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# Characterisation of Fmrp in zebrafish: evolutionary dynamics of the *fmr1* gene

**Abstract** Fragile X syndrome is the most common inherited form of mental retardation. It is caused by the lack of the Fragile X Mental Retardation Protein (FMRP), which is encoded by the *FMR1* gene. Although *Fmr1* knockout mice display some characteristics also found in fragile X patients, it is a complex animal model to study brain abnormalities, especially during early embryonic development. Interestingly, the ortholog of the *FMR1* gene has been identified not only in mouse, but also in zebrafish (*Danio rerio*). In this study, an amino acid sequence comparison of FMRP orthologs was performed to determine the similar regions of FMRP between several species, including human, mouse, frog, fruitfly and zebrafish. Further characterisation of Fmrp has been performed in both adults and embryos of zebrafish using immunohistochemistry and western blotting with specific antibodies raised against zebrafish Fmrp. We have demonstrated a strong Fmrp expression in neurons of the brain and only a very weak expression in the testis. In brain tissue, a different distribution of the isoforms of Fmrp, compared to human and mouse brain tissue, was shown using western blot analysis. Due to the high similarity between zebrafish Fmrp and human FMRP and their similar expression pattern, the zebrafish has great potential as a complementary animal model to study the pathogenesis of the fragile X syndrome, especially during embryonic development.

**Keywords** Zebrafish · *fmr1* · Fmrp · *Danio rerio* · Fragile X syndrome

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## Introduction

With a prevalence of 1 in 4,000 males and 1 in 6,000 females, the fragile X syndrome is the most common form of inherited mental retardation in man. Main characteristics are mild to severe mental retardation, macroorchidism, autistic-like behaviour and mild facial features (De Vries 1997). Fragile X syndrome is caused by the lack of the Fragile X Mental Retardation Protein (FMRP), which is encoded by the *FMR1* gene. The most common mutation in individuals with the fragile X syndrome is an expansion of a CGG trinucleotide repeat in the 5′ untranslated region of the *FMR1* gene that exceeds 200 units (full mutation). As a consequence the promoter region of the *FMR1* gene, including the CGG repeat, is methylated and *FMR1* transcription is repressed, which leads to the absence of the protein product of the *FMR1* gene (Verkerk et al. 1991; Verheij et al. 1993).

The *FMR1* gene, together with the two autosomal homologues, *FXR1* and *FXR2*, forms a small family of fragile-X-related genes. All three proteins contain a nuclear localisation signal (NLS), two KH (hnRNP K homology) domains, a nuclear export signal (NES) and an RGG box (arginine-glycine-glycine tripeptide repeat; Ashley et al. 1993; Eberhart et al. 1996). In human, FMRP is highly expressed in neurons of the brain and early spermatogonia in the testis (Devys et al. 1993; Tamanini et al. 1997). At the subcellular level, FMRP is present in mRNP (messenger ribonucleoprotein) particles associated with actively translating ribosomes (Tamanini et al. 1996; Willemsen et al. 1996; Feng et al. 1997; De Diego Otero et al. 2002). The precise cellular function of FMRP is still unclear; however, FMRP has been proposed to play a role in the regulation of transport/translation of a subset of dendritic mRNAs containing a G-quartet (Brown et al. 2001; Darnell et al. 2001; Laggerbauer et al. 2001; Li et al. 2001; Schaeffer et al. 2001). In neurons, selective targeting to dendrites and subsequent translation of specific mRNAs plays an important role in synaptic plasticity and is essential for learning and memory processes (reviewed in Willemsen et al. 2004). Misregulation or mistrafficking of FMRP-

associated mRNAs is thought to be the underlying cause of the fragile X syndrome.

Orthologs of the human *FMR1* gene have been identified in mouse *Mus musculus*, fruitfly *Drosophila melanogaster*, frog *Xenopus laevis* and zebrafish *Danio rerio*. The overall similarity between the functional domains indicates strong conservation of the *FMR1* gene, thus, apparently its function during evolution seems to be conserved as well. In mouse, frog and zebrafish all three members of the *FXR* genes have been found. Interestingly, the fruitfly contains a single, well-conserved d*FXR* gene representing homologues of the three *FXR* gene family members in mammals (Wan et al. 2000; Zhang et al. 2001). Very recently, the transcription of the zebrafish *FXR* family members was shown to be consistent with the expression pattern in mouse and human using whole-mount in situ hybridisation (Tucker et al. 2004). In addition, Engels et al. (2004) showed tissue-specific Fxr1p expression in skeletal muscle and brain of adult zebrafish at the translational level.

In order to study the pathogenesis of the fragile X syndrome and the physiological function of FMRP, an *Fmr1* knockout mouse has been generated. This mouse displays some characteristics in common with fragile X patients, including learning deficits and enlarged testes (Bakker et al. 1994). In addition, ultrastructural studies of the brain revealed the presence of abnormal dendritic spines illustrating reduced pruning and/or maturation of spines (Greenough et al. 2001; Nimchinsky et al. 2001; Irwin et al. 2002). Notably, spine abnormalities have already been reported in fragile X patients in earlier studies using brain autopsy material (Rudelli et al. 1985; Hinton et al. 1991). Compelling evidence suggests that these spine abnormalities result in altered synaptic development and plasticity and this is the proposed basis of mental retardation in fragile X syndrome. The process of pruning spines that are not frequently activated and further maturation of spines occurs during early embryonic development and continues after birth. Thus, fragile X syndrome can be classified as a human developmental disorder. In order to study the involvement of FMRP in the processes during (very) early embryonic development a more advantageous animal model than the mouse has been considered, that is, the zebrafish.

Here, we report the characterisation of Fmrp in both adult and embryonic stages of the zebrafish, including nucleotide sequence analysis, cellular distribution and detection of tissue-specific isoforms using specific antibodies raised against zebrafish Fmrp.

