

β 1 Integrins Regulate Myoblast Fusion and Sarcomere Assembly

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Summary

The mechanisms that regulate the formation of multinucleated muscle fibers from mononucleated myoblasts are not well understood. We show here that extracellular matrix (ECM) receptors of the β 1 integrin family regulate myoblast fusion. β 1-deficient myoblasts adhere to each other, but plasma membrane breakdown is defective. The integrin-associated tetraspanin CD9 that regulates cell fusion is no longer expressed at the cell surface of β 1-deficient myoblasts, suggesting that β 1 integrins regulate the formation of a protein complex important for fusion. Subsequent to fusion, β 1 integrins are required for the assembly of sarcomeres. Other ECM receptors such as the dystrophin glycoprotein complex are still expressed but cannot compensate for the loss of β 1 integrins, providing evidence that different ECM receptors have nonredundant functions in skeletal muscle fibers.

Introduction

During skeletal muscle development, myoblasts fuse to form syncytial skeletal muscle fibers. Little is known about the molecular mechanisms that regulate myoblast fusion and the subsequent differentiation events that lead to the formation of mature muscle fibers. The formation of muscle fibers can be divided temporally into a series of steps such as acquisition of fusion competence of myoblasts, initiation of myoblast-myoblast adhesion, membrane alignment, and formation of prefusion com-

plexes with electron-dense vesicles at cell-cell contact sites, followed by plasma membrane breakdown and assembly of the muscle fiber cytoskeleton (Doberstein et al., 1997; Ervasti, 2003).

Several cell surface receptors have been implicated in regulating muscle fiber formation in vertebrates, largely by function-blocking studies carried out with cells in culture. Adhesion molecules such as cadherins, the Ig superfamily members N-CAM and VCAM, the tetraspanins CD9 and CD81, and ADAMs, as well as ECM receptors, may regulate myoblast fusion (Menko and Boettiger, 1987; Dickson et al., 1990; Knudsen et al., 1990a, 1990b; Mege et al., 1992; Rosen et al., 1992; Yagami-Hiromasa et al., 1995; Zeschnigk et al., 1995; Sastry et al., 1996; Tachibana and Hemler, 1999). However, genetic tests have not confirmed an essential role for many of these molecules in myoblast fusion in vivo. The analysis of the role of ECM receptors in skeletal muscle has been particularly complex in this regard, as muscle fibers express several ECM receptors that may have redundant functions. These include the dystrophin glycoprotein complex (DGC) and members of the integrin family. The DGC is a protein complex that consists of the peripheral membrane protein α -dystroglycan; transmembrane proteins including β -dystroglycan and sarcoglycans; and cytoplasmic proteins such as dystrophin and syntrophins. Treatment of primary muscle cultures with antibodies that block α -dystroglycan interactions with laminin causes muscle fiber defects (Brown et al., 1999), but ES cells that lack dystroglycan form myotubes (Jacobson et al., 2001). This suggests that dystroglycan is not essential for myotube development, or that other receptors can compensate for its loss. However, the DGC is essential for muscle fiber maintenance. Accordingly, muscle fibers degenerate when genes encoding components of the DGC are mutated, leading to muscular dystrophy (Durbeej et al., 1998; Henry and Campbell, 1999; Parsons et al., 2002).

Integrins consist of α and β subunits and serve as receptors for many ECM ligands (Hemler, 1999). Vertebrate skeletal muscle fibers express many integrin subunits in developmentally regulated patterns, including the integrin β 1 subunit and its partners α 1, α 3, α 4, α 5, α 6, α 7, and α v (Gullberg et al., 1998). Genetic studies in mice have shown that α 7 β 1 is required to maintain myotendinous junctions (MTJs) (Mayer et al., 1997). Mutations that inactivate other integrins have been less informative. Mice that lack the β 1, α 4, and α 5 subunits die too early in embryogenesis to analyze muscle development (Fassler and Meyer, 1995; Stephens et al., 1995; Yang et al., 1993, 1995). No muscle defects have been reported for α 1-, α 3-, α 6-, and α v-deficient mice (Kreidberg et al., 1996; Gardner et al., 1996; Bader et al., 1998; Georges-Labouesse et al., 1998). However, several integrins may have redundant functions in muscle. In fact, when the β 1 gene is deleted in cardiac myocytes, myocardial integrity is affected (Shai et al., 2002). A similar defect has not been described for mice lacking single integrins.

Integrin functions in muscle have also been studied

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in cell culture and with chimeric mice, leading to some conflicting results. First, it is unclear whether integrins regulate myoblast fusion. Function-blocking antibodies to the integrin $\beta 1$ and $\alpha 4$ subunits, and antisense mRNA to the $\alpha 6$ subunit block myoblast fusion in vitro (Menko and Boettiger, 1987; Rosen et al., 1992; Sastry et al., 1996). However, $\alpha 4$ -, $\alpha 5$ -, and $\beta 1$ -deficient myoblasts and ES cells form myotubes in vitro, and are incorporated into chimeric muscle fibers in vivo (Fassler and Meyer, 1995; Fassler et al., 1996; Yang et al., 1996; Hirsch et al., 1998; Rohwedel et al., 1998; Taverna et al., 1998). Second, the function of integrins in the assembly of the muscle fiber cytoskeleton is unclear. The α PS2 integrin subunit of *D. melanogaster* is essential to form Z bands, and the β PAT3 integrin subunit of *C. elegans* to assemble a structural analog of Z bands, the dense bodies (Brown, 2000). Vertebrate muscles chimeric for cells that lack or express the integrin $\alpha 5\beta 1$ show dystrophic symptoms, suggesting that $\alpha 5\beta 1$ regulates muscle fiber integrity (Taverna et al., 1998). Antibodies to the vertebrate integrin $\beta 1$ subunit block sarcomere formation, but myotubes that are generated in vitro from $\beta 1$ -deficient myoblasts assemble sarcomeres (Hilenski et al., 1992; McDonald et al., 1995; Hirsch et al., 1998).

To clarify $\beta 1$ integrin functions in striated muscle in vivo, we have inactivated the mouse integrin $\beta 1$ subunit gene (*Itgb1*) in developing myoblasts. We show that $\beta 1$ integrins regulate myoblast fusion and sarcomere assembly. Surprisingly, $\beta 1$ integrins are dispensable for the formation of MTJs, and for the initial recruitment of ECM components to muscle fibers. ECM recruitment is likely mediated by dystroglycan, but this ECM receptor cannot substitute for the function of $\beta 1$ integrins to regulate muscle fiber development.

Results

Generation of HSA-Cre Mice and Inactivation of $\beta 1$ Integrins in Skeletal Muscle

We have described an *Itgb1*^{fllox} allele suitable for Cre/Lox-mediated gene inactivation of the *Itgb1* gene (Graus-Porta et al., 2001). To inactivate *Itgb1* in skeletal muscle, we have generated transgenic mouse lines that express Cre under control of the human skeletal α -actin (HSA) promoter (Brennan and Hardeman, 1993). To analyze the HSA-Cre mice, we crossed them to mice carrying a *Rosa26lacZ*^{fllox} gene. In this line, LacZ expression is induced by Cre-mediated recombination (Mao et al., 1999). Staining of whole mounts and sections revealed that one HSA-Cre line induced recombination in somites at embryonic day (E) 9.5 (Figure 1A). Costaining of E10.5 sections with an antibody to Cre and desmin, a marker for the myogenic lineage (Schaart et al., 1989), confirmed that Cre was expressed in cells of the myogenic lineage (Figure 1B). At E12.5, LacZ staining was evident in the myotome and at E14.5 throughout all skeletal muscle groups (Figure 1A). We also stained E18 hindlimb sections and dissociated muscle, and detected LacZ expression in essentially all muscle fibers (data not shown). We conclude that the HSA-Cre line induces efficient recombination in cells that are committed to the myogenic lineage. Cre activity was also seen in the trigeminal

ganglion, and in cells scattered throughout heart, lung, and skin (Figure 1A; data not shown).

