

Dystrophin is required for the formation of stable muscle attachments in the zebrafish embryo

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Summary

A class of recessive lethal zebrafish mutations has been identified in which normal skeletal muscle differentiation is followed by a tissue-specific degeneration that is reminiscent of the human muscular dystrophies. Here, we show that one of these mutations, *sapje*, disrupts the zebrafish orthologue of the X-linked human *Duchenne muscular dystrophy (DMD)* gene. Mutations in this locus cause Duchenne or Becker muscular dystrophies in human patients and are thought to result in a dystrophic pathology through disconnecting the cytoskeleton from the extracellular matrix in skeletal muscle by reducing the level of dystrophin protein at the sarcolemma. This is thought to allow tearing of this membrane, which in turn leads to cell death. Surprisingly, we have found that the progressive muscle degeneration phenotype of *sapje* mutant zebrafish

embryos is caused by the failure of embryonic muscle end attachments. Although a role for dystrophin in maintaining vertebrate myotendinous junctions (MTJs) has been postulated previously and MTJ structural abnormalities have been identified in the Dystrophin-deficient *mdx* mouse model, in vivo evidence of pathology based on muscle attachment failure has thus far been lacking. This zebrafish mutation may therefore provide a model for a novel pathological mechanism of Duchenne muscular dystrophy and other muscle diseases.

Key words: Myomuscular junctions, Myotendinous junctions, Dystrophin, *sapje*, Muscle attachments, Muscular dystrophy, Congenital myopathy

Introduction

The progressive degeneration of normally differentiated skeletal muscle is the main pathological feature of a diverse group of muscle-wasting conditions known as the muscular dystrophies, many of which result from genetic defects that disrupt genes encoding components of the Dystrophin-Associated Protein Complex (DAPC). For recent reviews see Spence et al. (Spence et al., 2002) and Blake et al. (Blake et al., 2002). In post-natal mammalian skeletal muscle, this complex links the actin cytoskeleton via the rod-like protein dystrophin and the laminin receptor dystroglycan to laminin- α 2, and is enriched at junctional as well as non-junctional specialisations of the sarcolemma such as the neuromuscular junction (NMJ), myotendinous junction (MTJ) (Clerk et al., 1993; Khurana et al., 1991) and myomuscular junction (MMJ) (Paul et al., 2002).

Although most attention has focused on the role of the DAPC in maintaining the integrity of the non-junctional sarcolemma, several mouse models of muscular dystrophies also show structural MTJ defects, although in vivo failure at this site has yet to be observed. Dystrophin-deficient *mdx* mice

(*Dmd* mice – Mouse Genome Informatics, however, possess a much milder muscle pathology than Duchenne muscular dystrophy (DMD) sufferers, probably because of a more efficient process of fibre regeneration and upregulation of the autosomal orthologue of dystrophin, utrophin, within the sarcolemma of *mdx* muscle fibres, which can compensate for dystrophin loss. However, *mdx* mice do show reduced membrane folding at MTJs (Law and Tidball, 1993; Ridge et al., 1994; Law et al., 1995) and mice deficient in another component of the DAPC complex, α -dystrobrevin, also show this relatively mild MTJ phenotype (Grady et al., 2003). *mdx/utrophin* double mutant and laminin- α 2-deficient (*dy*) mice, which more completely lack the DAPC as a mechanical link, show a striking near absence of folding at the MTJ (Grady et al., 1997; Deconinck et al., 1997; Deconinck et al., 1998; Desaki, 1992). These results clearly suggest that utrophin can compensate for dystrophin at junctional sarcolemmal sites within mammalian muscle fibres, and that the DAPC is required at least for normal MTJ formation. Mice lacking α 7-integrin also show changes at MTJs, but are viable and have not been reported to suffer MTJ failure. Despite these clear

abnormalities, however, there have been no reports that MTJs fail in any mouse models of muscular dystrophy or congenital myopathy. Indeed, mechanical testing of isolated muscle fibres has shown that in *mdx* mice there is no apparent loss of strength in the MTJ compared with wild-type (WT) (Law et al., 1995), although similar tests have not yet been performed on mice completely lacking DAPC function. Therefore, this lack of effect may represent a further area in which the *mdx* mouse differs from DMD, as utrophin has been postulated to substitute for dystrophin at the MTJ in mice to a greater extent than in human muscle (Grady et al., 1997; Deconinck et al., 1997; Deconinck et al., 1998).

However, to date, the MTJ has not been reported as a site of pathology in inherited disorders of human muscle, and thus it remains unclear as to what extent MTJ failure might contribute to Duchenne or other human muscular dystrophies and myopathies. This is in part because biopsies of patients' muscle tissue are often deliberately taken at a distance from the tendon in order to simplify histology and minimise the effects of removal. This necessary practice may have hitherto reduced opportunities for the observation of damage to this structure. However, there are indications from non-invasive methods of observation that the MTJ might be affected in DMD. Magnetic resonance imaging (MRI) studies are difficult in DMD patients because of the difficulties they encounter in maintaining an appropriate position, but there have been studies suggesting that damage in the thigh region is most severe towards the ends of muscles, near the MTJs (Nagao et al., 1991; Hasegawa et al., 1992).

Within some mammalian muscles, dystrophin is also enriched at specialised MMJs that transmit force between the ends of muscle fibres (Paul et al., 2002). These attachment sites occur as either intrafascicular fibre terminations (IFTs), connecting series of single fibres end-to-end or end-to-side (Snobl et al., 1998), or as fibrous sheets called tendinous intersections (TIs) that separate segmented blocks of non-overlapping fibres. Tendinous intersections such as those between blocks of the mammalian rectus abdominis bear a striking structural resemblance to the non-cellular embryonic attachments present between somites, and have been suggested to be evolutionarily analogous to somite boundaries (Snobl et al., 1998; Hijikata and Ishikawa, 1997).

