An evolutionary and functional analysis of *FoxL2* in rainbow trout gonad differentiation

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Abstract

FOXL2 is a forkhead transcription factor involved in ovarian development and function. Here, we have studied the evolution and pattern of expression of the *FOXL2* gene and its paralogs in fish. We found well conserved *FoxL2* sequences (*FoxL2a*) and divergent genes, whose forkhead domains belonged to the class L2 and were shown to be paralogs of the *FoxL2a* sequences (named *FoxL2b*). In the rainbow trout, *FoxL2a* and *FoxL2b* were specifically expressed in the ovary, but displayed different temporal patterns of expression. *FoxL2a* expression correlated with the level of aromatase, the key enzyme in estrogen production, and an estrogen treatment used to feminize genetically male individuals elicited the up-regulation of both paralogs. Conversely, androgens or an aromatase inhibitor down-regulated *FoxL2a* and *FoxL2b* in females. We speculate that there is a direct link between estrogens and *FoxL2* expression in fish, at least during the period where the identity of the gonad is sensitive to hormonal treatments.

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Introduction

FOXL2 is a putative winged helix/forkhead transcription factor gene involved in ovarian development and function. Its mutation leads to the blepharophimosis ptosis epicanthus inversus syndrome (BPES), a rare genetic disease involving eyelid malformations associated with premature ovarian failure (POF) (BPES type I) or occurring without premature ovarian failure (type II) (Zlotogora *et al.* 1983, Crisponi *et al.* 2001, De Baere *et al.* 2001). The expression of *FOXL2* has been studied extensively in mammals (Crisponi *et al.* 2001, Cocquet *et al.* 2002) and non-mammalian vertebrates (Loffler *et al.* 2003). *FOXL2* ovarian expression in mammals starts before the morphological differentiation of the gonad is recognizable and persists until adulthood. It is essentially restricted to the somatic compartment, thus follicular cells display a strong protein expression while the stroma shows a more diffuse protein expression. In the oocytes, no protein signal has been detected (Cocquet *et al.* 2002, Pannetier *et al.* 2003). However, in a recent study, *FoxL2* mRNA was observed in both granulosa cells and some oocytes of fetal and adult mouse ovaries (Loffler *et al.* 2003).

In previous studies, we have shown that *FOXL2* is highly conserved across divergent taxonomic groups. However, outside the DNA-binding forkhead domain, the C-terminal region is more conserved than the N-terminus (Cocquet *et al.* 2003). In mammals, *FOXL2* contains a polyalanine tract and other low-complexity repeats that are absent in fish sequences. In this paper, we focus on the *FoxL2* gene in fish with two main objectives: (1) we take advantage of the known abundance of duplicate genes in teleosts (Wittbrodt *et al.* 1998) to study the evolution of *FoxL2* paralogs in the rainbow trout (*Oncorhynchus mykiss*); and (2) from a functional perspective, we have studied the temporal pattern of expression of *FoxL2* paralogs (designated *a* and *b*) in the rainbow trout during normal gonadal development, as well as under

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masculinizing or feminizing treatments (leading to fertile males or females, respectively). We have detected a link between estrogens and *FoxL2* expression. Thus, we have also investigated the expression profile of aromatase, the key enzyme involved in estrogen production.

Materials and methods

Evolutionary analysis

The sequences of the *FoxL2* orthologs from *Tetraodon nigroviridis* and *Takifugu rubripes* (pufferfish), *Danio rerio* (zebrafish) and mammals are those described by Cocquet *et al.* (2003). BLAST queries of Genbank with these open reading frames (ORFs) detected high-scoring matches at the forkhead domain, but similarity was surprisingly low outside this domain (putative paralogs). The rainbow trout (*O. mykiss*) *FoxL2* sequences were obtained following BLAST searches against the ESTs available in GenBank. The partial sequences detected were then extended by PCR on cDNAs to obtain the full ORFs (Genbank AI: AY507927 for *FoxL2a*, AY507926 for *FoxL2b*).

For phylogenetic analysis of nucleotide and amino acid sequences, we used PhyML (Guindon & Gascuel 2003) and Tree-Puzzle (Schmidt *et al.* 2002) which implement fast heuristic tree-searching algorithms based on the maximum likelihood framework. Statistical support for alternative topologies was evaluated by the Kishino-Hasegawa test (Kishino & Hasegawa 1989) which has been implemented in Phylogenetic Analysis by Maximum Likelihood (PAML) (Yang 2002), Data Analysis in Molecular Biology and Evolution (DAMBE) (Xia 2001) and recently in Tree-Puzzle (Schmidt *et al.* 2002). Rate heterogeneity over sites was modeled by a discrete gamma distribution with the shape parameter α estimated from the sequences. The empirical Jones–Taylor–Thornton (JTT) substitution matrix (Jones *et al.* 1992) was used for analyzing amino acid sequences. For nucleotide sequences, the general time reversible (GTR) model was used with PhyML. As the GTR model has not yet been implemented in Tree-Puzzle, we used a more restrictive TN93 model (Tamura & Nei 1993) with one rate for transversions and two rates for A↔G and C↔T transitions.

