The zebrafish *candyfloss* mutant implicates extracellular matrix adhesion failure in laminin $\alpha 2$ -deficient congenital muscular dystrophy

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Mutations in the human *laminin $\alpha 2$* (*LAMA2*) gene result in the most common form of congenital muscular dystrophy (MDC1A). There are currently three models for the molecular basis of cellular pathology in MDC1A: (i) lack of *LAMA2* leads to sarcolemmal weakness and failure, followed by cellular necrosis, as is the case in Duchenne muscular dystrophy (DMD); (ii) loss of *LAMA2*-mediated signaling during the development and maintenance of muscle tissue results in myoblast proliferation and fusion defects; (iii) loss of *LAMA2* from the basement membrane of the Schwann cells surrounding the peripheral nerves results in a lack of motor stimulation, leading to effective denervation atrophy. Here we show that the degenerative muscle phenotype in the zebrafish dystrophic mutant, *candyfloss* (*caf*) results from mutations in the *laminin $\alpha 2$* (*lama2*) gene. *In vivo* time-lapse analysis of mechanically loaded fibers and membrane permeability assays suggest that, unlike DMD, fiber detachment is not initially associated with sarcolemmal rupture. Early muscle formation and myoblast fusion are normal, indicating that any deficiency in early *Lama2* signaling does not lead to muscle pathology. In addition, innervation by the primary motor neurons is unaffected, and fiber detachment stems from muscle contraction, demonstrating that muscle atrophy through lack of motor neuron activity does not contribute to pathology in this system. Using these and other analyses, we present a model of *lama2* function where fiber detachment external to the sarcolemma is mechanically induced, and retracted fibers with uncompromised membranes undergo subsequent apoptosis.

muscle | degeneration | fiber | time-lapse

Congenital muscular dystrophies (CMDs) are a group of neuromuscular disorders characterized by severe hypotonia, muscle weakness, and contractures (1). The incidence of CMDs has been estimated to be approximately one in 21,500 (2), with laminin $\alpha 2$ - (*LAMA2*) deficient CMD (MDC1A) accounting for ≈ 40 –50% of the CMD cases in European countries (3). MDC1A is caused by genetic lesions in the *LAMA2* gene (4). The classical phenotype is associated with complete *LAMA2* deficiency and pathological symptoms of muscle degeneration, fibrosis, and white matter abnormalities within the CNS (5).

Laminins are major structural components of basal laminae and exist as heterotrimeric complexes of one α , one β , and one γ chain. Specific complexes exhibit particular tissue specificities. There are currently 15 described mammalian complexes made up of varying combinations of five α , three β , and three γ chains (6, 7). In addition to their structural role, laminins also act as signaling molecules through receptors such as integrins (8). The most-studied complex to date has been laminin 1, largely for historical reasons, because it was the first to be identified. Laminin 1 consists of the laminin $\alpha 1$, $\beta 1$, and $\gamma 1$ chains. It appears early in epithelial morphogenesis in most embryonic tissues and is a major component of extracellular matrix (ECM) (9). In the zebrafish, the $\alpha 1$, $\beta 1$, and $\gamma 1$ chains that make up the laminin 1 complex are essential for normal embryonic develop-

ment and have been shown to be particularly important in notochord morphogenesis and maintenance (10, 11). Work in mammalian systems has shown that the $\alpha 2$ subunit is present in three complexes; laminins 2 and 4, expressed in the basal laminae of muscle fibers, and the Schwann cells surrounding the peripheral nerves (6, 12, 13); and laminin 12, a little-studied complex that is potentially the first non-basement membrane laminin (14).

