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Characterization of *Fxr1* in *Danio rerio*; a simple vertebrate model to study costamere development

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Summary

The X-linked *FMR1* gene, which is involved in the fragile X syndrome, forms a small gene family with its two autosomal homologs, *FXR1* and *FXR2*. Mouse models for the *FXR* genes have been generated and proved to be valuable in elucidating the function of these genes, particularly in adult mice. Unfortunately, *Fxr1* knockout mice die shortly after birth, necessitating an animal model that allows the study of the role of Fxr1p, the gene product of *Fxr1*, in early embryonic development. For gene function studies during early embryonic development the use of zebrafish as a model organism is highly advantageous.

In this paper the suitability of the zebrafish as a model organism to study Fxr1p function during early development is explored. As a first step, we present here the initial characterization of Fxr1p in zebrafish. Fxr1p is present in all the cells from zebrafish embryos from the 2/4-cell stage onward; however, during late development a more tissue-specific distribution is found, with the highest expression in developing muscle. In adult zebrafish, Fxr1p is localized at the myoseptum and in costamere-like granules in skeletal muscle. In the testis, Fxr1p is localized in immature spermatogenic cells and in brain tissue Fxr1p displays a predominantly nuclear staining in neurons throughout the brain. Finally, the different tissue-specific isoforms of Fxr1p are characterized.

Since the functional domains and the expression pattern of Fxr1p in zebrafish are comparable to those in higher vertebrates such as mouse and human, we conclude that the zebrafish is a highly suitable model for functional studies of Fxr1p.

Key words: zebrafish, *Danio rerio*, *Fxr1*, Fxr1p, costamere, striated muscle, fragile X syndrome.

Introduction

The small fragile X related (FXR*)* protein family consists of three proteins, FXR1P, FXR2P and FMRP, the lack of which in neurons causes the fragile X syndrome (Oostra and Willemsen, 2003; Willemsen et al., 2004). Both in human and mouse, the FXR-proteins are highly expressed in brain and testis and, uniquely, FXR1P is expressed in striated muscle tissue, including both skeletal and heart muscle (Bakker et al., 2000; De Diego Otero et al., 2000; Devys et al., 1993; Dube et al., 2000; Mientjes et al., 2004; Tamanini et al., 1997). The highly homologous FXR proteins share several functional domains, including two KH RNA binding domains and an RGG box, as well as nuclear localization and export signal sequences (Ashley et al., 1993; Eberhart et al., 1996; Fridell et al., 1996; Siomi et al., 1993b; Sittler et al., 1996). The association of both FMRP and FXR1P with mRNP particles present in actively translating ribosomes suggests that these proteins play a role in translational regulation (Dube et al., 2000; Huot et al., 2001; Laggerbauer et al., 2001; Siomi and Dreyfuss, 1997; Siomi et al., 1993a; Zalfa et al., 2003). FMRP binds to target mRNAs with high affinity and this binding appears to be mediated by G-quartet structures in target transcripts, whereas for both FXR2P and FXR1P, target transcripts have not yet been identified (Brown et al., 2001; Darnell et al., 2001; Darnell, 2004; Schaeffer et al., 2001). *In vitro* studies demonstrated the interaction of the three homologs with each other as homomers and heteromers (Zhang et al., 1995). Recently, *in vivo* studies showed the presence of both FMRP and FXR1P in specific granules involved in dendritic mRNA transport using a stably transfected PC12 cell line (neuronal cell line) with an inducible expression system (De Diego Otero et al., 2002).

Various animal models have been created to study the physiological function of the three genes. The *Fmr1* knockout mice display deficits in visual spatial performance and have macroorchidism, illustrating similarities between fragile X patients and this mouse model (Bakker et al., 1994). In addition, *Fmr1* knockout mice show altered dendritic spine morphology, indicating a reduced maturation/pruning of spines (Greenough et al., 2001). *Fxr2* knockout mice show a mild learning and behaviour phenotype (Bontekoe et al., 2002). Thus, both mouse models point to a mental retardation phenotype in the absence of Fmrp and Fxr2p, respectively. In

contrast, *Fxr1* knockout mice die shortly after birth and show a disruption of the cellular architecture and structure of both skeletal and cardiac muscle tissue (Mientjes et al., 2004). The absence of Fxr1p in E19 *Fxr1* knockout mice results in the reduced/abnormal expression pattern of costameric proteins like vinculin, dystrophin and α-actinin and it has been suggested that Fxr1p plays a role in transport/translational control of structural costameric mRNAs analogue to FMRP function for dendritic mRNAs (Mientjes et al., 2004).

In order to further study the function of FXR1P in the nervous system, testis and striated muscle tissue, particularly during embryonic development, it may be advantageous to use a model organism that allows avenues to study early developmental processes in more detail. The zebrafish *Danio rerio* is very suitable for developmental studies as it has a fast external development, and developing zebrafish remain translucent until the embryos are free-swimming and organogenesis is complete. Additionally the availability of techniques to manipulate gene expression, the vast knowledge base on zebrafish development and the near finished genome project make the zebrafish an attractive complementary vertebrate model. Importantly, orthologs of the three *FXR* genes have been identified in zebrafish (Wan et al., 2000).

In the present study an initial characterization of *Fxr1* in zebrafish has been conducted. We performed sequence analysis, embryonic and adult expression patterns using monospecific antibodies against Fxr1p, and western blotting to detect the different molecular forms of Fxr1p.

Materials and methods

Animals

Zebrafish *Danio rerio* (Hamilton 1822) used in this study were from a locally kept line that derives from the Wageningen ZF WT Zodiac F5 line. Fishes were maintained at 25°C in a 12 h:12 h light:dark cycle and fed *Artemia* 3 times a day.

Dissection of zebrafish

Male fish were euthanized in a 0.2 g l^{-1} solution of tricaine (3-amino-benzoic ethylester Sigma, St Louis, MO, USA) and brain, testis and a strip of dorsal skeletal muscle were dissected, snap frozen in liquid nitrogen and stored at –80°C till further use.

Fxr–EGFP fusion expression constructs

For total RNA, tissues were removed from –80°C and immediately homogenized in 1.0 ml trizol. The homogenate was chloroform extracted and RNA was precipitated according to standard protocols. Subsequently, cDNA was prepared from 1 μg RNA using AMV RT (Sigma, St Louis, MO, USA) with random hexamers and oligo dT according to the manufacturer's instructions.

