Molecular Pathogenesis of Genetic and Inherited Diseases

Deletion of Integrin-Linked Kinase from Skeletal Muscles of Mice Resembles Muscular Dystrophy Due to $\alpha 7\beta 1$ -Integrin Deficiency

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Integrin-linked kinase (Ilk) is a serine/threonine kinase and an adaptor protein that links integrins to the actin cytoskeleton and to a number of signaling pathways involved in integrin action. We hypothesized that Ilk may act as an important effector of integrins in skeletal muscle, where these receptors provide a critical link between the sarcolemma and the extracellular matrix. Using the cre/lox system, we deleted *Ilk* from skeletal muscles of mice. The resulting mutants developed a progressive muscular dystrophy with multiple degenerating and regenerating muscle fibers, increased central nuclei, and endomysial fibrosis. These defects were widespread but were most severe near myofascial junctions where *Ilk* mutants showed displacement of focal adhesion-related proteins, including vinculin, paxillin, focal adhesion kinase, dystrophin, and the $\alpha 7\beta 1$ -integrin subunits. Distal ends of mutant muscle fibers appeared irregular, and there was restructuring of the actin cytoskeleton. These findings resemble those seen in humans and mice lacking the $\alpha 7$ -integrin subunit and suggest that Ilk may act as a cytoplasmic effector of $\alpha 7\beta 1$ -integrin in the pathogenesis of these deficiencies. (*Am J Pathol* 2007, 171:1966–1977; DOI: 10.2353/ajpath.2007.070555)

most often followed by significant functional disability and early death. The majority of muscular dystrophies are caused by defects in complexes that link the cytoskeleton to the extracellular matrix, such as the dystrophin-glycoprotein complex in Duchenne muscular dystrophy. The consequence of these defects is commonly thought to be increased fragility of muscle cells and contraction-induced damage and death.¹

Integrins, which are $\alpha\beta$ -heterodimeric receptors, represent a parallel system to the dystrophin-glycoprotein complex by which the cytoskeleton is linked to the extracellular matrix in muscle. These receptors have increasingly been recognized to play a major role in the pathogenesis of muscular dystrophies. Expression of integrins is altered in muscular dystrophies caused by defects of the dystrophin-glycoprotein complex. Levels of $\alpha 7\beta 1$ -integrin are severely diminished in patients with laminin- $\alpha 2$ chain congenital muscular dystrophy and in *dy/dy* mice that lack the laminin- $\alpha 2$ chain.² Conversely, expression of $\alpha 7\beta 1$ -integrin is up-regulated in patients with Duchenne muscular dystrophy and in *mdx* mice that lack dystrophin.² Interestingly, overexpression of $\alpha 7\beta 1$ -integrin in *mdx/utr* ($-/-$) mice that lack both dystrophin and utrophin leads to symptomatic improvement and to an increase in viability.³ These findings suggest that integrins can compensate for absence of the dystrophin-glycoprotein complex. In fact, mice with deletion of both dystrophin and the $\alpha 7$ -integrin subunit^{4,5} or of both sarcoglycan and the $\alpha 7$ -integrin subunit⁶ display muscle damage that is much more severe than that in mice with single mutations of either integrins or dystrophin-glycoprotein complex compo-

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Muscular dystrophies are a diverse group of genetic diseases that lead to progressive muscle degeneration,

nents. Finally, mutations in the integrin genes themselves, including $\alpha 7$ -integrin (*itga7*) and $\alpha 5$ -integrin (*itga5*), lead to muscular dystrophies in humans and mice.^{7–10}

The downstream factors that mediate integrin function in skeletal muscle are not well understood. One candidate is integrin-linked kinase (Ilk), a serine/threonine kinase and adaptor protein that interacts with the cytoplasmic portion of the $\beta 1$ -integrin subunit.^{11–13} Ilk acts as part of a complex with PINCH and α/β -parvin, recruiting actin along with components of several signaling pathways to sites of focal adhesions.^{11–13} Ilk has been shown to regulate integrin-associated rearrangement of actin filaments through a phosphatidylinositol 3-kinase/Akt/Rac1 pathway.¹⁴ In addition, Ilk has kinase activity *in vitro* and is able to induce phosphorylation of signaling factors such as PKB/Akt and GSK-3 β .^{11–13}

Ilk may play a particularly important role in skeletal muscle function. Deletion of *Ilk* in both *Caenorhabditis elegans* and *Drosophila melanogaster* causes failure of muscle attachment to focal adhesion sites.^{15,16} Recent studies have highlighted the critical role of Ilk in cardiac development and function, particularly as a component of the cardiac stretch sensor.^{17–20} Ilk may also play a role in congenital muscular dystrophies. In recent studies, we have demonstrated that deletion of *Ilk* in the neocortex results in cobblestone lissencephaly, a developmental brain defect associated with congenital muscular dystrophies in humans.^{21,22} These studies point to the possibility that Ilk may also play a role in mammalian skeletal muscle function.

