Developmental defects in a zebrafish model for muscular dystrophies associated with the loss of fukutin-related protein (FKRP)

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A number of muscular dystrophies are associated with the defective glycosylation of \( \alpha \)-dystroglycan and many are now known to result from mutations in a number of genes encoding putative or known glycosyltransferases. These diseases include severe forms of congenital muscular dystrophy (CMD) such as Fukuyama type congenital muscular dystrophy (FCMD), Muscle-Eye-Brain disease (MEB) and Walker-Warburg syndrome (WWS), which are associated with brain and eye abnormalities. The defective glycosylation of \( \alpha \)-dystroglycan in these disorders leads to a failure of \( \alpha \)-dystroglycan to bind to extra-cellular matrix components and previous attempts to model these disorders have shown that the generation of fukutin- and Pomt1-deficient knockout mice results in early embryonic lethality due to basement membrane defects. We have used the zebrafish as an animal model to investigate the pathological consequences of downregulating the expression of the putative glycosyltransferase gene \( fukutin \)-related protein (FKRP) on embryonic development. We have found that downregulating FKRP in the zebrafish results in embryos which develop a range of abnormalities reminiscent of the developmental defects observed in human muscular dystrophies associated with mutations in FKRP. FKRP morphant embryos showed a spectrum of phenotypic severity involving alterations in somitic structure and muscle fibre organization as well as defects in developing neuronal structures and eye morphology. The pathological phenotype was found to correlate with a reduction in \( \alpha \)-dystroglycan glycosylation and reduced laminin binding. Further characterization of the developmental processes affected in FKRP morphant embryos may lead to a better understanding of the pathological spectrum observed in muscular dystrophies associated with mutations in the human FKRP gene.

Keywords: dystroglycan; glycosylation; muscular dystrophy; zebrafish

Abbreviations: CMD = congenital muscular dystrophy; DGC = dystrophin–glycoprotein complex; ECM = extra-cellular matrix; FCMD = Fukuyama congenital muscular dystrophy; MEB = Muscle–Eye–Brain disease; WWS = Walker-Warburg syndrome


Introduction

Over recent years, several forms of muscular dystrophy, ranging from severe congenital to mild limb girdle forms, have been associated with glycosylation defects of dystroglycan. Dystroglycan is a central component of the dystrophin–glycoprotein complex (DGC), a large protein complex implicated in maintaining the integrity of the sarcolemma by connecting components of the extra-cellular matrix (ECM) to the internal cytoskeleton of the muscle fibre. Dystroglycan genes from several species have been cloned and in humans the dystroglycan protein has been shown to consist of two subunits derived from post-translational cleavage of a precursor polypeptide encoded by the \( DAG1 \) gene (Ibraghimov-Beskrovnaya et al., 1993; Holt et al., 2000). The extra-cellular \( \alpha \)-dystroglycan subunit is capable of binding to a variety of laminin isoforms and the proteoglycan molecules agrin and perlecan. \( \beta \)-dystroglycan is membrane bound and connected to the actin cytoskeleton via dystrophin and utrophin. Both subunits undergo N-linked glycosylation and the \( \beta \)-dystroglycan subunit is detected at a molecular
mass ($M_r$) of 43 kDa. The molecular weight of α-dystroglycan varies (from 120 kDa in brain to 156 kDa in skeletal muscle) due to developmental- and tissue-specific O-glycosylation within a central mucin domain.

The forms of muscular dystrophy that have recently been associated with the defective glycosylation of α-dystroglycan are caused by mutations in six genes encoding proteins which are either known or putative O-linked glycosyltransferases. These diseases include severe forms of congenital muscular dystrophy (CMD) associated with structural brain defects and variable eye involvement such as Fukuyama congenital muscular dystrophy (FCMD), Muscle–Eye–Brain disease (MEB) and Walker–Warburg syndrome (WWS). Mutations in the same glycosyltransferase genes may also cause much milder muscular dystrophies such as the limb girdle muscular dystrophies types 2I (LGMD2I) and 2K (Straub and Bushby, 2006), in which only the skeletal muscles are affected. Sequencing studies have revealed that a spectrum of disease severity can result from different mutations in any one of the identified or putative glycosyltransferase genes: POMT1, POMT2, POMGnT1, Fukutin, FKRP (fukutin-related protein) and LARGE (Cohn, 2005; van Reeuwijk et al., 2005). The pathology of these disorders has been attributed to the failure of the resulting hypoglycosylated α-dystroglycan to bind to components of the extra-cellular matrix (Michele et al., 2002).