## Materials and methods

### Zebrafish

A zebrafish line was obtained from the Wageningen ZF WT Zodiac F5 line. Fish were kept at 25°C in a 12-h light/dark cycle and fed artemia three times a day. Zebrafish embryos were collected from spontaneous spawning.

### Amino acid sequence alignment

The amino acid sequences of FMRP from human, mouse, fruitfly and frog were taken from the GenBank database of NCBI. The accession numbers used for the alignment are NP_002015, NP_032057, NP_611645 and AAC59683, respectively. The accession number of zebrafish Fmrp is NP_694495. For retrieval of the zebrafish amino acid sequence of Fmrp, the zebrafish Ensembl Genome Browser has been used (Ensembl translation ID: ENSDARP000000 31163).

### Zebrafish Fmrp-specific antibody

A rabbit polyclonal antibody specific against zebrafish Fmrp (758) was purchased from Eurogentec according to the double X program (Herstal, Belgium). A synthetic peptide, SKLRPQEESRQIRID, was designed from the C-terminal end of the Fmrp protein, because of the low similarity in this area between the *fxr* genes. The antibody (named 758) against Fmrp was affinity purified against the synthetic peptide and used 1:400 for immunohistochemistry and 1:4,000 for western blotting.

### Zebrafish *fxr*-EGFP constructs

Total RNA was isolated from adult brain tissue and cDNA was synthesised using random hexamers and oligo dT. Amplification of *fmr1* was performed with the following PCR primers: 1F 5′-CGCTAGAATTCAATGGACGAG-3′ and 1R 5′-TGAATTCTAGCGCTACGAAAC-3′. Both primers contain an *Eco*RI restriction site and are located at the start and stop codon, respectively. The PCR product was digested with *Eco*RI and cloned into pEGFP-C1 vector from Clontech. For *fxr1*-EGFP (-enhanced green fluorescent protein) and *fxr2*-EGFP constructs, see Engels et al. (2004). All constructs were sequence-verified.

### Cell culture and transfection

COS-1 cells were maintained in DMEM (Gibco) supplemented with 10% foetal calf bovine serum (Gibco) and kept at 37°C in a 5% $CO_2$ atmosphere. A day before transfection, cells were seeded on cover slips in a 24-well plate at low density. Transfection with *fmr1*-EGFP, *fxr1*-EGFP and *fxr2*-EGFP constructs was performed according to the manufacturers instructions using Lipofectamine 2000 (Invitrogen). Twenty-four hours after transfection, cells were fixed with 4% paraformaldehyde for 10 min at room temperature, followed by a 100% methanol step for 20 min. Cells were immunostained with rabbit primary antibody 758 (see above) and subsequently with goat anti-rabbit immunoglobulins conjugated with TRITC (Nordic, Tilburg, The Netherlands).

## Immunohistochemistry

Zebrafish were euthanised in tricaine (3-amino-benzoic ethylester; Sigma, 25 g/l), fixed with 4% paraformaldehyde, decalcified with EDTA and embedded in paraffin. Embryos were harvested at 3, 6, 12 and 24 hpf, fixed with 4% paraformaldehyde and embedded in paraffin. Sections (5 μm) were deparaffinised, rehydrated and microwave-treated according to standard protocols (Bakker et al. 2000). Briefly, endogenous peroxidase activity was inhibited by 30 min incubation in PBS-hydrogen peroxide-sodium azide solution (100 ml 0.1 M PBS +2 ml 30% $H_2O_2$ +1 ml 12.5% sodium azide). Sections were incubated with primary antibody 758 for 1.5 h at room temperature. Subsequently, a 1-h incubation with a peroxidase-conjugated secondary antibody was performed. For signal detection 3,3,di-aminobenzidine was used as a substrate (Dako). Sections were counterstained with hematoxylin and mounted with Entellan (Merck).

## Western blot analysis

Zebrafish tissues (brain, testes and skeletal muscle) and transfected COS cells were homogenized in Hepes buffer (10 mM Hepes, 300 mM KCl, 5 mM $MgCl_2$, 0.45% Triton, 0.05% Tween, pH 7.6) containing Complete protease inhibitor cocktail (Roche), sonicated and centrifuged for 10 min at 10,000 rpm and 4°C. Protein concentrations were determined and equal amounts of protein were applied per lane. Proteins were separated on an 8% SDS-PAGE gel and were electroblotted onto nitrocellulose membrane (Schleicher & Schuell). Immunodetection was carried out using the zebrafish Fmrp-specific antibody 758. The secondary antibody (goat anti-rabbit Igs; 1:5,000) conjugated with peroxidase allowed detection with the chemiluminescence method (ECL Kit, Amersham).

# Results

## High similarity between orthologs of FMRP

The amino acid sequence alignment of FMRP from human, mouse, frog, zebrafish and fruitfly revealed high conservation at the N-terminal and showed lesser similarity at the C-terminal of the protein (Fig. 1). Overall identities between human-zebrafish, mouse-zebrafish, frog-zebrafish and fruitfly-zebrafish Fmrp are 74%, 70%, 72% and 38%, respectively. All proteins contained the important functional domains, including the nuclear localisation signal (NLS), two KH domains, the nuclear export signal (NES) and an RGG box. The 63-amino-acid region directly after the second KH domain that corresponds to exons 11 and 12 in human was missing in frog, fruitfly and zebrafish. Considering the mRNA no CGG repeats were found in the 5′ UTR sequences of the zebrafish *fmr1* gene, which is similar to frog and fruitfly.

