Abnormal vascular development in zebrafish models for fukutin and FKRP deficiency

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INTRODUCTION

Dystroglycan is a central component in the dystrophin–glycoprotein complex (DGC), responsible for maintaining skeletal muscle fibre integrity during cycles of contraction and relaxation (1–3). The dystroglycan gene (DAG1) has two exons split by a large intronic segment (4). The propeptide is translated from a single mRNA and proteolytically cleaved into two subunits. The subunits are then independently targeted to either the plasma membrane or the extracellular space, respectively, where they are held together by strong non-covalent interactions. Dystroglycan plays an important role in linking the outside and inside of muscle cells (5). The β-dystroglycan subunit interacts intracellularly with dystrophin (6), whereas the peripheral membrane protein α-dystroglycan binds to various extracellular ligands such as laminins through the central O-glycosylated mucin domain (7). The glycosylation state of dystroglycan is tissue-specific and variable, resulting in a range of dystroglycan glycoforms, each differing in molecular weight. The first major glycoform was identified in the brain in 1987 by Smalheiser and Schwartz (8) as cranin with a molecular mass of 120 kDa. In skeletal muscle, the major glycoform is 36 kDa larger than cranin as a result of tissue-specific O-glycosylation.

The muscular dystrophy phenotypes seen as a result of hypo-glycosylation of α-dystroglycan, also referred to as dystroglycanopathies, can be attributed to mutations in one of currently six known genes (9). The genes encode either known or putative glycosyltransferases: protein O-mannosyl transferase 1 and 2 (POMT1 and POMT2), protein O-mannosyl β-1,2-N-acetylglucosaminyltransferase (POMGnT1), acetylglucosaminyltransferase-like protein (LARGE), fukutin and fukutin-related protein (FKRP) (10,11). Recently, the first mutation in the dystroglycan gene itself has been shown to cause a muscular dystrophy (12). The diseases resulting from mutations in these genes can lie anywhere in a spectrum of severity.
from milder forms of limb girdle muscular dystrophies (LGMDs) to more severe forms of congenital muscular dystrophies (MDCs), some of which, like muscle–eye–brain (MEB) disease and Walker–Warburg syndrome (WWS), are also associated with brain and eye abnormalities (13, 14). A homozygous missense mutation in the FKRP gene (826C > A) that is more prevalent in Northern Europe and North America causes LGMD2I, a condition on the less severe end of the spectrum (15–17). Various mutations in other regions of the FKRP gene result in more severe conditions ranging from MDC1C to MEB and WWS (14, 18, 19). Another disorder of aberrant α-dystroglycan glycosylation with a high prevalence in Japan is Fukuyama congenital muscular dystrophy (FCMD) caused by mutations in the fukutin gene (20–22). Again, mutations in this gene can also be associated with a broad spectrum of clinical phenotypes, including LGMD2M (23).

A number of animal models have been generated to investigate the pathogenesis of dystroglycanopathies. However, due to embryonic lethality of several null mice (24–26), the successful generation of useful mammalian systems has been challenging and limited (27). The MORE-DG mouse circumvents embryonic lethality by expressing dystroglycan in extraembryonic membranes. Both the spontaneous myasthenia gravis mouse mutant (myd), which carries a deletion in the LARGE gene, and mice lacking POMGnT1 are viable and have been helpful in understanding the molecular defects underlying dystroglycanopathies (28–31). Mouse models that are viable to study FCMD are a chimeric mouse deficient in fukutin, which escapes embryonic lethality (32), and a fukutin knockin model, carrying the common fukutin retrotransposonal insertion (33).

Zebrafish are another system receiving increasing attention as a developmental model for muscular dystrophy and dystroglycanopathies. Many of the components of the DGC have orthologues in zebrafish (34, 35). In contrast to mice, zebrafish that lack dystroglycan expression progress through the early embryonic stages (36). Recent studies were able to successfully generate zebrafish by means of morpholino oligonucleotide (MO) knockdown of the fkrp and fukutin genes. Morphant fish show pathology of muscles, eyes and the brain and are therefore good animal models for the study of events in the pathogenesis of dystroglycan-associated muscular dystrophies (35–39).

