A Mouse Model for Dominant Collagen VI Disorders

HETEROZYGOUS DELETION OF Col6a3 EXON 16

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Te-Cheng Pan¹, Rui-Zhu Zhang¹, Machiko Arita¹, Sasha Bogdanovich¹, Sheila M. Adams³, Sudheer Kumar Gara¹, Raimund Wagener⁵, Tejvior S. Khurana¹, David E. Birk⁶, and Mon-Li Chu¹†

From the ¹Department of Dermatology and Cutaneous Biology, Thomas Jefferson University, Philadelphia, Pennsylvania 19107, ²Department of Physiology and Pennsylvania Muscle Institute, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104, ³Department of Molecular Pharmacology and Physiology, University of South Florida, Morsani College of Medicine, Tampa, Florida 33612, and ⁴Center for Biochemistry and Medicine, Medical Faculty Cologne, University of Cologne, Cologne D-50931, Germany

Background: Dominant collagen VI gene mutations cause the severe Ullrich congenital muscular dystrophy (UCMD) and mild Bethlem myopathy. A mutant mouse mimicking the most common molecular defect in dominant UCMD patients was generated and characterized.

Results: A mutant mouse mimicking the most common molecular defect in dominant UCMD patients was generated and characterized.

Conclusion: The mutant mouse displays muscle and connective tissue abnormalities.

Significance: The mutant mouse provides an animal model for dominant collagen VI disorders.

Dominant and recessive mutations in collagen VI genes, COL6A1, COL6A2, and COL6A3, cause a continuous spectrum of disorders characterized by muscle weakness and connective tissue abnormalities ranging from the severe Ullrich congenital muscular dystrophy to the mild Bethlem myopathy. Herein, we report the development of a mouse model for dominant collagen VI disorders by deleting exon 16 in the Col6a3 gene. The resulting heterozygous mouse, Col6a3+/d16, produced comparable amounts of normal Col6a3 mRNA and a mutant transcript with an in-frame deletion of 54 bp of triple-helical coding sequences, thus mimicking the most common molecular defect found in dominant Ullrich congenital muscular dystrophy patients. Biosynthetic studies of mutant fibroblasts indicated that the mutant α3(VI) collagen protein was produced and exerted a dominant-negative effect on collagen VI microfibrillar assembly. The distribution of the α3(VI)-like chains of collagen VI was not altered in mutant mice during development. The Col6a3+/d16 mice developed histopathologic signs of myopathy and showed ultrastructural alterations of mitochondria and sarcoplasmic reticulum in muscle and abnormal collagen fibrils in tendons. The Col6a3+/d16 mice displayed compromised muscle contractile functions and thereby provide an essential preclinical platform for developing treatment strategies for dominant collagen VI disorders.

Collagen VI is a microfibrillar extracellular matrix (ECM)² protein found in almost all tissues. Triple-helical collagen VI monomers consist of three different α chains (1). Monomers made up of the α1(VI), α2(VI), and α3(VI) chains are the most abundant and best characterized. Each α chain is composed of a central triple-helical domain of 335 or 336 amino acids with a repeating Gly-X-Y amino acid sequence (2), which is flanked by N- and C-terminal globular domains mainly composed of von Willebrand factor type A modules (3, 4) (Fig. 1A). The N- and C-globular domains of the α3(VI) chain are much larger than those in the α1(VI) and α2(VI) chains. As a consequence, the α3(VI) chain is about 3 times the size of the α1(VI) and α2(VI) chains (~3000 versus ~1000 amino acids). The triple-helical collagen VI monomers undergo intracellular assembly into dimers and tetramers and are then secreted (Fig. 1B). In the extracellular space, tetramers associated end-to-end into collagen VI microfibrils, which display a characteristic double-beaded structure (1). A strategic single cysteine residue in the triple-helical domain and short cysteine-rich segments flanking the triple-helical domain of each chain are essential for the assembly and stability of collagen VI dimers, tetramers, and microfibrils (2–4) (Fig. 1A and B). Three α3(VI)-like α chains, α4(VI), α5(VI), and α6(VI), have been identified in different animal species in recent years (5, 6) (Fig. 1A). Humans do not produce the α4(VI) protein because the gene is inactivated by a chromosomal translocation. These α3(VI)-like chains can substitute the α3(VI) chain in the assembly of collagen VI, but they have rather restricted tissue distributions (7).

Dominant and recessive mutations in the COL6A1, COL6A2, and COL6A3 genes lead to a continuous spectrum of disorders characterized by muscle weakness and connective tissue abnormalities. Ullrich congenital muscular dystrophy (UCMD, OMIM #254090), the severe end of the clinical spectrum caused by an absence or marked deficiency of collagen VI, is currently recognized as one of the most common types of congenital muscular dystrophies (8–11). Apart from muscle weakness,
UCMD patients display connective tissue defects, including joint contractures, distal hyperlaxity, and skeletal anomalies (scoliosis, kyphosis, torticollis, spinal stiffness, and Achilles ten- don contractures) (8, 9). In addition, skin abnormalities, e.g., velvety skin, hyperkeratosis, and keloid formation, are common. Severely affected patients either never achieve independent ambulation or can walk initially, but the ability is lost by the teen years (8, 12, 13). Respiratory insufficiency develops progressively, and ultimately patients almost invariably need ventilation support. Bethlem myopathy (OMIM #158810),
resulting from dominant collagen VI mutations primarily, is at the mild end of the clinical spectrum (8, 9). Due to a slowly progressive clinical course, more than two-thirds of the Bethlem myopathy patients over 50 years of age require aids for ambulation (14). Mutations in collagen VI also underlie disease phenotypes intermediate between classical UCMD and Bethlem myopathy, limb-girdle muscular dystrophy, and myosclerosis (OMIM #255600) (8, 9, 15, 16). Mutations in the COL6A5 and COL6A6 genes have not been described to date.

Severe UCMD phenotypes are caused by either recessive or dominant negative collagen VI gene mutations (17, 18). The recessive UCMD patients typically bear homozygous or compound heterozygous nonsense or frameshift mutations, which trigger nonsense-mediated mRNA decay and thus result in complete absence or drastic reduction of collagen VI (8, 9, 19). A mouse model for recessive UCMD (21) has been reported, suggesting the presence of genetic modifiers (20). A mouse mutant lacking the α1(VI) chain of collagen VI, the Col6a1 null mouse, has served as an animal model for recessive UCMD (21). Recently, we described a Col6a3 mutant mouse, Col6a3^hm/hm, which lacks normal α3(VI) chain but produces a low level of non-functional α3(VI) chain (22). The Col6a1 null and Col6a3^hm/hm mice both display relatively mild myopathic pathology and have normal life spans. Thus, the mouse models for recessive collagen VI disorders have substantially milder phenotypes than the patients.