To determine the role of Ilk in skeletal muscle, we used the cre/lox system to delete *Ilk* from skeletal muscle precursors in mice. The resulting *Ilk* mutants develop widespread progressive muscular dystrophy. The defect is initially most severe at the myofascial junctions (MFJs) with damage to surrounding muscle fibers, displacement of focal adhesion-related proteins, and actin cytoskeleton restructuring. In older mice, muscle damage becomes widespread with typical myopathic changes of muscular dystrophy. Our findings suggest that Ilk may be a cytoplasmic effector of $\alpha 7\beta 1$ -integrin in skeletal muscle function and may represent a novel factor in the pathogenesis of muscular dystrophies.

Materials and Methods

Mouse Lines

The *Ilk* floxed mice (*Ilk^{fl/fl}*) and the *Hsa-cre* mice have been described previously.^{23,24} The *Hsa-cre;Ilk^{fl/fl}* mutants were compared with sex-matched *Ilk^{fl/fl}* or *Ilk^{fl/+}* littermates in experiments with 6- and 9-month-old animals. *Ilk^{fl/fl}*, *Ilk^{fl/+}*, or *Hsa-cre;Ilk^{fl/+}* littermates were used as controls in 1-month-old animals. Genotyping for *Ilk* was performed by using the following primers: forward 1, 5'-CCAGGTGGCAGAGGTAAGTA-3'; reverse, 5'-CAAGGAATAAGGTGAGCTTCAGAA-3'; and forward 2, 5'-AAGGTGCTGAAGGTTTCAGAGA-3'. The expected pattern for forward 1 and reverse primers is as follows: wild-type, 1.9 kb; floxed allele, 2.1 kb; and cre excised allele, 230 bp. The expected pattern for forward 2 and reverse primers is

as follows: wild-type, 1.1 kb; and floxed allele, 1.3 kb. The animals examined were of mixed background. All animals were handled in accordance with protocols approved by the University of California at San Francisco Committee on Animal Research.

Human Tissue Samples

Biopsy specimens collected for diagnostic purposes and maintained by the University of California at San Francisco Department of Neuropathology were used with no identifying patient information according to guidelines and with approval of the University of California at San Francisco Committee on Human Research.

Antibodies

Antibodies were obtained from the following sources: Ilk monoclonal antibody (mAb) (sc-20019) and focal adhesion kinase polyclonal antibody (pAb) (A17; sc-557) (Santa Cruz Biotechnology, Santa Cruz, CA); integrin- $\alpha 7$ pAb (Randall Kramer, University of San Francisco, CA); $\beta 1$ D-integrin mAb (Arnoud Sonnenberg, Amsterdam, The Netherlands); $\beta 1$ D-integrin pAb (Ulrike Mayer, Norwich, UK); paxillin mAb (610619) (BD Transduction Laboratories, Lexington, KY); Englebreth-Holm-Swarm laminin pAb (L9393), dystrophin mAb (MANDRA1, D8043), vinculin mAb (V9131), and α -actin pAb (Sigma, St. Louis, MO); collagen IV pAb (Cosmo Bio, Tokyo, Japan); myosin heavy chain (MyHC F1.652, MyHC A4.840, MyHC and A4.74 mAbs) (Developmental Studies Hybridoma Bank, Iowa City, IA). Actin was visualized using rhodamine phalloidin (Molecular Probes, Eugene, OR).

Histological Analysis

Mice were sacrificed by cervical dislocation following 2.5% avertin anesthesia, and muscle tissue was dissected out immediately. Muscles were frozen using liquid nitrogen-chilled isopentane and used for cryostat sections (10 μ m). Alternatively, muscles were frozen directly in liquid nitrogen and used for cytoplasmic extract preparations. For visualization of damaged muscle fibers, animals were injected intraperitoneally with 1% Evans Blue dye (Sigma) resuspended in phosphate-buffered saline at 10 μ l/g body weight. Muscles were collected 20 hours after injection and processed as described above. H&E staining (Sigma) was performed using standard protocols. The creatine kinase assay (Catachem, Bridgeport, CT) and trichrome staining (Newcomer Supply, Middleton, WI) were performed using the manufacturer's instructions.

Immunohistochemistry

Frozen sections of muscle were fixed in cold acetone for 10 minutes and then blocked in 3% goat serum and 0.1% Triton X-100. Sections were preincubated in the Mouse on Mouse kit (Vector Laboratories, Burlingame, CA) when mouse antibodies were used. Sections were incubated in

primary antibodies overnight at 4°C and then incubated with goat anti-mouse, anti-rat, or anti-rabbit Alexa 488 or 594 for 2 hours (Molecular Probes).

Western Blotting

Muscles were dissected and lysed in modified radioimmunoprecipitation assay buffer [10 mmol/L Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 10 mmol/L Tris, pH 7.5, 100 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 10% glycerol, 1 mmol/L NaVO₄, 1 mmol/L NaF, and 1× Complete Mini protease cocktail (Roche Products, Welwyn Garden City, UK)]. Precleared lysates (20 μg) were resolved on 7.5% gradient SDS-PAGE gels (Bio-Rad, Hercules, CA). Proteins were transferred onto Immobilon-P transfer membranes (Millipore, Bedford, MA), blocked with 5% BSA, and incubated with primary antibodies overnight at 4°C. Membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA), followed by enhanced chemiluminescence reagent (Amersham Biosciences, Arlington Heights, IL). Membrane stripping was performed using the Restore Western Blot Stripping Buffer (Pierce, Rockford, IL).

