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Impairment of Caveolae Formation and T-System Disorganization in Human Muscular Dystrophy with Caveolin-3 Deficiency

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Caveolin-3, a muscle specific caveolin-related protein, is the principal structural protein of caveolar membranes. We have recently identified an autosomal dominant form of limb girdle muscular dystrophy (LGMD-1C) that is due to caveolin-3 deficiency and caveolin-3 gene mutations. Here, we studied by electron microscopy, including freeze-fracture and lanthanum staining, the distribution of caveolae and the organization of the T-tubule system in caveolin-3 deficient human muscle fibers. We found a severe impairment of caveolae formation at the muscle cell surface, demonstrating that caveolin-3 is essential for the formation and organization of caveolae in muscle fibers. In addition, we also detected a striking disorganization of the T-system openings at the subsarcolemmal level in LGMD-1C muscle fibers. These observations provide new perspectives in our understanding of the role of caveolin-3 in muscle and of the pathogenesis of muscle weakness in caveolin-3 deficient muscle. ***(Am J Pathol 2002, 160:265–270)***

Caveolae are 50 to 100 nm invaginations that represent an appendage or subcompartment of the plasma membrane. They are found in most cell types and are particularly abundant in striated muscle cells.[1–4] Caveolae have been implicated in many cellular functions, particularly in three major processes: endothelial transcytosis, potocytosis, and signal transduction.[1–4]

Caveolins are members of a gene family of 21- to 25-kd integral membrane proteins, that are the principal protein components of caveolar membranes. Caveolins play an important structural role in the formation of caveolar membranes, by acting as scaffolding proteins to organize and concentrate specific caveolin-interacting lipids and proteins within caveolae microdomains.[5–9] So far, three different caveolins have been recognized, termed caveolin-1, -2 and -3, which are the products of three different genes.[10–14]

Caveolin-3, the most recently recognized member of caveolin gene family, shows a muscle-specific tissue distribution and is the major caveolar protein of differentiated skeletal muscle cells. The expression of caveolin-3 is developmentally regulated, and the protein localizes to the sarcolemma in fully differentiated muscle fibers, where it interacts with dystrophin and with the dystrophin-associated glycoproteins.[12–14]

We have recently identified an autosomal dominant form of limb girdle muscular dystrophy (LGMD-1C) that is due to caveolin-3 deficiency and *CAV3* mutations. Analysis of genomic DNA revealed two distinct mutations in *CAV3*: a 9-base pair microdeletion that removes the sequence 63TFT65 from the caveolin scaffolding domain and a missense mutation that changes a proline to a leucine (P105L) in the transmembrane domain. Both mutations leaded to a severe deficiency (95%) of the caveolin-3 protein.[15]

It has long been hypothesized that muscle cell caveolae may play a role in the formation or organization of the T-tubule system. In agreement with this concept, recent studies on developing muscle have demonstrated that caveolin-3 associates with vescicles which eventually fuse to form the T-tubule openings at the surface of developing myotubes.[16]

To gain further insight into the distribution of caveolae and to evaluate the organization of the T-tubule system in caveolin-3 deficient muscle, we applied electron micro-

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scopic techniques, including freeze-fracture and lanthanum staining, to muscle biopsies from four patients with LGMD-1C and caveolin-3 deficiency.

Materials and Methods

Muscle Samples

We studied muscle biopsies (quadriceps) from four patients (case 1, age 45; case 2, age 15; case 3, age 13; case 4, age 10) from two different families with different LGMD-1C mutations. The clinical and molecular genetic aspects of these patients, together with other members of their families, were previously reported.[15] Two different mutations in CAV3 gene were found, a microdeletion in the scaffolding domain (patients 1 and 2) and a missense mutation in the membrane-spanning region (patients 3 and 4), leading to a severe caveolin-3 deficiency (~ 95%) in muscle fibers. As controls, we used samples of quadriceps muscle from four subjects of comparable age, with normal muscle histology. All samples were frozen in liquid nitrogen-cooled isopentane, sectioned for diagnostic purposes, and stored in liquid nitrogen until this study. A portion of each sample was fixed in glutaraldehyde and processed for ultrastructural examination as described below.