## Specificity of the polyclonal antibody against zebrafish Fmrp

The antibody 758 against zebrafish Fmrp has been developed against the C-terminal part of the protein, since similarity to the *fxr* proteins is low in that part of Fmrp (Fig. 1). To test the specificity of antibody 758, COS cells were transfected with *fmr1*-EGFP, *fxr1*-EGFP and *fxr2*-EGFP constructs and analysed by immunofluorescence microscopy using antibody 758. Using the *fmr1*-EGFP construct, a strong overlapping signal was observed for both the GFP fluorescence and staining with antibody 758 (Fig. 2a, b, respectively). On the other hand, in both *fxr1*-EGFP (Fig. 2c, d) and *fxr2*-EGFP (Fig. 2e, f) transfected cells only a clear GFP signal could be detected (Fig. 2c, e, respectively), whereas staining with 758 antibody showed a total lack of fluorescence signal. This demonstrates the absence of cross-reactivity of antibody 758 with Fxr1p-EGFP and Fxr2p-EGFP fusion protein (Fig. 2d, f, respectively).

Western blot analysis of the *fmr1*-EGFP transfected COS cell homogenate shows a band of approximately 100 kDa, that corresponds to the expected size of the Fmrp-EGFP fusion protein (Fig. 2g, lane 1; 27 kDa for EGFP and 73 kDa for Fmrp). In cell homogenates from COS cells overexpressing either Fxr1p-EGFP or Fxr2p-EGFP fusion proteins no cross-reactivity with antibody 758 could be detected (Fig. 2g, lanes 2 and 3, respectively).

## Localisation of Fmrp in adult and embryonic tissues of zebrafish

The expression pattern of Fmrp in adults and embryos of zebrafish was analysed by immunohistochemistry on paraffin sections using antibody 758. At 3 h post-fertilization (hpf), Fmrp was ubiquitously expressed throughout the embryo (Fig. 3a). Note the nuclear staining in the 3-hpf embryos (Fig. 3b, arrows). This ubiquitous expression continued in the embryos at the 6-, 12- and 24-hpf stages (Fig. 3c–d, e–f, g, respectively). However, in 72-hpf embryos the Fmrp expression became more tissue-specific, that is, restricted to neurons from the brain (Fig. 3h) and spinal cord (Fig. 3i), and from 48 hpf onwards no expression in skeletal muscle could be observed (for 72 hpf; Fig. 3i, h).

A comprehensive analysis of the different tissues of the adult zebrafish revealed that Fmrp expression was present in all the neurons of the brain, including telencephalon, diencephalon, metencephalon and spinal cord. An example of high Fmrp expression in the Purkinje cells of the cerebellum is shown in Fig. 4a and b. In sections of the testes, only a very weak signal could be observed in immature spermatogenic cells, including spermatogonia and early spermatocytes (Fig. 4c). No labelling was observed in skeletal muscle (Fig. 4d).
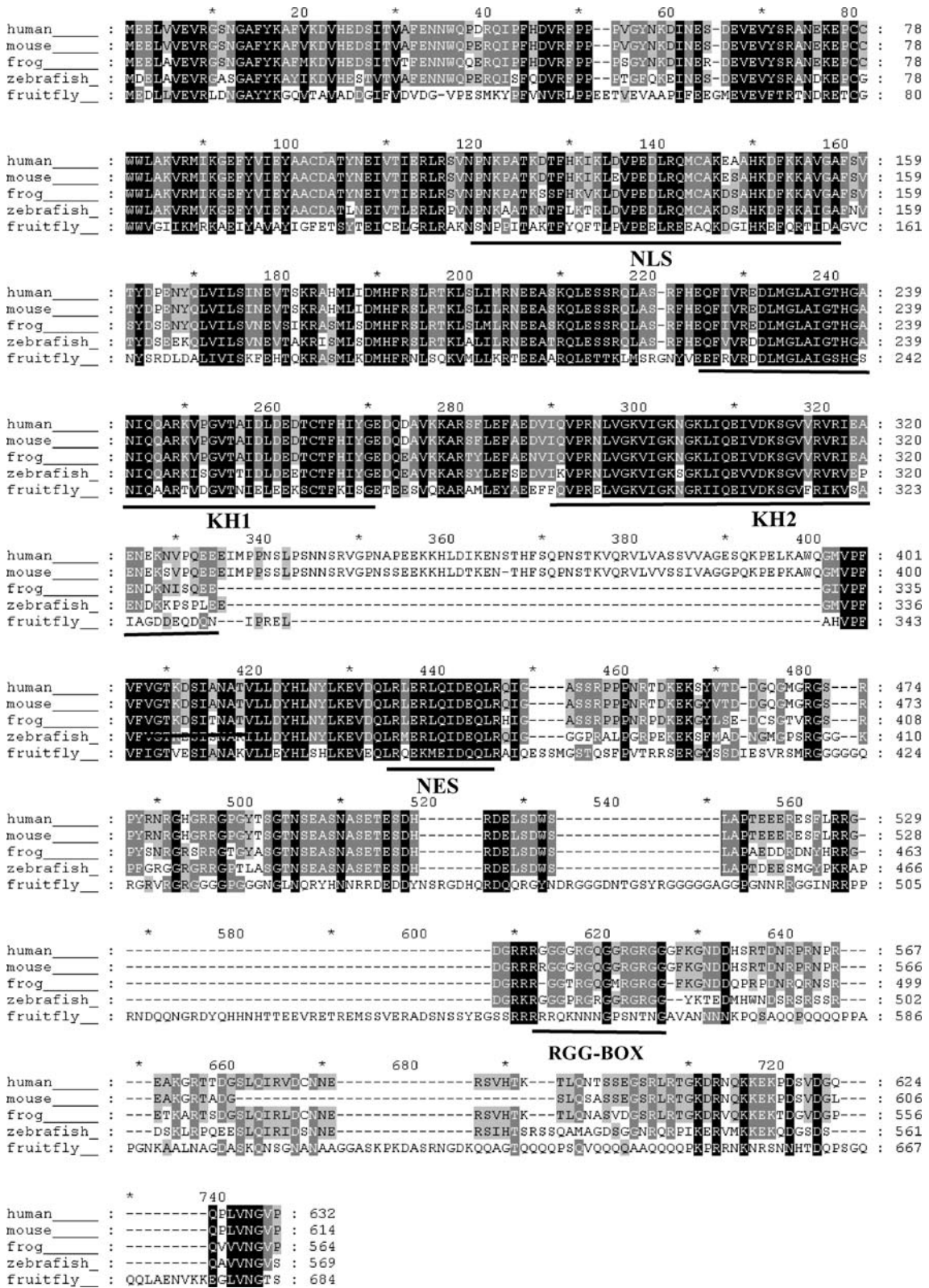
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                  *        20        *        40        *        60        *        80
human_____ : MEELVVEVRGSNGAFYKAFVKDVHEDSITVAFENNWQPDRQIPFHDVRFPP--PVGYNKDINES-DEVEVYSRANEKEPCC :  78
mouse_____ : MEELVVEVRGSNGAFYKAFVKDVHEDSITVAFENNWQPERQIPFHDVRFPP--PVGYNKDINES-DEVEVYSRANEKEPCC :  78
frog_____ : MEELVVEVRGSNGAFYKAFVKDVHEDSITVTFENNWQPERQIPFHDVRFPP--PSGYNKDINER-DEVEVYSRANEKEPCC :  78
zebrafish_ : MDELAVEVRGASGAFYKAYIKDVHESTVTVAFENNWQPERQSFQDVREPP--ETGEQKEINES-DEVEVYSRANDKEPCG :  78
fruitfly__ : MEDLVVEVRLNGAYYKGQVTAMADDGIFVDVDG-VPESMKYPRVNVRLPPEETVEVAAPIFEEGMEVEVFTRTNDRETCG :  80