Quantifications and Statistical Analyses

Statistical significance was determined using the Student's *t*-test. SEM was used for all quantification.

Results

Generation of Conditional Ilk Knockout Mice with Deletion Restricted to Mature Skeletal Muscles

To gain insight into the function of Ilk in skeletal muscles, we used the cre/lox system to generate conditional *Ilk* mutant mice with *Ilk* deletion restricted to skeletal muscle precursors. This approach was used to avoid early embryonic lethality that is observed with conventional *Ilk* knock-out mice.²⁵ We bred *Ilk^{fl/fl}* mice²³ with mice carrying cre recombinase under the human skeletal muscle actin (*Hsa*) promoter.²⁴ This promoter is first activated at embryonic day 9 in somites and leads to uniform expression of cre recombinase in approximately 90 to 95% of myofibers.²⁴ Cre recombinase is not expressed in other skeletal muscle components, including fibroblasts, muscle satellite cells, endothelial, and Schwann cells.²⁴ *Ilk* mutant mice were born at the expected Mendelian ratios, were viable, and did not exhibit any obvious behavioral defects. Deletion of the *Ilk* allele was confirmed by PCR, Western blotting, and immunohistochemistry (Figure 1). In Western blots of skeletal muscle extracts, a decrease of Ilk expression was observed in *Ilk* mutants compared with control littermates (mutant, 40.2 ± 9.4%, *N* = 3; normalized to controls at 100%, *N* = 3; Figure 1B). Ilk expression in skeletal muscle tissue sections from 1-month-old control animals was seen most intensely at the MFJ (Figure 1C) and in blood vessels and capillaries

Figure 1. Generation and analysis of 1-month-old skeletal muscle-specific *Hsa-cre;Ilk^{fl/fl}* mutant. **A:** PCR analysis of skeletal muscle DNA from *Ilk* mutant and control littermates. In *Ilk^{fl/+}* control extracts, two bands representing wild-type (1.9 kb) and floxed alleles (2.1 kb) are seen. In *Hsa-cre;Ilk^{fl/fl}* mutant extracts, only the *Ilk* floxed allele (2.1 kb) and the excised allele (230 bp) are detectable. The differing intensities of the two PCR products in the *Ilk* mutant reflect competition between differently sized templates. **B:** Western blots show a large decrease of Ilk protein expression in skeletal muscle extracts (mutant, 40.2 ± 9.4%, *N* = 3; normalized to controls at 100%, *N* = 3). Levels of Ilk expression were normalized to β-actin. **C:** Tissue sections from quadriceps muscles of control animals showed abundant expression of Ilk at the MFJs, particularly at the muscle epimysial interface. Ilk expression at the sarcolemma was low. **D:** In *Ilk* mutant sections, Ilk expression was significantly reduced at the MFJ. **E:** In control sections, strong Ilk expression was present in blood vessels and capillaries. **F:** In *Ilk* mutant sections, residual Ilk expression was detectable in untargeted components, including blood vessels, capillaries, nerves, fibroblasts, and satellite cells. These components were present at increased levels in *Ilk* mutants compared with control littermates, particularly around MFJs, and contributed to residual Ilk staining in mutant sections. **G:** A small level of background staining was detectable at the MFJs in sections stained with an antibody against embryonic myosin heavy chain. +/fl, *Ilk^{fl/+}* control; Cre;fl/fl, *Hsa-cre;Ilk^{fl/fl}* mutant; +/+, wild-type control; Con, control; Mut, mutant; Neg, negative control. Scale bar = 50 μm.

(Figure 1E). Levels of Ilk at the sarcolemma were low (Figure 1, C and E). In *Ilk* mutant sections, Ilk staining at the MFJ was significantly reduced (Figure 1D). Much of the residual Ilk staining in mutant sections was due to its presence in untargeted components, including blood vessels, capillaries, nerves, fibroblasts, and satellite cells (Figure 1F; data not shown). These components were present at increased levels in *Ilk* mutants compared with control littermates and manifested as increased cellularity and residual Ilk staining, particularly in MFJ regions (Figure 1, D and F).

Ilk Deletion in Skeletal Muscle Leads to Progressive Muscular Dystrophy

To determine the consequence of *Ilk* deletion on skeletal muscles, we examined H&E-stained cross sections of quadriceps muscles from *Ilk* mutants and littermate controls at 6 and 9 months of age. We found that deletion of *Ilk* led to widespread myopathic changes typical of muscular dystrophies, including degenerating and necrotic muscle fibers, fibers undergoing phagocytosis, clusters of regenerating fibers, large numbers of centrally nucleated fibers, increased variation of fiber size, split fibers, and endomysial fibrosis (Figure 2, A and B; H, I, and J).

