Epistatic dissection of laminin–receptor interactions in dystrophic zebrafish muscle

Tamar E. Sztal1,2, Carmen Sonntag1, Thomas E. Hall1,*,†,‡ and Peter D. Currie1,*,‡

1Australian Regenerative Medicine Institute, Monash University, Level 1, Building 75, Clayton, VIC 3800, Australia
2School of Biological Sciences, Monash University, Building 17, Clayton, VIC 3800, Australia

Received May 10, 2012; Revised July 5, 2012; Accepted July 24, 2012

Laminins form essential components of the basement membrane and are integral to forming and maintaining muscle integrity. Mutations in the human Laminin-alpha2 (LAMA2) gene result in the most common form of congenital muscular dystrophy, MDC1A. We have previously identified a zebrafish model of MDC1A called candyfloss (caf), carrying a loss-of-function mutation in the zebrafish lama2 gene. In the skeletal muscle, laminins connect the muscle cell to the extracellular matrix (ECM) by binding either dystroglycan or integrins at the cell membrane. Through epistasis experiments, we have established that both adhesion systems individually contribute to the maintenance of fibre adhesions and exhibit muscle detachment phenotypes. However, larval zebrafish in which both adhesion systems are simultaneously genetically inactivated possess a catastrophic failure of muscle attachment that is far greater than a simple addition of individual phenotypes would predict. We provide evidence that this is due to other crucial laminins present in addition to Lama2, which aid muscle cell attachments and integrity. We have found that lama1 is important for maintaining attachments, whereas lama4 is localized and up-regulated in damaged fibres, which appears to contribute to fibre survival. Importantly, our results show that endogenous secretion of laminins from the surrounding tissues has the potential to reinforce fibre attachments and strengthen laminin–ECM attachments. Collectively these findings provide a better understanding of the cellular pathology of MDC1A and help in designing effective therapies.

INTRODUCTION

Congenital muscular dystrophies (CMDs) are a heterogeneous group of inherited muscle disorders, causing severe muscle degeneration, fibrosis and muscle cell death. The incidence of CMDs has been estimated to be ~1 in 21 500, with mutations in Laminin-alpha2 (LAMA2) accounting for nearly half of these cases in European countries (1). Causative genetic changes in a number of loci have been associated with the CMDs. Strikingly, to date, almost all produce mutations in proteins which function at the interface between the muscle cell membrane and the extracellular matrix (ECM) (2) (http://www.dmd.nl/).

Laminins are heterotrimeric complexes forming major structural components of the ECM (3,4). Mammals possess at least 16 laminin complexes formed through different combinations of alpha (a), beta (b) and gamma (c) subunits, with additional trimers generated from alternative mRNA splicing of particular laminin isoforms (5–7). Mutations in different laminins result in a variety of human diseases, owing to their varied temporal and spatial distribution. Mutations in Lama3, Lamb3 and Lamc2 result in junctional or general epidermolysis (8,9), whereas mutations in Lama4 have been associated with human cardiomyopathy (10).

Laminins interact via two specific adhesion systems at the muscle cell membrane, both of which have been implicated in human muscle disease. Laminins interact through both integrins and the dystrophin-associated glycoprotein complex (DGC) and form an integral component of the basement membrane, serving as an anchor between the muscle cell membrane and the surrounding ECM. Dystroglycan (Dag1), consisting of an α and a β subunit, forms the integral membrane component

*To whom correspondence should be addressed. Tel: +61 73346 2100; Fax: +61 7 3346 2101; Email: t.hall5@uq.edu.au (T.E.H);
Tel: +61 399029602; Fax: +61 399029729; Email: peter.currie@monash.edu (P.D.C.)
†Contributed equally.
‡Present address: Institute for Molecular Bioscience, The University of Queensland, 306 Carmody Road, St Lucia 4067, Australia.

© The Author 2012. Published by Oxford University Press. All rights reserved.
For Permissions, please email: journals.permissions@oup.com
of the DGC in the muscle. The C-terminal cytoplasmic tail of β-dystroglycan (residing in the cell membrane) interacts with Dystrophin, which in turn binds to the actin cytoskeleton and α-dystroglycan acts as a receptor for several extracellular ligands, including laminins (11,12). Mutations in dystrophin result in the most prevalent form of muscular dystrophy, Duchenne muscular dystrophy (DMD) (13,14). Furthermore, several patients have been reported with missense mutations in α-dystroglycan (commonly referred to as dystroglycanopathies) leading to reduced laminin-2 binding (15). Dag1−/− null mice are early embryonic lethal and to date no human carriers of a null mutation have been identified (16).

In addition to the DGC, a second integrin-mediated mechanism of laminin dependant attachment is also thought to operate in muscle cells. Integrins bind their ligands as heterodimers, consisting of a combination of 1 of 18 alpha and 1 of 8 beta chains (17). Congenital MDs in humans have been associated with mutations in integrin alpha7 beta1 (itga7β1), the main integrin found in muscle cells (18). Analysis of the skeletal muscle of itga7−−/− null mice showed typical symptoms of progressive MD with a specific impairment of function at the myotendinous junction (19,20).

In recent years, a number of the mutations disrupting proteins involved in muscle–ECM attachments have been individually modelled in zebrafish. We have previously shown that the zebrafish orthologue of Lama2 is the most highly expressed laminin in the skeletal muscle throughout zebrafish development (21). Zebrafish lama2−/− mutants [synonym; candycross (caf)] show normal differentiation of muscle fibres, but by 3 days post-fertilization (dpf) fibres detach, retract and eventually die (22). Mutations in the zebrafish dystrophin orthologue have been identified in the dmd−−/− zebrafish mutant [synonym; sapje (sap)] causing severe muscle degeneration, reminiscent of the human DMD pathology (23,24). A similar phenotype was reported in zebrafish dystroglycan (dag1−−/−) mutants resulting in fibre detachment caused by a disruption in sarcolemma integrity (25,26). Furthermore, in zebrafish loss of either itga7 or integrin-linked kinase (ilk), an essential downstream mediator of integrin signalling results in fibre detachment, suggesting that integrins are also essential for the maintenance of muscle attachments (27). These numerous studies have shown that both DGC- and integrin-mediated attachments are critical; however, the relative contribution of each adhesion complex in normal and diseased muscle states remains an area of considerable debate (28−30).