In order to address the extent to which damage to muscle attachments might contribute to muscle degeneration and to fully understand the role of the DAPC in muscle attachment, it is necessary to study the effects of removal or reduction of the complex in vivo in animal models. However, whereas mammalian models have been complicated by the incomplete penetrance of the phenotype of dystrophin removal in vivo, invertebrate systems appear to lack an analogous function for the DAPC in muscle attachment. Genetic studies in both the nematode *Caenorhabditis elegans* and the fruit fly *Drosophila melanogaster* have implicated several genes in the formation and maintenance of muscle attachments. In *C. elegans*, mutations including the *mua* class affect attachments between the body wall muscles and epithelia, and those cloned so far correspond to structural components of an integrin complex analogous to the focal adhesion complex (Bercher et al., 2001; Plenefisch et al., 2000; Boshier et al., 2003). By contrast, the *C. elegans* homologues of dystrophin and utrophin, *dys-1* (Bessou et al., 1998), and other DAPC components (Grisoni

et al., 2002), are required for cholinergic signalling at neuromuscular junctions rather than muscle attachment or integrity. In *Drosophila*, mutations have been identified that affect the positioning and assembly of focal muscle attachments that resemble MTJs. Zinc finger transcription factors of the Broad-complex (Sandstrom and Restifo, 1999; Sandstrom et al., 1997), the EGF pathway and the integrin complex (Beumer et al., 1999; Martin-Bermudo, 2000; Becker et al., 1997; Volk, 1992; Volk, 1999; Yarnitzky et al., 1997) combine to direct these processes, with Broad-complex genes being required in the epithelial tendon cells to maintain correct muscle-tendon attachments. There is, however, no genetic evidence as to the function of the DAPC in *Drosophila*, although the reduced complement of components has been suggested to make the fly a suitable model for its study (Greener and Roberts, 2000). Similar data are not available in relation to vertebrate muscle attachment, so it is important to identify mutations with analogous phenotypes.

In the present study we have investigated *sapje* (*sap*), a member of a small class of recessive, lethal mutations which cause embryonic-onset, progressive degeneration of skeletal muscle in the zebrafish (*Danio rerio*) (Granato et al., 1996). We show that embryonically the mechanism of degeneration in *sap* homozygotes is the separation of somitic muscle fibres from their attachment points on myosepta, which are tendon-like sheets of extracellular matrix (ECM). Furthermore, we identify the *sapje* locus as *dmd* (Bolanos-Jimenez et al., 2001), the zebrafish orthologue of the human DMD gene, and show that dystrophin is required for the stability of the muscle attachments in the zebrafish embryo.

Materials and Methods

Immunohistochemistry

We performed immunohistochemistry as previously described (Macdonald et al., 1997). Anti-dystrophin MANDRA1 (Sigma) was diluted 1:1000. Anti- β DG (Novocastra) was diluted 1:10. Anti-MyHC A4.1025 (DSHB, University of Iowa) and anti-utrophin (N-19, Santa Cruz) were diluted 1:400. Alexa-labelled secondary antibodies and DAPI (Molecular Probes) were diluted 1:1000.

In situ hybridisation

We performed in situ hybridisation as previously described (Macdonald et al., 1997). Embryos were fixed in 4% paraformaldehyde in phosphate-buffered saline.

Evans blue dye (EBD) labelling

Dye (Sigma) was injected at 0.1 mg ml⁻¹ directly into the pre-cardiac sinus of anaesthetised embryos, which were examined and photographed 4-6 hours later.

Confocal microscopy

We used a Zeiss LSM 510 and Zeiss LSM software. Embryos were mounted in a cavity slide in 80% glycerol in phosphate-buffered saline.

Fish strains and maintenance

Complementation analysis of the dystrophic mutant class was performed on mutations obtained from the Tübingen Stock Centre. In this analysis *sapje*^{tm90c} and a second unnamed mutation, *ta222a*, failed to complement. Subsequent analysis has shown that extant stocks of both strains held in Edinburgh and Tübingen carry identical point mutations, suggesting that these strains may result from a single

founder mutation within the original mutant screen. We therefore refer here to *sapje*^{ta222a}, although analysis was performed on both strains.

Mapping

dmd was previously mapped using the LN54 radiation panel to between Z5508 and Z5085 (Bolanos-Jimenez et al., 2001). We established linkage between *sap* and *dmd* using Z5508 on individual embryos from a mapping cross versus the Wik strain.

Cloning of zebrafish *dmd* and identification of point mutation

Initial identification of zebrafish sequences was performed by comparing human dystrophin Dp427m with the zebrafish whole-genome shotgun-sequencing project (www.sanger.ac.uk). Exonic sequences were used to design primers for PCR from cDNA pools. We extracted mRNA from WT and mutant embryos using magnetic poly-T DynaBeads (Dynal) and made cDNA pools (using a Roche kit). PCR products were cloned in pGem-T (Promega), sequenced, and assembled and compared using Sequencher.

Morpholino antisense oligonucleotides

We purchased morpholino MO1 from GeneTools, (AAAGCGAAA-GCACCTGTGGCTGTGG), and injected a 0.5 mM solution at 1- and 2-cell stages in water using a Narishige pressure apparatus.

Sequence analysis

One dystrophin and one utrophin orthologue were identified in both *Danio rerio* and *Fugu rubripes*. Sequences were aligned using CLUSTALW v1.82, and positions in alignments containing gaps were omitted from subsequent analyses. All phylogenetic trees were constructed by the neighbour-joining method based on the proportion of amino acid sites at which sequences compared were different. The reliability of each interior branch of a given topology was assessed using the bootstrap interior branch test with 1000 bootstrap replications. Phylogenetic trees were constructed using MEGA v2.1 (www.megasoftware.net) and alignments were examined and formatted in GeneDoc (www.psc.edu/biomed/genedoc). The *Fugu* data has been provided freely by the Fugu Genome Consortium for use in this publication/correspondence only.

Electron microscopy

We used a Philips CM12 transmission electron microscope. Samples were fixed in 3% glutaraldehyde and embedded in Araldite.

GenBank accession numbers

Danio rerio 5' dystrophin (*dmd*) sequence is AY162403 and the *Fugu rubripes* predicted dystrophin 5' sequence is BK000643.