We next crossed *Itgb1*^{fllox/fllox} mice with mice carrying the HSA-Cre transgene on an *Itgb1*^{+/-} background, and analyzed whether the *Itgb1* gene was inactivated in *Itgb1*^{fllox/-}HSA-Cre offspring. The recombined *Itgb1*^{fllox} allele was detected in DNA isolated from E12.5 forelimbs and tails (Figure 1B; data not shown). We also observed the unrecombined allele because muscle cells comprise only a small percentage of all cell types in limb and tail somites. Immunohistochemistry confirmed that the integrin $\beta 1$ subunit and its $\alpha 7\beta$ partner were not expressed in the sarcolemma of intercostal muscle of mutant mice (Figure 1C). We conclude that we had inactivated $\beta 1$ expression in skeletal muscle, thereby also affecting the cell surface localization of $\alpha 7\beta$. In contrast, components of the DGC such as β -dystroglycan were still localized to the sarcolemma (Figure 1C).

To further confirm that we had inactivated $\beta 1$ expression, we analyzed extracts from E18 diaphragm muscle by Western blot (Supplemental Figure S1, available at <http://www.developmentalcell.com/cgi/content/full/4/5/673/DC1>). Expression of the $\beta 1$ subunit was below detection limit in the mutants. The expression of the integrin $\beta 3$ and $\beta 5$ subunits, and the αv subunit that heterodimerizes with many β subunits (Hemler, 1999), was unaltered.

Defective Skeletal Muscle Development

Mice that lacked $\beta 1$ integrins in muscle died at birth with noninflated lungs. At E18, the mutant embryos failed to straighten their body (Figures 2A and 2B). Defects in lungs and posture could be explained by muscle defects. We therefore crossed the *Itgb1*^{fllox} allele on a *Rosa26lacZ*^{fllox} background to obtain mice homozygous for both loci. This allowed us to identify muscles that had undergone Cre-mediated recombination by LacZ staining (Figures 2C–2H). We confirmed by PCR that HSA-Cre induced recombination of the *Itgb1*^{fllox} and *Rosa26lacZ*^{fllox} loci, but no transchromosomal recombination between the loci. No difference in phenotype was observed between mutant embryos obtained from intercrosses of HSA-Cre/*Itgb1*^{+/-} mice with *Itgb1*^{fllox/fllox} mice or with *Itgb1*^{fllox/fllox}/*Rosa26lacZ*^{fllox/fllox} mice (Figure 2; data not shown), confirming that transchromosomal recombination did not cause the observed defects.

Diaphragm muscle showed defects by E14.5 that were pronounced by E16.5 (Figures 2C–2F). LacZ-positive muscle fibers were present in the mutants, but their number and length was strongly reduced. LacZ-positive cells formed clumps that appeared to consist of individual cells or small groups of syncytial cells (Figure 2F). Similar observations were made with hypaxial (appendicular) and epaxial (body axis) muscle. For example, the length of intercostal muscle fibers was reduced (Figures 2G and 2H). We also analyzed sections stained with hematoxylin and eosin and confirmed the reduction in muscle mass (Figures 2I and 2J). Finally, high-resolution images of paraffin and semithin sections, as well as ultrastructural studies revealed an accumulation of unfused cells in mutant limb muscles (Figures 2K–2P, arrows).

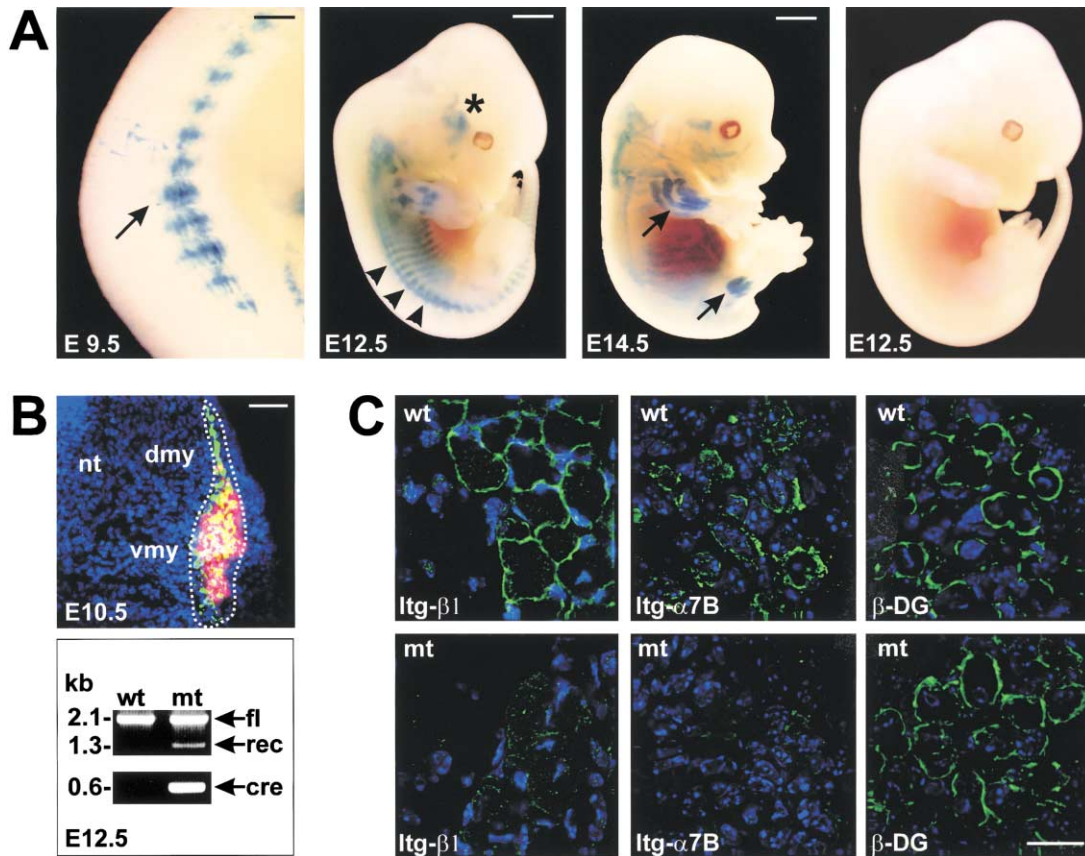


Figure 1. Analysis of HSA-Cre Mice

(A) HSA-Cre mice were crossed with *Rosa26lacZ^{fllox/flox}* mice and the Cre recombination pattern was analyzed by LacZ staining (blue). At E9.5, the rostral myotome was LacZ positive. At E12.5, LacZ was expressed in the caudal somites, in muscles that started to differentiate in the intercostal region (arrowheads), and in the trigeminal ganglion (asterisk). At E14.5, LacZ expression was visible in differentiating skeletal muscles (arrows point to limb muscles). No LacZ staining was evident in control littermates that lacked the HSA-Cre transgene.

(B) Upper panel: a transverse section through an E10.5 embryo was stained with DAPI (blue) to reveal nuclei, and with antibodies to Cre (red) and desmin (green) to reveal Cre-expressing cells and differentiating myogenic cells, respectively (vmy, ventral myotome; dmy, dorsal myotome; nt, neural tube).

(B) Lower panel: Cre-mediated recombination was analyzed by PCR with E12.5 tail DNA. A 2.1 kb band indicative of the unrecombined *Itgβ1^{fllox}* allele (fl) was visible in wild-type and mutant mice. A 1.3 kb band indicative of the recombined *Itgβ1^{fllox}* allele (rec) was evident in mutants. The presence of the HSA-Cre transgene (cre) was confirmed.

(C) Cross-sections of wild-type and mutant intercostal muscle stained with DAPI (blue) and antibodies to β1 (green) at E18.5, α7B (green) at E14.5, and β-dystroglycan (green) at E14.5. β1 and α7B were absent in the mutants.

The scale bars represent 200 μm ([A], E9.5); 1 mm ([A], E12.5); 2 mm ([A], E14.5); 200 μm (B); and 20 μm (C).