Here, we examine these questions through the systematic genetic analysis of zebrafish mutants that specifically affect muscle–ECM interactions. Through a series of epistatic experiments, we demonstrate the relative contribution of both the DGC and integrins to the maintenance of physical adhesions in the myotome. We further show that although Lama2 is a constituent of the main laminin complex, acting through both the DGC and integrins, Lama1- and Lama4-mediated interactions are important for muscle–ECM interactions in a Lama2-deficient context at different points in disease progression. Lama1 is important to strengthen early muscle–ECM attachments, whereas Lama4 appears to be a survival factor modulating muscle integrity through integrin signalling. Furthermore, we show that the delivery of exogenous laminin secreted from different non-muscle tissues can localize to zones of muscle attachment and is able to, in part, ameliorate the phenotype of Lama2-deficient zebrafish. We believe that these results reinforce the mechanistic validity for therapeutic interventions utilizing laminin-based therapeutics for the treatment of CMD.

RESULTS

DGC- and integrin-mediated adhesion both act to regulate laminin-mediated muscle attachment in the zebrafish myotome

To determine the relative role of the DGC and integrin signalling in laminin-mediated attachment to the ECM, we examined the contribution of each adhesion system in both laminin abundant and laminin-deficient environments. Since Lama2 trimer is postulated to be the main laminins contributing to muscle cell–ECM attachments, we hypothesized that no further decrease in attachments in lama2−/− mutants should be observed in the absence of various ECM components. We have analysed the level of muscle fibre detachment in a number of zebrafish mutants, which contain mutations in various adhesion system components (Fig. 1A and Table 1). Previous analyses have revealed that all the identified mutants demonstrate some level of fibre detachment, although they show normal muscle development and fibre elongation (22,27,31–33). We have previously shown that by 3 dpf, lama2−/− mutants possessed severe lesions in the myotome caused by the detachment of muscle fibres from the myosepta (22). Dystrophic fibre attachment phenotypes have also been reported in both dmd−−/− mutants and dag1 morphants (31,33). Similarly, ilk [synonym; loss of contact (loc)] mutants are characterized by consistent skeletal muscle fibre detachments along the trunk musculature. Unlike other classes of dystrophic mutants, ilk−−/− embryos initially exhibit normal swimming behaviour, but by 4.5 dpf, they become paralysed and develop severe cardiac oedema (27).

To determine the importance of each ECM component in mediating fibre attachments, we intercrossed the zebrafish dystrophic mutants and analysed the muscle integrity of all single and double mutant combinations during early zebrafish development. We chose to analyse the muscle both at 3 dpf, when fibre detachment is induced in dystrophic zebrafish models, and at 5 dpf, when the damage to the myotome appears to be irreversible, to clearly evaluate the progressive nature of the detachment.

We quantified the level of muscle damage observed in zebrafish mutants by analysing the level of birefringence (under polarized light) of the fully striated and elongated myotome. This technique has previously been used to assay myofibre disruption in dystrophic mutants (22,34–36). In wild-type siblings, the trunk muscle appears as a parallel fibrillar array of fibres (Fig. 1B). Thus, the birefringence of wild-type fish at each stage (in each genetic background) was used as a benchmark to which all other values were normalized. Of the single mutants analysed, lama2−−/− mutants showed the largest loss of birefringence and hence possessed the greatest level of muscle damage at both 3 and 5 dpf (Fig. 1C). ilk−−/−, dmd−−/− and dag1−−/− mutants all showed a reduced loss of
birefringence (Fig. 1B and C). As observed in the single mutants, the myotomes formed normally in all double mutant combinations, prior to their degeneration. Of the double mutant lines analysed, the \( \text{ilk}^{-/-} \)/\( \text{dag1}^{-/-} \) and \( \text{ilk}^{-/-} \)/\( \text{dmd}^{-/-} \) double mutants showed the lowest level of birefringence, despite each of the single mutants displaying only a mild dystrophic phenotype (Fig. 1B and C). This suggests that both integrin- and DGC-mediated mechanisms are essential for muscle attachment and that both adhesion complexes act redundantly to maintain the mechanical stability of fibres. To test the validity of this approach, we also measured the birefringence of the \( \text{dmd}^{-/-} \)/\( \text{dag1}^{-/-} \) mutant combination, which both form part of the DGC attachment complex. The birefringence measured for the \( \text{dmd}^{-/-} \)/\( \text{dag1}^{-/-} \) double mutants was equivalent to the \( \text{dmd}^{-/-} \) mutants alone, confirming the veracity of our experimental approach (Fig. 1C).