A number of animal models for these diseases are beginning to add to our understanding of their pathogenesis. Mice null for dystroglycan die around the time of implantation and fail to form Reichert’s membrane (Williamson et al., 1997) indicating the vital importance of dystroglycan in the development of the basement membrane. Brain-selective deletion of dystroglycan has been shown to cause brain malformations similar to the pathology observed in MEB and FCMD patients (Moore et al., 2002). Attempts to model the CMDs by the generation of mice null for fukutin or Pomt1 has resulted in early embryonic lethality attributed to basement membrane defects (Willer et al., 2004; Kurahashi et al., 2005). Moving closer to the human disease situation however, a chimeric mouse deficient in fukutin escapes embryonic lethality to develop a FCMD phenotype (Takeda et al., 2003; Chiyonobu et al., 2005), similar to that observed in the Large<sup>md</sup> mouse harbouring a deletion in the mouse Large gene (Grewal et al., 2001; Holzeind et al., 2002; Michele et al., 2002).

The zebrafish has recently received increasing attention as a model organism to investigate genes involved in muscle development and degeneration, and as a model for muscular dystrophy (Bassett and Currie, 2003; Kunkel et al., 2006; Guyon et al., 2007). The zebrafish expresses orthologues of many DGC components (Steffen et al., 2007) and a number of recent studies have investigated the consequences of genetic mutations or targeted downregulation of expression of muscular dystrophy associated genes on muscle fibre degeneration in the zebrafish (Parsons et al., 2002a; Bassett et al., 2003; Guyon et al., 2003, 2005; Nixon et al., 2005).

The histological hallmarks of muscular dystrophy in human patients and in mouse models for the diseases are ongoing muscle fibre de- and re-generation, variation in fibre size, centrally located nuclei and an increase in connective tissue (fibrosis) (Dubowitz, 1985). In the zebrafish on the other hand it appears as if the progressive muscle degeneration phenotype is caused by the failure of embryonic muscle end attachments, as shown for the sapje mutant zebrafish embryos, a model for Duchenne muscular dystrophy, the most common form of muscular dystrophy (Bassett et al., 2003). A degenerative muscle phenotype involving fibre detachment external to the sarcolemma has also been characterized in the zebrafish mutant caf resulting from mutations in the laminin α2 (lama2) gene (Hall et al., 2007). In a dystroglycan-deficient zebrafish model, in contrast to the mouse, the removal of dystroglycan expression was not essential for basement membrane formation during early stages of zebrafish development. Later in development however, the loss of dystroglycan led to a disruption of the DGC and loss of muscle integrity, accompanied with a loss of sarcomere and sarcoplasmic reticulum organization (Parsons et al., 2002a).

In this study, we have downregulated FKRP expression in the zebrafish and investigated the effects on embryonic development. FKRP morphant embryos showed defects in muscle organization resembling the degenerative phenotypes observed in other zebrafish models for muscular dystrophy (Bassett et al., 2003) and to human FKRP-related disorders. Using morpholino oligonucleotides to target FKRP translation, a spectrum of phenotypic severity was observed. This is the first study to utilize an animal model to investigate the pathogenesis associated with the FKRP gene and indicates that the zebrafish may be an important model for the identification of the precise role of FKRP in developmental processes associated with the glycosylation of α-dystroglycan.

**Materials and Methods**

**Fish strains and maintenance**

The wild-type strain *AB* was used in this study [Zebrafish International Resource Centre (ZIRC), Or, USA]. Zebrafish embryos were collected and raised at 28.5°C according to standard procedures (Westerfield, 2000) and staged in hours or days post-fertilization (hpf or dpf) according to standard criteria (Kimmel et al., 1995).

**Anti-sense morpholino oligonucleotide injection**

Anti-sense morpholino oligonucleotides (MO, Gene Tools LLC) targeted to interfere with FKRP translation were designed using the 5′ sequence around the putative start of translation of the zebrafish FKRP mRNA (accession number NM_001042689). The morpholino sequence was FKRPmo: 5′-ACTGATAACGATTATG GCTCTTGTG-3′. For control MO we used the Gene Tools standard control CONTMO: 5′-CCTCTTACCTGATTTAATAT A-3′. Morpholinos were re-suspended in 1× Danieu solution [58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO<sub>4</sub>, 0.6 mM Ca(NO<sub>3</sub>)<sub>2</sub>].
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5 mM HEPES; pH 7.6) with phenol red as an injection indicator. Morpholinos were injected into the yolk of one- to two-cell stage embryos. Embryos were injected with between 10 and 40 ng of FKRP and control morpholinos.