Intact Myoblast Migration and Proliferation

To determine whether defects in muscle size could result from perturbations in myoblast proliferation, we carried out BrdU labeling experiments in the myotome at E10.5 (data not shown), and at E12.5 in the semispinal capitis, the trapezius, and the lateral muscle (Figure 3). Proliferating myoblasts were identified by costaining for BrdU and desmin (Figures 3A and 3B). No difference in cell proliferation was detected between wild-type and mutant mice (Figure 3C). Two observations suggest that cell migration was also unaffected. First, muscle groups that are populated by migrating myoblasts, such as the semispinal capitis, contained comparable cell numbers early in muscle development in wild-type and mutant embryos (Figure 3C). Second, similar defects were observed in hypaxial and epaxial muscle, independent of the distance that myoblasts had to migrate (Figure 2).

We conclude that the muscle defects were not caused by defects in myoblast proliferation/migration, but we cannot exclude that β1 integrins regulate these events. Because the *Itgβ1* gene was inactivated in myogenic precursors, low levels of β1 protein may have persisted early after gene inactivation.

Defects in the Muscle Fiber Cytoskeleton but Not in MTJs

We next determined whether muscle fiber morphology and/or survival were affected. In sections of paraspinal and subscapularis longitudinalis muscle, wild-type muscle fibers had a regular appearance. The muscle in mutants contained fewer fibers, and some had an abnormally large diameter (Figure 4A). Similar defects were observed in hindlimb, intercostal, and diaphragm muscle (data not shown). TUNEL staining and staining for

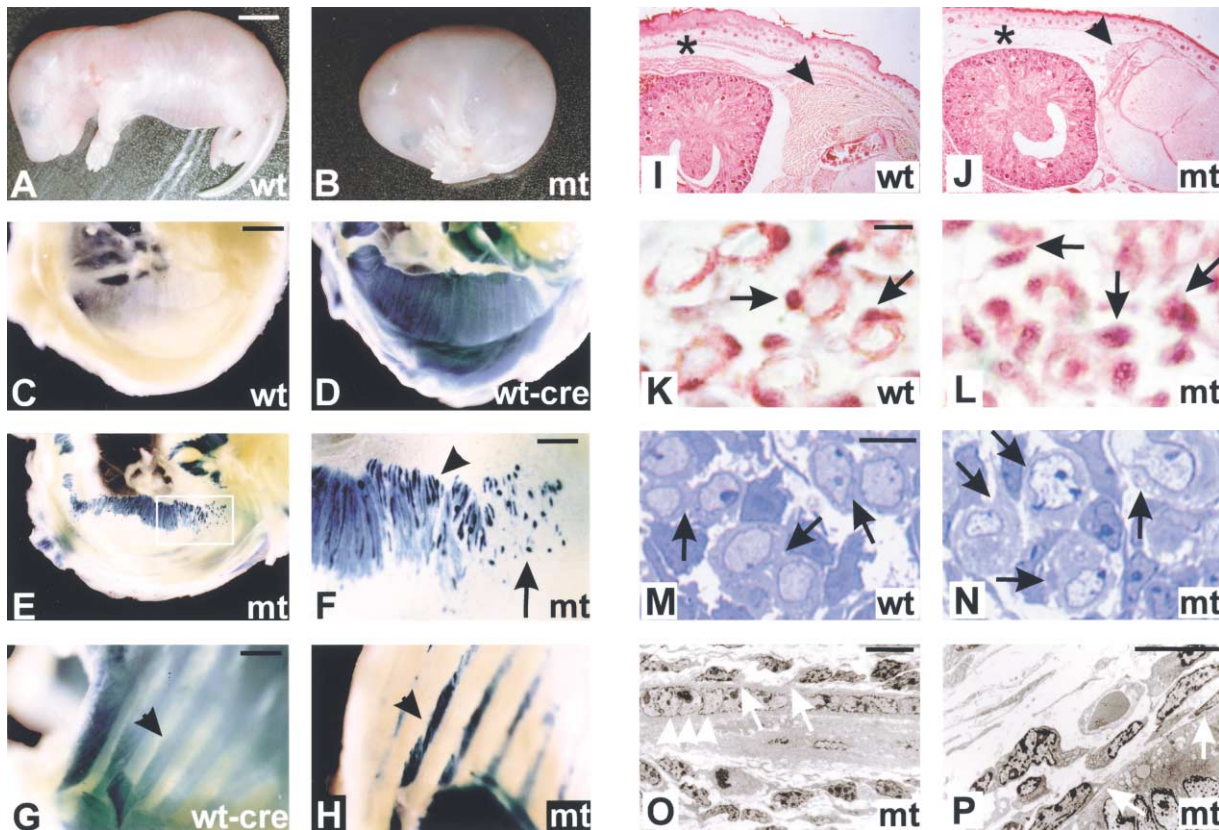


Figure 2. Skeletal Muscle Defects

(A and B) E18.5 wild-type and mutant embryos. Note the bent posture of mutant embryos.
(C–H) *HSA-Cre* mice were intercrossed with *Itgβ1^{flax/flax}/Rosa26lacZ^{flax/flax}* mice, and E16.5 offspring were analyzed by LacZ staining.
(C) Top view of half a diaphragm from a control embryo lacking Cre.
(D) Diaphragm from control embryos carrying a wild-type *Itgβ1* allele and a recombinant *Rosa26lacZ^{flax/flax}* allele.
(E) Diaphragm from an embryo that expresses LacZ, but not *Itgβ1*. The length of muscle fibers and muscle mass was reduced.
(F) Higher magnification view of the box shown in (E). Mutant muscle fibers were short and disorganized (arrowhead). Unfused cells accumulated in muscle (arrow).
(G and H) Intercostal muscle (arrowheads). Muscle fibers in the mutant were reduced in size.
(I and J) Transverse sections through E18.5 embryos stained with hematoxylin and eosin. The paraspinal (arrowhead) and body wall muscles (asterisk) were almost absent in mutant embryos.
(K–P) Unfused myoblasts (arrows) accumulated in mutant muscle as shown in cross-sections stained with hematoxylin and eosin (K and L), in semithin sections stained with toluidine blue (M and N), or in electron micrographs (O and P).
The scale bars represent 2.7 mm (A and B); 1 mm (C–E); 250 μ m (F); 0.5 mm (G–J); and 8 μ m (K–P).

activated caspase 3 revealed that some abnormal muscle fibers underwent apoptosis (Figure 4A). Morphological defects but not apoptosis were evident before E14.5, suggesting that development of β 1-deficient muscle fibers was perturbed, leading to their elimination by apoptosis.

At the ultrastructural level, wild-type muscle fibers showed a typical striated pattern, but mutant muscle fibers either lacked striation or showed a rudimentary striated pattern (Figure 4B). The assembly of Z bands progressed to some extent, but the spacing between Z bands was altered and M bands could hardly be detected. This suggests that in the mutants cytoskeletal assembly was initiated but not completed, or the cytoskeletal organization was not maintained.

The alterations in muscle fiber morphology could result from defects in muscle attachment at MTJs. However, staining for desmin, tenascin C, and laminin α 1

that become concentrated at MTJs (Daniloff et al., 1986; Tidball, 1992; Gullberg et al., 1998) was indistinguishable between wild-type and mutant mice at E12.5 and E14.5 (Figure 4C; Supplemental Figure S1). Integrin downstream effectors that localize to MTJs such as vinculin and integrin-linked kinase (Mackinnon et al., 2002; Shear and Bloch, 1985; Zervas et al., 2001) were present in the mutants (Figure 4C). The integrin α v subunit was also concentrated at MTJs (Figure 4C). Because α v also heterodimerizes with integrin β subunits other than β 1 (Hemler, 1999), α v-containing integrins may be sufficient for MTJ assembly. At E16.5, the diaphragm muscle detached from the tendon (Figure 2F). This was likely a consequence of growth of the animals leading to increased tension on the short muscle fibers and MTJ rupture. Because detachment occurs several days after the structural defects in muscle fibers, it cannot cause the defects.