De novo dominant collagen VI gene mutations have been found in more than half of the severely affected UCMD patients studied to date (8, 9). These patients often carry a heterozygous mutation in one of the three collagen VI genes that affects the amino acid sequence in the N-terminal region of the triple-helical domain before the single cysteine (18, 23–25). The mutations are either splice site mutations that cause small intron deletions in the triple-helical domains or missense changes that alter the obligatory glycine residues of the repetitive Gly-X-Y sequences. In contrast to the total absence or severe deficiency of collagen VI in recessive UCMD, abnormal collagen VI protein is abundantly present in the interstitial connective tissue between muscle fibers in the dominant UCMD patients (18). The pathological mechanisms and treatment strategies for the dominant and recessive patients are not identical. For instance, the presence of mutant collagen VI in the endomysium likely alters the muscle extracellular microenvironment, which in turn may influence the cellular activities of the adjacent muscle cells in a manner that differs from the total absence of collagen VI protein in recessive UCMD. Moreover, silencing the mutant allele can be explored as a treatment strategy for dominant UCMD and Bethlem myopathy patients. To our knowledge an animal model for dominant collagen VI disorders has not been described. In this study we generated a mutant mouse bearing the most common molecular defect found in dominant UCMD patients, i.e. skipping of exon 16 in the COL6A3 mRNA (11, 13, 23, 25). We show that mutant mice heterozygous for the Col6a3 exon 16 deletion, named Col6a3^+/d16, display muscle and connective tissue abnormalities resembling, albeit milder than, the dominant UCMD patients.

EXPERIMENTAL PROCEDURES

Col6a3 Gene Targeting—A mouse 129/Sv genomic cosmid clone encoding part of the Col6a3 gene was used to prepare the gene targeting construct (Fig. 2A). Exon 16 coding region was replaced by a neomycin resistance gene driven by the phosphoglycerol kinase promoter (PGK-Neo) and flanked by loxP sequences. The targeting vector contained a 6.1-kb DraIII fragment as the long arm, a 2.5-kb HindIII fragment as the short arm, and a diphtheria toxin A gene for negative selection. The targeting construct was linearized with SacI and electroporated into mouse 129/Sv embryonic stem cells. Neomycin-resistant cell clones were screened by Southern blotting using Bgl-cut genomic DNA and a 0.7-kb external probe generated by PCR from the genomic subclone with primers GAGAGGAGAGATGCCTGGGATTCG and GTGAGGCAAGAATGATG- TAGAG. Two correctly targeted embryonic stem clones (wild type, 5.8 kb; mutant, 3.6 kb) were injected into mouse C57BL/6 blastocysts to generate chimeric mice, which were crossed with wild-type C57BL/6 mice to obtain germ-line transmission of a targeted allele, Col6a3^d16N, which contained the neomycin resistance gene (Fig. 2A). The heterozygous Col6a3^+/d16N mice were crossed with transgenic mice bearing a Cre recombinase gene driven by a β-actin gene promoter to delete the neomycin resistance gene, yielding the final targeted allele, Col6a3^d16. Heterozygous mutant mice, Col6a3^+/d16, were backcrossed to C57BL/6 mice for 10 generations. Genotyping of the mice was performed by PCR. Forward primer CACTGCGGCCAGATTAGGACT and reverse primer CCAGAGCAGCTAACAAGTCTCCA yielded an ~450-bp product from the Col6a3^d16 allele and a faint 834-bp band from the wild type allele. Forward primer GCATACTAGGACGCTCAC and the same reverse primer generated a 399-bp PCR product from the wild type allele but no product from the targeted allele.

Fibroblasts, RNA Isolation, RT-PCR, and DNA Sequencing—Primary fibroblast cultures were established from littermates of the three genotypes at embryonic day 17 following standard protocols and grown in DMEM with 10% fetal bovine serum (Invitrogen). Total RNA was prepared from confluent fibroblasts using the RNAeasy mini kit (Qiagen). RT-PCR of the mRNA encompassing the exon 16 coding region was carried out using forward primer TCTTGAACGTTGTGGCTAACC and reverse primer TTTTCTCAGGAGAACAGGG. The resultant PCR products from homozygous and wild type cells were treated with ExoSAP-IT (Affymetrix) and subject to DNA sequencing using BigDye Terminator kit and 3730 DNA Sequencer (Applied Biosystems).

Collagen VI Biosynthesis—Embryonic fibroblasts were metabolically labeled with [35S]cysteine in serum-free medium containing 50 μg/ml sodium ascorbate overnight as described (18). Culture medium and cell lysate were immunoprecipitated with an antibody specific for the α1(VI) chain (26) as described previously (18). The precipitated material was analyzed after reduction with 25 mM dithiothreitol on 3–8% polyacrylamide gels (NuPAGE Novex Tris-acetate gel, Invitrogen) or without...
Western Blot Analysis—Tissues were frozen in liquid nitrogen and disrupted using a stainless steel pulverizer pre-chilled in liquid nitrogen. The resultant tissue powder was lysed in cold RIPA buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM PMSF, 1 mM Na₃VO₄, 1 mM NaF, and 1 μg/ml each of aprotinin, leupeptin, and pepstatin), incubated at 4 °C for 4 h with slow rotation, sonicated for 15 s twice, and then centrifuged at 14,000 × g for 10 min. Protein concentration of the tissue lysate was determined using the Bio-Rad protein assay kit. Equal amounts of total proteins were run on 4–12% NuPAGE Novex Bis-Tris gels and blotted onto PVDF membranes. The blots were incubated with primary antibodies at 4 °C overnight and developed with the ECL plus reagent (GE Healthcare). Antibodies used were polyclonal antibodies against \( \alpha \) (VI) and \( \alpha(\III) \) chains (26, 27) and \( \beta \)-actin (Sigma).

Histology and Immunostaining—Mouse embryos and tails were frozen in OCT compound (VWR International). Dissected muscles from postnatal mice were mounted on 10% gum tragacanth (Sigma) and frozen in liquid nitrogen-cooled isopentane. Sections of 6–8-μm were stained with hematoxylin-eosin. Measurement of single muscle fiber area and percentage...
of muscle fiber with central nuclei was performed as previously described (22). For immunostaining, cryosections were fixed with methanol and incubated with primary antibodies for 2 h at room temperature. The antibody reaction was detected with Cy3-labeled secondary antibodies (Jackson ImmunoResearch Laboratories). Fluorescence intensity of immunostained sections was measured using ImageJ software. Sections were also stained with DAPI to visualize nuclei. Immunostaining of fibroblasts was performed using cells grown in the presence of 50 μg/ml sodium ascorbate for 4 days post-confluency as described (18). Primary antibodies used were polyclonal antibodies against collagen VI α1, α3, α4, α5, and α6 chains (7, 26, 27), collagen I (Fitzgerald Industries International), tenascin-X (from Dr. Ken-Ichi Matsumoto), and periostin (R&D Systems). Samples were viewed using a Zeiss Axioskop epifluorescence microscope with a Toshiba 3CCD camera and ImagePro software (Media Cybernetics).

Physiological Analysis—Physiological studies were performed using female mice by methods described previously (28, 29). Briefly, weights of body, organs, and muscles were determined. Freshly dissected extensor digitorum longus (EDL) muscles were mounted between a force transducer and length controller in an organ bath containing oxygenated Ringer’s solution (pH 7.4) at room temperature. Muscle length was adjusted to achieve maximal twitch and tetanic response, and optimal length was determined. Muscles were subject to a series of three twitches, three tetanic contractions, and five eccentric contractions (ECCs) at a total duration of 700 ms with the muscle being lengthened by 10% of optimal length at velocity of 0.5 optimal length/s for mechanical property evaluation. ECC force decrement was calculated by the difference of isometric phase (500 ms) of the first and fifth ECCs. The cross-sectional area was determined using the Brooks-Faulkner approximation (30).