Results

Failure of muscle attachments in *sapje* mutant embryos

Myotomal lesions are evident in homozygous *sapje* (*sap*) mutant embryos by 48 hours post-fertilisation after an initial period of muscle development during which muscle fibres differentiate normally (Fig. 1A,B). Damage accumulates progressively, with death occurring after several weeks (median 31 days post-fertilisation, *n*=25). Histology revealed that fibres associated with lesions had detached from vertical myosepta at somite boundaries and dramatically shortened (Fig. 1C,D). Detachment and retraction of fibres was confirmed using electron microscopy (see below), revealing fibres that showed free ends, were often less than half their original length and contained compressed sarcomeres. The extent of damage to individual somites was examined using an α -actin promoter

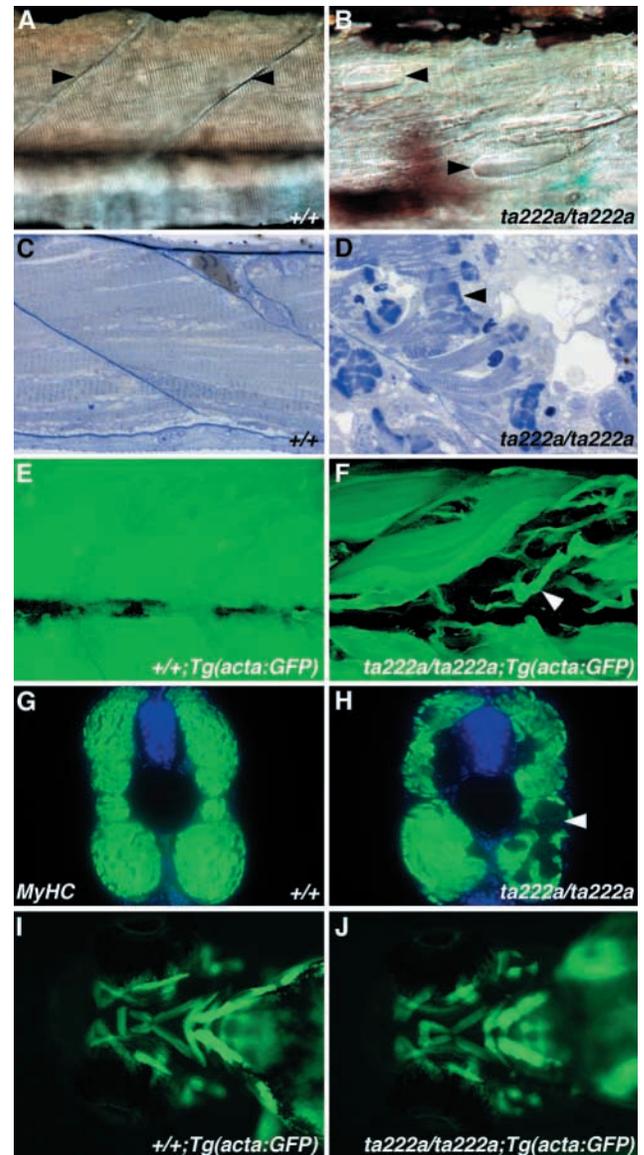


Fig. 1. *sap* mutants develop lesions in skeletal muscle where fibres detach from myosepta and retract. Fibres in wild type (WT) span the entire somite between myosepta (arrowheads in A; lateral views, 48 hours post-fertilisation), whereas lesions within *sap* somites are evident as cell-free spaces (arrowheads in B). Toluidine blue histology reveals detached, retracted fibres in association with lesions in *sap* (arrowhead in D; parasagittal sections, 72 hours post-fertilisation) but not in WT (C). Reconstruction of somites in 3D using confocal microscopy of fluorescence from the *Tg(acta:GFP)* transgene reveals extensive fibre loss in *sap* (arrowhead in F) but not WT (E). Anti-MyHC (green) reveals that whereas differentiation is normal in both WT (G) and *sap* (H), lesions in *sap* mutant somites lack contractile apparatus (arrowhead). Examination of head musculature using fluorescence from the *Tg(acta:GFP)* transgene shows that these muscles are unaffected in *sap* (J) compared with WT (I).

GFP transgene, *Tg(acta:GFP)* (Higashijima et al., 1997), under confocal microscopy. This revealed extensive fibre damage in some somites, whereas neighbouring somites remained almost unaffected, suggesting that factors such as

motor activity, fibre size and the variable spreading of damage may contribute to the pathology (Fig. 1E,F). Although this variability of effect between muscle fibres and between muscles is a common feature of human muscular dystrophies and their animal models, neither its cause nor its significance are understood. Examination of the expression of a myosin heavy chain (MyHC) within *sap* homozygotes confirmed that fibre differentiation occurs normally within these embryos. However, lesions were associated with fibre loss, producing MyHC-negative gaps within the differentiated myotome (Fig. 1G,H). Muscle damage in embryonic *sap* mutants appears to be restricted to somitic muscle and not to extend to the muscles of the jaw area, eyes or pectoral fins, as no lesions were observed here in vivo and the GFP in these muscles did not reveal any damage (Fig. 1I,J).

The DAPC is associated with junctional specialisations of the sarcolemma in WT zebrafish embryos but lacks dystrophin in *sapje* mutants

The similarity of the *sap* phenotype to muscular dystrophies led us to examine the effects of the *sap* mutation upon the DAPC in zebrafish. Identification of the zebrafish *dystrophin* (*dmd*) gene revealed an intimate association of its expression with fibre ends (Fig. 2A,B) (Bolanos-Jimenez et al., 2001; Guyon et al., 2003). *dmd* mRNA becomes intracellularly localised within somitic cells towards the somite boundaries before muscle fibre differentiation occurs, and remains so throughout development (Fig. 2A,B), being subsequently concentrated at the ends of muscle fibres. This pattern is preserved in *sap* (data not shown). Dystrophin protein was assayed using the monoclonal antibody Mandral1, which recognises a C-terminal epitope common to all isoforms. Dystrophin is localised in somitic muscle to the ends of fibres where they attach to vertical myosepta (Fig. 2C,D, Fig. 3A) (Parsons et al., 2002), which are comprised of fibrous sheets of ECM containing tenascin-C (Fig. 2D) (Roy et al., 1999; Waterman, 1969). Dystrophin was not detectable at the embryonic non-junctional sarcolemma before 5 days post-fertilisation, however it is known to be present within the adult zebrafish sarcolemma (Chambers et al., 2001). Dystrophin is also present at the embryonic NMJ in somitic muscle (Fig. 2E-G). Thus, dystrophin is initially localised to junctional specialisations of the sarcolemma in WT zebrafish embryos.

