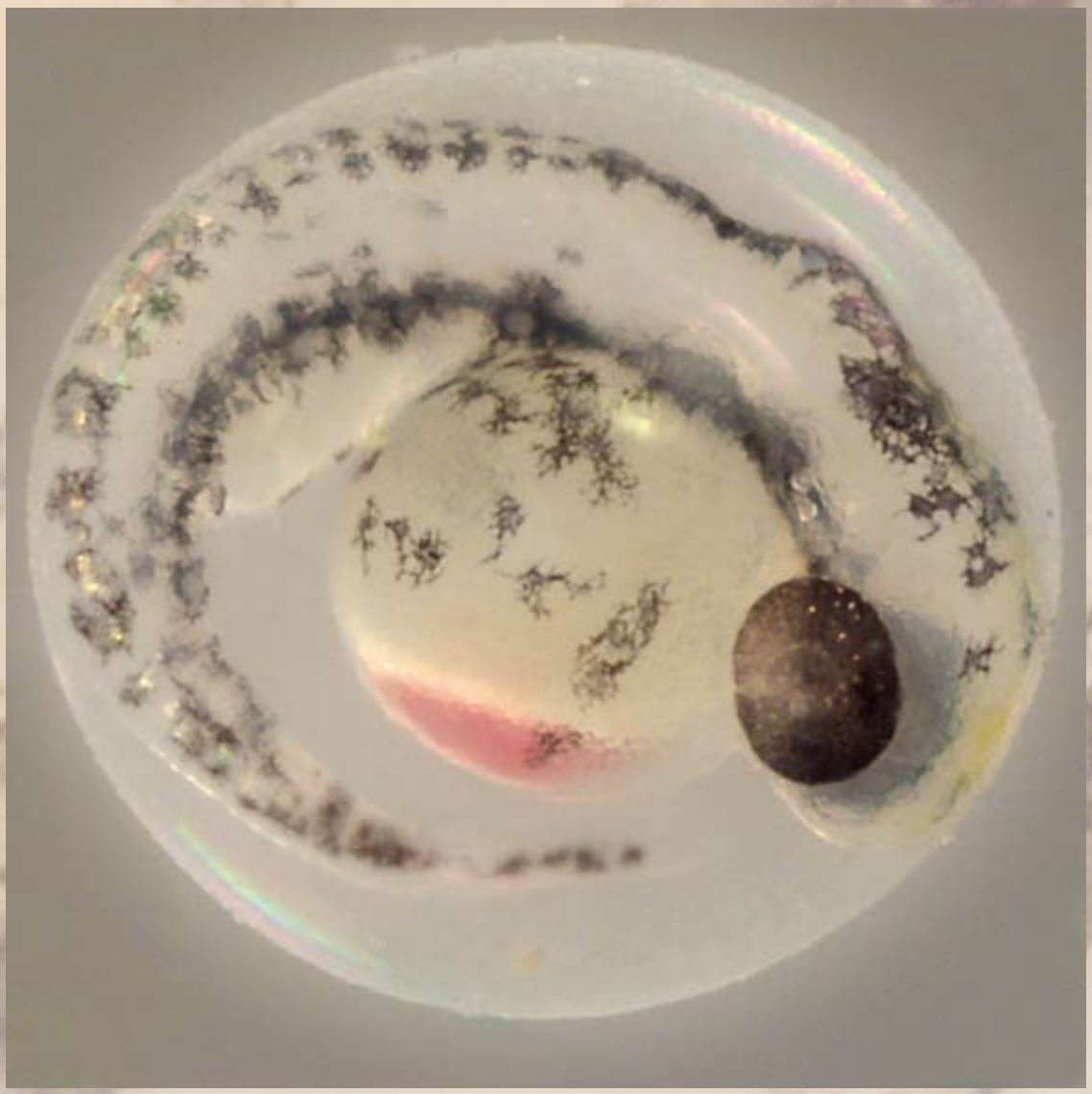
Zebrafish as a Model to study Human Disease

Functional Studies of the *FXR* Proteins



Sandra van ‘t Padje

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Functional Studies of the *FXR* Proteins

Zebravis als model voor het bestuderen van humane ziektebeelden
Functionele studies van de *FXR* eiwitten

Sandra van ‘t Padje

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Proefschrift

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Contents

Aim of this thesis

The aim of this thesis is to gain insight in the function of the *FXR* proteins, with special emphasis on their role during embryonic development. The *FXR* gene family is a small family of genes, including *FMR1, FXR1* and *FXR2,* of which *FMR1* is involved in the fragile X syndrome. The cellular function of the *FXR* proteins has been studied most thoroughly for FMRP using a knockout mouse model. Although no disease has been identified thus far involving FXR1P or FXR2P, the function is thought to be similar to FMRP, due to its high homology of important functional domains and similar expression patterns in the brain. The generation and characterisation of *Fmr1* and *Fxr1* KO mice suggest an important role of both FMRP and FXR1P during embryonic and postnatal development. However, the early embryogenesis is difficult to study in detail in mouse embryos due to their intrauterine development. This problem can be overcome by studying *FXR* gene function in zebrafish embryos.

The introduction (chapter 1) consists of 2 parts; the zebrafish (part 1) and the *FXR* protein family (part 2). Part 1 gives a general overview of the zebrafish as an animal model and the applications in zebrafish research. Some aspects will be described in more detail due to its relevance for research involved in this thesis, like chapter 1.5 Gene knockdown technologies. The second part of the introduction describes the *FXR* protein family. For more detailed descriptions regarding the fragile X phenotype, *FXR* mouse models and the function of FMRP in the brain, I refer to the publication of the corresponding thesis from our department [1-3]. In chapter 2, the *Fxr* proteins are identified in *Xenopus tropicalis,* another model organism suitable to study embryonic development. Chapter 3 and 4 describe the characterisation of Fmrp and Fxr1p in zebrafish. In chapter 5, the function of Fxr1p is studied using the antisense morpholino knockdown technique and the *fxr1* morphants are further characterised. The use of zebrafish as a model for human disease, the *fxr1* morphants and the possible cellular function of Fxr1p are discussed in chapter 6.

Chapter 1

Introduction

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Introduction

1 The zebrafish

1.1 The zebrafish as an animal model for human disease

The Latin name for zebrafish is *Danio* (formerly *Brachydanio*) *rerio*, which originates from the river Ganges in India and is common as an aquarium fish throughout the world [4]. Zebrafish belong to the cyprinid family of teleost fish (figure 1). In 1981, George Streisinger introduced the fresh water tropical zebrafish as a genetic model to study vertebrate development. Due to its transparent embryo that develops outside the mother's body the zebrafish represents an ideal vertebrate model system to study embryonic development. All developmental stages, including organogenesis, are clearly visible within the embryo and are described in detail by Kimmel *et al* and Haffter *et al* [5,6].

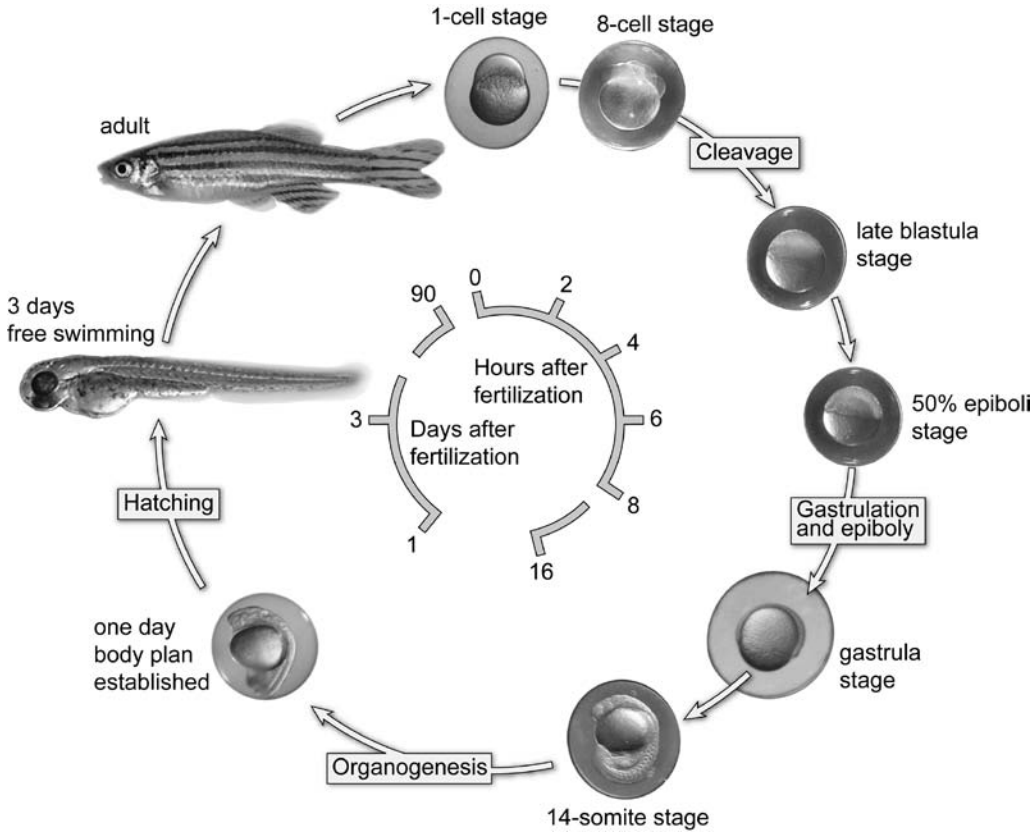

Figure 1. Phylogenetic trees of vertebrates and fish. A) Evolutionary tree of the chordates and vertebrates. B) Evolutionary tree of the bony fish (Osteichthyes). Zebrafish belongs to the euteleosti and ostariophysi. (Figure adapted from Campbell et al 1999).

Zebrafish is a relatively simple vertebrate and there is considerable conservation of pathways across species. This means that, despite teleost fish diverged more than 400 million years ago, they are more closely related to humans than invertebrates and thereby offer numerous advantages to researchers interested in many aspects of embryonic development, physiology and disease. By virtue of their being more closely related to humans, they have many biological functions comparable to human and hence many cellular processes have been conserved. Furthermore, it is becoming more and more clear that all vertebrates follow an evolutionary conserved developmental program.

The small size of the zebrafish (3-5 cm), their ability to be kept in large numbers, and the ease of breeding make them easy to maintain. Breeding and collecting eggs from the zebrafish is relatively easy. Since, zebrafish are photoperiodic in their breeding they are kept at a day-night cycle with an automatic timer (14 hrs light/10 hrs dark). The day before fertilisation, male and female fish are maintained separate using a special breeding tank with two separate compartments divided by a removable partition. By simply removing the partition in the morning, shortly after sunrise, embryos will be produced. As mentioned above, the fertilisation of eggs occurs externally and females are very fecund generating hundreds of eggs on a weekly basis.

The embryos develop quickly from a single cell to something that is recognizable as a fish after 24 hours of development. Between four and eight hours post fertilisation (hpf) several characteristic processes occur, including epiboly, involution and convergent extension. These processes start with the migration of the cells (approximately 1200 cells) over the yolk followed by extensive rearrangements. Subsequently, in the next three hours the three primary embryonic germ layers are formed through cell movements, a process called gastrulation. After gastrulation is complete, at around eleven hpf, the basic vertebrate body plan has developed and the formation of the first individual somites will begin. Somitogenesis starts anterior (close to the head) and sequentially moves towards the tail of the embryo (posterior). At 18 hpf, 18 somite pairs are formed and the total number that eventually forms is variable, from 30 to 34 pairs. At 24 hpf, a heartbeat and associated blood flow can be recognized. Within 48 hours after fertilisation all common vertebrate specific body features can be seen. Larvae hatch and are able to swim and search for food within 5 days. The generation time of the zebrafish is 2-3 months. For a schematic presentation see figure 2. The external fertilisation and the development outside the mother make it easy to access the embryos and to manipulate them, including exposure of larvae/fish to water-soluble chemicals and drugs. The transparency of the embryo enables us to follow the development *in vivo.* The accessibility and transparency in combination with a fluorescent marker make it possible to visualise detailed developmental cellular movements using real time imaging. In contrast, the embryonic development of a mouse takes 21 days. To study mouse embryos, the mother has to be sacrificed to get at the embryos, which sacrifices them as well. Due to these features the zebrafish has become an important model organism to determine the *in vivo* function of a gene (functional genomics) during embryonic development. Two general approaches are used to reveal the function of a gene: forward and reverse genetics. The process of forward genetics starts with a mutant phenotype and moves towards the gene; this was the first approach in functional genomics. The attractive features of zebrafish, as mentioned above, led to large-scale mutagenesis screenings in zebrafish [6-8]. It is easy to chemically induce mutations in the germ cells in the testis of adult zebrafish, which may give a phenotype in their

offspring. This procedure will be described in more detail in chapter 1.3. The process of reverse genetics starts with a particular gene and assays the effect of its disruption, that is, studying the phenotype associated with the mutant gene. This approach usually focuses on the inactivation of a gene and requires a way to selectively mutate a chosen gene. In general, the inactivation of the chosen gene is accomplished by homologous gene targeting, resulting in a complete (or sometimes conditional) knockout of the gene. Tools for reverse genetics typically include: gene knockouts/knock-ins using homologous recombination in embryonic stem cells (ES), gene knockdown using morpholino antisense technology (MO) or RNAi, and targeted induced local lesions in genomes (TILLING) technology. Some of these applications in zebrafish research will be discussed below in more detail (chapters 1.3, 1.4 and 1.5).

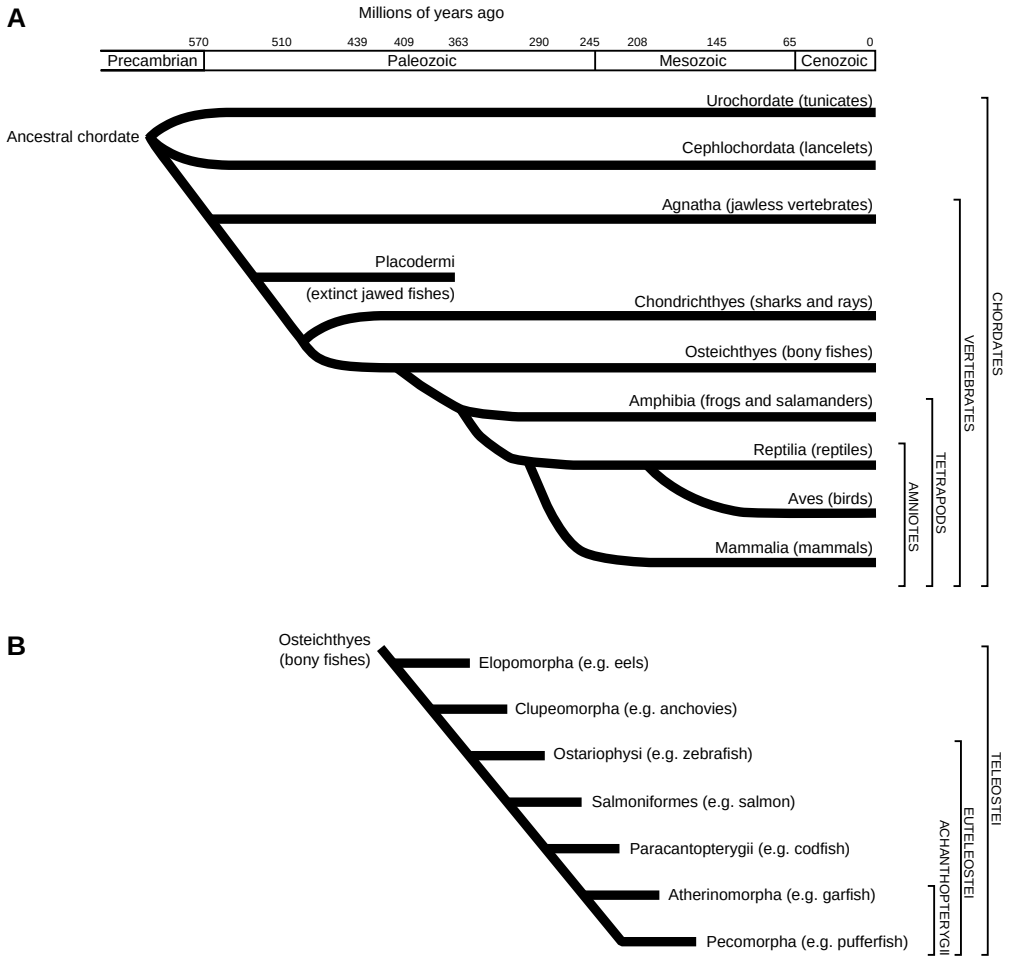

Figure 2. A schematic representation of the embryonic developmental stages of zebrafish. The cycle starts at the top with a fertilized single stage. The embryos develop quickly to a 14-somite stage within 16 hours. After 24 hours, the complete body plan of the embryo has been established meaning that all organs are present. Two days after fertilisation, the embryos hatch and become free swimming. Zebrafish are adult at 3 months of age and fertile.

Zebrafish models generated by both forward and reverse genetics are not only used as a genetic or developmental model system. The last decade, zebrafish has been successfully applied as an organism to elucidate the etiology of human disease. Zebrafish models of human disease are widely used in many different fields of medical research, like cancer, infectious diseases, cardiovascular disease, kidney disease, diabetes, blindness, deafness, digestive diseases, haematopoiesis, muscle disorders and neural disorders ([9] and references herein). For instance, features that make the zebrafish a good model for human disease are the haematopoiesis system and the heart. The haematopoietic process is conserved throughout vertebrate evolution and many homologues of blood specific genes have been identified in zebrafish. Like in all other vertebrates, haematopoiesis occurs in two phases namely a primitive and a definitive hematopoiesis. The use of mutagenesis screens has revealed mutants with defects in ventral versus dorsal cell fate patterning, which show either a lack or an expansion of haematopoietic cells. For instance, mutagenized zebrafish embryos for red blood cell deficiency resulted in the isolation of 26 different mutants [10]. The formation of the zebrafish embryonic heart resembles the first three weeks of gestation of the human heart and includes segregation of cardiogenic precursors (12 hpf), heart tube assembly (24 hpf), chamber formation (30 hpf), heart looping (36 hpf), valve formation (48 hpf) and cardiac function (30 hpf) [11]. Fortunately, embryonic heart function can easily be observed due to the transparency of the embryos. Another advantage of zebrafish is their ability to survive early development without blood circulation (reviewed in [12]). To date, many zebrafish mutants with cardiac defects affecting various aspects of heart development have been recognized. Further characterisation of these mutants has led to the identification of several key regulators in cardiac development. Importantly, the identified (disease) genes have shown to play a conserved role in human cardiac disease as well [13].
In summary, the zebrafish has become a well-established model organism, making important contributions to the identification and characterisation of genes and pathways involved in development, organ function and behaviour. Additionally it has become a valuable resource for identifying genes involved in human disease.

1.2 The zebrafish genome

The zebrafish is a vertebrate with a diploid genome consisting of 25 chromosomes (1n). Although the exact number of genes in zebrafish is currently unknown, estimates about the number of base pairs point to approximately 1.7 X 10^9 base pairs for the haploid genome [14]. The zebrafish is expected to have at least the same number of genes as the human. Almost all human genes can also be found in the zebrafish and approximately 20% of the human genes have two orthologs in zebrafish. This suggests genome duplication shortly before the teleost radiation, which was either partial or was followed by rapid gene loss. Importantly, if two orthologs are present they often show different expression patterns (both spatial and temporal). This suggests that the function of the ancestral gene has been divided up between two orthologs with more restricted (less complex) functions [15,16].
Sex determination in mammals is usually by the absence or presence of the Y chromosome. In fish, different mechanisms of sex determination have been found, however, the precise mechanisms are largely unknown. Some fishes have a homo/heterogametic system with 2 chromosomes, like the mammalian XX/XY system, while other fishes have 3 sex chromosomes.

Sex is usually determined genetically but can also be influenced by environmental factors such as temperature and pH. Other features seen in fishes are hermaphroditism and sex reversal, which can be accomplished by hormone treatment. The mechanism of sex determination in zebrafish is unknown. In zebrafish no sex chromosome or sex-linked genes have been identified. However, a number of genes have been linked to the process of sex determination and/or differentiation in zebrafish. These are autosomal genes of which allelic variants and dosage effects probably determine the sex (reviewed in Von Hofsten et al [17]).

In February 2001 the Sanger Institute started sequencing the genome of the zebrafish (www.sanger.ac.uk/Projects/D_rerio/). This whole genome-sequencing projects lead to the identification of genes of which the *in vivo* function is unknown. The assembled genomic zebrafish sequences are publicly available (http://www.ensembl.org/Danio_rerio/; http://vega.sanger.ac.uk/Danio_rerio/). In the last Ensembl release of June 2006 more than 21,000 genes were identified. Furthermore, the full sequence and assembly of the zebrafish genome facilitates evolutionary comparisons of the zebrafish genome with the mouse and human genomes.

1.3 Mutagenesis screens

The zebrafish is very well suitable for large-scale forward genetic screens in which phenotypic defects are identified before the identification of the gene causing these defects. This is due to its large quantity of eggs, short generation time and the external development of the transparent embryos. In addition, an important practical advantage is that zebrafish sperm can be frozen for future studies. The three techniques to induce mutations in zebrafish include chemical mutagenesis, gamma irradiation and insertional mutagenesis (reviewed in [9]).

Chemical mutagenesis is the most favourable and efficient method applied thus far [6,7]. The chemical mutagenesis by exposing adult zebrafish to N-ethyl-N-nitrosourea (ENU) is used for these screens. ENU, an alkylating agent, generates point mutations throughout the entire genome in premeiotic germ cells by transferring its ethyl group to individual bases of the DNA, which are misread by DNA polymerase in subsequent replications. However, most induced mutations are recessive and must be rendered to homozygosity to reveal a phenotype. This is accomplished by a multi-generation backcross model [6]. Mutagenized adult male zebrafish are crossed with wildtype females. The F1 offspring are heterozygous for individual mutations and are once more crossed with wildtype females. The resulting F2 generation is intercrossed randomly to produce F3 families in which homozygous mutations occur. In the F1 and F2 generation, rare dominant mutations might occur, but most ENU induced mutations are recessive. Finally, the F3 embryos are further selected on defects in organogenesis using microscopic examination between 1-5 days post fertilisation (dpf). Positional cloning can then identify the affected gene in isolated mutants. Large numbers of mutations that disrupt embryonic development have now been isolated in the zebrafish, many of which may serve as models for human diseases or syndromes. Further characterisation of these mutants (~2000) and identification of the genetic defect will advance our knowledge of the pathogenesis of the corresponding human disease. It will advance our understanding of the underlying molecular basis of the disease and ultimately may lead to the development of drugs aimed at treatment of the disease.

The second approach to induce mutations disrupting developmental processes in zebrafish is radiation, mainly gamma. Gamma-ray mutagenesis produces a very high locus mutation rate of

approximately 1:100 and has mainly been used in screens for morphological defects [5]. In contrast to chemical mutagenesis gamma rays induce translocations and large deletions at high frequency in the zebrafish genome and thus chemical mutagenesis is the method of first choice [18].
The third alternative approach to induce mutations in zebrafish is insertional mutagenesis, which can be established by injection of plasmid DNA, a mouse pseudotyped retrovirus or using a P-element transposon as insertional mutagens [8]. For retroviruses, a molecular tag at the site of the mutagenic lesion enables detection of the mutated gene. Although the efficiency of the mutagenesis is less than with ENU mutagenesis, the detection is 7 to 8 times higher than for ENU induced mutations [19,20]. These genetic screens allow the identification of novel genes and mutants for specific organs or processes. After examining the phenotypes by random mutagenesis, the mutation responsible for the specific defect has to be found using positional cloning. Major drawback of forward genetics is that it is slow and laborious due to positional cloning methods.