Electron Microscopy—Flexor digitorum longus (FDL) and Achilles tendons, and gastrocnemius muscles were dissected from 1-month-old mice and analyzed by transmission electron microscopy as previously described (31). Briefly, tissues were fixed in 0.1 M cacodylate buffer (pH 7.4) containing 4% paraformaldehyde (pH 3.2) and examined at 80 kV using a Tecnai 12 or post-fixed with 1% osmium tetroxide. Thin sections were post-fixed with 1% osmium tetroxide. Sections were also stained with DAPI to visualize nuclei. Immunostaining of the cell lysate and culture medium with an antibody specific for the α1(VI) chain (26). The anti-α1(VI) antibody should precipitate triple-helical collagen VI molecules containing the α1(VI) chain and also the free α1(VI) chain. When the immunoprecipitated material was analyzed on reduced polyacrylamide gels (Fig. 3A), all three collagen VI chains were found in the culture media of the wild type, heterozygous, and homozygous cells. The observation that the anti-α1(VI) antibody could immunoprecipitate all three collagen VI chains in the Col6a3<sup>d16/d16</sup> fibroblasts indicated that the mutant α3(VI) chain with an in-frame deletion of six Gly-X-Y repeats was synthesized, formed triple-helical collagen VI molecules with the other two chains, and was then secreted extracellularly. In the cell lysates, low levels of all three chains were found in all three genotypes (Fig. 3A, right panel), indicating that the majority of the triple-helical collagen VI molecules were secreted into the medium. When the immunoprecipitated products were analyzed under non-reduced conditions in composite agarose-polyacrylamide gels, collagen VI tetramers were detected in the culture media and cell lysates of all three genotypes, indicating that triple-helical collagen VI molecules containing the mutant α3(VI) chain could be assembled into tetramers and secreted (Fig. 3B). Notably, increased amounts of dimers and a product migrated below the monomers were seen in both the media and cell lysates of the Col6a3<sup>d16/d16</sup> fibroblasts and to a lesser extent in the Col6a3<sup>−/−</sup> counterparts (Fig. 3B, asterisks). The product migrated below the normal monomers could represent mutant monomers with alternative disulfide bonding. The result suggested that tetramer assembly from the mutant α3(VI) chain was somewhat compromised, resulting in the accumulation of dimers.

Collagen VI microfibrils are formed by an end-to-end association of the tetramers. To assess collagen VI microfibrillar formation, post-confluent fibroblasts from the Col6a3<sup>−/−</sup>, Col6a3<sup>+</sup><sup>+</sup><sup>/</sup><sup>d16</sup>, and Col6a3<sup>d16/d16</sup> mice were immunostained with antibodies specific for the α3(VI) or α1(VI) chain (26, 27). Collagen VI microfibrils were abundantly deposited in the ECM of the Col6a3<sup>−/−</sup> cells but not in the Col6a3<sup>+/−</sup> and Col6a3<sup>d16/d16</sup> fibroblasts (Fig. 4). The data indicated that abnormal collagen VI tetramers made up either the mutant α3(VI) chain exclusively (in Col6a3<sup>d16/d16</sup> cells) or a mixture of normal and mutant α3(VI) chain (in Col6a3<sup>+/−</sup> cells) could
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FIGURE 3. Collagen VI biosynthesis in fibroblasts from the Col6a3+/+, Col6a3+/d16, and Col6a3d16/d16 mice. Fibroblasts were grown in serum-free medium containing [35S]cysteine overnight. Culture medium and cell lysate were immunoprecipitated with anti-α1(VI) collagen antibody. A, immunoprecipitated samples were reduced with 25 mM DTT and separated on 3–8% polyacrylamide gels. Normal and mutant α3(VI) chains were synthesized, assembled into triple-helical collagen VI molecules with the α1(VI) and α2(VI) chains, and secreted. Note that fibronectin (FN), which is abundant in the culture medium, was co-precipitated. B, samples not treated with DTT were run on composite agarose-polyacrylamide gels. Collagen VI tetramers could be assembled from the mutant α3(VI) chains. Note the accumulation of dimers and the presence of a product below the normal monomers (asterisks) in the medium and cell fractions of the two mutant genotypes.

FIGURE 4. Immunostaining of collagen VI microfibrils deposited by the Col6a3+/+, Col6a3+/d16, Col6a3d16/d16, and Col6a3+/hm fibroblasts. Cells were grown for 4 days post-confluence and then immunostained with antibodies specific for the α3(VI) or α1(VI) chain. Microfibrils were barely detectable in the Col6a3d16/d16 and Col6a3d16/hm fibroblasts. The Col6a3+/hm fibroblasts deposited reduced amounts of collagen VI microfibrils. Magnification bar = 50 μm.

not assemble into long microfibrils. By contrast, fibroblasts from the Col6a3+/hm mice (22), which are equivalent to haploinsufficiency in Col6a3, deposited a reduced level of collagen VI microfibrils (Fig. 4).

Increased α3(VI) and α1(VI) Immunoreactivity in Mutant Embryos—To determine whether the mutant α3(VI) chain was produced in vivo, immunostaining of embryonic tissues during robust collagen VI synthesis at embryonic day 17 (E17) was performed. In coronal sections of the Col6a3+/+ embryos, both anti-α3(VI) and anti-α1(VI) antibodies showed a fibrous collagen VI staining pattern in the dermis, skeletal muscle, and around the ribs (Fig. 5A, a, d, and g). Surprisingly, increased immunoreactivity with both antibodies was observed in the Col6a3+/d16 and Col6a3d16/d16 embryos (Fig. 4A, b, c, e, and f). The strong immunoreactivity detected by the anti-α3(VI) chain antibody in the homozygous tissues indicated that the mutant α3(VI) chain was synthesized and deposited in tissues. High magnification images showed that the immunoreactivity in the mutant tissues did not exhibit the fibrous pattern but instead appeared amorphous (Fig. 5A, h and i). Western blot analysis of tissue extracts from hind limbs of E17 embryos showed that the α1(VI) and α3(VI) chains were increased in the heterozygous and homozygous mutant mice (Fig. 5B).

No Change in the Distribution of α3(VI)-like Chains in Mutant Tissues during Development—To determine whether the Col6a3 mutation resulted in a compensatory up-regulation of the three α3(VI)-like chains, the coronal sections of the E17 embryos were immunostained with chain-specific antibodies (7). The immunoreactivity and distribution of the α4(VI), α5(VI), and α6(VI) chains in the Col6a3+/d16 and Col6a3d16/d16 embryos were similar to the wild type embryos. In all three genotypes, the α4(VI) and α5(VI) chains were prominently expressed in the mucosal and submucosal layers of the gut but not detectable in the muscular skeletal tissues (data not shown). The α6(VI) chain was present in the skeletal muscle but absent in the dermis and ribs of all three genotypes, whereas the α3(VI) chain was abundant in all of these tissues (Fig. 6A). In addition, the α6(VI) chain was found in diaphragm but not in lung (except pleura) of all three genotypes (Fig. 6B), unlike the prominent expression of α3(VI) chain in lung tissue (not shown). Collagen VI is abundant in tendon tissue. Immunoreactivity of the α6(VI) chain in tendon was compared using 2-week-old mouse tails. Again, no difference was observed among the three
genotypes (Fig. 6C). The α6(VI) chain was expressed only in tendon sheaths but not within tendon fascicles, whereas the α3(VI) chain was robustly present in the tendon fascicles and less prominent in the tendon sheaths. In addition, the α6(VI) chain was absent in the nerves and vertebrae, in contrast to the α3(VI) chain.

