

DEVELOPMENT AND DISEASE

Removal of dystroglycan causes severe muscular dystrophy in zebrafish embryos

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SUMMARY

Muscular dystrophy is frequently caused by disruption of the dystrophin-glycoprotein complex (DGC), which links muscle cells to the extracellular matrix. Dystroglycan, a central component of the DGC, serves as a laminin receptor via its extracellular α subunit, and interacts with dystrophin (and thus the actin cytoskeleton) through its integral membrane β subunit. We have removed the function of dystroglycan in zebrafish embryos. In contrast to mouse, where dystroglycan mutations lead to peri-implantation lethality, dystroglycan is dispensable for basement membrane formation during early zebrafish development. At later stages, however, loss of dystroglycan leads to a disruption of the DGC, concurrent with loss of muscle integrity and necrosis. In addition, we find that loss

of the DGC leads to loss of sarcomere and sarcoplasmic reticulum organisation. The DGC is required for long-term survival of muscle cells in zebrafish, but is dispensable for muscle formation. Dystroglycan or the DGC is also required for normal sarcomere and sarcoplasmic reticulum organisation. Because zebrafish embryos lacking dystroglycan share several characteristics with human muscular dystrophy, they should serve as a useful model for the disease. In addition, knowing the dystroglycan null phenotype in zebrafish will facilitate the isolation of other molecules involved in muscular dystrophy pathogenesis.

Key words: Dystroglycan, Muscular dystrophy, Dystrophin, Zebrafish, *Danio rerio*, Sarcomere, Sarcoplasmic reticulum

INTRODUCTION

Most human muscular dystrophies result from deficiencies in components of the extracellular matrix (ECM) or the DGC, the cell anchorage system through which skeletal muscle cells transduce their contractile force (Campbell, 1995). A central component of the DGC is the receptor protein known as dystroglycan. The dystroglycan transcript encodes two polypeptides, α and β dystroglycan, which are separated by post-translational cleavage and associate with each other to form an integral membrane protein complex (Holt et al., 2000). The extracellular α subunit can bind a variety of laminin isoforms and the proteoglycan molecules agrin and perlecan. In the cytosol, the β subunit is connected to the actin cytoskeleton via dystrophin or utrophin (Winder, 2001). The mechanical linkage established by dystroglycan is important in skeletal muscle cells and is thought to prevent membrane damage during contraction. In ongoing studies of mutations that affect zebrafish notochord development, we have identified two loci that encode laminin chains (Parsons et al., 2002). As dystroglycan is a laminin receptor, we investigated its role during zebrafish embryogenesis.

Dystroglycan is required early in mammalian embryogenesis. Mice null for dystroglycan die around the time of implantation and fail to form the basement membrane (BM)

known as Reichert's membrane (Williamson et al., 1997). A major component of the BM is laminin, which polymerises to form a scaffold. Mice that lack the laminin $\gamma 1$ chain also fail to form Reichert's membrane and die around the time of implantation (Smyth et al., 1999). Not all BM formation, however, is dependent on dystroglycan. Chimaeric mice generated with dystroglycan-null ES cells display a dystrophic muscle phenotype but form a normal muscle BM (Cote et al., 1999). This suggests that dystroglycan is not essential for muscle cell BM assembly. Differences between early and late requirements for dystroglycan in BM formation may be explained by the action of other laminin receptors present in skeletal muscle.

Integrin dimers containing $\beta 1$ -integrin can act as laminin receptors. ES cells lacking $\beta 1$ -integrin can be made to differentiate in vitro into a variety of fates, including skeletal muscle. While $\beta 1$ -integrin mutant myotubes become surrounded by laminin, electron microscopy reveals that the basement membrane is thin or absent (Lohikangas et al., 2001). Taken together, these data suggest that different laminin receptors are instrumental in BM formation in distinct tissues and at different times during embryogenesis.

Dystroglycan is also thought to be important in both the peripheral and central nervous system (CNS). The only identified binding partners for dystroglycan in the CNS are the

neurexins, cell-surface proteins implicated in cell adhesion. This association suggests that dystroglycan may be involved in cell adhesion in the CNS (Sugita et al., 2001). Several studies have also implicated dystroglycan in the clustering of nicotinic acetylcholine receptors (AChR) in muscle cells, an essential feature of neuromuscular junctions (NMJ) (Chamberlain, 1999).

To investigate the action of dystroglycan in BM assembly and assess its importance in muscle integrity and innervation, we cloned zebrafish *dystroglycan* cDNA, ascertained its expression pattern during embryogenesis and removed its function using antisense morpholino oligonucleotides (MO).

