, L). Also at stage 21 a new site of expression is seen in the ectoderm of the hyoid and 3rd pharyngeal arches at sites from which the first gill buds will form (Fig.2H). Sections of a stage 22 embryo shows expression in the ectoderm of the hyoid and 3rd pharyngeal arch, and also in the mesodermal cores of each arch (Fig.2J, K). At this stage weak expression is also seen in the hinge of the

mandibular arch and this maintained up to stage 27 (Fig.2J, L, M). Stages 21 onwards sees the expression of *foxl2* in the prospective gill bud tissue of each arch, including those in the spiracular clefts (Fig.2M) and the mature gill filaments (Fig.2N, O, P). At stage 29 expression is seen in the mesenchyme around the eyes (Fig.2O, Q) and at stages 30 and 31 this expression restricts to the underlying mesenchyme at the outer edges of the developing eyelids (Fig.2R, S, T).

In summary expression of *S.canicula foxl2* marks the mandibular mesoderm, the mesodermal cores of the pharyngeal arches, the gill buds, the perioptic mesenchyme and the developing eyelids. Expression in the gill buds and mesodermal cores of the pharyngeal arches may be specific to the chondrichthian lineage however the gills have been identified as a site of expression by RT-PCR in the teleosts (Wang *et al.*, 2004). The expression of *foxl2* in the perioptic mesenchyme, eyelids and around the developing pituitary in mouse and *S. canicula* suggest these are conserved sites of gene expression in the gnathostomes (Crisponi *et al.*, 2001; Treier *et al.*, 1998). Despite this we note the apparent absence of eyelids from most fish. Finally *foxl2* has been reported as an early marker of ovary development in a range of tetrapods and teleosts, however we did not detect expression in the stages examined (Baron *et al.*, 2004; Loffler *et al.*, 2003; Nakamoto *et al.*, 2006; Pailhoux *et al.*, 2001; Wang *et al.*, 2004).

2. Experimental procedures

2.1 Obtaining *S. canicula* embryos

Eggs were collected from seaweed gardens at low tide from beaches in the Menai Strait in Wales, UK. The eggs were kept in saltwater tanks and allowed to develop. At selected developmental stages, see (Ballard *et al.*, 1993), they were fixed in 4% MOPS buffered paraformaldehyde at 4°C overnight then dehydrated into 100% methanol and stored at -20°C.

2.2 cDNA library screening

An *S. canicula* cDNA library in the vector lambdaZap Express was screened with an *S. canicula foxc1* gene fragment using Roche's DIG nucleic acid detection kit. One of the recovered cDNAs contained a forkhead domain with high similarity to the FoxL2 subfamily and was fully sequenced.

2.3 Phylogenetic analysis

Protein sequences were collected from the NCBI or the JGI websites (see Fig.1 for accession numbers). They were aligned and trimmed using BioEdit and ClustalX (Hall, 1999; Thompson *et al.*, 1997). Phylogenetic analyses were carried out using maximum likelihood implemented by ClustalX, and by PHYML (Guindon and Gascuel, 2003; Guindon *et al.*, 2005; Thompson *et al.*, 1997). Substitution models were predicted with ProtTest (Abascal *et al.*, 2005).

2.4 In situ hybridisation

In situ hybridisation of *S. canicula* embryos is based on a chick in situ protocol (Nieto *et al.*, 1996) with modifications described in (Freitas and Cohn, 2004). The protocol was carried out using a riboprobe derived from the whole cDNA and made using the Roche DIG RNA labelling kit.

2.5 Histology

Tissue was embedded in gelatin/albumin (0.45% gelatin, 25% albumin, 20% sucrose in PBS) and fixed with 2.5% glutaraldehyde. The embedded embryos were then sectioned at 50µm using a vibratome.