Muscle and brain cDNA was amplified with Pfx DNA polymerase (Invitrogen, Carlsbad, CA, USA) using the following *Fxr1* primers: f1, 5′-CCGATCGCATGGAGGAACTGACGGTGG-3′ and r1, 5′-GTACTCCAGCAGCACCTGTACG-3′. The PCR product was cloned into pCRtopo 2.1 TA (Invitrogen) and, in order to express Fxr1p as an enhanced green fluorescent protein (EGFP) fusionprotein, subcloned into pEGFP-C3 (Clontech, Palo Alto, CA, USA) using the *Eco*RI sites. Zebrafish *Fxr2* cDNA was available as an image clone (GenBank accession number: BC045999), which was ordered from MRC gene service and cloned into pCRtopo 2.1 TA by polymerase chain reaction (PCR) using the primers: f3, 5′-AAGCGACGACGAACATGGAC-3′ and r4, 5′-ATGCAAGCAGGGACAGAGTT-3′, and subsequently subcloned into pEGFP-C3 (Clontech) using the *Eco*RI sites. Both constructs were sequence verified.

Primary antibodies

Rabbit monospecific antibodies against Fxr1p were raised according to the double X program from Eurogentec (Herstal, Belgium). Briefly, synthetic peptides were produced from the C-terminal amino acid sequence from zebrafish Fxr1p: AESQSRQTNPRDTRK, and subsequently coupled to keyhole limpet hemocyanin (KLH). The final bleeding was used to produce an affinity-purified antibody using affinity purification against the synthetic peptide. The affinity-purified antibody (named affi 5) was used at a 1:500 dilution for immunoblotting and 1:25 dilution for immunohistochemistry. Antibodies against MANDRA1 (mouse anti-dystrophin; Sigma) and vinculin (goat anti-vinculin; Sanvertech, Santa Cruz, CA, USA) were used for immunohistochemistry at 1:1000 and 1:400 dilution, respectively. Antibodies against P0 and Staufen were from Immunovision (human; Bereldange, Luxembourg) and Chemicon (rabbit; Temecula, USA), respectively, and were used at a 1:100 dilution for immunohistochemistry. The secondary antibodies swine anti-rabbit conjugated with HRP and rabbit anti-mouse conjugated with horseradish peroxidase (HRP) were obtained from DAKO (Glostrup, Denmark). Goat anti-rabbit/mouse/human antibodies conjugated to TRITC or FITC were obtained from Sigma.

Cryosectioning and immunohistochemistry

Adult zebrafish were euthanized (see above) and embedded in Tissue-Tek (Sakura Finetek Europe BV, Zoeterwoude, The Netherlands). Using a Leica Jung CM3000 cryostat, 7 μm sections were cut and thaw-mounted on microscopic slides. Sections were fixed at room temperature for 10 min in 4% paraformaldehyde in 0.1 mol l^{-1} Sorrensen buffer, pH 7.3, followed by a permeabilization step in 100% methanol for 20 min. Sections were rinsed twice in PBS for 5 min and subsequent endogenous peroxidase activity was blocked for those slides that were incubated using the immunoperoxidase protocol with hydrogen peroxide (0.6%). After blocking, slides were washed twice in PBS+ (PBS containing 5 g non fat dry milk and 1.5 g glycine l^{-1}) for 5 min. Incubation with primary antibodies was for 1.5 h at room temperature or overnight at 4°C. Slides were rinsed three times in PBS+ for 5 min and incubated with secondary antibodies (both conjugated with FITC/TRITC or HRP) for 1 h at room temperature. After three washes with PBS+, slides were either covered with a coverslip