Immunohistochemistry

We used a monoclonal antibody directed against caveolin-3 (cl26) that was generated with a synthetic peptide corresponding to amino acids 3 to 24 of the rat caveolin-3 protein sequence. The immunological and immunohistochemical characteristics of this antibody have been previously described in detail.[14] For immunohistochemistry, 4 nm-thick unfixed serial frozen muscle sections were incubated with anti-caveolin-3 mAb diluted 1:1000 in phosphate-buffered saline, and processed for immunofluorescence microscopy as previously described.[15]

Table 1. Morphological Alterations in Caveolin-3-Deficient Muscle

Method	Observations
Optical microscopy	Mild myopathic pattern ([15]): scattered necrotic fibers, increased connective tissue
Conventional electron microscopy	Lack of caveolae at the muscle plasma membrane in most fibers Large membranous vacuoles close to the cell surface, generally empty or containing amorphous material
Freeze-fracture analysis of caveolae	Severe reduction (<5%) of caveolae at the cell surface
T-system lanthanum staining	Lack of caveolae at the muscle plasma membrane in most fibers Staining of large membranous vacuolated structures beneath the plasma membrane Staining of some vacuolated structures in continuity with plasma membrane Proliferation of the T-tubule system (honeycomb structures) at the subsarcolemmal level

Ultrastructural Study

For routine electron microscopy, samples were fixed in 2.5% glutaraldheyde, processed and embedded in epon-araldite. Ultrathin sections were cut using a Leica Ultracut UCT ultramicrotome, stained with uranyl acetate and lead citrate, and observed by a Zeiss 110 electron microscope. For lanthanum staining, small biopsy samples were fixed in 2.5% glutaraldheyde-2% paraformaldheyde in 0.1 mol/L cacodylate buffer, pH 7.2 for 30 minutes. Specimens were then reduced to 1 mm × 2 mm pieces, washed three times in buffer, and rinsed overnight at 4°C in 0.5 mol/L cacodylate buffer. The specimens were postfixed at room temperature by vibratory agitation for 2 hours in a medium containing 1.3% osmiun tetroxide in 0.2 s-collidine buffer at pH 7.2 and 2% lanthanum nitrate. They were rapidly dehydrated, washed in propylene oxide 100%, and finally embedded in epon-araldite.

Freeze-Fracture Analysis

The specimens were removed at rest length in a U-shaped muscle clamp or attached to a tick and fixed immediately in 3% glutaraldehyde in 0.1 mol/L phosphate buffer. The samples were cut into small blocks and gradually infiltrated with glycerol up to a concentration of 30% and then frozen in freon-liquid nitrogen. The specimens were fractured at −110°C in a freeze-fracture apparatus at a vacuum of 6×10^{-7} mmHg and immediately replicated with platinum and carbon using electron beam guns. The tissue was digested in sodium hypoclorite. The detached replicas were washed three times in distilled water and finally picked up on Formvar coated grids and examined/photographed using a Zeiss EM-10 transmission electron microscope.

Image Analysis and Quantitation

Tissue sections were examined using a Leitz Diaplan optical microscope equipped with epi-illumination. Images were obtained with an Optronics DEI-750 digital camera connected to a Power Macintosh. Image analysis was performed using Image Pro-Plus software. For electron microscopy and freeze fractures studies, pictures were scanned and analyzed using an Image Pro-Plus program. The density of caveolae was determined by counting the number of caveolae on the P face in at least 30 different fields from different fibers for each sample; 10 different samples were freeze-fractured from each muscle biopsy. The number of caveolae was expressed

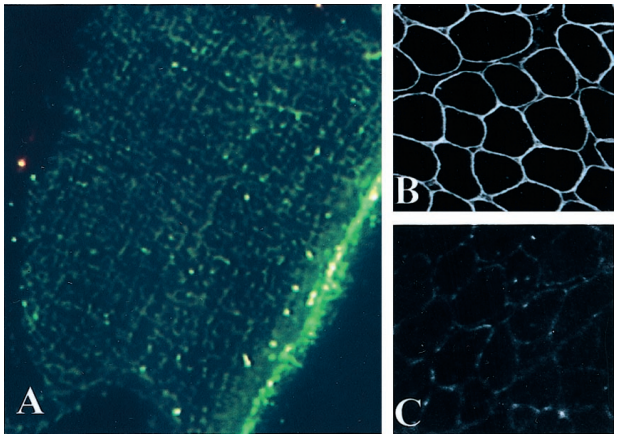

Figure 1. Immunofluorescent localization of caveolin-3 in the muscle fibers of normal and LGMD-1C patients. **A:** By high-resolution immunofluorescence, in longitudinal sections, caveolin-3 shows a network-like organization at the cell surface, with main bands running along the longitudinal axis of the fibers, interconnected by transverse bands. This pattern is reminiscent of the network organization of caveolae at the cell surface, as seen by freeze-fracture in normal muscle fibers (see Figure 3A). **B:** By immunofluorescence in normal muscle, in cross sections, caveolin-3 reveals a uniform staining pattern at the sarcolemma. **C:** In LGMD-1C patients, note that there is a severe deficiency of caveolin-3 at the cell surface. **A:** Original magnification, ×1200; **B:** Original magnification, ×40; **C:** Original magnification, ×40.

per square micrometer. Statistical analysis was performed using the two-tailed paired *t*-test.