Different hypotheses have been developed as to why *LAMA2* deficiency leads to the onset of CMD. Most commonly, the proposed mechanism of cellular pathology centers on the structural role of *LAMA2*, through its interaction with the dystrophin-associated glycoprotein complex (DGC), necessary for maintenance of sarcolemmal integrity (15). *LAMA2* is known to bind directly to α -dystroglycan, the component of the DGC most distal to the sarcolemma, and thereby to anchor the muscle cell membrane to the ECM (16). The notion of a structural link from the ECM through to the actin cytoskeleton being provided by the DGC is strengthened by the observation that a number of degenerative diseases of the skeletal muscle, including Duchenne muscular dystrophy (DMD) and certain limb girdle muscular dystrophies, are associated with abnormalities in components of the DGC (17). Traditionally, the accepted dogma regarding the cellular pathology of these diseases has been that loss of the structural link between the internal actin cytoskeleton and the cell membrane renders the sarcolemma vulnerable to mechanical damage that, in turn, leads to fiber apoptosis and/or necrosis [dystrophin (18) and δ -sarcoglycan (19)]. However, in recent years, mechanistic explanations of dystrophic pathologies have been challenged by hypotheses suggesting that signaling dysfunction could be more important than loss of sarcolemmal integrity. For instance, dystrophin, in addition to its structural role, serves as a scaffold for the assembly of a multicomponent signal transduction complex, members of which also form integral parts of the DGC (20). In the case of *LAMA2*, both mechanistic and dysfunctional signaling explanations have been

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Abbreviations: CMD, congenital muscle dystrophy; *LAMA2*, laminin $\alpha 2$; MDC1A, *LAMA2*-deficient CMD; ECM, extracellular matrix; DGC, dystrophin-associated glycoprotein complex; DMD, Duchenne muscular dystrophy; hpf, hours postfertilization; DIC, differential interference contrast microscopy; EBD, Evans blue dye; MTJ, myotendinous junction.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession no. EF467237).

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mooted for the pathology of MDC1A because, *in vitro* at least, laminin-integrin binding is involved in the regulation of myoblast proliferation and fusion (21). However, the laminin 2 complex is not strictly a DGC member and is found external to the sarcolemma, forming a link between the DGC and the ECM. As such, it is unclear whether fiber detachment and/or loss of membrane integrity contribute significantly to the pathology. In addition, LAMA2-deficient CMD is not normally associated with loss of DGC components or of dystrophin itself (5). Also unlike DMD, MDC1A involves peripheral nerve defects, leading to a third hypothesis, that impaired neural function results in relatively little electrical stimulation and contraction of myofibers, effectively causing denervation atrophy (7). Thus, the actual basis of the pathology evident in LAMA2-deficient CMD remains to be determined.

Here, we describe two null alleles of *lama2* in the zebrafish, in which we directly observe muscle pathology in real time by using time-lapse photomicroscopy. Our analyses lead to hypotheses of *lama2* function that are likely clinically significant. We clearly show that in the zebrafish model of MDC1A, the primary mechanism of pathology is through fiber detachment induced by mechanical loading of the fiber. In contrast to models of DMD, this fiber detachment occurs in the absence of major sarcolemmal disruption or loss of components of the DGC. Using transmission electron microscopy, we demonstrate a loss of integrity of the ECM and subsequent fibrosis. In addition, we show that early myoblast proliferation and fusion are unaffected, confirming that in this model, loss of *Lama2* does not lead to muscle differentiation abnormalities. Similarly, formation and function of the primary motor neurons are normal, and we find no difference in innervation between homozygous *caf* embryos and siblings. Furthermore, fiber detachment depends on motor activity, leading us to conclude that peripheral nerve defects also do not contribute to pathology in this system.

Results

Identification of the *candyfloss* Locus. The dystrophic mutants [class A4 (22)] from the Tübingen screen are characterized by muscle degeneration shortly after formation. They have impaired motility in the early larval phase and show reduced muscle birefringency under polarized light. Complementation was performed between mutants *sapje*^{ta222a} (*sap*), *softy*^{tm272a}, and “unresolved” alleles *tf212b*, *teg15a*, and *tk209*. Of these, only *teg15a* and *tk209* were found to be in the same complementation group and distinct from already-defined loci. We named this dystrophic mutant *candyfloss* (*caf*^{teg15a}, *caf*^{tk209}), because of the severe nature of the progressive muscle loss and the shape of dystrophic muscle fibers evident in homozygous mutants. The gross phenotype of the two *caf* alleles was indistinguishable. Consequently, all phenotypic analysis was performed on *caf*^{teg15a}.