In parallel to this analysis, we performed immunohistochemistry using antibodies against slow and fast myosin heavy chains to examine the extent of fibre detachment in single and double mutant combinations. As shown in Figure 2, wild-type siblings show an even array of fast and slow muscle fibres attached at each vertical myoseptum at 3 dpf (Fig. 2A) and 5 dpf (Fig. 2A'). In \( \text{lama2}^{-/-} \) mutants, there is severe detachment of both fast and slow fibres by 3 dpf (Fig. 2B), increasing in severity at 5 dpf (Fig. 2B'). As shown previously (22), the fibre detachment occurs stochastically and does not affect all somites equally. In comparison to \( \text{lama2}^{-/-} \) mutants, \( \text{dmd}^{-/-} \) (Fig. 2C and C'), \( \text{ilk}^{-/-} \) (Fig. 2D and D') and \( \text{dag1}^{-/-} \) (Fig. 2E and E') homozygous mutants show a much reduced level of muscle detachment. Although the extent of fibre detachment in these mutants also appears to become progressively worse by 5 dpf, it is still far less severe than is seen in \( \text{lama2} \) mutants (Fig. 2B and B'). In contrast to all single mutants, all double mutant combinations show a greater level of detachment of fast and slow fibres by 3 dpf (Fig. 2F–J), which again becomes more severe by
Table 1. Single and double mutant combinations analysed

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Genes affected</th>
<th>Pathway affected</th>
</tr>
</thead>
<tbody>
<tr>
<td>lama2</td>
<td>lama2</td>
<td>DGC and integrin</td>
</tr>
<tr>
<td>ilk</td>
<td>ILK</td>
<td>Integrin</td>
</tr>
<tr>
<td>dmd</td>
<td>dystrophin</td>
<td>DGC and integrin</td>
</tr>
<tr>
<td>dag1</td>
<td>dystroglycan</td>
<td>DGC and integrin</td>
</tr>
<tr>
<td>lama2/dmd</td>
<td>lama2/dystrophin</td>
<td>DGC and integrin</td>
</tr>
<tr>
<td>lama2/dag1</td>
<td>lama2/dystroglycan</td>
<td>DGC and integrin</td>
</tr>
<tr>
<td>lama2/ilk</td>
<td>lama2/ILK</td>
<td>DGC and integrin</td>
</tr>
<tr>
<td>dmd/ilk</td>
<td>dystrophin/ILK</td>
<td>DGC and integrin</td>
</tr>
<tr>
<td>dag1/ilk</td>
<td>dystroglycan/ILK</td>
<td>DGC and integrin</td>
</tr>
<tr>
<td>dmd/dag1</td>
<td>dystrophin/dystroglycan</td>
<td>DGC</td>
</tr>
</tbody>
</table>

5 dpf (Fig. 2F–J). Of the double mutant combinations, the ilk/dmd<sup>−/−</sup> (Fig. 2I) and ilk/dag1<sup>−/−</sup> (Fig. 2J) mutants show the greatest level of detachment, with the majority of fast and slow fibres completely detached by 5 dpf (Figs 1C and 2I and 2J).

Distinct roles for lama1 and lama4 in muscle attachment and survival in the zebrafish embryo

 Lama2 is clearly important for the integrity of muscle attachments since lama2 mutants measured the lowest level of birefringence compared with all other single muscle detachment mutants tested (Fig. 1C). However, the level of birefringence of the lama2/dmd<sup>−/−</sup>, lama2/ilk<sup>−/−</sup> and lama2/dag1<sup>−/−</sup> mutant combinations was significantly reduced compared with the lama2<sup>−/−</sup> single mutants alone (Fig. 1C). This suggests that other laminins must be involved in ECM adhesion.

We have previously shown that lama1, lama2 and lama4 are the only laminin alpha chains expressed in the embryonic and larval zebrafish myotomes (21), suggesting that Lama1 and Lama4 may also be involved in early muscle attachments. Lama2 is highly expressed throughout the fast and slow muscle, which is reflective of the severe dystrophic phenotype exhibited by lama2 mutants. Lama1 is expressed transiently in the early somites prior to 24 hpf, but after this time is detectable only in the notochord and tail bud, consistent with the observed defects noted in notochord differentiation in the lama1<sup>−/−</sup> mutant (21,32). Pollard et al. (32) previously demonstrated that morpholino knockdown of lama4 resulted in embryos with a mild brain defect and normal trunk appearance. It has been shown that Laminin111, Laminin 411 and Laminin 421 are incorporated into the basement membrane of muscle fibres (37), suggesting that lama1 and lama4 can also contribute to adhesion. However, their roles have not been comprehensively analysed during zebrafish muscle development.

To determine whether lama1 played a role in early muscle attachment, we injected a morpholino targeted against lama1 into both lama2<sup>−/−</sup> mutants and their wild-type siblings. The morpholino phenotype clearly recapitulated the notochord and central nervous system defects described previously (38) (Fig. 3A). The level of detachment was quantified by measuring the birefringence at 3 and 5 dpf. A significant decrease in birefringence was seen in lama1 morpholino-injected lama2<sup>−/−</sup> embryos compared with uninjected lama2<sup>−/−</sup> mutants (Fig. 5B), suggesting that lama1 plays a role in early muscle attachment. When we examined muscle fibre integrity directly, by injecting the lama1 morpholino (MO) into the lama2<sup>−/−</sup>; slow-myosin heavy chain-green fluorescent protein (GFP) line, we found a significant increase in the number of detached fibres at 3 dpf compared with uninjected lama2<sup>−/−</sup>; slow-myosin heavy chain-GFP fish (Fig. 3C). A significant increase in the number of detached fibres was also observed when the lama1 MO was injected into wild-type sibling controls (Fig. 3C), but these were localized adjacent to the dysmorphic sections of anterior notochord evident in these mutants which are likely to affect fibre integrity in adjacent myotomes. However, it is possible that loss of Lama1 directly affects fibre attachment in wild-type siblings, although this remains questionable given the nature of the localized fibre disruption. Previous studies in zebrafish have shown that loss of either Lama1 or Lama2 alone does not abolish laminin immunoreactivity in the zebrafish muscle (22,32). However, we found that simultaneous loss of Lama1 and Lama2 results in a complete loss of laminin immunoreactivity within the myotome, suggesting that both Lama1 and Lama2 contribute to the complement of laminins in the embryonic myotome and collectively constitute the major muscle isoforms of laminin (Fig. 3D).