sap mutants show a lack or reduction of dystrophin immunoreactivity at muscle fibre ends but not at other sites of expression, demonstrating a loss of muscle-specific isoforms (Fig. 3A,B,G,J). The transmembrane β -chain of dystroglycan (β DG) is also localised to the ends of WT muscle fibres, but unlike dystrophin is present in *sap* (Fig. 3H,K). Utrophin is detectable in embryonic epidermis cells at 72 hours post-fertilisation but not at either WT or *sap* mutant embryonic muscle fibre ends (Fig. 3I,L). Thus, a dystrophin-based form of the DAPC is localised to somitic muscle attachment sites in WT zebrafish embryos, and its loss in *sap* homozygotes does not result in a compensatory upregulation of utrophin expression at terminal fibre membranes, as is known to occur in other dystrophic models (Draper et al., 2001).

sapje corresponds to the zebrafish orthologue of the DMD gene

As loss of dystrophin and retention of dystroglycan also occur

in *mdx* mice and DMD (Spence et al., 2002), we considered *dmd* a strong candidate for *sap*. Comparing the human dystrophin protein with the zebrafish genome using BLAST (Altschul et al., 1997) identified fragments of the *dmd* gene, which we subsequently joined by PCR to recover cDNA sequences representing nearly the complete open reading frame. Analysis of vertebrate dystrophin and utrophin protein sequences, including *dmd* and novel predicted *Fugu rubripes* proteins which we identified, confirmed that zebrafish *dmd* is the true orthologue of human DMD (Fig. 4B). Only one orthologue each of DMD and Utrophin were identified in both the zebrafish and *Fugu rubripes*, each clustering with the mammalian genes in phylogenetic analyses. The previously reported radiation hybrid position of the C-terminal of zebrafish *dmd* is on Linkage Group/Chromosome 1 close to the markers Z5508 and Z5058 (Bolanos-Jimenez et al., 2001).

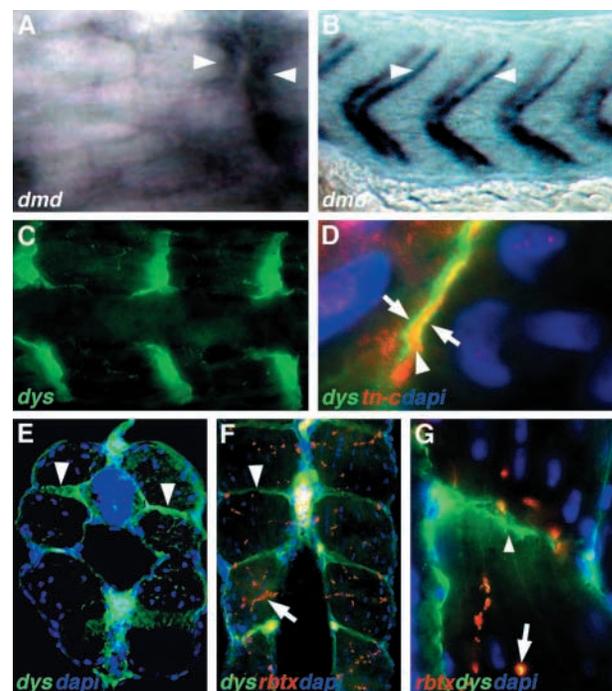


Fig. 2. Dystrophin is associated with the wild type (WT) embryonic muscle attachments. *dystrophin* mRNA (*dmd*) localises intracellularly to WT somite boundaries both before (19 hours post-fertilisation, A) and after (27 hours post-fertilisation, B) muscle fibre differentiation (arrowheads, lateral views). At 19 hours post-fertilisation, a crescent of mRNA is present at one side of undifferentiated cells that abut somite boundaries. Dystrophin protein (*dys*) is localised embryonically to fibre ends at somite boundaries, and at NMJs but not at the sarcolemma (C, horizontal section, 72 hours post-fertilisation). Dystrophin (green) in fibre ends sandwiches the ECM of the vertical myoseptum at somite boundaries, which contain tenascin-C (tn-c, red; arrowhead in D, horizontal section, 72 hours post-fertilisation). Dystrophin localises to fibre ends and NMJs but not to the sarcolemma embryonically (arrowheads in E, transverse section, 72 hours post-fertilisation). Dystrophin is detectable at muscle attachments (arrowheads in E-G), but triple labelling using anti-dystrophin (green), Alexa594- α -Bungarotoxin to label NMJs (rbtx, red), and DAPI (blue) reveals that dystrophin within the myotome is at NMJs, co-localising with rbtx to produce an overlapping yellow signal (arrows in F,G; horizontal sections 72 hours post-fertilisation).

Given the probable large size of the *dmd* locus, the degenerative phenotype of *sap* and the absence of dystrophin immunoreactivity, we tested for linkage between *sap* and *dmd* using the SSLP marker Z5508. We examined 124 homozygous mutant embryos from a cross against the Wik strain, as 4 pools of 25, and 24 individually, for the presence of the Wik-derived allele by PCR, but could not detect it in any pool or detect any recombinants among the individual embryos, indicating a genetic distance of within 2.4 cM. The lack of dystrophin from muscle but not other sites suggested that *sapje*^{ta222a} might be a mutation in an exon of *dmd* retained at embryonic stages exclusively in large, muscle-specific isoforms. This, and the large size of the human locus led us to initiate mutation detection within 5' exons of *dmd* homologous to those of Dp427m, the isoform that predominates in mammalian muscle (Spence et al., 2002). Within exon 4 of *dmd* from *sapje*^{ta222a} we found an A→T transversion causing a nonsense mutation at position K76 which segregates in the homozygous state exclusively with the *sap* phenotype (Fig. 4A,C). By comparison, a nonsense mutation in exon 4 of human *DMD* causes DMD, indicating that this exon is essential in human muscle (Sitnik et al., 1997). The N-terminal location of this mutation indicates that other shorter isoforms produced from downstream alternative first exons should be expressed normally in other tissues, as confirmed by immunohistochemistry (Fig. 3J).

To confirm that this mutation causes the *sap* phenotype we injected an antisense morpholino oligonucleotide (MO1). MO1 was targeted to overlap the boundary of zebrafish *dmd* exon 6 and its downstream intron and induce exon-skipping (Xu et al., 2002; Draper et al., 2001) from exon 5 to exon 7, resulting in a frameshift and premature termination in exon 7. Its effectiveness was evidenced by the reduction or absence of dystrophin C-terminal immunoreactivity in injected embryos (Fig. 3C). MO1 injections at 0.5 mM did not cause non-specific abnormalities above those seen in control injections, but phenocopied *sap* pathology to some extent in 29% of injected embryos (46/159), causing somitic lesions and uptake of EBD that were never seen among embryos injected with control morpholinos (Fig. 3F). Thus, dystrophin-deficiency causes muscle degeneration and membrane damage in zebrafish embryonic muscle. Together, these data show that a targeted loss of dystrophin causes similar changes to *sapje*, which in turn is co-inherited with a nonsense mutation in the dystrophin gene. Thus, we conclude that the K76stop mutation that we have identified in *dmd* causes the *sapje*^{ta222a} phenotype.