MATERIALS AND METHODS

Cloning and physical mapping

Using EST data from GenBank we obtained a 5' 720 bp (Accession Number, AW173854) and a 3' 650 bp (Accession Number, AW171079) fragment of zebrafish *dystroglycan* cDNA. Using a combination of RACE and RT-PCR, the remaining 2250 bp was obtained from cDNA derived from zebrafish embryos (15 somites). Primers were designed and overlapping PCR fragments cloned and sequenced. The forward primer 5'-CGGGAACAAAAGGAGAGGAT-3' and reverse primer 5'-AGCAGCACCACAAGAGTCCT-3' were used to clone the 5' untranslated region and start of translation. These same primers were used on the LN54 radiation panel (Hukriede et al., 1999) to map zebrafish *dystroglycan* physically. We mapped our sequence to linkage group 22, 0.1 cR from marker Z21243, adjacent to EST fb83d06, which is a 3' fragment of *dystroglycan* cDNA.

We predicted the start of translation to be a leucine because the DNA sequence indicates the presence of a consensus Kozak sequence and the first 22 residues of a signal peptide (Nielsen et al., 1997).

Whole-mount in situ hybridisation

For *dystroglycan*, a 492 bp product was amplified from cDNA, using 5'-AGAGCTGTAGAAGGGCGAGA-3' forward and 5'-AGTGCA-TAGACGCCTCCAAC-3' reverse primers, cloned and used as a template to generate a digoxigenin riboprobe. This region is approximately half 5'UTR and half ORF. For *dystrophin*, primers were designed to the published sequence (5'-GGATCTCCAGGCA-GAGATTG-3' forward and 5'-GGAGCTCCATCAGCCTCTC-3' reverse), to amplify a 599 bp cDNA fragment (Bolanos-Jimenez et al., 2001). The PCR product was cloned and used as a template.

Whole mount in situ hybridisation was carried out according to published protocols (Thisse et al., 1993).

Antisense morpholino oligonucleotide injections

Using the 5' sequence around the putative start of translation we designed an anti-sense morpholino oligonucleotide (MO, Gene Tools) to interfere with dystroglycan translation. The sequence used was 5'-CATGCCTGCTTTTATTTCCCTCGC-3'. A volume of 1.4 nl was injected through the chorion of single cell embryos to deliver 7 ng of MO. For control MO we used the Gene Tools standard control 5'-CCTCTTACCTCAGTTACAATTTATA-3'.

Immunohistochemistry

Protocols were standard with the following modifications:

Rabbit anti-laminin (Sigma L-9393, 1:400); embryos were fixed in 4% paraformaldehyde overnight at 4°C and then 100% methanol, followed by washes in phosphate-buffered saline, 0.1% Tween 20 (PBT) and digestion in 10 µg/ml proteinase K (10 minutes). Mouse anti-β-dystroglycan (Novocastra, 1:50); embryos were fixed for 2 hours at room temperature in 4% paraformaldehyde, then placed in 100% methanol. Anti-dystrophin (Sigma MANDRA-1, 1:100) or

monoclonal F59 anti-slow-twitch myosin (Devoto et al., 1996; Evans et al., 1988); embryos were placed straight into 100% methanol. Anti-acetyl-α-tubulin (Sigma T 6793, 1:1000); embryos were fixed in 2% TCA for 3 hours at room temperature, followed by washes in PBT and digestion in 0.025% trypsin in PBT (4 minutes on ice).

Electron microscopy

Whole zebrafish embryos were dechorionated manually and fixed overnight with 2% glutaraldehyde, 2% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.2 (SCB). They were washed for 10 minutes in SCB and post-fixed for 1 hour in 1% osmium tetroxide, SCB. They were washed again with SCB and stained en bloc with 1% aqueous uranyl acetate for 1 hour. The samples were then dehydrated through a graded ethanol series, followed by two changes of propylene oxide over 20 minutes and embedded in Epon resin (Agar Scientific). Ultra thin sections (50 nm) were cut and mounted on pioloform coated slot grids and stained with 1% aqueous uranyl acetate for 15 minutes followed by Reynold's lead citrate for 7 minutes. Sections were visualised in a Jeol 1200 EX electron microscope.

Detection of AChR clusters

Embryos were incubated in 5 µM tetramethylrhodamine-α-bungarotoxin (Molecular probes) in L15 medium (Life Technologies), 15% DMSO for 30 minutes at 4°C, then washed five times in L15 and viewed under fluorescence.

Accession Number

The full-length zebrafish *dystroglycan* cDNA sequence has been submitted to GenBank and the Accession Number is AF483476.

RESULTS

Sequence analysis and expression of dystroglycan

Dystroglycan transcripts encode a propeptide that is cleaved into α dystroglycan and β dystroglycan (Holt et al., 2000). We cloned and sequenced zebrafish *dystroglycan* cDNA and found a high degree of conservation between human and fish β-dystroglycan (75% identity, 85% similarity). The α dystroglycan chains, however, are less conserved between human and zebrafish (56% identity, 68% similarity). The regions showing the least conservation include the signal peptide and a region of low complexity (residues 312-447 in zebrafish).