For a long time, it had been postulated that vascular pathology might contribute to the phenotype seen in muscular dystrophy patients (40, 41). A ‘vascular hypothesis’ for the pathogenesis of muscular dystrophy had even been predicted before the identification of the first responsible disease gene (42, 43), and it was speculated that skeletal muscle necrosis was caused by muscle ischaemia (44). After the identification of the dystrophin gene, the vascular hypothesis became less popular and it was mainly the finding of abnormal nitric oxide synthase localization in Duchenne and Becker muscular dystrophy that the vascular hypothesis was revisited (45–47). Dystroglycan is also expressed in endothelial cells, and experiments by Hosokawa et al. (48) suggested a potential role of endothelial dystroglycan in the control of angiogenesis. To test whether dystroglycan modifications mediated by fukutin and FKRP are relevant for vascularization, we downregulated fukutin, FKRP and dystroglycan in fli-1 zebrafish, i.e. TG(fli-1:EGFP) (49). This zebrafish strain expresses the enhanced green fluorescent protein (EGFP) under the control of the promoter of the known transcription factor and early endothelial marker fli-1, thus allowing us to monitor blood vessel growth at several stages of embryonic development and to analyse the vasculature of the morphant phenotypes.

RESULTS

Characterization of fukutin Fli-1 embryos

The TG(fli-1:EGFP) strain was selected to study the effects of knocking down fukutin, FKRP and dystroglycan on vascular development in zebrafish. We injected antisense MOs to downregulate expression of fukutin, FKRP or dystroglycan in zebrafish embryos. Genes encoding zebrafish fkrp (37, 38), fukutin (39) and dag1 (35, 36) had been reported and respective morphants phenotypically described. Our database (ZDB-GENE-070410-96) search identified a single gene for fukutin in the zebrafish genome, encoding a 457-amino-acid polypeptide located on chromosome 5. There was 57% identical and 72% similar amino acid sequence homology between zebrafish and humans. To knock down fukutin, a novel antisense spliceosomal morpholino was designed to target the boundary between intron 3 and exon 4 (Fig. 1A). The ability of the fukutin MO to alter mRNA splicing was confirmed by reverse transcriptase–polymerase chain reaction (RT–PCR) analysis using primers flanking exons (Fig. 1B). Previously published morpholino antisense oligonucleotides were used to disrupt the translation of FKRP and dystroglycan (36, 37).

Fukutin knockdown embryos were viewed and sorted according to their overall body morphology. Morpholino injection produced a range of phenotypes as judged on morphological grounds with the most severe found dead at 1 day post-fertilization (dpf). The development of morphant fukutin embryos was slightly delayed in comparison to control-injected embryos at 1 dpf. Furthermore, their motility (tail coiling inside the chorion) at 1 dpf was reduced. We observed a slight ventral curvature of the distal portion of the tails in the morphant embryos with a mild phenotype (Fig. 2). The somite shape changed from V-shape to a more rounded appearance. Fish with less clearly outlined myosepta appeared more severely affected. In general, tail curvature was more pronounced in the fukutin morphants compared with dystroglycan or FKRP moderate morphants (Figs 1C and 2). The morphant embryos in the severe phenotype category had a clearly abnormal body shape and much shorter tails, and somite boundaries were no longer visible (Figs 1C and 2). From a morphological aspect, the fukutin knockdown embryos did also show structural brain and eye abnormalities, which were most prominent in fish with the severe phenotypes (Fig. 1). Overall, there was a clear overlap of abnormalities detected in the fukutin fish with abnormalities that had previously been described in fukutin, FKRP and dystroglycan morphants (Fig. 2) (35–37, 39).

The proportion of embryos found dead in the control morpholino-injected group was not significantly different from that of the non-injected controls, indicating that mortality was specific to the morpholino sequence. The fukutin morphants were sorted according to their phenotype.
Downregulation of FKRP and dystroglycan produced a range of phenotypes, which were very similar to the spectrum observed for fukutin (Table 1). No significant differences were found in morphant morphology between the wild-type AB strain and the transgenic fli-1 embryos (data not shown).