Reduced α3(VI) and α1(VI) Immunoreactivity in Postnatal Col6a3+/d16 and Col6a3d16/d16 Muscles—Both homozygous and heterozygous mutant mice had no obvious abnormal phenotypes. They were fertile and had normal life spans, surviving up to 2 years of age. To assess collagen VI protein expression in skeletal muscles of the postnatal mutant mice, limb and diaphragm muscles from young mice (2–6 weeks) were examined by immunohistochemistry using the antibodies against the α1(VI) and α3(VI) chains. As shown in the representative images of quadriceps muscles from 1-month-old mice (Fig. 7), the α3(VI) and α1(VI) immunoreactivities were somewhat decreased in the Col6a3+/d16 muscle. In the Col6a3d16/d16 muscles, whereas the α3(VI) immunoreactivity was substantially reduced compared with the Col6a3+/d16 muscle, the decrease in the α1(VI) immunoreactivity between the two mutant genotypes was less visible. The data suggested that 1) the abnormal collagen VI protein present at high levels at embryonic stages (Fig. 5) were unstable and prone to degradation over time when the growth slowed down, and 2) collagen VI molecules with a chain composition other than α1(VI)/α2(VI)/α3(VI) might be present. To determine whether the three α3(VI)-like α chains could substitute for the α3(VI) chain and assemble with the α1(VI) and α2(VI) chains in muscle, immunostaining with antibodies specific for the α4(VI), α5(VI), and α6(VI) chains was performed. The α6(VI) chain was found at comparable levels in all three genotypes (Fig. 7), whereas immunoreactivity with the α4(VI) or α5(VI) chains was not detectable (data not shown). The results suggested that the α6(VI) chain could form triple-helical collagen VI molecules with the α1(VI) and α2(VI) chains in muscle, but there was no significant compensatory up-regulation of the α6(VI) chain in the two mutant genotypes.
Col6a3<sup>+/d16</sup> and Col6a3<sup>d16/d16</sup> Mice Display Myopathic Histology—Limb and diaphragm muscles from the Col6a3<sup>+/+</sup>, Col6a3<sup>+/d16</sup>, and Col6a3<sup>d16/d16</sup> mice at different ages were examined by histology. Variation in muscle fiber size, myofibers with centrally located nuclei, and increased endomysial connective tissue were readily seen in the Col6a3<sup>+/d16</sup> and

**FIGURE 6.** Distribution of the α6(VI) collagen chain is not altered in the mutant mice during development. Cryosections were immunostained with antibodies against α3(VI) and α6(VI) chains and treated with DAPI to visualize nuclei. A and B, coronal sections of E17 Col6a3<sup>+/+</sup>, Col6a3<sup>+/d16</sup>, and Col6a3<sup>d16/d16</sup> embryos. A, the α3(VI) chain is present in the dermis (d), ribs (r), and skeletal muscle (m). The α6(VI) chain is present in skeletal muscle but not in ribs and dermis of all three genotypes. DAPI images corresponding to the α6(VI) stainings are shown in the third row. B, the α6(VI) chain is present in diaphragm (di), skeletal muscle (m), pleura (arrows) but not in lung (lu) of all three genotypes. DAPI images are shown in the second row. C, cross-sections of 2-week mouse tails. The α6(VI) chain is present in tendon sheath (arrowheads) and muscle (m) but not in tendon fascicles (t), nerve (n), and vertebrae (v). The α3(VI) chain is present in tendon fascicles, muscle, nerve, and vertebrae. Magnification bars = 50 μm.
Col6a3<sup>d16/d16</sup> mice at age 6 months (Fig. 8A). The muscle abnormalities were already noticeable in younger mice. As shown in Fig. 8B, the percentages of myofibers with central nuclei in the Col6a3<sup>+/H11001/H11001</sup> and Col6a3<sup>d16/d16</sup> mice were higher than the wild type controls at ages 1, 3, 6, and 12 months. Measurement of single muscle fiber areas in quadriceps muscles from the 6-month-old Col6a3<sup>+/H11001/H11001</sup> and Col6a3<sup>d16/d16</sup> mice showed the wide distribution in muscle fiber sizes (Fig. 8C). There were more small and large fibers in the mutant mice compared with the wild type animals. The results indicated that both Col6a3<sup>+/d16</sup> and Col6a3<sup>d16/d16</sup> exhibited progressive myopathic histopathology.

*ECM Changes in the Col6a3<sup>+/d16</sup> and Col6a3<sup>d16/d16</sup> Muscles—Histological analysis of muscles from 6-month-old Col6a3<sup>+/d16</sup> and Col6a3<sup>d16/d16</sup> mice above suggested substantial increases in endomysial matrix. Therefore, immunostaining with selected ECM molecules was performed. In quadriceps muscles from 6-month-old mice, the α3(VI) and α1(VI) immunoreactivities were decreased in the Col6a3<sup>+/d16</sup> and Col6a3<sup>d16/d16</sup> muscles compared with the wild type counterpart (Fig. 9), similar to the findings of the young mice described above (Fig. 7). Among the α3(VI)-like chains, the α5(VI) chain was readily seen, but the α6(VI) chain was barely detectable. The α6(VI) immunoreactivity was similar in all three genotypes.

Figure 7. Immunoreactivity of collagen VI chains in skeletal muscles of 1-month-old Col6a3<sup>+/+</sup>, Col6a3<sup>+/d16</sup> and Col6a3<sup>d16/d16</sup> mice. A, representative images of cryosections from quadriceps muscles immunostained with polyclonal antibodies specific for the α3(VI), α1(VI), and α6(VI) collagen chains. Magnification bar = 50 μm. B, relative fluorescence intensity of images from three sets of independent samples. The intensity of the Col6a3<sup>+/+</sup> sample was set as 1.00. Values of the two mutant genotypes were mean ± S.D. (error bars). *, p < 0.05 between Col6a3<sup>+/+</sup> and Col6a3<sup>+/d16</sup>; #, p < 0.05 between Col6a3<sup>+/+</sup> and Col6a3<sup>d16/d16</sup>. The immunoreactivities of α3(VI) and α1(VI) chains were reduced in the Col6a3<sup>+/d16</sup> and Col6a3<sup>d16/d16</sup> muscles. In the Col6a3<sup>d16/d16</sup> muscle, the α1(VI) chain was readily seen, but the α3(VI) chain was barely detectable. The α6(VI) immunoreactivity was similar in all three genotypes.
Ultrastructural Alterations in the Col6a3/H11001/d16 and Col6a3d16/d16 Muscles—A distinct pathological finding of the Col6a1 null mice is the morphological alteration of mitochondria and sarcoplasmic reticulum in the skeletal muscle (33). Therefore, transmission electron microscopy of gastrocnemius muscles from 1-month-old mice was performed. The analyses showed ultrastructural changes in the mitochondria and sarcoplasmic reticulum of the Col6a3/H11001/d16 and Col6a3d16/d16 mice. A substantial proportion of the mitochondria in both mutant genotypes were enlarged and irregularly shaped, and the sarcoplasmic reticula were distended (Fig. 10A). In addition, collagen fibrils in the endomysial connective tissue of the two mutant genotypes appeared disorganized (Fig. 10B).