Zebrafish *dystroglycan* is maternally expressed. Using whole-mount in situ hybridisation, we detected mRNA expression at the 128-cell stage, before the onset of zygotic transcription (Fig. 1A). The transcript is ubiquitously expressed throughout gastrulation, but by the tailbud stage expression is restricted to the midline and head (Fig. 1D,E). At the beginning of somitogenesis, we detected *dystroglycan* mRNA in adaxial cells and in the developing CNS. At this stage, midline expression is reduced (Fig. 1F). By the 12-somite stage, we detected expression throughout the paraxial mesoderm and developing CNS (Fig. 1H). Transverse sections reveal that *dystroglycan* mRNA is present in the immature notochord and adaxial cells in the tail (Fig. 1I, upper section) but not at the level of the hindbrain (Fig. 1I, lower section). CNS expression is seen at both axial levels. At 24 hours post-fertilisation (hpf) we observed expression in the CNS and mesoderm. Although there is little to no expression in the notochord, cells of the hypochord express *dystroglycan* at high levels (Fig. 1J).

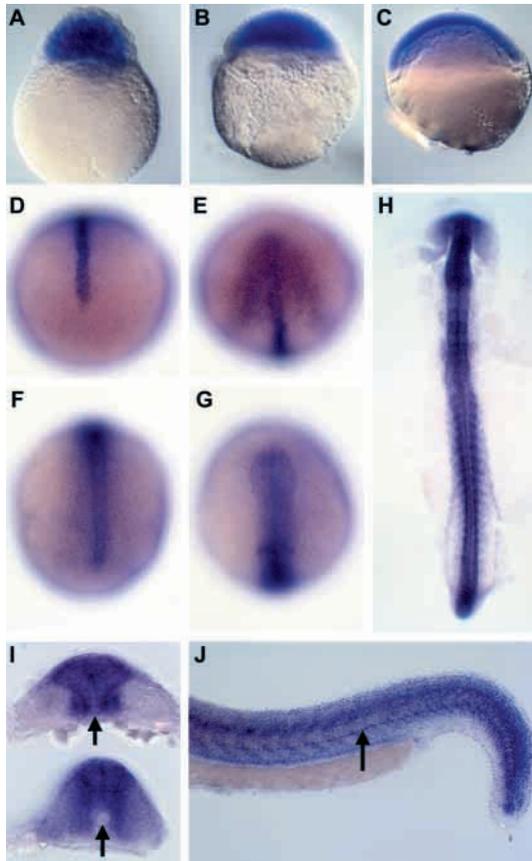


Fig. 1. Whole-mount in situ hybridisation for *dystroglycan*. (A) 128-cell stage and (B) 1000-cell stage, indicating maternal expression. (C) Shield stage and (D,E) tailbud stage; posterior (D) and anterior (E) views, indicating expression in the chordamesoderm and developing brain. (F,G) Five-somite stage, posterior (F) and anterior (G) views. (H) Flat mount of a 12-somite embryo, showing widespread expression. (I) Sections through tailbud and hindbrain level of the 12-somite embryo in H. Expression in immature (upper section) but not mature (lower section) notochord is indicated (arrow). (J) Trunk of 24 hpf embryo. High expression in the hypochord is indicated (arrow).

Muscle integrity is compromised in embryos lacking dystroglycan

We disrupted translation of dystroglycan using an anti-sense morpholino oligonucleotide (MO) approach (Nasevicius and Ekker, 2000). Following injection of *dystroglycan* MO at the one-cell stage, embryogenesis proceeds normally. By 24 hpf, however, morphant embryos appear to be developmentally delayed (Fig. 2B) compared with non-injected or control-MO-injected siblings (Fig. 2A). In addition, dystroglycan morphants have a hooked tail and smaller tail fin folds (Fig. 2B). Although dystroglycan morphants respond to touch, they appear to be less flexible than either non-injected or control MO-injected siblings. By 48 hpf, dystroglycan morphants are curved and dystrophic compared with controls (Fig. 2E,F) and move in an uncoordinated fashion.

To assess the level of dystroglycan protein production after injection of *dystroglycan* MO, we stained embryos using an anti-dystroglycan antibody. In control MO-injected embryos, at 24 hpf and 48 hpf, we detected dystroglycan in the transverse