These changes were particularly severe and well demonstrated in sections from 9-month-old animals [Figure 2, H (necrotic fibers, asterisks; fibrosis, arrow), I (clusters of regenerating fibers, arrows), and J (fiber undergoing phagocytosis, asterisk)].

Further studies of tissues from 6-month-old *Ilk* mutants highlighted the myopathic changes seen in histological sections. Multiple damaged muscle fibers in *Ilk* mutants were revealed by Evans blue dye taken up by cells with sarcolemmal defects (Figure 2D). No Evans blue-stained fibers were detectable in quadriceps of control littermates (Figure 2C). Active regeneration was highlighted in *Ilk* mutants by the presence of scattered muscle fiber clusters expressing embryonic myosin heavy chain, a marker of regeneration (Figure 2F).²⁶ Regenerating fibers were not detected in littermate control muscles (Figure 2E). The presence of ongoing regeneration in *Ilk* mutants was also supported by a large increase in the percentage of centrally nucleated muscle fibers, which indicate newly formed fibers (Figure 2G; mutant, $41.7 \pm 3.8\%$, $N = 3$; control, $0.8 \pm 0.5\%$, $N = 3$; $P < 0.001$). There was also increased variability of muscle fiber diameters in *Ilk* mutants, with an 8.5-fold increase in fibers smaller than $20 \mu\text{m}$ and a 10-fold increase in fibers larger than $65 \mu\text{m}$. These changes reflected an increase in small, regenerating fibers and large, abnormally hypertrophied fibers.

Interestingly, although there was no muscle damage in 9-month-old *Ilk*^{fl/+} or *Ilk*^{fl/fl} control littermates, 9-month-old *Hsa-cre;Ilk*^{fl/+} littermates revealed muscle damage comparable with the *Hsa-cre;Ilk*^{fl/fl} mutants (data not shown). This phenotype was not present in 1-month-old *Hsa-cre;Ilk*^{fl/+} or 1- or 6-month-old *Hsa-cre* animals. The appearance of muscle damage in *Hsa-cre;Ilk*^{fl/+} animals at 9 months most likely represents a dosage effect that does not become manifest until animals with one functional *Ilk* allele are aged. This finding further emphasizes the progressive nature of the *Ilk*-dependent myopathy.

Distribution of *Ilk*-Related Muscle Damage

Distinct forms of muscular dystrophy vary in the distribution of muscle damage among different muscle groups. In addition, some of these entities preferentially affect one muscle fiber type over another. To determine whether any specificity exists in the distribution of *Ilk*-related muscle damage, we analyzed *Ilk* mutant and control muscle sections from 6-month-old quadriceps, soleus, tibialis anterior, gastrocnemius, extensor digitorum longus, and diaphragm (Figure 3). These hindlimb muscles and the diaphragm are affected in various mouse models of muscular dystrophy and were selected to allow for comparison with other studies. We found that muscle damage in *Ilk* mutants was widespread in all muscles examined and tended to be more pronounced in peritendinous and perimysial regions. The most severe damage was found in the soleus, the diaphragm, and the vastus intermedius muscle of the quadriceps. In the soleus, damage was most pronounced at

the periphery of muscle fibers near the epimysium (Figure 3A, arrows). Particularly severe muscle damage was observed in the diaphragm of 6-month-old *Ilk* mutants where, in contrast to control littermates, an abundance of dystrophic fibers and endomysial fibrosis was found (Figure 3, E and F). Compromised integrity of the sarcolemma in *Ilk* mutant fibers of the diaphragm was highlighted by the high number of fibers that took up Evans blue dye (Figure 3H). Control littermates exhibited significantly smaller numbers of such fibers (Figure 3G). Other muscles examined showed various degrees of damage, with some regions within these muscles containing typical changes of muscular dystrophy and others showing nearly normal muscle histology (Figure 3, B–D).

In addition to the diaphragm, we consistently found that the vastus intermedius region of *Ilk* mutant quadriceps was more severely damaged than other muscles examined (Figure 2, A and B). In contrast, the vastus medialis, vastus lateralis, and rectus femoris regions were less severely affected by *Ilk* deletion (data not shown; Supplemental Figure 1, see <http://ajp.amjpathol.org>). Interestingly, the muscles most affected in our analysis, including the soleus and the diaphragm, have been shown to contain a high proportion of slow, type I oxidative muscle fibers.^{27,28} We hypothesized that the vastus intermedius may also contain a high percentage of type I fibers and that this may be the basis for the greater susceptibility of this muscle to *Ilk* related damage. We analyzed sections from 6-month-old quadriceps muscles from *Ilk* mutants and control littermates using myosin heavy chain antibodies and found that the vast majority of slow, type I muscle fibers are found in the vastus intermedius region (Figure 3, I and J, red fibers). In contrast, the vastus medialis, vastus lateralis, and rectus femoris muscles were made up of mostly fast, type IIB glycolytic fibers (Figure 3, K and L, unstained fibers). All regions of the quadriceps also contained type IIA fibers that were most abundant in the vastus intermedius region and near MFJs (Figure 3, I–L, green fibers). Although the fiber type composition of the vastus intermedius muscle was strikingly different from the rest of the quadriceps, we did not find any evidence of preferential fiber type damage in *Ilk* mutants. Both type I and type IIA fibers were found among degenerating and regenerating fibers of *Ilk* mutants, and there were no obvious changes in fiber type proportions or distribution in aged *Ilk* mutants (data not shown). The distinct fiber type composition of the vastus intermedius and the greater degree of damage in this region in *Ilk* mutants may reflect a functional specialization of this muscle that leads to greater dependence on *Ilk* to maintain fiber integrity. It has been hypothesized that skeletal muscles rich in slow muscle fibers, such as the vastus intermedius, may play an important role in sustaining neutral alignment of the lower extremity and reduce fatigue on larger adjacent muscles.²⁹ Further studies will reveal the mechanism by which *Ilk* may achieve this function.