                  *       100        *       120        *       140        *       160
human_____ : WWLAKVRMIKGEFYVIEYAACDATYNEIVTIERLRSVNPNKPATKDTFHRIKLDVPEDLRQMCAKEAAHKDFKKAVGAFSV : 159
mouse_____ : WWLAKVRMIKGEFYVIEYAACDATYNEIVTIERLRSVNPNKPATKDTFHKILEVPEDLRQMCAKESAHKDFKKAVGAFSV : 159
frog_____ : WWLAKVRMIKGEFYVIEYAACDATYNEIVTIERLRSVNPNKPATRSSFHKVKLDVPEDLRQMCAKDSAHKDFKKAVGAFSV : 159
zebrafish_ : WWLAKVRMVKGEFYVIEYAACDATLNEIVTLERLRPVNPNKAATNTFLTRLDVPEDLRQMCAKDSAHKDFKKAIGAFNV : 159
fruitfly__ : WWVGIIKMRKABIYAVAYIGFETSYTEICELGRLRAKNSNPEITAKTEYQFTLPVPEELREEAQKDGIHKEBQRTIDAGVC : 161
                                                                  NLS

                  *       180        *       200        *       220        *       240
human_____ : TYDPENYQLVILSINEVTSKRAHMLIDMHFRSLRTKLSLIMRNEEASKQLESSRQLAS-RFHEQFIVREDLMGLAIGTHGA : 239
mouse_____ : TYDPENYQLVILSINEVTSKRAHMLIDMHFRSLRTKLSLILRNEEASKQLESSRQLAS-RFHEQFIVREDLMGLAIGTHGA : 239
frog_____ : SYDSENYQLVILSVNEVSIKRASMLSDMHFRSLRTKLSLMLRNEEASKQLESSRQLAS-RFHEQFIVREDLMGLAIGTHGA : 239
zebrafish_ : TYDSEEKQLVILSVNEVTAKRISMLSDMHFRSLRTKLALILRNEEATRQLESSRQLAS-RFHEQFVVRDDLMGLAIGTHGA : 239
fruitfly__ : NMSRDLDALIVISKFEHTQKRASMLKDMHFRNLSQKVMLLKRTEEAARQLETTKLMSRGNYVEEFRVRDDLMGLAIGSHGS : 242

                  *       260        *       280        *       300        *       320
human_____ : NIQQARKVPGVTAIDLDEDTCTFHIYGEDQDAVKKARSFLEFAEDVIQVPRNLVGKVIGKNGKLIQEIVDKSGVVRVRIEA : 320
mouse_____ : NIQQARKVPGVTAIDLDEDTCTFHIYGEDQDAVKKARSFLEFAEDVIQVPRNLVGKVIGKNGKLIQEIVDKSGVVRVRIEA : 320
frog_____ : NIQQARKVPGVTAIDLDEDTCTFHIYGEDQEAVKKARTYLEFAENVIQVPRNLVGKVIGKNGKLIQEIVDKSGVVRVRIEA : 320
zebrafish_ : NIQQARKISGVTTIDLDEETCTFHIYGEDQEAVRKARSYLEFSEDVIKVPRNLVGKVIGKSGKLIQEVVDKSGVVRVRVEP : 320
fruitfly__ : NIQQAARTVDGVTNIELEBKSCTBKISGBTEESVQRARAMLEYABEFFQVPRELVGKVIGKNGRIIQEIVDKSGVFRIKVSA : 323
                   KH1                                               KH2

                  *       340        *       360        *       380        *       400
human_____ : ENEKNVPQEBEIMPPNSLPSNNSRVGPNAPEEKKHLDIKENSTHFSQPNSTKVQRVLVASSVVAGESQKPELKAWQGMVPF : 401
mouse_____ : ENEKSVPQEBEIMPPSSLPSNNSRVGPNSSEEKKHLDTKEN-THFSQPNSTKVQRVLVVSSIVAGGPQKPEPKAWQGMVPF : 400
frog_____ : ENDKNISQEB--------------------------------------------------------------GIVPF : 335
zebrafish_ : ENDKKPSPLEE-------------------------------------------------------------GMVPF : 336
fruitfly__ : IAGDBEQDQN---IPREL---------------------------------------------------------AHVPF : 343