Whole-mount antibody immunofluorescence

For whole-mount immunohistochemistry with IIH6 (a gift from K. P. Campbell, University of Iowa), embryos were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) at room temperature for 2 h and then transferred into 100% methanol. Embryos were blocked in 5% goat serum in PBS containing 0.1% Tween-20 (PBT). Embryos were incubated in blocking solution containing IIH6 overnight at 4°C followed by washing several times with PBT and incubation with secondary antibody (goat anti-mouse AlexaFluor-488, Invitrogen). Whole-mounted embryos were imaged using a Zeiss LSM510 laser scanning confocal microscope. Z-stack images were generated by scanning through one half of the myotome of the trunk (around somites 10–14) using a 40× objective (oil) at an optimal image slice distance. All z-stack images of control and FKRP morphant embryos were captured using identical gain and offset settings determined empirically for the optimal imaging of control MO embryos and included immunofluorescent staining controls lacking primary and secondary antibodies.

Western blotting and glycoprotein enrichment

Protein lysates were made from whole zebrafish embryos as previously described (Westerfield, 2000). Embryos were dechorionated and deyolled in ice-cold Ringer’s solution containing protease inhibitors (Complete protease inhibitor cocktail, Roche). Deyolled embryos were washed in Ringer’s solution followed by Tris-buffered saline (TBS) and homogenized in TBS containing 1% Triton X-100 and protease inhibitors followed by centrifugation at 13 000 r.p.m. at 4°C for 10 min. The concentration of protein in recovered supernatants was determined by bichinchoninic acid (BCA) assay (Sigma). For the purification of α-dystroglycan, wheat germ agglutinin (WGA)-agarose beads (Vector Labs) were added to lysates containing equal amounts of total protein and incubated overnight at 4°C followed by elution from the WGA-agarose with 0.11 g/ml N-acetylg glucosamine in TBS containing 0.1% Triton X-100 and protease inhibitors. Tissue homogenates were made from adult zebrafish and Bl/10 adult mouse muscle in TBS containing 1% Triton X-100 and protease inhibitors as described above. Proteins were separated on 4–12% Tris–acetate NuPage gels (Invitrogen) and transferred onto PVDF membrane (Hybond-P, GE Healthcare). Blots were blocked in TBS containing 0.1% Tween-20 (TBST) and 5% non-fat dry milk followed by incubation with mouse anti-β-dystroglycan (43DAG, Novocastra) overnight at 4°C. Blots were washed in TBST and incubated with anti-mouse horseradish peroxidase secondary antibody (GE Healthcare). Proteins were detected using ECL plus reagent (GE Healthcare).

Laminin overlay assay

Laminin overlay assays were performed on PVDF membranes using mouse Engelbreth-Holm-Swarm (EHS) laminin (Sigma) as previously described (Michele et al., 2002). PVDF membranes were blocked in laminin binding buffer (LBB: 10 mM triethanolamine, 140 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂, pH 7.6) containing 5% non-fat dry milk followed by incubation with EHS laminin overnight in LBB. Membranes were washed and incubated with rabbit anti-laminin (Sigma) followed by anti-rabbit IgG-horseradish peroxidase (GE Healthcare). Blots were developed using ECL plus reagent (GE Healthcare).

Electron microscopy and semi-thin sectioning

Embryos were fixed in 2% gluteraldehyde in Sorensons phosphate buffer followed by a secondary fix for 1 h in 1% osmium tetroxide in Sorensons buffer with subsequent washes in Sorensons buffer. Samples were then dehydrated through a graded acetone series and embedded in Epoxy resin (TAAB). Semi-thin sections (1 μm) were cut and stained with 1% Toluidine Blue in 1% Borax. Ultra-thin sections (80 nm) were cut and mounted on pioloform filmed copper grids and stained with 2% aqueous uranyl acetate followed by lead citrate. Sections were examined using a Philips CM 100 Compustage transmission electron microscope and digital images collected using an AMT CCD camera.