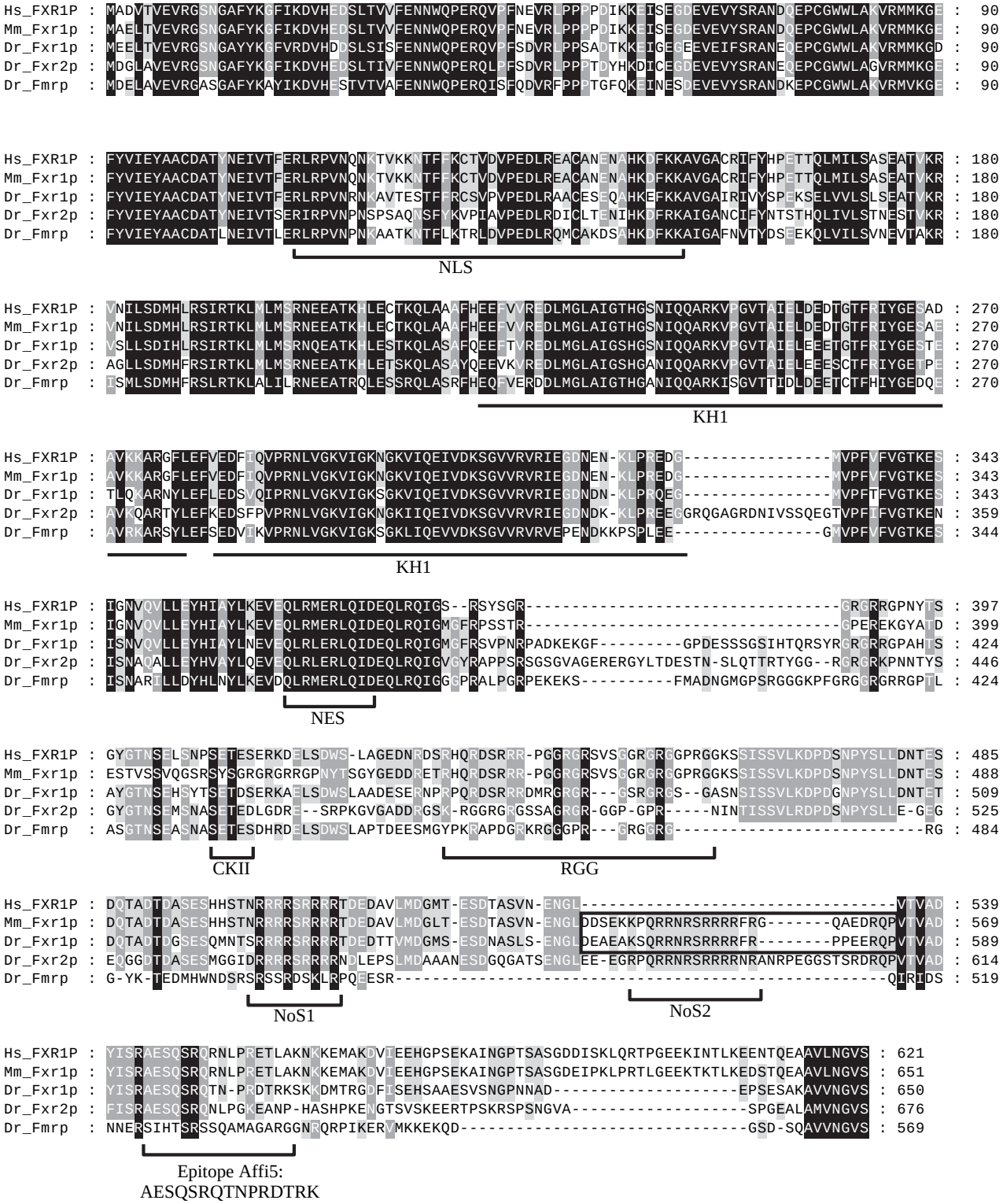

Fig. 1. Comparison of human FXR1P and the zebrafish Fxr-protein family. Identical residues are shaded in black, and conserved substitutions in grey. The following functional domains are indicated: the nuclear localization signal (NLS), the two KH1 RNA-binding domains (KH1), the nuclear export signal (NES), the conserved casein kinase II phosphorylation site found in *Drosophila* Fmrp (CKII), the region containing the RNA interaction RGG box (RGG) and two nucleolar targeting signals (NoS1 and NoS2). The boxed region of mouse and zebrafish Fxr1p that contains the second NoS indicates the alternatively spliced exon 15 of Fxr1p. The epitope of the affinity-purified anti-Fxr1p antibody Affi5 is depicted with the synthetic peptide.

using Vectashield containing Dapi (Vector Laboratories, Burlingame, USA) or further incubated with DAB-substrate (DAKO) for 6 min, followed by washing in tapwater. Finally, sections were counterstained using Haematoxylin and embedded in Entellan (Merck, Darmstadt, Germany). Slides were examined using either a fluorescence microscope or a bright field microscope.

Cell lines and transfection studies

Cos-1 cells were maintained in DMEM (Gibco Brl, Breda, The Netherlands) supplemented with 10% foetal calf serum (FCS; Gibco Brl) under 5% CO_2 at 37°C. Cells were seeded on coverslips or in 6-well plates at 75% confluence the night before transfection. Transient transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Cells were lysed the following day in Ripa (20 mmol l^{-1} Tris pH7.5, 140 mmol l^{-1} NaCl, 0.1% deoxycholate, 0.1% SDS, 0.5% Triton) and complete protease inhibitor cocktail (Roche, Basel, Switzerland) or, for immunocytochemistry, fixed in 4% paraformaldehyde in PBS for 10 min and permeabilized in 100% methanol for 20 min. For immunofluorescence the same protocol as for immunohistochemistry was followed.

Western blotting

Zebrafish tissues were homogenized in Ripa, centrifuged at 12 000 ***g*** for 15 min at 4°C and supernatants were stored at –80°C till further use. Homogenates from zebrafish brain, testis, muscle and Cos-1 cells transfected with the FXR1-pEGFP or FXR2-pEGFP constructs were size-separated by 7.5% SDS-PAGE and immunoblotted according to standard protocols.

Results

Zebrafish Fxr *genes are highly homologous*

Zebrafish FXR cDNAs have been cloned (Strausberg et al., 2002; Wan et al., 2000) and sequences are available in the public domain (NCBI accession no. NM_152963 for fmr1, BC055557 for fxr1 and BC045999 for fxr2). As shown in the protein alignment (Fig. 1), these sequences display a high degree of homology, encompassing all known functional domains. Strikingly, we were unable to identify CGG repeats within the zebrafish *Fmr1* 5′ untranslated region (5′UTR).

Cloning of zebrafish Fxr1

The PCR fragment cloned from zebrafish muscle cDNA spans the open reading frame and is identical to the published gene bank sequence, except that it also contains exon 15 (numbering according to Kirkpatrick et al., 2001; Fig. 1, boxed region).

Specificity of affinity-purified polyclonal antibody against zebrafish Fxr1p

According to the protein alignment in Fig. 1 the zebrafish Fxr proteins are highly homologous and the predicted molecular mass of both Fxr1p and Fxr2p are approximately identical. The Affi5 antibody was raised against the zebrafish Fxr1p using a synthetic peptide; however, part of the used peptide is also present in zebrafish Fxr2p. Therefore we examined whether Affi5 crossreacts with zebrafish Fxr2p. In order to determine the specificity of Affi5 we transiently overexpressed zebrafish *Fxr1*-EGFP and *Fxr2*-EGFP in Cos-1 cells and performed immunofluorescence using Affi5 antibody. In addition, cell homogenates were prepared for western blotting. For immunofluorescence, Affi5 showed a strong labelling of Cos-1 cells expressing Fxr1p-EGFP (Fig. 2A for GFP staining and 2B for Affi5 staining), whereas Cos-1 cells overexpressing Fxr2p-EGFP showed total absence