Mutagenic screening technology using a reverse genetic approach has been established as well. In 2002, due to the lack of a working protocol to produce an ES-cell-based knockout (or targeted gene expression) the TILLING technique was developed [21-23]. TILLING (Targeting Induced Local Lesions IN Genomes) involves random induced mutations by ENU and subsequent screening for mutations in target genes. This screen is an enzyme-mediated (CEL-I endonuclease) mismatch recognition procedure to detect heterozygous germ line mutations in the F1 generation. Further generation of embryos with mutant phenotypes is similar to the breeding scheme described above. TILLING can be performed in a high-throughput setup. The only disadvantage is that the mutations are randomly introduced. The TILLING method would lead to more null mutants in zebrafish than achieved by homologous recombination in mice [24,25]. Initially, TILLING was developed to reduce the time and costs of mutation detection using DNA sequencing [25]. However, recent advances in DNA sequencing technology make this method equal to TILLING [26]. Thus far several null mutants have been generated using the TILLING technology and recently a homozygous *fmr1* null mutant has been identified (E. Cuppen, Hubrecht Lab; personal communication). To exploit the advantages of the zebrafish to produce disease models a consortium of European zebrafish groups have been funded and includes the generation of targeted knockout zebrafish on request (see www.zf-models.org).

1.4 Transgenic and knockout zebrafish

Genetically modified animal models are widely used to characterize the function of many newly identified (disease) genes. Transgenic techniques in the mouse to generate transgenic and loss-of-function mutations are well established and have improved our understanding of the roles of specific gene products significantly. Genetically modified mice also serve as valuable models to study the pathogenesis of human disease and to test or develop experimental treatment regimes. However, with the zebrafish emerging as an important model organism to study human disease, the development of similar or additional genetic techniques specifically focused on zebrafish was needed. Methods for generating a transgenic zebrafish are pseudotyped retrovirus infection [19,27,28], transposons [29-31], transfection of sperm nuclei [32] and DNA microinjection. The latter is the most frequently used method for generating transgenic lines expressing a gene of

interest. DNA microinjection can be achieved by injection of plasmid DNA or bacterial artificial clones (BACs) into the cytoplasm of a 1-cell stage embryo. The frequency of DNA integration into the germline by microinjection in zebrafish is 1-30%, which is comparable to mouse [33]. Coinjection of I-SceI meganuclease and a construct flanked by meganuclease recognition sites has been shown to improve the integration in fish [34]. The gene of interest is randomly integrated or under the control of a general or tissue-specific promoter. An example of a gene of interest could be GFP under the control of a tissue-specific promoter and thereby generating a cell-specific fluorescent transgenic fish line. A number of fluorescent transgenic fish lines are commercially available (e.g. GFP-red blood cells or GFP-neurons). Fluorescent imaging using for instance confocal microscopy makes it possible to investigate detailed developmental processes like gene expression, single cell migration etc.

Another approach to investigate the function of a (disease) gene in an animal model is to inactivate or disrupt the gene of interest. A major advance in the ability to generate mouse disease models was the development of technology that makes it possible to introduce loss-of-function mutation into endogenous genes and then transmit these through the mouse germ line (reviewed in [35]). The desired null mutations are first created via homologous recombination in embryonic stem (ES) cells, which contribute to all cell lineages when injected into blastocysts. However, mouse developmental genetics is impeded by the high cost of maintenance of animals and by the intrauterine mode of development. Because of the expense and effort required to produce a genetically modified mouse and the inaccessibility of the embryos inside the mother, the zebrafish might be the vertebrate model to allow these genetic techniques.

The strategy to generate knockout zebrafish by the germ line transmission of targeted loss-of-function alleles using ES cells, as described for mice, has not yet been achieved. The only method to produce a knockout zebrafish was mutagenesis followed by targeted screening for point mutations as described above [21]. Pluripotent zebrafish ES cell lines have been established [36]. Recently, targeted incorporation of plasmid DNA into these cells by homologous recombination followed by *in vitro* drug selection was successful [37]. In addition, the authors were able to introduce these ES cells, expressing a marker gene such as GFP, into host embryos using microinjection techniques and achieved contribution to the germ line. Although the frequency of germline chimera production was 2-4%, the availability of large quantities of fertilized eggs makes it potential feasible to establish a knockout fish line in the near future [37]. Alternatively, Wu et al. described a novel method to generate site-directed knockout or knockin zebrafish without the use of embryonic stem (ES) cells. By microinjection of targeting plasmids, the endogenous growth hormone (GH) receptor was disrupted either by knockin of a ß–promoter or by knockout of the GH receptor. Correct integration of target genes was identified by dual selection using GFP and RFP for a positive and negative selection, respectively. They found 2-3% of potential founder knockout had the characteristic of germ line transmission [38].

In summary, these results show the potential for generating a knockout/knockin zebrafish targeted gene modification through homologous recombination, either with or without the culture of embryonic stem cells.

1.5 Gene knockdown technologies

Zebrafish reverse genetics is slowly catching up with *Drosophila* and/or mouse, as the techniques to perform gene-specific knockdowns, target-selected mutagenesis and transgenesis in zebrafish are quickly developing. Next to the ability to make a knockout (see above), the generation of a morpholino-mediated knockdown zebrafish was the favourite technology thus far to study gene function in zebrafish (reviewed by [39]). Currently the use of antisense modified oligonucleotides is still widely applicable due to their ease and quick results. Although morpholinos have been tested in different species, including sea urchin, ascidian, frog, chicken and mouse the most favourable model organism to test morpholinos has been carried out on zebrafish embryos. Morpholinos (MOs) are synthetic oligonucleotides of 25 bases, which hybridise specifically to complementary sequences of mRNA, disrupting translation initiation or pre-mRNA splicing. The backbone of MOs is similar to the backbone of DNA or RNA, but with some changes. In MOs, the ribose or deoxyribose sugar molecules that link the bases of the DNA or RNA are replaced by morpholino rings (hence the name). Anionic phosphates of bases replace non-ionic phosphorodiamidate linkages. Due to this modified backbone, the MO is uncharged, very stable and cannot be degraded by nucleases. (More details about MOs see www.gene-tools.com). The reduction of translation will never be 100%, but can be up to 90%, and is therefore called a knockdown (figure 3). Zebrafish embryos displaying a phenotype as a consequence from ectopic MO administration are called morphants [40].

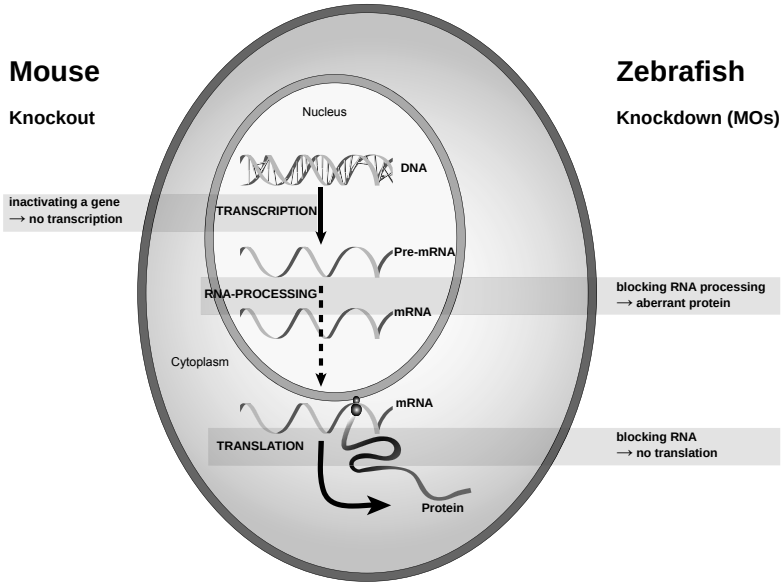

Figure 3. Differences between the gene knockout technologies, often used in mice, versus the gene knockdown technology in zebrafish. In case of knockout, the gene becomes fully inactivated at the level of transcription and subsequent total lack of protein. Knockdown by morpholinos occurs either on the level of RNA processing or translation. The injected morpholino technology can be designed against either a splice site or start site of the target mRNA. When a morpholino is targeted against a splice site, an exon could be spliced out or an intron could be spliced in during pre-mRNA processing. This incorrect splicing of the mRNA leads to aberrant or truncated protein. If the morpholino is targeted against the start site the translation initiation complex is blocked and results in reduced protein expression (up to 90%). Another important difference between knockout and knockdown is the effect, which is 100% for knockout and maximum 90% for knockdown.

The MOs can either be microinjected in the cytoplasm of a 1-cell stage embryo or into the yolk of a 1- to 16-cells stage embryo. MOs can diffuse into the cell until a 16 cells stage when a membrane is formed between the yolk and the cells that will form the actual embryo. MOs can be dissolved in Danieau buffer, which is thought to reduce lethality. As an injection tracer 0,05% Phenol Red can be added, which also works as a pH indicator and will turn yellow if the MO solution is acidic. MOs will directly bind to endogenous (also maternal) mRNAs at either the translational start site and disrupt translation initiation. However, MOs can also be designed against a splice junction, thereby preventing correct splicing into a mature mRNA [22,41]. This aberrant splicing includes exon deletion or intron insertion, which might lead to premature stop or a non-functional protein. Knockdown of gene function is transient and effective until 3 to 5 days of development due to dilution of the MOs. MOs exert their effect throughout embryogenesis in a dose-dependent fashion, thereby allowing the identification of morphants that might be masked or lethal in case of null mutations [40]. Interestingly, MOs against different genes of interest can be co-injected simultaneously to either study/exclude redundant function or assess interactions between gene products within a pathway. Large quantities of eggs can be microinjected in one experiment and due to the fast development of the embryos, a phenotype can often already be observed after 24 hours. MO microinjection might cause non-specific side effects thus control experiments are necessary. These controls could be microinjection of a second and/or mismatch MO or coinjection of target mRNA and MO to rescue the phenotype. The efficacy of depletion of the target protein is crucial and should be analysed by Western blotting using monospecific antibodies against the target protein. However, this can only be applied for MOs that disrupt translation. For MOs that result in aberrant spliced mRNA, all outcomes can be characterised and quantitated by RT-PCR [41].

Another approach for targeted knockdown is using RNA interference (RNAi). RNAi is the process of sequence-specific posttranscriptional gene silencing (PTGS), initiated by the introduction of double-stranded (ds) RNA. In invertebrates such as *C. elegans* and *D. melanogaster*, microinjection of dsRNA leads to specific silencing of genes highly homologous to these dsRNA, which activates a cascade leading to degradation of the mRNA (reviewed in [42,43]. However, in zebrafish the RNAi-mediated knockdown appeared only moderately efficient and moreover showed non-specific effects using dsRNAs [44-47]. To overcome these non-specific effects, mammalian specific gene silencing approaches have been developed for zebrafish using short interfering RNAs (siRNAs) and retroviral short hairpin RNA (shRNA) [48]. Recently, the approach of siRNAs-mediated gene silencing has been applied successfully for the zebrafish dystrophin gene [49]. This study demonstrates the effective use of gene silencing via siRNA in zebrafish, albeit in a brief temporal manner. Future research should be focused on the generation of stable lines of transgenic zebrafish expressing either siRNAs or shRNAs to overcome the transient siRNA.

1.6 Behavioural tests

The rapidly increasing number of zebrafish mutants from the mutagenesis screens and knockdown/transgenic strategies will result in zebrafish with defects in overall embryonic pattern, morphogenesis or organ formation. Initial phenotypic characterisation is done by microscopically screening all morphological features of embryos of 1-5 dpf. A screen of defects

in organogenesis is described by Haffter et al [6]. Next step is to characterize the phenotype by methods using molecular probes like in situ hybridisation and/or (immuno) histochemical techniques or biochemical tests. Other important aspects of characterisation of the phenotype are behavioural studies. For mice these tests are well defined, however, with respect to zebrafish behaviour surprisingly little is known.

Zebrafish is a typical diurnal schooling fish that prefers light to dark during the day. However, in response to danger they hide in the dark. Males exhibit territoriality, including dancing movements and agonistic behaviour. Zebrafish embryos (0-5 dpf) already exhibit simple sensory and locomotor abilities, whereas larvae (5-14dpf) possess many patterns of behaviour. Some simple behavioural tests concerning locomotion of the embryos/larvae have been described, like rhythmic tail movements, the escape response, equilibrium control and the touching assay. More sophisticated assays to identify defects in optokinetic and phototactic behaviours have been described as well [50-52]. Recently, a system to monitor behaviour in zebrafish has been established. Ethovision from Noldus is a computerized video tracking system that enables to record movement of animals including swimming patterns of small sized fish. The fish can be monitored in an open tank as well as a 96-well plate for high throughput screens. The coordinates of the swimming performance can be stored and used for further software analysis. Many behavioural tests can be performed due to automated locomotion recording and data analysis [53].

Learning and memory have been studied in many model organisms. Each species uses its own specific behavioural paradigms. Also for zebrafish a simple spatial alternation paradigm for evaluation of spatial learning and memory function has been developed [54]. This paradigm is based on an aquatic version of an alternation task (T maze) commonly used for rats and mice. The fish received a food reward after choosing the correct arm when they observed a light tap on the tank. A similar approach has been applied for the use of different colours to show visual discrimination learning [55]. Very recently, the aquatic T-maze has been combined with an automated tracking and analysis system (Ethovision) to track zebrafish reliably at a high sample rate.

These newly developed behavioural assays for zebrafish will help to characterize (new) zebrafish mutants produced by large-scale forward genetic screens and reverse genetics approaches.

1.7 Myogenesis in zebrafish

Myogenesis is a complex process that is conserved among vertebrate striated muscles. The formation of muscles within the vertebrate embryo starts during somitogenesis. Somites originate from the paraxial mesoderm. The dorsal part of an amniotic somite gives rise to a transient epithelial structure called the dermomyotome, which gives rise to the dermis and another compartment, the myotome. However, in non-amniots, like the zebrafish, no dermomyotomal stage could be identified for myotome formation. In zebrafish, initiation of muscle development occurs before the onset of somitogenesis within the adaxial cells [56]. These cells start the myogenic differentiation by expressing myogenic regulatory factors, such as *myf5*, *myoD* and *myogenin*, and myosin heavy chain genes [57-59]. The adaxial cells lie adjacent to the notochord, migrate laterally of the somite and elongate into mononucleated slow muscle fibres [56]. A subset of adaxial cells does not migrate and remain next to the notochord. These specific adaxial cells are called muscle pioneer cells and differentiate into the horizontal septum, which forms a

separation between the dorsal and ventral domains of the developing myotome [56,60]. With this adaxial cell migration, the rest of the cells follow to form the myotome. The slow muscle fibres act as a template for the differentiation of fast muscle fibres, which develop from the remainder of the presomitic cells [56,60-63]. The mechanism of myotomal growth resembles the amniotic program. First, the number of slow muscle fibres increases from a monolayer to a thicker layer and starts to grow towards the dorsal and ventral side of the myotome [64]. The next step is fusion of muscle fibres, which are mononucleated myoblasts and become multinucleated myotubes. The process of myofibre formation is called myofibrillogenesis.

The elongation of the muscle fibres is inhibited by boundaries. These boundaries are formed due to somite formation, which occurs as the myotome is formed. First, an initial epithelial somite boundary is formed, which changes into a transitional boundary. During this second stage of boundary formation, muscle fibres migrate laterally (as described above) and focal adhesion molecule appears at the boundary. In this stage the chevron-shaped somite becomes visible. The final stage is the formation of the myotome boundary, which is rich of extracellular, focal adhesion and dystrogyclan components [65-67].

During myofibrillogenesis, assembly of structural proteins is necessary to enable the development of highly organized striated muscle. These structural proteins include actin, alpha-actin, myosin and titin. The precise mechanism is still largely unknown and different models of myofibrillogenesis have been described (Reviewed in [68]. Most favourite model is currently the premyofibril model. This model proposes that premyofibrils act as a precursor of mature myofibrils. The premyofibril is a small sarcomere that contains sarcomeric proteins; actin, non-muscle myosin II, troponins, tropomyosin and Z-bodies, which is enriched with alpha-actinin. Premyofibrils will align and grow by assembly of titin and muscle myosin II. The myofibril is now called nascent [69]. In the transition from nascent to mature myofibril, the Z-bodies will form the Z-bands and the muscle myosin II will align into A-bands of the striated muscle. Although there is evidence in favour for this model, the precise mechanism of myotome formation is still unknown.

2 The *FXR* protein family

2.1 The *FXR* genes

The *FXR* genes are a small family of fragile X related genes, formed by *FMR1, FXR1* and *FXR2.* In 1991, the *FMR1* or Fragile X Mental Retardation 1 gene was found to be involved in the fragile X syndrome [70]. The *FMR1* gene is located at the long arm of chromosome X, at the position Xq27.3 [71]. *FMR1* has a genomic sequence of 40 kb and is composed of 17 exons, encoding an mRNA of 3,9 kb [72]. The two other members of the fragile X related family, *FXR1* and *FXR2,* are autosomal and map to chromosomes 3q28 and 17p13.1, respectively [73]. The amino acid sequence homology between the corresponding *FXR* proteins is very high at the N terminal domain and central regions (figure 4). At the C terminal domain both *FXR1* and *FXR2* lack exon 11 and 12 of the *FMR1* gene [74]. The high conservation of the gene structure among the three family members suggests that they may originate from a common ancestor gene [75].

Indeed, *Drosophila melanogaster* appeared to have one gene representing all three homologues of the *FXR* genes [76,77]. This will be described in more detail in chapter 2.5.
In the 5' untranslated region of the *FMR1* gene a CGG trinucleotide repeat is located, which cannot be found in the other members of the fragile X related genes. The number of repeats in normal individuals is highly variable (5-50 repeats) with an average of approximately 30 CGGs [78]. Normal alleles are stable upon transmission to the next generation [79]. A repeat of 50 to 200 CGG repeats is called a premutation and can be instable upon transmission. Once the repeat exceeds the 200 CGGs, called a full mutation, the CGG repeats and the promoter region of the *FMR1* gene is usually methylated. Due to this methylation, *FMR1* transcription is silenced and causing absence of the *FMR1* gene product, the Fragile X Mental Retardation Protein (FMRP). The absence of FMRP in neurons is the cause of the mental retardation in fragile X patients [80,81]. For both the *FXR1* and *FXR2* genes no human disease has been described so far.

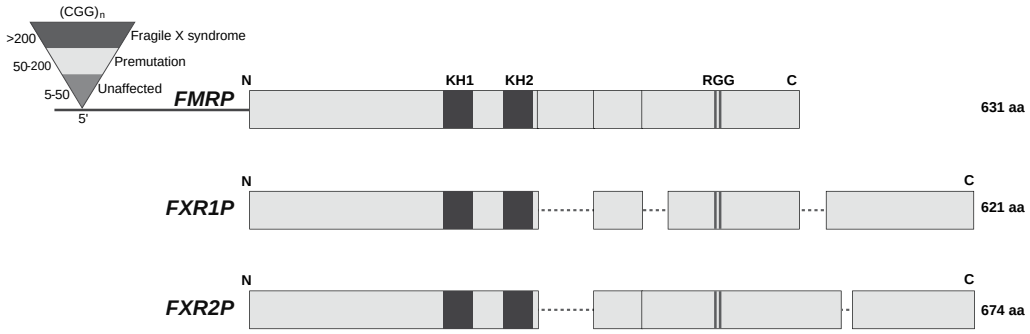

Figure 4. Schematic representation of the *FXR* protein family. The *FXR* proteins share a high homology, including the functional domains indicated by KH1, KH2 and RGG box. In the 5'UTR of the FMR1 gene, a CGG repeat is located, which normally varies between 5-50 repeats. A repeat size of 50-200 CGG repeats is called a premutation. If the repeat exceeds the number of 200,the *FMR1* gene becomes inactivated and results in total lack of the *FMR1* protein, FMRP. The lack of FMRP causes the fragile X syndrome

2.2 Clinical features of the fragile X syndrome

Martin and Bell first described the fragile X syndrome, also known as the Martin-Bell syndrome, in 1943 [82]. Fragile X syndrome owns its name to a fragile site on the X chromosome that can be visualised in some cells of fragile X patients under specific culture conditions [83]. With a prevalence of 1 in 4000 males and 1 in 6000 females, the fragile X syndrome is the most common form of inherited cognitive impairment. The fragile X syndrome has a wide range of clinical features, including moderate to severe mental retardation, macroorchidism and mild facial abnormalities, such as a long face, large everted ears and prominent jaws. In addition, many male patients exhibit autistic-like behaviour and other behavioural features like hand-flapping, hand-biting and poor eye contact. More than 20% of male patients develop seizures, particularly during childhood [84]. The molecular basis for the variability in the spectrum of

involvement is still largely unknown. Approximately 60% of females carrying a full mutation manifest learning disabilities, while the remaining 40% have normal intellectual capacity. For an extensive description of the phenotype of fragile X syndrome see thesis L.B.A. de Vries [3].

2.3 Unexpected phenotypes in *FMR1* premutation carriers; FXTAS and POF

Individuals with a premutation, which ranges from 50 to 200 CGG repeats, were initially thought to be asymptomatic because they produce relative normal levels of FMRP. Research has shown that in cells of male premutation carriers *FMR1* transcription is increased significantly, which results in elevated *FMR1* mRNA levels [85,86]. Typically, a 2-8 fold increase of *FMR1* mRNA levels can be detected that seems to be positively correlated with the size of the CGG repeat. These elevated *FMR1* mRNA levels have been proposed as the underlying molecular mechanism of a new neurological syndrome, fragile X-associated tremor/ataxia syndrome (FXTAS), in older male premutation carriers (reviewed in [87,88]).

FXTAS is a neurodegenerative disorder, characterised by progressive intention tremor and ataxia, but also memory and executive impairment, essential tremor, autonomic dysfunction, parkinsonism, anxiety and peripheral neuropathy can be observed [89]. The development of FXTAS in female premutation carriers is less evident than in males due to lyonisation in females.

The cause of FXTAS is still unclear. FMRP levels are mildly reduced, which led to the proposition that the CGG repeat in premutation individuals has become too long to be efficiently translated [85,86,90]. The CGG repeat causes a conformational change that leads to hampering of the ribosome during translation [91]. This leads to a reduced FMRP level, which in turn might activate a feedback mechanism to increase *FMR1* transcription. Another explanation might be a conformational change in the promoter region that leads to enhancement of transcription. However, further investigations are needed to understand the development of FXTAS.

Neurohistological studies on brains of FXTAS patients revealed eosinophilic intranuclear inclusions in neurons and astroglia [92]. These inclusions contain *FMR1* mRNA and are ubiquitin positive, linking the increased *FMR1* mRNA levels to the ubiquitin degradation pathway. A toxic gain of function effect by the elevated level of *FMR1* mRNA has been suggested to lead to formation of intranuclear inclusions.

On the other hand, nearly 20% of female premutation carriers manifest POF or premature ovarian failure, defined as menopause before the age of 40 [93]. A recent study reports early reproductive aging in regularly cycling premutation carriers [94]. These findings suggest that ovarian dysfunction in premutation carriers is related to a decreased number of ovarian follicles compared to controls. The role of elevated *FMR1* mRNA levels in the ovary and the development of POF in premutation carriers remain unclear. Importantly, POF and FXTAS are mechanistically distinct from the fragile X syndrome, that is, they are most likely caused by an RNA-gain-of-function whereas the fragile X syndrome is caused by a loss of function.