Muscle attachment failure in *sapje* occurs at the terminal membrane and can involve damage to the sarcolemma

In order to understand the cellular basis for muscle degeneration present within *sap* homozygotes, we continuously monitored mutant somites in vivo using both light microscopy and EBD, which in muscular dystrophies labels cells with compromised plasma membranes (Hamer et

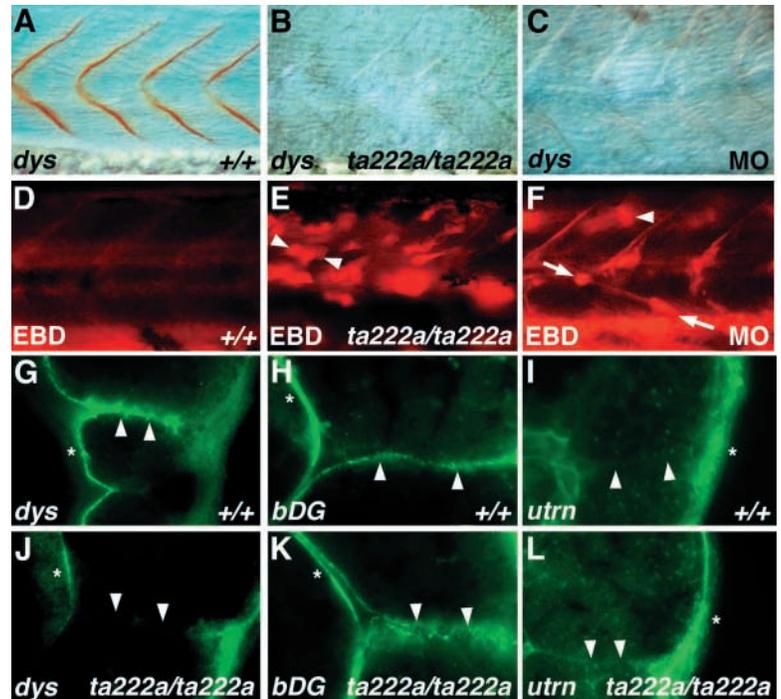
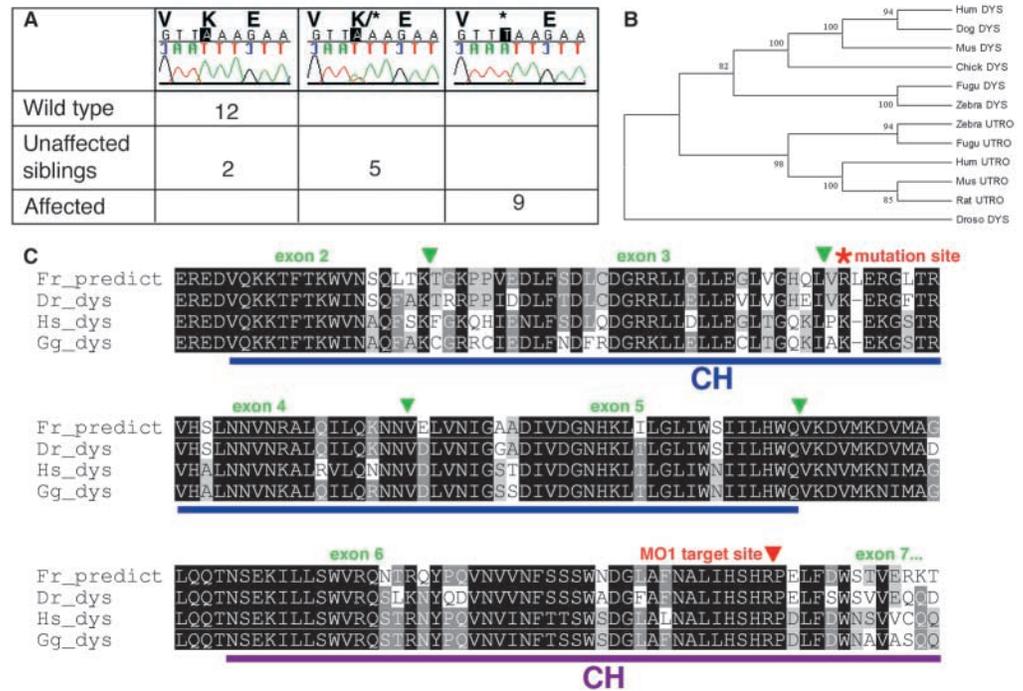


Fig. 3. *sap*^{ta222a} is a mutation of *dmd* that causes fibre detachment and is phenocopied by knocking down *dmd* function. Dystrophin C-terminal immunoreactivity is localised to somite boundaries in wild type (WT) (A, 27 hours post-fertilisation, lateral views) but lacking in both *sap* (B) and dystrophin-morphant embryos (C). Evans blue dye (EBD, red), does not appear in WT somites (D), but labels fibres in both *sap* (E) and dystrophin-morphant embryos (F). Labelled fibres are visible that have both detached and retracted (arrowheads) or still span a somite and show a retraction zone (between arrows in F, lateral views, 72 hours post-fertilisation). By 72 hours post-fertilisation, dystrophin is present in the neural tube and notochord in both WT (G) and *sap* (J, asterisks) but lacking from muscle attachments in *sap* (arrowheads in G,J, transverse sections). β -dystroglycan is also localised to muscle attachments (arrowheads) and other sites (asterisks) in WT (H), but unlike dystrophin is preserved at muscle attachments in *sap* (K, 72 hours post-fertilisation, transverse sections). Utrophin is not detectable at muscle attachments in either WT (I) or *sap* (L, arrowheads). Anti-utrophin immunoreactivity in the epidermis provides an internal positive control (asterisks).