Figure 3. Distribution of myopathic changes in *Ilk* mutant mice. Cross sections of soleus (A), gastrocnemius (B), extensor digitorum longus (C), tibialis anterior (D), diaphragm (E and F), vastus intermedius (I and J), and rectus femoris (K and L) from 6-month-old *Ilk* mutants (A–D, F, H, J, and L) and control littermates (E, G, I, and K). **A:** Soleus muscles of *Ilk* mutants revealed significant myopathic changes with most severe damage at the periphery of muscle fibers near epimysial regions (arrows). **B–D:** Various degrees of damage were present in the gastrocnemius, extensor digitorum longus, and tibialis anterior ranging from changes typical of muscular dystrophies to mild myopathic changes. **E and F:** Severe muscle damage was found in *Ilk* mutant diaphragms (F) compared with control littermates (E). **G and H:** Evans blue dye injection revealed large numbers of damaged muscle fibers taking up dye in the diaphragm of *Ilk* mutants (H). Control littermates exhibited much smaller numbers of these damaged fibers (G). **I–L:** Cross sections from quadriceps muscles of *Ilk* mutants (J and L) and control littermates (I and K) were examined for the presence of type I (red), type IIA (green), and type IIB (unstained) muscle fibers. Type I fibers were predominantly localized to the region of the vastus intermedius (I and J), with only rare type I fibers found outside this region. Type IIA fibers were most numerous in the region of the vastus intermedius (I and J) and in regions surrounding the MFJ in the rectus femoris (K and L). The remaining regions of the quadriceps consisted almost exclusively of type IIB fibers (not shown). No obvious differences in fiber type distribution or proportions were seen between *Ilk* mutants and control littermates. Gastroc, gastrocnemius; EDL, extensor digitorum longus; TA, tibialis anterior; v. intermedius, vastus intermedius; Con, control; Mut, mutant. Scale bars: 100 μ m (A–F); 250 μ m (I–L).

Muscle Damage in Young *Ilk* Mutants Is Limited to the MFJ

To determine the origin of muscle defects observed in 6- and 9-month-old *Ilk* mutants, we examined quadriceps muscles from *Ilk* mutants and control littermates at 1 month of age. We found that in contrast to aged animals, young *Ilk* mutants showed minimal damage in the majority of quadriceps muscle fibers (eg, rectus femoris; Supplemental Figure 1, see <http://ajp.amjpathol.org>). However, clear evidence of muscle damage was present around MFJs of *Ilk* mutants, particularly in epimysial regions (Figure 4, A and B). Histological findings included typical dystrophic changes similar to those seen in older

animals. There was a significant increase in centrally nucleated muscle fibers near the MFJs of *Ilk* mutants (mutant, $24.3 \pm 2.6\%$, $N = 4$; control, $1.0 \pm 1.0\%$, $N = 3$; $P < 0.001$; distance of 0 to 622 μ m from MFJ), indicating an increase in regeneration in this region (Figure 4C). Supporting this finding was the appearance in mutant quadriceps of increased numbers of muscle fibers expressing embryonic myosin heavy chain (mutant, $2.6 \pm 0.2\%$, $N = 2$; control, $0.1 \pm 0.1\%$, $N = 2$), as also seen in 6-month-old mutants (Figure 2, E and F). Centrally nucleated fibers were nearly absent, and the histology appeared normal in regions further away from the MFJ in both *Ilk* mutants and control littermates (data not shown). Creatine kinase levels were not elevated in *Ilk* mutants