2.4 *FXR* protein expression

The *FXR* gene family encodes the following proteins; FMRP, FXR1P and FXR2P. FMRP is maximally 632 amino acids long. Alternative splicing of exon 12, 14, 15 and 17 gives rise to many different isoforms of the protein [95]. The FMRP isoforms have a molecular weight of 70-80 kDa and do not show a tissue-specific expression pattern [81]. FMRP is expressed in a wide variety of

tissues with high expression in brain and testis. In the brain FMRP is expressed in differentiated neurons and concentrated in the perikaryon and the proximal dendrites. Most neurons of the brain show high labelling for FMRP, especially Purkinje cells and motorneurons. Generally, FMRP is localised in the cytoplasm, though nuclear localisation has been noted as well [96]. In the cytoplasm FMRP is predominantly associated with actively translating ribosomes [97-100]. Only a minority is present at synapses in the postsynaptic compartment [96,101] In primary neuronal cultures, FMRP could be detected in association with trafficking large mRNP particles involved in dendritic mRNA transport and translation [102,103] A recent study shows the presence of FMRP in the growth cones of developing axons in mature axons innervating dendrites ([104] and Fallon unpublished results). The testis shows a high expression of FMRP with prominent labelling in early spermatogonia located at the basal membrane of the seminiferous tubules [105].

In different mammalian cell lines and in the majority of mouse tissues, FXR1P consists of four major isoforms with a molecular mass of 70, 74, 78 and 80 kDa. In muscle, these isoforms of FXR1P are absent, while two other isoforms of 82 and 84 kDa have been identified [106,107]. In brain, FXR1P localisation is similar to FMRP, whereas FXR1P localisation in testis is not only restricted to early spermatogonia but is also localised in more mature spermatogenic cells including spermatocytes and spermatids [105]. Furthermore, at the subcellular level FXR1P has been found to be associated with ribosomes and microtubules in the flagella of the spermatozoa [100,108]. The muscle-specific isoforms were located to the costameric regions within the striated muscles [107,109].

Thus far only one isoform of FXR2P has been identified with a molecular weight of 95 kDa [105]. FXR2P is co-expressed with FMRP and FXR1P in brain tissue and at least partly associated with ribosomes [100]. In testis FXR2P is, although at low level, expressed in all spermatogenic cells of the tubules seminiferi [100,105].

Notably, FMRP expression in brain tissue from fragile X patients is totally absent while FXR1P and FXR2P expression was identical compared to normal brain tissue [105].

2.5 Orthologs of *FXR* genes

The *FXR* genes are highly conserved in evolution and orthologs of the human *FMR1* gene have been identified in mouse *Mus musculus*, chicken *Gallus gallus*, fruitfly *Drosophila melanogaster*, frog *Xenopus laevis* and zebrafish *Danio rerio* [76,110-112]. The overall homology 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 and zebrafish all three members of the *FXR* genes have been identified.

The three members of the *FXR* family in the mouse (*Mus musculus*) show high homology with the human *FMR/FXR* genes, except for exon 12, which is missing in mouse Fmrp. The murine homolog of FMRP has 97% homology [110]. The chicken (*Gallus gallus*) homologue of *FMR1* has nucleotide and amino acid identities with human *FMR1* of 85 % and 92%, respectively [111]. All three *FXR* members could be identified in zebrafish. The overall identities between human-zebrafish, mouse zebrafish, frog-zebrafish and fruitfly-zebrafish Fmrp are 74%, 70%, 72% and 38%, respectively [113]. In the frog (*Xenopus laevis*) the amino acid sequence of Fmrp is 86% identical to the human FMRP over the entire length of the protein. For *Xenopus tropicalis*

this homology with the human FMRP is 76%. In *X. tropicalis* only an ortholog for FMRP and FXR1P could be identified. Both genes contain all functional domains [112].

Interestingly, the fruitfly (*Drosophila melanogaster*) contains a single, well-conserved *dFxr* gene representing all three homologues of the *FXR* gene family in mammals [76,77]. The *dFxr* gene is homologous with all three *FXR* genes in human. The amino acid sequence of the KH domains is 85% homologous with human FMRP and also contains an RGG box. Even the subcellular localisation and the embryonic expression pattern are comparable to the expression pattern of FMRP in mammals [76].

In *Caenorhabditis elegans* and *Saccharomyces cerevisiae*, two lower eukaryotes, no homologues of the human *FMR1* could be identified [114,115]. Therefore it was thought that the *Drosophila melanogaster Fxr* gene is some kind of ancestral gene, which evolved into the *FXR* genes in mammals. However, recently an *FMR1* ortholog has been described in cnidarian hydroid *Hydractinia echinata*. An *Fxr1* or *Fxr2* ortholog could not be identified. Thus the origin of the *FXR* genes date back at least to a common ancestor of the cnidarians and bilaterans and that lack of *FXR* genes in invertebrates may have been due to gene loss in particular lineages during evolution [116].

2.6 *Fxr* knockout mouse models

In order to study the physiological function of a protein, animal models can be of great help. The high evolutionary conservation of *FXR* proteins between the different species makes this possible. In these functional studies the laboratory mouse is often the mammal of choice because embryonic stem cells are available and manipulation of gene expression is to date a standard technology. Indeed, knockout mice for all three genes were generated and characterised.

The *Fmr1* knockout mouse displays some characteristics in common with fragile X patients, including learning deficits and enlarged testis [117,118 for review]. In addition, ultrastructural studies of the brain revealed the presence of abnormal dendritic spines illustrating reduced pruning and/or maturation of spines [104,119-121]. Notably, spine abnormalities have already been reported in fragile X patients in earlier studies using brain autopsy material [122,123]. 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.

The *Fxr2* knockout mice show a discrete behavioural phenotype, like hyperactivity, impaired motor coordination, abnormal sensorimotor gating, and decreased response to heat stimulus and impaired learning and memory. Some features resemble the behavioural phenotype of the *Fmr1* KO mouse, while others are distinct for the *Fxr2* KO mouse [124]. Recently, an *Fmr1/Fxr2* double knockout mouse was generated to better understand the function, interaction and possible compensation between *Fmr1* and *Fxr2*. The *Fmr1/Fxr2* double knockout mice appeared to have exaggerated behavioural phenotypes compared to the *Fmr1* knockout mice and *Fxr2* knockout mice. This suggests that FMRP and FXR2P have common functions in some behavioural responses [125].

Fxr1 knockout mice die shortly after birth and appeared to have a striated muscle phenotype. Macroscopic examination reveals that some organs of the *Fxr1* knockout mice such as heart, skeletal muscle, lung, spleen and testis are less developed compared to wildtype mice and seem

to be missing large blood vessels at the surface of these organs. When examined microscopically they display a disruption of the cellular architecture of the striated muscle tissue. Due to loss of Fxr1p in the striated muscle, the costameric proteins vinculin, dystrophin and α-actinin are delocalised [109]. The neonatal death of the *Fxr1* KO mouse and the observed disruption of the cellular architecture of the striated muscle tissue illustrate the importance of Fxr1p during embryonic muscle development (figure 5).

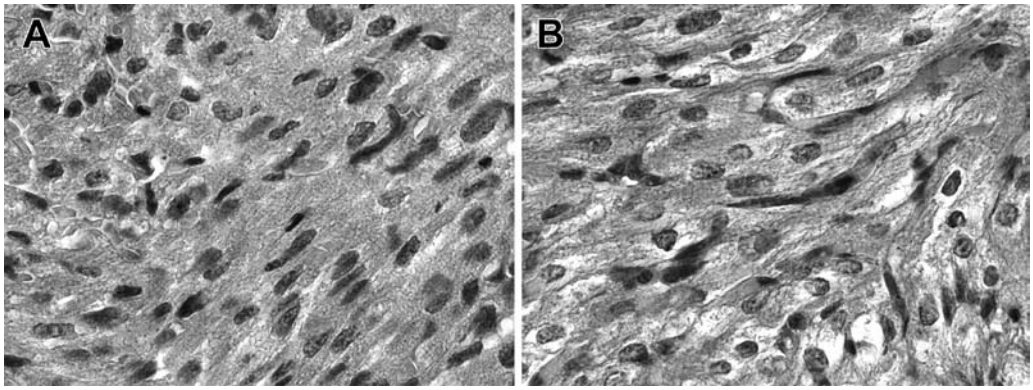

Figure 5. HE stained paraffin sections of neonatal WT (A) and *Fxr1* knockout (B) cardiac tissue. The overall cellular architecture of the cardiac muscle from *Fxr1* KO mice is disrupted.

2.7 Function of FMRP

FMRP has been extensively studied due to its involvement in the fragile X syndrome. The precise physiological function of FMRP is still unclear; however, compelling evidence supports a role of FMRP in synaptic plasticity. FMRP contains a nuclear localisation signal (NLS) and a nuclear export signal (NES), which are involved in shuttling of FMRP between the nucleus and cytoplasm [126,127]. Two hnRNP K homology domains (KH domains) and an arginin/glycin rich RNA binding motif (RGG box) have been identified within FMRP, which are motifs characteristic for RNA binding proteins [128,129]. A missense mutation in the second KH domain (Ile304Asn) causes a severe form of the fragile X syndrome illustrating the importance of this domain [130]. Searching for the target mRNAs for FMRP, a specific mRNA interaction motif was found in the RGG-box region. This high affinity-binding site for FMRP consists of a purine (G)-rich region that can fold into a tertiary structure, called the G-quartet structure. Also *FMR1* mRNA contains a G-quartet structure, which facilitates direct binding to FMRP [131]. Microarray technique on immunoprecipitated RNP complex containing FMRP from human and mouse tissues identified 12 potential target mRNAs of which eight transcripts were capable of forming a G-quartet. Interestingly, some of these target mRNAs are important neuronal proteins, like semaphorin, NAP22 and MAP1B [132-134]. Another RNA complex was identified as a target of the FMRP KH2 domain. This complex forms a stable loop-loop pseudoknot, or kissing complex (hence the name). In contrast to G-quartet RNAs, the kissing complex can compete with polyribosomes for the association of FMRP. RNAs within the kissing complex may play a role

in FMRP translation control and thereby related to the mental retardation in fragile X syndrome and the severe form associated with the I304N mutation [135]. An alternative target recognition mechanism has been proposed via a small non-translating RNA, named *BC1. BC1*-mediated recognition and translational control revealed Arc, MAP1B and α-CaMKII as potential target mRNAs for FMRP [136].

More and more data reveal the involvements of FMRP in synaptic plasticity. The long thin and tortuous spines in *Fmr1* KO mouse brains and correlated findings in Fragile X patients suggest that the lack of FMRP impairs maturation and pruning of spines [137,138]. At postsynaptic dendritic sites, FMRP and *FMR1* mRNA are also associated with polyribosomes [96,101]. In addition, a subset of the identified FMRP interacting mRNAs appeared to encode for a neuronal protein found at the postsynaptic site or is isolated from synaptoneurosomes. These mRNAs include MAP1B, Arc, CaMKIIa and glutamate receptors [139-142]. The observation of increased FMRP expression by activation of metabotropic glutamate receptor (mGluR) links FMRP to synaptic function [101]. Finally, measurements of long-term depression (LTD) in *Fmr1* KO mice showed an enhancement compared to wildtype mice. LTD is a long lasting decrease in synaptic connectivity, consistent with the role FMRP as a repressor of translation [143-145]. This leads to a model that suggests that when mGluR receptors are activated local protein synthesis in the postsynaptic compartment is stimulated to maintain LTD by (permanent) internalisation of AMPA receptors. One of the proteins synthesized is FMRP, which in turn has a negative feedback on the local protein synthesis (repressor). This inhibition of translation by FMRP is required to control LTD. In case of the absence of FMRP an increase in protein level at the synapse and an increased LTD is observed [143].

Thus, FMRP plays an important role in selective targeting of a subgroup of mRNAs to dendrites in an inactive state and subsequent translational control at the synapse. In this way FMRP is essential for synaptic plasticity and thus for learning and memory processes (reviewed in [146]). Misregulation or mistrafficking of FMRP-associated mRNAs is thought to be the underlying cause of the fragile X syndrome.

2.8 Possible functions of FXR1P and FXR2P

To date the cellular function of both FXR1P and FXR2P are less well understood. Sequence analysis of the FMRP homologues, FXR1P and FXR2P, show a high conservation, especially in the functional domains and an overlap in tissue distribution [73,74,100,105,147]. This suggests that FXR1P and FXR2P might have a similar function as FMRP in mRNA transport and translational control. Furthermore, all *FXR* proteins are associated with translating polyribosomes in the form of messenger ribonuclear particles (mRNP) [99,100]. FMRP, FXR1P and FXR2P are able to form homo- and heteromeres in vivo [148]. Apparently, FXR1P and FXR2P cannot completely compensate completely for the absence of FMRP in the fragile X syndrome. However, considering the importance of FMRP function in synaptic plasticity and the overall expression in brain tissue a more severe phenotype would be expected. The *Fmr1/Fxr2* double knockout mouse shows that Fxr2p could compensate only part of the Fmrp function in behaviour. This indicates that FXR1P and FXR2P may have their own specific function. [125] For example, CYFIP2 binds to all FXR proteins compared to CYFIP1, which binds only to FMRP [149].

As mentioned before all *FXR* proteins are expressed in the brain, however, they show distinct

localisation in the testis. FXR1P is the only family member expressed at high level in striated muscle tissue. [100,105-109]). As a consequence the other two proteins are not capable to compensate for the loss of FXR1P in striated muscle. A study of an *Fxr1* knockout mouse proposed a role for FXR1P in muscle mRNA transport/translation control and structural costameric mRNAs, particularly [109]. A recent article describes *Fxr1* knockdown experiments in *Xenopus laevis*. *Fxr1* knockdown in *Xenopus* showed a highly specific muscle phenotype. It is shown that Fxr1p regulates the somite formation in the frog and it is suggested that Fxr1p may be essential during the formation of functional muscle [150].

Although all *FXR* proteins bind RNA, associate with ribosomes and interact with each other and seem to play a role in translational inhibition and/or RNA transport, functional differences between FMRP and its homologues suggest that each protein does have its own tissue- or stage-specific function as well.

Chapter 2

Two members of the *Fxr* gene family, *Fmr1* and *Fxr1*, are differentially expressed in *Xenopus tropicalis*

Chapter 2

Two members of the *Fxr* gene family, *Fmr1* and *Fxr1*, are differentially expressed in *Xenopus tropicalis*

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Abstract

The *FXR* gene family is composed of three members, *FMR1, FXR1* and *FXR2*. The *FMR1* gene is involved in the fragile X syndrome, whereas for the other two members no human disorder has been identified yet. An appropriate animal model to study *in vivo* gene function is essential to unravel the cellular function of the gene products FMRP, FXR1P and FXR2P, respectively. In *Xenopus tropicalis* both *Fmr1* and *Fxr1* were identified; however, unexpectedly *Fxr2* not. Here we describe the characterisation of both Fmrp and Fxr1p in *Xenopus tropicalis.* Fmrp is expressed ubiquitously throughout the embryo during embryonic development, whereas Fxr1p shows a more tissue-specific expression particularly during late embryonic development. In adult frogs both proteins are highly expressed in most neurons of the central nervous system and in all spermatogenic cells in the testis. In addition, Fxr1p is also highly expressed in striated muscle tissue. Western blotting experiments revealed only one prominent isoform for both proteins using different tissue homogenates of adult frogs. Thus, for *in vivo* gene function studies this relative simple animal model may serve as a highly advantageous and complementary model.

FMRP, FXR1P and FXR2P belong to a small family of fragile X-related proteins (*FXR* family). Absence of FMRP in neurons of the central nervous system is the cause of the fragile X syndrome, the most common inherited form of mental retardation in humans [1]. The fragile X syndrome is a neurodevelopmental disorder characterised by immature dendritic spines and altered synaptic strength [2]. The absence or dysfunction of both FXR1P and FXR2P has not yet been defined to a human disease; however, a function of FXR1P in striated muscle development has been suggested [3,4]. The cellular function of FXR2P, like FMRP, seems to be related to learning processes [5]. For all three genes mouse models have been generated and specific phenotypes have been reported [4-6]. Unfortunately, *in vivo* gene function studies in mammalians, including mice, focusing on the period of embryonic development are difficult. As a first step to generate a more suitable vertebrate animal model to study the physiological function of the three *Fxr* genes during embryonic development an initial characterisation of the *Fxr* gene family has been performed in *Xenopus tropicalis*. Unlike the slow-growing, tetraploid *Xenopus laevis*, however, *Xenopus tropicalis* is diploid and has a relatively short (<5 months) life cycle.

A high level of sequence conservation of the *FXR* genes throughout various vertebrates (mouse, rat, zebrafish) has been demonstrated [7]. This was used to screen the Sanger Institute *X. tropicalis* EST database with sequences from the human *FXR* family members to find orthologues of the *X. tropicalis Fxr* genes. Interestingly, only complete coding sequences for both *XtFmr1* and *XtFxr1* could be compiled from overlapping ESTs. Using the JGI *X. tropicalis* genome assembly release v3.0 which includes a total of 33,749 gene models at a coverage of 7.4X, combined with the Sanger Institute *X. tropicalis* EST database did not yield a potential *XtFxr2* gene. Apparently, an ortholog of *FXR2* is absent in this particular species. Figure 1a illustrates a comparison of human FMRP and FXR1P with the *X. tropicalis* Fxr-protein family. Overall identities between human and *X. tropicalis* FMRP and between human and *X. tropicalis* FXR1P was 76% and 82%, respectively. Both *X. tropicalis* 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 exon 11 and 12 in human was missing from *FMR1* in *X. tropicalis*, which is in accordance with earlier notions of *FMR1* obtaining these sequences during mammalian evolution. In contrast to mouse, both human and *X. tropicalis* show the presence of the largest transcript containing the 51 basepairs of exon 17. Interestingly, no CGG repeats were found in the 5' UTR sequences of the *XtFmr1* gene. CGG repeat expansion to over 200 units is the mutational mechanism found in the vast majority of fragile X patients. In addition, a phylogenetic analysis was performed, including human, mouse, zebrafish and frog proteins. A clear clustering of the respective *X. tropicalis* proteins with their predicted orthologues could be demonstrated (Figure 1b). There is no indication for a separate development of the *X. tropicalis* Fxr family. The similar branch evolution of the three clusters makes an absence of *XtFxr2p* unexpected. Further Blast search through the JGI *X. tropicalis* genome database version 2.0 with the longest coding sequences for both *XtFmr1* and *XtFxr1* revealed the genomic structure of both genes. Alignments of these virtual gene structures with known human/mouse data showed a 100% identical exonic structure (data not shown).

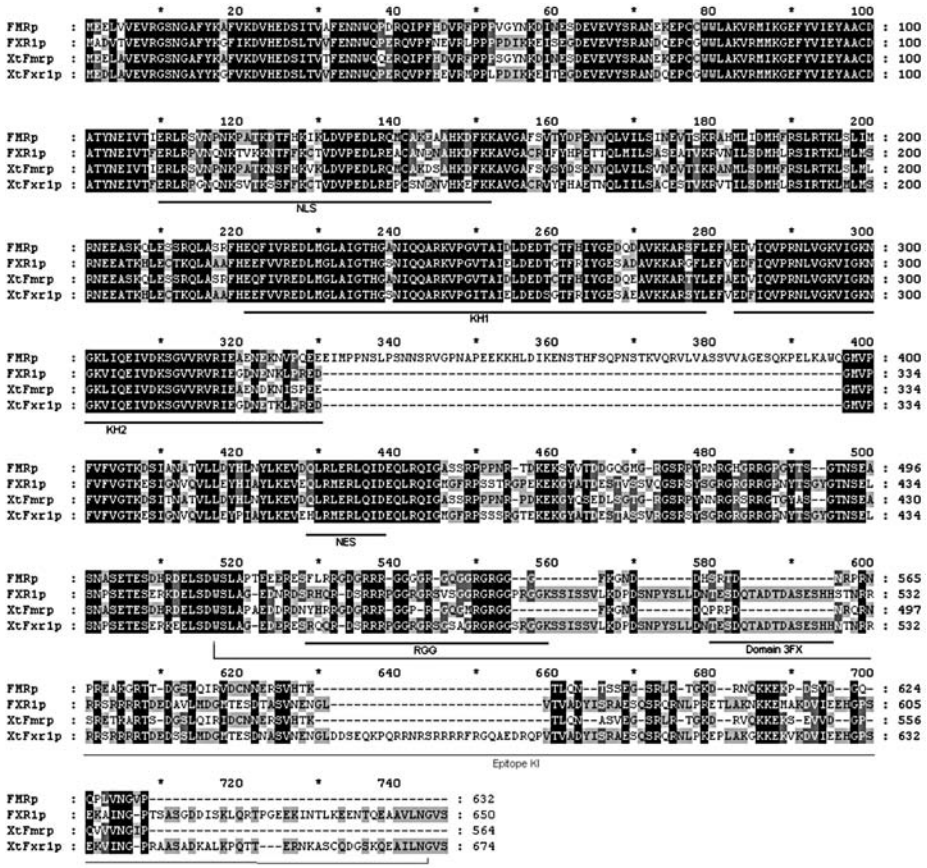

Figure 1A. Amino acid sequence alignment of *X. tropicalis Fmrp* and Fxr1p with their human orthologues. Identical residues are shaded in black and conserved substitutions in grey. The alignment shows highly homologous regions of both Fmrp and Fxr1p between human and *X. tropicalis*. All orthologues contain two KH domains, a nuclear localisation signal (NLS), a nuclear export signal (NES) and an RGG box. In addition, the peptide sequence of the epitopes of the antibodies are depicted.

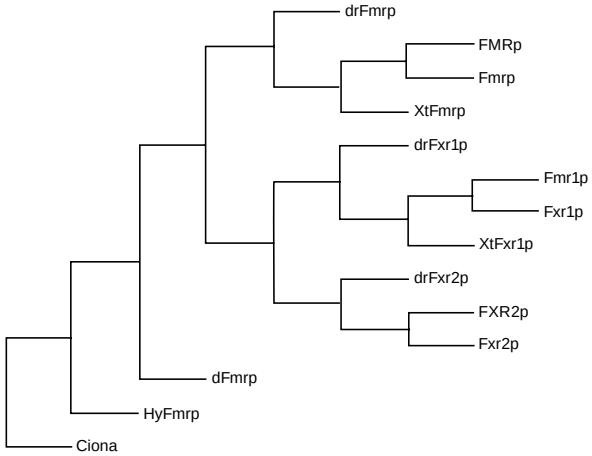

Figure 1B. Phylogenetic analysis of the Fxr gene family members for human (*FXR*), mouse (*Fxr*), zebrafish (*drFxr*) and frog (*XtFxr*).
The early evolutionary origin of this family is indicated by the *Fmr1* like genes from Ciona, Hydractinia (*hyFmr1*) and fruitfly (*dFmr1*)

Protein expression studies were performed with specific antibodies against both Fmrp and Fxr1p. First, transfection studies were performed to study the occurrence of cross-reactivity of our antibodies between the different Fxr-proteins. Both proteins share a high homology and knockout tissue from frogs is not available, thus, HEK293T cells were transfected with an expression construct containing either *XtFmr1* or *XtFxr1* preceded by an EGFP sequence. Expression of the construct was monitored by the presence of green fluorescent HEK293T cells. For the two fusionproteins (EGFP-Fmrp and EGFP-Fxr1p) no cross-reactivity with our antibodies could be observed (Figure 2).