Results

FKRP morphant morphology

Database searching identified a single gene for FKRP in the zebrafish genome on chromosome 15 encoding a peptide sequence of 536 amino acids with homology to human and mouse FKRP (56% AA identity and 69% AA similarity). To study the developmental functions of FKRP in the zebrafish embryo, we used anti-sense morpholino oligonucleotides (MO) to disrupt the translation of FKRP (Nasevicius and Ekker, 2000). At 24 hpf, following injection of FKRP MO, morphant embryos were developmentally delayed in comparison to control MO injected embryos. FKRP morphants displayed bent tails and alterations in somite morphology observed as curved somite boundaries that are distinct from the regular V-shaped pattern observed in control MO injected embryos (Fig. 1). FKRP morphant embryos were found to have a range of phenotypic severity and were categorized into mild, moderate and severe phenotypes according to morphological characteristics (Table 1). At 24 hpf, mildly affected morphant embryos were found to have curved tails and a slight curvature of the somite boundaries. More severely affected FKRP morphant embryos displayed a more severe curvature of the tail and somite boundaries along with eyes of a smaller diameter and abnormalities in neuronal morphology observed as alterations in the morphology of the midbrain-hindbrain boundary, composed of cells with an opaque cellular appearance (Fig. 1). The most severely affected embryos were found to die within 24 hpf. The frequency of moderate and severe phenotypes was found to increase with injection of an increased amount of FKRP MO (Table 1). By 48 hpf, the bent appearance of the tail became more prominent with more severely affected embryos possessing a tightly curled tail which was unable to extend (Fig. 2). FKRP morphant embryos lacked normal locomotor activity and did not respond to touch with the normal escape response observed in control embryos.
The tails of moderate and severely affected morphants appeared rigid and fasciculated rapidly both spontaneously and in response to touch. Overall, FKRP morphants appeared to have less somitic muscle mass and eyes of smaller diameter compared to controls. These morphological alterations were consistently observed at up to 5 dpf (Fig. 3). Morphometric analysis demonstrated a significant reduction in average eye diameter at 48 hpf over the observed phenotypic range in comparison to control [Control = 0.2619 mm; FKRP mild = 0.2416 mm (Students t-test \( P < 0.01 \)); FKRP moderate = 0.2199 mm (\( P < 0.01 \)); FKRP severe = 0.2021 mm (\( P < 0.01 \)) n = 29].

**Histological examinations of somitic, muscle and eye morphology**

To investigate further the effects of downregulating FKRP on muscle fibre structure and organization we sectioned FKRP morphant and control embryos at 24 hpf. Transverse sections of the trunk region demonstrated widespread disruption of muscle fibre structure and organization (Fig. 4A and B). FKRP morphant embryos also showed an abnormal notochord structure and a loss of the horizontal myoseptum. The organization of the neural tube was also seen to be disrupted. Although the notochord was seen to be surrounded by a thick basement membrane sheath, the overall structure of the notochord appeared disorganized in FKRP morphant embryos, containing cells with smaller vacuoles than control notochords.

Alterations in somitic structure and a loss of the horizontal myoseptum were also observed by differential interference contrast (DIC) microscopy of lateral views of the trunk region of moderately affected morphant embryos at 48 hpf (Fig. 5A and B) and in parasagittal sections of the trunk region at 3 dpf (Fig. 5C and D). FKRP morphant embryos were found to have an irregular arrangement of
muscle fibres compared to control embryos (Figure 5C and D), where an average of 5% of fibres were found to have detached and contracted away from their sites of attachment at the vertical myoseptum in moderately affected FKRP morphants compared to 0% in control embryos. Ultrastructural analysis of FKRP morphants found a disorganization of the vertical myosepta separating adjacent fibres (Fig. 5E and F). The myosepta of morphant embryos appeared fragmented with regions of separated extra-cellular matrix, some of which projected outwards into the adjacent fibres. Localized regions of detached basement membrane were also seen around individual fibres in morphant embryos (Fig. 5G and H). Light micrographs of transverse sections of eyes at 3 dpf found disorganized layering of the different cell types forming the retina of FKRP morphant embryos (Fig. 4C and D). To determine whether neuromuscular junction formation was affected in FKRP morphant embryos we studied the co-localization of acetylcholine receptors and pre-synaptic terminals, at 48 hpf, using fluorescently conjugated α-bungarotoxin and SV2 antibody which demonstrated no significant difference between FKRP morphant and control embryos (see Supplementary material). No significant differences in neuromuscular junction formation were also previously seen in dystroglycan morphant embryos (Parsons et al., 2002a).