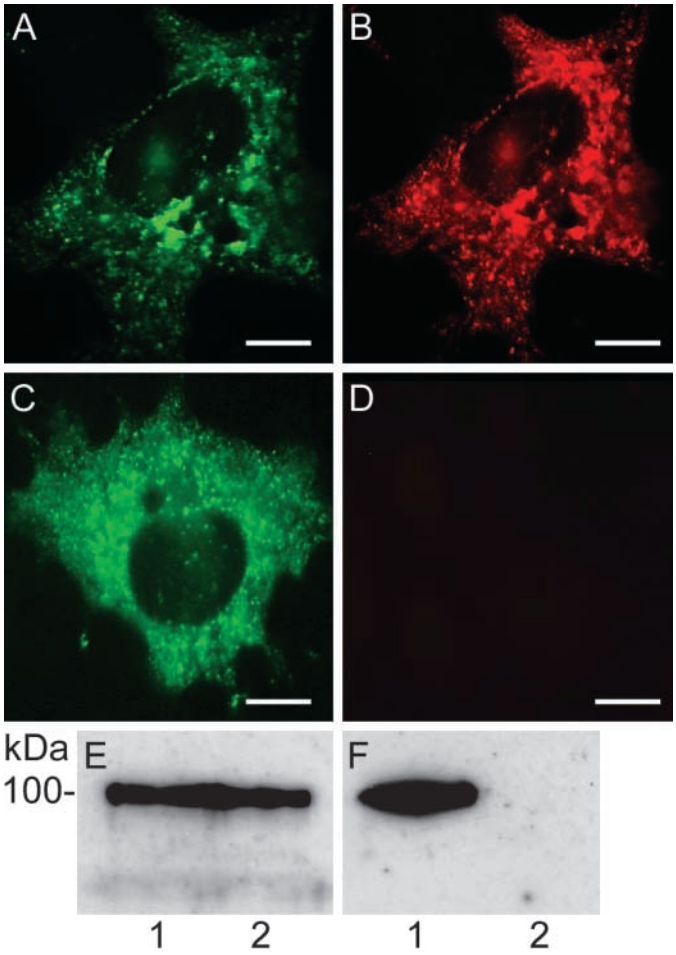

Fig. 2. Specificity of affinity-purified antibody Affi5. Cos-1 cells were transiently transfected with EGFP-Fxr1 (A,B) or with EGFP-Fxr2 (C,D), and stained with Affi5. The EGFP signal (A,C, green) is present in both transfected Cos-1 cells, while Affi5 labelling is only present in EGFP-Fxr1 transfected Cos-1 cells (B, red). Cos-1 cells transfected with EGFP-Fxr2 show absence of labelling after Affi5 incubation (D). Lysates of EGFP-Fxr1 (lanes 1) and EGFP-Fxr2 (lanes 2) transfected Cos-1 cells were immunoblotted using antibodies against EGFP (E, lanes 1 and 2) and Affi5 (F, lanes 1 and 2). Note the absence of cross reactive material in the lane with EGFP-Fxr2 transfected Cos-1 cells using Affi5 antibodies (F, lane 2). Bars, 10 μm.

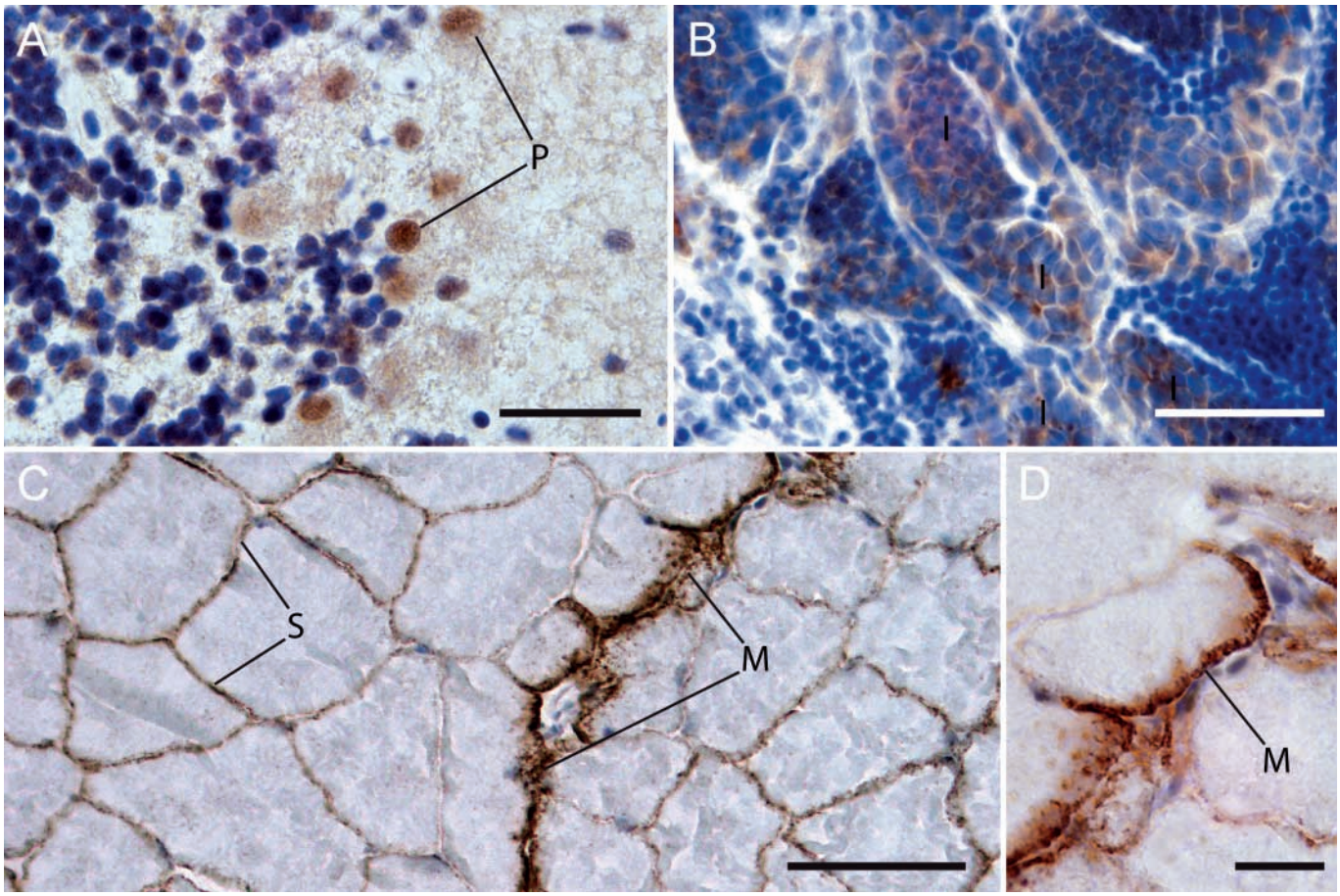

Fig. 3. Immunohistochemical analysis of Fxr1p expression in adult zebrafish tissues. Cryostat sections from adult zebrafish were immuno-incubated using Affi5 antibodies. Significant Fxr1p expression was present in the brain (A), the testis (B) and in skeletal muscle tissue (C). Note the nuclear labelling in Purkinje cells of the cerebellum (A). All immature spermatogenic cells of the testis showed Fxr1p expression (B). A transversal section of skeletal muscle shows that Fxr1p expression is mainly present at the sarcolemma and at the myoseptum (C). A higher magnification of the myoseptum is shown in (D). P, Purkinje cells; I, immature spermatogenic cells; S, sarcolemma; M, myoseptum. Scale bars, 3.5 μm (A); 30 μm (B); 80 μm (C); 320 μm (D).

of labelling for Affi5 (Fig. 2D) while an intense staining could be detected for GFP fluorescence signal (Fig. 2C).