We next examined the role of Lama4 during muscle attachment via injection of an oligonucleotide antisense morpholino (32) against lama4. To verify the efficacy of the knockdown considering the mild muscle phenotype observed in Figure 4B, we synthesized control RNA for a fluorescent reporter; the 5′ sequence of which had been modified to contain the transcriptional start site for the endogenous lama4 open reading frame. When this control RNA was injected in the absence of the lama4 MO, fluorescence was detected in morpholino-injected larvae until 6 dpf (Fig. 4A). Co-injection of the control RNA together with the lama4 MO resulted in no fluorescence, indicative of effective blocking of the lama4 transcription initiation sequence (Fig. 4A). Knockdown of lama4 did not show any additional decrease in birefringence in wild-type siblings or lama2<sup>−/−</sup> embryos at both 3 and 5 dpf (Fig. 4C). We also found no significant difference in the number of detached slow muscle fibres between lama4 morpholino-injected lama2<sup>−/−</sup> mutants compared with uninjected lama2<sup>−/−</sup> mutants (Fig. 4D), nor did knockdown of lama4 decrease Laminin immunoreactivity within the myotome (Fig. 3D). These results suggest that Lama4 heterotrimeric complexes play little role in generating initial fibre attachments in the zebrafish embryo.

To examine this question more closely, we directly compared the ability of Lama4 and Lama2 to localize to the sites of fibre attachment and rescue the pathology of the lama2-deficient myotome. In order to achieve this, the open reading frames for lama2 and lama4 were fused with mCherry and placed under the control of the muscle-specific acta1 (skeletal muscle alpha-actin) promoter, in both wild-type siblings and lama2<sup>−/−</sup> mutants. When lama2 was mosaically overexpressed from individual muscle fibres, it was efficiently secreted into the myotome and localized specifically to the myosepta (Fig. 5A). The expression of even a low level of mosaicism was sufficient to significantly increase
the level of birefringence in \textit{lama2}^{-/-} mutants at 3 and 5 dpf (Fig. 5C) and also significantly reduce the number of detached muscle fibres in \textit{lama2}^{-/-} mutants compared with control myotomes (where \textit{lama2} is not overexpressed) (Fig. 5B). However, when Lama4 was overexpressed from within muscle fibres in the identical manner, it was secreted into the myotome but localized to small cells between the fibres and along the myosepta (Fig. 5A), a pattern reminiscent of the \textit{lama4} mRNA expression pattern observed previously (Fig. 4G and G′) (21). Overexpression of \textit{lama4} did not significantly alter the level of birefringence (Fig. 5C) or the number of detached muscle fibres in \textit{lama2}^{-/-} embryos compared with controls (Fig. 5B). Given that overexpression of \textit{lama2} can effectively rescue the \textit{lama2}^{-/-} dystrophic phenotype [similar C-terminal yellow fluorescent protein fusions to \textit{lama3} were shown to be effectively secreted and function appropriately in human keratinocytes (39)] our results therefore suggest that Lama4 does not appear to play any role in promoting fibre attachment in either the wild-type or \textit{lama2}^{-/-} contexts.

Laminin deposition at the somite boundaries is closely followed by ILK recruitment from the cytoplasm to the myotendinous junction (MTJ), and injection of mRNA encoding the mCherry–ILK fusion protein results in its deposition at the MTJ (27). Thus, the deposition of mCherry–ILK protein at the MTJ can be used as readout of the level of integrin signalling activated at the MTJ. Upon injection of the mCherry–ILK fusion construct into \textit{lama2}^{-/-} mutants, we observed ILK expression localized appropriately to the myosepta, and at the end of retracted detached fibres, indicating that loss of \textit{lama2} on its own does not dramatically alter the levels of integrin-mediated signalling within the MTJ environment (Fig. 4E). However, in \textit{lama4} morpholino-injected \textit{lama2}^{-/-} mutants, ILK localization is severely reduced within the myosepta, but not from the ends of detached fibres (Fig. 4E). Given that ILK also requires the presence of both Lama4 and Itga7 at the sarcolemma for strengthening of muscle attachments (27), we analysed the expression of \textit{lama4} and \textit{itga7} by \textit{in situ} hybridization in \textit{lama2} mutants. \textit{Itga7} was up-regulated specifically within the detached fibres of \textit{lama2}^{-/-} mutants, and reduced in muscle fibres that had not undergone detachment (Fig. 4F), and thus overall \textit{itga7} RNA levels appeared unchanged (Fig. 4H). This suggests that \textit{itga7} is down-regulated in \textit{lama2}^{-/-} mutant muscle prior to detachment, as is the case in MDC1A patients (40) and up-regulated specifically within detached fibres. In contrast, \textit{lama4} was up-regulated in \textit{lama2}^{-/-} mutants both by quantitative real-time (RT) polymerase chain reaction (PCR) at 3 dpf (Fig. 4H), and by \textit{in situ} hybridization experiments which also showed that this upregulation specifically occurred within detached fibres of

Figure 2. Confocal images of single and double mutant combinations stained with antibodies directed against slow myosin heavy chain (A–J) and fast/slow myosin heavy chain (A′–F′) at 3 and 5 dpf.
Itga6 was previously shown to be up-regulated in regenerating muscle of dy/dy mice (41); however, itga6a and itga6b expression was unchanged in \( \text{lama2}^{-/-} \) zebrafish embryos (Fig. 4F). Collectively, these results suggest that Lama4 may act through an Itga7 integrin-mediated signalling mechanism to modify detached fibres.