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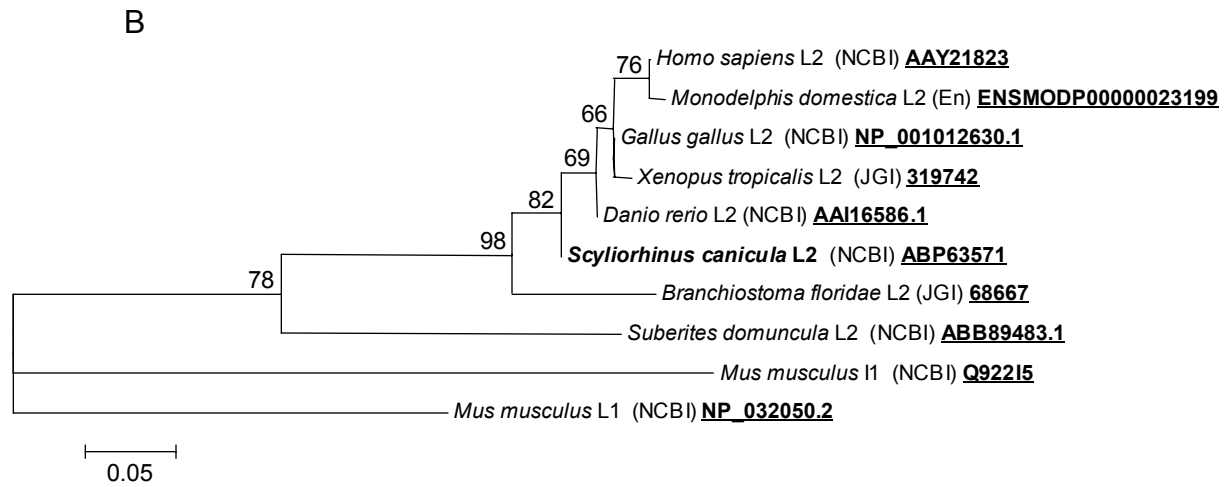
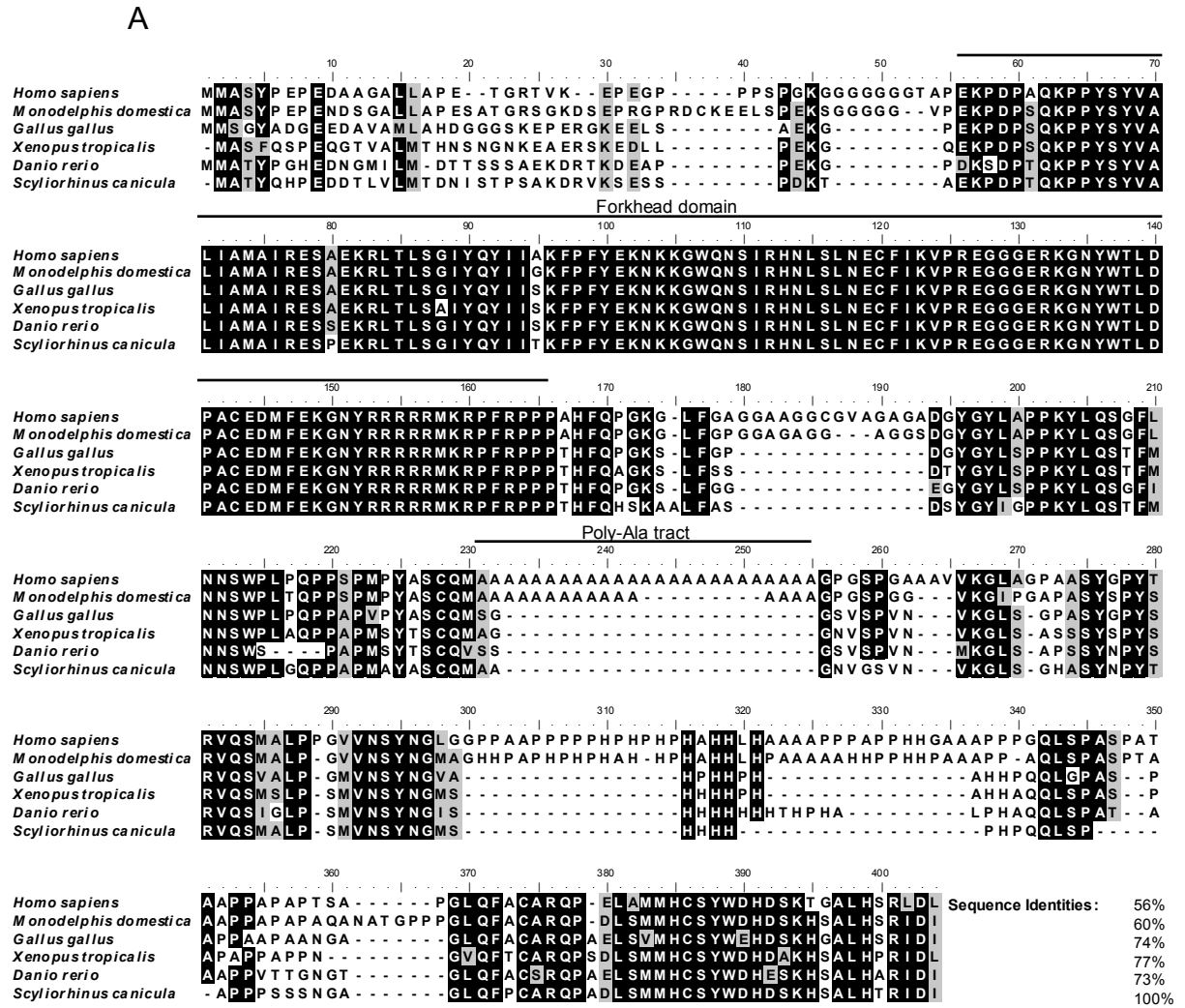
Figure Legends