Animals and sampling

Research involving animal experimentation has been approved by the authors' institution (authorization number 35–14) and conforms to NIH guidelines. Exclusively male and female rainbow trout populations were obtained from the INRA experimental fish farm (Drennec, France) as previously described (Guiguen *et al.* 1999). Fifty-five days post-fertilization (55 dpf), five batches of 1500 fry each, corresponding to the five experimental groups described below, were transferred to 0·3 m^3 tanks. They were held at 12 °C and fed *ad libitum* with a commercial diet (dry pellet food, Biomar, Brande, Denmark). Steroid (11β-hydroxyandrostenedione (11βOHΔ4, Sigma, St Louis, MO, USA), 17α-ethynylestradiol (EE2, Sigma, St Louis, MO, USA)) and anti-aromatase (Androstentrione (ATD, Steraloids, Newport, RI, USA) treatments were carried out by dietary administration for 2 to 3 months (11βOHΔ4, 10 mg/kg diet for 3 months; EE2, 20 mg/kg diet for 2 months; ATD, 50 mg/kg diet for 3 months) starting from the first feeding. Briefly, the steroid and anti-aromatase were added to the food in ethanolic solution (40 ml/kg of food), which was then evaporated to dryness. Ethanol was also added to the food of the control groups in the same proportion. These molecules have been previously shown to be potent feminizing (Govoroun *et al.* 2001*a*) and masculinizing (see Govoroun *et al.* 2001*b*, for 11βOHΔ4; Guiguen *et al.* 1999, for ATD) treatments when administered at the mentioned doses to rainbow trout. Gonads were sampled, starting at the onset of the free swimming period (day 0, d0) and at d7, d16 (around the first occurrence of oocyte meiosis), d30 (first previtellogenic oocytes), d63, d91 and d111 in duplicate (20–100 pooled gonads, depending on the age of fish) and stored at −80 °C until RNA extraction. Additional gonads were sampled for the five groups on the same dates for histological analysis.

Total RNA extraction and reverse transcription (RT)

Total RNA was isolated using TRIzol reagent (Invitrogen) as previously described (Govoroun *et al.* 2001*b*). Total RNA concentration was determined with an Agilent 2100 Bioanalyzer and the RNA 6000 LabChip kit (Agilent Technologies, Colorado

Springs, CO, USA) according to the manufacturer's instructions. For cDNA synthesis, 1 μg of RNA was denaturated in the presence of random hexamers (0·5 μg) for 5 min at 70 °C, and then chilled on ice for 5 min. RT was performed at 37 °C for 1 h using Moloney, Munine Leukemia Virus (M-MLV) reverse transcriptase (Promega) as described by the manufacturer.

Real-time PCR

Real-time PCR was carried out on an iCycler iQTM (BioRad, Hercules, CA, USA). Reactions were performed in 20 μl solution, with 300 nM of each primer, 5 μl of a 1/50 dilution of the RT reaction and the SYBER-Green PCR master Mix (Eurogentec, Seraing, Belgium) according to the manufacturer's instructions. The first two incubation steps (50 °C for 10 min, 95 °C for 2 min), were followed by the thermal cycling protocol which included 10 min at 95 °C followed by 40 cycles of PCR (95 °C for 30 s, 60 °C for 1 min). For each primer set (see below) the efficiency of the PCR was measured in triplicate on serial dilutions of the same cDNA sample (pool of reverse transcribed RNA samples). Melting curve analysis was also performed for all genes to check the specificity and identity of the RT-PCR products. The relative amount of the target RNA was then determined using the I-Cycler IQ software (BioRad) by comparison with the corresponding standard curve for each sample run in duplicate. Each transcript level was then normalized on the basis of the expression values of the constitutive elongation factor 1α (EF1α). The primers used were: P450 aro-up, CTCTCCTCTCATACCT CAGGTT; P450 aro-dwn, AGAGGA ACTGCT GAGTATGAAT; FOXL2a-up, TGTGCTGGAT TTGTTTTTT GTT; FOXL2a-dwn, GTGTCGT GGACCATCA GGGCCA; FOXL2b-up, CGAG GAAGATTTA AACTACATG; FOXL2b-dwn, GAGGACGAG TCGGTTAGATCCA; EF1α-up, AGCGCAATC AGCCTGAGAGGTA and EF1α-dwn, GCTGG ACAAGCTGAAGGCTGAG

