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Genetic isolation and characterization of a splicing mutant of zebrafish dystrophin

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Sapje-like (*sap^{cl100}*) was one of eight potential zebrafish muscle mutants isolated as part of an early-pressure screen of 500 families. This mutant shows a muscle tearing phenotype similar to *sapje* (dys−/−) and both mutants fail to genetically complement suggesting they have a mutation in the same gene. Protein analysis confirms a lack of dystrophin in developing *sapje-like* embryos. Sequence analysis of the *sapje-like* dystrophin mRNA shows that exon 62 is missing in the dystrophin transcript causing exon 63 to be translated out of frame terminating translation at a premature stop codon at the end of exon 63. Sequence analysis of *sapje-like* genomic DNA identified a mutation in the donor splice junction at the end of dystrophin exon 62. This mutation is similar to splicing mutations associated with human forms of Duchenne Muscular Dystrophy. *Sapje-like* is the first zebrafish dystrophin splicing mutant identified to date and represents a novel disease model which can be used in future studies to identify therapeutic compounds for treating diseases caused by splicing defects.

INTRODUCTION

Muscular dystrophy is the one of the most common X-linked human genetic disorders. Mutations in the dystrophin gene account for almost 90% of the human forms of the disease (1–3). Depending on disease severity, these mutations can cause Duchenne (more severe) or Becker (less severe) Muscular Dystrophy (4). While many different types of dystrophin mutations can cause muscular dystrophy, the most common are deletions (65%), mutations (30%) and duplications (5%) (5).

Dystrophin is a large protein (427 kDa) that localizes in healthy muscle to the inside of the sarcolemmal membrane and is thought to function by connecting transmembrane proteins with the actin cytoskeleton inside muscle (6–8). Loss of dystrophin can destabilize the dystrophin-associated protein complex (DAPC) thereby increasing the fragility of the diseased muscle cell membrane. Mutations in many DAPC proteins have also been linked to different forms of muscular dystrophy (9–13). Once the membrane is compromised, calcium infiltrates the muscle and the fiber degenerates, eliciting a large immune response and the regeneration of additional diseased muscle fibers. While specific mutations in dystrophin were identified in 1986 as the cause of Duchenne Muscular Dystrophy (3), new treatments are only recently emerging based on either dystrophin replacement or gene correction. These therapies seem promising and are currently being evaluated (reviewed in 14,15). Despite these advances, approaches to therapy would benefit from the establishment of additional new disease models adapted to high-throughput drug screening.

Over the last eight years, muscular dystrophy has been investigated in zebrafish as a potential disease model (reviewed in 16). Morpholino experiments have shown that dystroglycan, dystrophin, δ-sarcoglycan, titin and caveolin-3 are all required for normal muscle development in fish during the first seven days of development (17–21). In addition, four of the five available zebrafish muscle degeneration mutants isolated from the 1996 Tuebingen screen (22) have now been characterized. Bassett *et al*. characterized the

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first of these fish in 2003 and showed that *sapje* carries a nonsense mutation in exon 4 of the dystrophin gene (23). The second and third mutants (both allelic and designated *candyfloss*) were shown to carry mutations in laminin α2 (24), whereas the mutation in *runzel* has been linked to the titin locus (25). In humans, mutations in laminin α2 have been associated with a congenital muscular dystrophy (26), and mutations in titin have been linked with various muscle disorders including Tibial muscular dystrophy, Familial Dilated Cardiomyopathy and limb-girdle muscular dystrophy type 2J (27–31).

All four of the currently characterized zebrafish dystrophy mutants were originally isolated as part of the 1996 Tuebingen screen (22). These dystrophic mutants show a phenotype of muscle degeneration between two and five days post-fertilization (dpf). Since zebrafish embryos are transparent at early stages, Granato *et al*. used muscle birefringence to assay muscle integrity. Birefringence is a physical property in which light is rotated as it passes through ordered matter and was used in the initial characterizations of muscle in the 1950s (32).

To isolate additional zebrafish dystrophic mutants, an early-pressure screen was completed, looking for mutants showing decreased birefringence over time, decreased activity and bending. Early pressure is a technique in which the haploid genome of the mother can be screened by making gynogenetically diploid embryos (reviewed in 33). In this technique, the final phase of meiosis is inhibited using pressure to prevent the extrusion of the polar body after egg fertilization. Using this technique, offspring from 500 female carriers were screened for defects in muscle birefringence, activity and posture. Eight potential muscle mutants were isolated and one (*sapje-like*) showed, upon using the birefringence assay, muscle lesions very similar to that of *sapje*, a known dystrophin mutant. In this report, we show that the *sapje-like* muscular dystrophy mutant has a mutation within the dystrophin exon 62 donor splice junction causing the resulting mRNA to be translated out of frame and protein translation to be prematurely terminated. *Sapje-like* (sap^{cl100}) is the first zebrafish dystrophy mutant isolated to date with a dystrophin splice site mutation making it an ideal model for investigating corrective therapies for muscle disorders associated with splicing.