Initial formation of myotomal muscle is normal in *caf* embryos (Fig. 1A). However, immunohistochemistry using antibodies against slow and fast myosin heavy chains revealed that at 36 h postfertilization (hpf), shortly after elongation and fusion of myofibers, the first pathology becomes evident (Fig. 1B and C). The dystrophic appearance of the muscle is caused by detachment and retraction of muscle fibers from the vertical myosepta that form the somite boundaries. Detachment first occurs in the slow muscle layer at the periphery of the myotome, which are the first fibers in the embryo to differentiate and function (Fig. 1B). This is closely followed by detachment in the deeper fast muscle layer (Fig. 1C). In live embryos, the *caf* phenotype is first visible under differential interference contrast microscopy (DIC) at 48 hpf. At this time, the first myotomal lesions can be seen in a small proportion of embryos within a clutch. There is some variability in the severity of the phenotype among homozygotes, and the phenotype becomes fully penetrant only after 72 hpf, around the time of hatching (Fig. 1D). Mutant embryos often need to be

Fig. 1. Initial muscle formation and degeneration in *caf* embryos. (A) Myoblasts fuse and elongate to form multinucleate fibers (arrowheads) that span the myotome between vertical myosepta. Single confocal scan through epaxial somite at 36 hpf. Green, f310 anti-fast MyHC; red, propidium iodide. (B) Degeneration in the slow layer, which is the first to elongate and fuse. Confocal projection of somite at 36 hpf. f59 anti-slow MyHC. (C) Degeneration in the deeper fast muscle layer. Arrowhead indicates a site of fiber loss. Confocal projection of somite at 36 hpf, f310 anti-fast MyHC. (D) Myotomal lesions at 72 hpf. DIC brightfield image of epaxial somite. (E) Stills from a time-lapse DIC movie showing fiber detachment and retraction in a homozygous *caf* embryo at 72 hpf, captured by using the anesthetic recovery technique. Time is indicated in seconds. The full movie sequence is available as [SI Movie 1](#). In all images, anterior is to the left.

manually dechorionated at this time, because many are unable to extricate themselves and otherwise die within the chorion. Muscle damage does not affect all somites equally. Whereas a particular somite may appear normal, its neighbor might contain virtually no intact fibers. Such stochastic fiber damage is a hallmark of muscular dystrophy of human patients and mammalian animal models alike, as well as the dystrophin-deficient zebrafish mutation *sap*. The majority of mortality was found to occur suddenly around days 14–16. However, a small number of homozygote mutants survived this critical period and reached adulthood (2/80), although these individuals have so far failed to reproduce [[supporting information \(SI\) Fig. 7](#)].

Muscle Fiber Detachment Is Induced by Motor Activity. The stochastic pattern of muscle damage between somites led us to investigate whether muscle damage was related to motor activity. Raising embryos under anesthetic resulted in complete suppression of the phenotype up to 72 hpf ([SI Table 1](#)). Conversely, mechanically overloading the muscle of mutant larvae greatly increased both the severity and incidence of the fiber pathology within these animals. Mechanical loading of fibers was achieved by stimulating larvae to swim through raising media to which had been added the inert cellulose polymer, methyl-cellulose, which increased the viscosity of the surrounding media through which the larvae were required to swim. Raising embryos in 0.6% methyl-cellulose led to an increased severity of phenotype, with *caf* homozygous mutants displaying detached fibers in virtually every somite ([SI Tables 1 and 2](#)). The nature of this fiber loss could be captured in real time by the use of an anesthetic recovery protocol. Mutants previously anesthetized in 0.001% tricaine were transferred into a highly viscous 3% methyl-cellulose solution, dissolved in raising media that contained no anesthetic. Upon anesthetic recovery, the partially immobilized muscle began to contract against the high viscosity of the mounting media inducing a rapid fiber pathology. Fiber detachment induced under these conditions could be visualized by using time-lapse photomicroscopy (Fig. 1E and [SI Movie 1](#)). It is clear from these analyses that the phenotype of homozygous *caf* mutants results from contraction-induced fiber detachment from the ends of the muscle fibers, and that the severity of this