Glycosylation of α-dystroglycan
To investigate the glycosylation status of α-dystroglycan in FKRP morphant embryos we immunostained embryos at 24 hpf with the antibody IIH6, which reacts specifically to glycosylated isoforms of α-dystroglycan (Fig. 6). At 24 hpf, IIH6 staining was observed mainly at the somite boundaries and also less intensely along the lengths of muscle fibres, which run longitudinally and are attached between the vertical myosepta which form the somite boundaries. The vertical myosepta are composed of extra-cellular matrix components providing attachment sites for the developing muscle fibres. IIH6 staining of viable FKRP morphant embryos revealed an irregular structure of the somite boundaries with a decreased reactivity to IIH6. In FKRP morphant embryos, a decreasing reactivity of IIH6 was found to correlate with an increasing degree of phenotypic severity. Somite boundaries were misshapen with a curved appearance and a less distinct V-shaped structure with localized variation in reactivity to IIH6 along the lengths of the boundaries.
We investigated the consequences of a reduction in the glycosylation of \(\alpha\)-dystroglycan on extra-cellular matrix binding in FKRP morphant embryos using a laminin overlay assay to study the binding of EHS laminin to purified \(\alpha\)-dystroglycan. We first studied the binding characteristics of laminin to \(\alpha\)-dystroglycan purified from zebrafish tissue at different developmental stages; between 1 and 5 dpf (Fig. 7A). Laminin was found to be capable of binding to \(\alpha\)-dystroglycan species present throughout embryonic and larval stages of development which increased up to 5 dpf. The binding of laminin to \(\alpha\)-dystroglycan from embryonic and larval tissue was found to be at a significantly lower molecular weight than to adult zebrafish tissue. This difference in apparent molecular weight may be due to differential regulation of glycosylation of \(\alpha\)-dystroglycan during zebrafish development. Laminin binding to \(\alpha\)-dystroglycan from mouse muscle was found to be at a significantly higher molecular weight compared to adult zebrafish tissue and this may result from species differences in the glycosylation status of \(\alpha\)-dystroglycan. Although the binding of laminin to \(\alpha\)-dystroglycan purified from developing zebrafish tissue was found to be at a significantly lower molecular weight in comparison to adult tissues, the binding of laminin suggests that these isoforms of \(\alpha\)-dystroglycan have a functional role in binding to laminin and possibly other components of the extra-cellular matrix.

To investigate the laminin-binding properties of \(\alpha\)-dystroglycan from FKRP morphant embryos we compared morphant embryos of mild and moderate severity to control and uninjected embryos at 3 dpf. Laminin binding was found to be significantly reduced in morphant embryos of mild and moderate severity compared to control and uninjected embryos (Fig. 7B).

**Discussion**

The zebrafish has recently been utilized as a model organism for investigating genes associated with the pathogenesis of muscular dystrophy (Parsons et al., 2002a; Bassett and Currie, 2003; Bassett et al., 2003; Guyon et al., 2005; Nixon et al., 2005). Here, we have shown the pathological consequences of downregulating the expression of FKRP in the developing zebrafish and the effects on the glycosylation of \(\alpha\)-dystroglycan and on binding to the extra-cellular matrix ligand laminin. There is some consistency across various zebrafish mutants involving known \(\alpha\)-dystroglycan binding proteins indicating that this model may be particularly appropriate for this group of diseases. The zebrafish mutant *candyfloss (caf)* has a degenerative muscle phenotype resulting from mutations in the *laminin \(\alpha_2\) (lama2)* gene. Although the initial formation of myotomal muscle was found to be normal, muscle fibres were found to subsequently detach and retract from the
vertical myosepta leading to cell death (Hall et al., 2007). A muscle degenerative phenotype has also been described in dystroglycan knockdown embryos in which there is a disruption of the DGC and abnormalities in muscle fibre organization (Parsons et al., 2002a). The laminin mutants bashful (bal), grumpy (gup) and sleepy (sly), whose loci encode laminin α1, β1 and γ1 respectively, have defects in notochord differentiation and eye development (Parsons et al., 2002b; Pollard et al., 2006). In addition, agrin morphant embryos have been shown to exhibit a range of axon outgrowth defects as well as inhibited tail development, defects in the formation of the midbrain-hindbrain boundary and alterations in eye morphology (Kim et al., 2007).