RESULTS

Genetic isolation of zebrafish muscle mutants

An early-pressure screen was performed to genetically isolate zebrafish mutants with symptoms of muscle disease. Briefly, as described in Supplementary Material, Figure S1, the screen was performed by mutating the spermatagonia of 80 adult male zebrafish with ethylnitrosylurea (ENU). Sperm from ENU-treated males were cleared by outcrossing with wild-type females. The mutation rate was then determined to be 1×10^{-3} mutations/allele as assayed by complementation with albino heterozygous mutants. ENU-treated males were outcrossed to wild-type females to generate a stock of heterozygous females. Upon reaching sexual maturity, these females were squeezed, and the eggs were fertilized with irradiated sperm and subjected to high pressure to create gynogenetically diploid embryos. Fertilized eggs were scored at 5 dpf for birefringence defects, decreased activity and a bent posture. The offspring of 500 females were screened and 8 distinct mutants with abnormal birefringence of muscle were isolated. Carrier females were outcrossed to maintain the line and the resultant progeny incrossed to verify phenotype.

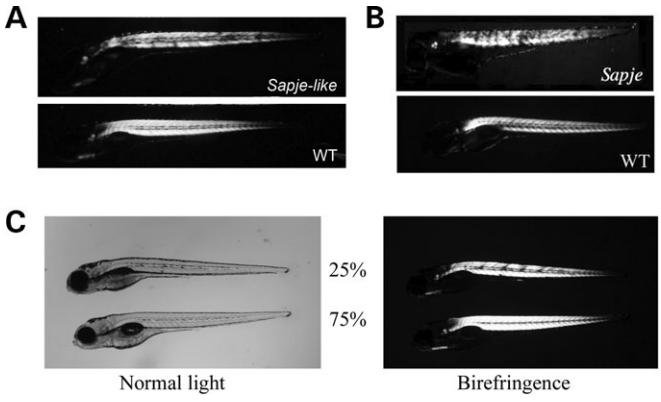

Figure 1. *Sapje-like* mutant offspring show muscle defects which can be assayed using birefringence. (**A**) Muscle degeneration in *sapje-like* mutants is clearly visible as small dark spots at 5 dpf using birefringence. These spots represent areas of muscle tearing. (**B**) The *sapje-like* birefringence is very similar to that seen for *sapje*, a known zebrafish dystrophin mutant. (**C**) *Sapje* and *sapje-like* fail to complement each other. Heterozygous *sapje* mutants (±) were crossed with heterozygous *sapje-like* mutants (±) and the offspring assayed at 4 dpf for defects in muscle using birefringence. From this cross, 25% of the offspring showed muscle lesions (top fish, right) suggesting that the two fish have mutations in the same gene. All panels are shown at approximately 10-fold magnification.

Phenotype of the *sapje-like* mutant

Of the eight isolated muscle mutants, one mutant (*sapje-like*) showed a muscle birefringence phenotype very similar to *sapje*, a known dystrophin mutant. Both mutants show normal muscle birefringence at 2 dpf (not shown), but then the muscle degenerates such that by 5 dpf muscle lesions are clearly apparent as assayed using muscle birefringence (Fig. 1A). Since *sapje-like* showed a muscle phenotype very similar to that of *sapje* (Fig. 1B), complementation analysis was performed. When *sapje-like* heterozygous fish were crossed with *sapje* heterozygous fish, 25% of the offspring showed the muscle birefringence phenotype suggesting that the mutation in each of the mutants resided in the same gene (Fig. 1C). This cross was repeated twice to verify our results. Since *sapje* was shown previously to carry a nonsense mutation in exon 4 of the zebrafish dystrophin gene (23), this evidence strongly suggested that *sapje-like* also carried a recessive mutation in the dystrophin gene.

Immunohistochemical analysis confirms loss of dystrophin protein in the *sapje-like* mutant

In human Duchenne Muscular Dystrophy patients and in the zebrafish dystrophin morphant (19), dystrophin protein is missing and its associated proteins are often partially down-regulated at the muscle sarcolemma. To assay for overall dystrophin protein expression in the *sapje-like* mutant, wild-type

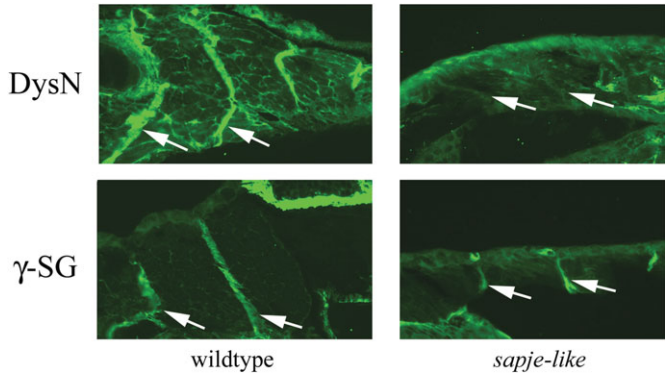

Figure 2. Immunohistochemical analysis of *sapje-like* horizontal sections at five days post-fertilization (dpf). Dystrophin protein localizes in unaffected siblings to the muscle myosepta (top left), but is absent in the mutant (top right). γ-Sarcoglycan is also strongly expressed in unaffected siblings (bottom left) and also in the *sapje-like* mutant (bottom right). From this analysis, it is difficult to determine if γ-sarcoglycan is down-regulated in the mutant. The myoseptas are designated with white arrows. For each antibody, pictures were taken at the same exposure level for both the wild-type and *sapje-like* mutant. All panels are shown at approximately 80-fold magnification.

and *sapje-like* mutants were analyzed by immunohistochemistry. Sections from 5 dpf embryos were immunostained with antibodies designed specifically against zebrafish dystrophin and in all cases, dystrophin protein was absent at the myosepta of developing *sapje-like* mutants (Fig. 2, top).