Fig. 2. Unlike in dystrophin-deficient *sap* embryos, detached fibers in *caf* embryos do not show uptake of EBD, indicating that the sarcolemma is not compromised. (A) EBD within *caf* homozygotes is not taken up by a recently detached fiber in a homozygous *caf* embryo (arrowhead). (B) EBD can be seen to pool in a large myotomal lesion created by multiple retracting fibers (arrowhead). Retracted fibers themselves remain impermeable to EBD (arrow). (C) Damaged fibers in homozygous *sap* embryos are infiltrated by EBD before detachment. (D) EBD-positive retracted fiber in a *sap* embryo. (i) fluorescence, (ii) DIC, and (iii) merge. All images represent 72-hpf embryos, lateral views with anterior to the left.

detachment phenotype is proportional to the load under which muscle fibers are placed.

Fiber Detachment Is Not Associated with Loss of Sarcolemmal Integrity. The similarity of the *caf* phenotype to models of DMD led us to investigate whether sarcolemmal integrity was also compromised in these animals. Evans blue dye (EBD) is a small-

molecular-weight dye that fluoresces in the red channel under UV light. Although the sarcolemma of physiologically normal cells is impermeable to EBD, it selectively accumulates in cells in which the sarcolemma has been torn. Injection of EBD into the precardiac sinus results in the passage of dye through the larval circulatory system and consequent uptake by cells with compromised membranes. Unlike in *sap* fish [Fig. 2C (23)], no uptake of EBD was seen in *caf* homozygotes at 72 hpf by retracted or nonretracted fibers (Fig. 2A). On the contrary, EBD fluorescence was seen to pool in the interfiber myotomal lesions created by fiber retraction, indicating that sarcolemmal integrity was maintained (Fig. 2B).

The *caf^{teg15a}* and *caf^{tk209}* Alleles Map to a Region Containing the Zebrafish Orthologue of LAMA2. A first-pass map position for *caf^{teg15a}* was established by using standard bulked segregant analysis (24) to markers z6804 and z10056 on linkage group 20. Using a fine mapping strategy, this region was further refined to the flanking markers z9708 and z7603 (Fig. 3A), approximating a genomic region of ≈ 0.89 Mb, containing 23 transcripts. In the center of this region was a portion of the zebrafish orthologue of laminin $\alpha 2$ (*lama2*), which in humans is causative of LAMA2-deficient CMD. To confirm the genomic position of zebrafish *lama2*, radiation hybrid mapping was carried out on the LN54 panel (25) by using gene-specific primers to a portion of the *lama2* ORF. Using this approach, significant linkage was found to marker z6804 on linkage group 20 (one of those implicated in the initial bulked segregant analysis).

***lama2* mRNA Expression Is Reduced in Mutant Embryos.** To investigate *lama2* as a candidate causative of the *caf* phenotype, we