Histology

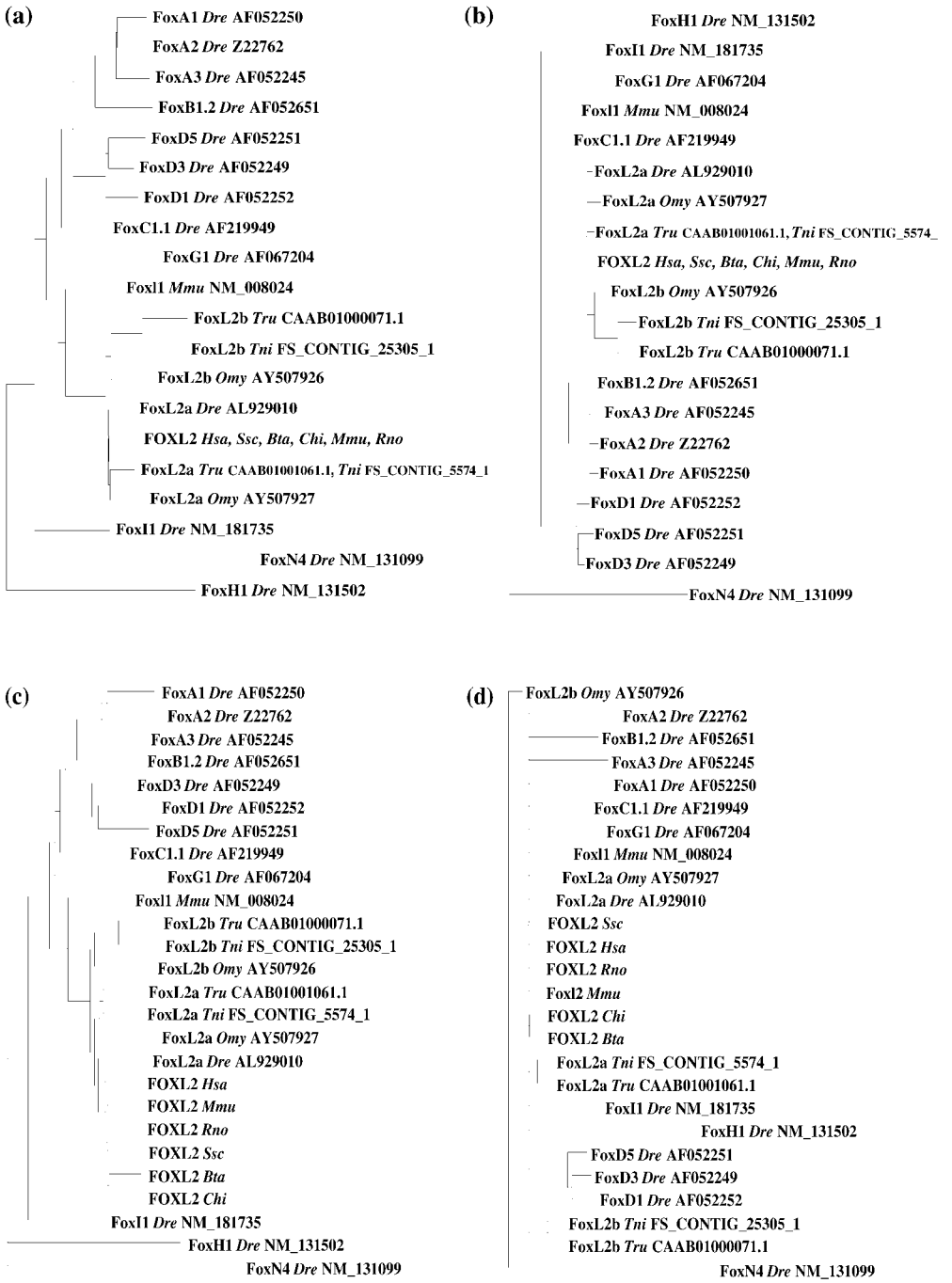
Gonads were fixed for 2 h in 0·13 M sodium cacodylate buffer (OP 320 mOsm; pH 7·4) containing 2·5% glutaraldehyde and 20 mM $CaCl_2$, then postfixed for 1 h with 1% osmium tetroxide and 1·5% potassium ferricyanide in the same buffer. After the postfixation, samples were washed in sodium cacodylate buffer. Subsequently, they were dehydrated in increasing concentrations of ethanol solutions (30%–100%), substituted in propylene oxide and embedded in epoxy resin. Semi-thin sections were cut, collected on glass slides and stained with toluidine blue.

Results and discussion

FoxL2 orthologs and paralogs in fish

Pufferfish, *T. nigroviridis*, and zebrafish carry sequences highly similar to the mammalian *FOXL2* ORFs and are considered to be conserved orthologs (Cocquet *et al.* 2003). Notably, they are without introns. Using BLAST searches, we have detected additional sequences with lower degrees of similarity and decided to analyze the highest scoring sequences, as they might be divergent paralogs. In fact, in the latter, conservation was essentially confined to the forkhead domain. We also isolated two *FoxL2*-related sequences from the rainbow trout (see Material and methods). One sequence was very close to well characterized *FOXL2* orthologs and the other was more divergent. In order to show a potential evolutionary affiliation of these forkhead genes to the *FoxL2* family, we gathered 13 well-known forkhead sequences from the zebrafish genome for phylogenetic analyses (as described in Kaestner *et al.* 2000). We also included the mammalian *FOXL1* and *FOXL2* sequences. The maximum likelihood trees from PhyML (Fig. 1a) and Tree-Puzzle (Fig. 1b) based on the amino acid sequences of the forkhead domains were largely congruent (see also online Fig. 1). The fully resolved PhyML tree (Fig. 1a) was significantly better ($P=0·01$) than the consensus tree from Tree-Puzzle (Fig. 1b) when evaluated by the Kishino–Hasegawa test, with $\ln L_{\text{PhyML}} = -2025·20$, and $\ln L_{\text{Tree-Puzzle}} = -2051·26$ ($\Delta \ln L = 26·06$ and the variance of the difference, estimated by bootstrapping 10000 times, equals 11·13). The phylogenetic trees from nucleotide sequences were consistent with the tree from amino acid sequences, except that the tree from Tree-Puzzle had little resolution. These results suggest that Tree-Puzzle is limited in searching through the tree space and our interpretation below will be based on the PhyML

