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Isolation and expression analysis of *foxj1* and *foxj1.2* in zebrafish embryos

Emil Aamar and **Igor B. Dawid***
Laboratory of Molecular Genetics, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda MD, USA

Abstract

In this report we present the isolation and identification of a zebrafish homolog of the winged helix \forkhead transcription factor Foxj1. Foxj1 was identified in other species but not in zebrafish. Foxj1 was shown in mice to be required in ciliogenesis and left-right asymmetry establishment. Here we present a spatio-temporal expression pattern of zebrafish *foxj1*, showing that this gene is expressed in dorsal forerunner cells, Kupffer's vesicle, the floor plate, pronephric ducts and kidney. This expression pattern is overlapping but different from that of the *foxj1.2*, the closest related gene in zebrafish. Foxj1 in zebrafish appears to have similar functions as those reported in other species connected to ciliogenesis and left-right asymmetry.

Keywords

Foxj1; Foxj1.2; dorsal forerunner cells; Kupffer's vesicle; floor plate; pronephric duct

Hepatocyte nuclear factor-3 (HNF-3)/forkhead homologue 4 (HFH-4)/Foxj1 is a winged helix/forkhead transcription factor. A 100-amino-acid DNA-binding motif, known as the winged helix, was first identified in mammalian hepatocyte nuclear factor-3 (HNF-3) and Drosophila Forkhead transcription factors (Avraham et al., 1995; Lai et al., 1993). Subsequently, many additional proteins containing the winged helix motif have been identified (Avraham et al., 1995). In humans (Pelletier et al., 1998) rats (Hackett et al., 1995) and mice, Foxj1 is expressed in ciliated cells of various tissues including the respiratory tract, brain, and ependyma in late development through adulthood, as well as in oviduct, testis, embryonic kidney (Blatt et al., 1999; Brody et al., 2000; Tichelaar et al., 1999a; Tichelaar et al., 1999b) and the choroid plexus (Blatt et al., 1999; Brody et al., 1997; Lim et al., 1997; Swetloff and Ferretti, 2005).

Foxj1 is involved in the regulation of ciliogenesis and axonemal structural proteins. Foxj1 regulates basal body anchoring to the cytoskeleton of ciliated pulmonary epithelial cells, and is required for apical localization of ezrin and the formation of axonemal structures (Gomperts et al., 2004; Gomperts et al., 2007). Further, Foxj1 promotes RhoA-mediated apical actin enrichment required for ciliogenesis (Pan et al., 2007). In *Foxj1* null mice, classic motile type cilia with a 9+2 microtubule ultrastructure were absent in epithelial cells, resulting in defective ciliogenesis in the airways. In other organs, sensory cilia with a 9+0 microtubule pattern, such as those on olfactory neuroepithelial cells, were still present. *Foxj1* is expressed in the ciliated cells of Hensen's node in the chick, and is required for left/right asymmetry determination (Blatt et al., 1999; Chen et al., 1998; Feistel and Blum, 2006; Maiti et al., 2000; Tamakoshi et al., 2006; Zhang et al., 2004). However analysis of the node in *Foxj1* null mice revealed that,

*Corresponding author: Laboratory of Molecular Genetics, National Institute of Child Health and Human Development, National Institutes of Health, Building 6B, Room 413, Bethesda, MD, 20892 USA, E-mail address: idawid@nih.gov aamaremi@mail.nih.gov

in contrast to the absence of airway cilia, node cilia were present. These observations indicate that there are independent regulatory pathways for node ciliogenesis compared with 9+2 type ciliogenesis in airways, and support a central role for Foxj1 in ciliogenesis and left-right axis formation (Brody et al., 2000; Chen et al., 1998). At high levels of expression, Foxj1/HFH-4 altered epithelial cell differentiation and inhibited branching morphogenesis in the developing mouse lung, restricting the expression of markers typical of nonciliated cells of the distal lung parenchyma (Blatt et al., 1999; Maiti et al., 2000).

Foxj1 plays a critical role in the immune system, influencing thymocyte export and T cell tolerance (Jin et al., 2006; Srivatsan and Peng, 2005). Foxj1 modulates Th1 activation, and inhibits NF-kappaB activation and interferon-gamma secretion (Lin and Peng, 2006; Sela et al., 2006). Foxj1 also restrains B cell activation and the maturation of humoral responses (Lin et al., 2005). *Foxj1* seems to be regulated by different factors like Foxd1 in the immune system (Lin and Peng, 2006), Sox17 in epithelial airway cells (Park et al., 2006), and by both Foxa1 and Foxa2 in branching morphogenesis in the pulmonary mesenchyme (Wan et al., 2005). *Foxj1* expression in lung bud morphogenesis is further regulated by BMP4 signaling (Hyatt et al., 2004).