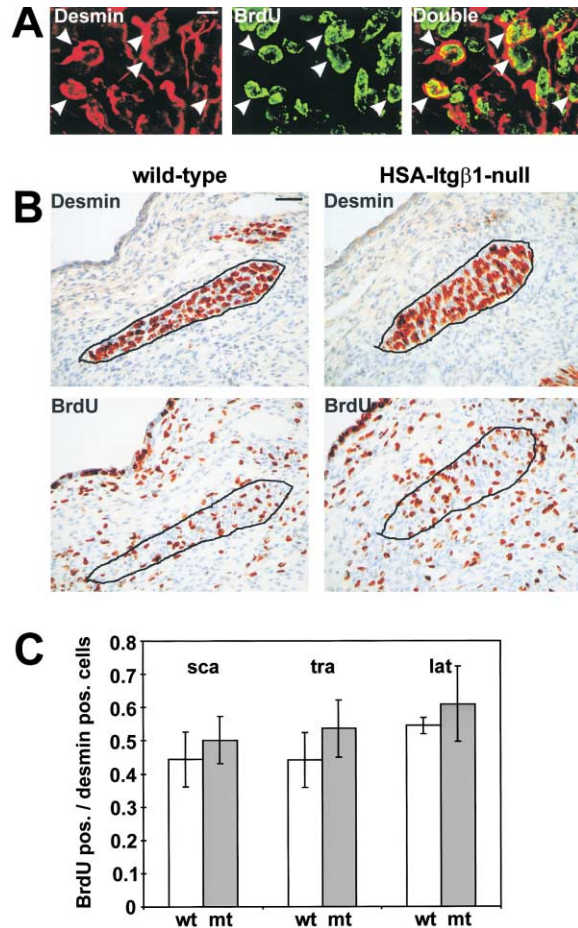


Figure 3. Normal Myoblast Proliferation

HSA-Cre mice were intercrossed with *Itgβ1^{flax/flax}* mice. Pregnant mice were injected with BrdU 2 hr before embryos were recovered. (A) Sections of E12.5 trapezius muscle were stained for desmin and BrdU. The majority of BrdU-positive cells expressed desmin (arrowheads). (B) Adjacent sections of trapezius muscle from E12.5 wild-type and mutant embryos were stained for desmin (upper panels) and BrdU (lower panels). The sections were counterstained with hematoxylin (blue). (C) The proliferation rate was quantified as the ratio of BrdU- versus desmin-positive cells. sca, semispinalis capitis; tra, trapezius; la, lateral muscle. The scale bars represent 10 μm (A); and 40 μm (B).

Defects in Costameric Complexes

Defects in the lateral linkages of the muscle fiber cytoskeleton via costameric complexes to the sarcolemma and/or perturbations in ECM recruitment could lead to muscle fiber defects. We therefore analyzed whether integrin downstream effectors, components of the DGC, and ECM proteins were localized to the sarcolemma. Strikingly, vinculin and talin were absent from the costameres in the mutants (Figure 5A; data not shown). In contrast, the expression of β-dystroglycan, dystrophin, laminin α2, collagen IV, entactin/nidogen, and perlecan was unaffected (Figures 1C and 5A–5C). At E14.5, the distribution of ECM components was patchy in wild-type and mutant muscle (Figure 5A). By E18.5, muscle fibers were surrounded by a layer of laminin α2 and

collagen IV (Figure 5C), but ultrastructural studies demonstrated that ECM components in the mutants were not assembled into a continuous BM (Figure 5B). We conclude that the recruitment of proteins such as vinculin and talin to costameric complexes is defective in the absence of β1 class integrins. ECM molecules are still recruited to β1-deficient muscle fibers, likely in part through interactions with the DGC, but an intact BM is not present.

Defective Myoblast Fusion In Vitro

In the absence of β1 integrins short muscle fibers developed in vivo, and unfused cells accumulated (Figures 2K–2P), suggesting that myoblast fusion was defective. We therefore analyzed myoblast fusion in vitro. As a control, myoblasts were isolated from E18.5 wild-type embryos transgenic for *HSA-Cre* and the *Rosa26lacZ^{flax}* reporter. Myoblasts derived from these mice expressed Cre-recombinase prior to fusion (Supplemental Figure S2). Whereas myoblast fusion was observed when wild-type cells were cultured for 1 or 3 days (Figures 6A and 6C), many unfused myoblasts and only few short myotubes were visible in cultures with β1-deficient cells (Figures 6B and 6D). Even when β1-deficient myoblasts aggregated into clusters, they did not fuse (Figure 6E). Fusion of wild-type myoblasts was enhanced when cells were plated at higher density, but fusion of β1-deficient myoblasts was still impaired (Figure 6I). Fusion defects were not caused by a developmental delay. The mutant myoblasts expressed myogenic differentiation markers such as MyoD, sarcomeric α-actinin, and fast isoforms of myosin heavy chain (MHCf) in a temporal pattern comparable to wild-type myoblasts (Figures 6C and 6D; Supplemental Figure S2). The defects were not caused by lack of adhesion to the fibroblast layer. β1-deficient myoblasts spread on the feeder layer, formed filopodia (Supplemental Figure S2), and could not be removed by liquid shear force. Because the efficiency of myoblast fusion is dependent on culture conditions (Hirsch et al., 1998), we performed fusion experiments with five different combinations of medium and serum, but could not rescue fusion (data not shown). However, the fusion defect was rescued when β1-deficient (Cre-positive) and β1-expressing (Cre-negative) myoblasts were co-cultured. Mixed muscle fibers containing both Cre-positive and -negative nuclei readily formed (Figures 6F–6H). The fusion defect of β1-deficient myoblasts could not be rescued by conditioned medium from wild-type cultures, suggesting that fusion was not dependent on a soluble factor whose expression is β1 dependent (data not shown).

To define the step in cell fusion that was β1 dependent, we analyzed the cell cultures by electron microscopy (Figures 6L–6N). We identified in cultures of β1-deficient myoblasts characteristic features resembling the consecutive subcellular events that have been described for myoblast fusion in *Drosophila* (Doberstein et al., 1997), such as membrane alignment, formation of prefusion complexes with electron-dense vesicles at cell-cell contact sites (Figure 6L), and electron-dense plaques (Figure 6M). Electron-dense plaques were prominent only in cultures with β1-deficient myoblasts. In contrast, cultures with wild-type cells contained large