In immunoblotting, Affi5 recognizes the Fxr1p-EGFP fusion protein. The size of the band is approximately 100 kDa, which reflects a molecular mass of 73 kDa for Fxr1p plus 27 kDa for EGFP. In contrast, Affi5 staining did not detect the Fxr2p-EGFP fusion protein (Fig. 2F, compare lanes 1 and 2). Note that equal amounts of Fxr1-EGFP and Fxr2-EGFP fusion protein were present as shown by immunoblotting using antibodies against GFP (Fig. 2E, lanes 1 and 2).

Distribution of Fxr1p in adult zebrafish

In man and in mouse Fxr1p is highly expressed in skeletal muscle, heart, testis and brain. As an indication whether Fxr1p has a role in zebrafish comparable to that in mouse or in human, and whether it would therefore be a suitable model to study the function of Fxr1p, we examined the distribution of Fxr1p in the zebrafish using Affi5 antibodies in cryostat sections from adult zebrafish.

Immunoreactivity of Affi5 was observed in the brain, most notably in the Purkinje cells of the cerebellum and a number of neurons in the brainstem. Surprisingly, a significant number of neurons throughout the brain display nuclear staining (Fig. 3A).

Although Fxr1p is highly expressed in the testis, the signal is restricted to the immature spermatogenic cells (Fig. 3B). Skeletal muscle showed the highest level of Fxr1p expression, where it was localized in granular structures throughout the muscle fiber and intensely at the sarcolemma. Furthermore, a very intense and granular staining was observed bordering the myoseptum (Fig. 3C,D). The signal of Fxr1p at the myoseptum extends somewhat into the cytoplasm, more so than at the sarcolemma.

Fxr1p in the developing zebrafish

To study the expression of Fxr1p during embryonic development and to test whether it was localized in a similar pattern as in adult tissues, zebrafish embryos at different stages

were embedded in Tissue Tek and cryosections were immunoincubated with Affi5.

Fxr1p is already detectable at the 2/4 cell stage, where it is distributed evenly over the cell mass (data not shown). At the dome/epiboly stage at 6 h.p.f. (hours post fertilization), Fxr1p is present at high levels in all the cells (Fig. 4A). From early somitogenesis onward Fxr1p is expressed at very high levels in myoblasts throughout the somites. During the maturation of the embryos (1–5 d.p.f.) the immunoreactivity gradually concentrates at the myosepta and at the sarcolemma in regularly placed granular structures. Fig. 4B illustrates the weak Fxr1p expression in the head of an embryo 1 d.p.f., whereas the tail from 1 d.p.f. embryos showed a high Fxr1p expression in myoblasts (Fig. 4C). In embryos at 3 d.p.f. Fxr1p expression level is moderate in the brain (Fig. 4D) and very high in myoblasts (Fig. 4E).

Fxr1p isoforms are differentially expressed in zebrafish tissues.

In the mouse, several different isoforms of Fxr1p have been described, due to extensive alternative splicing. We examined the presence of different isoforms of Fxr1p in zebrafish by western blot analysis using different tissues, including brain, skeletal muscle and testis. In brain, the most prominent isoform is approximately 74 kDa (Fig. 5, lane 1). In muscle tissue we could detect Fxr1p isoforms (two major bands) of approximately 80–88 kDa (Fig. 5, lane 2) and in testis the most prominent isoform was 72 kDa (Fig. 5, lane 3).

Colocalization of Fxr1p with components of the translational machinery

In mammals, Fxr1p is incorporated in mRNP particles within actively translating ribosomes (Ceman et al., 1999; Dube et al., 2000; Khandjian et al., 1998; Tamanini et al., 1999). Furthermore, it has been described that (poly)ribosomes are located at the myoseptum, and that transcripts can be translated locally (Horne and Hesketh, 1990; Ovalle, 1987). We therefore set out to determine whether P0, a protein component of ribosomes, and Staufen, which can form complexes with both FMRP and FXR1P and is known to be involved in transport/translation of mRNAs, are also localized at the myoseptum.