The glycosylation properties of α-dystroglycan in fish embryos at 1 dpf were investigated by immunostaining with the antibody IIH6, which specifically binds to glycosylated forms of α-dystroglycan (Fig. 3). In control fish, IIH6 stained the somite boundaries with the greatest intensity. Muscle fibres run longitudinally and attach to the vertical myosepta, and only weak IIH6 staining was observed along the length of the fibres. In fukutin knockdown fish, we found a similar reduction as seen and described previously in the FKRP morphants (Fig. 3) (37). As expected, no staining was observed in the dystroglycan morphants (Fig. 3). To investigate the consequences of a reduction in glycosylation on the overall expression of the dystroglycan complex, 1 dpf embryos were immunostained with the 43DAG antibody recognizing β-dystroglycan. In control embryos, the 43DAG antibody stained the myosepta, which have a classical chevron appearance. In the fukutin morphants, the intensity of the stain in the myosepta was reduced, but interestingly, β-dystroglycan staining seemed less affected than in the FKRP and dystroglycan morphants, in which it was barely visible or completely abolished, respectively (Fig. 3).

To ascertain structural changes in the muscle, 1 dpf embryos were stained with phalloidin to detect f-actin. In control fish, the fibres between the myosepta were straight and filled most of the myotome trunk. In mild fukutin, FKRP, dystroglycan and the control glucosamine-fructose-6-phosphate aminotransferase 1 (gfat1) morphant embryos, on the other hand, muscle fibres were not straight and regular (Fig. 4). The gfat1 morpholino knockdown has been used as control for our downstream studies of vascular changes. GFAT1 is essential in the hexamine pathway that eventually yields the amino sugar uridine diphosphate-N-acetylglucosamine. Mutations in GFAT/GFPT1 have been shown to cause a congenital myasthenic syndrome (50). In the mild FKRP, fukutin and dystroglycan morphants, muscle fibres became more spaced out and thinner than those in controls, while the myosepta kept their classic chevron shape. As the morphant severity increased, the fibres also became more distorted. In the most severe phenotypes, the myosepta lost their chevron appearance and became more rounded (Fig. 4).

Abnormal somitic vasculature in fukutin, FKRP and dystroglycan morphants

Fli-1 embryos were imaged using a confocal microscope to capture images through the myotome trunk. During the pharyngeal phase, the segmental arteries of zebrafish embryos arise from the dorsal aorta, following the course of the
In fukutin, FKRP and dystroglycan morphants with a mild phenotype, intersegmental vessels failed to reach the dorsal longitudinal anastomotic vessel pectorally at 24 hpf (Fig. 5A). In some instances, the intersegmental vessels of the FKRP morphants branched from the top of the segmental arteries at the midline. With increasing phenotypic severity of the fukutin and FKRP morphants, the intersegmental vessels were found to be missing with greater frequency. The intersegmental vessels of moderate-to-severe fukutin morphants were more disorganized with increasing severity of the phenotypes.

We downregulated the expression of the GFAT1 protein as controls for these findings. We wanted to see whether the induction of muscle fibre pathology causes unspecific vascular changes or whether this effect is specific for FKRP, fukutin and dystroglycan. The gata1 morpholino has been used by us previously (50), and GFAT1-deficient zebrafish embryos were shown to have muscle fibre abnormalities and a curled tail very similar to the tail phenotype caused by the FKRP and fukutin knockdown. The GFAT1-deficient embryos had vascular morphology identical to control morpholino-injected and wild-type fli-1 embryos. The dorsal aorta in most cases was the only distinguishable vessel in the vasculature of the severe fukutin, FKRP and dystroglycan morphants within the myotome trunk region. There were a few instances in which intersegmental artery branching occurred below the midline in the morphants with severe phenotypes. In several fukutin or dystroglycan knockdown embryos with moderate phenotypes, the vessels joined together via anastomoses in the embryo midline (Supplementary Material, Fig. S1).

The vertical height of the segmental vessels above the dorsal aorta was measured and expressed as a proportion of body height in 12 independent experiments. Ten embryos and five intersegmental vessel heights from each embryo were measured for each phenotype (Fig. 5B). All morphants were smaller than controls. In each severity spectrum of the morphant phenotypes, there was a progressive decrease in the size of the segmental vessel with increasing phenotype severity. Blood vessels in the FKRP morphants were shorter across each phenotype than those in the comparable dystroglycan or fukutin morphants, whereas blood vessels in the dystroglycan morphants were larger than those in FKRP morphants of comparable phenotypic severity.