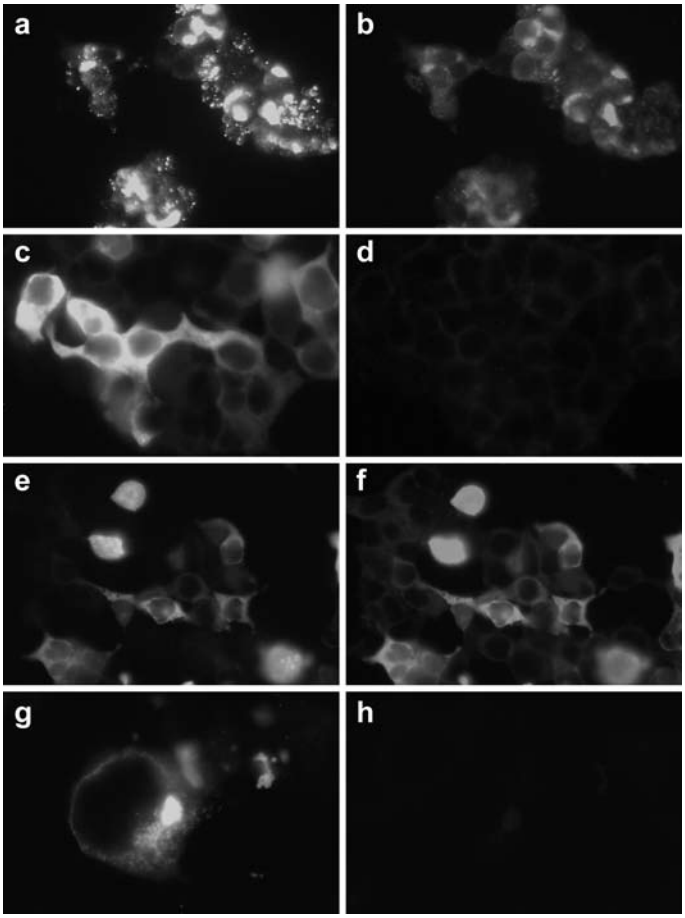

Figure 2. Specificity of antibodies KI and 3FX against XtFmrp and XtFxr1p, respectively.
GFP signal in HEK293T cells that were transfected with either *XtFmr1*-EGFP (a and g) or *XtFxr1*-EGFP (c and e). Cells were simultaneously stained immuno-cytochemically (red) using either antibody KI (b and d) or 3FX (f and h). Specific labelling of antibody KI was only observed in the *XtFmr1*-EGFP transfected cells (b). The absence of KI positive-labelling in the *XtFxr1*-EGFP transfected cells (d), indicates lack of cross-reaction of this antibody with Fxr1p. Similarly, antibody 3FX shows only a specific labelling with Fxr1p (f) and no cross-reactivity with Fmrp (h).

Antibody KI specifically recognized Fmrp in HEK293T cells transfected with the EGFP-*Fmr1* expression construct (Figure 2b; red), whereas Fxr1p labelling was totally absent in HEK293T cells expressing EGFP-Fxr1p (Figure 2d). Similar results were obtained with antibody 3FX against Fxr1p. Figure 2f (red) illustrates the presence of Fxr1p with antibody 3FX using expression construct EGFP-*Fxr1* and the lack of cross-reactivity with EGFP-Fmrp using construct EGFP-*Fmr1*(Figure 2h).

Next, the cellular distribution of Fmrp was studied in embryonic stages and adult frogs using an indirect immunoperoxidase technique (Figure 3).

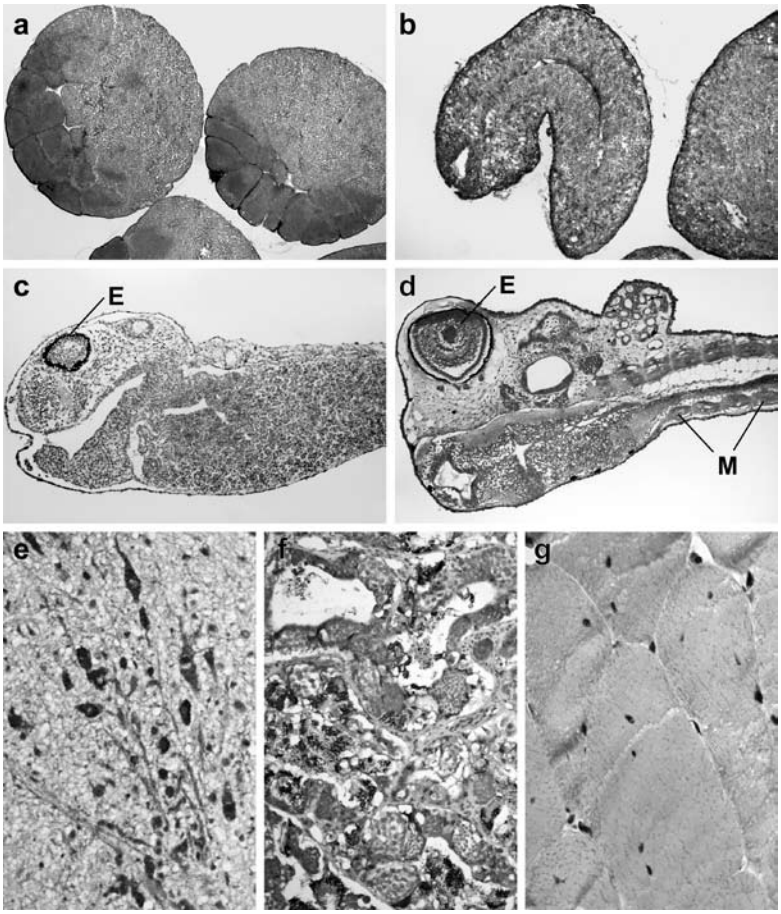

Figure 3. Fmrp distribution in *X. tropicalis* embryos and adult frogs using antibody KI.
Fmrp staining is present in the nuclei in stage 6 (a). The cells composing the animal pole show the typical large nucleus. At stage 23, when internally segregation of the brain occurs, Fmrp expression is evenly distributed in the cytoplasm of most cells (b). At stages 37 (c) and 46 (d), Fmrp expression remains ubiquitous with high expression in the eye, brain, intestine and skeletal muscle tissue. In contrast, adult *X. tropicalis* show a tissue-specifc expression pattern with high expression in the cytoplasm of most neurons in the brain (e) and all the spermatogenic cells of the testis (f). Note the absence of Fmrp in skeletal muscle (g).

Paraffin sections of embryos at stages 6, 23, 37 and 46 of development representing 2hrs, 1d, 2d and 7d after fertilisation, respectively, were studied. Fmrp showed a nuclear staining at 2 hours post fertilisation (2 hpf; Figure 3a) that changed to a cytoplasmic labelling throughout all cells in the later stages (Figures 3b-3d).

In adult frogs, Fmrp distribution showed a tissue-specific pattern. Fmrp was abundantly present in the cytoplasm of most neurons in the brain (Figure 3e) and in all the spermatogenic cells of the testis (Figure 3f). Notably, Fmrp could not be detected in striated muscle tissue (Figure 3g for skeletal muscle). Our results for embryonic Fmrp expression in *X. tropicalis* are not completely in line with previous reports on mouse and zebrafish expression patterns that showed

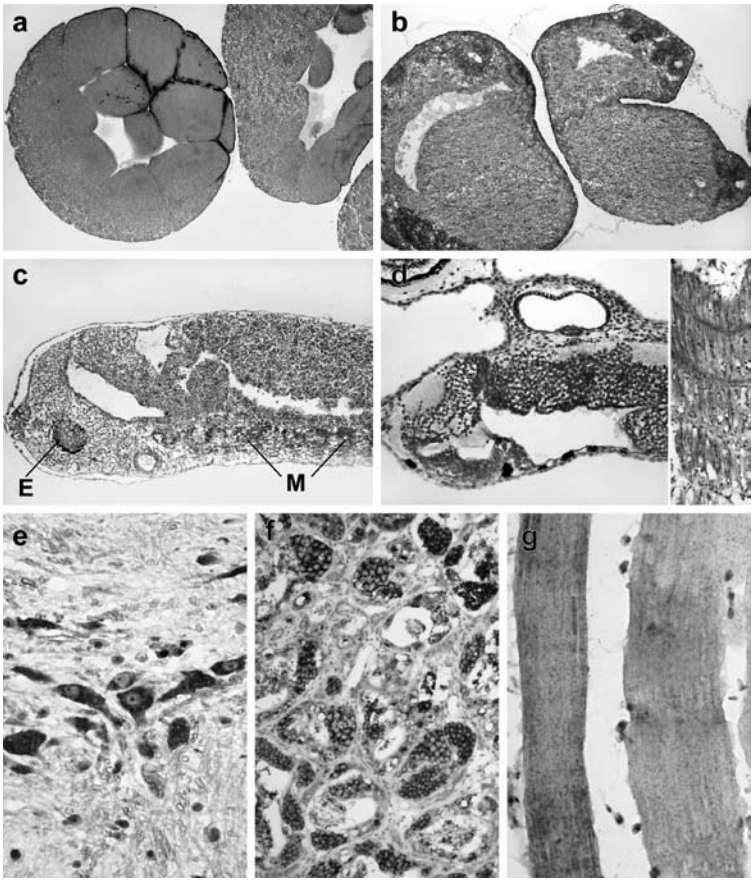

Figure 4. Fxr1p distribution in *X. tropicalis* embryos and adult frogs using antibody 3FX.
Fxr1p staining is detected in the nuclei of pre MBT cells (a, (=morula); stage 6). At stage 23, high Fxr1p expression is predominantly present in structures that will develop into muscle and brain tissue (b), although a weak labelling is also present in the other cell types. At stages 37 (c) and 46 (d), Fxr1p shows a tissue-specific expression pattern with a high expression in myoblasts of muscle tissue and neurons of the brain. The inset in (d) shows a high magnification of Fxr1p labelled myoblasts. In adult *X. tropicalis* , Fxr1p expression is defined to the cytoplasm of neurons in the brain (e) and all the spermatogenic cells in the testis (f). In addition, a granular labelling pattern is observed in skeletal muscle tissue (g).

an overall expression during very early embryonic development that became tissue-specific at later stages [8](van't Padje, personal comm.). Apparently, the tissue-specific Fmrp expression in frogs occurs later in development compared to mouse and fish. Interestingly, the nuclear distribution of Fmrp during very early development (2 hpf) has been described for zebrafish as well (van't Padje, personal comm.). In adult frogs, a similar tissue-specific expression of Fmrp in brain and testis has been reported in human, mouse and fish. However, the presence of Fmrp in all spermatogenic cells of the testis has not been observed in the other vertebrates that showed only Fmrp expression in spermatogonia at the basal membrane of the seminiferous tubules [8,9].
In contrast, Fxr1p distribution shows a more tissue-specific pattern during embryonic development. At a very early stage (2 hpf), Fxr1p showed a nuclear staining like Fmrp (Figure 4a). However, at stage 23 when internally segregation of the brain occurs a spatial organisation of Fxr1p developed more preferentially in structures that will develop into muscle (mesodermal) and neural (ectodermal) tissue (Figure 4b). From stage 37 onwards a further tissue-specific expression was observed with a high expression in the cytoplasm of neurons and skeletal muscle tissue (Figure 4c-4d). In adult frogs, Fxr1p distribution remained tissue-specific. A strong labelling is observed in the cytoplasm of neurons (Figure 4e), all the spermatogenic cells of the testis (Figure 4f) and skeletal muscle tissue (Figure 4g). This Fxr1p expression pattern during embryonic development and adult stages showed high similarities with the expression patterns described for man, mice and fish [3,7,9,10].

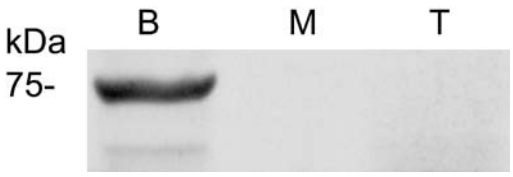

Figure 5. Western blot analysis of adult *X. tropicalis* tissues using antibodies KI (a) and 3FX (b).
In homogenates from brain (a; lane B) and testis (a; lane T) a single isoform of approximately 72kDa could be detected. Importantly, homogenates of HEK293T cells transfected with *XtFmr1-EGFP* showed a prominent band representing the Fmrp-EGFP fusion protein of approximately 99 kDa (a; lane 2), whereas transfection with *XtFxr1-EGFP* did not results in any detectable isoform (a; lane 1). For Fxr1p, a weak band of approximately 80 kDa could be detected in brain (b; lane B) and in testis this band was more prominent (b; lane T). In skeletal muscle homogenates a single isoform of approximately 84 kDa appeared (b; lane M). The specificity of antibody 3FX is illustrated by the detection of the Fx1p-EGFP fusionprotein in *XtFxr1-EGFP* transfected HEK293T cells with a size of approximately 107 kDa (b; lane 1) and the absence of any isoform in *XtFmr1-EGFP* transfected HEK293T cells (b, lane 2).

Both Fmrp and Fxr1p share the nuclear labelling during very early development, which suggests a specific nuclear function during this short period of development. Most likely, translation of maternally provided transcripts account for these gene products. The high expression of Fxr1p in skeletal muscle tissue at late embryonic stages and in adult frogs suggests a role for Fxr1p in myogenesis. Similar to striated muscle tissue from mouse and zebrafish, the characteristic

granular labelling of Fxr1p present in skeletal muscle from frogs suggests that Fxr1p is located in specific structures of the muscle tissue, called costameres [3,4,7].

In man and mice, different isoforms of Fxr1p have been described for the different tissues, due to alternative splicing [8-11]. In contrast, Fmrp isoforms were identical for all the different tissues tested, that is, the presence of four major isoforms. Here, Western blotting was performed to study the presence of specific isoforms in frog tissues. Using antibody KI, against Fmrp, only one major isoform of 72 kDa could be detected in both brain and testis tissue from *X. tropicalis*. As a control, homogenates from HEK293T cells transfected with an expression construct containing EGFP-*Fmr1* or EGFP-*Fxr1* were incubated with antibody KI. The lane loaded with the homogenate containing EGFP-Fmrp fusion proteins revealed a band of 99 kDa (72kDa for Fmrp and 27 kDa for EGFP), whereas the other lane loaded with EGFP-Fxr1p fusion proteins was totally devoid of specific isoforms (Figure 5a). Antibody 3FX, against Fxr1p, showed the presence of a single molecular form of approximately 80 kDa in homogenates from both brain and testis tissue. In skeletal muscle tissue Fxr1p is present as a prominent single isoform of approximately 84 kDa. The specificity of the 3FX antibody was illustrated by the presence and absence of cross reactive material in the lane loaded with a homogenate from HEK293T cells transfected with *XtFxr1* and *XtFmr1*, respectively (Figure 5b). These results demonstrate that *X. tropicalis* expresses fewer *Fxr* isoforms compared to man and mice.

In conclusion, we report here the characterisation of Fmrp and Fxr1p in *Xenopus tropicalis*. Both proteins share the major functional domains with their murine and human orthologues and show a differential expression pattern. Importantly, the tissue-specific expression of both proteins in adult frogs is comparable to the other vertebrate systems; however, the number of isoforms is restricted to one major isoform, which makes this model more simple to study *in vivo* gene function. Additionally, *Xenopus* is a widely used developmental model organism with a fast external (transluscent) development, many eggs per breeding and the recent development of novel gene knockdown technology using the morpholino strategy. All together, this makes *X. tropicalis* an attractive complementary model system to study the physiological function of Fmrp and Fxr1p, especially during embryonic development.

Experimental procedures

Xenopus tropicalis were obtained from the Hubrecht laboratory, Utrecht and originate from a 6th generation Nigeria inbred line. Methods of egg collection, fertilisation, and embryo culture were as described [12]. Developmental stages were assigned according to Nieuwkoop and Faber [13].

The Sanger Institute *Xenopus tropicalis* EST database was searched for orthologues (www.sanger.ac.uk/Projects/X. _tropicalis/).

Sets of oligo nucleotide primers were designed and used to extract mRNA sequences by RT-PCR from either adult brain for *XtFmr1* or whole embryos, stage 37. For *XtFxr1* adult skeletal muscle was used.

Phylogenetic analysis has been performed using the Treetop phylogenetic tree prediction program from GeneBee Services (http://www.genebee.msu.su)Rabbit antibody KI was raised against the C-terminus of FMRP [14,15] while monoclonal 3FX antibody was raised against a synthetic polypeptide that was common to the long and short isoforms of FXR1P [10]. HEK293T cells were

transfected with lipofectamine PLUS according to the manufacturer. For immunofluorescence analysis of transfected cells either an anti-rabbit or anti-mouse secondary antibody conjugated with TRITC was used. The details of the Western blotting procedure and the indirect immunoperoxidase method on paraffin sections has been described before [8].

Acknowledgements

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Chapter 3

Characterisation of Fmrp in zebrafish; evolutionary dynamics of the *fmr1* gene

Chapter 3

Characterisation of Fmrp in zebrafish; evolutionary dynamics of the *fmr1* gene

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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 adult and embryos of zebrafish using immunohistochemistry and Western blotting with specific antibodies raised against zebrafish Fmrp. We 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 the zebrafish Fmrp and the 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.

Introduction

With a prevalence of 1 in 4000 males and 1 in 6000 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 [1]. 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 [2,3].

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)[4,5]. In human, FMRP is highly expressed in neurons of the brain and early spermatogonia in the testis [6,7]. At the subcellular level, FMRP is present in mRNP (messenger ribonucleoprotein) particles associated with actively translating ribosomes [8-11]. 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. [12-16]. 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 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 [17,18]. 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. showed tissue-specific Fxr1p expression in skeletal muscle and brain of adult zebrafish at the translational level (Engels et al., 2004).

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 testis [19]. In addition, ultrastructural studies of the brain revealed the presence of abnormal dendritic spines illustrating reduced pruning and/or maturation of spines [20-22]. Notably, spine abnormalities have already been reported in fragile X patients in earlier studies using brain autopsy material [23,24]. 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

Zebrafish line was obtained from the Wageningen ZF WT Zodiac F5 line. Fish were kept at 25°C in a 12-hour light/dark cycle and fed artemia 3 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: ENSDARP00000031163).

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 part between the *fxr* genes. The antibody (named 758) against Fmrp was affinity purified against the synthetic peptide and used 1: 400 for immunohistochemistry and 1:4000 for Western blotting.

Zebrafish *fxr*-EGFP constructs

Total RNA was isolated from adult brain tissue and cDNA was synthesized 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 EcoRI restriction site and are located at the start- and stopcodon, respectively. The PCR product was digested with EcoRI and cloned into pEGFP-C1 vector from Clontech. For *fxr1*-EGFP and *fxr2*-EGFP constructs, see Engels *et al.* [25]. 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-wells plate at low density. Transfection with *fmr1*-EGFP,

fxr1-EGFP and *fxr2*-EGFP constructs has been 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 minutes. 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 euthanized in tricaine (3-amino-benzoic ethylester, Sigma, 25g/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 (5µm) were deparaffinized, rehydrated and microwave-treated according to standard protocols [26]. 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 hour at room temperature. Subsequently, a 1-hour incubation with a peroxidase conjugated secondary antibody was performed. For signal detection 3,3, di-amino-benzidine was used as a substrate (DAKO). Sections were counterstained with hematoxylin and mounted with Entellan (Merck).

Western blot analysis

Zebrafish tissues (brain, testis and skeletal muscle) and transfected COS cells were homogenized in Hepes buffer (10mM Hepes, 300mM KCL, 5mM MgCl2, 0.45% Triton, 0.05% Tween, pH 7.6) containing Complete protease inhibitor cocktail (Roche), sonicated and centrifuged for 10 minutes 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; 1:5000) 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 part and showed lesser similarity at the C-terminal part of the protein (figure 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 exon 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.

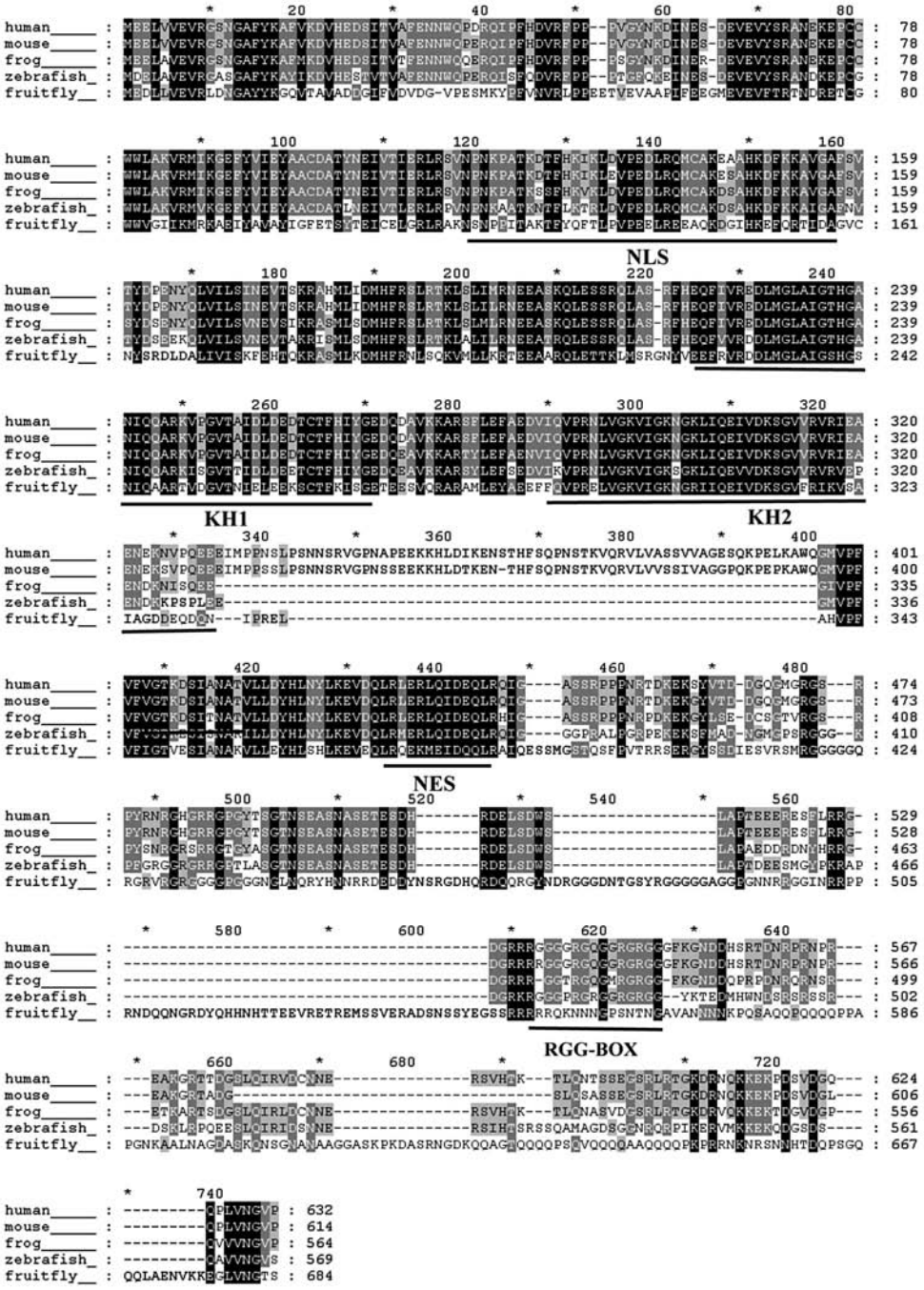
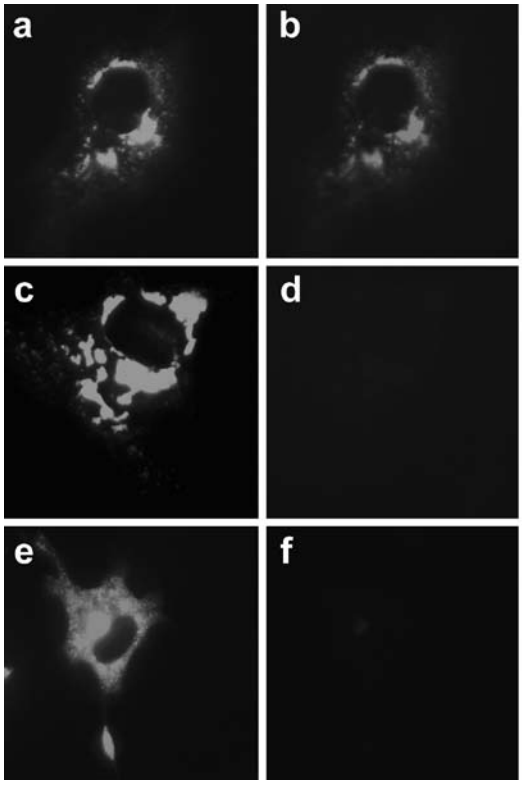


Figure 1. Amino acid sequence alignment of *FMR1* proteins (Fmrp) of several species.
Identical residues are shaded in black and conserved substitutions in grey. The alignment shows highly homologous regions of Fmrp between human, mouse, frog, fruitfly and zebrafish. All orthologs contain the nuclear localisation signal (NLS), two KH domains, nuclear export signal (NES) and an RGG box.