trees (other details in online Fig. 2). The maximum likelihood trees from PhyML (Fig. 1a and online Fig. 2a) clustered all FOXL2 sequences together, including the highly diverged (presumed) paralogs. The murine *Foxl1* sequence was weakly but consistently clustered with the *FOXL2* sequences in the nucleotide-based tree (Fig. 1a and online Fig. 2a). The tree shows that *T. nigroviridis*, pufferfish, and rainbow trout have at least two *FoxL2* paralogs, and justify our naming them as *FoxL2a* and *FoxL2b* genes. Recently, a forkhead gene from the ascidian *Ciona intestinalis* has been considered as the potential ortholog of *FoxL2* based on a phylogenetic analysis (Mazet *et al.* 2003). It would be interesting to study



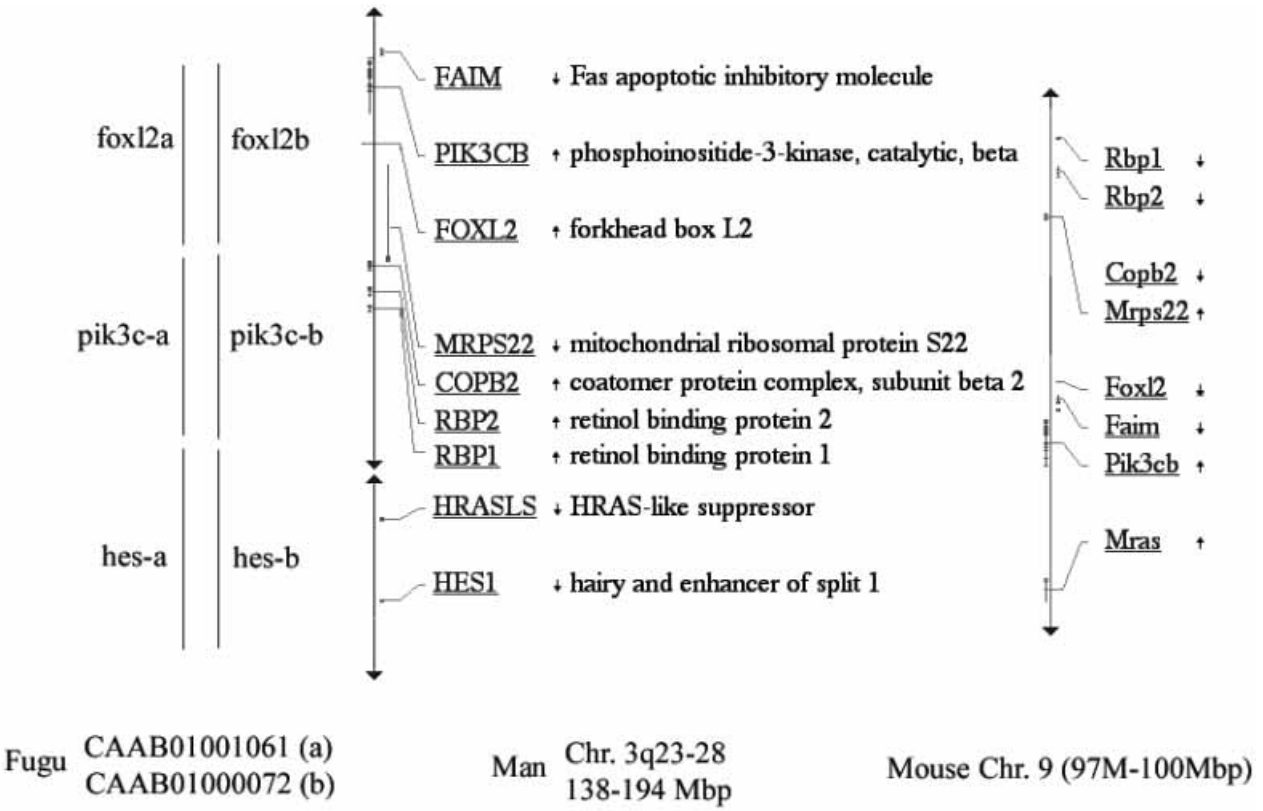


Figure 2 Conservation of synteny around *FoxL2a* and *FoxL2b* genes in pufferfish. Notice also the conservation in man.

the expression profile of the putative ancestor of *Foxl2* in *Ciona*, which is a hermaphroditic organism.