Here we report the cloning of *foxj1* in zebrafish and the protein encoded by it, and we compare the predicted zebrafish protein sequence to Foxj1 sequences of other species. We further present the temporal and spatial expression of *foxj1* in zebrafish embryos. Zebrafish *foxj1* is expressed in the dorsal forerunner cells, floor plate, Kupffer's vesicle, and in the pronephric duct and developing kidney. These sites are similar but not identical to the sites of expression of *foxj1* in other vertebrate species. We compare this expression pattern with that of *foxj1.2* and find that they have distinct and partially overlapping patterns. Foxj1.2 is the closest to Foxj1 in *Danio reri*o. Even though a sequence for Foxj1.2 is available, no work has been done to describe it's expression pattern or function in zebrafish or other species, except for *Xenopus tropicalis*, where a very brief expression pattern is described. In *X. tropicalis* FoxJ1.2 is expressed in the otic vesicle during late neurula stages and is then also expressed in the presumptive nephrostomes of the pronephros during tailbud stages (Choi et al., 2006).

Isolation of zebrafish foxj1 and comparison of vertebrate Foxj1 proteins

Our studies started with cDNA clone #5134 isolated in a previous gene expression screen (Kudoh et al., 2001). This clone was extended by 5′RACE, resulting in a 3786bp long cDNA sequence with 1377bp long open reading frame (ORF). The full sequence was submitted to GenBank under accession number EU201184. This sequence aligns at the 99% level to the uncharacterized mRNA sequence available under accession number BC124228 (1690bp long). The ORF predicts a protein that shares 48-51% identity with Foxj1 in human, mouse, rat, *Xenopus laevis* and *Xenopus tropicalis* (Fig. 1, 2), and contains a Forkhead/winged helix DNA binding domain with over 90% sequence identity to the same domain in Foxj1 proteins of other species (boxed in Fig. 1). The evolutionary relationship between vertebrate Foxj1 and Foxj1.2 proteins and some of the other Foxj proteins is illustrated in Figure 2.

Expression of foxj1 *during zebrafish embryogenesis*

Expression of *foxj1* in zebrafish embryos was examined by RT-PCR and whole-mount in situ hybridization. *Foxj1* mRNA is not detected maternally and begins to be expressed weakly at high-dome stages as shown by RT-PCR (Fig. 3A lane 3). The earliest expression that could be detected by in-situ hybridization occurs at about 30% epiboly (Fig. 3B) in dorsal forerunner cells. This expression continues through shield stage (Fig. 3C) and is maintained until late epiboly stages (Fig. 3D). Consistently with the fact that dorsal forerunner cells are the precursors of Kupffer's vesicle we see that *foxj1* is strongly expressed in Kupffer's vesicle starting at bud stage (Fig. 3F, H and J). *Foxj1* transcripts are also detected in the presumptive

floor plate at the dorsal midline, with increasing extent and intensity as development proceeds (Fig. 3E-K). This expression pattern might correspond to that described in rabbits where *foxj1* is expressed in the notochordal plate as the cells migrate from Hensen's node anteriorly, and eventually is expressed in the notochord (Feistel and Blum, 2006). However, in zebrafish, *foxj1* expression was not detected in the notochord but in the floor plate as visualized by DIC optics (not shown) and by double in-situ staining with *ntl* as a notochord marker (Fig. 3G, R and S). This expression in the floor plate is maintained through somitogenesis stages up to about 2 days where it becomes weaker in the floor plate and shows weakening expression at the posterior neural tube at the tail tip. Both expression domains weaken or disappear by three and four days of age (not shown). Although at 16 somites (16s) to 24hpf stages the expression in the floor plate is relatively weaker in the trunk and most of the tail, it is strongly maintained in the tail tip in the floor plate and neural tube, and at the ventral edge of the mid-hindbrain boundary (MHB) (Fig. 3L-S). The MHB is the anterior limit of *foxj1* expression (Fig. 3P, Q), as also seen by double in-situ staining with pax2.1 which marks the MHB (Fig. 3E, L-N). Expression in Kupffer's vesicle is no longer detected at about 16s stages since the vesicle is much smaller (Fig. 3L).

By bud stage, *foxj1* expression is also detected in the presumptive pronephric ducts (Fig. 3E-F). This expression is continues through the 24hpf stage where it is detected also in the forming kidney (Fig. 3H-L, H-P). Double in-situ hybridization using the *foxj1* and *pax2.1* confirmed expression in the pronephric duct and its precursors, as both markers align in this region (Fig. 3E-F, K-M and O). *Foxj1* expression in the pronephros at the 24hpf stage is usually weaker as compared to the 16s stage.