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 (figure 1). To test the specificity of the antibody 758, COS cells have been 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 is observed for both the GFP-fluorescence and staining with antibody 758 (figures 2a and 2b, respectively). On the other hand, in both *fxr1*-EGFP (figures 2c and 2d) and *fxr2*-EGFP (figures 2e and 2f) transfected cells only a clear GFP signal could be detected (figures 2c and 2e, 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 (figures 2d and 2f, respectively).

Western blotting analysis of the *fmr1*-EGFP transfected COS cells homogenate shows a band of approximately 100 kDa, that corresponds to the expected size of the Fmrp-EGFP fusion protein (figure 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 (figure 2g, lane 2 and 3, respectively).



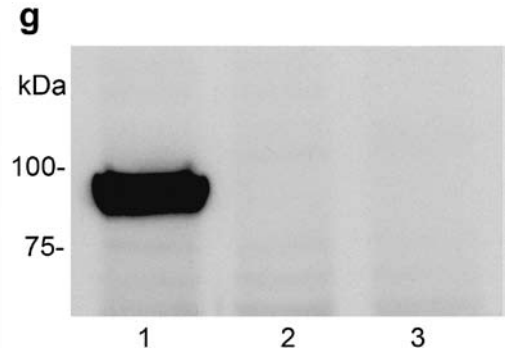


Figure 2. Specificity of antibody 758 against zebrafish Fmrp.
GFP signal in COS cells that were transfected with *fmr1*-EGFP (a), *fxr1*-EGFP (c) and *fxr2*–EGFP constructs (e). Cells were also stained immunocytochemically using antibody 758 against zebrafish Fmrp. Specific labelling by antibody 758 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 and f). Western blot analysis of homogenates of the *fmr1*-EGFP, *fxr1*-EGFP and *fxr2*–EGFP over expressing COS cells (g, lanes 1, 2 and 3, respectively), only shows a band in lane 1, confirming these results.

Localisation of Fmrp in adult and embryonic tissues of zebrafish

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

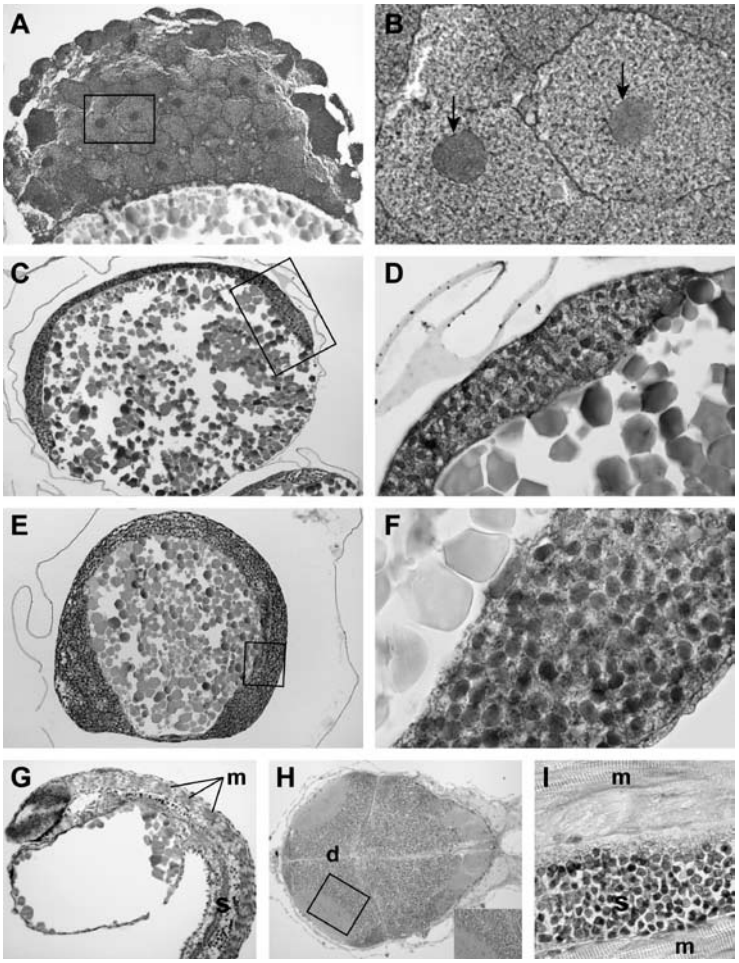


Figure 3. Immunohistochemistry on zebrafish embryos using antibody 758.
In 3 hours post fertilisation (hpf) (a and b), 6hpf (c and d), 12 hpf(e and f) and 24 hpf (g) embryos, Fmrp was ubiquitously expressed throughout the embryo. Note the nuclear staining in cells of the 3 hpf embryo (b, arrows). In 72 hpf (h and i) embryos 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

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 figures 4a and 4b. In sections of the testis, only a very weak signal could be observed in immature spermatogenic cells, including spermatogonia and early spermatocytes (figure 4c). No labelling was observed in skeletal muscle (figure 4d).

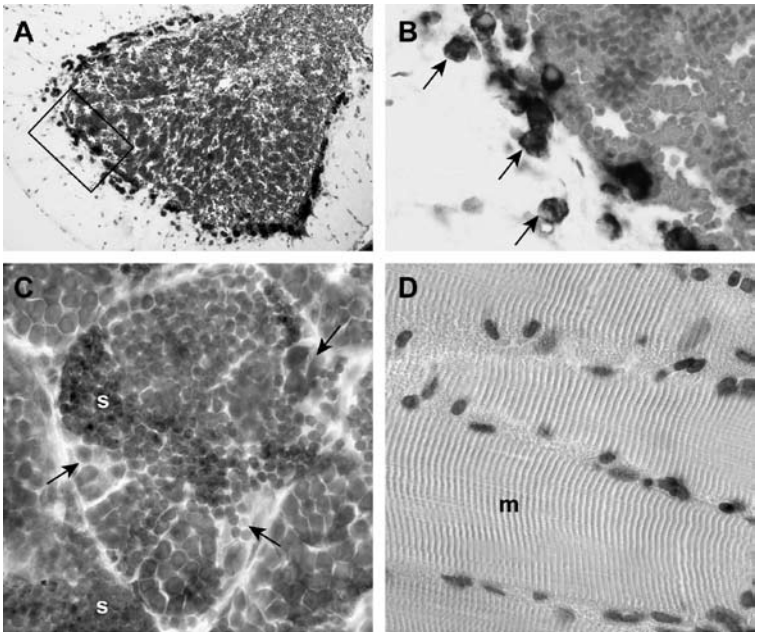
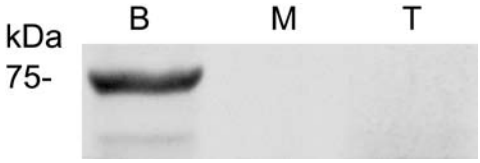


Figure 4. Immunohistochemistry on adult tissues of zebrafish using antibody 758.
In adult zebrafish tissues, high Fmrp expression could be detected in all the neurons throughout the brain. Here we show, as an example, Fmrp expression in Purkinje cells of the cerebellum (arrows). In testis, a very weak 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).

Detection of isoforms in adult zebrafish tissues

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

Figure 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 testis, no isoforms could be detected.



Discussion

After identification of the *FMR1* gene as the gene involved in fragile X syndrome, fundamental research focused 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 the aspects of the human disease. Thus far, often the mouse is 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 [19]. Interestingly, morphological spine abnormalities and delayed maturation of spines were already present directly after birth in *Fmr1* knockout mice [20,21]. 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 characterisation 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 part of the protein. This confirms the high degree of evolutionary conservation of the *FMR1* gene [27,28].

We raised a specific antibody (758) against zebrafish Fmrp to study the expression pattern and the presence of the different molecular isoforms in several tissues of both adult 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 (3hpf) 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 been demonstrated only in transfected COS cells and in some neurons of adult murine brain [9,26]. In addition, small amounts of FMRP were present in the nucleus after leptomycin B treatment of transfected COS cells [29]. It has been suggested that FMRP shuttles 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 [5]. Apparently, this specific function of Fmrp is more prominent during this early period of embryonic development. After 72 hours 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 [30]. In a recent study Tucker et al. 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 (Tucker et al., 2004). Although our present study focused 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 doesn't necessary 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 the tissue-specific expression in later stages and adult zebrafish is the result of tissue-specific transcription.

Although FMRP is widely expressed, the localisation of high quantities of FMRP has been restricted to most neurons of human and mouse adult brain and spermatogonia within the testis [6,7,26]. In adult zebrafish tissues, Fmrp appeared to be primarily expressed in brain. Labelling was found in all differentiated neurons of the brain, including motorneurons, 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 extreme low Fmrp expression in immature spermatogenic cells in the testis of zebrafish and total lack of Fmrp in Western blot studies using testis homogenates from zebrafish differs from human and mouse. This suggests a less important Fmrp function within this tissue; however, we cannot exclude that testis 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 [3,26]. In Western blot analysis, antibody 758 revealed 2 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 localisation and functional domains, however, FXR1P is highly expressed in striated muscle tissue whereas FMRP and FXR2P are absent in striated muscle tissue. 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 localised in the costameric protein network. Apparently, Fxr1p has an 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. Considering 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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Chapter 4

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

Chapter 4

Characterisation 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 proven to be valuable in elucidating the function of these genes, particularly in adult mice. Unfortunately, *Fxr1* knock out 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 characterisation 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 localised at the myoseptum and in costamere-like granules in skeletal muscle. In the testis, Fxr1p is localised 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 characterised.

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

Introduction

The small family of fragile X related (*FXR)* proteins consists of three proteins, including FXR1P, FXR2P and FMRP, the lack of which in neurons causes the fragile X syndrome [1,2]. 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 [3-8]. The highly homologous *FXR* proteins share several functional domains among which two KH RNA binding domains and an RGG box, as well as nuclear localisation and export signal sequences [9-13]. 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 [7,14-18]. 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 [19-22]. *In vitro* studies demonstrated the interaction of the three homologs with each other as homomers and heteromers [23]. 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 [24].

To study the physiological function of the three genes animal models have been created. The *Fmr1* knock out mice display deficits in visual spatial performance and have macroorchidism, illustrating similarities between fragile X patients and this mouse model [25]. In addition, *Fmr1* knockout mice show altered dendritic spine morphology, indicating a reduced maturation/pruning of spines [26]. *Fxr2* knockout mice show a mild learning and behaviour phenotype [27]. 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 [8]. 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 [8].

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 in regard to developmental studies as it has a fast external development and developing zebrafish remain translucent until the embryos are free swimming and organogenesis is completed. 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 [28].

In the present study an initial characterisation 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

Fish 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 hr light/dark cycle and fed artemia 3 times a day.

Dissection of zebrafish

Male fish were euthanized in a 0.2 g/l solution of tricaine (3-amino-benzoic ethylester Sigma, St. Louis, 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) with random hexamers and oligo dT according the manufacturer's instructions.
Muscle and brain cDNA was amplified with Pfx DNA polymerase (Invitrogen, Carlsbad, 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 intopEGFP-C3 (Clontech, Palo Alto, USA) using the EcoR1 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 PCR using the primers: f3: 5'-AAGCGACGACGAACATGGAC-3' and r4: 5'-ATGCAAGCAGGGACAGAGTT-3', and subsequently subcloned into pEGFP-C3 (Clontech) using the EcoR1 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 sequence from zebrafish Fxr1p: AESQSRQTNPRDTRK that subsequently were coupled to 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 in a 1:500 dilution for immunoblotting and 1:25 dilution for immunohistochemistry. Antibodies against MANDRA1 (mouse anti-dystrophin; Sigma) and vinculin (goat anti-vinculin; Santa Cruz) were used for immuno-histochemistry 1: 1000 and 1: 400, respectively. Antibodies against P0 and Staufen were from Immunovision (human; Bereldange, Luxembourg) and Chemicon (rabbit; Temecula, USA), respectively, and were used in a 1: 100 dilution for immunohistochemistry. The secondary antibodies swine anti-rabbit conjugated with HRP and rabbit anti-mouse conjugated with HRP were obtained from DAKO (Glostrup, Denmark). Goat anti-rabbit/mouse/human conjugated with TRITC or FITC was obtained from Sigma.

Cryosectioning and immunohistochemistry

Adult zebrafish were euthanized (see above) and embedded in Tissue-Tek (Sakura Finetek Europe B.V.). Using a Leica Jung CM3000 cryostat, 7µm sections were cut and thaw-mounted on microscopic slides. Sections were fixed at room temperature for 10 min in 4% paraformaldehyde in 0.1 M Sorrensen buffer, pH 7.3 followed by a permeabilisation 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 using 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) for 5 min. Incubation with primary antibodies was 1.5 hr 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 hr at room temperature. After three washes with PBS+, slides were either covered with a coverslip using Vectashield containing Dapi (Vector Laboratories, Burlingame, USA) or further incubated with DAB-substrate (DAKO) for 6 min, followed by washing in tap water. Finally, sections were counterstained using haematoxylin and embedded in Entellan (Merck, Darmstadt, Germany). Slides were examined with 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% FCS (Gibco Brl) under 5% CO_2 at 37 °C. Cells were seeded on coverslips or in 6 wells 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 mM Tris pH7.5, 140 mM NaCl, 0.1% deoxycholate, 0.1% sodium dodecylsulphate, 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 was followed as for the immunohistochemistry.

Western blotting

Zebrafish tissues were homogenized in Ripa, centrifuged at 12000 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 on 7.5% SDS-PAGE and immunoblotted according to standard protocols.

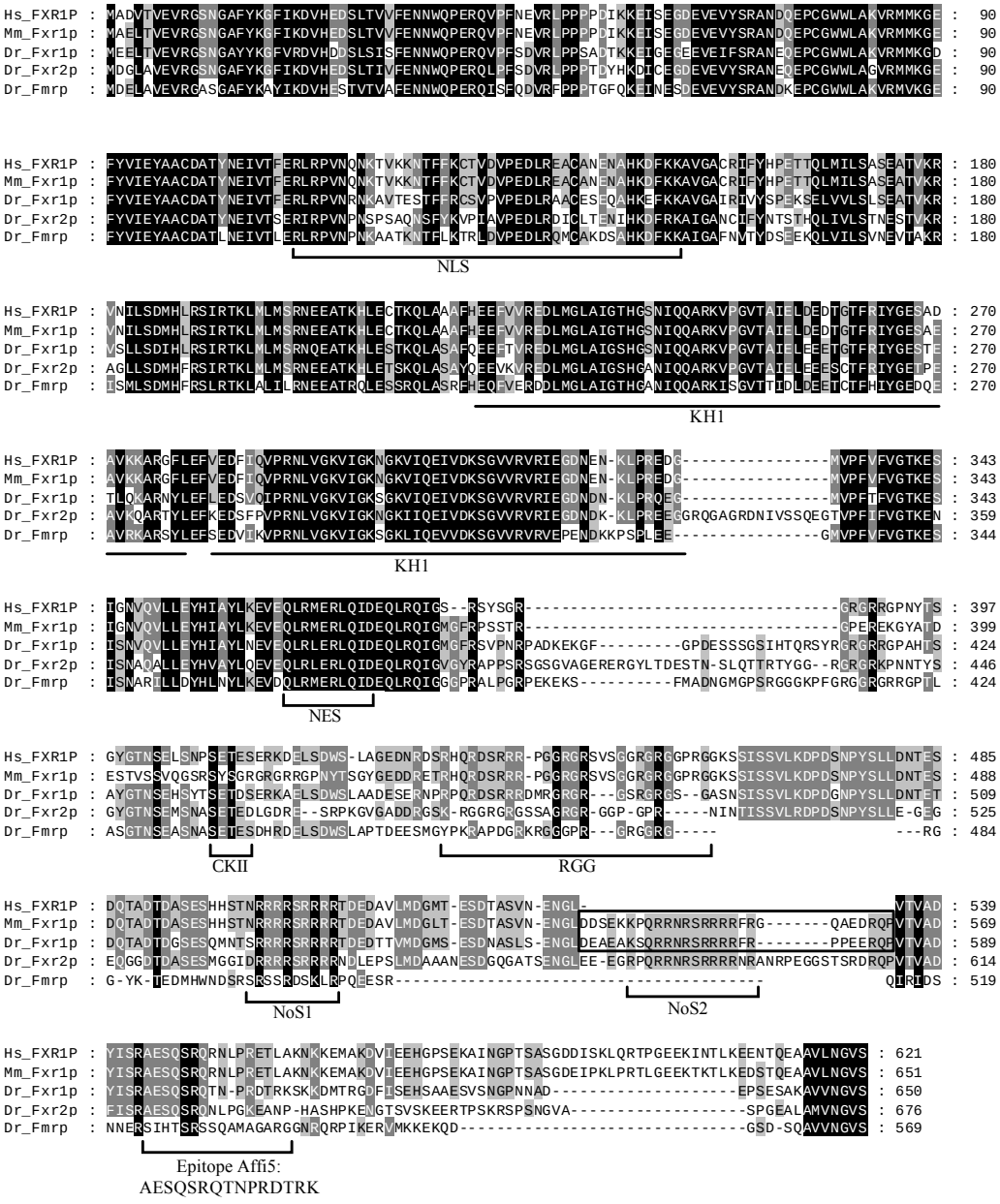

Figure 1. Comparison of human FXR1P and the zebrafish *Fxr*-protein family.
Identical residues are shaded in black, and conserved substitutions in grey. The following functional domains are indicated: the nuclear localisation signal (NLS), the two KH1 RNA-binding domains (KH1), the nuclear export signal (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.

Results

Zebrafish *Fxr* genes are highly homologous

Zebrafish FXR cDNAs have been cloned [28,29] and sequences are available in the public domain (NCBI accession: NM_152963 for *fmr1*, BC055557 for *fxr1* and BC045999 for *fxr2*). As shown in the protein alignment (Figure 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 [30], Figure 1, boxed region).

Specificity of affinity purified polyclonal antibody against zebrafish Fxr1p

According to the protein alignment in Figure 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 cross reacts 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 (Figure 2A for GFP staining and 2B for Affi5 staining), whereas Cos-1 cells overexpressing Fxr2p-EGFP showed total absence of labelling for Affi5 (Figure 2D) while an intense staining could be detected for GFP fluorescence signal (Figure 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, and 27 kDa for EGFP. In contrast, Affi5 staining did not detect the Fxr2p-EGFP fusion protein (panel F, compare lanes1 and 2). Note that equal amounts of Fxr1-EGFP and Fxr2-EGFP fusion protein were present as shown by immunoblotting using antibodies against GFP (panel E, 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 using 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 (Figure 3A).

Although Fxr1p is highly expressed in the testis, the signal is restricted to the immature

spermatogenic cells (Figure 3B). Skeletal muscle showed the highest level of Fxr1p expression, where it was localised in granular structures throughout the muscle fiber and intensely at the sarcolemma. Furthermore, a very intense and granular staining was observed bordering the myoseptum (Figures 3C and 3D). The signal of Fxr1p at the myoseptum extends somewhat into the cytoplasm, more so than at the sarcolemma.

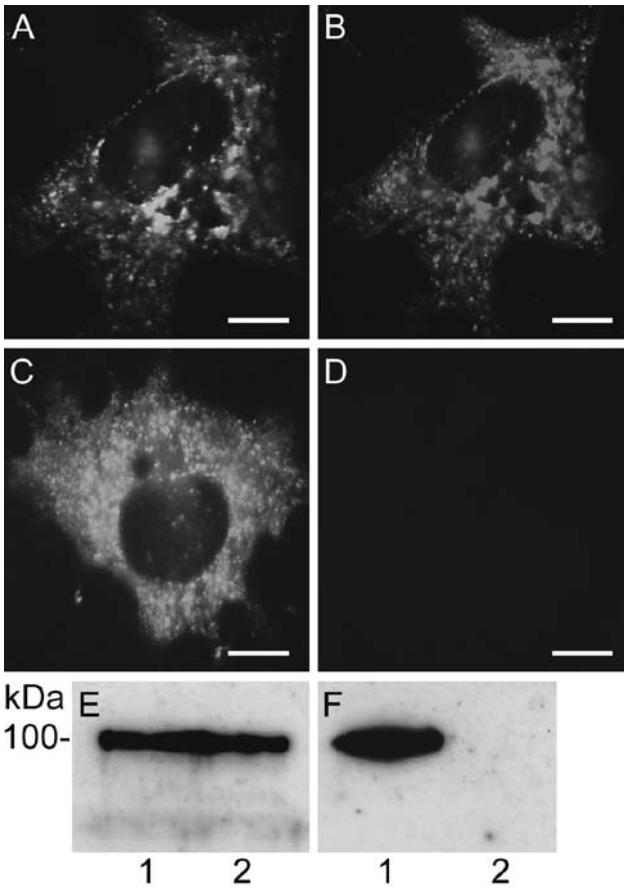

Figure 2. Specificity of affinity-purified antibody Affi5.
Cos-1 cells were transiently transfected with EGFP-*Fxr1* (A and B) or with EGFP-*Fxr2* (C and D), and stained with Affi5. The EGFP signal (A and C, green) is present in both transfected Cos-1 cells, while Affi5 labelling is only present in EGFP-*Fxr1* 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 10μm

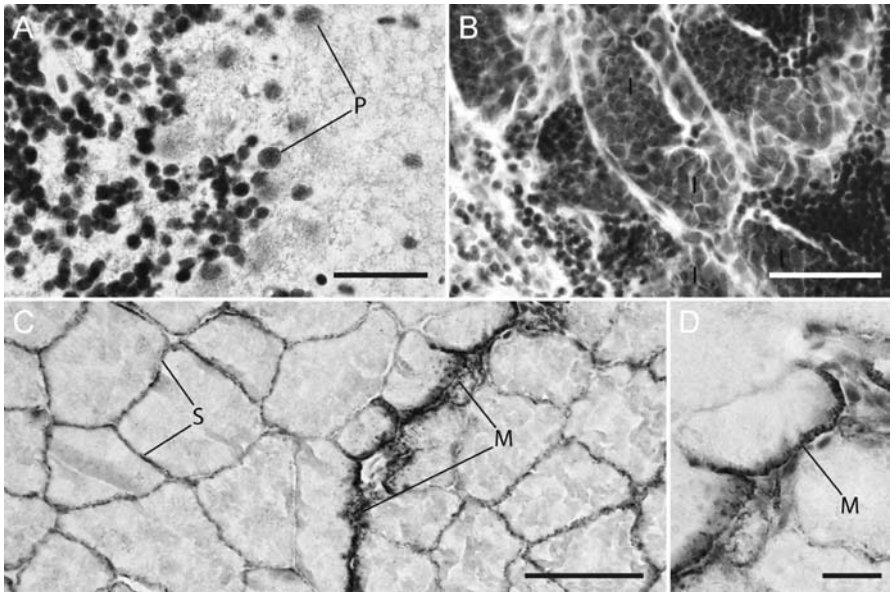

Figure 3. Immunohistochemical analysis of Fxr1p expression in adult zebrafish tissues.
Cryostat sections from adult zebrafish were immuno-incubated using Affi5 antibodies. In the brain, the testis and in skeletal muscle tissue significant Fxr1p expression was present. Note the nuclear labelling in Purkinje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

Fxr1p in the developing zebrafish

To study the expression of Fxr1p during embryonic development and to test whether it was localised 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 can already be detected in the 2/4 cell stage where it is distributed evenly over the cell mass (data not shown). At the dome /epiboly stage at 6 hpf (hours post fertilisation), Fxr1p was present at high levels in all the cells (Figure 4A). From early somitogenesis onward Fxr1p is expressed at very high levels in myoblasts throughout the somites. During the maturation of the embryos (1dpf - 5 dpf) the immunoreactivity gradually concentrates at the myosepta and at the sarcolemma in regularly placed granular structures. Figure 4B illustrates the weak Fxr1p expression in the head of an embryo 1 dpf, whereas the tail from 1 dpf embryos showed a high Fxr1p expression in myoblasts (Figure 4C). In embryos at 3 dpf Fxr1p expression level is moderate in the brain and very high in myoblasts (Figures 4D and 4E, respectively).