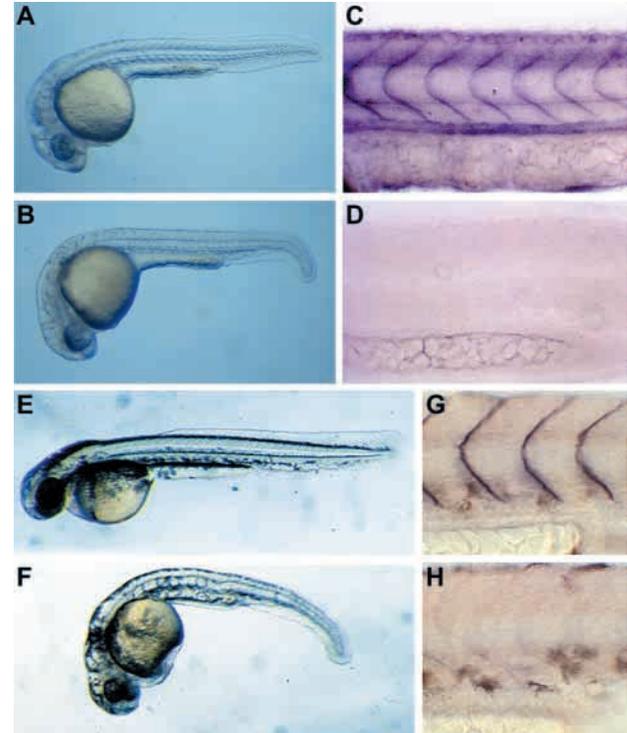


Fig. 2. Removal of dystroglycan protein leads to abnormal development. Embryos injected with control MO at 24 hpf (A) and 48 hpf (E) and with a dystroglycan MO at 24 hpf (B) and 48 hpf (F). (C,D,G,H) Staining for dystroglycan in control (C,G) and dystroglycan morphant (D,H) embryos at 24 hpf (C,D) and 48 hpf (G,H).

myosepta and various other regions (Fig. 2C,G). By contrast, *dystroglycan* MO-injected embryos completely lacked dystroglycan (Fig. 2D,H). This indicates that the antisense morpholino oligonucleotide completely disrupts protein production of the intended target even as late as 48 hpf.

We focused our attention on the skeletal muscle development of dystroglycan morphants, as this is a tissue known to be affected in mouse dystroglycan mutants (Cote et al., 1999). The F59 antibody recognises a myosin isoform expressed by slow-twitch muscle fibres (Devoto et al., 1996; Evans et al., 1988). In embryos that lack dystroglycan, the overall morphology of the slow-twitch muscle is normal (Fig. 3A,B). Closer inspection reveals, however, that muscle fibres in morphants are less fasciculated than in control MO-injected embryos. This indicates that removal of dystroglycan has no effect on slow-twitch muscle differentiation per se but does affect organisation of the muscle tissue.

Dystroglycan is central to the DGC. We therefore investigated the localisation of dystrophin in dystroglycan morphants. Normally, dystrophin is located at the transverse myoseptum, consistent with its interaction with dystroglycan (Fig. 3C). In embryos that lack dystroglycan, however, localisation of dystrophin at the plasma membrane of the transverse myoseptum is lost (Fig. 3D). As neither dystroglycan nor dystrophin could be detected in dystroglycan morphants, we wanted to know if there was any effect on the transcription of these two genes. We performed whole-mount

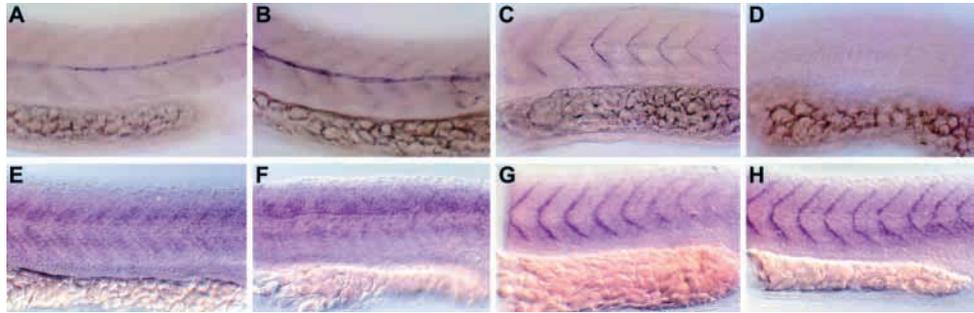


Fig. 3. Molecular characterisation of the dystroglycan morphant phenotype. (A,C,E,G) Embryos injected with a control MO. (B,D,F,H) Dystroglycan morphants. (A,B) Staining for slow twitch myosin. Dystrophin is clearly localised to myosepta of control-MO-injected embryos (C) and is undetectable in dystroglycan morphants (D). Dystroglycan morphants do not show a decrease of either dystroglycan (E,F) or dystrophin (G,H) mRNA expression.

in situ hybridisation on 28 hpf embryos. In 28 hpf non-injected or control MO-injected embryos, *dystroglycan* mRNA has a widespread distribution in the somite, with faint stripes apparent in the middle of the somite, probably due to perinuclear localisation of transcript (Fig. 3E). Levels of *dystroglycan* mRNA expression were similar in controls and morphants. We did not, however, observe the stripes in morphant embryos, which is presumably due to the disorganisation of cells within the somite (Fig. 3F). In control MO-injected embryos, we detected *dystrophin* mRNA at the transverse myoseptum, as previously reported (Bolanos-Jimenez et al., 2001) (Fig. 3G). In contrast to its protein localisation, we found that *dystrophin* mRNA is expressed at normal levels and is localised at the transverse myosepta in *dystroglycan* MO-injected embryos (Fig. 3H). In conclusion, even though the loss of dystroglycan caused by MO treatment affects neither its own transcription nor that of *dystrophin*, neither protein is detectable by immunostaining.