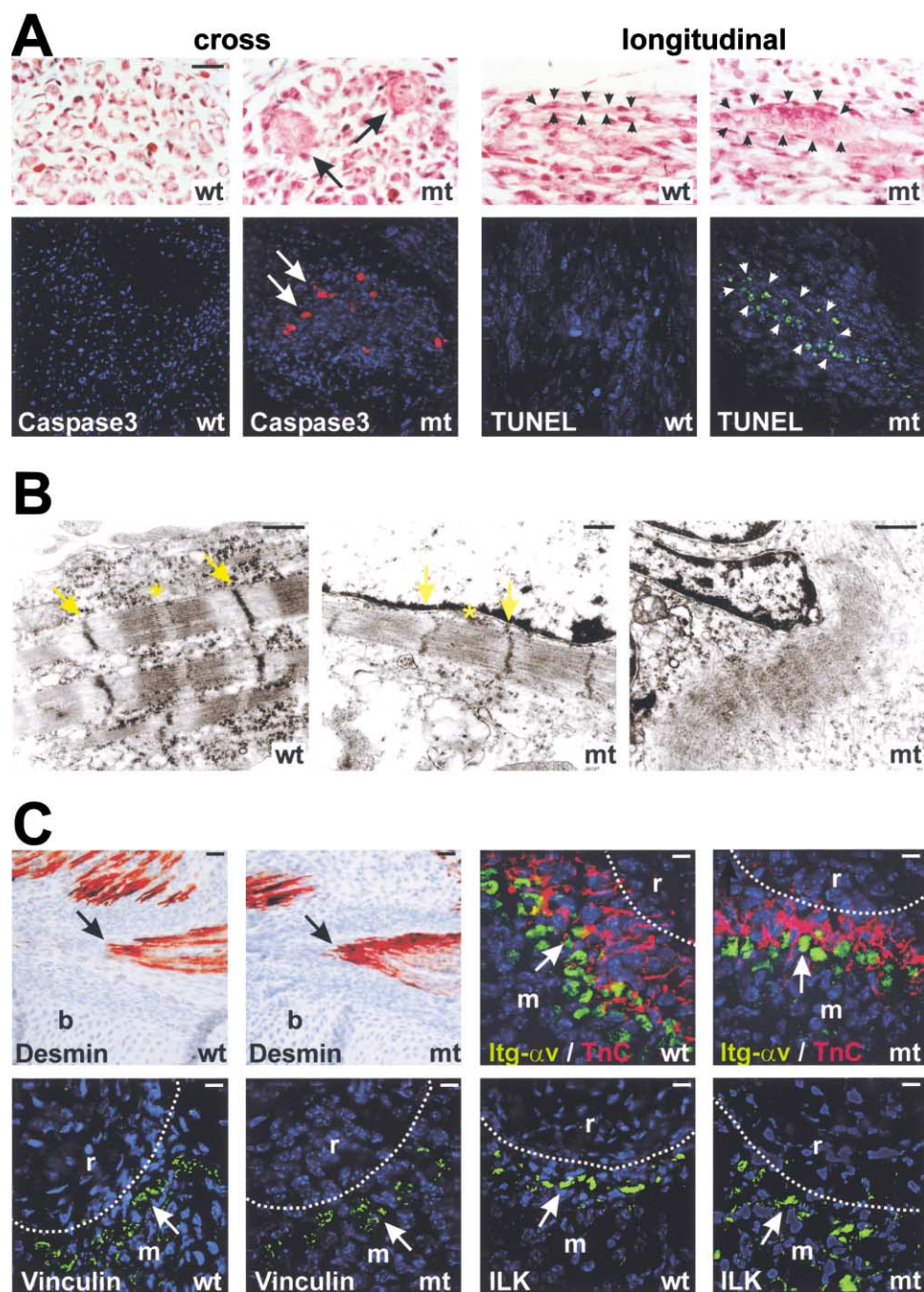


Figure 4. Defects in Muscle Fiber Morphology and Survival, but Not in MTJs

(A) Upper panels: cross-sections through paraspinous muscles, and longitudinal sections through subscapularis muscle at E14.5. Muscle fibers with an abnormally large diameter (arrows) were present in mutant embryos. Wild-type muscle fibers (outlined by arrowheads) were regular in morphology and contained rows of myonuclei (dark red). Muscle fibers in the mutants were irregular in shape.

(A) Lower panels: apoptotic cells were detected in mutant but not wild-type muscle, by staining for activated caspase 3 (red, arrows) and by the TUNEL method (green, arrowheads).

(B) Ultrastructural analysis of E14.5 forelimb muscle fibers. The sarcomeric organization with Z lines (arrows) and M lines (asterisk) was evident in wild-type muscle fibers. In the mutants, muscle fibers contained a rudimentary sarcomeric organization with Z, but not M lines, or no striation.

(C) Cross-sections through intercostal muscle were stained with the indicated antibodies to reveal MTJs. Upper left panels: antibody to desmin (brown) to visualize muscle cells, and hematoxylin (blue) to reveal tissue morphology (b, bone). MTJs (arrows) were visible in wild-type and mutant embryos. Upper right panels: antibody to the integrin α v subunit (green) and tenascin C (red), and DAPI (blue). Borders between rib bones (r) and muscle (m) are outlined with a dotted line. Lower left panels: antibody to vinculin (green), and DAPI (blue). Lower right panels: antibodies to integrin-linked kinase (green), and DAPI (blue).

The scale bars represent 15 μ m (A); 1 μ m (B); 50 μ m (C, upper left panels); and 10 μ m (C, all other panels).

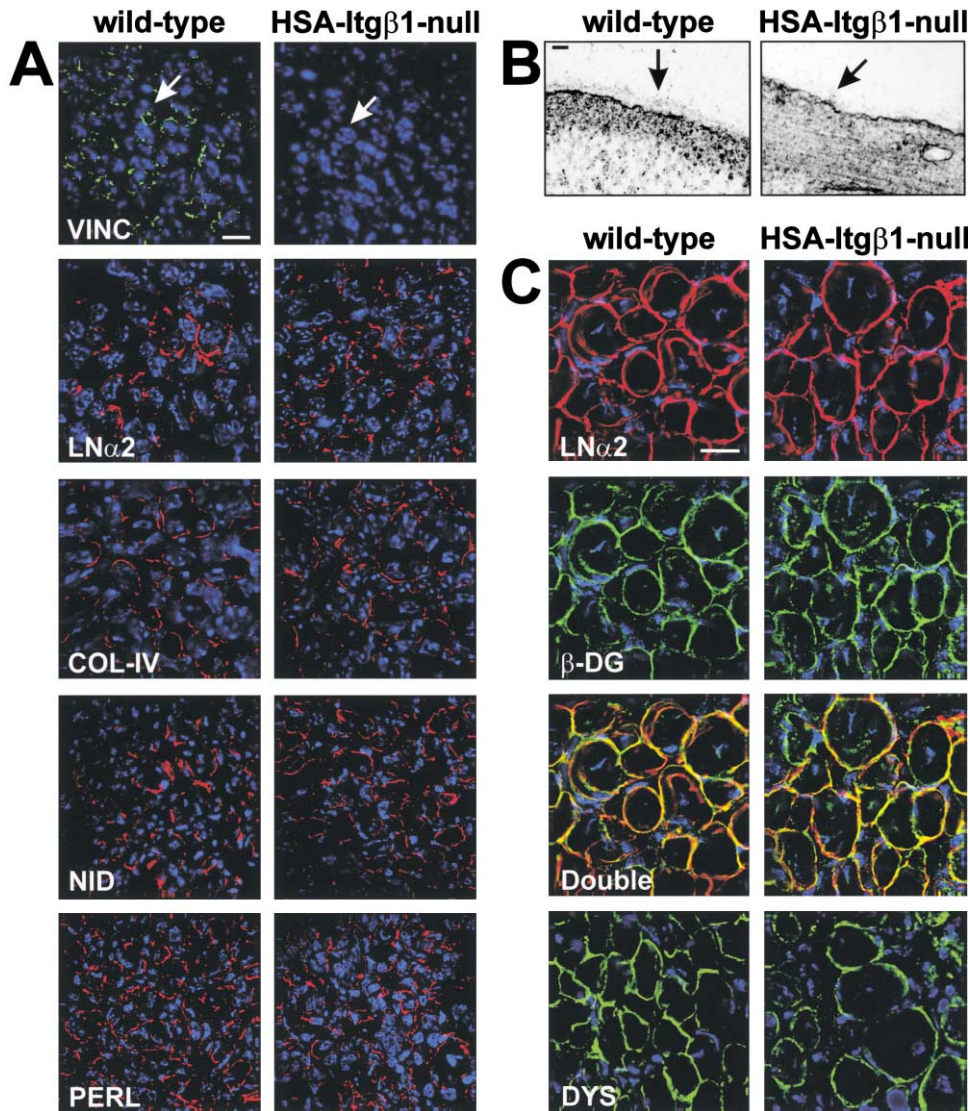


Figure 5. Expression of Costameric Proteins, ECM, and Dystrophin

(A) Cross-sections of E14.5 intercostal muscle were stained with DAPI (blue) and antibodies to vinculin (green). The lattice-like vinculin staining characteristic of costameres (arrows) was absent in the mutants. Cross-sections of E14.5 forelimb muscle were stained with DAPI (blue) and antibodies to laminin α2, collagen IV, entactin/nidogen, and perlecan (all in red). ECM components were recruited in a patchy pattern to the membrane of wild-type and mutant muscle fibers.

(B) Electron micrographs of forelimb muscle from E18.5 embryos. A continuous BM was present only at the surface of wild-type muscle fibers (arrow), but not in the mutants (arrow).

(C) At E18.5, laminin α2 (red) was incorporated into a continuous layer surrounding wild-type and mutant muscle fibers. β-dystroglycan (green) colocalized with laminin α2. Dystrophin (green) was also localized to the sarcolemma of wild-type and mutant muscle fibers.

The scale bars represent 15 μm (A); 0.2 μm (B); and 15 μm (C).

numbers of myoblasts that progressed to the next step of membrane breakdown, which creates cytoplasmic continuity between the fusing cells (Figure 6N). Signs of membrane breakdown were rarely detected in cultures with mutant cells.