Fig. 3. The *caf* phenotype is a result of mutations in the zebrafish homologue of the LAMA2 gene. (A) Initial bulked segregant analysis implicated markers z6804 and z10056 as being linked to the *caf* phenotype. Further fine mapping resolved flanking markers z9708 and z7603, delineating a candidate genomic region of ≈ 0.89 Mb, containing sequence with homology to human LAMA2. Single base changes in both *caf^{teg15a}* and *caf^{tk209}* were found in the homologue of human exon 60, resulting in the introduction of premature stop codons to the zebrafish *lama2* ORF. (B) *In situ* hybridization to the *lama2* mRNA at 48 hpf shows expression in the myotomal muscle. Strikingly, there is a severe reduction of message in *caf* homozygous embryos, suggestive of nonsense-mediated decay. Brackets indicate region of myotome. (i) Homozygous *caf* embryo, (ii) wild-type embryo, and (iii) transverse methacrylate sections through the trunk of *caf* (Left) and wild-type (Right) embryos. (C) Structure of the human LAMA2 protein. The positions of mutations that result in the MDC1A disease are indicated by black arrowheads. The homologous positions of the *caf^{teg15a}* and *caf^{tk209}* stop codons are indicated by red arrowheads. Both *caf* mutations occur in close proximity to known human MDC1A mutations, within the globular domain, which is essential for dystroglycan binding. Domains represented are laminin VI (red), laminin IV (blue), EGF-like (gray), and globular (green). (D) Amino acid alignment between zebrafish (*Dr*), human (*Hs*), and mouse (*Mm*) sequences for the mutant exon. Both *caf^{teg15a}* and *caf^{tk209}* mutations occur within completely conserved amino acids. (E) Injection of antisense morpholino oligonucleotides against the *lama2* mRNA phenocopies the *caf* homozygous mutants. Seventy-two hpf, lateral view, anterior to the left.

carried out *in situ* hybridization for the *lama2* mRNA on *caf* mutant and sib embryos (Fig. 3*B*). Expression of the transcript closely follows the sites of muscle differentiation in the embryos and larvae (Fig. 3*B*; SI Fig. 8), being first detected in an adaxial cell pattern, which are the first muscle cells to differentiate and express myofibrillar markers such as *sMyHC1* (26). By 48 hpf, the transcript is expressed predominantly in the skeletal muscle, supporting the notion of a role in muscle cell maintenance. Importantly, we noted that $\approx 25\%$ of embryos from an incross of *caf* heterozygote parents showed a weaker staining pattern for the *lama2* expression than their siblings. At 72 hpf, this lower level of *lama2* message correlated with embryos exhibiting the *caf* phenotype ($n = 6/20$), suggesting that *lama2* transcripts undergo nonsense-mediated decay in *caf* homozygotes.

The *caf^{teg15a}* and *caf^{tk209}* Strains Contain Stop Mutations in the *lama2* ORF and Are Phenocopied by Anti-*lama2* Morpholinos. The high degree of misassembly in the genomic region necessitated rearrangement of the region by using the mouse and human LAMA2 amino acid sequences as references. Once a contiguous genomic sequence was created, 55 putative coding exons of the zebrafish *lama2* gene were predicted by using Genewise (27). All putative exons were successfully sequenced by using flanking primers within adjacent introns. Premature stop codons were found in the zebrafish homologue of human exon 60 in both *caf^{teg15a}* and *caf^{tk209}* (Fig. 3*A* and *D*; GenBank accession no. EF467237). This region encodes the globular domain of the Lama2 protein that is essential for dystroglycan binding and is in close proximity to human mutations causative for MDC1A (Fig. 3*C*). Twenty-four affected and 24 unaffected progeny from each allele strain were genotyped by initial restriction analysis, followed by sequencing, demonstrating absolute segregation of the mutations with the dystrophic phenotypes (SI Table 3).

To further demonstrate that mutations in the zebrafish *lama2* gene cause a dystrophic phenotype, we injected antisense morpholino oligonucleotides into the first blastomere of wild-type embryos. MO-*Lama2*-1 was a translation blocking morpholino designed to cover the initiation codon, and MO-*Lama2*-60 was designed to overlap the boundary of the zebrafish homologues of human exons 59 and 60, inducing exon skipping of exon 60 and a frameshift in exon 61, to result in a truncated protein. Injection of MO-*Lama2*-1 at 50 ng/ μ l and MO-*Lama2*-60 at 10 ng/ μ l did not cause nonspecific abnormalities at levels above sham-injected embryos and phenocopied the *caf* phenotype at 59% (10/17) and 52% (15/29) respectively (Fig. 3*E*). Thus, we concluded that the stop mutations we have identified in *lama2* cause the *caf^{teg15a}* and *caf^{tk209}* phenotypes, respectively.