Abnormal Collagen Fibrils in the Col6a3/H11001/d16 and Col6a3d16/d16 Tendons—Patients affected with collagen VI disorders have tendon defects. Therefore, FDL and Achilles tendons from 1-month-old mice of the three genotypes were assessed by transmission electron microscopy. Cross-sectional images of both types of tendons revealed abnormal collagen fibril morphology in the mutant mice (Fig. 11). Collagen fibrils in the Col6a3/H11001/d16 and Col6a3d16/d16 tendons were less organized and sparse in some areas. Moreover, there were unusually large fibrils with cauliflower-like fibril contours and many small diameter fibrils. Abnormal collagen fibrils were mostly located in the pericellular regions (Fig. 11) and less...
abundant in areas distant from the cells (not shown). In addition, tenocyte organization was disrupted in the mutant genotypes with cellular processes defining extracellular domains containing collagen fiber poorly organized. The abnormal cellular organization is consistent with altered cell-matrix interactions in the presence of mutant collagen VI.

Muscle Function of the Col6a3\(^{+/d16}\) Mice Is Compromised—Histological and ultrastructural analyses indicated that the Col6a3\(^{+/d16}\) mice displayed notable muscle abnormalities. To further evaluable whether the Col6a3\(^{+/d16}\) mouse is a suitable animal model for dominant UCMD, physiological assessment of the Col6a3\(^{+/d16}\) muscles at ages 3 and 8 months was performed. The Col6a3\(^{+/d16}\) mice showed significantly reduced muscle contractile function at age 8 months (Table 1) but not at 3 months (data not shown). In the 8-month-old Col6a3\(^{+/d16}\) mice, growth and weight of some muscle groups were reduced (Table 1). EDL muscles from the Col6a3\(^{+/d16}\) and control mice were used to measure peak force. Twitch contraction force of the EDL muscle from the Col6a3\(^{+/d16}\) was significantly reduced. Tetanus contraction force of the Col6a3\(^{+/d16}\) EDL muscle also was decreased, but statistical significance was not reached. No significant differences were observed in ECC-induced force decrement and cross-sectional area of the EDL muscles.

DISCUSSION

Skipping of COL6A3 exon 16, due to splice site mutations or small deletion, is the most common molecular defect in dominant UCMD patients (11, 13, 23, 25). In this study we deleted exon 16 and its flanking sequence in the mouse Col6a3 gene by gene targeting. We show that the heterozygous mutant mice, Col6a3\(^{+/d16}\) mice, produce comparable amounts of the normal and exon 16-deleted Col6a3 mRNA similar to those patients with the heterozygous exon 16 skipping mutations (23, 25). The Col6a3\(^{+/d16}\) mice thus represent a mouse model of dominant UCMD.

The deletion of Col6a3 exon 16 removes 6 Gly-X-Y repeats in the N terminus of the triple-helical domain of the \(\alpha3(\text{VI})\) collagen chain (amino acids 16–33 counting from the beginning of the triple-helical domain) but preserves the unique cysteine residue (amino acid 50) essential for collagen VI tetramer assembly (Fig. 1, A and B). Current evidence suggests that folding of the three collagen VI chains into triple-helical monomers proceeds from the C to N terminus, similar to collagen type I (18, 34). Therefore, the mutant \(\alpha3(\text{VI})\) chain with a small in-frame deletion in the N terminus should be able to assemble into collagen VI triple-helical monomers. Examination of the amino acid sequence indicates that the deleted Gly-X-Y repeats are of low triple-helix propensity (35) (Fig. 2D). Therefore, the N terminus of the normal collagen VI triple helix is likely to be reduced.
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seen in fibroblasts from mice heterozygous for the nonfunctional Col6a3 mutant allele (Col6a3+/hm, Fig. 4), the results demonstrate that heterozygous exon 16 deletion in Col6a3 exerts a dominant negative effect on collagen VI microfibrillar assembly. In the homozygous cells, all of the tetramers are defective and cannot assemble microfibrils. Therefore, the Col6a3+/d16 mice should be equivalent to the collagen VI null mice reported previously (21).

An unexpected finding is that high levels of the α3(VI)-like chains in skeletal muscle and other organs a relatively unstable region. When a small in-frame deletion is present in the N-terminal triple-helical region of the α3(VI) chain, it is possible that the triple-helix folding continues upon reaching the deleted region, although out of register, until the end of the α3(VI) collagenous domain, thereby leaving overlaps of unfolded α1(VI) and α2(VI) chains. Alternatively, the triple helix folding could stop at the deleted region, leaving all three chains unfolded. In either case, the N terminus of the triple helix would be misfolded. However, dimers and tetramers are expected to be assembled, as the single cysteines in the three chains essential for oligomer assembly/stabilization are preserved (Fig. 1B). Biosynthetic studies of mouse fibroblasts indeed confirm that monomers, dimers, and tetramers are formed when either a half or all of the α3(VI) chains contain the deletion in the heterozygous or homozygous cells, respectively (Fig. 3, A and B). Theoretically, in the heterozygous cells, 15 out of 16 tetramers contain at least one mutant α3(VI) chain and the abnormal tetramers with defective N termini are unable to assemble end-to-end into microfibrils (18) (Fig. 1C). The effects of the N-terminal mutations are further amplified during the formation of long microfibrils as this process requires the association of multiple tetramers via their N termini. Consistent with this proposition, we show that collagen VI microfibrils are barely detectable in both heterozygous and homozygous fibroblasts (Fig. 4). Given that collagen VI microfibrils are readily

table

| Genotype | Number of mice | EDL Absolute force (mN) | EDL Specific force (mN/mm²) | EDL Specific force (mN/mg) |
|----------|----------------|-------------------------|----------------------------|----------------------------|
| +/-      | 5              | 102.2 ± 19.64           | 55.9 ± 12.66               | 9.6 ± 2.16                 |
| +/-d16   | 5              | 67.1 ± 22.04            | 42.9 ± 10.98               | 7.0 ± 2.18                 |
| d16/d16  | 5              | 200.7 ± 56.36           | 123.1 ± 32.67              | 84.7 ± 20.12               |

*Statistically significant between wild type and heterozygous mutant (p < 0.05).
terns of the three α3(VI)-like chains. Previous studies have shown that the α4(VI) chain is not, and the α5(VI) chain is only weakly expressed in the skeletal muscle (7). The α6(VI) chain is present in the endomysium and perimysium of fetal and adult skeletal muscle, but its expression level is much lower than the α3(VI) chain (6, 7). Our studies extend these previous findings and demonstrate the distinctively restricted expression of the α6(VI) chain in the musculoskeletal tissue as opposed to the ubiquitous distribution of the α3(VI) chain. The absence of the α6(VI) chain in tendon proper, bone/cartilage, and dermis suggests that the Col6a3 and Col6a6 genes are independently regulated and implies that COL6A6 mutations, if any, would result in a disease manifestation distinct from UCMD. On the other hand, we found increased immunoreactivities of the α5(VI) and α6(VI) chains in a subset of muscle fibers in mutant mice at age 6 months, a stage when muscle fibrosis has developed in the mutant mice. Our results partially agree with a recent finding in patients with Duchenne muscular dystrophy, demonstrating that α6(VI), but not α5(VI), chain is up-regulated in fibrotic areas of muscle biopsies (36). Together, these observations support an association of the α5(VI) and α6(VI) chains with muscle fibrosis.