Phylogenetic analysis of homologous sequences provides only limited support for paralogy, and extrinsic evidence of synteny has long been considered essential to support paralogy (Comings 1972). For this reason, we studied the conservation of synteny around *FoxL2* in the completely sequenced pufferfish genome. We retrieved the genomic sequences containing the presumed *FoxL2a* and *FoxL2b* and searched for coding regions using the Genscan program (Burge & Karlin 1997). In one of the genomic contigs, this tool recognized an intronless ORF corresponding to *FoxL2b*. Moreover, both *FoxL2a* and *FoxL2b* were found to be linked to copies of the *PI3K* (*phosphoinositide-3 kinase*, catalytic sub-unit) and *Hes* (*Hairy and enhancer of split-1*) genes. Remarkably, this group of synteny has been conserved up to man (Fig. 2). This clearly shows that the highly diverged *FoxL2b* is indeed the paralog of

Figure 1 Maximum likelihood trees based on the amino acid sequences of the forkhead domain, with the JTT model and a discrete gamma distribution to fit variable rates of substitutions (a) from PhyML, with the estimated α=0·761 (b) from Tree-Puzzle, with the estimated α=0·70. The amino acid sequences of *FOXL2* for the six mammalian species are practically identical and grouped together as *FOXL2*, so are pufferfish and *Tetraodon nigroviridis*. The Genbank accession numbers are included along with the names of the genes. (c) Maximum likelihood tree based on the nucleotide sequences of the forkhead domain from PhyML with the GTR model and the estimated α=0·553. (d) From Tree-Puzzle, with the estimated α=0·56. The fully resolved PhyML tree is significantly better ($P < 0{\cdot}001$) than the poorly resolved tree from Tree-Puzzle, when evaluated by the Kishino–Hasegawa test, with $\ln L_{PhyML} = -4939{\cdot}864$, and $\ln L_{Tree\text{-}Puzzle} = -5375{\cdot}076$. The difference in lnL is –435·212 and the variance of the difference, estimated by bootstrapping 10 000 times, equals 44·857. *Bta, Bos taurus* (cow); *Chi, Capra hircus* (goat); *Dre, Danio rerio* (zebrafish); *Hsa, Homo sapiens* (man); *Mmu, Mus musculus* (mouse); *Omy, Oncorhynchus mykiss* (rainbow trout) ; *Rno, Rattus norvegicus* (rat); *Ssc, Sus scrofa* (pig); *Tni, Tetraodon nigorviridis* (pufferfish).

Table 1 Isoelectric points (pI) for the forkhead domain and the whole sequence of *FOXL2* orthologs, and several other forkhead proteins. The increased usage of basic amino acids in the forkhead domain leads to a significantly higher pI in this region with respect to the whole sequences

	pI-forkhead	whole sequence
Sequence name		
FOXL2 Mammals	10·64	9·87
FoxA1 *Danio rerio* AF052250	10·33	10·34
FoxA2 *Danio rerio* Z22762	10·42	9·17
FoxA3 *Danio rerio* AF052245	10·33	7·50
FoxB1.2 *Danio rerio* AF052651	10·24	10·32
FoxC1.1 *Danio rerio* AF219949	10·60	9·16
FoxD1 *Danio rerio* AF052252	10·35	7·07
FoxD3 *Danio rerio* AF052249	10·36	6·37
FoxD5 *Danio rerio* AF052251	10·33	6·98
FoxG1 *Danio rerio* AF067204	10·56	8·95
FoxH1 *Danio rerio* NM_131502	10·49	9·93
FoxI1 *Danio rerio* NM_181735	10·31	7·75
FoxI1 Mouse NM_008024	10·63	10·54
FoxL2a *Danio rerio* AL929010	10·64	9·85
FoxL2a *Takifugu*, Tetraodon	10·64	9·70
FoxL2a *Oncorhynchus mykiss*	10·51	9·70
FoxL2b *Oncorhynchus mykiss* CA354643	10·82	8·35
FoxL2b *Takifugu rubripes* CAAB01000071_1	10·76	8·63
FoxL2b *Tetraodon nigroviridis* FS_CONTIG_25305_1	10·40	9·88
FoxN4 *Danio rerio* NM_131099	10·30	7·83
Mean	10·48	8·89
Standard deviation	0·17	1·26

t-test, P<0·0001

FoxL2a. We next compared the *FoxL2b* ORF detected by Genscan in pufferfish with the intronless ORF containing the *FoxL2*-related forkhead of the close species *Tetraodon*. Only weak similarities were found in the 5′ region outside the forkhead domain (available for analysis). This shows that paralogs of *FoxL2* in fish are evolving at very high rates, to a point that similarity is virtually undetectable by traditional means. This dissimilarity was particularly striking in the comparison with the rainbow trout *FoxL2b* sequence. This contrasts with the good conservation of many other paralogous pairs in fish (Robinson-Rechavi & Laudet 2001). It is conceivable that *FoxL2* duplicates are degenerating. However, it is more likely that paralogs may have acquired novel functions. As *FoxL2* is involved in fertility, the paralogs may contribute to reproductive isolation, which would explain their rapid evolution. A similar idea has been prompted to explain the divergence of the sex determining gene *Sry* in mammals (see Pamilo & O'Neill 1997 and references therein).

The analysis of the forkhead domains of the *FoxL2* homologs, and also from other genes, provided two lines of evidence suggesting that selection favors a high isoelectric point (pI) in the forkhead domain. First, we detected a significant increase in pI in this domain with respect to those of the whole proteins (i.e. 10·48 vs 8·89 respectively) (Table 1). Secondly, the standard deviation of the pI is much smaller among the forkhead domains than among the whole sequences (i.e. 0·17 vs 1·26 respectively) (Table 1). The former finding can be explained by an increased usage of positively charged amino acids (Lys and Arg) in the DNA-binding domain with respect to the corresponding whole proteins (not shown). Indeed, clusters of positively charged amino acids can be important for DNA-binding as documented for the basic region in the leucine zipper (bZIP) gene family of transcription factors (Metallo *et al.* 1997). In the case of the forkhead domain, the positively charged amino acid residues may be important for both DNA binding and nuclear localization (Romanelli *et al.* 2003).