To test if the dystrophin-associated proteins were partially down-regulated in the *sapje-like* mutant, sections from 5 dpf wild-type and *sapje-like* mutants were stained with antibodies against the following DAPC components: β-sarcoglycan, δ-sarcoglycan and γ-sarcoglycan. Due to similar staining patterns, only the panels from the γ-sarcoglycan staining are shown in Figure 2 (bottom). This analysis showed little difference in the intensities of the sarcoglycan myoseptal staining patterns in the 5 dpf and 4-week-old mutant fish (data not shown).

Western blot analysis shows that the DAPC is partially destabilized in the *sapje-like* mutant

Since protein expression levels are difficult to compare using immunohistochemical analysis, immunoblot analysis was performed to compare expression of dystrophin and dystrophin-associated proteins in the *sapje-like* mutant. Protein extracts from wild-type and *sapje-like* mutants at 5 dpf were separated by gel electrophoresis and transferred to nitrocellulose membrane. Using antibodies developed against both the N- and C- terminus of zebrafish dystrophin, immunoblot analysis confirmed the loss of the dystrophin protein specifically in the *sapje-like* mutant (Fig. 3A, see arrows). While the dystrophin protein appeared to be completely absent in the blot using the C-terminus dystrophin antibody, the N-terminus antibody detected a reduced (although present) smaller dystrophin protein in the mutant. This band likely represents the truncated dystrophin isoform resulting from the splice site mutation (see later).

To determine if expression of the dystrophin-associated proteins was affected in the *sapje-like* mutant, western blot analysis was also performed for the sarcoglycans. In the *sapje-like* mutant, the β-, δ- and γ-sarcoglycan proteins were moderately although specifically reduced (Fig. 3B). This data suggest that the sarcoglycan subcomplex is weakly destabilized in the *sapje-like* mutant, similar to that seen in some human Duchenne patients (sarcoglycan destabilization can be variable). Western analysis was also performed for β-dystroglycan, a member of the DAPC that is not typically reduced in human Duchenne patients. While subtle, β-dystroglycan also appears to be slightly reduced, suggesting the integrity of the entire complex might be partially compromised in the mutant. Equal staining of the actin control in both the wild-type and mutant embryos confirms equal loading across the wells (Fig. 3B).

sapje-like carries a mutation in the donor splice junction of exon 62 of the zebrafish dystrophin gene

The birefringence phenotype, complementation results and protein analysis strongly suggest that the *sapje-like* causative mutation resides in the dystrophin gene. In humans, the dystrophin gene spans 2.5 million bases of the human X chromosome and previous work suggests the gene might be equally as large in zebrafish (19). Because the zebrafish dystrophin gene has not been cloned, it was difficult to conclusively identify zebrafish orthologues to the 79 human dystrophin exons. To facilitate sequencing the dystrophin coding sequence, sections of the zebrafish dystrophin gene transcript were PCR amplified in sections from cDNA using primers designed to the more conserved sequences. Resulting PCR products were sequenced from *sapje-like* mutant fish and compared with the sequence from unaffected siblings. Sequencing these products revealed that *sapje-like* mRNA was missing dystrophin exon 62 since the sequence went directly from exon 61 to 63 (Fig. 4A). In addition, using primers homologous to exons 57 and 63, a clear size difference is seen in the *sapje-like* PCR product, suggesting a portion of the cDNA is missing in the mutant transcript (Fig. 4B). The smaller mutant band can still be faintly seen in the unaffected siblings, some of which are heterozygous for the mutation. For both the sequencing analysis (Fig. 4A) and the PCR experiment (Fig. 4B), the same cDNA preparations were used and were made by phenotypically grouping the embryos. The removal of exon 62 results in a translational frameshift yielding an inadvertent termination signal near the end of exon 63 (Fig. 4C).

To identify the mutation, splice sites between exons 61-62 and exons 62-63 were sequenced from genomic DNA in mutant and wild-type embryos. A mutation was found in the +5 position of the exon 62 splice donor (Fig. 4D). Based on the human consensus splice site (Fig. 4E), this base is normally present 80% of the time (34). This base change was homozygous in only affected *sapje-like* embryos and was not found in any of the other strains of mutants that were analyzed (20 individual fish were tested). The location of the mutation within the gene is diagrammatically shown in Figure 4F.