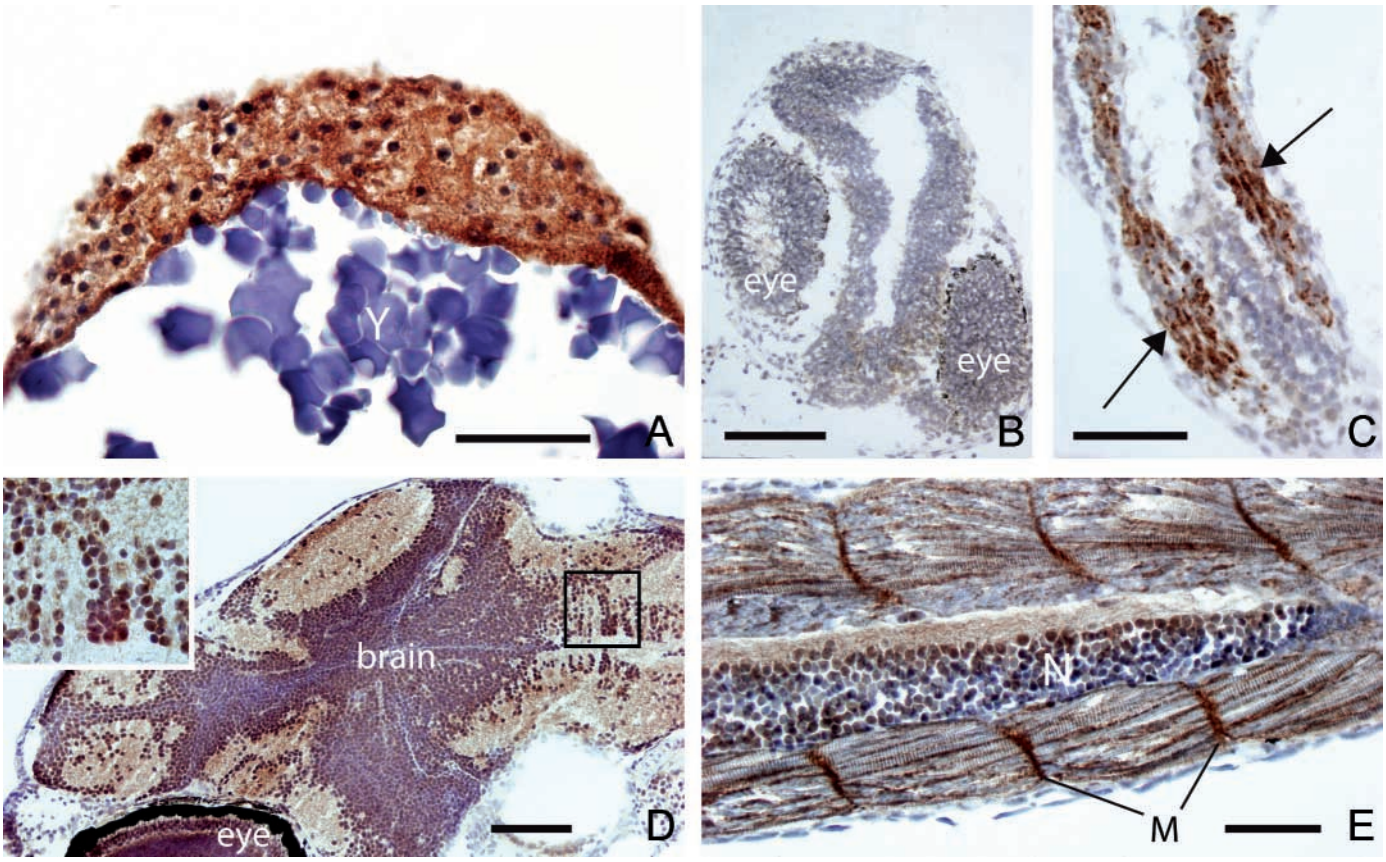

Fig. 4. Immunohistochemical analysis of Fxr1p expression in zebrafish embryos during embryonic development. (A) Longitudinal cryostat sections of 6 h.p.f. embryos, (B,C) head (B) and tail (C) of 1-day-old embryos, and (D,E) head (D) and tail (E) of 3 d.p.f. embryos stained for zebrafish Fxr1p using Affi5. Inset in D shows a higher magnification of the boxed region in the hindbrain from D. Note the nuclear staining in neurons from 3 d.p.f. embryos (D) and the very intense staining of myoblasts in muscle tissue within the somites (E). Y, yolk sac; M, myoseptum; N, neural tube. Arrows in C point to Fxr1p immunoreactive myoblasts in the developing somite. Scale bars, 15 μm (C); 30 μm (B); 35 μm (E); 70 μm (A); 100 μm (D).

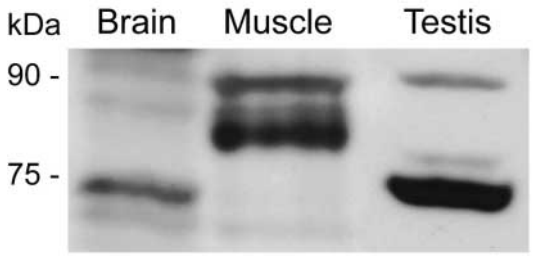

Fig. 5. Analysis of Fxr1p isoforms in adult zebrafish tissues, Homogenates of adult zebrafish brain, muscle and testis were immunoblotted using Affi5 antibodies to detect the presence of molecular forms from Fxr1p. In brain a prominent band of approximately 73 kDa and a low-intensity band of approximately 70 kDa are present. In skeletal muscle high molecular mass isoforms of Fxr1p are present of approximately 86–88 kDa. The detection of Fxr1p isoforms in testis results in a prominent band of 73 kDa and weaker bands of 77 kDa and 88 kDa.

To this end, cryosections of adult zebrafish were immunoincubated simultaneously with Affi5 in combination with anti-Staufen or anti-P0 antibodies. Both the Staufen antibody and the P0 antibody recognize the zebrafish orthologs and show a strong immunoreactivity at the myoseptum and at the sarcolemma. Staufen immunoreactivity appears to be more concentrated around junctions of fibers (Fig. 6A), whereas P0 appears to be relatively more localized at the myoseptum (Fig. 6E). Simultaneous distribution with Fxr1p (Fig. 6B,D) illustrates the colocalization with Staufen to some extent (Fig. 6C, merge) and with P0 in higher quantities (Fig. 6F, merge).

Fxr1p is localized next to dystrophin and vinculin at the myoseptum

The localization of Fxr1p at the myoseptum is reminiscent of that of dystrophin in zebrafish. Additionally, Fxr1p has been reported to be localized in costameres (Dube et al., 2000). In order to study whether Fxr1p is colocalized with vinculin and dystrophin, two proteins of the costameric protein network, and to explore a potential role of Fxr1p in the maintenance of the structural integrity of costamers, we examined the localization of Fxr1p in relation to dystrophin by double immunofluorescence in combination with confocal microscopy using cryosections from muscle tissue.

Frx1p, vinculin and dystrophin are all three localized at the myoseptum. However, this close localization at the myoseptum of Fxr1p on the one hand and vinculin and dystrophin on the other is not an exact colocalization, as shown in Fig. 7 using confocal analysis. Closer examination reveals that the signals only partly overlap. Both vinculin and dystrophin are localized more to the centre of the myoseptum than is Fxr1p.

Discussion

In the present study we performed an initial characterization of *Fxr1* expression in zebrafish. *In vitro* studies in both human cells and mouse models have revealed important data about the physiological function of Fxr1p. Nevertheless, the precise