Expression of *FoxL2a*, *FoxL2b* and aromatase in the rainbow trout differentiating gonad

Quantitative real-time PCR analysis of the expression of the two rainbow trout *FoxL2* paralogs in conditions of natural gonadal differentiation showed that their mRNA appears specifically during ovarian differentiation (Fig. 3). The steady state mRNA levels during testicular differentiation were barely detectable (from 10 to 100 times less than in female gonads). However, the kinetics of expression of these two *FoxL2* paralogs differed substantially. Namely, *FoxL2a* was highly expressed since the very beginning of the free swimming period (d0) and remained expressed roughly at the same level from d0 to d111 (Fig. 3a). On the contrary, *FoxL2b* expression increased from d0 to d16, concomitantly with the first oocyte meiosis period (Fig. 4) and then dropped to levels similar to those detected at d0 (Fig. 3b). Female-specific expression of *FoxL2* genes have also been found in other non-mammalian species. In the chicken, it is expressed in the two female gonads (ZW) at early stages of development, at least from day 5 to 8, whereas no expression is detected in male gonads at comparable stages. In the turtle, a species undergoing temperature-dependent sex determination, *FoxL2* is expressed in the developing gonads at a higher level in female-promoting temperatures than in male-promoting temperatures (Loffler *et al.* 2003).

Sex determination in the rainbow trout is primarily governed by sex chromosomes. However, successful development of the gonads may be influenced by exposure to exogenous steroids during a critical period of gonadal differentiation. This may lead to reversal of the expected phenotypic sex, with the resulting population being fertile (Guiguen *et al.* 1999, Govoroun *et al.* 2001*b*). In our context, following EE2 treatment of males leading to an effective feminization (Fig. 4), the steady-state mRNA levels of *FoxL2a* and *FoxL2b* increased in the gonad, with important differences in their expression profiles. Specifically, *FoxL2a* expression was up-regulated very quickly (d7) to levels similar to those detected during natural ovarian differentiation (Fig. 3a). This up-regulation persisted after the end of the treatment (d63). We suggest that exogenous estrogens are able to rapidly trigger *FoxL2a* expression in a male background (mRNA is detected as early as d7). This also supports the notion that *FoxL2* is involved in early ovarian development, in an evolutionary scale ranging from fish to man. Notice that in the case of the feminizing treatment, the gonad is forced to differentiate into an ovary and *FoxL2a* expression seems to be required for this process. *FoxL2b* was also clearly up-regulated when compared with the basal male levels (Fig. 3b). However, when compared with natural female differentiation, it only reached similar levels at d30 and higher levels at d63. After completion of the EE2 treatment, expression levels were similar to those of females. Our results suggest that *FoxL2b* is still functional in the rainbow trout as it has kept an intact ORF encoding a basic protein expressed in the female gonad (as its paralog) and is responsive to hormonal treatments. However, *FoxL2b* may have acquired a novel function, which explains its divergence in sequence and expression pattern. *FoxL2b* expression peaks at about d16, concomitantly with the onset of oocyte meiosis and may well be involved in this process. A possible neofunctionalization would be consistent with the maintenance of many duplicate genes in fish (Govoroun *et al.* 2001*a*).

The expression profile of aromatase, a key gene of estrogen synthesis, was also followed by real-time RT-PCR (Fig. 3c). In accordance with previous results, aromatase expression during natural differentiation was only detected in the female (Guiguen *et al.* 1999). The comparison of the aromatase expression profiles during natural differentiation with those of *FoxL2a* demonstrated a high correlation ($R = 0{\cdot}98$, $P < 10^{-8}$) which suggests that both genes are close in a regulatory cascade, or that they respond to the same regulators. The feminizing EE2 treatment in males did not up-regulate aromatase expression, which suggests that the exogenous estrogens are enough to drive the feminization process, including early expression of *FoxL2a* (i.e. in absence of aromatase transcription). Accordingly, aromatase and *FoxL2a* mRNA expression profiles did not correlate during the feminizing treatment. After completion of the treatment (d63), expression increased slightly. On the contrary, the specific ovarian expression of aromatase during differentiation was very quickly down-regulated by masculinizing treatments in females, either with androgens (11βOHΔ4) or with an aromatase inhibitor (ATD), a situation that persisted after completion of the treatments (d91) as a functional testis had developed (Fig. 4). Following