We have previously reported that cell death within detached fibres in \( \text{lama2}^{-/-} \) larvae is delayed in comparison to fibres within homozygous \( \text{dmd}^{-/-} \) mutants, suggesting that inhibition of apoptosis may be an important mechanism for increasing muscle integrity and fibre survival in \( \text{lama2}^{-/-} \) mutants (22). These observations have suggested that specific survival pathways may be activated within detached fibres to facilitate their survival. Integrin signalling is well placed to stimulate fibre survival, and the up-regulation of Lama4 specifically within detached fibres of \( \text{lama2}^{-/-} \) mutants suggested that it could play a role in this process. In order to examine this possibility, we determined whether knockdown of Lama4 increased the number of apoptotic muscle cells in \( \text{lama2}^{-/-} \) mutants. Our analysis determined that a reduction in Lama4 activity indeed increased apoptosis in the detached fibres of \( \text{lama2}^{-/-} \) mutants at 5 dpf (Fig. 4I and J), revealing that the up-regulation of Lama4 within these fibres contributes to fibre survival and remodelling. Collectively, these observations suggest that stimulation of integrin activity may well have therapeutic benefit in the treatment of CMD specifically in promoting fibre survival rather than attachment.

**Laminin can be secreted into the myotome from other tissues to aid muscle attachment**

The knockdown of \( \text{lama1} \) suggests that it plays an important role in muscle attachment during embryogenesis. However, \( \text{lama1} \) is only expressed transiently in the muscle precursors prior to 24 h post-fertilization (hpf) and is largely restricted to the notochord during the post-embryonic and larval development (21). Furthermore, we have shown that overexpression of Lama2 in muscle fibres allows it to translocate freely into the myotome to sufficiently rescue muscle detachment in \( \text{lama2}^{-/-} \) mutants, suggesting that laminin could be provided in a non-cell autonomous fashion to modulate the \( \text{lama2} \)
loss-of-function phenotype. We therefore wished to determine the source of laminin that is provided to the myotome during early zebrafish larval development and to determine whether laminin could be secreted from non-muscle sources to aid muscle attachments during these stages.

In order to examine this question, we mosaically overexpressed lama2 in wild-type embryos under the control of either the ubiquitous \( \beta \)-actin (\( \beta \)act2) promoter (42,43) or the tissue-specific \( \text{tw} \text{hh} \) (tiggy-winkle hedgehog) promoter (44), which drives expression specifically in the notochord and floor plate cells. Using the \( \beta \)act2 promoter, we were able to select mosaic clones expressing lama2 from a variety of
different cell types including dermal (Fig. 6A and A') and notochord cells (Fig. 6C and C'). Expression within these clones led to Lama2 secretion into the muscle where it consequently localized to the myosepta (Fig. 6A–D), suggesting that this ectopic expression is sufficient to contribute to muscle attachments.

We next examined mosaic notochord clones expressing lama2 under the control of the twhh promoter in both wild-type and lama2−/− embryos. In these animals, we observed that the Lama2 secreted from notochord clones entered the myotome and deposited laminin fusion protein at the myosepta (Fig. 6E). Furthermore, in lama2−/− mutants, Lama2 secretion from the notochord into adjacent myotomes resulted in significantly decreased number of damaged and retracted fibres compared with lama2−/− embryos injected with the control construct (Fig. 6E), suggesting that this may be an important and therapeutically relevant source of laminin in the developing myotome.

**DISCUSSION**

**Integrins and the DGC are both necessary for laminin-mediated attachments**

Previous studies have demonstrated that both integrin and DGC complexes are critical for maintaining muscle attachments. We now provide a systematic genetic evaluation of their individual and compound contributions to muscle integrity in zebrafish. We have made use of the tractability of this model, and availability of zebrafish mutants in genes coding for essential muscle/ECM proteins, to quantitatively analyse the relative contribution of each adhesion system to muscle attachment. Through epistasis experiments, we have shown that while mutations that are specific to downstream components of either adhesion pathway produce relatively mild defects in muscle adhesion, double mutant combinations produce a very severe dystrophic pathology, which are not indicative...
of a simple additive phenotype. This analysis confirms that integrin- and DGC-mediated adhesions are both essential to stabilize muscle attachments in the zebrafish myotome, but neither are required for the formation of initial attachments. Specifically, the ilk/dmd\(^{-/-}\) and ilk/dag1\(^{-/-}\) double mutants showed near complete detachment of slow and fast fibres, suggesting that both mechanisms are essential for fibre adhesion. Reflecting the progressive pathology of many MDs, the dystrophy evident in these zebrafish increases with many larvae showing complete fibre detachment by 5 dpf. This synergistic relationship has also been revealed in double mutant mouse studies, where mice containing mutations in either dystrophin or dystroglycan and itga7, although indistinguishable from their littermates at birth, died soon after from a severe early onset of muscular dystrophy (30,45). Similarly, mice containing mutations in both itga7 and \(\gamma\)-sarcoglycan, which is intimately associated with the DGC, displayed a weakened cellular attachment to the ECM (28). In zebrafish, morpholino knockdown of dystroglycan in ilk\(^{-/-}\) mutants caused detachment of muscle fibres much earlier in embryogenesis, than previously observed in each of the single mutants (27). Indeed, our results also suggest that an important interplay exists between these two adhesion systems that is required to maintain muscle attachment in vivo.