At 2-4 days after fertilization, *foxj1* expression fades or has disappeared, except in the kidney (Fig. 3T-W) and around the mid-hindbrain boundary, which strengthens in intensity and extends to the tectal ventricle (Fig. 3T-V). Expression in the tectal ventricle decreases by 3 days (not shown) and 4days of development (Fig. 3V vs. T), while expression in the olfactory pits starts at about 3days and increases by 4days (Fig. 3V). By 4days a weak expression is also seen in the otoliths of the otic vesicles (Fig. 3W top).

Expression of foxj1.2 *is overlapping but different from* foxj1

The closest sequence in *Danio rerio* to Foxj1 is listed under accession number NP_001008648.1 as a hypothetical protein. The predicted protein is most closely related to Foxj1.2 in *X. tropicalis*, and we therefore identify this sequence as zebrafish Foxj1.2. Zebrafish *foxj1* and *foxj1.2* genes are located on chromosomes 3 (ch3, NC_007114 REGION: 63826357..63840654) and 12 (ch12, NC_007123 REGION: 19967418..19975131), respectively, and their ORFs are similarly encoded by two exons (Fig. 2C; the exon start and end positions are given). *Foxj1* and *foxj1.2* share very limited homology at the level of cDNA sequence which is mostly restricted to a part of the forkhead box (FH box) domain (82% identity in 293nt). The proteins share 35% identity mainly in the FH box (Fig. 2B). Zebrafish Foxj1 has higher similarity to Foxj1 proteins from other species than Foxj1.2 does (Fig. 2).

Foxj1.2 mRNA is not detected maternally and begins to be expressed at about 40% epiboly, as shown by RT-PCR (Fig. 4Q lane 4). In situ hybridization shows strong expression in the epiblast by shield stage (Fig. 4A), and at 80% epiboly (Fig. 4B), different from the pattern of *foxj1* which is restricted to the dorsal forerunner cells (compare Fig. 4A and B to Fig. 3B-D). During the bud through early somite stages, the expression of *foxj1.2* overlaps with *foxj1* in the floor plate and pronephric duct. Unlike *foxj1* which is strongly expressed from bud stage until about 2days, *foxj1.2* expression in the floor plate is seen mainly from bud through early somite stages and is faint at later stages (Fig. 4C-M). Unlike *foxj1*, *foxj1.2* is also strongly expressed in the otic vesicles (Fig. 4F, H, I), becomes restricted to the otoliths by 1-2 days (Fig.

4K, L, M, M2), and fades subsequently. Expression in the posterior neural tube also differs where *foxj1.2* is strongly expressed, and in Kupffer's vesicle where *foxj1*, but not *foxj1.2*, is strongly expressed (Fig. 4D, F, G, I-K, L2, M, compared to Fig. 3F, H, J). At late somite stages through 24hr, *foxj1.2* is expressed in the olfactory pits, where *foxj1* is detected only by 3-4 days of age (Fig. 4K, L, M4, N). Further, *foxj1.2* is expressed in the ventral diencephalon (Fig. 4L, M, M4) and weakly in the MHB and dorsal midline of the tectum, resembling the wnt1 pattern in 1day old embryos (Fig. 4M, M1). This anterior-most expression overlaps with that of the pineal markers *aanat2* and *otx5* (data not shown). Staining in this area is weaker by 2 days and moved to the ventral side of the pineal gland (Fig. 4N), as seen in double-staining with pineal markers (not shown). By 3 and 4days, *foxj1.2* staining in this region seems to be restricted to the anterior edge of the tectal ventricle ventral to the pineal gland (arrows in Fig. 4O), providing an additional difference from *foxj1* which is widely expressed in this region (compare to Fig. 3T-V). *Foxj1.2* is expressed in the trigeminal ganglia at the 15somite stage but not at other stages examined (Fig. 4P). In summary, *foxj1.2* is strongly expressed in the shield, the floor plate in early somitogenesis, the posterior neural plate, the otic vesicles and later in the otoliths, the pronephric ducts, diencephalon, olfactory pits, briefly in the trigeminal ganglion, and in an anterior brain region that overlaps the pineal gland and later corresponds to the anterior tectal ventricle. This expression pattern is partially overlapping with that described for *foxj1.2* in *Xenopus tropicalis* (Choi et al., 2006).

As conclusion, the expression pattern of Foxj1.2 is different from that of Foxj1, which supports the conclusion that our sequence is the one which is more likely to be called Foxj1.