Results

Immunohistochemistry

In cross sections, immunofluorescent staining of normal control muscle showed that caveolin-3 is localized at the sarcolemma of human muscle fibers (Figure 1B). In longitudinal sections and using high-resolution immunofluorescence, caveolin-3 showed an organized network at the cell surface. This network consisted of a set of major bands running along the longitudinal axis of the fibers and a set of minor interconnecting transverse bands (Figure 1A). In contrast, muscle sections prepared from LGMD-1C patients showed a severe reduction in caveolin-3 staining at the sarcolemma (Figure 1C). This caveolin-3 deficiency was previously confirmed by immunoblot analysis (data not shown, see Reference 15).

Conventional Electron Microscopy

In normal control muscle fibers, caveolae appeared as flask-shaped profiles located beneath the plasma membrane (Figure 2A). Muscle samples from LGMD-1C patients showed that caveolae were virtually absent from most muscle fibers. Only in a few areas we could detect isolated caveolae-like structures adjacent to the muscle plasma membrane. Quantitative analysis of more than 30 different fields revealed that the number of caveolae was less than 5% in muscle fibers from LGMD-1C patients. Furthermore, LGMD-1C fibers contained in several areas large vacuoles close to the cell surface. These vacuolated structures revealed the presence of a membrane at the outskirt and were generally empty, or partially containing amorphous material (Figure 2, B and C).

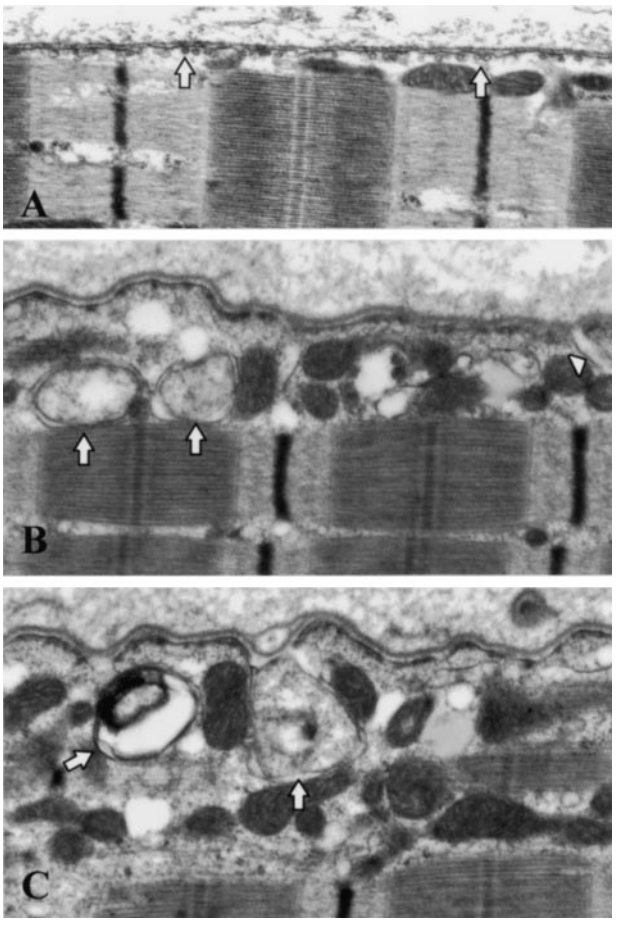

Figure 2. Transmission electron microscopic analysis of muscle fibers from normal and LGMD-1C patients. By routine transmission electron microscopy, caveolae in normal muscle fibers appear as flask shaped vesicles localized subjacent to the subsarcolemmal membrane (**A**, **arrows**). In LGMD-1C muscle fibers (patient 1), very few caveolae were found near to the cell surface (**B, C**). Furthermore, in LGMD-1C fibers, we observed large vacuolated structures close to the cell surface. These vacuolated structures appeared membranous and were generally empty, or containing amorphous material. (**B, C**, **arrows**). Some of these structures revealed continuity with the membrane (**B**, **arrowhead**). **A–C:** Original magnification, ×12,500.