al., 2002). Injection of EBD into the pre-cardiac sinus results in the passage of the dye through the larval circulatory system, and consequent uptake by damaged fibres in *sap* homozygotes. Uptake was never seen within muscle fibres of WT embryos (Fig. 3D). Within *sap* homozygotes most EBD-positive cells extended only a small distance across the somite from a myoseptum, indicating that they had become detached at their opposite end (Fig. 3E). However, EBD did label a few fibres prior to detachment and we were consequently able to observe single mutant fibres in vivo detaching from myosepta (Fig. 5A,B). Some of these cells exhibited a dumb-bell morphology reminiscent of that of mammalian muscle fibres mechanically injured and subsequently overloaded to induce severing and retraction of the contractile apparatus, leaving behind a collapsed sleeve of sarcolemma known as a retraction zone (Tidball and Chan, 1989). This suggests that in *sap* mutants fibre degeneration sometimes involves separation of the terminal sarcomeres from the

Fig. 4. (A) Homozygosity for the nonsense mutation (AAA→TAA) in *sap*^{ta222a} *dmd* exon 4 segregated exclusively with the *sap* phenotype. (B) Rooted tree showing dystrophin and utrophin proteins in vertebrates. The tree is rooted using *Drosophila melanogaster* dystrophin. The numbers represent the percentage of 1000 bootstrap trials that support the branch. Protein accession numbers: XP_081212 NP_000100 O97592 NP_031894 CAA31746 NP_009055 CAA58496. The Fugu sequences are manually corrected GENSCAN predictions from genomic scaffolds (www.jgi.doe.gov/fugu, Scaffold 234). The zebrafish utrophin sequence is predicted from the zebrafish genome project. The tree has been made from partial sequences corresponding to the zebrafish protein published in this paper. (C) Partial alignment of zebrafish (*Dr_dys*), predicted *Fugu rubripes* (*Fr_predict*) and chicken (*Gg_dys*) dystrophin proteins including the two N-terminal calponin homology domains (CH, underlined). The position of the stop codon in *sap*^{ta222a} *dmd* is marked by an asterisk. Exon boundaries 2 to 7 are marked by green arrowheads, except between exons 6 and 7 (red) against which MO1 was directed. Chick, *Gallus gallus*; Dog, *Canis familiaris*; DYS, dystrophin; Fugu, *Fugu rubripes*; Hum, *Homo sapiens*; Mus, *Mus musculus*; Rat, *Rattus norvegicus*; UTRO, utrophin; Zebra, *Danio rerio*.



terminal sarcolemma, as well as tearing and detachment of the terminal sarcolemma from the myoseptum (Fig. 3E, Fig. 5A,B). Utilising three-dimensional confocal microscopy of fixed GFP-transgenic animals to trace detached fibres along the entirety of their length revealed sporadic lesions and many detached fibres. These often displayed a normal diameter and a distinct blunt or multi-faceted appearance to their free ends, suggesting that detachment in these fibres involved separation of the terminal sarcolemma from the basal lamina of the myoseptum (Fig. 5C,D). Thus, the degeneration of muscle tissue in *sap* mutants is because of the detachment of muscle fibre ends, which occurs with an associated loss of membrane integrity. In order to determine precisely where detachment and membrane damage occurs we examined *sap* homozygotes at single-cell resolution using immunohistochemistry to detect proteins normally localised either within the terminal membrane or immediately intracellular to it. In *sap* mutants β -dystroglycan protein remains associated with the myoseptum when fibres detach, consistent with failure at the level of dystrophin, which lies immediately intracellular to the sarcolemma (Fig. 5E,F). As a marker of terminal cytoplasm, we used an antibody specific to tyrosine-397-phosphorylated focal adhesion kinase, a cytoplasmic protein enriched at ends of muscle fibres in integrin complexes (Henry et al., 2001). Anti-p(Tyr397)FAK immunoreactivity is retained in the stumps of detached fibres, indicating that failure occurs within the membrane plane of the sarcolemma (Fig. 5G,H). These observations are consistent with a structural failure of the dystrophin linkage between the actin cytoskeleton and the basal lamina of the

myoseptum in the region of the sarcolemma causing the observed detachments.

Muscle attachment failure causes collapse of the contractile apparatus and precedes cell death in *sapje* mutant embryos

Electron microscopy shows that WT embryonic myofibrils align to form regular sarcomeric arrays that attach to the obliquely oriented myosepta in a stepwise fashion (Fig. 6A). In *sap* homozygotes, fibres showing detached ends and shortening of both the entire fibre and the sarcomeres are visible (Fig. 6B,C). In these cells, the separation and regularity of sarcomeric banding is greatly reduced or collapsed by comparison to intact neighbouring cells, and in some regions is absent entirely. Actin filaments run longitudinally from the terminal sarcomeres to the myoseptum in both WT and intact *sap* mutant muscle fibres (Fig. 6D,E). Similar filaments run within the membrane folds of mammalian MTJs and are present in the *mdx* mouse (Law et al., 1995; Law and Tidball, 1993; Tidball and Law, 1991), whereas the simple, laminar structure of embryonic somitic attachments in the zebrafish precludes the use of lateral actin linkages within folds of membrane, such as are present in mammalian MTJs but absent in the *mdx* mouse. The somitic muscle attachments of teleost fish can take a more complex three-dimensional form as they mature (Bremner and Hallett, 1985; Hallett, 1987; Bartels, 1987; Gembella and Vogel, 2002), indicating that such linkages may exist in the adult zebrafish.

In order to examine whether detachment precedes or follows cell death in *sapje* mutants, nuclear changes were also