                  *       420        *       440        *       460        *       480
human_____ : VFVGTKDSIANATVLLDYHLNYLKEVDQLRLERLQIDEQLRQIG----ASSRPPENRTDKEKSYVTD-DGQGMGRGS---R : 474
mouse_____ : VFVGTKDSIANATVLLDYHLNYLKEVDQLRLERLQIDEQLRQIG----ASSRPPENRTDKEKGYVTD-DGQGMGRGS---R : 473
frog_____ : VFVGTKDSITNATVLLDYHLNYLKEVDQLRLERLQIDEQLRHIG----ASSRPPNRPDKEKGYLSE-DCSGTVRGS---R : 408
zebrafish_ : VPVGTKGISNATVILLDYHLNYLKEVDQLRMERLQIDEQLRAIG----GGPRALEGRPEKEKSFMAD-NGMGPSRGG--K : 410
fruitfly__ : VFIGTVESIANAKVLLEYHLSHLKEVEVDQLRAIQESSMGSTQSFEVTRRSERGYSSDIESVRSMRGGGGGQ : 424
                                              NES

                  *       500        *       520        *       540        *       560
human_____ : PYRNRGHGRRGPGYTSGTNSEASNASETESDH-------RDELSDWS--------------------LAPTEEERESELRRG- : 529
mouse_____ : PYRNRGHGRRGPGYTSGTNSEASNASETESDH-------RDELSDWS--------------------LAPTEEERESELRRG- : 528
frog_____ : PYSNSRGRRGTCYASGTNSEASNASETESDH-------RDELSDWS--------------------LAPAEDDRDN-HRRG- : 463
zebrafish_ : PEGRGGRGRRGPTLASGTNSEASNASETESDH-------RDELSDWS--------------------LAPTDEESMGLPKRAP : 466
fruitfly__ : RGRVRGRCGGGPGGGNGLNQRYHNNRRDEDDYNSRGDHQRDQQRGYNDRGGGDNTGSYRGGGGGAGGEGNNRRGGINRRPP : 505

                  *       580        *       600        *       620        *       640
human_____ : ----------------------------------DGRRRGGGGRGQGRGRGGGFKGNDDHSRTDNRPRNPR--- : 567
mouse_____ : ----------------------------------DGRRRRGGGRGQGRGRGGGFKGNDDHSRTDNRPRNPR--- : 566
frog_____ : ----------------------------------DGRGQGMRGRGG-FKGNDLDQPRPDNRQRNSR--- : 499
zebrafish_ : ----------------------------------DGRKRGGGPRGRGGGRGG--YKTEDMHWNLSRSRSSR--- : 502
fruitfly__ : RNDQQNGRDYQHHNHTTEEVRETREMSSVERADSNSSYEGSSRRRRRQKNNNGPSNTNGAVANNNNKPQSAQQPQQQQPPA : 586
                                             RGG-BOX

                  *       660        *       680        *       720
human_____ : ---EAKGRTTDGSLQIRVDCNNE---------------RSVHTK---TLQNTSSEGSRLRTGKDRNQKKEKPDSVDGQ--- : 624
mouse_____ : ---EAKGRTADG----------------------------------SLGSASSEGSRLRTGKDRNQKKEKPDSVDGL--- : 606
frog_____ : ---ETKARTSDGSLQIRLDCNNE---------------RSVHTK---TLQNASVDGSRLRTGKDRVQKKEKTDGVDGP--- : 556
zebrafish_ : ---DSKLRPQEESLQIRIDSNNE---------------RSIHASRSSQAMAGDSGNRQRPIKERVMKKEKQDGSES---- : 561
fruitfly__ : PGNKAALNAGDASKQNSGNANAAGGASKPKDASRNGDKQQAGTQQQQPSCVQQQQAAQQQQPKPRRNKNRSNNHTDQPSGQ : 667

                  *       740
human_____ : ---------QPLVNGVP : 632
mouse_____ : ---------QPLVNGVP : 614
frog_____ : ---------QVVNGVP : 564
zebrafish_ : ---------QAVVNGVS : 569
fruitfly__ : QQLAENVKKEGLVNGTS : 684
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**Fig. 1** Amino acid sequence alignment of *Fragile X Mental Retardation 1* (*FMR1*) proteins (*FMRP*) of several species. Identical residues are shaded in *black* and conserved substitutions in *grey*. The alignment shows highly homologous regions of Fmrp between human, mouse, frog, fruitfly and zebrafish. All orthologs contain the nuclear localisation signal (*NLS*), two KH domains, nuclear export signal (*NES*) and an RGG box
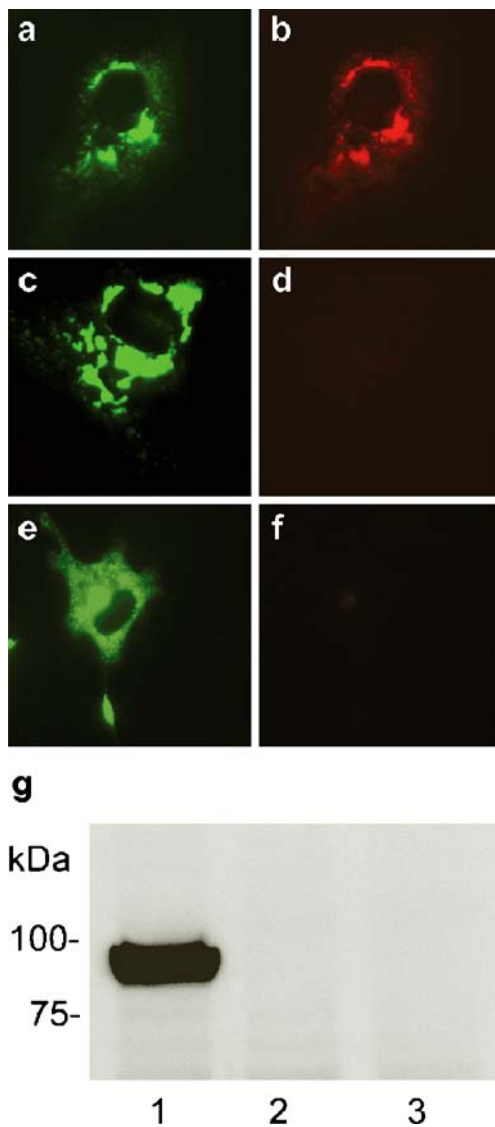
**Fig. 2** Specificity of antibody 758 against zebrafish Fmrp. Green fluorescent protein (GFP) signal in COS cells that were transfected with *fmr1*-enhanced GFP (**a**), *fxr1*-EGFP (**c**) and *fxr2*-EGFP constructs (**e**). Cells were also stained immunocytochemically using antibody 758 against zebrafish Fmrp. Specific labelling by antibody 758 is only observed in the *fmr1*-EGFP transfected cells (**b**). The absence of 758-labelling in both the *fxr1*-EGFP and *fxr2*-EGFP transfected cells indicates lack of cross-reaction of antibody 758 with either Fxr1p or Fxr2p (**d**, **f**). **g** Western blot analysis of homogenates of the *fmr1*-EGFP, *fxr1*-EGFP and *fxr2*-EGFP overexpressing COS cells (*lanes 1, 2* and *3*, respectively), only showing a band in lane 1, confirming these results