Like mouse models for Duchenne muscular dystrophy (mdx mouse) and for recessive UCMD (Col6a1 null and Col6a3<sup>hm/hm</sup> mice), the Col6a3<sup>+/d16</sup> mice display much milder gross and myopathic phenotypes compared with patients with heterozygous exon 16 skipping mutations. The milder muscle phenotype in mdx mice compared with human patients is thought to be related at least in part to the intrinsic difference in muscle regeneration capacity between humans and mice (37). Even though the Col6a3<sup>+/d16</sup> mice have mild phenotypes, we show that the myopathic pathology, such as changes in muscle fiber size and percentage of muscle fibers with central nuclei, can be detected in young mice. Moreover, dilated sarcoplasmic reticulum and enlarged mitochondria are apparent in muscles from both mutant genotypes at age 1 month. The histological and ultrastructural abnormalities of the Col6a3<sup>+/d16</sup> muscles can be used to follow disease progression.

Significant decrease in muscle contractile function can be seen when the Col6a3<sup>+/d16</sup> mice reach age 8 months but not in young adult mice at 3 months. The reduction in muscle force was overall proportional to the degree of reduction in muscle growth and muscle weight (EDL and quadriceps). ECC force decrement is considered to be a hallmark of Duchenne muscular dystrophy (29) and, therefore, is not expected to be observed in mouse models other than the mdx mice. We have previously shown that the Col6a3<sup>hm/hm</sup> mice display reduced muscle contractile forces after twitch and tetanic stimulation at age 4 months (22). In UCMD patients, whereas recessive mutations causing an absence of collagen VI almost invariably display an “early-severe (never acquired ambulation)” phenotype, dominant mutations causing skipping of COL6A3 exon 16 result in both early-severe and “moderate-progressive (loss of ambulation at a mean age of 10 years)” manifestations (13). These observations suggest that dominant exon skipping mutation may result in a somewhat less severe phenotype compared with recessive mutations that lead to mRNA decay and that modifier genes likely contribute to the phenotypic variations of dominant exon skipping mutations. Consistent with this suggestion is the finding that the Col6a3<sup>d16/d16</sup> mice, which are equivalent to collagen VI null mice, show more pronounced muscle and tendon histopathology than the Col6a3<sup>+/d16</sup> mice. Our studies show that the Col6a3<sup>+/d16</sup> mice exhibit ultrastructural abnormalities in mitochondria and sarcoplasmic reticulum similar to the Col6a1 null mice and patients carrying collagen VI mutations (33, 38). Whether the Col6a3<sup>+/d16</sup> mice display mitochondrial transition pore defects that can be reversed by cyclosporine A treatment remains to be investigated (33, 39). Nevertheless, the Col6a3<sup>+/d16</sup> mice are ideally suited for testing allele-specific silencing strategies to treat dominant collagen VI disorders. For instance, an antisense oligoribonucleotide has been successfully employed to deplete mutant COL6A2 mRNA in fibroblasts from a UCMD patient with a heterozygous COL6A2 mutation (40).

Histological and ultrastructural analyses indicate alteration of the endomysium in the mutant mice, suggesting that collagen VI plays a role in modulating muscle ECM. Immunohistochemistry studies (Fig. 9) show increased expression of ECM proteins that are associated with tissue fibrosis (collagen I and periostin) or regulate collagen fibrillogenesis (tenascin-X, peristin) (32, 41). The observed changes in ECM composition likely influence the mechanical properties of the endomysial connective tissue and thereby alter cellular activities. Consistent with this notion is the recent finding that muscles from the Col6a1 null mice display reduced stiffness, which leads to compromised activity of muscle satellite cells (42). Mutations of tenascin-X underlie a form of Ehlers-Danlos syndrome, characterized by joint hypermobility and skin laxity similar to patients with collagen VI muscle disorders (43, 44). Fibroblasts from tenascin-X-deficient mice have reduced levels of collagen VI mRNA and protein (45), suggesting coordinate regulation of collagen VI and tenascin-X. Interestingly, mice lacking tenascin-X exhibit mild myopathy (46). Periostin is a matricellular protein capable of directly interacting with collagen I and regulating collagen I fibrillogenesis (32). Periostin is up-regulated in the δ-sarcoglycan null mouse model of muscular dystrophy, and its absence markedly improves the skeletal muscle abnormalities of the mutant mice (47). It is unclear whether the increases in these ECM proteins are compensatory responses to the deficiency of collagen VI. Nevertheless, these proteins have potential to serve as biomarkers for disease progression or targets for developing therapies.
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collagen VI regulates collagen fibril morphology remains unclear, the ability of collagen VI to mediate cell-matrix and matrix-matrix interactions likely plays a central role.

In summary, we have generated a Col6a3 exon 16 deletion mouse strain to serve as an animal model for dominant UCMD. We have demonstrated that the heterozygous mice exhibit myopathic histopathology, ultrastructural abnormalities in muscle and tendon and compromised muscle functions. Overall, the heterozygous Col6a3 exon 16 mice display a milder phenotype than UCMD patients with an analogous molecular defect. Nevertheless, the mutant mouse recapitulates many features of UCMD and will be a valuable tool to better understand the pathogenic mechanisms of dominant UCMD and develop treatment strategies.

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REFERENCES

1. Engel, J., Furthmayr, H., Odermatt, E., von der Mark, H., Aumailley, M., Fleischmajer, R., and Timpl, R. (1985) Structure and macromolecular organization of type VI collagen. Ann. N.Y. Acad. Sci. 460, 25–37

2. Chu, M. L., Conway, D., Pan, T. C., Baldwin, C., Mann, K., Deutzmann, R., and Timpl, R. (1988) Amino acid sequence of the triple-helical domain of human collagen type VI. J. Biol. Chem. 263, 18601–18606

3. Chu, M. L., Zhang, R. Z., Pan, T. C., Stokes, D., Conway, D., Kuo, H. I., Glanville, R., Mayer, U., Mann, K., and Deutzmann, R. (1990) Mosaic structure of globular domains in the human type VI collagen α3 chain. Similarity to von Willebrand factor, fibronectin, actin, salivary proteins, and apotinin type protease inhibitors. EMBO J. 9, 385–393

4. Chu, M. L., Pan, T. C., Conway, D., Kuo, H. I., Glanville, R. W., Timpl, R., Mann, K., and Deutzmann, R. (1989) Sequence analysis of 3(VI) and α2(VI) chains of human type VI collagen reveals internal triplication of globular domains similar to the A domains of von Willebrand factor and two α2(VI) chain variants that differ in the carboxy terminus. EMBO J. 8, 1939–1946

5. Gara, S. K., Grumati, P., Urciuolo, A., Bonaldo, P., Kobbe, B., Koch, M., Paulsson, M., and Wagener, R. (2008) Three novel collagen VI chains with high homology to the α3 chain. J. Biol. Chem. 283, 10658–10670