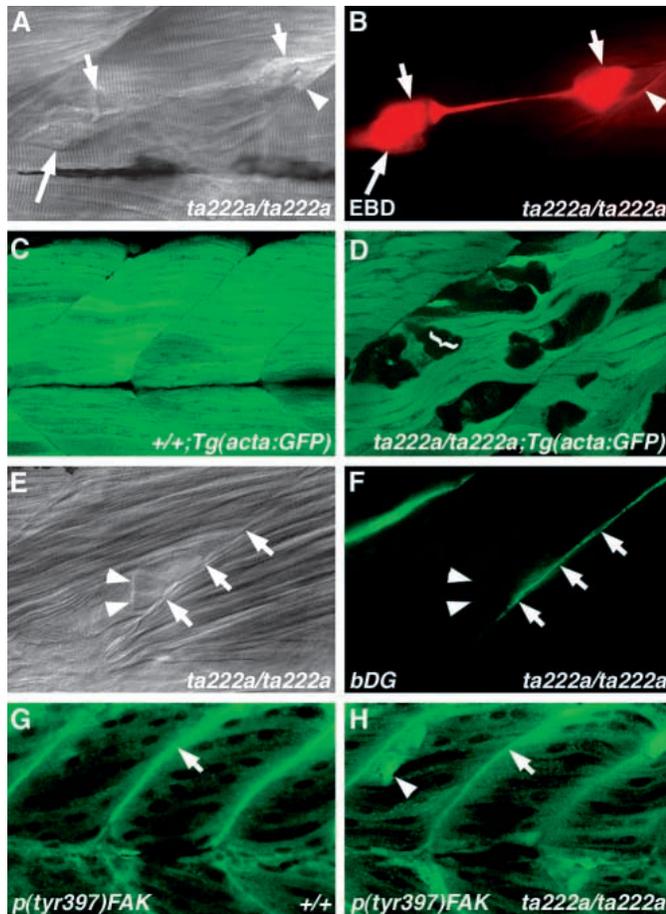


Fig. 5. In vivo observation of muscle attachment failure and molecular analysis of detached free ends. A single fibre (A,B short arrows) viewed in vivo in the process of detaching myosepta, under differential interference contrast (A, lateral view, 5 days post-fertilisation) and labelled with EBD (B). A gap is visible between the separating posterior end of the fibre (right, short arrow) and the myoseptum (arrowhead). A narrowed retraction zone has formed where the contractile apparatus has withdrawn from the centre of the fibre (between the short arrows). The anterior end of the fibre (left, short arrow) is partly obscured by a second dye-positive detached cell (long arrow). Confocal microscopy of GFP revealed this example of a free end of a single detached fibre (D, bracket, lateral view, 6 days post-fertilisation). Stacked confocal images allow tracing of individual fibres to their insertion points at the muscle attachments. Fibres within *sap* homozygotes (D) exhibit a club-like or faceted appearance at their newly detached membranes, not evident in wild-type embryos (C). β DG protein at the muscle attachments is not retained in the fibre membranes once detachment occurs. Two neighbouring detached and retracted mutant cells visible under DIC (arrowheads in E, 72 hours post-fertilisation, lateral view) are still attached to their posterior (right) myoseptum (arrows in E,F). Labelling with anti- β DG (F) shows that this integral membrane protein has been lost from their free (left) ends upon detachment, possibly maintaining its binding to α -dystroglycan and laminin at the myoseptum. Phosphorylated focal adhesion kinase is enriched in terminal cytoplasm at muscle attachments (arrows in G,H, lateral confocal images, 72 hours post-fertilisation). Unlike β DG, however, p(tyr397)FAK remains visibly localised to the free end of detached cells, indicating the retention of terminal cytoplasm by detached and retracted fibres (arrowheads in H).

examined by electron microscopy. Nuclear condensations indicative of apoptosis were present in many of the nuclei in detached and retracted mutant fibres (37/50, 74%) but were not observed in either intact mutant (0/50) or WT (0/50) fibres. Thus, muscle attachment failure is not a secondary process that results from either apoptosis or necrosis of muscle fibres, but is likely to precede fibre death (Fig. 6F,G).

Discussion

Our results, reveal that dystrophin-deficiency in zebrafish embryos causes a progressive loss of muscle integrity because of muscle attachment failure. We have found that the primary pathology of the lethal *sapje* mutation is the failure of the specialised sarcolemmal junctions that attach embryonic somitic muscle fibres to the myosepta, which share several characteristics of both mammalian myotendinous and myomuscular junctions. We further show that *sapje* mutant embryos lack dystrophin in somitic muscle, and that the *sapje*^{ta222a} mutation disrupts the zebrafish orthologue of the DMD gene. The lack of dystrophin from the DAPC at this site leads to failure of the cells at the level of the terminal membrane, which precedes muscle fibre death. Our data provide the first in vivo evidence of vertebrate muscle attachment failure by revealing a novel requirement for dystrophin in these structures and a novel pathological mechanism that may contribute to Duchenne and other muscle diseases.

The role of the DAPC in zebrafish

Translation-blocking morpholinos targeted to a zebrafish dystrophin exon 1 sequence were recently reported to cause an uncharacterised disorganisation of the somites similar to that seen in degenerating *sap* mutant embryos, and reduced levels of the DAPC component δ -sarcoglycan, although no specific defect in muscle fibre integrity was reported (Guyon et al., 2003). However, these also cause reduced activity and curvature of the body axis that are not present in either *sap*-homozygote or MO1-injected morpholinos. This may suggest that there are multiple isoforms of dystrophin, possibly using different promoters, that are affected differently in these three deficiencies, all of which perturb different exons of this highly alternatively spliced gene. Body curvature might alternatively reflect a midline defect resulting in the removal of an isoform expressed in the notochord or neural tube or, as suggested by Guyon et al., a genetic background effect. Several products of zebrafish *dmd* have been detected by western blotting (Guyon et al., 2003; Chambers et al., 2001; Bolanos-Jimenez et al., 2001), including the short Dp71 isoform, which should not be affected in any of these cases, and which has been detected by in situ hybridisation in both embryonic somites and notochord. It remains to be seen where other isoforms are required. In addition, the behaviour and efficacy of splice-blocking morpholinos are not yet well characterised, so these data may not be directly comparable with the MO1 phenotype presented here (for a review, see Bassett et al., 2003).

Compensation by utrophin is unlikely to affect the *sapje* phenotype

The surprising effect of dystrophin deficiency in zebrafish may be because of several factors, including the lack of

compensation by utrophin, and the simple nature of muscle attachment sites in the zebrafish somites by comparison with the complex array of cell types and ECM within bone-tendon-muscle found in the head or limbs of fish and mammalian muscle attachments. Within *sap* homozygotes, compensation for loss of dystrophin by utrophin is unlikely to affect the stability of the embryonic muscle attachment, as utrophin is not present at detectable levels at this site. Thus, as in severe mouse models (Sicinski et al., 1989; Deconinck et al., 1997; Grady et al., 1997), *sap* mutant muscle attachments lack significant DAPC-mediated linkage, exposing them to failure. Equivalent abnormalities, and therefore possibly fibre detachment, may be present in human muscular dystrophies or myopathies. One other possible contributory factor to the severe effects of loss of dystrophin in zebrafish embryos is their

precocious use of locomotion, which may strain early DAPC-mediated linkages at muscle attachments before the deposition of other structural protein complexes such as the integrins.