As a means of confirming the nature of the splice mutation, the Leiden Muscular Dystrophy database (http://www.dmd.nl/) (35) was searched to determine if similar mutations were causative in human patient populations. A +5G→A splice

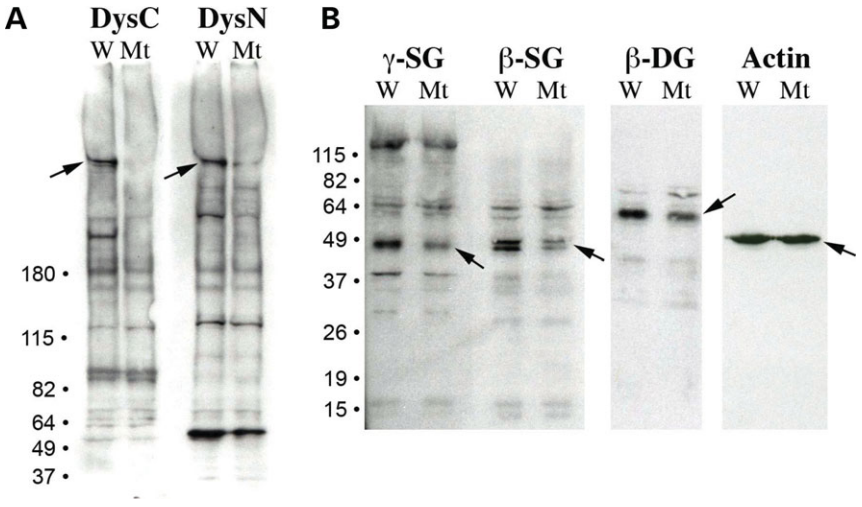

Figure 3. Immunoblot analysis shows that dystrophin and the sarcoglycans are reduced in the *sapje-like* mutant. (**A**) Antibodies against both the C- and N-terminus of dystrophin (DysC and DysN, respectively) show that dystrophin is strongly down-regulated in the *sapje-like* mutant ('Mt') relative to the unaffected wild-type siblings ('W'). (**B**) γ- and β-Sarcoglycan are down-regulated in the *sapje-like* mutant. β-Dystroglycan also appears to be affected in the *sapje-like* mutant, but the actin control and background non-specific bands seen in all of the blots show that the lanes were loaded equally. Size markers are shown on the left and each of the proteins is identified with an arrow on the blot.

site mutation was identified in humans for seven different exons (exons 4, 7, 32, 41, 43, 55 and 67), suggesting that such changes can cause disease. Importantly, the wild-type 5′ exon 62 zebrafish splice junction does not conform to the consensus human splice site at the −1 position (A versus T). This position is present in 80% of the splice sites although A is present 10% of the time (34). It is possible that this difference in the wild-type splice junction at the −1 position may make the zebrafish 5′ exon 62 splice junction more sensitive to the +5G→A mutation. Importantly, the human exon 55 mutation (which has a +5G→A splice site mutation; 36) has the same change at the −1 position and is identical to the zebrafish exon 62 splice donor suggesting that this may be the case.

Sequence conservation of the zebrafish dystrophin gene

As part of identifying the *sapje-like* mutation, the entire dystrophin coding sequence was determined by designing primers to conserved exons and using PCR amplification of the dystrophin coding sequence from cDNA. Since the sequence was assembled by overlapping ends of PCR products rather than sequencing an entire EST, it is possible (although unlikely) that portions of two highly conserved genes could be assembled together although all coding sequences position on the same genomic sequence suggesting that the entire sequence is part of the same large gene. To fill in gaps in the zebrafish cDNA, five small missing sections were added by including zebrafish EST sequences from the Genbank database. Blast homology results suggest that zebrafish dystrophin is more closely related to human dystrophin (57% identical/74% similar from exons 2–78) than human utrophin (33% identical/53% similar from exons 2–57 and 38% identical/56% similar from exons 34–78). When zebrafish dystrophin was compared with the known sequences of zebrafish utrophin (37), there was no significant homology. Taken together, these data suggest that all of the amplified exons likely belong to the zebrafish dystrophin gene.

To our knowledge, zebrafish are the most evolutionarily diverged vertebrate from human from which the dystrophin sequence has been fully sequenced. As a means of determining which portions of the dystrophin sequence were most conserved among vertebrates, the zebrafish dystrophin sequence was compared with the human sequence using the Tcoffee algorithm (Fig. 5) (38–42). In this figure, highly conserved sequences are shown in red and less-conserved regions in blue. As is clearly evident, the N- (the actin binding region) and C-terminus (the DAPC interacting region) of the zebrafish dystrophin sequence are highly conserved with the most variability present in the middle spectrin repeat section of the protein. Interestingly, when zebrafish cDNA sequence was compared with the genomic sequence, all intron/exon boundaries found in humans were conserved in zebrafish with the exception that zebrafish exon 79 does not encode protein (data not shown). Given the low degree of conservation of the spectrin repeat region of the protein, it was somewhat unexpected to see that the intron/exon boundaries were maintained even in this genomic region.

DISCUSSION

Because of their small size, proliferative capacity and transparency, zebrafish are quickly becoming an ideal complementary model to mice for the study of human disease. While further removed from humans than mice, zebrafish offer many advantages for identifying new diseases genes and investigating potential therapies through cell transfer experiments or large-scale chemical screens. Over the last eight years, a number of laboratories have established the zebrafish as a disease model for muscular dystrophy and morpholino experiments have confirmed the phenotype of a dystrophic