Detection of isoforms in adult zebrafish tissues

Immunoblot analysis of total protein of brain, skeletal muscle and testes from adult zebrafish has been performed using the antibody 758. For brain, a prominent and a much weaker band were observed of approximately 75 and 71 kDa, respectively. For skeletal muscle and testes, no Fmrp isoforms could be detected (Fig. 5).

## Discussion

After identification of the *FMR1* gene as the gene involved in fragile X syndrome, fundamental research focussed on the cellular function of the gene product, FMRP. A logical tool in these studies was the use of a mammalian model system, including the generation of an animal model representing all aspects of the human disease. Thus far the mouse is often the animal of choice because genetic modification of the genome of the mouse is a rather established technology. In addition, the availability of many different behavioural tests to study cognitive functions makes the mouse a valid animal model for further studies to unravel the pathogenesis of the fragile X syndrome. Indeed, the fragile X knockout mice show both learning deficits and macroorchidism, illustrating similarities between fragile X patients and this mouse model (Bakker et al. 1994). Interestingly, morphological spine abnormalities and delayed maturation of spines were already present directly after birth in *Fmr1* knockout mice (Greenough et al. 2001; Nimchinsky et al. 2001). We looked for an alternative animal model system that would allow easy access to embryos and we propose here the zebrafish (*Danio rerio*) as a new complementary animal model system to study Fmrp function during (early) embryonic development. Importantly, zebrafish possess all three *fxr* genes showing very similar amino acid sequence patterns compared with humans and mice. Here we report the initial characterization of zebrafish Fmrp to establish it as a model for functional studies.

The presence of the same functional domains within FMRP between the different orthologs indicates preservation of its function. Comparison of the *FMR1* amino acid sequences of human, mouse, frog and fruitfly revealed strong similarity, especially at the N-terminal of the protein. This confirms the high degree of evolutionary conservation of the *FMR1* gene (Pieretti et al. 1991; Deelen et al. 1994).

We raised a specific antibody (758) against zebrafish Fmrp to study the expression pattern and presence of the different molecular isoforms in several tissues of both adults and embryos of zebrafish. The specificity of the antibody has been demonstrated in transfection experiments with constructs containing the different *fxr* gene using COS cells. No cross-reactivity was found with its homologs, Fxr1p and Fxr2p. Western blotting analysis confirmed this specificity for Fmrp. Immunohistochemical studies with antibody 758 showed that in early stages of embryonic development Fmrp is ubiquitously expressed in all cells using paraffin sections of zebrafish embryos. However, during the very early period of embryonic development (3 hpf) Fmrp labelling was not only observed in the cytoplasm, but also present within the nucleus where it showed a random distribution. Thus far, a nuclear distribution of FMRP has only been demonstrated in transfected COS cells and in some neurons of adult murine brain (Willemsen et al. 1996; Bakker et al. 2000). In addition, small amounts of FMRP were present in the nucleus after leptomycin B treatment of transfected COS cells (Tamanini et al. 1999). It has been suggested that FMRP shuttles