Figure 2. Deletion of *Ilk* leads to progressive myopathic changes in skeletal muscle of mice. Cross sections of quadriceps muscles from 6-month-old control littermates (A, C, and E) and *Ilk* mutants (B, D, and F) and from 9-month-old *Ilk* mutants (H–J). **A and B:** H&E-stained sections of *Ilk* mutant quadriceps showed widespread muscle damage with changes typical of muscular dystrophies (B). Damage was most severe in the region of the vastus intermedius, shown. **C and D:** Analysis of Evans blue-injected quadriceps muscles by fluorescent imaging revealed a large number of damaged fibers in *Ilk* mutants (D), whereas no such fibers were detectable in littermate controls (C). **E and F:** Multiple clusters of regenerating muscle fibers expressing embryonic myosin heavy chain (eMyHC) were found in *Ilk* mutant quadriceps (F), but no such fibers were identified in control littermates (E). **G:** Quantification of centrally nucleated fibers in quadriceps of *Ilk* mutants and control littermates showed a significant increase of these fibers in *Ilk* mutants (41.7 ± 3.8 versus $0.8 \pm 0.5\%$, $N = 3$). **H–J:** H&E-stained sections from 9-month-old *Ilk* mutants showed severe muscle damage, including necrotic fibers (asterisks, H), focal areas of fibrosis (arrow, H), large clusters of regenerating fibers (arrows, I), and fibers undergoing phagocytosis (asterisk, J). Con, control; Mut, mutant. Scale bars: 100 μ m (A, B, E, F, and H–J); 250 μ m (C and D).

compared with control littermates or to standard normal values for mice (mutant, 90 ± 30 U/L, $N = 4$; control, 90 ± 31 U/L, $N = 2$; standard values, 24 to 195 U/L). These findings indicate that the MFJ is the initial site of damage in the absence of Ilk and suggest the possibility that Ilk plays a significant role at sites of greatest muscle tension.

Focal Adhesion Proteins Are Disrupted at the MFJ of Ilk Mutants

MFJs are stabilized by protein complexes that link the actin cytoskeleton to the extracellular matrix. Components of these complexes, which along with Ilk form focal adhesions in other systems, are found at increased levels at the MFJs.³⁰ To determine whether the absence of Ilk leads to disruption of these complexes, we examined the distribution of several focal adhesion proteins at the MFJ of 1-month-old *Ilk* mutants and control littermates. In control animals, focal adhesion proteins, including vinculin, paxillin, focal adhesion kinase, β 1D-integrin, dystrophin, and α 7-integrin, were strongly expressed in muscle fibers adjacent to the MFJ, with intense expression at the muscle-tendon interface (Figure 4D, low and high power images). In contrast, these junctional proteins in *Ilk* mutants were no longer confined to the interface between muscle and tendon but were found to encircle multiple muscle fibers in an uneven pattern near the MFJ (Figure 4D). In addition, there was increased staining in the interstitium around regions of increased cellularity and vascularity (Figure 4D). The pattern appeared similar for all focal adhesion proteins examined as represented by colocalization studies of dystrophin and α 7-integrin (Figure 4D, bottom panels).

To better understand the nature of the MFJ defect that occurs in the absence of Ilk, we examined changes to the extracellular matrix and the actin cytoskeleton in longitudinal sections of 1-month-old *Ilk* mutants. We found that whereas laminin evenly outlined muscle fibers in control sections with no enhancement at the MFJ, laminin was abnormally thickened near damaged junctional fibers of *Ilk* mutants (Figure 5B, arrow). These damaged fibers showed a striking restructuring of the actin cytoskeleton, with multiple "flames" of actin present at the fiber ends (Figure 5D). In contrast, control sections showed enhancement of actin staining in a tight linear pattern that was confined to the muscle-tendon interface (Figure 5C). Muscle fiber regions that were not immediately adjacent to the MFJ showed a normal striated pattern of costameric actin in both mutants and controls, indicating that

the cytoskeletal defect was specific for muscle fiber ends (Figure 5, C and D).

The abnormalities of laminin and actin at MFJs of *Ilk* mutants coincided with abnormal distribution of focal adhesion-related proteins in this region. Both vinculin and dystrophin showed uneven distribution and abnormal spreading away from the muscle-tendon interface (Figure 5, E–H). Fibers appeared jagged and detached from the tendon (Figure 5J). The abnormality of the actin cytoskeleton was confined to fiber ends and colocalized with abnormalities of focal adhesion proteins like dystrophin (Figure 5L).

The abnormal distribution of focal adhesion proteins and restructuring of the actin cytoskeleton at the MFJ indicates that Ilk is critical for the proper formation and/or maintenance of adhesion sites between muscle fiber ends and the tendon in muscle.

Discussion

In this study, we demonstrated an important role of Ilk in skeletal muscle function. Mice lacking Ilk developed progressive muscular dystrophy that began near myofascial junctions and became widespread throughout remaining muscle regions. Damage near the MFJ was associated with displacement of focal adhesion-related factors and restructuring of the actin cytoskeleton. Muscle fiber ends appeared detached from the tendon, suggesting that absence of Ilk may lead to abnormal adhesion of fibers in this region.