Freeze-Fracture Analysis of Caveolae

To evaluate the number and distribution of caveolae in the plasma membrane of the muscle fibers, we examined the two leaflets of the surface membrane, the protoplasmic leaflet (P face) and the external leaflet (E face). In normal muscle plasma membrane, both the P and E faces contained caveolae which appeared relatively uniform in size and shape, and they were characteristically distributed in rows of longitudinal and transverse bands (Figure 3A). This highly organized array of caveolae seen in freeze-fracture preparations appeared to correspond to the pattern of longitudinal and transverse bands that we observed by high-resolution immunofluorescence (Figure 1B). The mean density of caveolae in normal

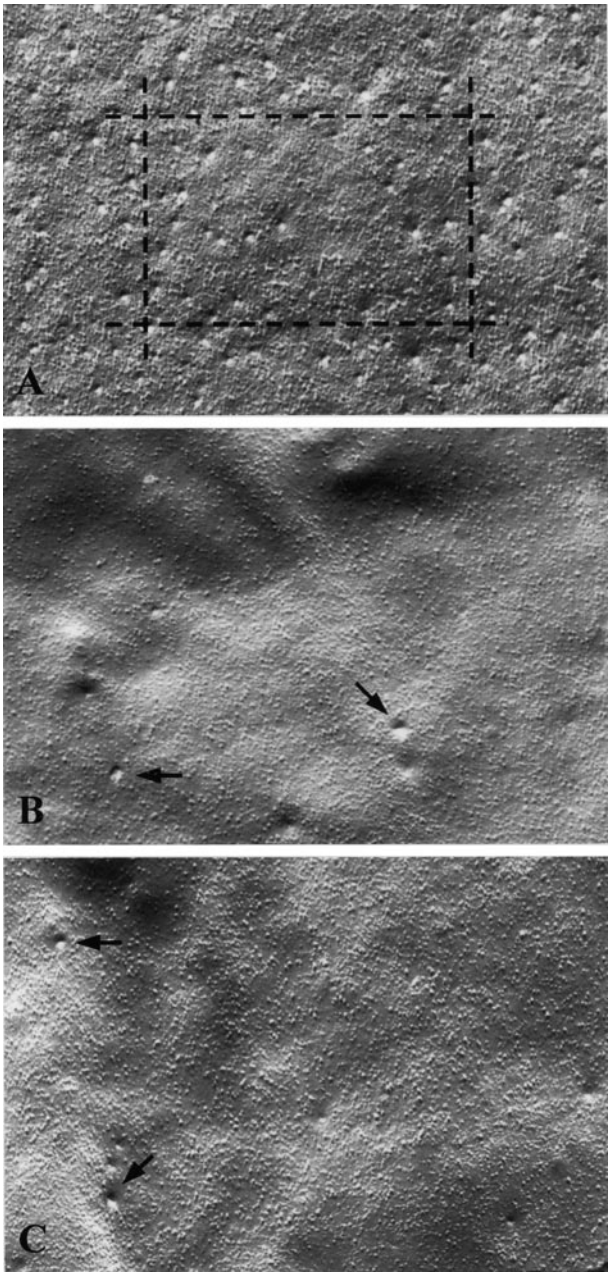

Figure 3. Freeze-fracture analysis of muscle fibers from normal and LGMD-1C patients. In freeze-fracture preparations of normal muscle cell plasma membrane, caveolae appeared as small invaginations or indentations of the plasma membrane (**A**). The distribution of caveolae at the cell surface was not homogeneous; caveolae were present in ordered arrays or rows that may correspond to the horizontal and vertical band-like structures we observed by immunofluorescence microscopy (see Figure 1A). In striking contrast, in LGMD-1C muscle fibers, we observed very few scattered caveolae at the cell surface (**B**, patient 1 and **C**, patient 2, **arrows**). **A–C:** Original magnification, ×12,500.

muscle plasma membrane was 9.68 (SD ± 2.05) per square micron. In sharp contrast, in LGMD-1C muscle plasma membrane we observed very few caveolae in both P and E faces of the membrane. (Figure 3, B and C). Quantitative analysis showed that the number of caveolae in LGMD-1C muscle fibers was less than 5%, as compared to normal control muscle.

T-System Lanthanum Staining

To study the organization of the T-tubule system in muscle fibers, we used lanthanum nitrate staining combined with transmission electron microscopy. Lanthanum is an electron-dense tracer that, in muscle, stains the extracellular space, and also outlines the caveolae and the T-system for ultrastructural studies.[17,18] Previous reports have shown that lanthanum does not penetrate the sarcoplasmic reticulum, except in specific pathological conditions such as thermal injury.[19]

In our experiments, muscle samples from normal controls and from LGMD-1C patients were pre-treated with lanthanum nitrate, and then processed for electron microscopy. In normal muscle, the extracellular space as well as the caveolae were labeled with electron-dense material. The T-tubules were also stained by lanthanum and were observed at the I and A band junction (Figure 4A).