Dystroglycan is an integral component of the DGC and in muscle is implicated in maintaining muscle fibre structure and integrity. Dystroglycan also plays important roles in the structure and function of the central nervous system (Moore et al., 2002), myelination and nodal architecture of peripheral nerves (Saito et al., 2003), cell adhesion (Matsumura et al., 1997) and signalling (Langenbach and Rando, 2002; Spence et al., 2004). Dystroglycan is

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**Fig. 4** Histological analysis of control and FKRP morphant embryos. (A and B) Toluindine blue-stained transverse sections of proximal regions of the trunk showing cellular morphology in Control MO (A) and FKRP morphant embryos (B) at 24 hpf. The structures of the neural tube (NT), notochord (NC) and horizontal myoseptum (HM) are clearly defined in control embryos. FKRP morphant embryos have disorganized muscle fibres, lack the horizontal myoseptum (arrow) and have a disrupted notochord (arrowhead). (C and D) Toluindine blue-stained transverse sections showing eye morphology in FKRP morphant (D) and control embryos (C) at 3 dpf. FKRP morphants have disrupted layering of the retina. L = lens; ON = optic nerve; GCL = ganglion cell layer; IPL = inner plexiform layer; INL = inner nuclear cell layer; PR = photoreceptor cell layer. Scale bars = 50 µm.

**Fig. 5** Analysis of somitic structure and muscle morphology in control and FKRP morphant embryos. (A and B) Differential interference contrast (DIC) images of proximal regions of the trunk at 48 hpf. FKRP morphant embryos (B) show alterations in somite shape and a loss of the horizontal myoseptum (arrow in A) in comparison to control (A). (C and D) Toluindine blue-stained parasagittal sections of proximal regions of the trunk in FKRP morphant (D) and control (C) embryos at 3 dpf. FKRP morphant embryos have an altered somitic structure with disorganized muscle fibres and lack the horizontal myoseptum (arrow in C). (E–H) Electron micrographs of parasagittal sections through muscle fibres at 3 dpf. FKRP morphants have disrupted vertical myosepta (F) and basement membranes (H) in comparison to control embryos (E and G). Scale bars = 50 µm (A–D), 2 µm (E–H).
essential for early embryonic development in the mouse (Williamson et al., 1997) and attempts to model congenital muscular dystrophies by the complete knock out of fukutin and Pomt1 in mice have resulted in early embryonic lethality due to defects in Reichert’s membrane (Willer et al., 2004; Kurahashi et al., 2005). No animal model has previously been described to investigate the developmental abnormalities associated with defects in the FKRP gene. We have found that downregulating FKRP in the zebrafish results in embryos which develop a range of abnormalities reminiscent of the developmental defects observed in the forms of human muscular dystrophy associated with mutations in the FKRP gene. Mutations in the FKRP gene in humans are associated with a spectrum of disease ranging from mild LGMD2I to congenital muscular dystrophy type 1C and more severe forms including MEB and WWS, which are associated with neurological and ocular abnormalities (Brockington et al., 2001a, b; Mercuri et al., 2003, 2006). The most severely affected morphant embryos were found to die within 24 hpf of development, however less severely affected embryos developed a range of phenotypic severity that included morphological alterations in somitic and sarcomeric integrity (Fig. 5), muscle fibre organization and basement membrane structure (Fig. 5D, F and H). Similar defects have been described in patients with severe forms of FKRP deficiency (Ishii et al., 1997; Vajsar et al., 2000). Pathology was also observed in developing neuronal structures and alterations in eye morphology. There is known to be a correspondingly wide spectrum of clinical variability associated with mutations in the human FKRP gene. Dilated cardiomyopathy has been described in patients with LGMD2I in which cardiac involvement does not always correlate with the severity of muscle weakness (Poppe et al., 2003, 2004; Muller et al., 2005; Gaul et al., 2006).