We next analyzed the expression of proteins implicated in myoblast fusion such as N-CAM and the tetraspanin CD9 (Dickson et al., 1990; Knudsen et al., 1990a; Tachibana and Hemler, 1999). N-CAM was concentrated at the cell-cell interaction surface of β1-deficient myoblasts (Figures 6J and 6K), suggesting that it mediates myoblast adhesion. Strikingly, CD9 was strongly expressed only at the cell surface of wild-type

myoblasts, but could hardly be detected in the mutants (Figures 6O–6R; Supplemental Figure S2). Taken together, the data suggest that β1 integrins are not essential to mediate cell-cell interactions between myoblasts, but they are required at a subsequent step in cell fusion. Because CD9 expression was defective in the mutants, the data suggest that β1 integrins and CD9 act in a common molecular pathway at a late step in cell fusion.

Defective Sarcomere Assembly In Vitro

The muscle fiber cytoskeleton in the mutant mice was disorganized, suggesting that cytoskeletal assembly

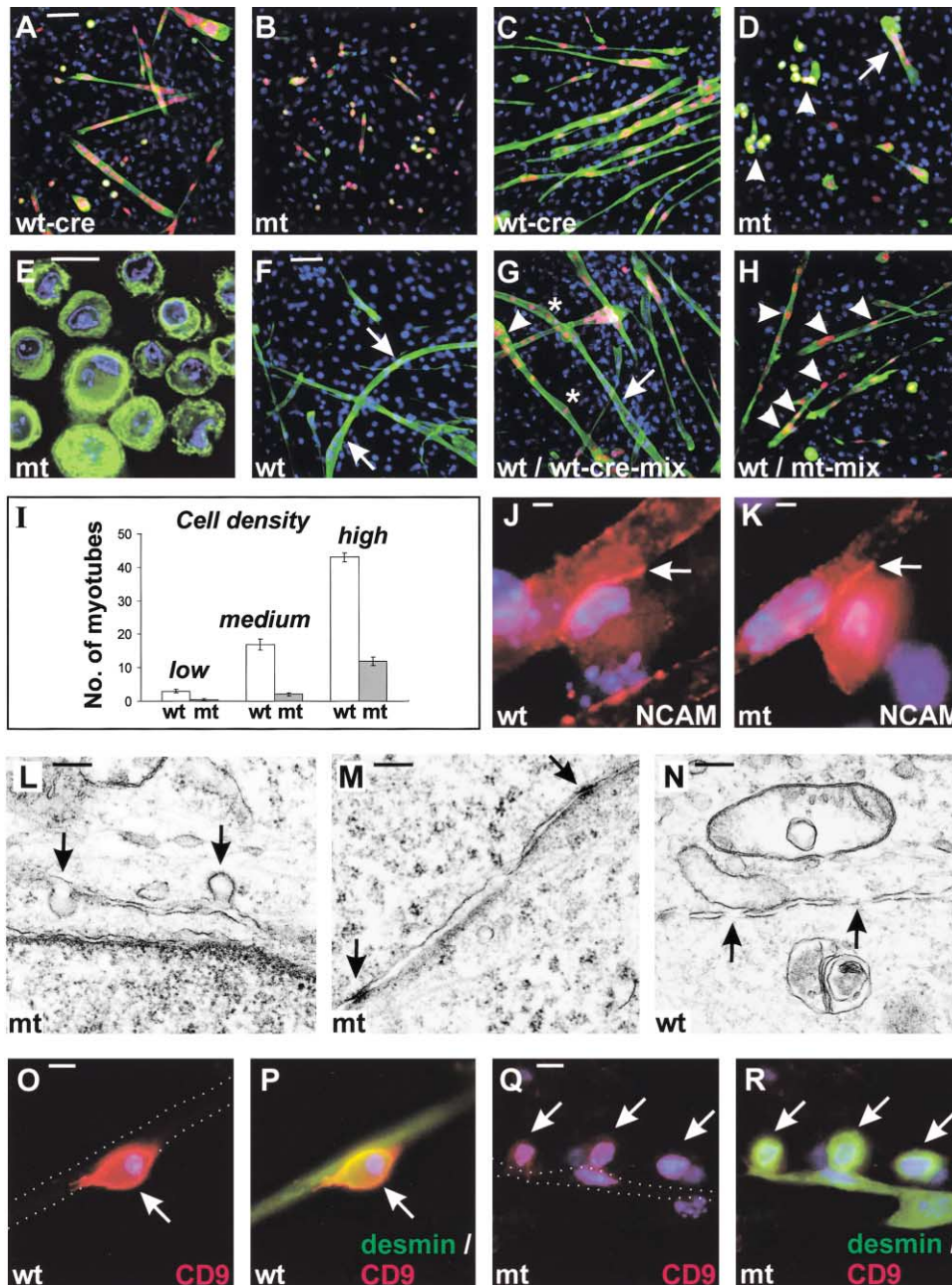


Figure 6. Defective Myoblast Fusion

(A–D) Muscle cells were isolated from E18.5 wild-type and mutant embryos expressing Cre. The cells were cultured on fibroblast layers for 1 (A and B) and 3 (C and D) days. Differentiating myoblasts and myotubes were stained with antibodies to sarcomeric α -actinin (green), Cre-recombinase (red), and DAPI (blue). Myotube formation was impaired in cultures with mutant cells. Only a few short myotubes (arrow in [D]) were present in the cultures, and many myoblasts remained unfused (arrowheads). Note that some of the cells outlined by an arrowhead could be dividing cells, not cells that try to fuse.

(E) Higher magnification view of clustered, unfused β 1-deficient myoblasts stained for α -actinin (green) and DAPI (blue).

(F–H) The fusion capability of a mix of wild-type and β 1-deficient myoblasts was analyzed. Myoblasts and myotubes were visualized by staining with sarcomeric α -actinin (green), and Cre-expressing cells were identified by staining with antibodies to Cre (red) to reveal nuclear Cre.

(F) Cre-negative wild-type cells were cultured alone and formed myotubes.

(G) Cre-negative wild-type cells were mixed with Cre-expressing wild-type cells; myotubes formed that contained Cre-positive (arrowhead) and -negative (arrow) nuclei. Cre diffused into Cre-negative nuclei (asterisks).

(H) Cre-negative wild-type cells were mixed with Cre-positive mutant cells; the mutant cells (containing nuclear Cre, arrowheads) readily fused with the wild-type cells.

(I) Quantification of the number of myotubes per unit area (0.1 mm²) that formed when cells from wild-type mice or β 1-deficient mice were plated at different cell densities (n = 2; plating densities: low = 200; medium = 400; high = 800 myoblasts/mm²).

(J and K) Myoblast cultures of wild-type and β 1-deficient cells were stained with DAPI (blue) and antibodies to N-CAM (red). N-CAM was

and/or maintenance were defective. To distinguish between these possibilities, we analyzed cytoskeletal assembly in the few β 1-deficient myotubes that formed in vitro. Sarcomeres are assembled in multiple steps. Premyofibrils develop at the growing ends of muscle fibers that contain α -actinin in punctate aggregates called Z-bodies. Z-bodies subsequently align into Z bands that span the cell (Wang et al., 1992; Franzini-Armstrong and Fischman, 1994; Dabiri et al., 1997). At this stage, other proteins including MHCf are recruited into a striated pattern (Isaacs et al., 1992). In wild-type myotubes, α -actinin and MHCf were assembled into a striated pattern (Figure 7A). In the mutants, α -actinin but not MHCf accumulated in a rudimentary striated pattern (Figure 7A). Vinculin was detectable at costameres only in wild-type, but not in β 1-deficient myotubes (Figure 7A, arrowheads). The distribution of α -actinin in mutant myotubes was reminiscent of its distribution at the growing ends of wild-type myotubes, where assembly of the cytoskeleton was initiated (Figure 7B). The data suggest that β 1 integrins are not required for the assembly of premyofibrils and Z-bodies, but for the formation of a mature striated pattern. Because the recruitment of integrin downstream effectors such as vinculin to costameres is perturbed, the defects are likely caused by perturbations in the interaction of premyofibrils with costameric complexes.