Role of Ilk in Mechanotransduction at the MFJ

Skeletal muscle fibers rely on a proper connection with the extracellular matrix to maintain cell integrity and to transduce muscle tension into intracellular signals that activate a variety of signaling pathways. This link is performed by laminin receptors present on the cell surface that include the dystrophin-glycoprotein complex and the integrin complex. Our study and others^{7,31} suggest that the integrin complex is particularly important at the site of greatest muscle tension, the myotendinous junction, which includes the myofascial component at the muscle fiber/epimysial interface. The fascial component is continuous with the tendinous region and contributes to muscular force transmission through both intramuscular and intermuscular pathways.³² The main integrin of mature skeletal muscle, α 7 β 1, is strongly expressed at both the myotendinous and myo-

Figure 4. Deletion of *Ilk* leads to muscle damage at the MFJ and mislocalization of focal adhesion-related proteins in 1-month-old animals. **A** and **B**: H&E-stained cross sections of quadriceps from *Ilk* mutants showed severe muscle fiber damage near the MFJ and in the region of the vastus intermedius (**B**). No damage was found in control littermate sections in this region. Shown is a section from an *Hsa-cre;Ilk^{fl/y}* animal that was indistinguishable from *Ilk^{fl/y}* or *Ilk^{fl/y}* littermates at this age (**A**). **C**: Quantification of centrally nucleated fibers in the region of the MFJ (distance of 0 to 622 μ m from MFJ) showed a significant increase of these fibers in *Ilk* mutants (24.3 ± 2.6 versus $1.0 \pm 1.0\%$; $N = 3$, control; $N = 4$, mutant). **D**: Cross sections from quadriceps muscles of *Ilk* mutants and control littermates shown at low and high magnification. Immunostaining for focal adhesion proteins, including vinculin, paxillin, focal adhesion kinase, β 1D-integrin, dystrophin, and α 7-integrin, showed an abnormal distribution of these proteins at MFJ of *Ilk* mutants. Whereas in control sections, these proteins were limited to the muscle-tendon interface, in *Ilk* mutants, multiple fibers were completely encircled by these proteins, and increased expression was found in fibers that were not immediately adjacent to the MFJ. Panels labeled "Merge" show colocalization of dystrophin and α 7-integrin and are representative of the high degree of colocalization between all focal adhesion proteins tested. Con, control; Mut, mutant. **Asterisk**, MFJ. Scale bars: 100 μ m (**A** and **B**); 50 μ m (**D**).

Figure 5. Damage to distal ends of muscle fibers and actin cytoskeleton restructuring in 1-month-old *Ilk* mutants. Longitudinal sections of quadriceps muscles from *Ilk* mutants (**B, F, J, D, H, and L**) and control littermates (**A, E, I, C, G, and K**) were co-immunostained for laminin and vinculin (**A, B, E, and F**) or dystrophin and actin (**C, D, G, and H**). Merged images are shown in **I–L**. **A and B:** In control sections, laminin evenly outlines each muscle fiber (**A**), whereas mutant sections show thickening of laminin in damaged fibers at the MFJ (**B, arrow**). **C and D:** In sections from control littermates, actin is found in a linear costameric pattern throughout the muscle fibers (**C, arrow**) and in a tight, intense linear pattern at fiber ends near the tendinous insertion (**C, asterisks**). In *Ilk* mutants, actin restructuring is observed as “flames” of actin at fiber ends (**D, asterisk**), whereas the regular costameric staining is preserved in the remainder of each mutant fiber (**D, arrow**). **D: Inset** shows the actin flames at lower intensity fluorescence to highlight the thread-like morphology of actin fibers. **E and F:** In control littermates, vinculin shows a tight enhancement of staining in muscle fiber ends bordering the MFJ (**E**). In *Ilk* mutants, there is an abnormal distribution of vinculin, with spreading and displacement of vinculin from the MFJ (**F**). In merged sections, muscle fibers appear jagged and displaced from the tendinous insertion of *Ilk* mutants (**J**), whereas a tight alignment is seen in control fibers (**I**). **G and H:** Dystrophin is found intensely expressed at the muscle-tendon interface of control littermates (**G, asterisks**). At the distal ends of *Ilk* mutant fibers, dystrophin mirrors the abnormal structure of actin (**H**). Merged images highlight that actin cytoskeleton abnormalities are confined to terminal regions of muscle fibers where focal adhesion proteins are abnormally distributed (**K and L**). Scale bars = 50 μ m.

epimysial junctions. *Ilk* follows a similar pattern of expression and plays an important role at this location, as documented by the results we obtained in this study.

Ilk is particularly well positioned for a role in mechanotransduction because it is both directly and indirectly involved in the connection of integrins to the actin cytoskeleton. *Ilk* links the cytoplasmic portion of β 1-integrins to parvin and through kindlin and migfilin to filamin, which in turn bind directly to the actin cytoskeleton.^{11–13} *Ilk* also regulates actin dynamics through the parvin/ α -PIX/Rho-family GTPase and the PINCH/Nck/WASp/Arp 2/3 pathways.^{11–13} Our study showed major abnormalities of the actin cytoskeleton at the MFJ of *Ilk* mutants, suggesting that the absence of *Ilk* may preclude normal actin dynamics by disrupting one or both of these pathways.