Concluding remarks

Here we present the isolation of the zebrafish *foxj1* gene; we illustrate its dynamic expression during development and compare it to *foxj1.2*. Zebrafish *foxj1* is expressed in three major domains, dorsal forerunner cells and Kupffer's vesicle, the floor plate, and the pronephros. This pattern is similar to that of *Foxj1* genes in other vertebrates. Foxj1 function has been connected to ciliogenesis and the regulation of left/right asymmetry, which fits the major expression sites in zebrafish embryos, Kupffer's vesicle and the pronephric ducts, both of which are known to contain highly ciliated cells. Therefore it is likely that zebrafish Foxj1 functions similarly to Foxj1 of other vertebrate.

Experimental Procedures

Embryos

Zebrafish (*Danio rerio*) were raised and maintained according to standard procedure (Westerfield, 2000). Embryos were raised at 28.5°C and staged as described (Kimmel et al., 1995).

RT-PCR

RNA isolation was performed using the RNeasy Mini Kit (Qiagen, http://www1.qiagen.com). Reverse transcription and PCR were performed as described in the SuperScript™ II Reverse Transcriptase manual (Invitrogen, http://www.invitrogen.com). The expression levels of zebrafish *foxj1* were compared to *histone 4* (*H4*, AM422106). The primers used for *H4* were: forward, 5′- GAAGAGGCAAAGGAAGCAAA -3′ and reverse, 5′- TGGCGTGCTCTGTGTAGGTA -3′ (58 °C, 25 cycles). The primers used for *foxj1* were: forward, 5′- GATTCCAGCCAGGATTTTCA -3′ and reverse, 5′- AATGCAAATGTGCCAACAAA -3′ (58 °C, 30 cycles). The expression levels of zebrafish *foxj1.2* were compared to *β-actin* (BC154531). The primers used for *β-actin* were: forward, 5′- GAGGAGCACCCCGTCCTGC -3′ and reverse, 5′- GATGGCTGGAACAGGGCC -3′ (58

°C, 25 cycles). The primers used for *foxj1.2* were: forward, 5′- cgtgaagccaccctattcat-3′ and reverse, 5′- ggattgagttctgccagctc -3′ (58 °C, 35 cycles)

Cloning and construction of expression plasmid

A partial zebrafish *foxj1* clone (#5134) was identified in an expression pattern screen (Kudoh et al., 2001). The 5′ end of *foxj1* was subsequently generated by 5′- RACE using the SMART RACE Kit (Clontech) with a gene-specific primer (5′-CGTGTTCGTGTGGGCGATTTTAAGAG -3′), and Nested-GSP primer 5′-AGCTCGAATGTTAGCGGGAATTGGAC -3′, according to the manufacturer's instructions.

Whole-mount in situ hybridization

In situ hybridizations were performed as described by Thisse and Thisse (http://zfin.org/zf_info/zfbook/chapt9/9.82.html) (Westerfield, 2000). Antisense digoxigenin or fluorescein-labeled probes were synthesized for the following zebrafish markers: *Foxj*, using the original EST clone (5134), which is a 3′UTR of the gene; *Foxj1.2*, using the clone IMAGE: 7228406 (http://www.openbiosystems.com/), *ntl* (Schulte-Merker et al., 1994), *pax2a/2.1* (Krauss et al., 1991; Pfeffer et al., 1998), *charon* (a gift from Dr. Rebagliati Michael) (Gourronc et al., 2007), *aanat2* (Falcon et al., 2003; Ziv et al., 2005), and *otx5* (Gamse et al., 2002; Ziv et al., 2005). The labeling kit from Roche Molecular Biochemicals was used as described (Westerfield, 2000).