**Fig. 3** Immunohistochemistry on zebrafish embryos using antibody 758. In 3 h post-fertilisation (hpf; **a**, **b**), 6 hpf (**c**, **d**), 12 hpf (**e**, **f**) and 24 hpf (**g**) embryos, Fmrp was ubiquitously expressed throughout the embryo. Note the nuclear staining in cells of the 3-hpf embryo (**b**, *arrows*). In 72-hpf embryos (**h**, **i**) Fmrp expression was restricted to neurons throughout the brain and spinal cord (*s*); however, skeletal muscle (*m*) was totally devoid of Fmrp. A dorsal view of the brain of a 72-hpf embryo is shown in **h**; the *inset* shows a higher magnification of the indicated region of the brain (*d* diencephalon)
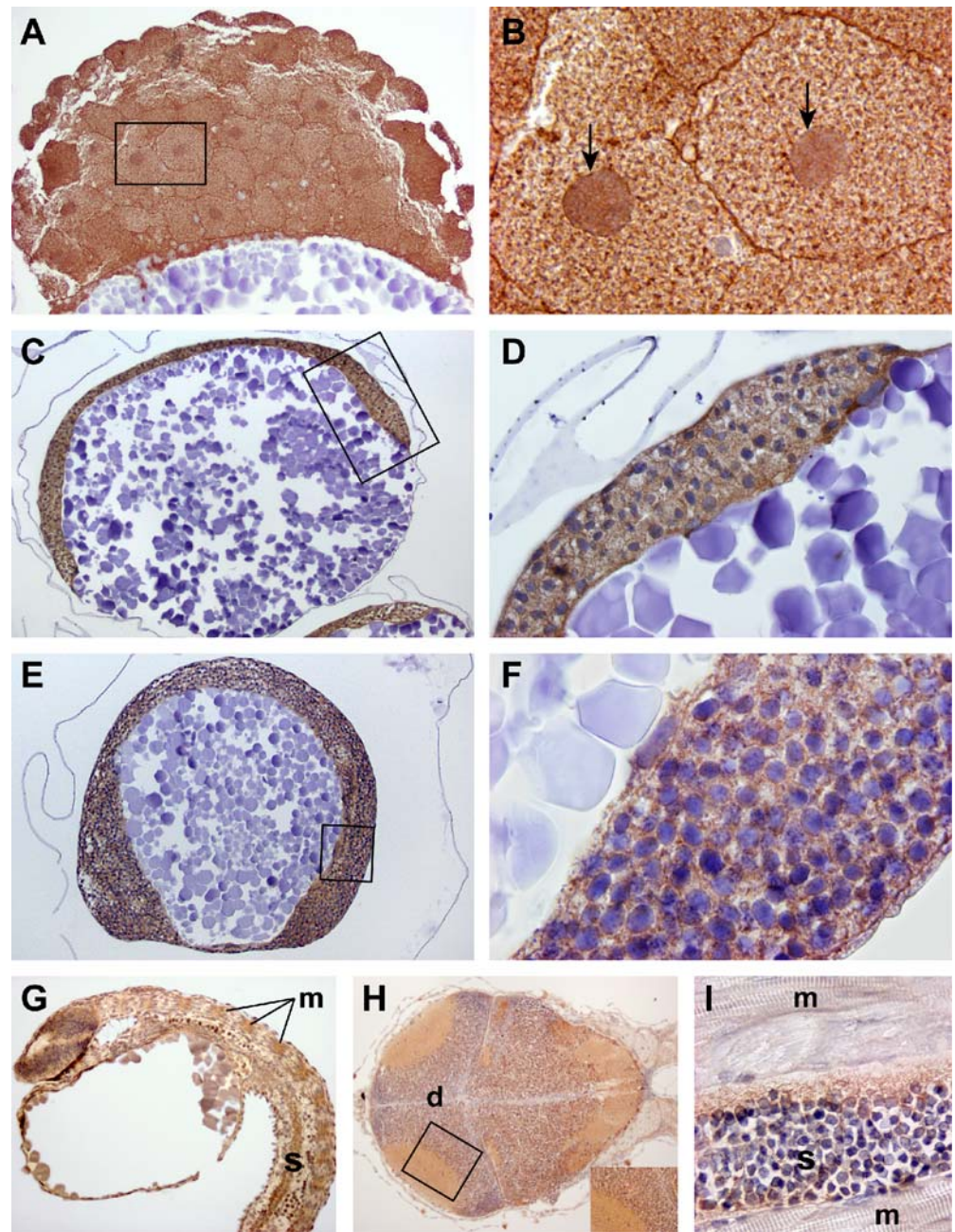


between the cytoplasm and the nucleus in a regulated manner using a nuclear localisation signal (NLS) and nuclear export signal (NES), two functional domains present within the protein (Eberhart et al. 1996). Apparently, this specific function of Fmrp is more prominent during this early period of embryonic development. After 72 h of development, Fmrp expression is restricted to neurons in the brain and spinal cord, illustrating a change from an ubiquitous expression to a more tissue-specific expression pattern at this developmental stage. This phenomenon has been found during embryonic development of the mouse as well (De Diego Otero et al. 2000). In a recent study Tucker et al. (2004) described the expression of the three zebrafish orthologs of the *FXR* family using whole-mount in situ hybridisation. During early embryonic development

(0–12 hpf) *fmr1* transcripts were distributed ubiquitously with a higher expression in the anterior of the embryo. At 18 hpf *fmr1* expression was at a low level, whereas at 24 hpf a high *fmr1* expression was present in the brain. Although our present study focussed on expression of Fmrp at the protein level and not on *fmr1* transcripts the results of both studies are consistent. We could not demonstrate a higher Fmrp expression in the anterior of the embryo at 12 hpf; however, this can be explained by differences in half-life between *fmr1* mRNAs and Fmrp. Furthermore, the presence of high quantities of mRNAs does not necessarily implicate the (immediate) translation of these mRNAs in proteins. We propose that during early embryonic development *fmr1* mRNAs, of presumably maternal origin, are responsible for the ubiquitous expression of Fmrp and that