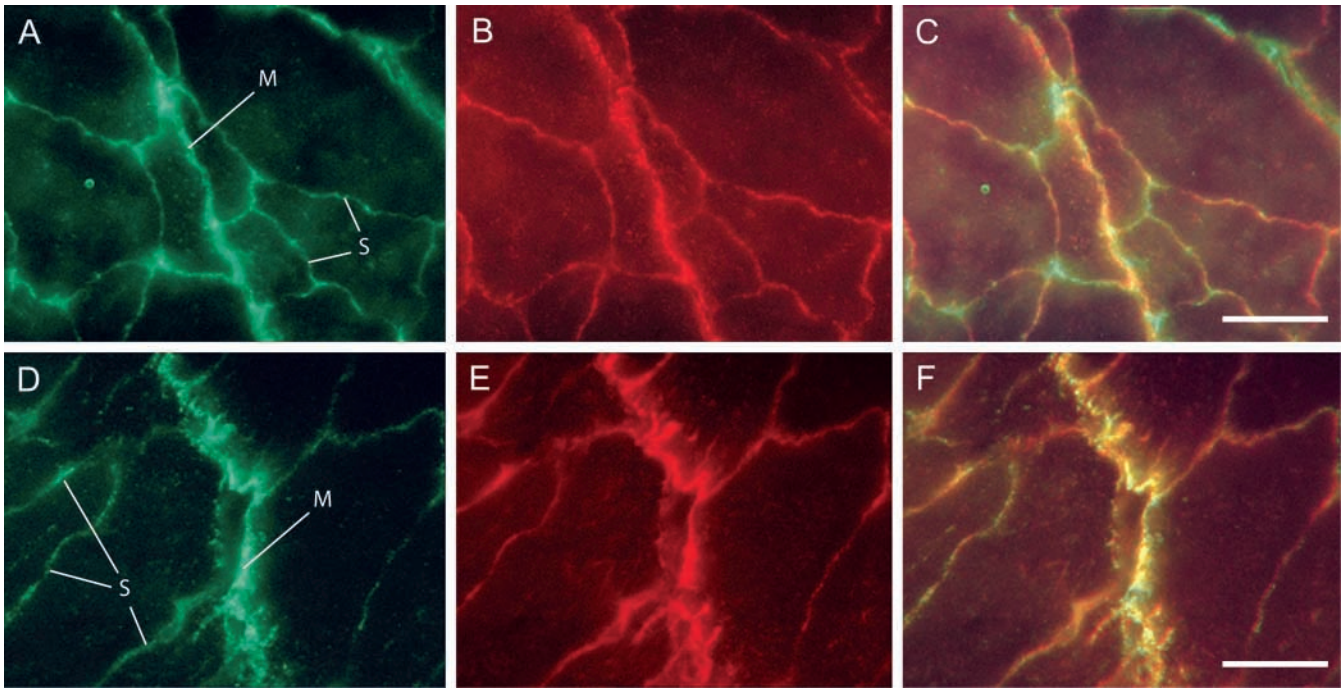

Fig. 6. Colocalization of Fxr1p with components of the translational machinery. Transverse cryostat sections of skeletal muscle tissue from adult zebrafish were stained for Fxr1p (B, red; D, green), Staufen (A, green) and P0 (E, red). (C) and (F) The merged images of A,B and D,E, respectively. Colocalization is shown by yellow staining. M, myoseptum; S, sarcolemma. Bars, 40 μm.

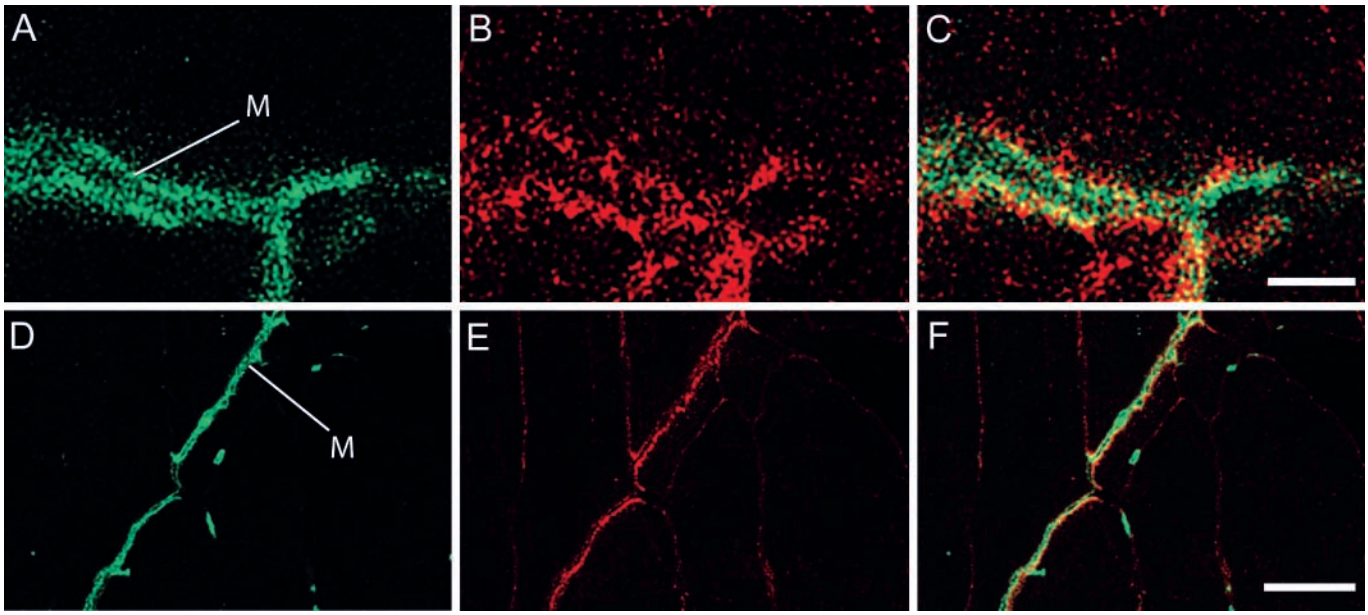

Fig. 7. Fxr1p is localized next to vinculin and dystrophin. Longitudinal cryosections of skeletal muscle tissue from adult zebrafish were stained for Fxr1p (A and D, green), vinculin (B, red) and dystrophin (E, red) and examined using confocal microscopy. Images were taken in one confocal plane. (C) and (F) show overlays of Fxr1p with vinculin (bar, 5 μm) and dystrophin (bar, 50 μm), respectively. Note that only partial overlap is observed. M, myosepta.

cellular function of Fxr1p remains unknown. The main reason for this is that *Fxr1* knockout mice die shortly after birth, suggesting an important cellular function of Fxr1p during embryonic development or neonatally. Unfortunately, the mouse is not an ideal model for the study of gene function during early embryonic development. Thus, knowledge about Fxr1p function during (early) embryonic development is essential to gather knowledge about Fxr1p function and to understand the demise of the neonatal knockout mice. The zebrafish is a widely used developmental model organism that combines the attractive features of invertebrate models on the one hand and higher model systems such as mouse, on the other (Briggs, 2002; Haffter et al., 1996). The diploid zebrafish embryos, like those of the invertebrates, have a fast external development and are produced in high numbers per breeding. These characteristics make large-scale forward genetic approaches very effective. In addition, the zebrafish has basically the same anatomical organization as man, and data on gene function obtained from zebrafish are highly applicable in the context of human diseases and development (Dodd et al., 2000; Dooley and Zon, 2000).

To examine the feasibility of the zebrafish as a model for Fxr1p function we first compared the zebrafish Fxr1p sequence with that from human and mouse. Fxr1p is highly conserved between these different species and all major domains that have been described to play a role in the function of the FXR family of proteins are present in zebrafish Fxr1p, although the alignment for the RGG-box domain in zebrafish Fxr1p was not unambiguously clear (Fig. 1). Nevertheless, the evolutionarily conserved domains in zebrafish Fxr1p suggest that Fxr1p has a cellular function in zebrafish similar to that in human and mouse.