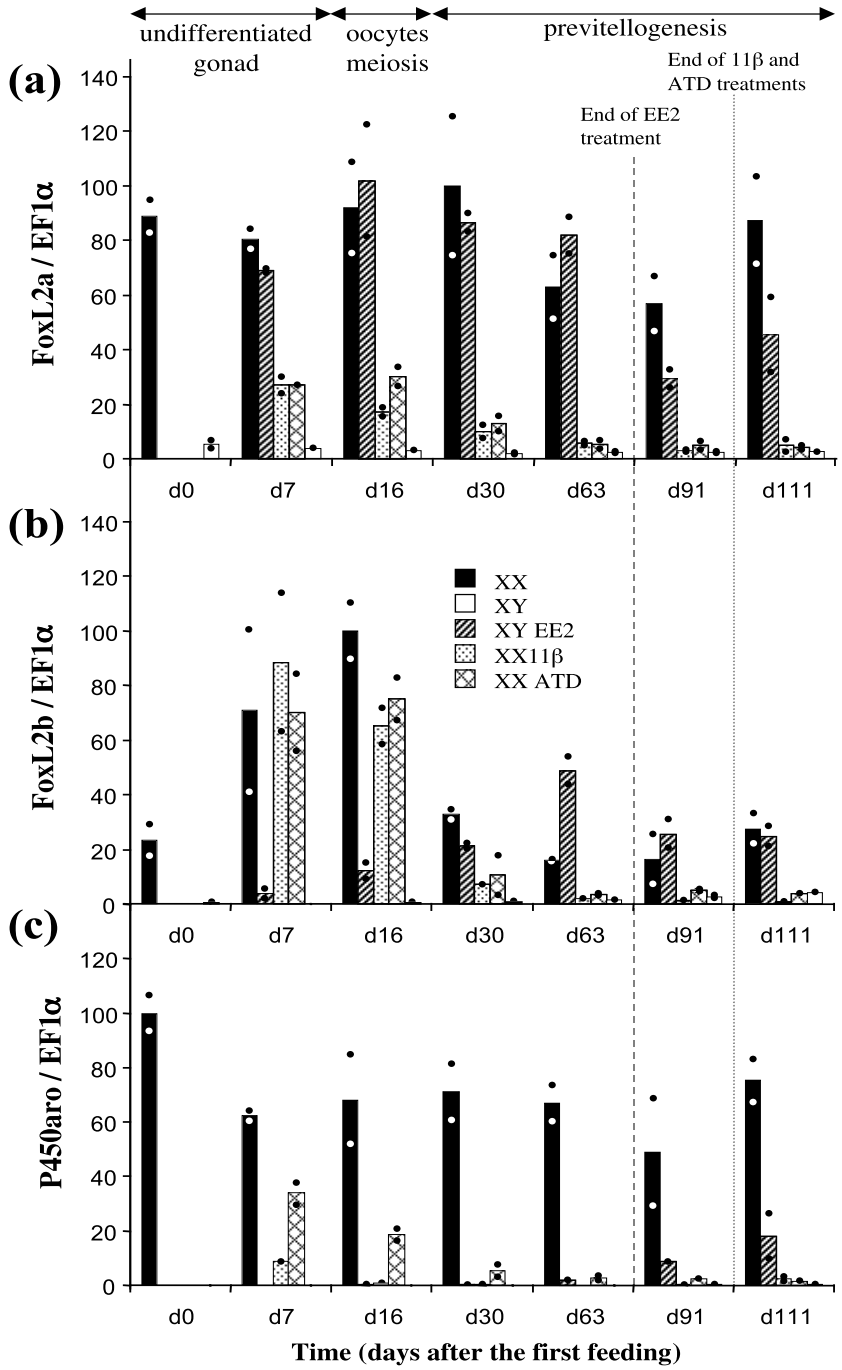


Figure 3 Expression profiles of *FoxL2* paralogs (*FoxL2a or FoxL2b*) and aromatase during natural differentiation and steroid induced differentiation. Kinetics of *FoxL2a* (3a), *FoxL2b* (3b) and aromatase (P450 aro) (3c) in female control group (XX, black bar), male control group (XY, white bar), EE2 treated group (XY EE2, hatched bar), 11βOHΔ 4 treated group (XX 11β, dotted bar) and ATD treated group (XX ATD, trekked bar). Results of RT-PCR are represented as the ratios between the expression of the specific gene and that of the EF1α (arbitrary scale). Each bar represents the mean of two independent measures using pooled gonads. The particular values are represented for each bar by black or white dots. Major events of ovarian natural differentiation are depicted on the top of the figure.