**Figure 2.** Light microscopic images of embryos at 24 hpf. Embryos were either untreated fli-1 or injected with 10 pg of control/FKRP, 2 ng fukutin and 5 ng dystroglycan morpholinos. Embryos were staged according to Kimmel 

### DISCUSSION

Eye abnormalities have been described in the severe end of the spectrum of patients with mutations in the genes involved in glycosylation of α-dystroglycan. For this reason and because the vasculature acts as a structural marker, the blood vessels of the eyes in the morphant zebrafish were investigated. The inner optic circle vessels of embryonic zebrafish eyes at 1 dpf are spherical in shape (Figs 6 and 7). The eyes of fukutin and FKRP morphant embryos with a mild phenotype had smaller and more oval inner optic circle vessels than the eyes of control fish. The eye vasculature of fukutin morphant phenotype became increasingly distorted with increasing severity of the phenotype (Fig. 6). The eye vasculature of the most severely affected embryos was too distorted to be clearly identified. When we investigated the eye vasculature in dystroglycan morphant fish, we surprisingly did not find any significant changes compared with control fish.

The eye vasculature of 3 dpf morphant embryos was measured in comparison to the areas of the eyes of the morphants (Fig. 7). A minimum of three independent injections was carried out for each of the phenotypes, and 15 eyes were scanned on average in each experiment. In control morpholino-injected embryos, the eyes were slightly smaller than those in the non-injected embryos. The mean area of the mild morphant fukutin and FKRP embryo eyes was smaller than that of the control-injected eyes. The most severe fukutin and FKRP morphant embryos had the smallest eye areas. The eyes of the FKRP morphant larvae were larger than those found in the fukutin morphants across each phenotype. In dystroglycan morphant embryos, eye size across all morphant phenotypes was similar to that of the control morpholino-injected embryos.

**Perturbation of eye vasculature in fukutin and FKRP morphant fish**

Recently, we and others investigated the effect of FKRP and fukutin deficiency on muscle, eye and brain development in zebrafish (37–39). FKRP morphant fish showed a very similar...
phenotype to the dystroglycan-deficient zebrafish model (35,36). As dystroglycan had been reported to play a role in angiogenesis, we examined whether the morpholino-induced downregulation of fukutin, FKRP and dystroglycan affected vascularization of muscle and eyes in zebrafish. As both FKRP and fukutin have been suggested to be involved not only in dystroglycan glycosylation but also in protein secretion, we were particularly interested to learn whether knockdown of fukutin or FKRP expression would reflect the vascular phenotype of dystroglycan zebrafish models. For our studies of vascular development, we used the transgenic zebrafish line TG(fli-1:EGFP) (52). The morphological appearance of the FKRP, dystroglycan and fukutin fli-1 morphants was similar to that of the previously published morphants on the wild-type AB background (35–37,39).

Fukutin morphants appeared slightly more severe than the others not only morphologically but also microscopically, showing damaged muscle fibre structure by staining the α-actin architecture of somites. Muscle fibre structure in the fukutin morphants was more distorted and irregular than that in FKRP morphants across comparable morphological phenotype groups. The somitic vascular morphology of the fukutin morphants was also more abnormal than that seen in FKRP or dystroglycan embryos, even when general body morphology was directly comparable between the morphants. Overall, the general similarity of the FKRP and fukutin morphant phenotypes added weight to the argument that the two proteins have related functions, not only in mammalian tissues but also in fish (37).

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Transient knockdown of fukutin and FKRP using morpholinos was successful in reducing glycosylation of α-dystroglycan in the morphants, as shown by immunostaining. A weak laminin–dystroglycan interaction may cause a destabilization of the sarcolemma (39), and this could account for the muscle pathology of the morphants. Previous studies have shown that correctly glycosylated α-dystroglycan is important for sarcolemma stability (37,39,53). Surprisingly, we also detected a simultaneous reduction of β-dystroglycan immunostaining in FKRP and fukutin morphants. This observation was in contradiction to previously published work (38). It is possible that DGC proteins are lost from the sarcolemma and degraded as a consequence of structural defects caused by incorrect or incomplete glycosylation of α-dystroglycan, thereby leading to a secondary disappearance of β-dystroglycan (30). However, the reduction of β-dystroglycan was an inconsistent feature that we did not see in all examined morphants.