Dystroglycan provides a link between the intra-cellular cytoskeleton and the extra-cellular matrix mediated through the binding of α-dystroglycan to a number of extra-cellular matrix proteins such as laminin, perlecan, neurexin and agrin. These interactions rely on the effective glycosylation of α-dystroglycan and in the dystroglycanopathies carbohydrate modifications are either absent or reduced resulting in decreased binding of α-dystroglycan to its ligands (Barresi and Campbell, 2006; Martin, 2006). In FKRP morphant embryos we found a reduction in the glycosylation of α-dystroglycan, which correlated with an increasing phenotypic severity. A reduced binding of α-dystroglycan to laminin was also found in FKRP morphant embryos, consistent with a disruption in extra-cellular matrix binding associated with the human disorders.

Laminin mutant and dystroglycan morphant zebrafish appear to share some similarities in phenotype compared to FKRP morphant embryos. Dystroglycan morphant embryos have been demonstrated to develop a curved body axis similar to that observed in FKRP morphants in comparison to the shortened body axes of sly and gup.

Fig. 6 Altered somitic structure and decreased immunoreactivity to glycosylated α-dystroglycan in FKRP morphant embryos at 24 hpf. Anti-glycosylated α-dystroglycan antibody IIH6 reactivity in Control MO (A) and FKRP morphant embryos of mild (B), moderate (C) and severe (D) phenotypes. Increased phenotypic severity and disruption in somite structure correlates with decreased IIH6 immunoreactivity in FKRP morphant embryos. Scale bars = 50 μm.
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laminin mutants (Parsons et al., 2002a, b; Pollard et al., 2006). The laminin mutants gup and sly (and less severely affected bal mutant) have defects in notochord differentiation involving basement membrane defects (Parsons et al., 2002b; Pollard et al., 2006) and all three laminin mutants have defects in eye development including lens abnormalities (Semia et al., 2006; Zinkevich et al., 2006; Biehlmaier et al., 2007). FKRP morphant embryos appear to have defects in retinal layering however there appears to be no pronounced defect in lens development in comparison to the laminin mutants. A xenopus dystroglycan morphant, however, displays eye malformations similar to the FKRP morphants, characterized by a disruption of the basal lamina layers, microphntalmia and retinal delayering (Lunardi et al., 2006). Such defects are reminiscent of the ocular and cortical layering abnormalities characterized in mouse models involving mutation or genetic manipulation of the fukutin, POMGnT1, Large and DAG genes (Holzein et al., 2002; Michele et al., 2002; Moore et al., 2002; Takeda et al., 2003; Liu et al., 2006) and in patients with severe forms of dystroglycanopathies (Muntoni and Voit, 2004).

The precise role of FKRP in the glycosylation of α-dystroglycan remains unclear. Although FKRP has been proposed as a putative glycosyltransferase based on sequence similarity to fukutin and other glycosyltransferase genes it has not yet been proven to possess transferase activity (Aravind and Koonin, 1999). FKRP has recently been shown to localize with the sarcolemmal DGC (Beedle et al., 2007) and further investigations using a zebrafish knockdown model may help to elucidate the precise role of FKRP within the DGC and in the glycosylation of α-dystroglycan. Further studies aimed at investigating the spectrum of disease pathogenesis associated with mutations in the human FKRP gene could utilize the zebrafish to study the consequences of FKRP downregulation on cardiac development. Morpholino oligonucleotides are a useful tool for targeting gene expression in studies of early development. In contrast, the development of a mutant zebrafish model for FKRP by the process of TILLING (targeting induced local lesions in genomes) using ENU-induced mutagenesis would allow the opportunity to study later degenerative phenotypes involving the skeletal muscle, CNS and heart that are not possible to investigate using a morpholino approach. These studies should aid our understanding of the phenotypic spectrum seen in human muscular dystrophies associated with mutations in the FKRP gene.

Supplementary material
Supplementary material is available at Brain online.

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References

Fig. 7 Characterization of laminin binding to α-dystroglycan purified from developing zebrafish and FKRP morphant embryos. (A) Increased laminin binding to species of α-dystroglycan purified from developing zebrafish embryos between 1 and 5 dpf, which show different binding profiles to α-dystroglycan from adult zebrafish and adult mouse muscle although all species of α-dystroglycan are capable of laminin binding. (B) Decreased laminin binding to α-dystroglycan purified from FKRP morphant embryos of mild and moderate severity compared to control and non-injected embryos at 3 dpf. Anti-β-dystroglycan (43 DAG) reactivity demonstrates protein loading.