To investigate the effect of removing dystroglycan on muscle integrity, we sectioned developing somites of 28 hpf and 48 hpf control MO- and *dystroglycan* MO-injected embryos (Fig. 4). Light micrographs of transverse sections revealed that the cellular appearance of muscle in dystroglycan morphants was less organised than in control embryos. We observed numerous lesions within the somitic tissue (Fig. 4B). In the ventral region of morphant embryos, we found darkly stained cells possessing the morphology of apoptotic cells. Electron micrographs reveal that the lesions we observed are

swollen cells with intact nuclear membranes and normal mitochondria, which are characteristics of necrotic cells (Fig. 4D). Hence, in dystroglycan morphants we found cells dying via each of the two known mechanisms: apoptosis and necrosis. In muscle tissue, however, we found cells dying only via necrosis. Electron microscopy also revealed damage to the subcellular organisation of dystroglycan-deficient muscle cells. Cross-sections through morphant muscle cells showed a clear presence of actin-myosin filament bundles (Fig. 4F); however,

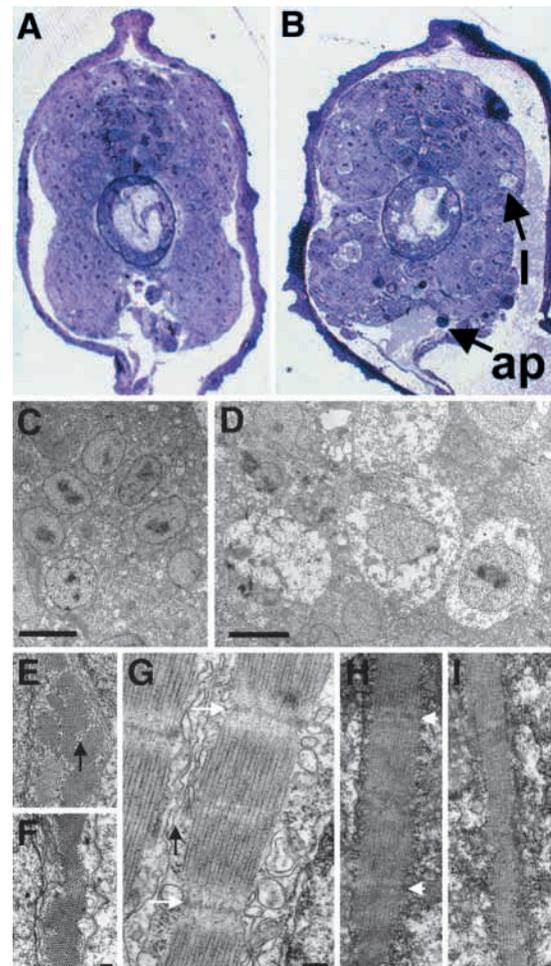
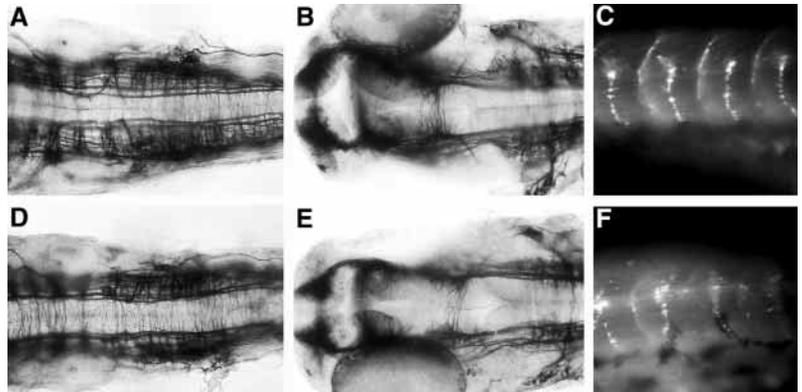


Fig. 4. Histological analysis of dystroglycan morphants. Sections of control MO-injected (A,C,E,G) and dystroglycan morphant (B,D,F,H,I) embryos, all at 28 hpf. (A,B) Light micrographs of transverse sections. l, lesions; ap, apoptotic cells. (C,D) Electron micrographs of transverse sections through muscle. Lesions seen in B are confirmed as swollen, necrotic cells shown in D. (E-I) Subcellular structure of muscle cells at higher magnification. (E,F) Typical hexagonal array of actin and myosin in transverse sections. (G-I) Longitudinal sections. Z-discs are indicated, where detected, by white arrows. In the few identifiable sarcomeres in dystroglycan morphants, muscle fibre organisation is disrupted and sarcoplasmic reticulum is difficult to discern (H). The majority of muscle fibres in dystroglycan morphants possessed no sarcomeres (I). Sarcoplasmic reticulum in control embryos is indicated by black arrows. Scale bars: 5 μ m in C,D; in F, 0.2 μ m for E,F; in G, 0.2 μ m for G-I.