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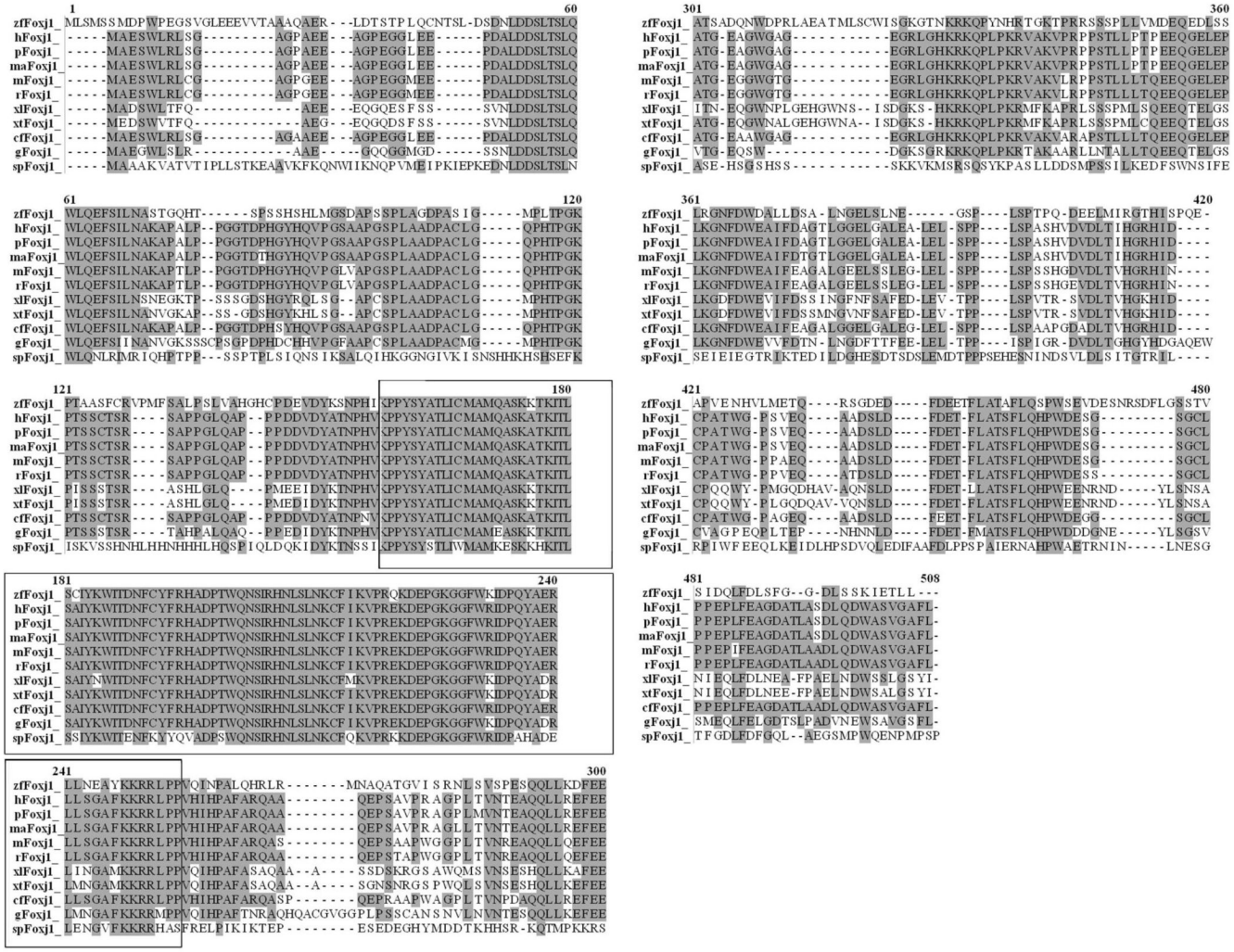

Fig. 1. Molecular analysis of Foxj1

Amino acid alignment of predicted zebrafish Foxj1 protein with Foxj1 in other species, using the http://molbio.info.nih.gov/molbio/gcglite/clustal17.html online program. Consensus amino acids are shaded. The Winged helix domain is boxed. Zebrafish Foxj1 (ABW82682) aligns at a level of 99% to the hypothetical sequence (AAI24229). h, Homo sapiens (AAB09039); p, Pan troglodytes (XP_511694); ma, Macaca mulatta (XP_001104114); m, Mus musculus (AAH82543); r, Rattus norvegicus (NP_446284); xl, Xenopus laevis (AAH77846); xt, Xenopus tropicalis (CAJ82756); cf, Canis familiaris (XP_533124); g, Gallus gallus (XP_001233327); and sp, Strongylocentrotus purpuratus (NP_001073013).