6. Fitzgerald, J., Rich, C., Zhou, F. H., and Hansen, U. (2008) Three novel collagen VI chains, α4(VI), α5(VI), and α6(VI). J. Biol. Chem. 283, 20170–20180

7. Gara, S. K., Grumati, P., Squarzoni, S., Sabatelli, P., Urciuolo, A., Bonaldo, P., Paulsson, M., and Wagener, R. (2011) Differential and restricted expression of novel collagen VI chains in mouse. Matrix Biol. 30, 248–257

8. Bönnemann, C. G. (2011) The collagen VI-related myopathies. Muscle meets its matrix. Nat. Rev. Neuro. 7, 379–390

9. Allamand, V., Briñas, L., Richard, P., Stojkovic, T., Quijano-Roy, S., and Bonne, G. (2011) ColVI myopathies. Where do we stand, where do we go? Skelet. Muscle 1, 30

10. Norwood, F. L., Harling, C., Chinnery, P. F., Eagle, M., Bushby, K., and Straub, V. (2009) Prevalence of genetic muscle disease in Northern England. J. Neurol. 256, 3175–3186

11. Okada, M., Kawahara, G., Noguchi, S., Sugie, K., Murayama, K., Nonaka, I., Janssen, P. Y., Merlini, L., Navarro, C., Toutain, A., Chaigne, D., Desguerre, I., de Die-Smulders, C., Dunand, M., Echene, B., Eyemd, B., Kuntzer, T., Maincent, K., Mayer, M., Plessis, G., Rivier, F., Roelens, F., Stojkovic, T., Taratuto, A. L., Lubieniecki, F., Monges, S., Tranchant, C., Vioillet, L., Romero, N. B., Estournet, B., Guicheney, P., and Allamand, V. (2010) Early onset collagen VI myopathies. Genetic and clinical correlations. Ann. Neurol. 68, 511–520

12. Jöbsis, G. J., Boers, J. M., Barth, P. G., and de Visser, M. (1999) Bethlem myopathy. A slowly progressive congenital muscular dystrophy with contractures. Brain 122, 649–655

13. Merlini, L., Martoni, E., Grumati, P., Sabatelli, P., Squarzoni, S., Urciuolo, A., Ferlini, A., Gualandi, F., and Bonalbo, P. (2008) Autosomal recessive myosclerotic myopathy is a collagen VI disorder. Neurology 71, 1245–1253

14. Scacheri, P. C., Gillanders, E. M., Subramony, S. H., Vedenarayavan, V., Crowe, C. A., Thakore, N., Bingler, M., and Hoffman, E. P. (2002) Novel mutations in collagen VI genes. Expansion of the Bethlem myopathy phenotype. Neurology 58, 593–602

15. Camacho Vanegas, O., Bertini, E., Zhang, R. Z., Petriti, S., Minosse, C., Sabatelli, P., Giusti, B., Chu, M. L., and Pepe, G. (2001) Ullrich scleroatonic muscular dystrophy is caused by recessive mutations in collagen type VI. Proc. Natl. Acad. Sci. U.S.A. 98, 7516–7521

16. Pan, T. C., Zhang, R. Z., Sudano, D. G., Marie, S. K., Bönnemann, C. G., and Chu, M. L. (2003) New molecular mechanism for Ullrich congenital muscular dystrophy. A heterozygous in-frame deletion in the COL6A1 gene causes a severe phenotype. Ann. Hum. Genet. 73, 355–369

17. Zhang, R. Z., Sabatelli, P., Pan, T. C., Squarzoni, S., Mattioli, E., Bertini, E., Pepe, G., and Chu, M. L. (2002) Effects on collagen VI mRNA stability and microfibrillar assembly of three COL6A2 mutations in two families with Ullrich congenital muscular dystrophy. J. Biol. Chem. 277, 43557–43564

18. Peat, R. A., Baker, N. L., Jones, K. J., North, K. N., and Lamannde, S. R. (2007) Variable penetrance of COL6A1 null mutations. Implications for prenatal diagnosis and genetic counselling in Ullrich congenital muscular dystrophy families. Neuromuscul. Disord. 17, 547–557

19. Bonaldo, P., Braghetta, P., Zanetti, M., Piccolo, S., Volpin, D., and Bressan, G. M. (1998) Collagen VI deficiency induces early onset myopathy in the mouse. An animal model for Bethlem myopathy. Hum. Mol. Genet. 7, 2135–2140

20. Pan, T. C., Zhang, R. Z., Markova, D., Arita, M., Zhang, Y., Bogdanovich, S., Khurana, T. S., Bönnemann, C. G., Birk, D. E., and Chu, M. L. (2013) COL6A3 protein deficiency in mice leads to muscle and tendon defects similar to human collagen VI congenital muscular dystrophy. J. Biol. Chem. 288, 14320–14331

21. Lampe, A. K., Zou, Y., Sudano, D., O’Brien, K. K., Hicks, D., Laval, S. H., Charlton, R., Jimenez-Mallebrera, C., Zhang, R. Z., Finkel, R. S., Tenkkoon, G., Schreiber, G., van der Knaap, M. S., Marks, H., Straub, V., Flanigan, K. M., Chu, M. L., Muntoni, F., Bushby, K. M., and Bönnemann, C. G. (2008) Exon skipping mutations in collagen VI are common and are predictive for severity and inheritance. Hum. Mutat. 29, 809–822

22. Giusti, B., Lucarini, L., Pietroni, V., Lucioli, S., Bandinelli, B., Sabatelli, P., Squarzoni, S., Petriti, S., Gartioux, C., Talim, B., Roelens, F., Merlini, L., Topaloglu, H., Bertini, E., Guicheney, P., and Pepe, G. (2005) Dominant and recessive COL6A1 mutations in Ullrich scleroatonic muscular dystrophy. Ann. Neurol. 58, 400–410

23. Baker, N. L., Mörgelin, M., Peat, R., Goemens, N., North, K. N., Bateman, J. F., and Lamannde, S. R. (2005) Dominant collagen VI mutations are a common cause of Ullrich congenital muscular dystrophy. Hum. Mol. Genet. 14, 279–293

24. Tillet, E., Wiedemann, H., Golibek, R., Pan, T. C., Zhang, R. Z., Mann, K., Chu, M. L., and Timpl, R. (1994) Reombinant expression and structural and binding properties of α1(VI) and α2(VI) chains of human collagen type VI. Eur. J. Biochem. 221, 177–185

25. Specks, U., Mayer, U., Nischot, R., Spiessing, T., Mann, K., Timpl, R., Engel, J., and Chu, M. L. (1992) Structure of recombinant N-terminal globule of type VI collagen α3 chain and its binding to heparin and hyaluronan. EMBO J. 11, 4281–4290

26. Bogdanovich, S., Krag, T. O., Barton, E. R., Morris, L. D., Whitemore, L. A., Ahima, R. S., and Khurana, T. S. (2002) Functional improvement of dystrophic muscle by myostatin blockade. Nature 420, 418–421