Fig. 5. CNS and neuromuscular junction organisation is unaffected in dystroglycan morphants. Acetylated tubulin staining shows the pattern of axonal scaffolds in control MO-injected (A,B) and dystroglycan morphant (D,E) embryos. (A,D) Hindbrain views. (B,E) Anterior views. We could find no consistent morphological difference between control MO-injected (C) and dystroglycan morphant (F) after staining for AChRs. All embryos are at 48 hpf.



longitudinal sections revealed a huge reduction in the number of sarcomeres (Fig. 4I) and the sarcomeres that could be discerned were clearly aberrant (Fig. 4H). Finally, the abundant sarcoplasmic reticulum clearly visible in both longitudinal and cross-sections of control muscle is virtually absent in dystroglycan morphants.

CNS and NMJ development is apparently unaffected by dystroglycan removal

To determine whether dystroglycan removal affects cell adhesion within the developing CNS, we examined the gross morphology of the brain and the localisation of acetylated α tubulin in 48 hpf morphant fish (Fig. 5A,B,D,E). We could observe no obvious difference between dystroglycan morphant and control embryos. Innervation of the skeletal muscle also appeared to be normal when compared with controls, as judged by a comparable appearance and number of motoneurons (data not shown) and AChR clusters (Fig. 5C,F). We therefore conclude that removing dystroglycan in zebrafish embryos does not radically affect embryonic CNS morphology or NMJ formation.

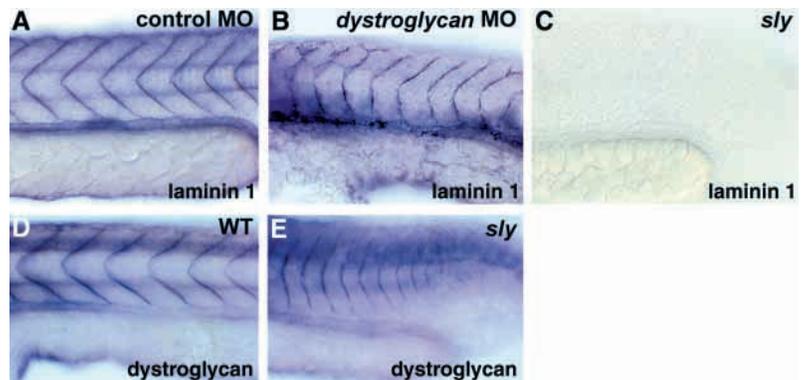
Localisation of dystroglycan and laminin 1 are mutually independent

Dystroglycan is known to be a receptor for laminin (Campbell, 1995). Mouse mutant studies suggest that dystroglycan may organise the assembly of laminin. It was therefore of interest to know what effect the loss of dystroglycan had on laminin levels and localisation. In control MO-injected embryos, laminin is concentrated in transverse myosepta and in the membranes surrounding the yolk extension (Fig. 6A). The

notochordal sheath also includes laminin 1 (Parsons et al., 2002). In dystroglycan morphants, we detected laminin 1 expression at control levels in all these tissues (Fig. 6B). Hence, formation of laminin 1 polymer is independent of dystroglycan. Laminin 1 localisation, however, is somewhat disrupted in dystroglycan morphants. For example, we found that the laminin expression pattern in the transverse myoseptum was irregular and bifurcated abnormally (Fig. 6B). Expression of *laminin C1* and *laminin B1* are unaffected in dystroglycan morphants (data not shown), hence the abnormal localisation of laminin 1 immunoreactivity is probably a secondary effect of the disruption of the muscle morphology caused by the removal of dystroglycan.

Laminin complexes are heterotrimers of α , β and γ chains. Mutations in the *laminin A2* gene, which encodes laminin $\alpha 2$, cause congenital muscular dystrophy in both mouse and human (Campbell, 1995). Extracellular α dystroglycan is known to bind the laminin $\alpha 1$ and $\alpha 2$ chains (Colognato and Yurchenco, 2000). Furthermore, the addition of laminin can strongly influence the localisation of dystroglycan in cultured cells (Cohen et al., 1997; Li et al., 2001). To test whether any similar interactions occur in developing zebrafish, we examined the effect of removing laminins on dystroglycan localisation. A zebrafish *laminin A2* mutant has yet to be isolated. The gene encoding laminin $\gamma 1$, however, is disrupted in zebrafish *sleepy (sly)* mutants (Parsons et al., 2002). The laminin $\gamma 1$ chain is a component of 10 out of the 12 known laminin isoforms. In *sly* mutants, laminin 1 ($\alpha 1 \beta 1 \gamma 1$) is undetectable at 24 hours (Fig. 6C), indicating that removing one chain disrupts the stability of the entire heterotrimeric complex. Using anti-dystroglycan antibody on *sly* mutant and wild-type