Fxr1p isoforms are differentially expressed in zebrafish tissues.

In the mouse, a number of different isoforms of Fxr1p has been described, due to extensive alternative splicing. We examined the presence of different isoforms of Fxr1p in zebrafish by

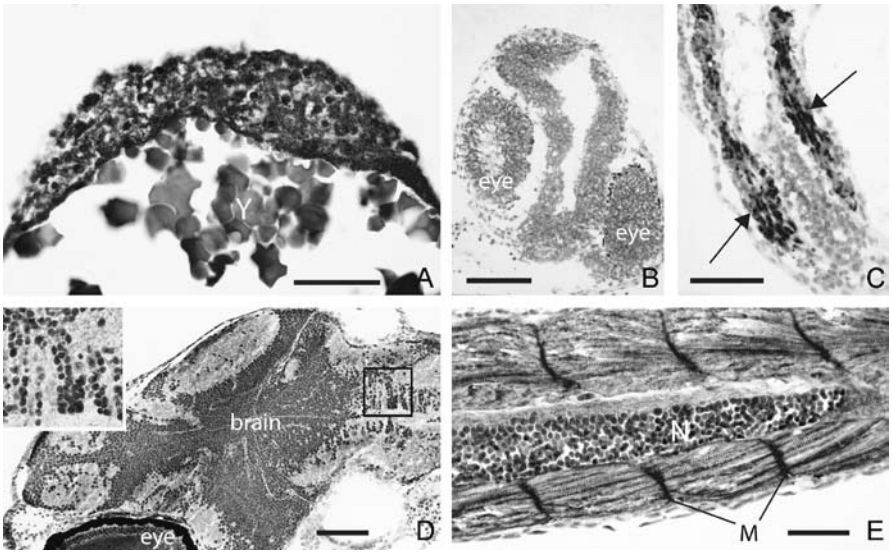

Figure 4. Immunohistochemical analysis of Fxr1p expression in zebrafish embryos during embryonic development
Longitudinal cryostat sections of 6 hpf embryos (A; bar 70 μm)), 1 day old embryos (B; head and C; tail; bars 30 and 15 μm, respectively) and 3 dpf embryos (D; head and E; tail; bars 100 and 35 μm, respectively) were stained for zebrafish Fxr1p using Affi5. The inset in D shows a higher magnification of the boxed region in the hindbrain from D. Note the nuclear staining in neurons from 3 dpf embryos (D) and the very intense staining of myoblasts in muscle tissue within the somites (E). The yolk sac visible in panel A is denoted by Y, the myoseptum and the neural tube in panel E are indicated with M and N respectively. Arrows in C point to Fxr1p immunoreactive myoblasts in the developing somite.

Western blot analysis using different tissues, including brain, skeletal muscle and testis. In brain, the most prominent isoform is approximately 74 kDa (Figure 5, lane 1). In muscle tissue we could detect Fxr1p isoforms (two major bands) of approximately 80 - 88 kDa (Figure 5, lane, 2) and in testis the most prominent isoform was 72 kDa (Figure 5, lane 3).

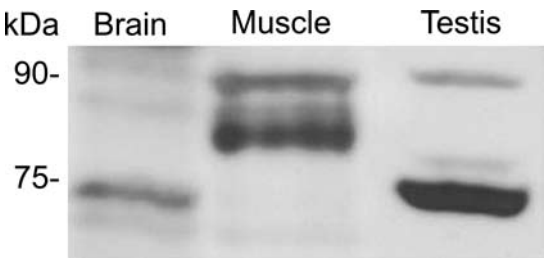

Figure 5. Analysis of Fxr1p isoforms in adult zebrafish tissues
Homogenates of adult zebrafish brain, muscle and testis were immunoblotted using Affi5 antibodies to detect the presence of molecular forms from Fxr1p. In brain a prominent band of approximately 73 kDa and a low-intensity band of approximately 70 kDa are present. In skeletal muscle high molecular weight 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.

Colocalisation of Fxr1p with components of the translational machinery

In mammals, Fxr1p is incorporated in mRNP particles within actively translating ribosomes [7,31-33]. Furthermore, it has been described that (poly)ribosomes are located at the myoseptum, and that transcripts can be translated locally [34,35]. 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 localised at the myoseptum.

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

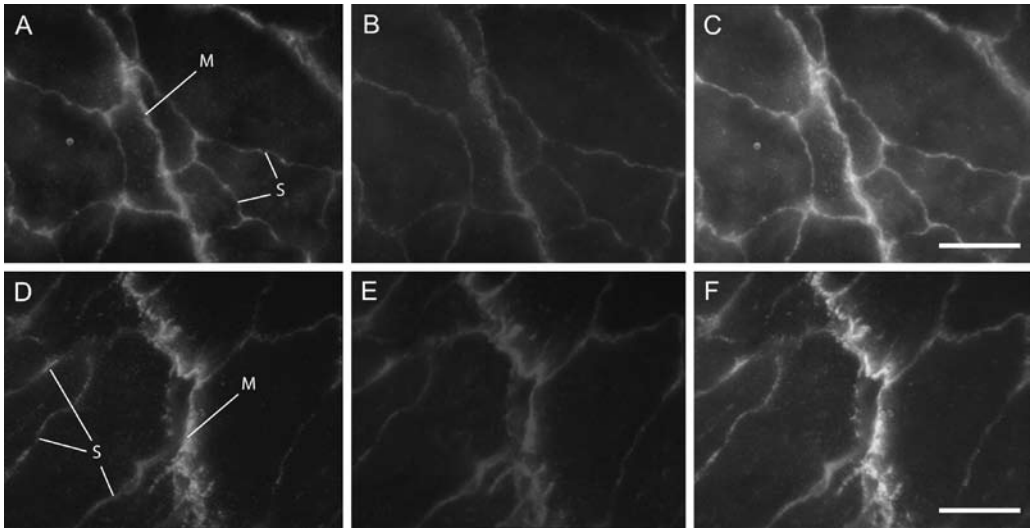

Figure 6. Colocalisation of Fxr1p with components of the translational machinery
Transverse cryostat sections of skeletal muscle tissue from adult zebrafish were stained for Fxr1p (B, red and D, green), Staufen (A, green) and P0 (E, red). Figures C and F depict the merge images of A,B and D,E, respectively.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

Fxr1p is localised next to dystrophin and vinculin at the myoseptum

The localisation of Fxr1p at the myoseptum is reminiscent of that of dystrophin in zebrafish. Additionally, Fxr1p has been reported to be localised in costameres [7]. Next we would like to study whether Fxr1p is colocalised 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 therefore examined the localisation 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 localised at the myoseptum. However, this close localisation at the myoseptum of Fxr1p on the one hand and vinculin and dystrophin on the other hand is not a exact co localisation as shown in Figure 7 using confocal analysis. Closer examination reveals that the signals only partly overlap. Both vinculin and dystrophin are localised more to the centre of the myoseptum than is Fxr1p.

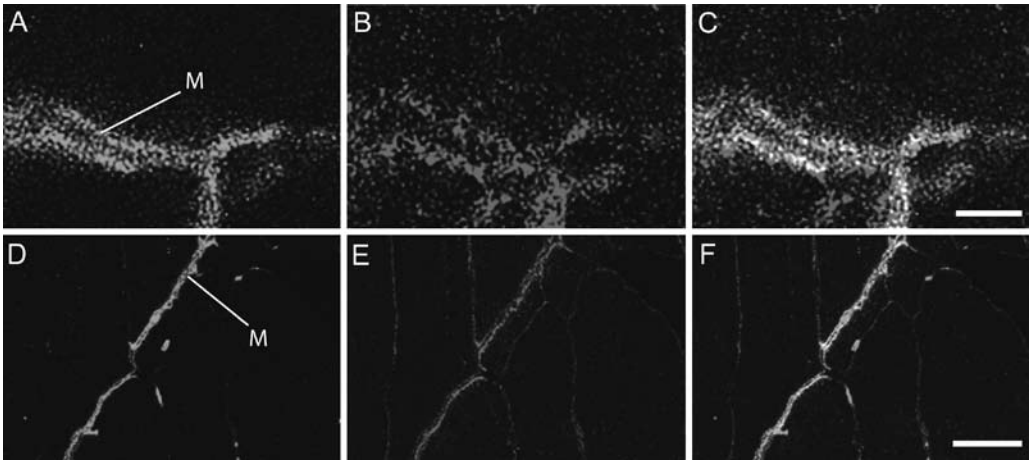

Figure 7. Fxr1p is localised next to vinculin and dystrophin
Longitudinal cryosections of skeletal muscle tissue from adult zebrafish were stained for Fxr1p (A and D, green), vinculin (B, red) and dystrophin (E, red) and examined using confocal microscopy. Images were taken in one confocal plane. Figures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

Discussion

In the present study we performed an initial characterisation 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 cellular function of Fxr1p remains unknown. The main reason for this is that *Fxr1* knock-out 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 to study 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 the invertebrate models on the one hand and the higher model systems such as mouse, on the other hand [36,37]. 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 genetics approaches very effective. In addition, the zebrafish has basically the same anatomical organisation as man, and data on gene function obtained from zebrafish are highly applicable in the context of human diseases and development [38,39].

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 (figure 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, as in mammals, predominantly in skeletal muscle, testis and brain. In embryos, Fxr1p is ubiquitously expressed in all cells between 0 hpf and 6 hpf. From 1 dpf 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 [6]. In 3 dpf 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 been reported for FXR1P in human fetal brain (18 weeks) as well [4].

The labelling intensity of Fxr1p in skeletal muscle tissue from both 1 and 3 dpf 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 localisation. In the 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 observed in 3dpf embryos as well. This is in contrast with the human and mouse subcellular Fxr1p localisation in neurons, which is predominantly cytoplasmic [5,31].

The difference in subcellular localisation of Fxr1p indicates that the cellular context of adult zebrafish neurons may share characteristics with that of human fetal neurons (Tamanini et al., 1997).

The subcellular localisation 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 [40]. This is in agreement with previous reports in mice that also described Fxr1p staining in granular structures in costameres [7,8]. Most striking is the predominant localisation 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 [41,42]. This localisation 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 [43].

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 localisation of Staufen and P0 in double labelling experiments. Indeed, both proteins showed a co localisation 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 [43].

Staufen has been reported to be present in RNP particles that also contain FMR1 and FXR1p [44]. Recent data show that Staufen protein is localised at the neuromuscular junctions (NMJ) and may be involved in maturation and plasticity of the NMJ [45]. 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 musclefibers. These concentrations of Staufen immunoreactivity could correspond to the NMJs. This suggests that, although Fxr1p and Staufen can be both present in RNP particles in the brain and may partially colocalise in zebrafish muscle, both proteins have distinct roles in zebrafish skeletal muscle tissue.

The localisation of Fxr1p at the myoseptum is reminiscent of the localisation of dystrophin and vinculin in zebrafish. Both proteins are components of the myoseptum and provide a connection between the extra cellular matrix (ECM) and the intracellular cytoskeleton [40,46,47].

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 colocalisation of dystrophin and vinculin protein using confocal immunofluorescent imaging. As both vinculin and dystrophin are distinctly more centrally localised 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 localisation bordering at the myoseptum from 19 hpf onward and appears to be located immediately outside the myoseptum itself, where dystrophin protein is located [46]. Comparing these findings to our confocal study on the possible colocalisation of Fxr1p with dystrophin or vinculin, it appears likely that Fxr1p may colocalise with dystrophin mRNA. Further in situ hybridisation studies are necessary to establish this colocalisation.

The Fxr1p localisation 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 [4,16].

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. Especially, gene knockdown experiments using the morpholino gene-targeting strategy and transgenic techniques using expression plasmids with *Fxr1*-EGFP may open new avenues that will lead to knowledge about the *in vivo* function of Fxr1p.

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Chapter 5

Fxr1 morphants display abnormal somite formation and absence of cardiac looping

Chapter 5

Fxr1 morphants display abnormal somite formation and absence of cardiac looping

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Manuscript in preparation

Abstract

The *FXR* gene family consists of three members; *FMR1, FXR1* and *FXR2*. Lack of the *FMR1* gene product (FMRP) results in the fragile X syndrome, whereas for the other two genes no human disease has been identified yet. Although mouse models have been generated for all three *FXR* genes our knowledge about the *in vivo* gene function during embryonic development is limited. Interestingly, the *Fxr1* knockout mice display a striated muscle phenotype and die shortly after birth. We applied the zebrafish as a new genetic model system to study *FXR* gene function during embryonic development. Here we report antisense morpholino knockdown studies to study the function of Fxr1p during embryonic development. The *fxr1* morphants display a striated muscle phenotype and severe heart malformations. Initial characterisation of Fxr1p in zebrafish shows an abnormal somite formation and, in contrast to other Fxr1p animal models, a failure in looping of the heart.

Introduction

The *FXR* protein family of RNA binding proteins consists of three members; FMRP, FXR1P and FXR2P. The *FMR1* gene product, FMRP, has been extensively studied due to its involvement in the fragile X syndrome, which is mainly characterised by mental retardation, macroorchidism and mild facial dysmorphologies. The gene affected in this syndrome, *FMR1*, is transcriptionally silenced by the methylation of its promoter due to the expansion of a CGG repeat in the 5' UTR of the gene. Thus the fragile X syndrome is caused by the absence of FMRP. For FXR1P and FXR2P no human disease has been described so far.

The *FXR* proteins have RNA-binding capacities via two KH domains and one RGG box. Further they exhibit a nuclear export signal (NES) and a nuclear localisation signal (NLS), which allow the protein to shuttle between the nucleus and cytoplasm. All three *FXR* proteins show a high expression in the brain and testis. In addition, FXR1P is also highly expressed in striated muscle tissue and was found to localise along the Z-lines and costameres [1-6]. Furthermore, all *FXR* proteins are found to be associated with ribosomes in the form of messenger ribonuclear particles (mRNP) [1,7]. Both the high conservation among the *FXR* proteins and the overlap in tissue distribution, suggest that FXR1P and FXR2P might have a similar function as FMRP in the regulation of transport and/or translation of specific mRNAs. The overlap in cellular function between the three members and the exaggerated phenotypes observed in *Fmr1/Fxr2* double knockout mice support a mechanism in which Fmrp and Fxr2p modulate each others function [8]. Since FXR1P is the only family member expressed at high level in striated muscle tissue the other two members are not able to compensate for the lack of FXR1P in this tissue.

To study the *in vivo* function of FXR1P, animal models are crucial. The high evolutionary conservation between the different species of the *FXR1* gene enables such studies. As a first step, *Fxr1* knockout mice have been generated [6]. These mice died shortly after birth and appeared to suffer from a striated muscle phenotype. They display a disruption of the cellular architecture of striated muscle tissue. Due to the absence of Fxr1p in costameric regions of striated muscle tissue, the costameric proteins vinculin, dystrophin and alpha-actinin are delocalised [6]. Recently, *Fxr1* knockdown experiments in *Xenopus laevis* have been reported. *Fxr1* knockdown in *Xenopus* showed a muscle-specific phenotype, including abnormal formation of the somites suggesting that Fxr1p is required during the formation of functional muscle [9]. The early lethality of *Fxr1* KO neonates prompted us to study Fxr1p function during embryonic development in a more suitable animal model; the zebrafish (*Danio rerio*).

Zebrafish muscle development starts early during somitogenesis with adaxial cells that express myogenic factors, like MyoD. These adaxial cells lie adjacent to the notochord, migrate laterally of the somite and elongate into mononucleated slow muscle fibres [Devoto, 1996 #141]. The slow muscle fibres act as a template for the differentiation of fast muscle fibres, which develop from the remainder of the presomitic cells [Devoto, 1996 #141; Stickney, 2000 #12; Wolff, 2003 #149; Blagden, 1997 #145; Henry, 2004 #150].

We have previously shown that the functional domains of *FXR* genes are conserved in zebrafish and that the expression pattern of Fxr1p in zebrafish is similar to human and mouse [10]. In adult zebrafish, Fxr1p is localised at the myosept and in costamere-like structures in striated muscle tissue. In addition, Fxr1p is present in immature spermatogenic cells and in brain tissue. In situ hydridisation studies has shown that *fxr1* mRNA expression in zebrafish is high in the anterior of the embryo, and *fxr1* is raised in adaxial and somitic cells from 12 till 24 hpf [11].

We performed *fxr1* knockdown experiments using morpholino technology to study the role of Fxr1p, especially during early embryonic development. The *fxr1* morphant showed a disorganisation of the striated muscle and a severe heart phenotype. The heart failed to loop and became string-like when higher concentrations of *fxr1* morpholino are injected. In addition, an abnormal somite formation was observed with a disruption of the MyoD pattern, suggesting an aberrant migration of the adaxial cells.

Material & methods

Zebrafish strains and care

The zebrafish (*Danio rerio*) strain used for this work was obtained from Wageningen ZF WT Zodiac F5 line. Fish were maintained at 25 °C on a 14 h light/10 h dark cycle.
Embryos were raised at 28.5 °C and different developmental stages were determined according to [12].

Morpholino injection

Morpholino antisense oligonucleotides targeting the ATG (MO-ATG), the 5'UTR (MO-UTR) and the mismatch morpholino (MOmm), containing 7 mismatches, were purchased from Gene Tools. Sequences were as follows: MO-ATG 5'-gtcagttcctccatgttgagcgcga-3', MO-UTR 5'-gatgctgggtaaagttccagaac-3' and MOmm 5'-gcctttgcgtttccaacatggagga-3'. Morpholinos were dissolved to a stock concentration in distilled water and further diluted in 1x Danieau solution (58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM Ca $(NO_3)_2$, 5 mM Hepes, pH 7.6) and a 0,05% solution of Phenol Red (Sigma). Zebrafish embryos were injected into the yolk at one to four cell stages using a pneumatic picopump (World Precision Instruments). Different concentrations of *fxr1* MO were injected to determine the optimum concentration of *fxr1* MOs (1-15 ng). As the observed phenotype was dosage-dependent, most of the experiments were carried out with embryos injected with 4 and 8 ng of *fxr1* MO.

Western blot analysis

MO (8 ng) injected and WT zebrafish embryos were analysed at 1, 2 and 3 dpf. Western blot analysis was carried out as previously described [13]. For immunodetection a zebrafish specific antibody against Fxr1p (affi5) was used in a concentration of 1: 500 [10]. The secondary antibody (goat anti-rabbit Igs; 1:5000) conjugated with peroxidase allowed detection with the chemiluminescence method (ECL Kit, Amersham). As a control for equal amounts per lane, the same samples were stained for actin (AC-40 from Sigma, 1:1000).

Histology, immunocytochemistry and in situ hybridisation

Routine histology and immunohistochemistry were performed as previously described [13]. The primary antibodies used to stain were actin (AC-40 from Sigma), dystrophin (Mandra1 from Sigma) and vinculin (Santa Cruz). For all stainings, an anti-mouse or anti-goat Igs conjugated with peroxidase as secondary antibody (1: 100) was used.
Whole mount in situ hybridisation was performed with digoxigenin (DIG) labeled RNA probes.

The probes *MyoD* and *Cmlc* were kindly provided by the Hubrecht Laboratory [14,15]. In situ hybridisation was performed as described [16].

Results

Characterisation of the *fxr1* morphant phenotype

The role of Fxr1p during early zebrafish development was studied using two different morpholinos (MOs). The MOs were designed against the start site (MO-ATG) and against the 5′ untranslated regions (MO-UTR) of the zebrafish *fxr1* cDNA, and both gave a similar phenotype when injected into the yolk of a 1-4 cell stage. Also further characterisation of the *fxr1* morphants, using histology, in situ hybridisation and immunocytochemistry, showed a similar result between the two MOs. Embryos injected with MO-ATG and MO-UTR displayed both a curled and/or reduced tail and cardiac abnormalities, whereas embryos injected with MOmm showed no apparent phenotype (data not shown). The *fxr1* morphant phenotype showed a dosage-dependent effect, as the severity of the phenotype increased with higher MO concentrations, which is illustrated in figures 1A-1C for MO-ATG. In embryos injected with 4 ng MO the characteristic phenotype became clearly recognizable at 24 hpf (Figure 1B) and more pronounced with 8 ng MO (Figure 1C). The reduction of the tail was already clearly visible at 24 hpf and varied from a curly tail, reduction of the tail to complete absence of the tail (Figures 1B and 1C). All *fxr1* morphants showed at 24 hpf U-shaped somites (Figure 1E for 8 ng MO-ATG), compared to the V-shaped myotomes in WT embryos (Figure 1D). In addition, the horizontal myosept was lacking. All morphants displayed a poor motility (data not shown). The cardiac defects observed in *fxr1* morphants include the absence of looping and pericardial oedema. When lower concentrations of MOs (4 ng) were injected, both the ventricle and atrium could still be identified in the linear heart, however, both were dilated (Figure 1G), while at higher concentrations (8 ng) only a linear heart tube without dilatations could be observed (Figure 1H). At 3 dpf, the rate of the heartbeat of embryos injected with 4 ng or 8 ng was significantly reduced to 155 and 66 beats/minute, respectively (normal ~190 beats/ minute). In general, embryos injected with either 4 ng or 8 ng of MOs causes a severe phenotype and is embryonically lethal at 4 dpf and 6 dpf, respectively.