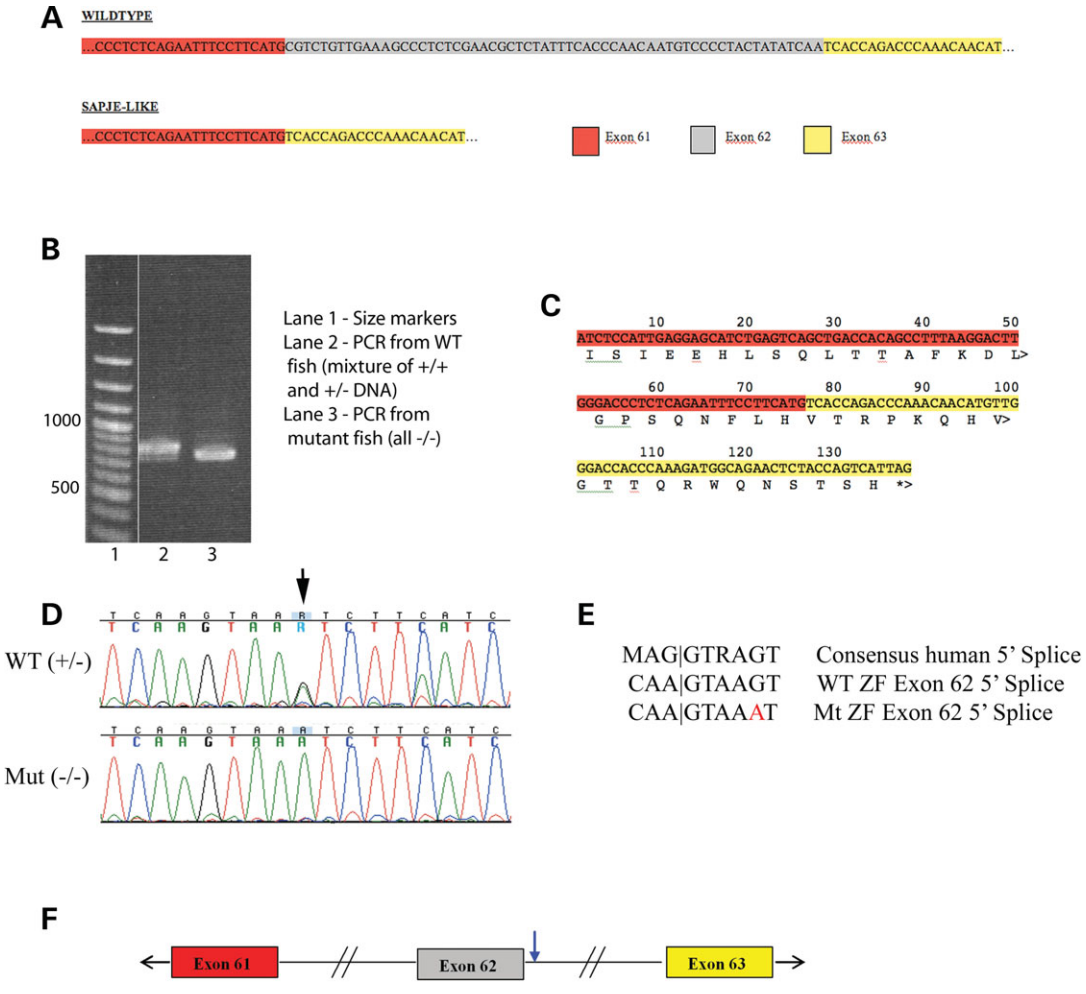

Figure 4. *Sapje-like* has a mutation in the donor splice junction of dystrophin exon 62. (**A**) Based on sequencing the zebrafish dystrophin cDNA, zebrafish exon 61 is directly adjacent to exon 63 in the *sapje-like* mutant and missing exon 62. (**B**) Using primers within zebrafish dystrophin exons 57 and 63, PCR amplification of the zebrafish cDNA templates produces a smaller PCR product in the *sapje-like* mutant than in the unaffected siblings as assayed by agarose gel electrophoresis. (**C**) When exon 61 is directly adjacent to exon 63 as in *sapje-like*, this shifts the reading frame of exon 63 resulting in a premature stop codon at the end of exon 63. (**D**) Sequencing of the 5′ splice site adjacent to exon 62 reveals a G to A base change (see arrow). (**E**) Comparison of the human consensus 5′ splice site with the wild-type and mutant zebrafish dystrophin exon 62 5′ splice site. The *sapje-like* mutation resides at position +5 (G→A) and is highlighted in red. (**F**) The sapje-like mutation is located in the Exon 62 donor splice junction (blue arrow).

fish at 5 dpf as inactive, bent and having disorganized muscle (reviewed in 16).

In 1996, a large scale genetic screen was completed in which a small group of muscle degenerative mutants were labeled as potentially dystrophic (22). Bassett *et al*. characterized the first of these mutants and showed that *sapje* carries a nonsense mutation in exon 4 of the dystrophin gene (23). Three other mutants have since been characterized. Two carry a mutation in laminin α2 and one carries a mutation in titin (24,25). Because this group of mutants is limited, an early-pressure genetic screen was conducted to identify additional zebrafish muscle degenerative mutants. One of these mutants (*sapje-like*) has a phenotype similar to *sapje* and our analysis shows that this recessive mutant carries a splice mutation in exon 62 of the zebrafish dystrophin gene. In addition to being a unique dystrophin mutant, *sapje-like* also confirms the dystrophin null-mutation described previously for *sapje* (23).