Despite these data supporting the generalized concept of the redundant action of these two pathways, there are however intriguing differences in the pathology of the skeletal muscle when dystrophin, dystroglycan and integrins are individually mutated in zebrafish. In dmd\(^{-/-}\) mutants, detachment of both fast and slow muscle fibres was evident by 3 dpf, producing a 35% reduction in birefringence, with the birefringence decreasing as low as 50% by 5 dpf. In contrast, ilk\(^{-/-}\) and dag1\(^{-/-}\) mutants displayed a 10–20% loss of birefringence at 3 dpf, which was caused by the detachment of only a small number of fibres in one or two myotomes along the trunk. At 5 dpf, degeneration of fast and slow muscle fibres in dag1\(^{-/-}\) mutants was more evident, with the level of birefringence nearing that measured for lama2\(^{-/-}\) and dmd\(^{-/-}\) dystrophic mutants. However, by 5 dpf, dag1\(^{-/-}\) mutants possessed a greater level of muscle integrity than in ilk mutants, suggesting that there are other mechanisms that may compensate for the loss of integrin binding. There is evidence to suggest that integrin signalling is also modulated in the absence of proper DGC function. In DMD patients and mdx\(^{-/-}\) mice, both containing mutations in the dystrophin gene, expression of the itga7b1 receptor was increased specifically in skeletal muscle (40), suggesting a complicated synergism in muscle–ECM attachments. It is also important to acknowledge that there are other intracellular adhesion systems that mediate muscle cells attachments in independently of the DGC and integrins. Transmembrane receptor such as integrins can be localized to muscle attachment sites in the absence of laminins. Recently, MIBP, a muscle-specific \(\beta1\) integrin-binding protein (46), a splice variant of Nrk2 (Nicotinamide riboside kinase 2), was shown to mediate adhesions to Laminins (47). Nrk2b is required for normal Laminin polymerization at the MTJ, which can be rescued by exogenous NAD+ treatment (47).

**Figure 6.** Expression of lama2 in clones using either the (A–D) ubiquitous bact2 or (E) tissue-specific twhh promoter. (A–C) Secretion and localization of lama2 (red) to the myosepta from various cell types (green) including (A', overlap with bright field in A) dermal cells in wild-type siblings, (B', overlap with bright field in B) muscle fibres in wild-type siblings and (C', overlap with bright field C) notochord cells in lama2\(^{-/-}\) mutants. (D) Percentage of cells showing myoseptal localization of lama2 from expressing and secreting dermal, muscle and notochord cell wild-type sibling myotomes. (E) Quantification of the percentage of retracted fibres when lama2 expressed from notochord clones using the twhh promoter (twhh-lama2) compared with the control (twhh-control) in lama2\(^{-/-}\) mutants and wild-type sibling myotomes. Error bars represent ± SEM, *P < 0.05.

**Table 1.** Tissue type and % expressing lama2 and myoseptal localization.

<table>
<thead>
<tr>
<th>Tissue type</th>
<th># expressing lama2</th>
<th>% myoseptal localization</th>
</tr>
</thead>
<tbody>
<tr>
<td>dermal</td>
<td>15</td>
<td>67%</td>
</tr>
<tr>
<td>muscle</td>
<td>27</td>
<td>100%</td>
</tr>
<tr>
<td>notochord</td>
<td>9</td>
<td>78%</td>
</tr>
</tbody>
</table>

**Distinct roles for laminins in muscle attachment and maintenance in the zebrafish myotome**

Laminins are thought to be the primary basal lamina-binding proteins mediating both integrin- and DGC-mediated...
attachment during muscle development and disease (3,4). Mutations in zebrafish lama2, the major muscle isoform of laminin in all vertebrates so far studied, produce a 50% loss in birefringence resulting in a muscle pathology, more severe than the dmd<sup>−/−</sup>, ilk<sup>−/−</sup> or dag1<sup>−/−</sup> single mutants, suggesting that lama2 is essential for muscle attachment via both the integrin and DGC pathways. Despite this, the level of birefringence measured for lama2<sup>−/−</sup> mutants is still greater than the level measured for lama2/ilk<sup>−/−</sup>, lama2/dmd<sup>−/−</sup> or lama2/dag1<sup>−/−</sup> mutants, suggesting that there are additional laminins involved in attachment.

We have previously detected expression of lama1, lama2 and lama4 in muscle during zebrafish development (21). lama2 is most highly expressed in the developing skeletal muscle and is required to maintain attachments during muscle contractions. Our epistasis experiments indicated that lama1- and lama4-mediated interactions contribute to the relative lack of severity in the lama2-null context. Specifically, knockdown of lama1 in lama2<sup>−/−</sup> mutants abolished laminin immunoreactivity at the myosepta (Fig. 3D) and reduced the birefringence to a level comparable with the ilk/dag1<sup>−/−</sup> and ilk/dmd<sup>−/−</sup> double mutants (Figs 1C and 3B). Lamal has not been shown to play any significant role in the skeletal muscle until now as it is thought to be required for early somite differentiation and basement membrane assembly (48). However, reduced laminin immunoreactivity at the myosepta has been noted in bal mutants and lama1 morphants, suggesting that lama1 may be required at the somite boundaries (38,49). Furthermore, a number of studies have shown that Laminin111 intramuscular or systemic injection reduces muscular dystrophy and restores peripheral nerve defects in mouse models, suggesting that Laminin111 can compensate for the loss of Laminin211 (50–52). Interestingly, in vitro studies in myogenic cell lines found that both lama1 and lama2 possess similar myogenic properties when expressed in the skeletal muscle (53). Lama1 is most structurally similar to lama2 and, in addition to lama2, is the only other alpha chain that also binds dystroglycan and itg<sup>a7</sup> (54). Expression of lama1 in dy<sup>5k</sup>/dy<sup>5k</sup> mice restores itg<sup>a7b1</sup> expression at the sarcolemma suppressing pathology (55). Interestingly, a Lama1 chain devoid of the dystroglycan-binding site but retaining the integrin-binding domain was able to significantly increase the lifespan of dy<sup>5k</sup>/dy<sup>5k</sup> mice and partially rescue dystrophic muscles (56). Our results demonstrate a requirement for Lama1 in early muscle attachment, which may explain why it is able to compensate for lama2 in some contexts.