Discussion

We show here that β 1 integrins regulate myoblast fusion and the assembly of the muscle fiber cytoskeleton. Unfused myoblasts and syncytia consisting of few cells accumulate in vivo in β 1-deficient muscle, and β 1-deficient myoblasts have an impaired ability to undergo fusion in vitro. β 1 integrins are required at a step subsequent to myoblast adhesion when plasma membrane breakdown establishes cytoplasmic continuity between fusing cells. Cell surface expression of the tetraspanin CD9 that has been implicated in regulating cell fusion is abolished in β 1-deficient myoblasts, suggesting that β 1 integrins and CD9 act in a common pathway. Subsequent to fusion, β 1 integrins regulate the assembly of the myofiber cytoskeleton, and our data provide strong evidence that the final assembly of premyofibrils into sarcomeres is β 1 dependent. Unexpectedly, β 1 integrins are not essential for the assembly of MTJs and for the initial recruitment of ECM components to muscle fibers. ECM recruitment is likely mediated by the DGC, but the DGC cannot compensate for the loss of β 1 integrins during myofiber development.

Defects in fusion are likely not caused by defects in cell differentiation, because β 1-deficient myoblasts

express myogenic lineage markers such as Myo-D, α -actinin, and MHCf. The β 1-deficient myoblasts also establish in vitro adhesive interactions with each other and with the underlying fibroblast layer, but they are trapped at a step in cell fusion preceding plasma membrane breakdown. N-CAM is concentrated at the contact surface between myoblasts even in the mutants, and likely mediates adhesive interactions. In contrast, the tetraspanin CD9 that forms a complex with many receptors including β 1 integrins (Hemler, 2001) is expressed at the cell surface of wild-type but not β 1-deficient myoblasts. CD9 regulates in vitro myoblast and sperm-egg fusion (Hemler, 2001). In vivo it is essential for sperm-egg but not myoblast fusion (Kaji et al., 2000), but another member(s) of the tetraspanin family may compensate for a loss of CD9 in myoblasts. This is consistent with the observation that antibodies to CD9 and a second tetraspanin, CD81, have additive effects on myotube formation in vitro (Tachibana and Hemler, 1999). Intriguingly, during sperm-egg fusion, CD9 is required for a step subsequent to adhesion (Kaji et al., 2000). We show here that β 1 integrins are also required to regulate myoblast fusion at a step subsequent to myoblast adhesion. Taken together, the data suggest that β 1 integrins and CD9 act in a common pathway to regulate cell fusion subsequent to cell adhesion.

Fusion defects are rescued when β 1-deficient and wild-type myoblasts are mixed, suggesting that heterophilic interactions between β 1 integrins and a yet to be defined cell surface receptor may be important. One candidate class of receptors is the ADAMs proteins. Some ADAMs proteins interact with β 1 integrins, and ADAM12 and ADAM1/2/3 regulate myoblast and sperm-egg fusion, respectively (Seals and Courtneidge, 2003). Other candidate receptors are members of the Ig-CAM family, including VCAM-1, which has been implicated as a receptor for integrin α 4 β 1 in cell fusion (Rosen et al., 1992). However, in vivo evidence confirming a role for ADAMs and Ig-CAMs in myoblast fusion is missing. Given the complexity of the cell fusion process, it is likely that several receptors, including β 1 integrins, ADAMs, and IgCAMs cooperate to regulate myoblast fusion.

Previous studies have shown that β 1-deficient ES cells induced to differentiate into myotubes can fuse in vitro, but fusion was dependent on the culture conditions (Hirsch et al., 1998). We carried out in vitro fusion experiments under all culture conditions described by Hirsch et al. (1998), but could not rescue the fusion defects of primary β 1-deficient myoblasts. A possible explanation for the difference in our results is that during long-term culture in an ex vivo environment used in previous experiments, other cell surface receptors may

concentrated at the cell-cell interaction surface. Staining with desmin (not shown) confirmed that the cells were of the myogenic lineage.

(L–N) Electron micrographs of myoblasts in culture.

(L) Prefusion complexes with electron-dense vesicles were readily visible at the plasma membrane of mutant myoblasts.

(M) In cultures with β 1-deficient cells, most myoblasts accumulated at an intermediate stage in cell fusion, characterized by the formation of electron-dense adhesion plaques.

(N) In cultures with wild-type myoblasts, the most widespread morphological feature was membrane breakdown indicative of active cell fusion.

(O–R) Cells in culture were stained with DAPI (blue), and antibodies to CD9 (red) and desmin (green). CD9 was expressed in wild-type myoblasts (arrows) that aligned with myotubes in preparation for fusion. CD9 was not expressed in β 1-deficient myoblasts.

The scale bars represent 40 μ m (A–D); 15 μ m (E); 40 μ m (F–H); 2 μ m (J and K); 0.4 μ m (L–N); 5 μ m (O–R).

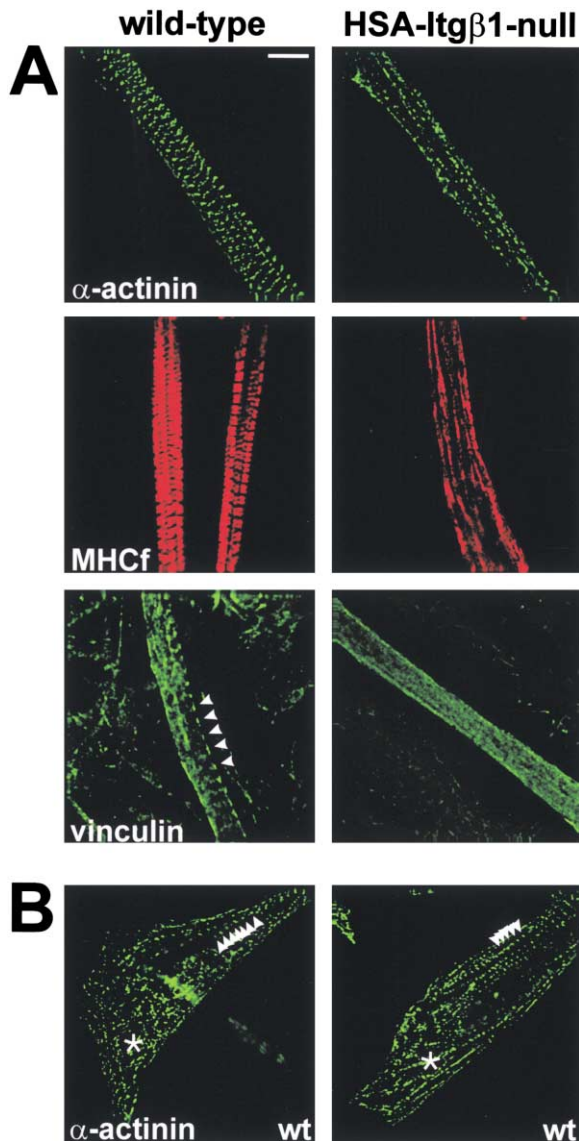


Figure 7. Defective Sarcomere Assembly

(A) Wild-type and mutant myoblasts were cultured for 5 days in differentiation medium and stained with antibodies: sarcomeric α -actinin (green) for Z lines and Z-bodies; MHCf (red) for M lines; vinculin for costameres (green, arrowheads). Wild-type myotubes showed a striated pattern of α -actinin, MHCf, and vinculin. In β 1-deficient myotubes, α -actinin was assembled only into dots resembling Z-bodies, and MHCf and vinculin were not properly localized. The scale bar represents 10 μ m.