Supporting the function of *Ilk* in mechanotransduction is its critical role as a component of the cardiac stretch sensor in mice and zebrafish.^{17,18} Conditional *Ilk* mutant mice develop dilated cardiomyopathy and spontaneous heart failure,¹⁷ whereas *Ilk*^{L308P} mutant zebrafish display progressive reduction of cardiac contractility and severe down-regulation of the stretch-responsive genes *anf* and *vegf*.¹⁸ These effects may be comparable with those found at the MFJ of *Ilk* mouse mutants, where continued use of skeletal muscle causes progressive damage at the site of greatest muscle tension. Further studies will reveal the mechanisms involved in this process.

Our results also indicate that *Ilk* is not required for recruitment of junctional proteins to the MFJ, including its direct binding partners β 1-integrin and paxillin, because these proteins were present, albeit in a disorganized

Figure 6. Ilk expression is enhanced at the MFJ and in regenerating muscle fibers from muscular dystrophy patients. **A–C:** Five-year-old boy with Duchenne muscular dystrophy. Typical histological changes of muscular dystrophy were present in this dystrophin-negative patient. In addition, histological sections showed a myofascial insertion with multiple regenerating fibers flanking the fascia (**A**, arrow). Ilk expression was significantly enhanced in muscle fibers lining the MFJ (**B**). Many fibers near the MFJ had abnormally enhanced Ilk staining at the sarcolemma and within individual muscle fibers (**C**). **D–F:** Six-year-old boy with Becker dystrophy. This patient had typical histological changes of muscular dystrophy with multiple clusters of regenerating muscle fibers (**D**). These regenerating fibers showed abundant Ilk expression (**E**) compared with normal appearing fibers in other regions (**F**). Ilk expression was also high in blood vessels (**F**) and nerves (data not shown). Scale bars: 100 μ m (**A**, **B**, and **F**); 50 μ m (**D**, **E**, and **C**).

pattern, at the MFJ of *Ilk* mutants. Nevertheless, mislocalization of junctional proteins may make them less effective at stabilizing MFJs and may result in detachment and death of fibers in this region.

Comparison of *Ilk* Mutants to Integrin- α 7 and - β 1 Mutants

The phenotype of mice lacking *Ilk* in skeletal muscle described in this study are similar to those found in mice lacking the integrin- α 7 β 1 (*Itga7*^{-/-}).⁷ Both the targeted deletion of *Ilk* and the null mutation of α 7-integrin result in viable animals with no apparent defects of muscle development. However, both mutants show muscle damage that most prominently and severely affects the MFJ.^{31,33} The close similarity between the *Ilk* mutant and the α 7-integrin mutant suggests that *Ilk* may represent a cytoplasmic effector of α 7 β 1-integrin function for maintenance of the MFJ and is a candidate factor in the pathogenesis of α 7-integrin deficiency in humans.

Our findings in *Ilk* mutants are in contrast to those in conditional mutants of β 1-integrin. The β 1-integrin mutants, which were generated using the cre/lox system and an *Hsa-cre* line similar to the one used in our study, show abnormal skeletal muscle development and perinatal death associated with defects of myocyte fusion and sarcomere assembly.³⁴ In contrast, such defects are ab-

sent in *Ilk* mutants. This finding is surprising because, as an important effector of β 1-integrin function, *Ilk* might have been expected to play similar roles in muscle development. In addition, cell culture studies have implicated *Ilk* in muscle cell differentiation.^{35,36} However, our findings do not support a major role for *Ilk* in muscle development. It is possible that in place of *Ilk*, other adaptor proteins, such as focal adhesion kinase, perform integrin functions during muscle development.³⁷ Instead, *Ilk* appears to be specifically adapted for the maintenance of high-tension regions such as the myofascial insertions in adult muscle.

Interestingly, myotendinous abnormalities have been described in mouse models of other muscular dystrophies, including animals lacking dystrophin,^{38,39} α -dystrobrevin,⁴⁰ and α 2-laminin.⁴¹ These findings suggest that damage to the MFJ may be an underappreciated feature of many muscular dystrophies involving the dystrophin-glycoprotein complex. Our preliminary studies of patients with dystrophin-negative muscular dystrophies show high *Ilk* expression in fibers lining tendinous insertions (Figure 6B) and in abnormal fibers surrounding these regions (Figure 6C), suggesting that *Ilk* may compensate partially for the absence of constituents of the dystrophin-glycoprotein complex at the MFJ. In addition to its role at the MFJ, *Ilk* may also be involved in regeneration of damaged myofibers of dystrophic muscle. We

have observed increased expression of Ilk in regenerating muscles from muscular dystrophy patients (Figure 6E). Future studies will address whether deletion of *Ilk* specifically from satellite cells will affect the regenerative capacity of skeletal muscle.

Our finding that Ilk is critical for proper skeletal muscle function opens the way to dissecting out integrin-mediated pathways involved in muscular dystrophies. Such studies promise to clarify the mechanisms by which integrins play compensatory roles in dystrophin- and laminin-related muscular dystrophies. In addition, they will provide insight into mechanisms by which abnormalities of integrin function, such as integrin- α 7 deficiency, lead to the development of muscular dystrophies.

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