**Lama4 and integrin signalling promote fibre survival**

Our previous studies have determined that during early embryonic development, lama4 is expressed in the anterior somite at 24 hpf, but as development proceeds, lama4 becomes restricted to interstitial cells between the muscle fibres and to the cells at the apices of the myotome (21). In mammalian muscle, Laminin411 and Laminin421 are also incorporated in the basement membrane (37), suggesting that Lama4 in addition to Lama2 is required for muscle development and maintenance. Structurally, trimers containing Lama4 have a limited capacity for incorporation into a higher-order lattice (57), and thus, overexpression of lama4 alone in CMD is unlikely to entirely compensate for the loss of Lama2.

Strikingly, knockdown of lama4 caused a greater level of muscle specific apoptosis in the lama2<sup>−/−</sup> mutant background, suggesting that Lama4 protects against programmed cell death of muscle fibres in lama2 deficiency. How exactly Lama4 may provide this protective function is not yet known but studies in the mouse have shown that Lama4 is highly expressed in microvessels of the vasculature and is thought to be important for stabilizing blood vessels that form with injury and inflammation (58,59), which may be essential in the repair and regeneration of the dystrophic muscle. Furthermore, damaged fibres are able to reseal membranes though Dysferlin-mediated repair processes (60,61) and to a certain extent through fusion (62,63) which Lama4 may mediate. Alternatively, Lama4 may protect muscle from apoptosis through the prevention of ‘abortive regeneration’ of fibres, a process known to be active in lama2-deficient mice (64).

In either case, Lama4 likely acts through integrin-mediated signalling in the myotome as suggested (27). Previous studies have demonstrated that although DGC complexes seem to remain stable in dystrophic models (65,66), laminin and integrins are modulated in a more complex way. Therefore, stimulation of integrin signalling within dystrophic muscle presents as a strong candidate pathway for therapeutic intervention. Knockdown of lama4 caused a loss of Ilk at the MTJ, confirming that lama4 regulates integrin signalling at the somite boundaries (27). In lama2<sup>−/−</sup> mutants, lama4 along with itg<sup>a7</sup> and its downstream effector ilk were up-regulated specifically by damaged fibres (Fig. 4F). In dystrophic mouse models, lama4 along with itg<sup>a6</sup> and itg<sup>a7</sup> have been shown to be up-regulated specifically on detached fibres implying a role in regeneration and repair, in line with our observations (41,57). Collectively, our analyses suggest a model whereby Lama4 directly secreted from damaged fibres binds integrins to trigger signalling within detached muscle cell that prevents apoptosis in the Lama2-deficient muscle.

**Distinct sources of Laminin can contribute to fibre survival**

Mammalian cell culture experiments have shown that the alpha laminin monomers can be secreted alone, whereas secretion of beta and gamma chains requires simultaneous expression of all three chains and their assembly into heterotrimeres (67). However, the current model, which has recently been corroborated in Drosophila (68), is that a transitional dimeric configuration composed of a beta and a gamma chain is first assembled intracellularly before incorporation of an alpha chain allows secretion (69–72). Our results show that heterotrimeres comprising lama2 can be produced in multiple tissues of the body and secreted into the myotome to aid muscle formation and attachment. Three lines of evidence support this statement. First, lamal contributes to myotome stability and muscle attachment in the absence of lama2, despite only being expressed within the notochord at the time when these attachments are being formed. Secondly, Laminin secreted from notochord and skin cells can localize to the sites of muscle attachment deep within the myotome in wild-type embryos. Thirdly, lama2 expressed from a notochord-specific promoter is secreted...
into the myotome and can significantly decrease the amount of fibre detachment evident in lama2-/- mutants. These series of striking results indicate that direct intramuscular delivery of laminin is not necessarily required to correct the pathology evident in laminin2-deficient CMD. In support of this assumption, Rooney et al. (51) have recently shown that systemic injections of Laminin111 protein ameliorates the pathology evident in the dyw/dyw MDCIA mouse model. Thus, we believe that our results substantiate that a more generalized approach can be deployed in laminin replacement strategies for the treatment of CMD.

**MATERIALS AND METHODS**

**Zebrafish strains, maintenance and genotyping of mutant alleles**

Previously described zebrafish strains were lama2/cafreg15a (22), dmd/sap222a (31), dag1hu3072 (26), loc/ilhu801 (27), TgBAC_ZC227E6(smyhc1:EGFP)i108 (73) and acta1-EBF P2p5 (74). Zebrafish maintenance and embryo collection were carried out using established protocols (75). Embryos produced from an incross of cafreg15a, sap222a, dag1hu3072 or ilhu801 compound heterozygotes were genotyped using derived cleaved amplified polymorphic sequence (dCAPS) analysis (76) (Table 2).

**Production of transgenic constructs and acta1-h2afv-mCherry fish strain**

The acta1:lama2-mCherryT2A-EGFPcaax, acta1:lama4-mCherryT2A-EGFPcaax-pA, bact2:lama2-mCherryT2A-EGFPcaax-pA, twhh:lama2-mCherryT2A-EGFPcaax-pA, acta1:ilk-mCherry-pA and acta1-h2afv-mCherry constructs were created using the tol2kit (43). Additional entry clones used for the reactions were p5E-acta1 (GenBank accession number JN689239), p5E-twhh (JX261972), pME-ilk-NS (JX261971), pME-lama4-NS (JX217820), pME-lama2-NS (JN689241) and p3E-mCherry-T2A-EGFPcaax-pA (JN71 7246). p5E-acta1 has been previously described (35), p5E-twhh was subcloned from twhh-EGFP (44), pME-ilk-NS was subcloned from GFP-ilk (27), pME-lama2-NS was cloned using RACE (BD Clontech) followed by infusion cloning (BD Clontech), pME-lama4-NS was subcloned from IMAGE clone 9038736 (Imagene) and p3E-mCherry-T2A-EGFPcaax was cloned using Infusion (BD Clontech). The acta1-h2afv-mCherrypc6 strain was produced by microinjection of acta1-h2afv-mCherry and screening of founders according to standard procedures (77).