(B) Wild-type cells were cultured for 3 days. The growing ends of myotubes are shown. They contained punctate accumulations of α -actinin (green, asterisk), resembling the distribution of α -actinin throughout β 1-deficient myotubes after 5 days in culture. In wild-type myotubes, the α -actinin pattern became organized into a striated pattern (Z discs) in more distal locations (arrowheads).

have been upregulated that rescued fusion. Consistent with this finding, in previous but not in our experiments, vinculin was recruited to costameres, probably by an alternative receptor. In our experiments, expression of β 1 integrins was inactivated acutely in myoblasts and there may have not been sufficient time to activate compensatory mechanisms. To search for compensatory

mechanisms, we studied expression of the integrin subunits β 3, β 5, and α v in β 1-deficient diaphragm muscle. No upregulation was observed. It would be interesting to investigate whether integrins or other receptor molecules were upregulated in embryoid bodies. We also attempted to analyze whether fusion defects could be rescued by culturing β 1-deficient myoblasts for extended periods of time. Fusion defects were not rescued, even after 7 days in culture. However, whereas β 1-deficient myoblasts initially adhered and spread on the feeder cell layer, they started to round up after 4–5 days and were overgrown by replicating fibroblasts, preventing a conclusive interpretation of the results. Overall, the previous and our results suggest that cell fusion can be achieved by different pathways, at least in vitro. We demonstrate here in addition fusion defects in vivo, providing strong evidence that cell fusion during primary myogenesis in vivo is critically dependent on β 1 integrins.

We also provide evidence that β 1 integrins regulate the assembly of the muscle fiber cytoskeleton. Our data suggest that β 1 integrins are not essential for the generation of premyofibrils, but for their subsequent assembly into mature sarcomeric structures. The defects are reminiscent of those in invertebrate muscle fibers devoid of the integrins α PS2 and β PAT3. In *C. elegans*, perlecan is required to localize β PAT3, which in turn recruits talin, vinculin, and α -actinin. Mutations that disrupt these interactions lead to defects in the formation of sarcomeric structures (Brown, 2000). We saw similar defects in the recruitment of vinculin and talin to costameres in β 1-deficient muscle fibers, suggesting that integrins have an evolutionarily conserved function to link myofibrils during assembly via costameres to the sarcolemma.

Earlier studies with β 1-deficient embryoid bodies have led to the conclusion that β 1 integrins are dispensable for the formation of sarcomeres. Vinculin was still normally localized in these myotubes (Hirsch et al., 1998). In contrast, we observed that vinculin localization to costameres is greatly perturbed when β 1 integrins are acutely inactivated in myoblasts. As discussed above, it is possible that in the ex vivo embryoid body differentiation model, other receptor(s) were upregulated that recruited vinculin, leading to functional compensation for the loss of β 1 integrins.

Our data demonstrate that β 1 integrins are required for muscle fiber development, but they do not exclude a role for components of the DGC. The function of components of the DGC in muscle fiber development has been controversial. Treatment of primary muscle cultures with antibodies that block the binding of α -dystroglycan to laminin causes defects in myotubes (Brown et al., 1999). However, differentiation and sarcomeric organization is unaffected in myotubes derived from ES cells that lack dystroglycan (Jacobson et al., 2001). Unfortunately, chimeric mice and mice in which dystroglycan has been inactivated at late stages of muscle fiber development are not suitable tools to study its function in the early development of skeletal muscle fibers (Cote et al., 1999; Cohn et al., 2002). However, in dystroglycan-deficient zebrafish sarcomeres the sarcolemmal reticulum is disorganized, suggesting that it may regulate muscle fiber development in vivo (Parsons et al., 2002). This raises the intriguing possibility that β 1

integrins and components of the DGC have specialized functions to regulate different steps during the complex process that leads from myoblast to muscle fibers.

Precedence for the cooperative action of β1 integrins and dystroglycan comes from studies on BM assembly. Previous studies have shown that dystroglycan-deficient myotubes recruit ECM components (Cote et al., 1999; Cohn et al., 2002). We show that ECM components are still recruited to β1-deficient muscle fibers, but an intact BM is not assembled, suggesting that both dystroglycan and β1 integrins are required. This is consistent with studies carried out with myotubes in culture, where BM assembly is dependent on interactions between ECM molecules, their interaction with β1 integrins and dystroglycan, and the proper organization of the muscle fiber cytoskeleton (Colognato et al., 1999; Henry et al., 2001). In the mutants described here, the muscle fiber cytoskeleton was disorganized, suggesting that the regulatory crosstalk between the cell exterior and interior was disrupted.

Finally, we show that β1 integrins are dispensable for the initial assembly of MTJs. Because the α_v subunit and integrin downstream effectors are localized to MTJs of β1-deficient muscle fibers, α_v as a heterodimer with β subunits other than β1 may have important functions in this process. However, no defects in muscle have been reported for α_v-deficient mice (Bader et al., 1998). Clearly, β1 integrins are important to maintain the integrity of MTJs in the adult. Mice that lack the integrin α7β1 develop dystrophic symptoms that are caused at least in part through the instability of MTJs (Mayer et al., 1997).

Taken together, our data provide strong evidence that different ECM receptors within the same cell (syncytium) have specific nonredundant functions. β1 integrins function to regulate myoblast fusion, the assembly of muscle fiber cytoskeleton, and the maintenance of MTJs. The DGC is important to maintain the integrity of muscle fibers. β1 integrins and dystroglycan are required for BM assembly. Future studies will be necessary to analyze whether components of the DGC also cooperate with β1 integrins in muscle fiber development. An interesting possibility is that β1 integrins and components of the DGC regulate independent steps during myoblast fusion and cytoskeletal assembly.

Experimental Procedures

Mouse Strains

The *Rosa26lacZ^{flax}*, *Itgβ1^{flax}*, and *Itgβ1^{+/-}* mouse strains have been described (Stephens et al., 1995; Mao et al., 1999; Graus-Porta et al., 2001). Transgenic *HSA-Cre* mouse lines, where a 2.2 kb fragment of the human skeletal α-actin (*HSA*) promoter drives *Cre*, were generated as described (Brennan and Hardeman, 1993).

Histology, Immunohistochemistry, and Electron Microscopy

Hematoxylin/eosin and LacZ staining, and electron microscopy were carried out as described (Farinas et al., 1996; Müller et al., 1997). For immunohistochemistry with antibodies to desmin (Sigma), Cre-recombinase (Babco), β1 (Graus-Porta et al., 2001), α7B (provided by U. Mayer), α_v (Chemicon), vinculin (Sigma), talin (Sigma), ILK (provided by C. Wu), laminin α2 (provided by L. Sorokin), collagen IV (Chemicon), entactin/nidogen (Chemicon), perlecan (provided by I. Farinas), β-dystroglycan (provided by K. Campbell and L. Anderson), sarcomeric α-actinin (Sigma), and MHCf (Sigma), embryos were embedded in OCT (Tissue Tek), cryosectioned at 10 μm, and

fixed in 2% paraformaldehyde for 7 min at room temperature, and permeabilized in methanol for 5 min at -20°C. Caspase 3 antibody (Pharmingen) was used on frozen sections treated with acetone for 30 s at -20°C. For BrdU labeling, pregnant mothers were injected with 100 μg BrdU/g body weight, embryos were isolated 2 hr later, fixed in 4% paraformaldehyde, and processed for paraffin sectioning. For staining with anti-desmin or anti-BrdU (Pharmingen) antibodies, sections were incubated for 10 min in 0.1 M citrate buffer (pH 6.0) at 95°C.

Sections or cells were incubated at room temperature for 30 min in 3% H₂O₂ in methanol, for 10 min in 100 mM glycine, for 30 min in 1% BSA in PBS, and for 2 hr at room temperature with primary antibodies in 1% BSA in PBS. FITC- or TRITC-labeled secondary antibodies (Jackson ImmunoResearch) were used, or peroxidase-coupled secondary antibodies and the Vectastain ABC kit (Vector Laboratories). Images were collected on a Deltavision microscope and processed by deconvolution.

To quantify proliferation, the number of BrdU-positive cells and desmin-positive cells was counted on ten sections, and the ratio between the values was calculated. A small fraction of the BrdU-positive cells was desmin negative, but the cells were not further considered. The mean and standard deviation were determined.

Muscle Cell Cultures

Myoblast fusion experiments with primary cells from hindlimb muscle were carried out as described (Dorchies et al., 2001). For fusion rescue experiments, equal numbers of wild-type and β1-deficient cells were mixed. The number of myotubes was determined by counting α-actinin-positive myotubes with two or more nuclei on ten fields. The mean and standard deviation were calculated.

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