Innervation Is Unaffected in *caf* Embryos. One model of the cellular pathology in LAMA2 deficiency is that innervation defects lead to secondary muscle tissue defects such as denervation atrophy. To investigate the effect of Lama2 deficiency on innervation in the context of the *caf* model we used TRITC conjugated bungarotoxin, which binds irreversibly to the neuromuscular junction, and fluoresces in the red channel. In addition, we examined the outgrowth of the primary motor neurons from the neural tube by crossing *caf* embryos with a transgenic line *Tg(cmet:EGFP)^{ed6}*, which expresses EGFP in the middle primary, rostral primary, and caudal primary neurons (T.E.H. and P.D.C., unpublished observations). Using confocal microscopy, we detected no difference in the extent of innervation or neuromuscular junction formation between homozygous mutant and sibling embryos (SI Movies 2 and 3). Similarly, formation of the primary motor neurons in homozygous *caf* embryos was normal (SI Movies 4 and 5).

Fig. 4. Immunohistochemistry at 72 hpf shows retraction of epitopes with detaching fibers, demonstrating that failure occurs within the extracellular matrix rather than at the sarcolemma. (A) *caf*, anti-dystrophin. (B) Wild-type anti-dystrophin. (C) *caf*, anti- β -dystroglycan. (D) Wild-type anti- β -dystroglycan. (E) *caf*, anti-laminin 1. (F) Wild-type anti-laminin 1. (i) Fluorescence, (ii) DIC, and (iii) merge. Arrowheads indicate retraction of epitopes with individual fibers. Images show lateral views, anterior to the left.

Fiber Detachment Occurs on the Extracellular Side of the Membrane at the Myotendinous Junction (MTJ), Rather Than at the Sarcolemma.

The maintenance of membrane integrity in retracted fibers led us to investigate the effects of the *caf* phenotype on DGC-associated proteins at the sarcolemma. Dystrophin and β -dystroglycan (β DG) proteins are known to be expressed at the junctional sarcolemma after 36 h, and in the dystrophin-deficient *sap* fish, β DG remains at the MTJ during fiber detachment (23, 28). In addition, laminin 1 (α 1, β 1, and γ 1) immunoreactivity is detectable within the ECM of the vertical myoseptum at this time (10). Dystrophin, β DG, and laminin 1 expression at the MTJ were unaffected in *caf* embryos. Furthermore, all epitopes showed a retraction with the detached fiber ends (Fig. 4), consistent with attachment failure occurring within the ECM rather than at the sarcolemma.

***lama2* Mutants Display Ultrastructural Defects at the MTJ.** The vertical myoseptum, dividing the trunk somites, is composed mainly of dense collagen, and its structure and function are similar to that of the mammalian tendon. As such, it is regarded as the homologous or analogous tissue (29). To investigate the detachment of fibers at the zebrafish MTJ, in the context of Lama2 deficiency, we used transmission electron microscopy on *caf* embryos. We compared mutant and unaffected sibling embryos at two separate periods of development, first at 72 hpf, when the phenotype is still relatively mild, and at 120 hpf, when the phenotype is relatively severe.

Fig. 5. Transmission electron micrographs of the vertical myosepta in *caf* and wild-type embryos at 72 and 120 hpf. (A) *caf*, 72 hpf. The extracellular matrix phenotype is subtle and under low magnification (*i*; $\times 2,400$) the myosepta appear normal. However, under higher magnification (*ii*; $\times 7,100$ and *iii*; $\times 54,000$), tearing of the myosepta can be seen that is not apparent in wild-type siblings (arrowheads). (B) Wild-type, 72 hpf. (C) *caf*, 120 hpf. Even under low magnification, the myosepta are grossly distorted and fibrotic. Under medium magnification, portions of extracellular matrix can be seen to infiltrate the myotome (arrowhead), pulled with the detaching fibers. Under high magnification, the myosepta are greatly increased in diameter and show condensed collagen fibers, indicative of a fibrotic response. (D) Wild-type, 120 hpf.