**Fig. 4** Immunohistochemistry on adult tissues of zebrafish using antibody 758. In adult zebrafish tissues, high Fmrp expression could be detected in all the neurons throughout the brain. Here we show, as an example, Fmrp expression in Purkinje cells of the cerebellum (*arrows*). In testes, a very weak labelling is observed in immature sperm cells (**c**; *arrows*; *s* mature sperm cells). In skeletal muscle, no Fmrp signal could be detected (**d**; *m* skeletal muscle)
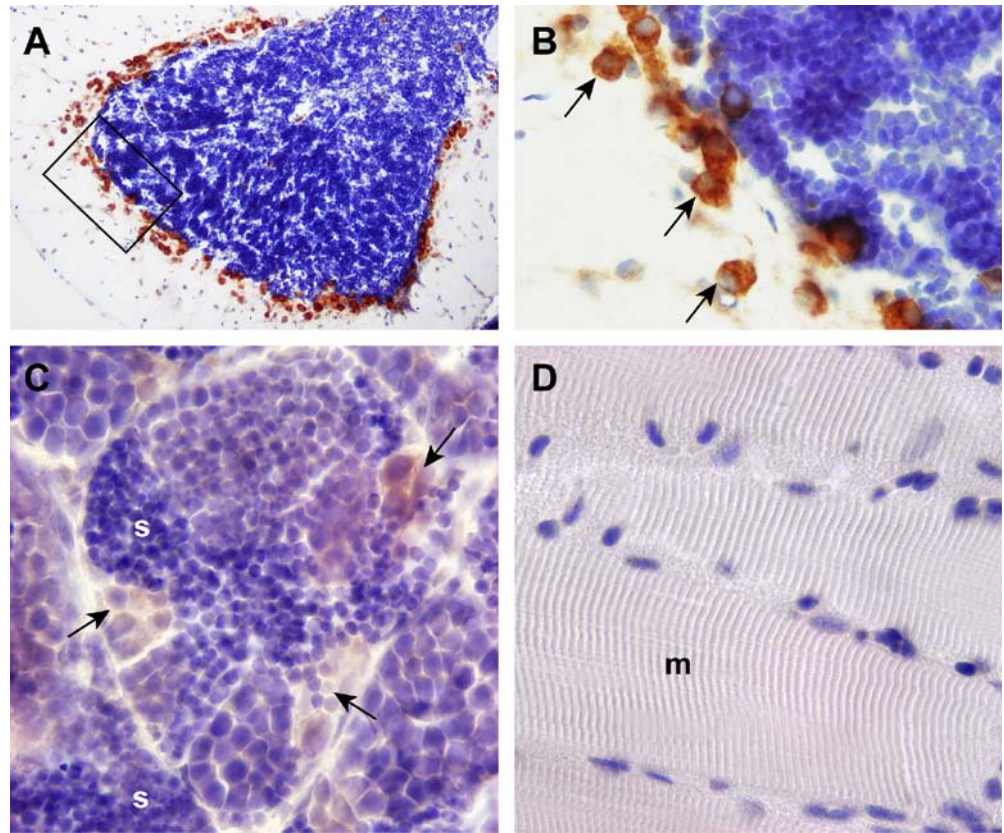
the tissue-specific expression in later stages and adult zebrafish is the result of tissue-specific transcription.

Although FMRP is widely expressed, the localization of high quantities of FMRP has been restricted to most neurons of human and mouse adult brain and spermatogonia within the testes (Devys et al. 1993; Tamanini et al. 1997; Bakker et al. 2000). In adult zebrafish tissues, Fmrp appeared to be primarily expressed in brain. Labelling was found in all differentiated neurons of the brain, including motoneurons, neurons of the telencephalon and Purkinje cells of the cerebellum. The Fmrp expression in brain is corresponding to the FMRP expression in human and mouse. In contrast, the extremely low Fmrp expression in immature spermatogenic cells in the testes of zebrafish and total lack of Fmrp in western blot studies using testes homogenates from zebrafish differs from human and mouse. This suggests a less important Fmrp function within this tissue; however, we cannot exclude



**Fig. 5** Western blot analysis of different adult zebrafish tissues. In brain homogenates a very prominent isoform of 75 kDa is present using antibody 758. In addition, a very weak band of 71 kDa could be detected. In skeletal muscle and testes, no isoforms could be detected

that testes-specific isoforms from zebrafish are not well recognized by the zebrafish-specific antibodies used in our immunohistochemical experiments.

In human and mouse brain four prominent isoforms are present with molecular weights ranging between 70 and 80 kDa (Verheij et al. 1993; Bakker et al. 2000). In western blot analysis, antibody 758 revealed two bands in zebrafish brain; a prominent isoform of approximately 75 kDa and a much weaker isoform of approximately 70 kDa. This indicates alternative isoforms to be present in brain or a differential distribution of these two isoforms compared to the four isoforms present in human and mouse brain. Apparently the importance of specific isoforms, mediated by different splicing events, has changed during evolution. No specific role for individual isoforms has been identified.

The *FMR1* gene, together with *FXR1* and *FXR2*, forms a small gene family of highly homologous genes that can interact with each other. In mouse and man the three gene products, FMRP, FXR1P and FXR2P, are closely related with respect to subcellular localization and functional domains; however, FXR1P is highly expressed in striated muscle tissue whereas FMRP and FXR2P are absent. In a recent study we have described the initial characterisation of Fxr1p in zebrafish (Engels et al. 2004). As Fmrp, Fxr1p was expressed in both the neurons of the brain and immature spermatogenic cells. In addition, Fxr1p expression was very high in striated muscle tissue from zebrafish, both in embryonic and adult stages, where it was localized in the costameric protein network. Apparently, Fxr1p has a unique function in myogenesis compared to
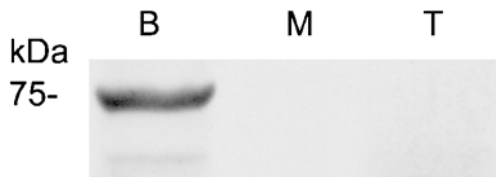
the other two members of the small *FXR* family. This is further illustrated by the early demise of *Fxr1* knockout mice (shortly after birth), as a consequence of striated muscle abnormalities, and the normal life expectancy of both the *Fmr1* and *Fxr2* knockout mice (Mientjes et al. 2004).

In conclusion, the strong conservation of important functional domains and an almost similar localisation pattern of Fmrp (especially in brain) when compared with humans and mice makes the zebrafish a suitable model for studying *fmr1* protein function. Recent developments in manipulation of gene expression in zebrafish, such as (over)expression vectors using GFP-fusion constructs and morpholino gene-targeting strategy to generate "knockdown" fish, opens new perspectives for understanding complex developmental neurogenetic disorders, including fragile X syndrome. Therefore, we propose the zebrafish as an attractive complementary vertebrate model to study *fmr1* gene function during early embryonic development, especially in the brain.

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