27. Bogdanovich, S., McNally, E. M., and Khurana, T. S. (2008) Myostatin
blockade improves function but not histopathology in a murine model of limb-girdle muscular dystrophy 2C. *Muscle Nerve* **37**, 308–316
30. Brooks, S. V., and Faulkner, J. A. (1990) Contraction-induced injury. Recovery of skeletal muscles in young and old mice. *Am. J. Physiol.* **258**, C436–C447
31. Izu, Y., Ansonje, H. L., Zhang, G., Soslowsky, L. J., Bonaldo, P., Chu, M. L., and Birk, D. E. (2011) Dysfunctional tendon collagen fibrillogenesis in collagen VI null mice. *Matrix Biol.* **30**, 53–61
32. Norris, R. A., Damon, B., Mironov, V., Kasayanov, V., Ramamurthi, A., Moreno-Rodriguez, R., Trusk, T., Potts, J. D., Goodwin, R. L., Davis, J., Hoffman, S., Wen, X., Sugì, Y., Kern, C. B., Mjaatvedt, C. H., Turner, D. K., Oka, T., Conway, S. J., Molkentin, J. D., Forgacs, G., and Markwald, R. R. (2007) Periostin regulates collagen fibrillogenesis and the biomechanical properties of connective tissues. *J. Cell Biochem.* **101**, 695–711
33. Irwin, W. A., Bergamin, N., Sabatelli, P., Reggiani, C., Megighian, A., Merlini, L., Braghetto, P., Columbaro, M., Volpin, D., Bressan, G. M., Bernardi, P., and Bonaldo, P. (2003) Mitochondrial dysfunction and apoptosis in myopathic mice with collagen VI deficiency. *Nat. Genet.* **35**, 367–371
34. Lamandé, S. R., Mörgelin, M., Selan, C., Jöbsis, G. J., Baas, F., and Bateman, A. (2012) Expression of collagen VI in muscle biopsies of patients with collagen VI myopathies. *J. Biol. Chem.* **287**, 1949–1956
35. Persikov, A. V., Ramshaw, J. A., Kirkpatrick, A., and Brodsky, B. (2000) Amino acid propensities for the collagen triple-helix. *Biochemistry* **39**, 14960–14967
36. Sabatelli, P., Gualandi, F., Gara, S. K., Grumati, P., Zamparelli, A., Maraldi, N. M., Paulsson, M., Squarzoni, S., and Wagener, R. (2012) Expression of collagen VI α5 and α6 chains in human muscle and in Duchenne muscular dystrophy-related muscle fibrosis. *Matrix Biol.* **31**, 187–196
37. Sacco, A., Mourikiotis, F., Tran, R., Choi, J., Llewellyn, M., Kraft, P., Shkreli, M., Delp, S., Pomerantz, J. H., Artandi, S. E., and Blau, H. M. (2010) Short telomeres and stem cell exhaustion model Duchenne muscular dystrophy in mdx/mTR mice. *Cell* **143**, 1059–1071
38. Angelin, A., Tiepolo, T., Sabatelli, P., Grumati, P., Bergamin, N., Golfieri, C., Mattioli, E., Gualandi, F., Ferlini, A., Merlini, L., Maraldi, N. M., Bonaldo, P., and Bernardi, P. (2007) Mitochondrial dysfunction in the pathogenesis of Ullrich congenital muscular dystrophy and prospective therapy with cyclosporins. *Proc. Natl. Acad. Sci. U.S.A.* **104**, 991–996
39. Merlini, L., Angelin, A., Tiepolo, T., Braghetto, P., Sabatelli, P., Zamparelli, A., Ferlini, A., Maraldi, N. M., Bonaldo, P., and Bernardi, P. (2008) Cyclosporin A corrects mitochondrial dysfunction and muscle apoptosis in patients with collagen VI myopathies. *Proc. Natl. Acad. Sci. U.S.A.* **105**, 5225–5229
40. Gualandi, F., Manzati, E., Sabatelli, P., Passarelli, C., Bovolenta, M., Pellegrini, C., Perrone, D., Squarzoni, S., Pegoraro, E., Bonaldo, P., and Ferlini, A. (2012) Antisense-induced messenger depletion corrects a COL6A2 dominant mutation in Ullrich myopathy. *Hum. Gene Ther.* **23**, 1313–1318
41. Minamitani, T., Ikuta, T., Saito, Y., Takebe, G., Sato, M., Sawa, H., Nishimura, T., Nakamura, F., Takeda, K., Ariga, H., and Matsumoto, K. (2004) Modulation of collagen fibrillogenesis by tenascin-X and type VI collagen. *Exp. Cell Res.* **298**, 305–315
42. Urciuolo, A., Quarta, M., Morbidoni, V., Gattazzo, F., Molon, S., Grumati, P., Montemurro, F., Tedesco, F. S., Blauw, B., Cossu, G., Vozi, G., Rando, T. A., and Bonaldo, P. (2013) Collagen VI regulates satellite cell self-renewal and muscle regeneration. *Nat. Commun.* **4**, 1964
43. Schalkwijk, J., Zweigs, M. C., Steijlen, P. M., Dean, W. B., Taylor, G., van Vlijmen, I. M., van Haren, B., Miller, W. L., and Bristow, J. (2001) A recessive form of the Ehlers-Danlos syndrome caused by tenascin-X deficiency. *N. Engl. J. Med.* **345**, 1167–1175
44. Kirschner, J., Hausser, L., Zou, Y., Schreiber, G., Christen, H. J., Brown, S. C., Anton-Lamprecht, I., Muntoni, F., Hanefeld, F., and Bönnemann, C. G. (2005) Ullrich congenital muscular dystrophy. Connective tissue abnormalities in the skin support overlap with Ehlers-Danlos syndromes. *Am. J. Med. Genet.* **A 132A**, 296–301
45. Minamitani, T., Ariga, H., and Matsumoto, K. (2004) Deficiency of tenascin-X causes a decrease in the level of expression of type VI collagen. *Exp. Cell Res.* **297**, 49–60
46. Voermans, N. C., Verrijp, K., Eshuis, L., Balemans, M. C., Egging, D., Sterrenburg, E., van Rooij, I. A., van der Laak, J. A., Schalkwijk, J., van der Maarel, S. M., Lammens, M., and van Engelen, B. G. (2011) Mild muscular features in tenascin-X knockout mice, a model of Ehlers-danlos syndrome. *Connect. Tissue Res.* **52**, 422–432
47. Lorts, A., Schwanejamp, J. A., Baudino, T. A., McNally, E. M., and Molkentin, J. D. (2012) Deletion of peristin reduces muscular dystrophy and fibrosis in mice by modulating the transforming growth factor-β pathway. *Proc. Natl. Acad. Sci. U.S.A.* **109**, 10978–10983
48. Ritty, T. M., Roth, R., and Heuser, J. E. (2003) Tendon cell array isolation reveals a previously unknown fibrillin-2-containing macromolecular assembly. *Structure* **11**, 1179–1188
49. Wilberg, C., Hedbom, E., Khaireeula, A., Lamandé, S. R., Oldberg, A., Timpl, R., Mörgelin, M., and Heinegard, D. (2001) Biglycan and decorin bind close to the N-terminal region of the collagen VI triple helix. *J. Biol. Chem.* **276**, 18947–18952
50. Chen, S., and Birk, D. E. (2013) The regulatory roles of small leucine-rich proteoglycans in extracellular matrix assembly. *FEBS J.* **280**, 2120–2137