Reduced levels of Fxr1p

The effectiveness of the MOs was determined by quantitative Western blot analyses using monospecific antibodies raised against zebrafish Fxr1p. For this experiment either MO-ATG or MO-UTR (both 8 ng) were injected and compared with WT embryos at 1, 2 and 3 dpf (Figure 2). In WT homogenates of 1dpf two Fxr1p isoforms can be detected of 73 and 75 kDa from which the 73 kDa form is the most prominent (Figure 2A). In contrast, WT homogenates of 2 and 3 dpf showed a more equal expression level between the two isoforms. Homogenates from MO-injected embryos showed at all three time points a clear reduction in Fxr1p expression levels. To ensure equal gel loading an anti-actin staining was performed on the same blot (Figure 2B).

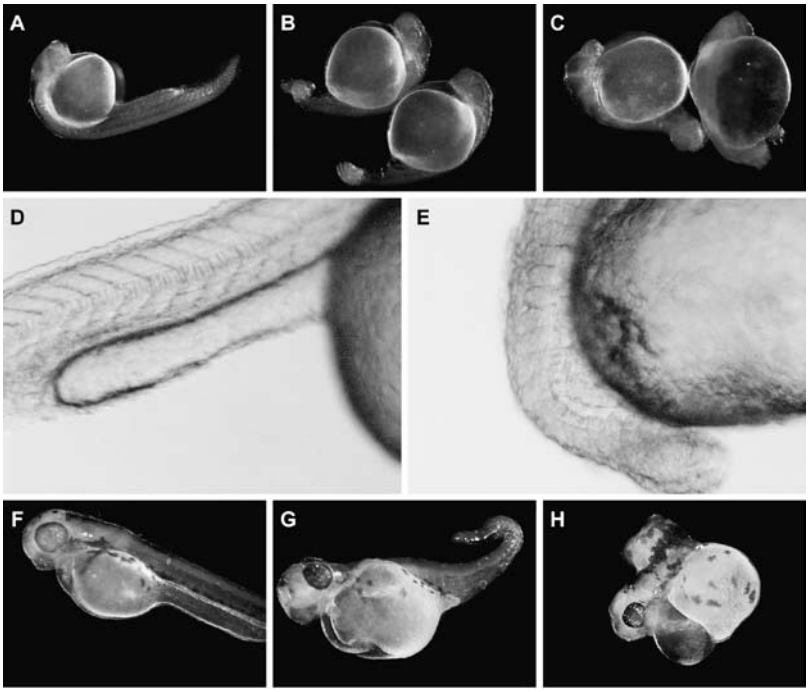

Figure 1. *Fxr1* morphants display a striated muscle phenotype and severe cardiac malformations. Embryos were injected with 4 ng (B and G) and 8 ng MO-ATG (C, E and H). The *fxr1* morphants at 1 dpf (B and C) show a curled and/or reduced tail compared to the WT embryos (A). The reduction of the tail is more severe in the embryos injected with 8 ng of *fxr1* MO-ATG (B) than 4 ng (C). Details of the tail (D and E) of the *fxr1* morphants (E) show U-shaped somites instead of V-shaped somites in WT embryos at 1dpf (D). At 3 dpf, the muscle phenotype in *fxr1* morphants is more pronounced and, in addition, a heart defect can be observed (G and H), whereas WT embryos show a normal heart development (F). Embryos injected with 4 ng *fxr1* MO-ATG display an unlooped heart with a distinct ventricle and atrium at 3 dpf (G), while embryos injected with 8 ng MO-ATG lack the complete tail and displays a linear string-like heart with severe pericardial oedema (H). Similar results were obtained with the second MO (MO-UTR) that was designed against the UTR of *fxr1*.

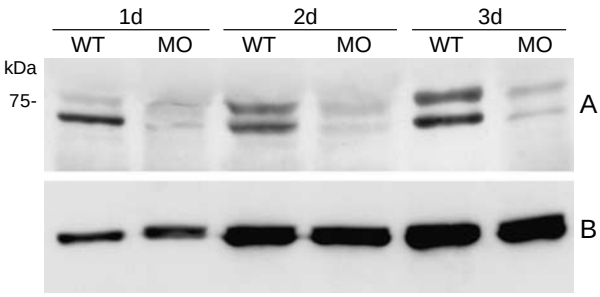

Figure 2. Western blot analysis of homogenates from wildtype embryos (WT) and MO-ATG (8 ng) injected embryos (MO) at 1, 2 or 3 dpf using monospecific antibodies against zebrafish Fxr1p. A) In WT homogenates two isoforms of Fxr1p (73 and 75 kDa) are recognized at this gestational age. Notably, WT embryos show a prominent 73 kDa form of Fxr1p at 1 dpf, whereas the 75 kDa form is almost absent. At later stages (2 and 3 days), the ratio between the two isoforms has changed. Homogenates from embryos injected with MO-ATG show a significant reduction in Fxr1p expression. B) Actin expression levels are used as a control for equal gel loading

Overall pathology of the *fxr1* morphant

The overall pathology, with special emphasis on muscle development and cardiac dysfunction, was examined by HE staining of both paraffin and GMA sections of MO-injected embryos (4 ng) and compared with WT embryos at 2 dpf. The paraffin section of an *fxr1* morphant clearly showed the disorganisation of striated muscle (Figure 3A and B). At the anterior part of the tail, some myosepts are still recognizable, however, more posterior of the tail no myosepts are visible and no striations can be seen (figure 3B). GMA sections were made to study the morphology of sarcomeres. The nuclei of the sarcomeres showed an oval shape in WT embryos (Figure 3C) and also at the proximal part of the tail of the *fxr1* morphants (Figure 3D). However, the distal part of the tail of the *fxr1* morphants showed round rather than oval shaped nuclei of the sarcomeres, which indicates a delay in muscle differentiation. The paraffin sections of WT heart showed a looped heart with a distinct ventricle and atrium (Figure 3E), and although the heart of *fxr1* morphants showed a distinct ventricle and atrium as well, no cardiac looping could be observed (Figure 3F). In addition, these histological sections showed the presence of a dilated atrium and a dilated ventricle with a very thin muscle wall.

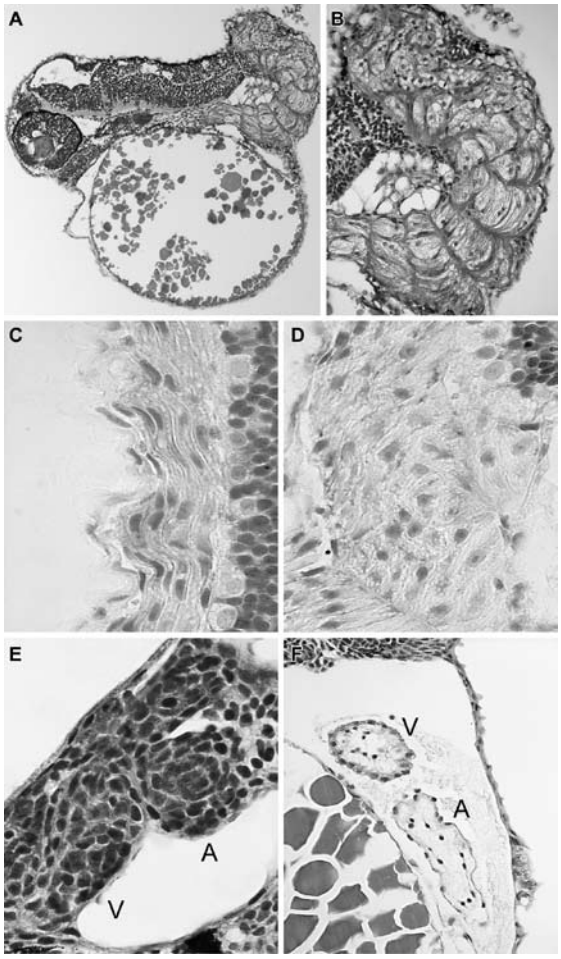

Figure 3. HE stained muscle and heart sections of WT embryos (C and E) and *fxr1* morphants (MO-ATG, 4 ng; A, B, D and F) at 3 dpf. Longitudinal paraffin section of an *fxr1* morphant is showing the striated muscle phenotype (A). Detail of the severely reduced tail in the *fxr1* morphant (B). At the anterior part of the tail myosepts can be observed, however, more posterior of the tail muscle organization is completely lost. GMA section of skeletal muscle from a WT embryo (C) and an *fxr1* morphant (D) illustrating the normal presence of oval nuclei in WT embryos at this stage (3 dpf), whereas embryos injected with MO-ATG show predominantly round nuclei instead of oval, suggesting a delay in development. In paraffin sections of WT embryos (E) at 3 dpf the looped heart can be recognized with the ventricle (v) and atrium (a). *fxr1* morphants at 3 dpf show a distinct ventricle (v) and atrium (a), but no cardiac looping.

Structural proteins present but delocalised

To further characterize the muscle tissue of *fxr1* morphants, paraffin sections of 3 dpf embryos (both WT and *fxr1* morphants) were stained using specific antibodies against important structural striated muscle proteins, including actin, dystrophin and vinculin. In WT embryos, the three proteins showed a differential expression pattern. Actin is expressed at high levels within the muscle fibres (Figure 4A), whereas dystrophin expression is located in the V-shaped myosepts (Figure 4B) and vinculin showed expression in both the muscle fibres and in the myosepts (Figure 4C). In *fxr1* morphants, all three proteins were expressed at similar levels compared with WT, but the distribution was dramatically altered (Figures 4D-E). The normal architecture of the muscle tissue at the distal part of the morphants was completely lacking and muscle fibres were in different orientations. Strikingly, higher concentrations of the MO injected, resulted in disruption of the muscle architecture at the most anterior parts of the tail.

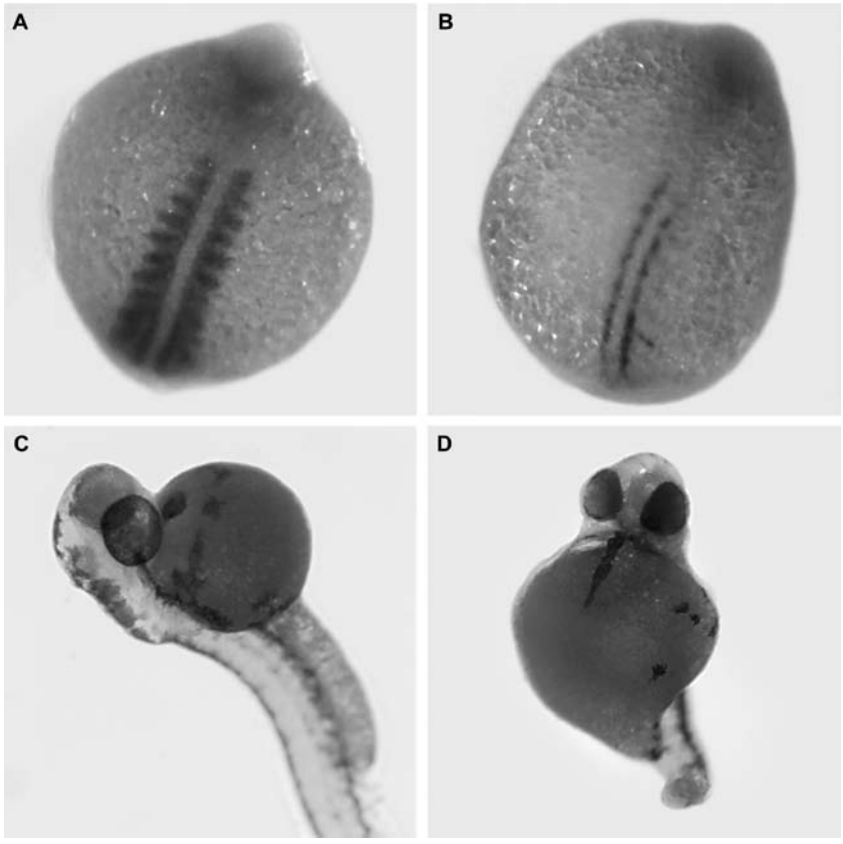

Figure 4. Immunocytochemical distribution of structural proteins in striated muscle tissue of WT embryos (A-C) and *fxr1* morphants (D-F) using paraffin sections. Sections are stained for actin (A and D), dystrophin (B and E) and vinculin (C and F).

MyoD and *Cmlc* expression

To determine whether other genes known to be involved in myogenesis were altered whole mount in situ hybridisation was performed. Myogenesis initiates during early somitogenesis with the expression of the muscle specific marker *Myo*D. *MyoD* plays a crucial role in the expression of muscle structural proteins and the assembly of myofibres. *MyoD* expression was examined in *fxr1* morphants between 10 and 16 hpf. Figure 5A illustrates MyoD expression in WT embryos of 14 hpf (10-11 somite stage). Two longitudinal stripes along the notochord with lateral bands are clearly visible. In contrast, *fxr1* morphants of 14 hpf showed less prominent and shorter lateral bands compared to WT (Figure 5B).

The thin muscle wall of the heart of *fxr1* morphants might be caused by altered expression of sarcomeric proteins. Disruption of the heart marker cardiac myosin light chain (*Cmlc*) causes disturbance of cardiac myofibrillar assembly and was used to study the expression pattern in our *fxr1* morphants. In WT embryos (2 dpf), *Cmlc* stained both cardiac chambers, the ventricle and the atrium. Figure 5C shows a normal looping of the heart. In the *fxr1* morphants (8 ng) the heart tube showed *Cmlc* expression, however, no distinct ventricle and atrium could be discriminated (Figure 5D).

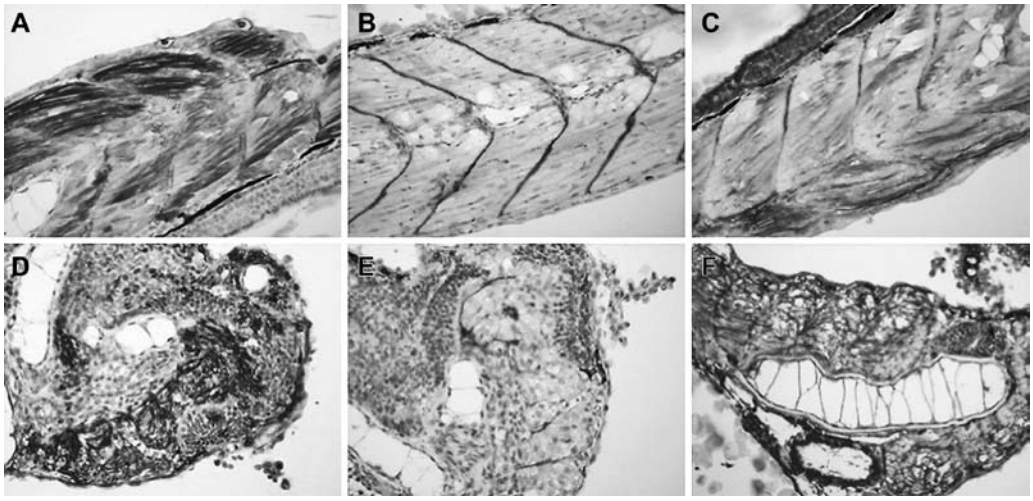

Figure 5. Whole mount in situ hybridization for *MyoD* and *Cmlc* gene expression in WT embryos and *fxr1* morphants (MO-ATG, 8 ng). Characteristic MyoD expression in a WT embryo at 14 hpf (A) and altered MyoD expression in an *fxr1* morphant (MO-ATG; 8ng) at 14 hpf showing less prominent and shorter lateral bands. *Cmlc* gene expression in a WT embryo at 2dpf visualises both the atrium and ventricle and show at this stage in development that the heart has undergone looping (C). *Cmlc* gene expression in an *fxr1* morphant visualises that looping did not occur and instead the heart remains as a linear tube (D).

Discussion

The *FXR* gene family is a small family of genes, including *FMR1, FXR1* and *FXR2,* of which *FMR1* is involved in the fragile X syndrome. Although no disease has been identified thus far involving FXR1P, the function is thought in part to be similar to FMRP, due to its high homology of important functional domains and similar expression patterns in the brain. FXR1P is the only family member that shows high expression in striated muscle tissue. Generation of *Fxr1* knockout mice that die shortly after birth pointed towards a striated muscle phenotype, suggesting FXR1P plays an important role in the development of striated muscle tissue. However, the early embryogenesis is difficult to study in mouse embryos due to their intrauterine development. In the current study, this problem was overcome by studying FXR1P in zebrafish embryos. Zebrafish have a relatively simple musculature. In zebrafish embryos skeletal muscle arises from the somites and the first muscle fibres are slow [17]. Here we report *fxr1* knockdown experiments using the antisense morpholino technology and further characterisation of the *fxr1* morphants.

The *fxr1* morphants displayed a striated muscle phenotype and developed congenital heart malformations. The severity of this striated muscle phenotype of the *fxr1* morphants was dosage-dependent, the higher the concentration of *fxr1* MO, the more severe the phenotype. The muscle development in the tail was disturbed resulting in a reduced curled tail to even a totally absent tail and poor motility. The remainder of the tail showed complete loss of the characteristic striated muscle architecture. The presence of U-shaped tail somites in *fxr1* morphants suggests a defect in the formation of the horizontal myosept and is characteristic of defects in slow muscle development [17].

In situ hybridisation studies in WT zebrafish embryos have shown that *fxr1* mRNA is expressed in adaxial cells [11]. Most of the adaxial cells migrate laterally from the notochord region to the entire surface of the myotome [18]. A few of the adaxial cells remain medially located and differentiate to slow myosin heavy chain (MyHC) expressing muscle cells. These presomitic adaxial cells initiate muscle differentiation by expressing the myogenic regulatory factor MyoD. The *fxr1* morphants display an aberrant MyoD pattern with shorter and less prominent lateral bands. This suggests that the adaxial cells do not migrate properly from notochord region to the lateral surface of the myotome. Thus, the *fxr1* morphants have a very early myogenesis defect, which results in a phenotype involving midline structures. This disorganisation of the skeletal muscle tissue was also demonstrated by the immunostaining of the structural striated muscle proteins actin, dystrophin and vinculin. These structural proteins appeared to be expressed in *fxr1* morphants of 3 dpf, but not in the organized fashion seen in WT embryos. The lack of organisation of the striated muscle seen in *fxr1* zebrafish morphants corresponds to the phenotype observed in *Fxr1* knockout mice [6]. The *Fxr1* knockout mouse also displayed disruption of the cellular architecture of striated muscle, including the heart. *Fxr1* knockout mice die shortly after birth, while a second mouse model with reduced levels of Fxr1p (*Fxr1*-neo) could overcome this lethality after birth. The zebrafish *fxr1* morphants are lethal at 4-6 dpf depending on the dosage of *fxr1* MOs injected. Thus, like in the *Fxr1* mouse models, the phenotype of zebrafish *fxr1* morphants is correlated with the level of reduced Fxr1p expression.

Next to the muscle phenotype, disturbed cardiac development could be observed in our *fxr1* morphants. The severity of the heart malformations was also dosage-dependent. Between 24-

48 hpf, the heart failed to undergo cardiac looping when treated with low concentration of MOs (4 ng), although the two chambers were normally formed. Embryos treated with higher concentration of MOs (8 ng) showed only the presence of a single heart tube without recognizable chambers and with extensive pericardial oedema. As a consequence of these abnormalities the heart function was reduced illustrated by a reduced heartbeat of both chambers. The cause of the pericardial oedema is probably a secondary event caused by the hypoplastic ventricular wall combined with poor cardiac output. Interestingly, no heart phenotype was reported in *Xenopus Fxr1* MO knockdown experiments [9]. These experiments in *Xenopus* showed that Fxr1p has an early role during somitogenesis. In the *Fxr1* MO injected frog embryos, segmentation is inhibited and myotome development is abnormal. The fact that the authors did not observe a heart phenotype could be due to the knockdown of *Fxr1* in only one side of the frog. Although *Fxr1* knockout mice showed a disrupted architecture of the heart striated muscle, cardiac looping occurred and overall pathology was less pronounced compared to zebrafish [6].

The molecular mechanisms of heart tube formation and subsequent cardiac looping remains poorly understood. Cardiac development requires interaction of several transcription factors in a tightly regulated fashion, including GATA factors. Recently, Walton et al. reported that the repression motif of *Fog1,* a multi-type zinc finger protein, was required for cardiac looping in zebrafish [19]. Thus, the normal cellular function of *Fog1* in cardiac development in zebrafish can be thought in the regulation of transcription of specific genes involved in cardiac looping. Fxr1p, an RNA binding protein, is present in mRNP particles and might be involved in transport and/or regulation of specific transcripts in striated muscle tissue, analogous to FMRP function in neurons [6,20,21]. Based on this study we hypothesize that Fxr1p transports and/or regulates translation of mRNAs encoding proteins involved in early muscle morphogenesis, including costameric proteins and proteins involved in heart morphogenesis. Misrouting or reduced translation of these target mRNAs results in delocalised or reduced protein expression and as a consequence congenital abnormalities in striated muscle morphogenesis develop. Indeed, in our *fxr1* morphants we observed delocalised costameric proteins in skeletal muscle tissue. Other factors involved in looping are diverse as microtubules, asymmetrically located bundles of actin, pressure of the cardiac jelly, and changes in the shape of individual myocardiac cells. One of the mechanisms of looping is active myocardial cell shape change, possibly due to actin polymerisation [22]. Interestingly, Fxr1p associates with CYFIP2 (for Cytoplasmic FMRP Interacting Proteins). This protein interacts with the small GTPase Rac1, which in turn plays a role in the dynamic reorganisation of the actin cytoskeleton [23,24]. Future research should be focused on the identification of the target mRNAs of *fxr1* in striated muscle tissue during embryonic development. The results of these studies will lead to increased knowledge about the molecular mechanisms underlying normal and genetic disease of skeletal muscle, including muscular dystrophies and congenital myopathies.

In conclusion, *fxr1* plays a role in the differentiation and/or migration of adaxial cells within the myotome and lack of Fxr1p results in U-shaped somites and a defect in the formation of the horizontal myosept in zebrafish. In addition, *fxr1* is required for normal cardiac looping in zebrafish.

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Chapter 6

Discussion

Chapter 6

Discussion

Animal models are often used to study the cellular function of specific genes *in vivo*. To study the function of the *FXR* genes, knockout (KO) mice have been generated for all three genes. *Fmr1* KO mice display the characteristic features as seen in fragile X patients, like learning deficits and macroorchidism [117]. In addition, they showed reduced pruning and/or maturation of the spines, which occurs during embryonic and early postnatal development [104,119-121]. Therefore, the fragile X syndrome can be characterised as a developmental disorder. Also *Fxr2* KO mice showed a mild learning and behavioural phenotype, however, further characterisation is necessary [124]. *Fxr1* KO mice showed a striated muscle phenotype and died shortly after birth [109]. This suggests that Fxr1p plays an essential role during normal embryonic development. Thus an animal model that would allow functional studies during embryonic development would be beneficial to study *FXR* gene function. A good vertebrate model to study (early) embryonic development is the zebrafish and this thesis describes functional studies of the *FXR* genes in zebrafish.