Fig. 6. Expression of laminin 1 and dystroglycan are mutually independent. Expression of laminin 1 in control MO-injected (A), dystroglycan morphant (B) and *sly* mutant (C) embryos. In the absence of dystroglycan, laminin 1 is still localised to the transverse myoseptum (B). Expression of dystroglycan in wild-type (D) and *sly* mutant (E) embryos. As indicated in C, *sly* mutants lack laminin 1, which does not affect dystroglycan localisation to the transverse myoseptum (E).



siblings embryos, we find that dystroglycan is localised correctly to the transverse myoseptum in the absence of laminin 1 and by implication, the vast majority of other laminin isoforms (Fig. 6E). Therefore, if disruption of a specific laminin heterotrimer were to elicit a muscular dystrophy phenotype in zebrafish, it is unlikely to occur via the disruption of dystroglycan localisation.

DISCUSSION

We find embryonic zebrafish muscles can be properly specified and differentiate to a contractile state in the complete absence of dystroglycan or properly localised dystrophin. Without dystroglycan, however, muscles eventually undergo necrosis. The mode of muscle cell loss in dystroglycan morphants is similar to the loss of muscle in human congenital muscular dystrophy in several important ways. First, muscle cell necrosis appears to coincide with the onset of spontaneous muscle contraction in the embryo. Second, the gross morphological phenotype of embryos that lack dystroglycan increases in severity over time, suggesting that the phenotype is progressive. Although dystroglycan is widely expressed at 24 hpf, by 48 hpf we could find dystroglycan immunoreactivity only at the transverse myoseptum. No phenotype, other than the muscle morphological phenotype, was apparent in the dystroglycan morphants, suggesting that either dystroglycan has no function in other embryonic tissue or that it is redundant to other molecules in non-muscle tissues.

It is well known that mutations in the Duchenne muscular dystrophy (*DMD*) gene, which encodes dystrophin, lead to muscular dystrophies and ultimately premature death in humans (Koenig et al., 1987). The *Mdx* mouse is null for dystrophin but displays a milder form of muscular dystrophy, displaying the muscle pathology typical of the human disease only in the diaphragm (Stedman et al., 1991). Removing both dystrophin and the closely related utrophin provides a better mouse model for Duchenne muscular dystrophy (Deconinck et al., 1997; Grady et al., 1997). By removing dystroglycan, we have disrupted the complex that links both dystrophin and utrophin to the extracellular space. We have shown that removing dystroglycan leads to loss or mislocalisation of dystrophin in skeletal muscles concurrent with a general disruption of muscle integrity, loss of sarcomere organisation and necrosis of the developing muscle. These phenotypes are very similar to those observed in muscle degeneration that occurs in humans suffering from muscular dystrophy or the mouse models of the disease. For example, disruption of the interaction of α dystroglycan and laminin in a mouse cell culture system has been shown to disrupt sarcomere organisation (Brown et al., 1999).

Although the *dystroglycan* gene itself has not been identified as a cause of human muscular dystrophy (because mutations probably lead to embryonic lethality) there are several human muscular dystrophy genes known to affect dystroglycan function. For example, Limb-girdle muscular dystrophy 1C (LGMD-1C) is caused by mutations in caveolin 3 (*CAV3*), which is believed to be important for the normal organisation of T-tubules and correct membrane localisation of dystroglycan (Galbiati et al., 2001; Minetti et al., 1998). When caveolin 3 is overexpressed in muscle of transgenic mice, it leads to

downregulation of β dystroglycan expression and a Duchenne-like muscular dystrophy (Galbiati et al., 2000). Altering the glycosylation state of α dystroglycan has also been found to cause muscular dystrophy. For example, Muscle-eye-brain disease (MEB) is caused by mutations in a glycosyltransferase, POMGnT1, which is likely to mediate the O-mannosyl glycosylation of α dystroglycan (Kano et al., 2002; Yoshida et al., 2001). Similarly, markedly reduced glycosylation of α dystroglycan is thought to be responsible for the phenotype of myodystrophic (*Myd*) mice, which carry a deficiency in the gene encoding a glycosyltransferase-like protein called large (Grewal et al., 2001). Thus, in mammals there is a correlation between compromised dystroglycan function and muscular dystrophy.