Under low-powered EM ($\times 2,400$) at 72 hpf, the thickness and architecture of the vertical myosepta were indistinguishable between mutant and sibs. However, under higher magnification ($\times 7,100$ – $\times 54,000$), tearing and detachment at the myosepta were apparent in the mutants (Fig. 5 *A* and *B*). By 120 hpf, the myoseptal architecture in the mutant embryos was grossly distorted. Most significantly, portions of connective tissue were seen to infiltrate the myotome carried with the ends of retracting fibers. The myoseptum itself was greatly expanded in thickness and contained an irregular array of collagen fibers (Fig. 5 *C* and *D*).

Death of Detached Fibers Is Delayed in *caf* Compared with *sap* Embryos. In models of DMD, cells sustaining catastrophic sarcolemmal damage are thought to undergo immediate necrosis, whereas cells with minor damage undergo either a program of sarcolemmal repair or apoptosis. To investigate the time course of cell death-associated DNA fragmentation in retracted fibers, we compared *caf* and *sap* embryos by using acridine orange staining. Acridine orange, when injected into the embryonic circulation, is taken up by the fragmenting nuclei of cells undergoing apoptosis/necrosis (30). *caf* and *sap* embryos were anesthetized between 24 and 60 hpf to suppress extensive fiber detachment. At 60 hpf, embryos were injected with acridine orange, and severe fiber detachment was induced by using the anesthetic recovery protocol previously described. By 72 hpf, acridine orange positive cells were present in *sap* embryos, with staining becoming extensive by 96 hpf (Fig. 6 *D* and *E*). However, in *caf* embryos acridine orange uptake by retracted fibers did not occur until 96 hpf and was not extensive until 120 hpf (Fig. 6 *A–C*). We concluded that retracted fiber cell death is delayed in *caf* embryos in comparison to *sap* embryos.

Fig. 6. Cell death after artificially induced fiber detachment is delayed in *caf* embryos when compared with dystrophin-deficient *sap* embryos. Fiber detachment was induced at 60 hpf and the time course of cell death followed by using acridine orange. Acridine orange uptake by apoptotic/necrotic cells is first seen at 72 hpf in *sap* embryos but not until 96 hpf in *caf* embryos. In addition, uptake does not peak until 120 hpf in *caf* embryos compared with 96 hpf in *sap*. (A–C) *caf*, 72–120 hpf. (D and E) *sap*, 72–96 hpf. Time-lapse analysis of acridine orange uptake between 60 and 72 hpf on individual embryos confirms uptake in *sap*, but not in *caf* embryos. (F and G) Images show apical somites, anterior to the left. Time-lapse movies are available as [SI Movies 6 and 7](#).

Discussion

In this paper, we present a zebrafish model of MDC1A and examine the cellular phenotype with respect to three current hypotheses, that pathology occurs because of (i) sarcolemmal weakness, leading to loss of membrane integrity and fiber necrosis (15); (ii) loss of Lama2-mediated signaling, leading to defects in myoblast proliferation and fusion (21); and (iii) effective denervation atrophy because of defects in the peripheral nervous system (7). We demonstrate that none of these explanations adequately accounts for the early dystrophic muscle phenotype in homozygous *lama2*-null mutant zebrafish embryos. We believe that damage to the muscle tissue occurs by mechanically induced fiber detachment in the absence of sarcolemmal rupture, and that detached fibers undergo subsequent cell death.