The observed differences in severity between the fukutin and the FKRP phenotypes might be attributed to either a higher efficiency of fukutin knockdown, which would be an unspecific effect, or specific differences in the function of the two proteins. However, as the FKRP and fukutin morpholinos were not directly comparable due to differences in sequence and modes of actions (spliceosomal versus translation blocking MO), the precise reason for the increased severity observed in fukutin morphant embryos compared with FKRP morphant embryos remained unclear.

When we examined blood vessel development in tail muscles of fli-1 embryos after downregulation of fukutin, FKRP or dystroglycan, we observed absence or delayed growth of intersegmental vessels in the somites of all three morphants. This finding raised several questions about the underlying cause of the abnormal vascularization we detected. Was vascular pathology in the dystroglycanopathy fish models secondary to the observed changes in the muscle, or was muscle necrosis an inevitable consequence of hypoxia in somites? Or were both muscle and vascular development primarily affected in the morphant fish?

Table 1. Morphology of morphant embryos at 1 dpf

<table>
<thead>
<tr>
<th></th>
<th>Normal (%)</th>
<th>Mild (%)</th>
<th>Moderate (%)</th>
<th>Severe (%)</th>
<th>Dead (%)</th>
<th>Total (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fukutin MO 2 ng</td>
<td>10</td>
<td>9</td>
<td>7</td>
<td>12</td>
<td>62</td>
<td>252</td>
</tr>
<tr>
<td>FKRP MO 10 ng</td>
<td>12</td>
<td>17</td>
<td>14</td>
<td>11</td>
<td>46</td>
<td>286</td>
</tr>
<tr>
<td>Dystroglycan MO 5 ng</td>
<td>40</td>
<td>9</td>
<td>2</td>
<td>3</td>
<td>46</td>
<td>384</td>
</tr>
<tr>
<td>CoMo 10 ng</td>
<td>84</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>16</td>
<td>346</td>
</tr>
<tr>
<td>Uninjected</td>
<td>85</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>15</td>
<td>427</td>
</tr>
</tbody>
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The phenotypic data shown were obtained from a total of 12 independent experiments, three for each morpholino used. The proportion of embryos observed for each phenotypic class is presented as a percentage of the total number of embryos studied for each morpholino.

Figure 3. IIH6 and β-dystroglycan staining of 1 dpf embryos. Projections from confocal microscopy through myotome trunk. White scale bar represents 100 μm. Upper panel, IIH6 staining; lower panel, β-dystroglycan. From left to right: uninjected fli-1 control, embryo injected with control morpholino, embryo injected with fukutin MO, embryo injected with FKRP MO and embryo injected with dystroglycan MO.
Based on zebrafish models in which proteins important for angiogenesis were knocked down, such as the endothelial cell-specific molecule 2 (ECSM2) (54) and the netrin receptor UNC5B (55), it is unlikely that the vascular phenotype observed in the dystroglycanopathy models is responsible for muscle damage, as both the ECSM2 and the UNC5B models have poor intersegmental vessel growth without any muscle pathology. Similarly, knocking down the myotubulin family gene Mtmr8 in zebrafish resulted in reduced segmental vessel development without a curly tail phenotype (56). Mtmr8 is expressed in the mesoderm, a tissue that both muscle and endothelial cells are derived from during development; however, Mtmr8 downregulation only resulted in vascular abnormalities. These findings make it unlikely that the muscle phenotype seen in the fukutin, FKRP and dystroglycan morphants was a secondary result of abnormal vascularization in the fish.

The question remained whether abnormal vascularization seen in the somites could still be a secondary consequence of the muscle defect. To our knowledge, vascularization has not been studied in other zebrafish models for muscle disease to see whether the described vascular abnormalities were an unspecific response to the somitic pathology. We knocked down the ubiquitously expressed GFAT1 protein and showed that a curly tail phenotype with abnormal muscle fibres has no vascular phenotype in the somites. Generally, vascularization is enhanced in regenerating muscles, and there is no evidence from mouse models or patients with muscular dystrophy that muscle pathology affects vascular morphology.