Figure 1. Sequence and phylogeny of FoxL2 proteins. (A) Sequence alignment of FoxL2 proteins. The forkhead domain and polyalanine tracts are shown and percentage identity values to the *S. canicula* sequence are indicated. (B) Phylogenetic tree of FoxL2 subfamily proteins constructed using 106 amino acids of the forkhead domain. We chose foxL1 and FoxI sequences as outgroups as they often associate with the FoxL2 branch in analyses including all Fox classes (Kaestner *et al.*, 2000; Mazet *et al.*, 2003). *S. canicula* foxl2 groups within the vertebrate FoxL2 sequences with high bootstrap support. Accession numbers are shown and can be retrieved from the following genome websites: NCBI <http://www.ncbi.nlm.nih.gov/>, JGI <http://www.jgi.doe.gov/> or Ensembl <http://www.ensembl.org/index.html> (indicated by the bracketed abbreviation En).

Figure 2. Developmental expression of *S. canicula* foxl2. Stage numbers (Ballard *et al.*, 1993) are shown in the bottom right corner, arrows indicate planes of section. (A) Stage 17 expression in the mandibular head mesoderm (mm). (B) Section of embryo in (A) showing expression in the mandibular mesoderm (mm) and the mandibular head cavity (mhc). (C) Stage 19 expression in the mandibular arch (ma) and mandibular head mesenchyme (mm). (D, E, F, G) Serial sections of embryo in (C),

(D, E) show expression in the mandibular head mesoderm (mm) (F) shows expression in the mesenchyme around the head cavities (mhc) and Rathke's pouch (rp) and (G) shows expression in the mandibular arch blood vessels (aa). (H) Stage 21 expression in the future gill bud forming regions of the hyoid arch (ha) and arch 3 (a3). (I) Sagittal section of embryo in (H) showing expression in the head mesenchyme relative to the developing brain. (J) Stage 22 expression in the gill bud forming regions of the hyoid arch and arch 3. (K) Section of embryo in (J) showing expression can be seen in the mesodermal cores of the pharyngeal arches (mc) and the ectoderm (ec) of the gill bud forming regions. (L) Stage 24 embryo showing expression in arch 2 and 3 gill buds (gb) and arch 4 and 5 gill buds forming regions. Expression is also seen in mandibular arch hinge region (h). (M) Stage 27 embryo showing expression in the mandibular hinge region (h) and gill buds (gb) on arches 2-5 and the early forming gill buds of the mandibular arch in the spiracular cleft (sgb). Expression is also seen in the gill buds forming region of arch 6. (N) Stage 28 expression in the lengthening gill buds and gill filaments (gf). (O) Stage 29 expression in the gill filaments and in the mesenchyme around the eye (pom). (P, Q) Sections of embryo in (N) showing expression in the gill filaments and the perioptic mesenchyme. (R, S, T) Expression in the developing eyelid (el) at stages 30 and 31. (S) Section through the eye of the embryo in (R), note that the lens has been removed. Labels: (aa) aortic arch, (bg) buccal groove, (c1) hyomandibular or spiracular cleft, (c2) pharyngeal cleft 2, (di) diencephalon, (ec) ectoderm, (el) eyelid, (en) endoderm, (gb) gill buds, (gf) gill filaments, (h) hinge of mandibular arch, (ha) hyoid arch, (in) infundibulum, (lp) lens placode, (ma) mandibular arch, (mc) mesodermal core of pharyngeal arch, (me) mesencephalon, (mhc) mandibular head cavity, (mm) mandibular mesoderm, (mt) metencephalon, (my) myelencephalon, (olf) olfactory placode, (ot) otic placode, (ov)

optic vesicle, (p1-p5) pharyngeal pouches, (ph) pharynx, (rp) Rathke's pouch (sgb)
spiracular cleft gill bud, (te) telencephalon.



Figure