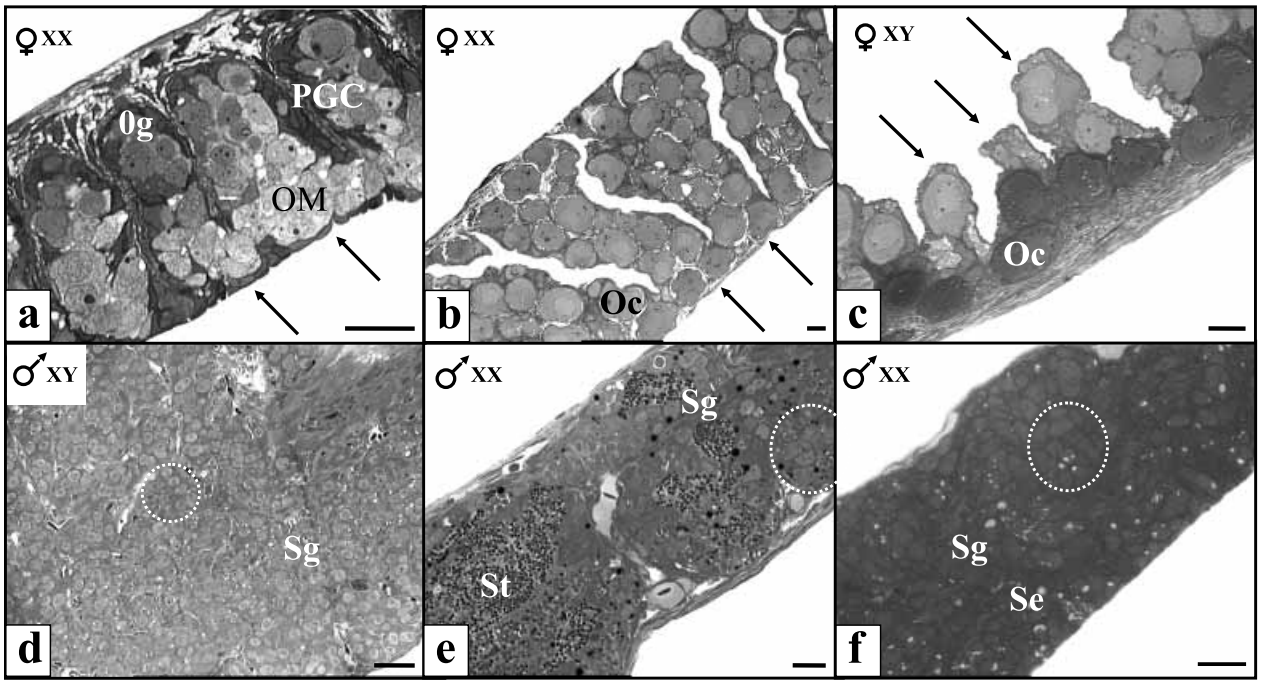


Figure 4 Histology of the gonads from control (a, b, d) and treated groups (c, e, f). Phenotypes of animals are indicated by symbols on the top left (genotypes, XX for genetic females and XY for genetic males). Panel (a) depicts the appearance of the first ovarian meiosis (OM) in a normal female at d16, concomitantly with the appearance of lamellar structures (arrows). At this time (d16), these lamellar structures still contain primordial germ cells (PGC) and oogonia (Og), whereas they contain mainly previtellogenic oocytes (Oc) at d111 (panel b). Compared with normal females (in panel b), XY EE2 gonads (panel c) have differentiated into typical ovaries in which lamellar structures contain fully developed oocytes at the end of the experiment (d111). Compared with normal males (panel d), 11βOHΔ 4-treated XX (panel e) and ATD-treated XX (panel f) gonads have differentiated into typical testes at d111, filled with seminiferous lobules (highlighted by circles) containing mainly spermatogonias (Sg) and Sertoli cells (Se). Notice that in the 11βOHΔ 4-treated XX group (panel e) spermatogenesis seems to be more engaged with spermatids (St), probably due to androgen treatment. Scale bars=50 μm.

effective masculinization of females, we detected a decrease in the expression levels of both *FoxL2* paralogs. These two masculinizing treatments had largely similar effects. The decrease of *FoxL2a* was detected as early as d7 and then reached a level similar to that of a normal male. Moreover, high and significant correlation coefficients between aromatase and *FoxL2a* expression were found for masculinizing treatments (for 11βOHΔ4: $R=0{\cdot}81$, $P<0{\cdot}05$; for ATD: $R=0{\cdot}88$, $P<0{\cdot}02$) when the gonad stops producing estrogens (as expected from the inhibition of aromatase transcription). This co-variation may be explained by a concerted repression of both *FoxL2a* and aromatase, but is also compatible with the notion that estrogens stimulate *FoxL2a* transcription. Thus, a decrease in estrogen production due to aromatase repression or inhibition would induce *FoxL2a* down-regulation. After completion of the masculinizing treatments (d91), the expression levels of *FoxL2a* remained as low as in normal males. This is the result of the long-term transformation of the gonad into a functional testis. For *FoxL2b* there is no relevant repression detectable before d30. After completion of the treatments, expression levels remained low and similar to those in males. The masculinizing effects of androgens are probably triggered by the suppression of estrogen synthesis through inhibition of aromatase gene expression. The inhibition of aromatase following androgen treatment in females has already been reported in rainbow trout (Govoroun *et al.* 2001*b*). Here we have shown that an aromatase inhibitor can also induce a similar expression profile. These data taken together suggest that estrogens are key regulators of ovarian differentiation in fish and androgens would

interfere with ovarian differentiation by inhibiting their synthesis.

Sex determination in birds and reptiles is thought to depend on the initiation of estrogen synthesis in the undifferentiated gonad, which would inhibit male differentiation and stimulate ovarian development. In the absence of this estrogenic signal, a testis would develop. However, further analyses are required to assess if the mere activation of aromatase is the initial trigger of gonadal differentiation (Gabriel *et al.* 2001). Here we have documented a similar process in fish. From a wider perspective, it has been demonstrated that female mutant mice homozygous for the targeted disruption of two estrogen receptors exhibit some degree of morphological gonadal sex reversal (Couse *et al.* 1999). This strongly suggests that, even in mammals, the estrogen response can also lead to a perturbation of gonad differentiation. Although early *FOXL2* expression in mammals is thought to be steroid independent, it would be interesting to explore this connection at later stages of gonad development and in adults.

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