**Birefringence assays**

Birefringence assays are based on methods developed in Berger et al. (78). Three-day-old zebrafish larvae were sorted from each cross and placed on the Nikon Abrio Polaris imaging microscope stage in a glass bottom FluoroDish (World Precision Instruments, FL, USA). These were photographed using the automated fully rotating polarizers within the Abrio system and then raised in individual dishes until 5 dpf, at which time they were re-photographed and genotyped. The obtained birefringence pictures are saved as tiff files and subsequently analysed for their brightness values. The area of the 2nd to the 21st somite of a pictured larva is selected in the software ImageJ and the average grey value of the pixels in this area is measured, resulting in the mean grey value. For statistical analysis, a minimum of 15 larvae from each condition from independent breeding pairs were analysed for their muscle birefringence. Data are represented as means ± SEM. Genotyping assays are outlined in Table 2.

**Quantitative RT-PCR**

Total RNA was extracted using TRIzol reagent (Invitrogen Life Technologies). RNA samples were treated with RNase-free DNase (Promega). cDNA was synthesized from 1 μg of each RNA sample in a 20 μl reaction using SuperScript III Reverse Transcriptase (Invitrogen Life Technologies) and oligo(dT)20 primer following the supplier’s instructions. Quantitative PCR was performed as previously described using beta actin as a housekeeping gene (b-act-RT-F: GCATTGCTGACCGTATGCAG and b-act-RT-R: GATCCACATCTGCTGGAGGTGG). Primers for quantitative PCR of lama4 were lama4-RT-F: TCTGTCTGGTAATCAGCTGCT and lama4-RT-R: CGGTGATGTCCCTCACAG. Primers for quantitative PCR of itga7 were itga7-RT-F: TTCTCGTTGGCTCATA and itga7-RT-R: CGAACACAGAGTTTG.

**Injections and morpholinos**

A lama1 (32) or lama4 morpholin (32) antisense oligonucleotides (MOs) (Gene Tools, Philomath, OR, USA) were diluted in distilled water and injected into one- to four-cell embryos.

<table>
<thead>
<tr>
<th>Table 2. Genotyping of single and double mutant combinations analysed</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mutant</strong></td>
</tr>
<tr>
<td>---------------------</td>
</tr>
<tr>
<td>cafreg15a</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>sap222a</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>dag1hu3072</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>ilkhu801</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Genes</strong></th>
<th><strong>Alleles</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>acta1</td>
<td>mutants</td>
</tr>
<tr>
<td>lama1</td>
<td>mutants</td>
</tr>
<tr>
<td>lama2</td>
<td>mutations</td>
</tr>
<tr>
<td>lama4</td>
<td>mutations</td>
</tr>
<tr>
<td>itga7</td>
<td>mutations</td>
</tr>
</tbody>
</table>

*Note: Specific primers and enzymes used for genotyping and analysis.*
Immunohistochemistry and in situ hybridization

Whole-mount immunohistochemistry was performed by standard procedures (79). Primary antibodies were used at the following dilutions: antimyosin heavy chain, slow isoform 1:10 (F59; DSHB, Iowa City, IA, USA), antimyosin heavy chain 1:10 (1025; DSHB) and anti-laminin 1:100 (L9393; Sigma). Alexa-fluor-488- and Alexa-fluor-568-conjugated fluorescent secondary antibodies (Invitrogen, Carlsbad, CA, USA) were diluted 1:500. Whole-mount secondary antibodies (Invitrogen, Carlsbad, CA, USA) were Alexa-fluor-488- and Alexa-fluor-568-conjugated fluorescent antibodies (Invitrogen, Carlsbad, CA, USA) were diluted 1:500. Whole-mount in situ hybridization was carried out as described previously (80). The lama1 (21), lama2 (22), lama4 (21), itga6b (F: AAGCCAGGGCCTTTACCAAAT, R: CCGAACATGGAGTCTTTGGT), itga6b and (F: TTCAG GAACTGCGTGACTTTG, R: ATCCATGTTCCCAGCAAG AG) itga7 (27) riboprobes were synthesized from a mixed-stage zebrafish larval cDNA.

Muscle-specific terminal deoxynucleotidyl transferase dUTP nick end labeling assay

The lama4 morpholino was injected into embryos produced from a lama2+/−; actal-h2afv-mCherrype6 heterozygous incross. These embryos were sorted for lama2+/− mutants and wild-type siblings by birefringence at 3 dpf and for those that showed mCherry expression throughout all skeletal muscle nuclei. Embryos were processed using an In situ Cell Death Kit (Roche), with a Alexa-fluor-488 secondary (Invitrogen) and imaged using a Zeiss LSM-710 confocal.

ACKNOWLEDGEMENTS

We would like to thank Jeroen Bakkers (Hubrecht Institute) for the Ilkkn401 strain and plasmid, Hans Georg Frohnhoefler (Max Plank Institute, Tubingen) for the lama2meg15a and dmdm222u strains, Derek Stemple (Sanger Centre) for the dag1m372 strain, Phil Ingham (A-Star Institute of Molecular and Cell Biology) for the Tg(BAC ZC227 E6(smyh- c1:EGFP) strains, Jin Du (University of Maryland) for the tvdh strain and Kristen Kwan and Chi-Bin Chien (University of Utah) for the Tol2 kit plasmids.

Conflict of Interest statement. None declared.

FUNDING

This work was supported by a National Health and Medical Research Council Project grant (#573709) to P.D.C. and T.E.H. The Australian Regenerative Medicine Institute is supported by grants from the State Government of Victoria and the Australian Government.

REFERENCES


Native chick laminin-4 containing the beta 2 chain (s-laminin) promotes behavior of the zebrafish embryo and larva. _Development_, 123, 399–413.