We argue that the disruption of angiogenesis in somites was a primary result directly caused by the knockdown of fukutin, FKRP or dystroglycan, and not a secondary consequence of muscle damage. Vice versa, aberrant vascular development did not result in abnormal somite or tail structure in other zebrafish models (54,55), with vascularization defects. This suggested that vascular and muscle pathologies seen in our morphants were two unrelated separate pathogenic consequences of the loss of fukutin, FKRP or dystroglycan expression. This hypothesis was supported by our findings of the eye vascularization patterns in the three morphant fish models.

In addition to the skeletal muscle pathology, patients with FCMD, MEB and WWS caused by mutations in the fukutin and FKRP genes also present with severe structural eye...
abnormalities (14,57). Eye abnormalities reminiscent of the human condition were also described in FKRP- and fukutin-deficient animal models (26,37,38). When studying vascular morphology in the eyes of the fukutin and FKRP morphants, we noticed abnormal eye vasculature development in both models. The findings were in accordance with POMT1 and
POMT2 morphant zebrafish, which also showed eye abnormalities (34). The dystroglycan morphant eyes, on the other hand, had the same appearance and size as the controls. This suggested that eye vascular development in zebrafish embryos and early larvae did not depend on dystroglycan expression. Studies that have investigated myd, FKRP and MORE-DG mutant mice suggest that not only the presence of α-dystroglycan but also its glycosylation is important for eye development (27,30,58). These studies might indicate that there are differences between eye development in fish (35) and mice or that dystroglycan deficiency in fish affects eye development at a later stage, as indicated by Gupta et al. (35). The differences in eye vasculature between fukutin and FKRP morphant fish and dystroglycan morphants are in contrast to the somitic vascular findings, in which the intersegmental vessels were reduced in size in all three dystroglycanopathy models. This suggests that the effects of fukutin and FKRP knockdown are not mediated by dystroglycan in the development of the eye in zebrafish and indicates that vascular development may be dependent on correctly glycosylated α-dystroglycan in muscle and other unknown factors in the eye. Our findings suggest that both FKRP and fukutin are important in vascular development and have divergent roles in muscle and eye.

The relevance of vascular defects for patients affected by dystroglycanopathies has so far not been investigated in great detail. Vascular abnormalities have been reported in a series of cases with FCMD (41) and more recently also in patients with MEB disease (59,60). Most interestingly, the vascular abnormalities that were described in the MEB patients were detected in the eyes and showed similarities to our findings in the zebrafish models. Patients were affected by an abnormal distribution of retinal vessels, choroidal atrophy, in one case an avascular retina and vessels with reduced calibre (59,60). In our own cohort of patients with LGMD2I, we also had one with retinal bleeding and one with intracerebral haemorrhage caused by a vascular aneurysm (Supplementary Material, Fig. S3). Vascular pathology might therefore contribute to the phenotype of patients with dystroglycanopathies and needs to be studied in more detail.

Our work is the first report of vascular defects in animal models for dystroglycanopathies. Understanding the implications of the vascular component of pathology may also improve our understanding of the human conditions.
MATERIALS AND METHODS

Fish strains and maintenance

The two fish strains used in the study were the wild-type AB\(^+\) strain [Zebrafish International Resource Centre (ZIRC), Eugene, OR, USA] and the transgenic line TG (fli-1: EGFP) (52) expressing EGFP in the blood vessel endothelium. Zebrafish embryos were collected and raised at 28.5\(^\circ\)C in E3 medium using established procedures (61) and staged in hours or days post-fertilization, according to Kimmel et al’s criteria (62).