Next we examined the expression pattern of Fxr1p in zebrafish to establish whether it is expressed predominantly in skeletal muscle, testis and brain, as in mammals. In embryos, Fxr1p is ubiquitously expressed in all cells between 0 h.p.f. and 6 h.p.f. From 1 d.p.f. onward, Fxr1p showed a more tissue-specific expression with a very high expression in myoblasts and a moderate expression level in neurons from the central nervous system. This more differential expression was also observed during late embryonic development in the mouse (De Diego Otero et al., 2000). In 3 d.p.f. embryos, Fxr1p was present in almost all the neurons of the central nervous system with a high expression in the Purkinje cells of the cerebellum, but, surprisingly, a significant number of neurons displayed a predominantly nuclear staining. Interestingly, a nuclear staining of neurons has also been reported for FXR1P in human foetal brain (18 weeks; Tamanini et al., 1997).

The labelling intensity of Fxr1p in skeletal muscle tissue from both 1 and 3 d.p.f. embryos was very high compared to the brain tissue and suggests an important role for Fxr1p in myogenesis. At this stage of development Fxr1p already showed the characteristic costamere localization. In adult zebrafish, Fxr1p expression was tissue-specific and similar to the differential expression in man and mouse, that is, high expression in brain, striated muscle tissue and testis. However, the subcellular distribution of Fxr1p in neurons from adult zebrafish was predominantly nuclear as also observed in 3 d.p.f. embryos. This contrasts with the human and mouse subcellular Fxr1p localization in neurons, which is predominantly cytoplasmic (Bakker et al., 2000; Khandjian et al., 1998). The difference in subcellular localization of Fxr1p indicates that the cellular context of adult zebrafish neurons

may share characteristics with that of human foetal neurons (Tamanini et al., 1997).

The subcellular localization of Fxr1p in striated muscle tissue was in granular structures at the sarcolemma, which appear to be costameres as the granular Fxr1p staining overlaps with that of vinculin and dystrophin, both are components of the costameric protein network (Morris and Fulton, 1994; Bassett et al., 2003; Bassett and Currie, 2003; Costa et al., 2003). This is in agreement with previous reports in mice that also described Fxr1p staining in granular structures in costameres (Dube et al., 2000; Mientjes et al., 2004). Most striking is the predominant localization of Fxr1p at the myosepta. These structures have been linked to laminar tendons and serve to transmit the force of the contracting muscle segments to the vertebral column (Gemballa and Roder, 2004; Gemballa and Vogel, 2002). This localization of Fxr1p is, however, not entirely surprising as Fxr1p is probably, like FMR1P, involved in transport and/or regulation of translation of specific mRNAs. It has been described that both at the myoseptum and next to the costameres large numbers of actively transcribing (poly)ribosomes are located (Morris and Fulton, 1994). Considering these findings it is tempting to hypothesize that Fxr1p is involved in local translation of transcripts encoding proteins that are of importance for these structures. Further exploring this notion, we determined the localization of Staufen and P0 in double-labelling experiments. Indeed, both proteins showed a colocalization with Fxr1p, albeit with different intensities. The presence of P0, a component of the 60S ribosomal precursor unit, illustrates the presence of ribosomes at the myoseptum and at the sarcolemma in zebrafish skeletal muscle. Thus, Fxr1p might be associated to (poly)ribosomes in zebrafish muscle, which is in line with a role for Fxr1p in transport and/or translation of specific mRNAs in the vicinity of costameres (Morris and Fulton, 1994).

Staufen has been reported to be present in RNP particles that also contain FMR1 and FXR1p (Ohashi et al., 2002). Recent data show that Staufen protein is localized at the neuromuscular junctions (NMJ) and may be involved in maturation and plasticity of the NMJ (Belanger et al., 2003). Although Staufen is, like Fxr1p, present both at the sarcolemma and at the myoseptum in zebrafish muscle, it has a distinctly different pattern of signal intensity, being more concentrated around junctions of fibers with the myoseptum and other muscle fibers. These concentrations of Staufen immunoreactivity could correspond to the NMJs. This suggests that, although Fxr1p and Staufen can both be present in RNP particles in the brain and may partially colocalize in zebrafish muscle, both proteins have distinct roles in zebrafish skeletal muscle tissue.

The localization of Fxr1p at the myoseptum is reminiscent of the localization of dystrophin and vinculin in zebrafish. Both proteins are components of the myoseptum and provide a connection between the extracellular matrix (ECM) and the intracellular cytoskeleton (Bassett et al., 2003; Bassett and Currie, 2003; Costa et al., 2003).

Hypothetically, Fxr1p could be involved in maintaining muscle fiber integrity by a direct binding to components of the myoseptum, such as dystrophin or vinculin. We therefore examined the possible colocalization of dystrophin and vinculin protein using confocal immunofluorescence imaging. As both vinculin and dystrophin are distinctly more centrally localized at the myoseptum than Fxr1p, it is unlikely that Fxr1p is part of the dystrophin-containing complex that anchors the muscle fiber to the ECM. However, dystrophin mRNA shows a distinct localization bordering at the myoseptum from 19 h.p.f. onward and appears to be located immediately outside the myoseptum itself, where dystrophin protein is located (Bassett et al., 2003). Comparing these findings to our confocal study on the possible colocalization of Fxr1p with dystrophin or vinculin, it appears likely that Fxr1p may colocalize with dystrophin mRNA. Further *in situ* hybridisation studies are necessary to establish this colocalization.

The Fxr1p localization in zebrafish testis appears to be predominantly in all the immature spermatogenic cells, which has also been observed in mouse and human, albeit Fxr1p immunoreactivity has also been reported in the tails of murine sperm using antibodies against high molecular isoforms of Fxr1p (Huot et al., 2001; Tamanini et al., 1997).

In conclusion, the functional domains of Fxr1p are evolutionary conserved in zebrafish and the expression pattern of zebrafish Fxr1p is consistent with the expression of the Fxr1p orthologs in mouse and man. Thus, zebrafish should be an outstanding model organism to study the cellular function of Fxr1p, particularly during embryonic development and neonatally. Gene knockdown experiments using the morpholino gene-targeting strategy and transgenic techniques using expression plasmids with *Fxr1*-EGFP especially may open new avenues that will lead to knowledge about the *in vivo* function of Fxr1p.

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