Superficially, the *caf* and *sap* phenotypes have a similar appearance, because there is no impairment of new muscle formation [consistent with *dy/dy* (31) and *mdx* (32) phenotypes respectively], and both involve fiber detachment. Dystrophin is enriched both at mammalian MTJs and myomuscular junctions, end-to-end/end-to-side muscle-to-muscle attachments that form without intervening tendons (33). Analysis of the *sap* model suggested that in larger mammalian muscles, loss of junctional integrity at intrafascicular fiber terminations and tendinous intersections are likely to contribute to the pathology of DMD (23). Similarly, the *caf* model shows a loss of junctional integrity at the embryonic MTJ, suggesting that Lama2 is necessary for the stability of vertebrate muscle attachments, and that fiber detachment is the primary cause of muscle degeneration. However, in direct contrast to the *sap* and other models of DMD, where fiber detachment is associated with sarcolemmal rupture, in *caf* embryos fiber detachment occurs without compromise of membrane integrity. Attachment failure occurs within the ECM itself, such that ECM components retract into the myotome with the fiber. Furthermore, we find that postdetachment cell death begins substantially later in *caf* compared with *sap* embryos. Detached fibers in *sap* embryos show more rapid acridine orange staining, consistent with swift necrosis after sarcolemmal rupture.

These observations provide a mechanistic explanation for previous studies in the Lama2-deficient *dy/dy* mouse, which show lower EBD accumulation in the skeletal muscles than the *mdx* mouse (34) and, furthermore, that down-regulation of the proapoptosis factor Bax or up-regulation of the antiapoptosis protein Bcl2, results in amelioration of the dystrophic phenotype (35, 36). Importantly, the same treatment appears to produce no effect in the *mdx* mouse. It can be envisaged that, in the case of fiber retraction without loss of membrane integrity, a retracted cell would remain in a viable but afunctional state and would undergo subsequent apoptosis. Amelioration of the Lama2-deficient phenotype might then occur either by tempering the inflammatory response, which is known to exacerbate dystrophic pathology (37), or by fiber reattachment during subsequent tissue growth. However, our model suggests that muscle atrophy is driven primarily by the absence of functional connections with the ECM rather than by apoptosis itself. The understanding of such mechanisms will likely implicate new targets for gene therapy and for therapeutic drugs.

Zebrafish models of disease are particularly valuable because they develop *ex utero* and are optically clear, highly manipulable, and genetically tractable. We show direct visual observations of the pathology of muscular dystrophy by using time-lapse photomicroscopy in a manner hitherto impossible in other animal models of muscle disease. This makes possible a number of avenues of investigation. Until now, the only zebrafish model of muscular dystrophy has been the *sap* model of DMD, which is progressive and homozygous lethal. Consistent with human MDC1A, we have shown that *caf* zebrafish, despite an initially more severe phenotype, can reach adulthood in the homozygous

state. This presents opportunity for the study of later phenotypes such as CNS and peripheral nerve effects (38) and cardiac involvement, which has been implicated in MDC1A (39).

Materials and Methods

Bulked segregant analysis and mapping were performed according to current protocols (24). Antibody staining was performed as described (28), with the exception of anti-laminin 1, for which methanol fixation was used in place of 4% paraformaldehyde in PBS. Primary antibodies used were anti-dystrophin MANDRA (Sigma, St. Louis, MO), anti- β dystroglycan 7D11 (Developmental Studies Hybridoma Bank, Iowa City, IA), anti-laminin 1 (Sigma). Alexa-labeled secondary antibodies were diluted 1:200. *In situ* hybridization was performed as described, as was the use of TRITC-conjugated bungarotoxin (23). Evans blue (23), acridine orange (30), and morpholino injections (28) were performed as described. For electron micrographs, we used a Philips (Eindhoven, The Netherlands) EM410 transmission electron microscope. Samples were fixed in 2.5% glutaraldehyde and embedded in Spurr's resin. For production of transgenic lines (40), a 5.5-kb region upstream of the *cmr* promoter was amplified by PCR, fused to a GAL4-UAS-EGFP reporter, and placed into the pT2XIG Δ in plasmid. Additional details are provided in *SI Materials and Methods*.

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