Antisense MO injection

We designed a morpholino antisense oligonucleotide (MO, Gene Tools LLC) targeted to the putative start of translation of the \(fkrp\) transcript. The \(fkrp\) MO 5’-ACTGATACGCA TTATGGCTCTTGTG-3’ produces a muscle, eye and brain

Figure 7. (A) Three-day post-fertilization eye vasculature using projections from confocal microscopy through the eye. Red dots show how area is calculated in (B); white bar represents 100 \(\mu\)m. IOC, inner optic circle; DCV, dorsal ciliary vein; NCA, nasociliary vessel; OV, optic vein. (B) Relative area of 3 dpf eye vasculature. The FKRP- and fukutin-injected embryos were staged according to the phenotype at 2 dpf. Bars represent 95% confidence interval, and y-axis is in arbitrary units. Results for each phenotype from a minimum of three separate experiments.
phenotype in AB zebrafish embryos (37). The fukutin splice MO 5′-GC CCCAGAAACCTCTTGAGATGC-3′ is directed against the splice donor site of exon 4, leading to the skipping of the exon and consequently to a frame shift that results in premature translation termination. The dystroglycan translation blocking MO 5′-CATGCCTGCTTTATTTTCCCTGC-3′ has previously been described by Parsons et al. (36). The gfa1 splice MO was used as described in Senderek et al. (50). The Gene Tools standard control morpholino 5′-CCTCTTACCTCAGTAATTTATA-3′ was used for control MO injections. The morpholinos were re-suspended in 1× Danieau solution [0.4 mM MgSO4, 58 mM NaCl, 0.7 mM KCl, 5 mM HEPES, 0.6 mM Ca(NO3)2; pH 7.6] (61). The embryos were then injected into the yolk at the one- to two-cell stage with 10 ng of either FKRP or control, 2 ng of fukutin and 7 ng of dystroglycan morpholino using phenol red as an injection indicator. The dose for the generation of fukutin morphants was optimized by injecting various amounts of morpholino until a phenotype range similar to the FKRP morphants was reached.

RNA isolation and RT–PCR
RNA was collected from approximately 30 zebrafish embryos following morpholino injection for RT–PCR analysis. The RNA was isolated with Trizol reagent (Invitrogen, Paisley, UK) and converted into cDNA. PCR using gene-specific primer pairs was carried out: fukutin: exon 2 forward: 5′-TGTTCTGGATCGCTCTAATG-3′ and exon 3 reverse: 5′-CATTAAAGCGGACAGGAAG-3′; individual bands were purified and sequenced.

Whole-mount antibody immunofluorescence staining
Embryos were fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) overnight at 4°C and then permeabilized with pre-cooled acetone at −20°C. Non-specific antibody binding sites were blocked in PBS containing 5% horse serum and 0.1% Tween-20 (PBT). Embryos were incubated overnight at 4°C in blocking solution containing either of the following primary antibodies: IIH6 (anti α-dystroglycan mouse IgM 1:50, a kind gift from Kevin Campbell) and 43DAG (anti β-dystroglycan mouse IgG 1:50, Novocasta). The embryos were washed several times with PBT and incubated with the secondary antibody: goat anti-mouse IgM Alexa Fluor 594, 1:500 (Invitrogen) and goat anti-mouse IgG Alexa Fluor 488, 1:500 (Invitrogen). Texas red-conjugated phalloidin 1:100 (Invitrogen) was used to label f-actin. A minimum of 20 embryos split between three independent injections were stained and scanned out for each phenotype.

Microscopy
Embryos at day 1 post-fertilization were dechorionated manually, and all fish, including 3 dpf larvae, were euthanized in 4 mg/ml tricine methanesulphate, E3 medium mix (50:50). Embryos were washed in PBS and then fixed in 4% PFA overnight at 4°C before mounting on slides in Vectashield (Vector Laboratories, Peterborough, UK). Samples were analysed on a Zeiss LSM 510 Meta laser scanning confocal microscope, using the argon laser line at 488 nm and the HeNe 543 nm ×20 or oil ×40 objectives and analysed with LSM software V4.2.0.121. An epifluorescence stereo microscope (Leica MZ16F) was used to image live embryos.

Image software
Image J software was used to calculate the area of the 3 dpf embryos eyes that were derived from projections of the eye vasculature. The projections came from complete cross-sections of the eyes (z-stacks of 35 slices). The intersegmental vessel heights from the dorsal aorta and expressed as a proportion of total somite height were measured using image J.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG online.

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Conflict of Interest statement. The authors declare no conflicts of interest.

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