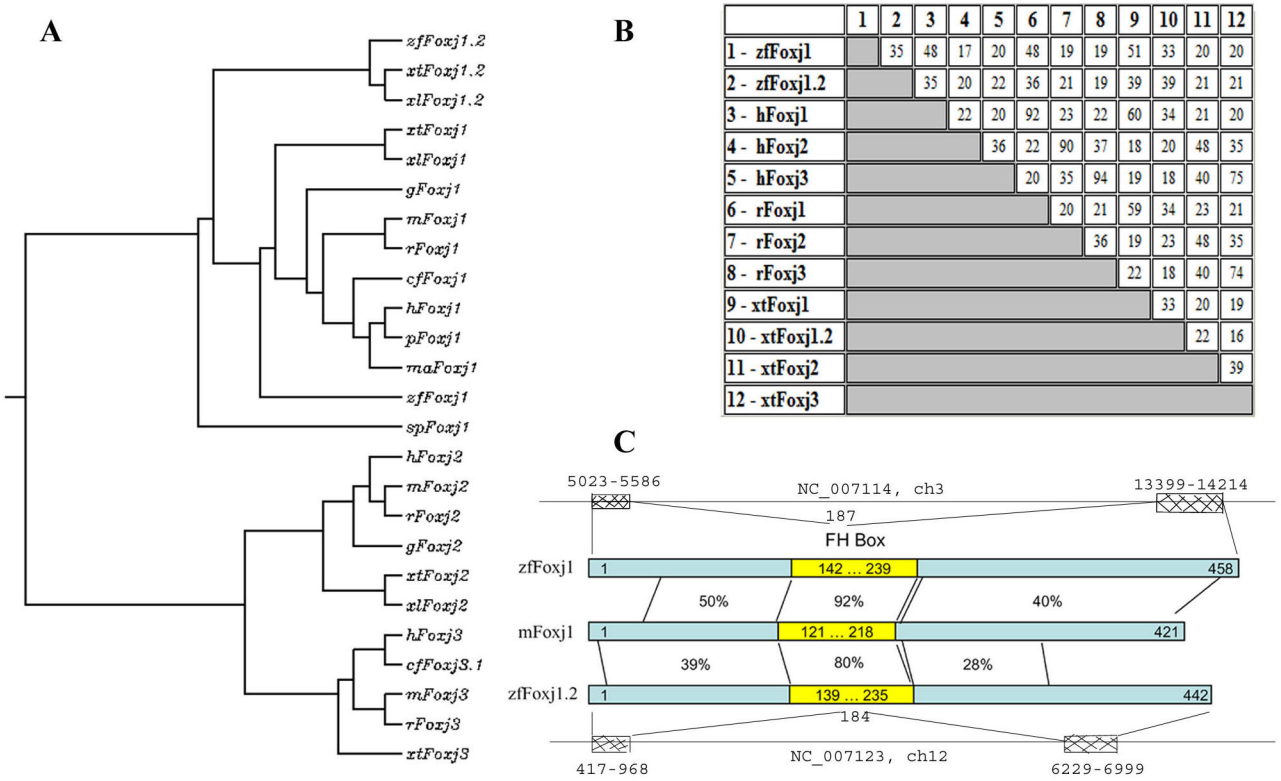

Fig. 2. Comparison of Foxj protein sequences

A) Phylogenic tree of Foxj1/1.2/2/ and 3 proteins, given by http://align.genome.jp/CLUSTALW. B) "PairWise" Comparisons Scores (percentage). C) Schematic drawing of identities between zebrafish Foxj1 and Foxj1.2 and mouse Foxj1. The forkhead box is in yellow (FH Box), and chromosomal locations of zebrafish genes are indicated; proteins are encoded by two exons.

The accession numbers used in these comparisons are as follows (the numbers for Foxj1 are as in Figure 1): Danio rerio (zebrafish, zf) Foxj1.2 (NP_001008648.1); Homo sapiens (h) Foxj2 (NP_060886) and Foxj3 (Q9UPW0); Mus musculus (m), Foxj2 (NP_068699.1), and Foxj3 (Q8BUR3); Rattus norvegicus (r), Foxj2 (XP_578387.2), and Foxj3 (NP_001101441); Xenopus tropicalis (xt), Foxj1.2 (Q66IG8), Foxj2 (Q28EM1), and Foxj3 (NP_001103516); Xenopus laevis (xl), Foxj1.2 (Q32NH9), and Foxj2 (NP_001087521.1), Gallus gallus (g), Foxj2 (XP_416484); Canis familiaris (cf), Foxj3.1 (XP_532540).