While mutations in the human muscle dystrophin isoform cause muscular dystrophy, there are many other human forms of dystrophin that are transcribed from alternative promoters (43). In humans, there are four full-length 427 kDa dystrophin isoforms that are transcribed in brain (Dp427c), lymphocyte (Dp427l), purkinje cells (Dp427p) and muscle (Dp427m). In addition, there are many other shorter dystrophin isoforms including Dp260 (retinal dystrophin), Dp140 (brain), Dp116 (Schwann cells), Dp71 (ubiquitous) and Dp40 (ubiquitous). While expression of these isoforms have not yet been reported in zebrafish, the western blot using the C-terminus antibody (recognizes the region conserved in all isoforms) specifically recognizes a 260 kDa dystrophin isoform down-regulated in the *sapje-like* mutant (Fig. 3A). This suggests that zebrafish express at least full-length and Dp260 dystrophin isoforms. Since the *sapje-like* mutation lies just upstream of where the DP71 promoter would be (in the intron between exon 62 and 63), all dystrophin isoforms

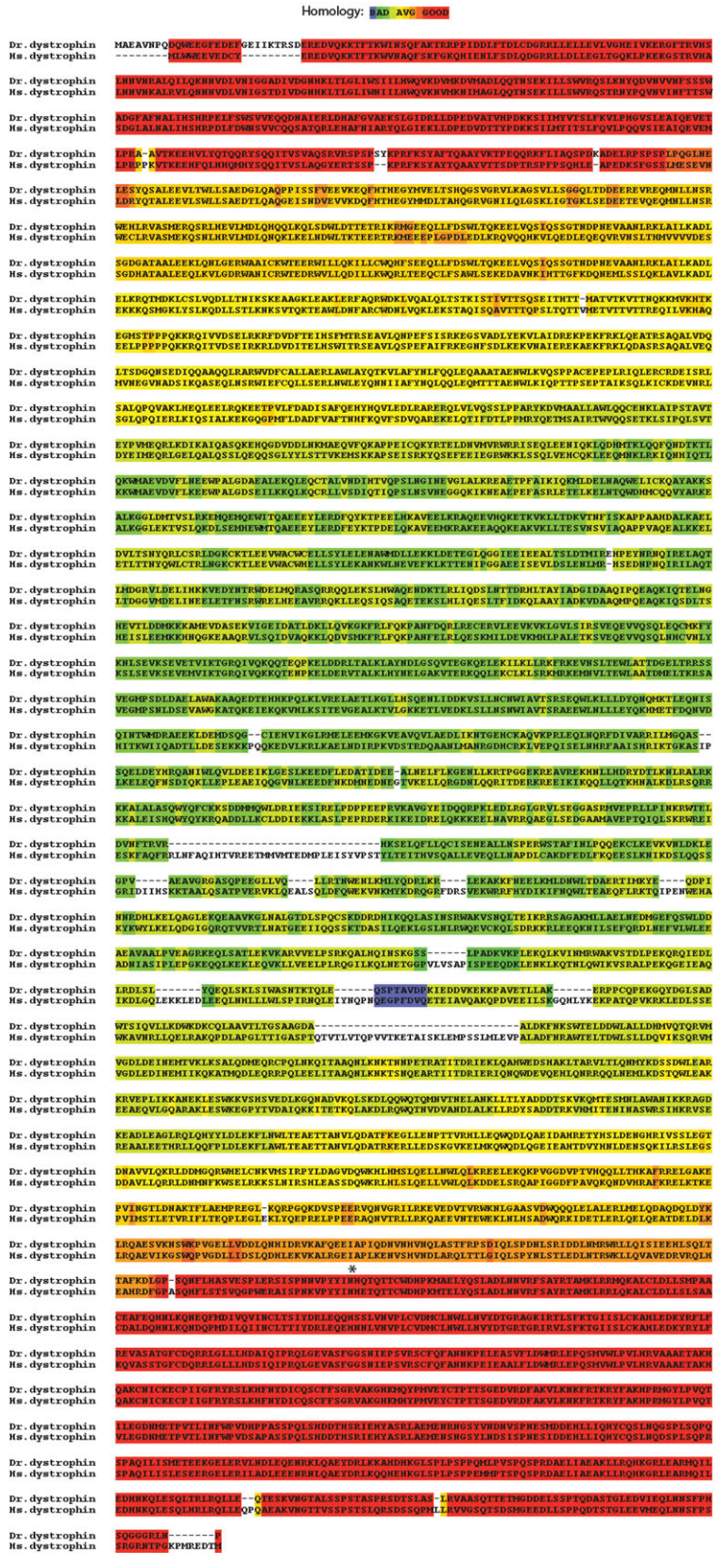

Figure 5. Conservation of the zebrafish dystrophin protein sequence. The zebrafish dystrophin sequence is designated 'Dr. dystrophin' and the human sequence is designated 'Hs. Dystrophin'. The degree of homology has been evaluated by the Tcoffee alignment program (38,39) on a blue (not conserved) to red (highly conserved) scale. The location of the zebrafish *sapje-like* mutation is shown with an asterisk.

larger than DP71 should be affected. As in human, defects in muscle Dp427m cause the most overt phenotype (muscular dystrophy) although there are additional defects in dystrophic patients including cognitive defects which may be caused by changes in other dystrophin isoforms. It is unclear whether the *sapje-like* mutation causes cognitive impairment or visual defects although direct physical manifestations resulting from the mutation are almost identical to *sapje* in which only the largest dystrophin isoform is affected.