Zebrafish as a model to study human disease

Zebrafish is a well-established animal model to study development due to its small size, fast ex-utero development and optical clarity (see chapter 1.1). The last decades, the zebrafish has become increasingly popular as a vertebrate model to study human disease, because technology to manipulate gene expression has been further developed for zebrafish as well. There are some disadvantages to the use of zebrafish as a model organism. The zebrafish is a vertebrate and evolutionary more distant from human, which makes it that some processes need to be studied in a mammalian model, like the mouse model. However, there is a strong similarity between the zebrafish and the human genome and many developmental processes are conserved. Although, reverse and forward genetics in zebrafish is developing very quickly, generating specific mutations is still rather difficult in zebrafish. In ENU-mutagenized zebrafish screens, many zebrafish mutants have been identified, but the transgenic techniques are not as established as in the mouse. However, it is very difficult to study the early embryonic development in mouse due to the development of the embryos in utero. Another advantage of zebrafish is the ability to absorb small molecules from the water. Until 12-14 dpf, their oxygen supply is by diffusion across the skin. These features make the zebrafish suitable for pharmacological use. They can live in these early days of their life in a 96-wells plate, which make high throughput drug screens possible.

In the next paragraphs, I will discuss a number of examples that are relevant in the light of the research discussed in this thesis. To date, many zebrafish have been identified which display a phenotype that resembles a human disease. Zebrafish are attractive as model of myopathy because they have high skeletal muscle content and express orthologs of most human DGC proteins with similar membrane localisation [151,152]. A nice example is the zebrafish mutant, called

sapje, which has a mutation in the ortholog of dystrophin, which causes Duchenne or Becker muscular dystrophies in human patients [50]. The degeneration of normally differentiated skeletal muscle tissue in these patients is thought to be caused by disconnection of the cytoskeleton from the extracellular matrix by reduction. Dystrophin is part of the Dystrophin-Associated Protein Complex (DAPC), which transmits forces between the ends of the muscle fibres [153,154]. This complex has been shown to be present in zebrafish [151]. The *sapje* mutant zebrafish displays progressive degeneration of skeletal muscle with embryonic onset [50]. Dystrophin has also been knocked down in zebrafish using antisense morpholino knockdown technology. These morphants showed a bent and curved phenotype and were less motile compared to wildtype fish. Experiments on the *sapje* mutant showed that the degeneration was caused by separation of somitic muscle fibres from their attachments point on the myosept [151]. Myosepts are tendon-like sheets of extracellular matrix that separate segmented blocks of non-overlapping fibres. In mammalian muscles, these structures are called myotendinous junction. The requirement of dystrophin at sites of muscle attachment in the zebrafish suggested that the myotendinous junction of the human muscle might be an important site for the pathogenesis of DMD. The phenotype observed in zebrafish is more severe than seen in the mouse dystrophin mutant *mdx*. This is due to the level of utrophin, which protects the muscle of the mouse and is not present in human and zebrafish [155]. Despite the use of other animal models for DMD, the zebrafish is a valuable complementary animal model to the study of muscular dystrophies.

The zebrafish has a relatively simple neuro-muscular organisation and its nervous system is well characterised. Therefore, the zebrafish was used as a model to study spinal muscular atrophy (SMA), which is a neuromuscular disease with infant mortality. It is caused by low level of survival motor neuron (SMN) protein resulting in loss of α motor neurons in the spinal cord [156]. Morpholino knockdown studies suggest that reduced levels of SMN cause a defect in the developing motor neuron. Characterisation of the SMN morphants showed an axon-specific path finding and outgrowth defect leading to loss of motor neurons [157].

Neurodegenerative diseases, such as inherited forms of Parkinson's disease (PD), have been associated with mutations in specific genes. Genetic analyses of familial PD have identified several genes that lead to PD when mutated. These genes include α-synuclein, parkin, DJ-1, PINK1 and LRRK2 [158]. The advantages of zebrafish as a model organism to study neurodegeneration are the ability to study the *in vivo* function using fluorescent markers and the availability of techniques that allow efficient generation of transgenic animals. Furthermore, as mentioned above, they can absorb small molecules from the water, which makes them suitable for high throughput drug screens to test potential drugs for PD patients. Moreover, the dopaminergic system is complex and well developed in zebrafish [159] and a region anatomically similar to the striatum was identified in the forebrain [160]. Parkinson's disease (PD) is characterised by the progressive loss of dopaminergic neurons in the substantia nigra and movement defects. Protein deposits characterize Parkinson brains and zebrafish can be used as a model to understand mechanisms of neurotoxicity of these deposits. Next to the advantage of genetic screens, the zebrafish can be given with PD-inducing neurotoxins (MPTP, rotenone, and paraquat), which eventually leads to loss of DA neurons [161]. Recently, it was shown that DJ-1 is evolutionarily conserved and expressed in dopaminergic neurons of zebrafish [162].

Thus, the zebrafish is an ideal vertebrate animal model to study (disease) gene function during early embryonic development and, in addition, very suitable to be used as a complementary system to the mouse.

Zebrafish versus *Xenopus*

Although *Xenopus* is also widely used as a model to study embryonic development, we chose to use the zebrafish to study the function of FXR1P. A major advantage of using *Xenopus* as a model is the phenomena of the development each cell of a two-cell stage into either the left or the right side of the embryos. When only one cell is injected, the other halve of the embryo can be used as an internal control. However, the *Xenopus laevis* is a tetraploid and relatively slow growing compared to *Xenopus tropicalis*, which is diploid. For *Xenopus tropicalis*, *Fmr1* and *Fxr1* genes could be identified, but *Fxr2* was not [112]. Both the frog and zebrafish are established animal models for embryonic development. In addition, the zebrafish has been used as a model for myogenesis and myopathies [67,163]. Therefore, we used the zebrafish as a model to study the cellular function of FXR1P.

Evolutionary conservation of Fxr1p

To be able to use the zebrafish as an animal model to study the *FXR* genes, these genes have to be present in the zebrafish and expressed in the same tissues as in human, preferably. In the zebrafish genome, all three *fxr* genes could be identified and appear to be conserved during evolution. The domains important for the function of the *fxr* genes are highly homologous to the human genes, which suggests that the function of the *fxr* proteins in zebrafish is similar to the function in human (Chapter 5, [164]; [165]). The expression pattern of both Fmrp and Fxr1p in zebrafish has been thoroughly studied and appeared to have similar distributions as in human and mouse. Fmrp is predominantly expressed in the neurons of the brain. In skeletal muscle no Fmrp expression could be detected, similar to the human situation (Chapter 4, [165]). Fxr1p is moderately expressed in the neurons of the brain, immature spermatogenic cells of the testis and highly expressed at the myosept and costamere-like structure of skeletal muscle tissue (Chapter 5, [164]). With the *fmr1* and *fxr1* orthologs being evolutionary conserved and showing similar expression patterns, zebrafish can be used as a model to study the function of Fmrp and Fxr1p during embryonic development.

Fxr1 morphants

The *fxr1* MO injected zebrafish embryos or *fxr1* morphants showed disorganisation of the striated muscle and a severe heart phenotype, which includes the absence of looping and the presence of pericardial oedema. The phenotype showed a dosage-dependent effect and *fxr1* morphants died at 4-6 dpf depending on the concentration of injected *fxr1* MO. Characterisation of the *fxr1* morphants showed an aberrant migration of adaxial cells, indicating a defect in early steps of muscle development. Although the structural organisation is lost, structural proteins, like dystrophin, vinculin and actin, were present but delocalised. The phenotype of the *fxr1* morphants corresponds to the main characteristics observed in *Fxr1* KO mice, which lack the structural organisation of both skeletal muscle and the heart [109]. Experiments with *Fxr1* MO injection in the *Xenopus laevis* showed similar muscle specific defects [150]. Striking is the heart

phenotype that is observed in the *fxr1* morphants. Dependent on the dosage, it varied from a linear heart for low concentrations of *fxr1* MO to a string-like heart with no distinct ventricle and atrium for higher concentrations of *fxr1* MO. In the *Fxr1* KO mice, the heart lacks the organisation, but is normally looped. In the *Fxr1* MO injected frog, no heart phenotype has been described. Apparently, Fxr1p plays a role in the differentiation and/or migration of adaxial cells within the myotome and lack of Fxr1p results in U-shaped somites and a defect in the formation of the horizontal myosept in zebrafish. In addition, Fxr1p is required for normal cardiac looping in zebrafish.

Considering the advantages of zebrafish to study early embryonic development, the *fxr1* morphant is a good vertebrate model to further study the development of striated muscle and the function of Fxr1p in this process.

Fxr1p function

From mouse studies we have learned that Fxr1p is highly expressed in a punctuated pattern in skeletal muscle. This pattern is thought to be reflecting the localisation of Fxr1p in costameric structures [107,109]. Costameres are protein complexes consisting of cytoskeleton, transmembrane glycoproteins, and extracellular matrix. They connect the extracellular matrix to the Z-disks of the muscle and thereby transfer tension from contractile elements to connective tissue [153]. In zebrafish, a similar punctuated expression pattern was observed in skeletal muscle, especially at the myosept (Chapter 5, [164]). Colocalisation studies in zebrafish muscle demonstrated that Fxr1p expression partially overlaps with dystrophin and vinculin expression. Both dystrophin and vinculin are components of costameric structures [67]. Thus, considering its functional domains and its expression pattern in muscle, Fxr1p is hypothesized to play a role in transport and/or translation of specific mRNAs at the costamere. In line with this hypothesis, large numbers of actively transcribing polyribosomes have been identified at the myosept and also at costameric sites [166,167]. Translational control at costameric sites has already been shown to occur for the protein Raver1 [168]. Raver1 is thought to coordinate RNA processing and targeting of vinculin and α-actinin.

Due to the premature death of the *Fxr1* KO mice and *fxr1* zebrafish morphants, Fxr1p is thought to have an essential function during early muscle development. Muscle development involves many changes in cell morphology and cytoskeletal arrangements. During myofibrillogenesis, assembly of structural proteins is necessary to enable the development of highly organized striated muscle. Soon after myofibril formation, they become highly structured and in distinct stages will develop into mature striated muscle (reviewed in [68]). Little is known about how muscles achieve their highly structured organisation. Recently, this process of myofibrillogenesis was shown to be tightly coupled to costamere formation. Although the structural organisation of the muscle is lost, all structural proteins are present. Within this, the role of Fxr1p is translational control and transport mRNAs encoding costameric proteins during muscle development. Misrouting and/or perturbed translation of target mRNAs might result in muscle lacking structural organisation.

The heart phenotype observed in the *fxr1* morphants shines a new light on the function of Fxr1p. Characterisation of the cellular basis for cardiac looping has proven very difficult. Factors involved in cardiac looping are diverse as microtubules, asymmetrically located bundles of actin, pressure of the cardiac jelly, and changes in the shape of individual myocardiac cells.

One of the mechanisms of looping is active myocardial cell shape change, possibly due to actin polymerisation [169]. Interestingly, Fxr1p associates with CYFIP2 (for Cytoplasmic FMRP Interacting Proteins). This protein interacts with the small GTPase Rac1, which in turn plays a role in the dynamic reorganisation of the actin cytoskeleton [170,171].

Future experiments

Another way to study specific gene function is to overexpress Fxr1p in zebrafish. Transgenic fish with an inserted fusion protein, like GFP-Fxr1p, under the control of a specific promoter enable to monitor gene expression and other cellular processes *in vivo.*

FMRP is involved in pruning and/or maturation of the spines, a process that occurs during embryonic and early postnatal development [104,119-121]. It might therefore be interesting to use the zebrafish as a model to study the function of FMRP as well. Extrapolation of the data in mouse studies, suggests an *Fmr1* knockout/knockdown zebrafish to have altered spine morphology and affected synapse formation. When *fmr1* MOs are used to knockdown *fmr1,* changing the amount of MO injected could vary the level of Fmrp. In addition, it would be interesting to see if there is a phenotype and whether the phenotype is reversible when the effect of the MOs stops after 4 days and normal Fmrp production starts. Recently, an *fmr1* KO fish was generated using the TILLING technique (personal communication R. Ketting/E. Cuppen, Hubrecht Laboratory). Like the *Fmr1* KO mouse model, this *fmr1* KO fish could be tested for certain behavioural abnormalities. Ethovision from Noldus is a computerized video tracking system that enables to record movement of animals including swimming patterns of small sized fish. Learning and memory of fish can be studied using a T-maze test combined with the Ethovision system. This behavioural test can also be used to study Fxr2p function in zebrafish using *fxr2* morphants.

An advantage of MOs is the possibility to inject more than one MO simultaneously. For instance, by injecting MOs against the *FXR* proteins simultaneously, all three proteins can be knocked down in many combinations. In this way the level of redundancy among the *FXR* proteins can be studied.

In conclusion, the zebrafish is a suitable vertebrate model system to study the *in vivo FXR* protein function and especially attractive to study gene function during embryonic and neonatal development. Thereby, the zebrafish is a good complementary animal model to the mouse. Elucidating the cellular function of Fxr1p helps to understand the functions of Fmrp and/or Fxr2p as well. Although, no human disease has been linked to Fxr1p up till now, zebrafish can be used to investigate early muscle development.

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Summary

The *FMR1* protein, FMRP, has been extensively studied due to its involvement in the fragile X syndrome, which is mainly characterised by mental retardation, macroorchidism and facial dysmorphologies. The *FMR1* gene is transcriptionally inactivated by the methylation of its promoter region due to the expansion of a CGG repeat. Thus the fragile X syndrome is caused by the absence of the protein FMRP. FMRP is part of a small RNA binding protein family, which also include FXR1P and FXR2P. Due to the high homology of important functional domains and similar expression patterns in the brain, FXR1P and FXR2P are thought to have a similar function to FMRP. All three proteins are ubiquitously expressed with high expression in brain and testis. In addition, FXR1P is highly expressed in striated muscle tissue. The cellular function of the *FXR* proteins has been studied by the generation of knockout mouse models for all three genes. The *Fmr1* KO mice display similar features as fragile X patients, including learning and memory abnormalities and macroorchidism. Further characterisation of the *Fmr1* KO mice showed a reduced pruning and/or maturation of spines, which normally occurs during late embryonic and postnatal development. *Fxr1* KO mice showed a severe striated muscle phenotype and died shortly after birth. This early lethality indicates that cellular Fxr1p function is critical during embryonic development. *Fxr2* KO mice showed a discrete behavioural phenotype, but needs further characterisation to determine whether Fxr2p has a role during embryonic development. Processes during embryonic development are difficult to study in the mouse model due to *in utero* development of the embryos. For this reason, we have chosen the zebrafish (*Danio rerio*) as a model to study the *FXR* protein function especially during embryonic development.

The zebrafish is a well-established simple vertebrate animal model. Their ability to be kept in large numbers and the ease of breeding make them easy to maintain. Due to its transparent embryos that develop externally the zebrafish represents an ideal model to study processes during embryonic development. In addition, the embryos develop quickly from a single cell to something that is recognizable as a fish after 24 hours of development. The favourite method to study gene function in zebrafish is the generation of a morpholino-mediated knockdown zebrafish. This morpholino technology is based on the injection of synthetic antisense oligonucleotides into a 1-8 cell stage embryo, which subsequently binds to the mRNA molecule and thereby preventing translation. The introduction of this thesis (chapter 1) describes the use of zebrafish as a vertebrate animal model with special emphasis to human disease (part 1) and introduces the *FXR* protein family with its three individual members (part 2). The aim of this thesis is to gain insight in the function of the *FXR* proteins, and their role during embryonic development particularly using the zebrafish as a model system.

Another simple animal model organism suitable to study embryonic development is the frog *Xenopus tropicalis*. Characterisation of the *FXR* proteins in the *Xenopus tropicalis* led only to the identification of Fmrp and Fxr1p and not Fxr2p. In adult frogs both proteins are highly expressed in most neurons of the central nervous system and in all spermatogenic cells in the testis. In addition, Fxr1p is also highly expressed in striated muscle tissue (chapter 2).

A first step in studying the *FXR* proteins in zebrafish is the characterisation of these proteins in this model. All *FXR* proteins are evolutionary conserved in zebrafish. The expression pattern

of both Fmrp and Fxr1p was studied using specific antibodies against the zebrafish proteins. Fmrp expression is high in specific neurons of the brain and very weak in the testis. In brain tissue, only two prominent isoforms of Fmrp could be detected, compared to four prominent isoforms in human and mouse brain tissue. Fxr1p is expressed in neurons throughout the brain, in immature cells of the testis and in skeletal muscle. The high expression of Fxr1p in skeletal muscle is localised at the myosept and in costamere-like granules. For Fxr1p in zebrafish, different tissue-specific isoforms were characterised compared to human and mouse. Due to similar expression patterns and the high homology of the functional domains of both Fmrp and Fxr1p make the zebrafish a suitable animal model to study the cellular function of FMRP and FXR1P during embryonic development (chapter 3 and 4).

The function of Fxr1p was studied using the antisense morpholino knockdown technique. The *fxr1* knockdown zebrafish display a striated muscle phenotype and severe heart malformations. Initial characterisation of the *fxr1* morphants showed an abnormal somite formation, which resulted in delocalisation of muscle proteins, including dystrophin, vinculin and actin. In contrast to other Fxr1p animal models, a failure in looping of the heart was also observed. FXR1P is thought to be involved in transport and/or regulation of specific transcripts in striated muscle, analogous to FMRP function in neurons (chapter 5).

In conclusion, the zebrafish appeared to be a suitable animal model to study the cellular function of both FMRP and FXR1P during embryonic development. The use of zebrafish as a model for human disease, the *fxr1* morphants and the possible cellular function of FXR1P are discussed in chapter 6.

Samenvatting

Het *FMR1* eiwit, FMRP, is uitgebreid bestudeerd vanwege zijn betrokkenheid bij het fragiele X syndroom, dat voornamelijk gekenmerkt wordt door mentale retardatie, macroorchidisme en dysmorfologieën in het gezicht. Een verlenging van een CGG repeat in het *FMR1* gen zorgt voor de methylatie van het promotorgebied en voorkomt daarmee transcriptie. De afwezigheid van FMRP veroorzaakt het fragiele X syndroom. FMRP is onderdeel van een kleine familie van RNA bindende eiwitten, waartoe ook FXR1P en FXR2P behoren. Vanwege de hoge homologie in de belangrijke functionele domeinen en een vergelijkbaar expressiepatroon van FXR1P en FXR2P in het brein, wordt aangenomen dat zij dezelfde functie hebben als FMRP. De 3 eiwitten komen hoog tot expressie in brein en testis. Daarnaast komt FXR1P hoog tot expressie in dwarsgestreept spierweefsel. De cellulaire functie van de *FXR* eiwitten is bestudeerd met behulp van knockout muis modellen voor de 3 genen. De *Fmr1* KO muizen hebben kenmerken vergelijkbaar met een fragiele X patiënt, zoals leer- en geheugenproblemen en macroorchidism. Verdere karakterisatie van deze muizen laat zien dat zij verminderde volgroeiing van de zenuwen hebben, dat normaal tijdens de laat embryonale en vroege postnatale ontwikkeling gebeurt. *Fxr1* KO muizen hebben een ernstig spierfenotype, waarbij het dwarsgestreepte spierweefsel aangedaan is. De pups overlijden vlak na de geboorte, dat aangeeft dat Fxr1p een belangrijke rol speelt bij de embryonale ontwikkeling. De *Fxr2* KO muizen hebben een discreet gedragsfenotype, dat verder gekarakteriseerd moet worden om te bepalen of Fxr2p een rol speelt in de embryonale ontwikkeling. Doordat de embryonale ontwikkeling van de muis plaatsvindt in de baarmoeder is het moeilijk deze te bestuderen in muis modellen. Daarom hebben wij gekozen voor de zebravis als diermodel voor de bestudering van de functie van de *FXR* eiwitten, met name tijdens de embryonale ontwikkeling.
De zebravis is een simpel vertebraat diermodel, dat zich bewezen heeft voor onderzoek. De mogelijkheid om ze in grote aantallen te huisvesten en het gemak van voortplanten, maakt ze eenvoudig te houden. Doordat de embryo's transparant zijn en buiten de moeder groeien is de zebravis een ideaal model om embryonale ontwikkeling te bestuderen. Daarnaast ontwikkelt een embryo zich binnen 24 uur van eencellige tot een complete vis. De favoriete methode om genfunctie te bestuderen in zebravis is het maken van een morpholino-geinduceerde knockdown zebravis. De morpholino technologie is gebaseerd op het injecteren van synthetische antisense oligonucleotieden in een 1 tot 8-cellig embryo. De morpholino bindt vervolgens aan het mRNA molecuul en voorkomt hiermee translatie. De introductie van dit proefschrift (hoofdstuk1) beschrijft het gebruik van de zebravis als vertebraat diermodel met name voor humane ziektebeelden (deel 1) en introduceert de drie leden van de *FXR* eiwit familie (deel 2). Het doel van dit proefschrift is om inzicht te krijgen in de functie van de *FXR* eiwitten met name tijdens de embryonale ontwikkeling door het gebruik van de zebravis als diermodel.
Een ander simpel diermodel dat geschikt is voor het bestuderen van de embryonale ontwikkeling is de kikker *Xenopus tropicalis*. Karakterisatie van de *FXR* eiwitten in de kikker heeft geleid tot de identificatie van Fmrp en Fxr1p, maar niet van Fxr2p. In volwassen kikkers komen beide eiwitten hoog tot expressie in de meeste neuronen van het centrale zenuwstelsel en in alle spermacellen van de testis. Daarnaast komt Fxr1p ook hoog tot expressie in dwarsgestreept spierweefsel (hoofdstuk 2).

De eerste stap in het bestuderen van de *FXR* eiwitten in de zebravis is de karakterisatie van deze eiwitten in dit model. Alle *FXR* eiwitten zijn tijdens de evolutie geconserveerd in de zebravis. Het expressiepatroon van zowel Fmrp en Fxr1p is bestudeerd met behulp van antilichamen specifiek tegen de zebravis eiwitten. De Fmrp expressie is hoog in specifieke neuronen van het brein en is zwak in de testis. In het brein zijn slechts twee isovormen van Fmrp gedetecteerd in vergelijking met de vier isovormen die voorkomen in het brein van mens en muis. Fxr1p komt tot expressie in de neuronen van het brein, onvolwassen cellen van de testis en skeletspier. De hoge expressie van Fxr1p in skeletspier wordt voornamelijk gezien in de myosepten en in costameer-achtige granulen. In vergelijking met mens en muis heeft Fxr1p in zebravis andere weefsel-specifieke isovormen. Vanwege de gelijkende expressiepatronen en de hoge homologie van de functionele domeinen van zowel Fmrp en Fxr1p is de zebravis een geschikt diermodel voor het bestuderen van de cellulaire functie van FMRP en FXR1P tijdens de embryonale ontwikkeling (hoofdstuk 3 en 4).

De functie van Fxr1p is bestudeerd met behulp van antisense morpholino knockdown techniek. De *fxr1* knockdown vissen hebben een fenotype van het dwarsgestreepte spierweefsel en het hart. Een eerste karakterisatie van deze *fxr1* knockdown vissen laat een abnormale somietvorming zien, dat resulteert in delocalisatie van spiereiwitten als dystrofine, vinculine en actine. In tegenstelling tot andere Fxr1p diermodellen laten de *fxr1* knockdown vissen ook een misvorming aan het hart zien, waarbij het hart niet gevouwen is. FXR1P is wellicht betrokken bij het transport en regulatie van specifieke transcripten in dwarsgestreept spierweefsel, analoog aan de functie van FMRP in neuronen (hoofdstuk 5).

De zebravis bleek geschikt als diermodel voor de bestudering van de cellulaire functie van zowel FMRP als FXR1P tijdens de embryonale ontwikkeling. Het gebruik van de zebravis als diermodel voor humane ziektebeelden, de *fxr1* knockdown vissen en de mogelijke functie van FXR1P worden bediscussieerd in hoofdstuk 6.

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