***sapje* provides a model of a novel pathological mechanism that may contribute to human muscle disease**

The specificity of the phenotype of *sap* mutant embryos to the somite boundaries is suggestive of an essential role for the DAPC in the stability of the highly similar MMJs, as those head and fin muscles unaffected embryonically are of the MTJ-attached type and work against the endoskeleton as opposed to the myosepta and notochord. If the failure of MMJs were a significant factor in muscle disease, their differential deployment might contribute to the observed variations in pathology between individual muscles, and between different dystrophic animal models. Our data strongly suggest that the MMJ, and possibly the MTJ, warrants further examination as a site of pathology in human muscular dystrophies.

Collectively, these results provide the first *in vivo* genetic evidence that dystrophin can be required not only for the normal morphology and ultrastructure of vertebrate muscle attachments, as has been found in *mdx* mice, but also for their stability, and that failure of these attachments can lead to a progressive muscular dystrophy. *sap* mutants therefore provide a model for the novel pathological mechanism of junctional failure that could well contribute to some of the many different human muscular dystrophies or congenital myopathies. The treatment of muscular dystrophies using pharmaceuticals remains elusive in part because of a lack of suitable targets, however, such proteins might be identified using *sapje* in future via a genetic approach. The amenity of the zebrafish to large-scale genetic screening makes the prospect of screening for

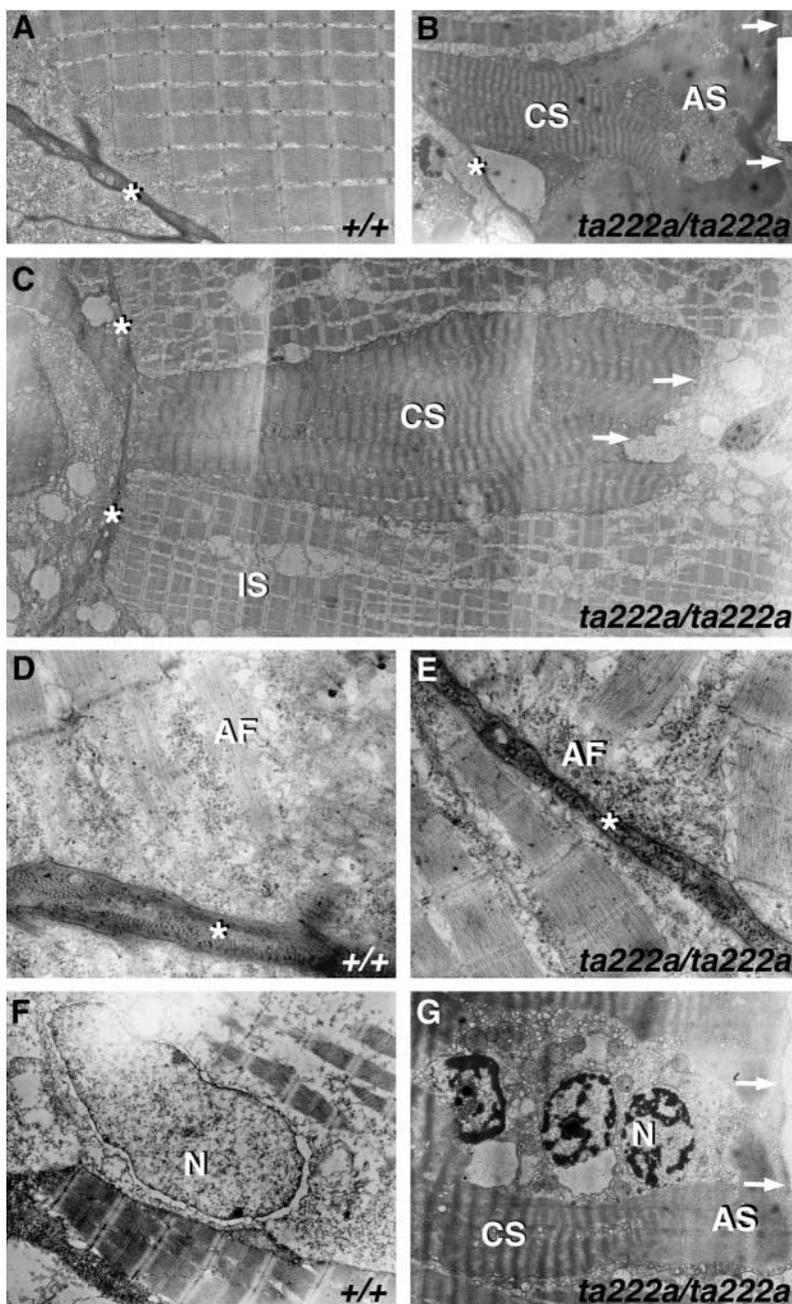


Fig. 6. Electron microscopy shows that wild-type (WT) embryonic myofibrils align to form a regular sarcomeric array that attaches obliquely to the myosepta (asterisks) (A). In *sap* homozygotes, fibres showing detached ends (arrows in B,C,G) and shortening of both the entire fibre and the sarcomeres, are visible. In these cells, the separation and regularity of sarcomeric banding is greatly reduced or collapsed compared with that in intact neighbouring cells, and absent in some places (B,C). Actin filaments (AF) run longitudinally from the terminal sarcomeres to the vertical myoseptum in both WT and intact *sap* mutant muscle fibres (D,E). Nuclear changes were also followed by electron microscopy in order to examine whether detachment precedes or follows cell death in *sapje* mutants. Nuclear condensations indicative of apoptosis were only present in detached mutant fibres (G), but were not observed in either intact mutant or WT (F) fibres, demonstrating that detachment is not a secondary process resulting from apoptosis of muscle fibres. Four days post-fertilisation, parasagittal sections. AS, absent sarcomeres; CS, collapsed sarcomeres; IS, intact sarcomeres; N, nucleus.

second-site mutations that suppress or enhance dystrophin-deficiency in a vertebrate feasible. These results suggest that *sap* is a useful addition to our range of models for understanding the roles of the dystrophin complex, in particular at muscle attachments, and for increasing the range of approaches available to the question of relevant therapies.

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