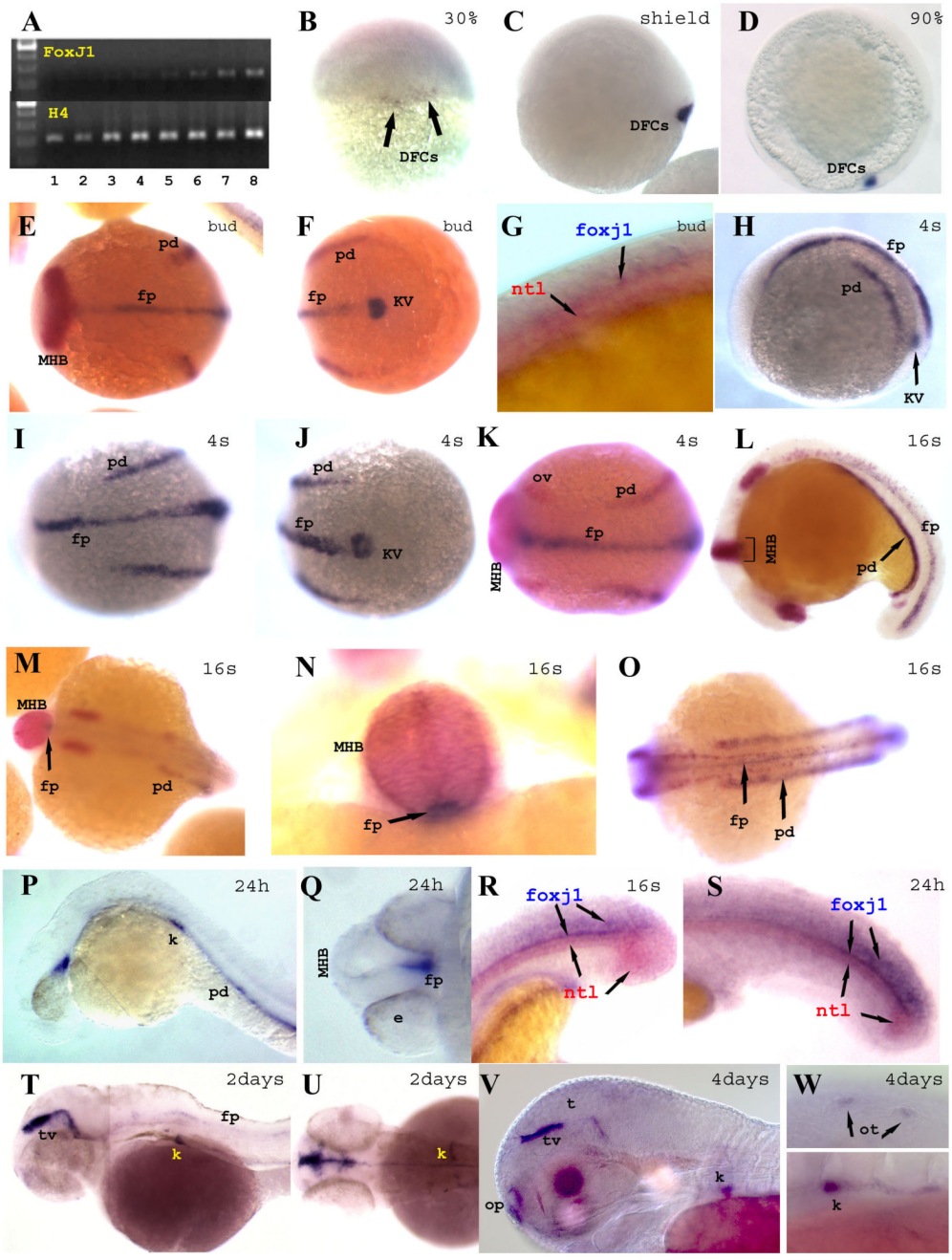

Fig. 3. Expression pattern of *foxj1*

A)RT-PCR expression analysis of zebrafish *foxj1* and *histone 4* (H4) as control was performed for different stages (1-8: unfertilized eggs, 100-200 cells, high-dome, 40-50% epiboly, 80-90%, bud, 6-somites, and 24h embryos, respectively). **B-W)** Spatio-temporal expression pattern of zebrafish *foxj1*. Whole-mount in situ hybridization with zebrafish *foxj1* probe alone (B-D, H-J, P-Q, T-W), or combined with either *pax2.1* (red: E-F, K-O) or *ntl* (red: G, R-S). Stages are indicated at top right, with "s" referring to somite number, and hpf referring to hours post-fertilization. Views are as follows: (B) dorsal, (C, D) lateral with dorsal to the right, (E, I, K, M, O, U) dorsal with anterior to the left, (F, J) posterior with dorsal to the left, (Q) anterior with dorsal to the left, (G, H, L, P, R, S, T, V, W) lateral with anterior to the left, and (N) is

posterior with dorsal up. DFCs, dorsal forerunner cells; e, eye; fp, floor plate; k, kidney; KV, Kupffer's vesicle; MHB, mid-hindbrain boundary; op, olfactory pits; ot; otolith; ov, otic vesicle; pd, pronephric duct; t, tectum; tv, tectal ventricle.