Comparing the phenotype of zebrafish dystroglycan morphants with standard mammalian models of muscular dystrophy, such as *Mdx* mutant mice, a key difference is the timing of the dystrophic phenotype. There are several possible explanations for this. The early onset of muscle pathology seen in zebrafish dystroglycan morphants may relate to a developmental role of dystroglycan, perhaps in basement membrane organisation. There may be a similar early requirement for dystroglycan in mouse muscle development, but this has not been addressed because of the peri-implantation lethality (Williamson et al., 1997). Alternatively, in human muscular dystrophy and in the mouse models of the disease, the comparatively late onset of the disease may be the result of regeneration that does not occur in zebrafish. It is known that overexpression of a muscle-specific isoform of the insulin-like growth factor, IGF1, can sustain hypertrophy of muscle and prevent muscle loss because of age-related muscle atrophy (Musaro et al., 2001). Indeed, IGF1 is found to be upregulated in *Mdx* mutant muscle (De Luca et al., 1999). Hence, the reduced severity of the mouse *Mdx* mutant compared with human Duchenne or Becker muscular dystrophy may be due, in part, to enhanced regenerative capacity of mouse muscle. Zebrafish embryonic muscle may not possess the regenerative capacity of mouse or human muscle. While we cannot examine the role of regeneration in dystroglycan morphants, because of the transient nature of the morpholino-based approach, the knowledge of the morphant phenotype will now guide us to identify dystroglycan mutants as well as other muscular dystrophy mutants that were probably observed in chemical mutagenesis screens (Driever et al., 1996; Haffter et al., 1996).

What remains clear is that there is a sharp contrast between the mouse and zebrafish in the onset of a requirement for dystroglycan. In the zebrafish there is no equivalent need to form a BM such as the mammalian Reichert's membrane until organogenesis is under way. This is also apparent in laminin mutants, which appear morphologically normal prior to displaying a notochord differentiation defect (Parsons et al., 2002). Zebrafish are therefore a useful model to study the role of dystroglycan and other ECM components during late embryogenesis. Dystroglycan is maternally expressed and later becomes restricted to the midline prior to somitogenesis, when the first requirement for a BM and laminin 1 occurs in zebrafish. Although dystroglycan is a laminin receptor, removing dystroglycan has no effect on notochord differentiation. Dystroglycan morphant embryos do not resemble zebrafish *laminin* mutants (*sly*, *gup*) at 24 hpf

(Parsons et al., 2002). Hence, dystroglycan is not required for laminin 1 accumulation or BM formation in zebrafish embryos. Moreover, we find that laminin 1 is not required for correct dystroglycan localisation. It is possible that there is functional redundancy between dystroglycan and other laminin receptors expressed in the midline such as the integrins (Whittaker and DeSimone, 1993).

Dystroglycan can bind other components of the ECM and the role of laminin may be redundant to other proteins containing laminin G-like modules such as agrin or perlecan (Winder, 2001). Congenital muscular dystrophy in humans and mice has been linked to mutations in laminin chain $\alpha 2$ (Campbell, 1995). The antibody we used in this study recognises the heterotrimer laminin 1 ($\alpha 1 \beta 1 \gamma 1$). It is possible that another laminin heterotrimer consisting of completely different chains from those found in laminin 1 may be important in the biology of dystroglycan.

Dystroglycan has been implicated in the establishment of AChR clusters at the NMJ. Work in tissue culture suggests that removing dystroglycan abrogates AChR clustering (Jacobson et al., 1998). However, other studies using dystroglycan mutant myotubes in culture have shown AChR clusters do form in the absence of dystroglycan (Grady et al., 2000). In the zebrafish, we found that dystroglycan removal has no effect on the localisation or appearance of AChR clusters. Indeed, we do not observe dystroglycan in the synaptic region of muscle cells, but rather at the transverse myoseptum. From these data, we conclude that dystroglycan is unlikely to have a role in the establishment of AChR clusters in the embryonic zebrafish. Nevertheless the dystrophin-glycoprotein complex may be required for the maintenance of AChR clustering as has been suggested (Grady et al., 2000). It has also been suggested that dystroglycan is important for cell adhesion in the CNS through its association with neurexins (Sugita et al., 2001). In zebrafish dystroglycan morphants, however, the gross morphology and axonal scaffold of the brain is unaffected.

There are several features of zebrafish embryogenesis and genetics that make it well suited for the study of muscular dystrophy. Zebrafish embryonic skeletal muscle is simply organised, with single myotubes extending across each somite and attached at either end to the ECM of the transverse myoseptum. Loss of dystroglycan and the DGC leads to a less pleiotrophic phenotype than in mouse. Finally, generation of zebrafish embryos that lack dystroglycan is convenient and inexpensive. These embryos should facilitate the study of the cellular pathogenesis associated with muscular dystrophy and can be used to examine interactions of dystroglycan with other gene products that have known or suspected roles in muscular dystrophy aetiology. In addition, dystroglycan morphant or mutant zebrafish could be used to screen for agents capable of suppressing the dystrophic phenotype. Finally, several human muscular dystrophies, such as facioscapulohumeral muscular dystrophy (FSHD), have been mapped but the underlying disease gene remains unknown (van Geel et al., 2002). Zebrafish homologues of candidate human genes from a critical region can be readily identified using the zebrafish genome and EST sequence. The fact that we can now recognise muscular dystrophy in zebrafish combined with the efficacy of antisense morpholino oligonucleotides make zebrafish a fast and effective means with which to identify the specific genetic causes of a variety of muscular dystrophies.

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