While genomic deletions cause the vast majority of the cases of Duchenne Muscular Dystrophy, splicing defects can cause muscular dystrophy and many other diseases. *Sapje-like* represents the first zebrafish dystrophic mutant characterized to date that has dystrophy due to a splicing defect. As such, future drug screens using *sapje-like* are likely to uncover therapeutics that work in a completely different manner than drugs which would be able to correct *sapje* which carries a nonsense mutation in exon 4 of the zebrafish dystrophin gene. Since the muscular dystrophy phenotype in *sapje-like* can be easily and specifically assayed using birefringence, this makes *sapje-like* valuable not just for finding drugs capable of correcting muscular dystrophy, but potentially any other disease caused by splicing defects.

The location of the *sapje-like* splicing defect was identified in the donor splice site of exon 62. The loss of exon 62 was first noted experimentally by sequencing the complete zebrafish dystrophin cDNA in affected and unaffected siblings. The fragment that was initially sequenced was amplified using primers in exons flanking exon 62 (primers located in exon 57 and 63). In this scenario, the only product that could amplify in the splicing mutant was a transcript that skipped exon 62 (since that exon was incapable of splicing and would yield a PCR product too big for amplification and sequencing). While spliced (exon 61 to exon 63) and unspliced (exon 62 to intron to exon 63) transcript may exist in the *sapje-like* mutant, no full-length dystrophin protein is produced and the muscle degenerates due to the dystrophic phenotype.

Sapje-like is a novel zebrafish dystrophin mutant isolated as part of a large genetic screen. Its characterization has shown that it has symptoms of muscle degeneration similar to that seen in human patients with Duchenne Muscular Dystrophy. We have identified the molecular lesion as a G→A mutation in the donor splice junction of zebrafish dystrophin exon 62. *Sapje-like* is the first characterized dystrophic mutant which was not isolated as part of the 1996 Tuebingen screen. Since many of the Tuebingen dystrophic mutants have already been characterized and there are at least 30 different forms of muscular dystrophy, this suggests that additional dystrophic mutants remain to be identified and validates our approach to genetically isolate additional mutants as part of our own genetic screen. The identification of *sapje-like* suggests that our screen was successful and that the characterization of our remaining muscle mutants will help elucidate additional disease mechanisms.

MATERIALS AND METHODS

Early-pressure screen

As outlined in Supplementary Material, Figure S1 and described in (44), 80 male AB strain adult zebrafish were mutagenized with three treatments of 3 mM ENU (one treatment per week). Following treatment, 27 males were found to be fertile. After clearing the males by random outcrosses for a month, the mutation rate was assessed by complementation analysis with heterozygous albino mutants. Of 2400 scored embryos, 2 albinos were identified suggesting a mutation rate of approximately 1.2×10^{-3} mutations per albino locus. This mutation rate is similar to that from previously published reports. Mutagenized males were crossed with wild-type AB females to create a stock of heterozygous carriers which were grown to adulthood.

To perform the early-pressure screen, heterozygous females were separated from the males. The night before squeezing, the females were primed by placing in a tank with males although each sex was separated by a screen barrier. In the morning, the males were sacrificed and the testes were ground in Hanks Buffer to make a sperm stock. The sperm was subjected to UV irradiation for 3 min to crosslink the DNA. The females were anesthetized and systematically squeezed to extract the eggs and the eggs were fertilized with the UV-irradiated sperm. After squeezing, the females were placed in numbered tanks to be later correlated with the clutch of scored eggs. The sperm was activated by adding 1 ml of fish water and after 1 min 24 s, the fertilized eggs were subjected to high pressure (8 000 pounds/inch) for an additional 4 min 36 s to inhibit the second phase of meiosis (polar body extrusion). Pressure was gently released and the eggs were placed in a plate with fish water. The following day, the dead eggs were removed and the clutch was counted if more than five eggs were viable (norm estimated at 10–30 eggs). Gross phenotype was noticed at 1 dpf and muscle structure, activity and bending confirmation assayed at 5 dpf. If the clutch showed any mutants of interest (recombination can make telomeric mutations hard to score by EP), the squeezed mother was outcrossed to wild-type AB males and the resultant progeny incrossed to genetically verify phenotype. The screen was complete when 500 viable clutches were scored. Of these, eight showed a muscle-specific phenotype (not gross abnormalities) including three which show the hallmarks of muscular dystrophy (muscle degeneration over time).

Birefringence

Muscle birefringence was analyzed by placing anesthesized embryos on a glass polarizing filter and covering the embryos with second polarizing filter elevated to prevent the embryos from being harmed. The filters were then placed on an underlit dissecting scope (Leica, model MZ75) and the top polarizing filter twisted until only the light refracting through the striated muscle was visible. Since the degree of birefringence is affected by the horizontal orientation of the fish, the fish were gently oscillated back and forth to account for differences in positioning.

Complementation analysis

Complementation analysis was performed twice. First to assay the mutation rate by crossing mutagenized males with heterozygous albino females. Second, complementation was used to

determine if *sapje* and *sapje-like* were allelic by crossing heterozygous *sapje-like* mutants with heterozygous *sapje* mutants. Resultant progeny were scored using birefringence.