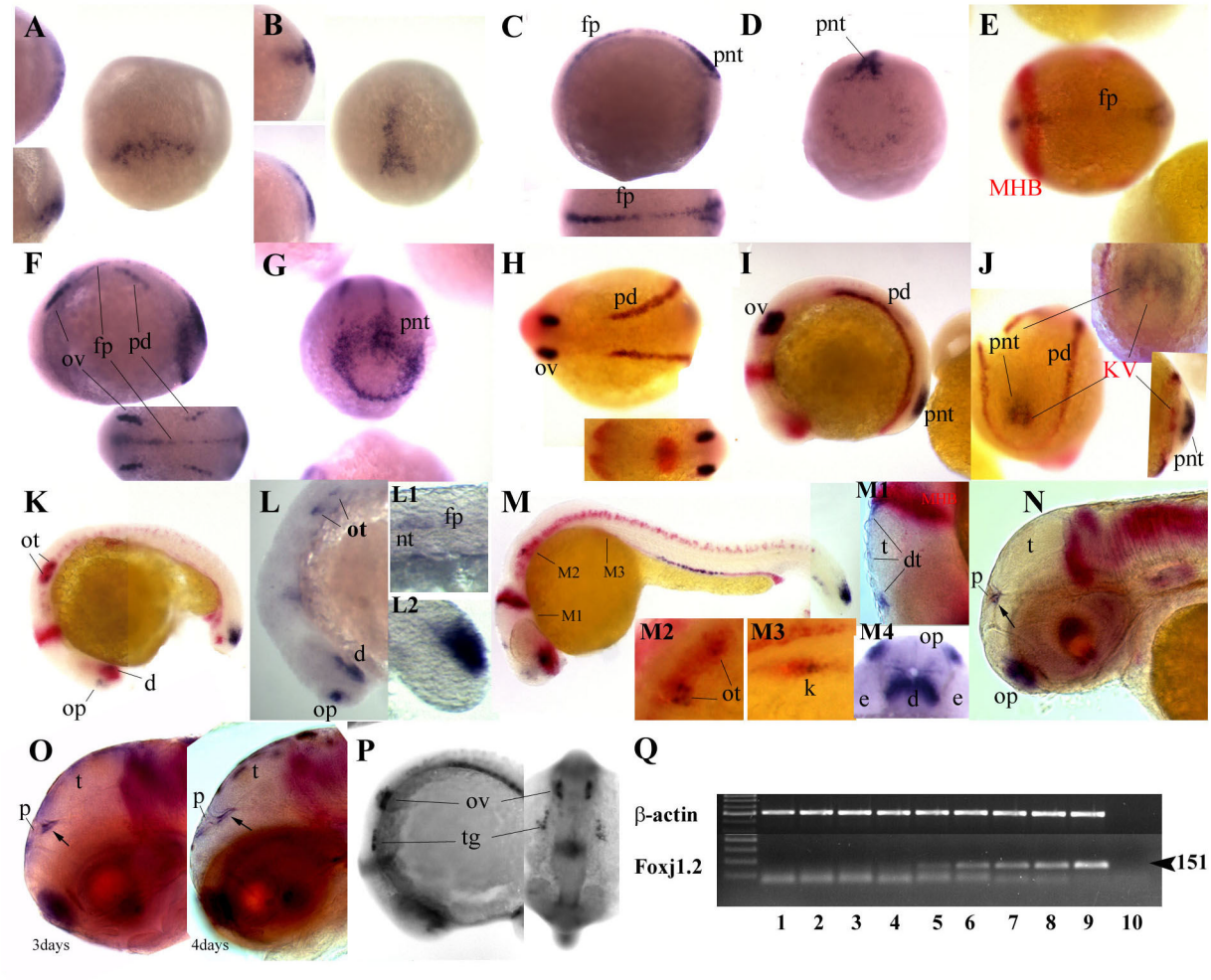

Fig. 4. Zebrafish foxj1.2 expression

A-P) Spatio-temporal expression pattern of *foxj1.2*. Whole-mount in situ hybridization with *foxj1.2* probe alone (A-D, F-G, L, M4), or combined with *pax2.1 and charon* (both red: E, H-K, M-P). Shield-60% epiboly (A), 80% epiboly (B), bud (C-E), 4 somites (F-G), 7 somites (H-J), 15 somites (K, P), 16 somites (L), 1 day (M), 2 days (N), 3 and 4 days (P left and right respectively). Views are as follows: dorsal (A, B); dorsal with anterior to the left (C bottom, E, F bottom, H and H bottom), dorsal with anterior up (P right), lateral with dorsal to the right, (A left bottom, B left bottom), lateral with anterior to left (C top, F, I, K-O except for M4, P left), posterior with dorsal up (D, G, J), anterior ventral with dorsal up (M4), animal view (A left top, B left top), ventral with posterior to the right (J right bottom). **Q)** RT-PCR expression analysis of *foxj1.2* and *β-actin* as control was performed for different stages (1-8: unfertilized eggs, 100-200 cells, high-dome, 40-50% epiboly, 80-90%, bud, 13-somites, 24hpf and 3days old embryos respectively, and -RT in lane 10). d, diencephalon; e, eye; fp, floor plate; k, kidney; KV, Kupffer's vesicle; MHB, mid-hindbrain boundary; nt, notochord; op, olfactory pit; ot; otolith; ov, otic vesicle; pd, pronephric duct; pnt, posterior neural tube; t, tectum; tv, tectal ventricle.