Immunoblot analysis

For separation of large proteins like dystrophin, 3–8% Tris–Acetate gradient gels were used and electrophoresis was at 150 V for ~2 h. Following separation, proteins were transferred to 0.45 μm nitrocellulose membranes in a submerged transfer apparatus according to the instructions provided by the manufacturer (Invitrogen). For separation of smaller proteins (<100 kDa), 12% Tris–Glycine gradient gels (4% stacking) were used and electrophoresis was at 130 V for ~2 h. Following separation, the proteins were transferred to 0.45 μm nitrocellulose using a Semi-Dry Transfer Apparatus according to the instructions provided by the manufacturer (Bio-Rad). All antibodies used have either been described previously or are commercially available.

Membranes were incubated with the following antibodies in a cocktail of 1× PBS (phosphate-buffered saline), 0.1% Tween-20 and 5% non-fat dry milk (NFDM): 'DysC' (1:333 dilution) (19), 'DysN' (1:333 dilution) (18), δ-sarcoglycan (1:300 dilution) (12,19), γ-sarcoglycan 'γ-SG' (1:333 dilution) (18), β-sarcoglycan 'β-SG' (Novocastra) (1:20 dilution) (19), β-dystroglycan 'β-DG' (Novocastra) (1:10 dilution) (19) and actin 'A2066 '(Sigma) (1:300 dilution) (19). Membranes were incubated with primary antibodies for 1 h at 25°C then washed two times quickly and three times for 10 min in a solution of 1× PBS with 0.1% Tween-20.

An anti-rabbit-conjugated goat HRP secondary antibody (1:5000 dilution) (Jackson ImmunoResearch Lab) was used to detect the following primary antibodies: 'DysC', 'DysN', 'δ-SG', 'γ-SG' and actin 'A2066'. An anti-mouse-conjugated goat HRP secondary antibody (1:5000 dilution) (Jackson ImmunoResearch Lab) was used to detect the 'β-SG' and 'β-DG' primary antibodies. The membranes were incubated with 1:5000 dilution of secondary antibody in a solution of 1× PBS with 0.1% Tween-20 and 5% NFDM for 1 h at 25°C before washing two times quickly and three times for 10 min in a solution of 1× PBS with 0.1% Tween-20. The blots were incubated with Western Lighting Chemiluminescence Reagent Plus (Perkin Elmer) as described by the distributor and exposed to film for up to 5 min.

Immunohistochemistry

Juvenile (5-day-old) zebrafish were anesthetized in 0.3% tricaine solution for 2–10 min, coated in Optimal Cutting Temperature Compound (Sakura) and frozen in liquid nitrogen-cooled 4-methyl butane. 5–10 μm sections (as indicated) were cut at −20°C using a Microm HM 505 E cryostat, transferred to silane-coated microscope slides and fixed by immersing the slides in methanol for 3 min before equilibrating in 1× PBS at 4°C overnight.

To block non-specific binding of the antibodies, sections were incubated for 30 min at room temperature in PBS+10% horse serum. Following blocking, the slides were incubated overnight at 4°C using the same antibodies described for immunoblot analysis. Slides were washed three times in 1× PBS and sections incubated with FITC-conjugated secondary antibodies (Jackson ImmunoResearch Lab) at a 1:100 dilution for 1 h at room temperature. Slides were washed three times in 1× PBS before mounting in 25 μl vectashield. Slides were analyzed using a Zeiss Axioplan II microscope with a triple filter and the images stored using either IP Lab or OpenLab Software.

Sequencing the zebrafish dystrophin gene

Zebrafish cDNA was synthesized for both wild-type and *sapje-like* 5 dpf embryos by first isolating total RNA with a QIAGEN RNA isolation kit. The poly A containing mRNA was used as a template for cDNA synthesis using a One-Cycle cDNA Synthesis Kit (Affymetrix). To specifically amplify segments of the zebrafish dystrophin gene, PCR primers were designed by finding regions of zebrafish chromosome 1 exon sequences which could encode peptide fragments having high homology to the analogous section of the human dystrophin protein sequence. Using this approach, 20 PCR reactions were designed to amplify the complete dystrophin cDNA in overlapping fragments. Overall, 12 000 bases of the zebrafish dystrophin cDNA were sequenced with a few remaining bases filled in using pre-existing EST sequences available in Genbank. For identification of the splice site mutation, primers were designed from genomic sequence outside the splice regions and the region of interest amplified from wild-type and mutant genomic DNA.

PCR reactions were performed at 35 cycles of 1 min at 94°C, 2 min at 52°C, and 1 min at 72°C using a GC 2 PCR kit (Clontech). Finished PCR reactions were purified with a QIAGEN PCR Cleanup Kit and prepared for sequencing.

All DNA sequencing was performed in The Mental Retardation Research Center Sequencing Core Facility located within Children's Hospital Boston. Sequencing reactions were prepared with ~50 ng of PCR product and 10 pm of primer in a 12 μl reaction volume. DNA was fluorescently labeled and analyzed on an Applied Biosystems 3730 DNA Analyzer. The sequences were imported into Sequencher and comparisons made between wild-type and *sapje-like* DNA and cDNA sequence to identify the exon missing in the mutant fish and the causative mutation. In addition, at least 10 linked SNPs were also identified as part of this analysis.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* Online.

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Conflict of Interest statement. None declared.

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