In LGMD-1C muscle samples, most of the fibers did not show caveolae, while lanthanum-stained caveolae were seen in adjacent fibroblasts or endothelial cells. (Figure 4B). This may be explained by the observation that non-muscle cells predominantly express caveolin-1, a more ubiquitously expressed product of the caveolin gene family.[8–11] Interestingly, in LGMD-1C muscle fibers, we also observed beneath the plasma membrane several large vacuolated structures which were labeled by electron-dense lanthanum (Figure 4C). These structures may correspond to the large vacuoles seen in subsarcolemmal regions by conventional electron microscopy in LGMD-1C muscle fibers (Figure 2, B and C). In addition, some of the large vacuolated structures showed continuity with the plasma membrane, possibly representing abnormal caveolae-like membrane invaginations or disrupted T-tubule openings. Furthermore, some of the vacuolated structures were also associated with lanthanum positive honeycomb structures, a known indicator of abnormal proliferation of the T-tubule system (Figure 4D).[17,18] The described abnormalities were seen in all LGMD-1C muscle samples.

For comparative purposes and to exclude the possibility that our observations may be due to technical artifacts, we also examined by lanthanum staining muscle biopsies from two patients affected with dermatomyositis. It has been reported that in this disorder, the T-system is generally abnormal and that it proliferates in affected muscle fibers.[20] In these biopsies, we found disorganization and proliferation of the T-tubules, but we did not detect lanthanum-positive large vacuoles beneath the plasma membrane, similar to the structures we noted in LGMD-1C muscle fibers (data not shown).

Discussion

This is the first demonstration that caveolin-3 deficiency, due to mutations in the caveolin-3 gene, leads in human muscle fibers to a severe impairment of caveolae formation at the cell surface. The immediate consequence of this observation is the conclusion that caveolin-3 is es-

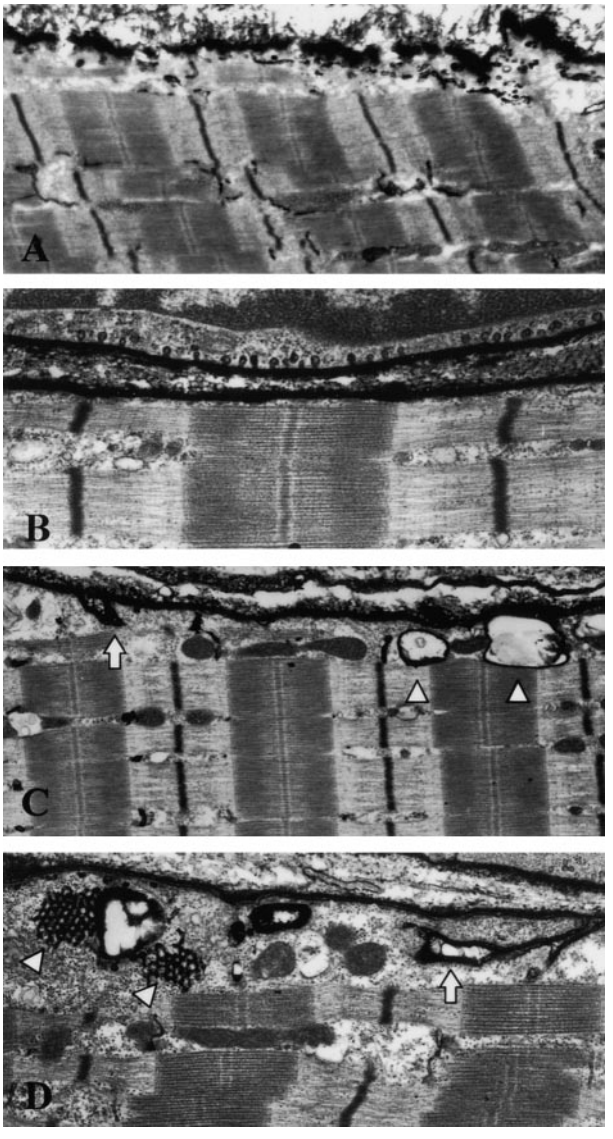

Figure 4. Lanthanum nitrate staining of muscle fibers from normal and LGMD-1C patients. After lanthanum nitrate treatment, normal control muscle samples showed dense staining of the T-tubules, which were generally observed between the I- and A-band. Lanthanum penetration was able to stain the first few sarcomeres close to the membrane (**A**). In LGMC-1C muscle fibers, we confirmed that only very few caveolae are found near to the cell surface, in contrast with the large number of caveolae that are present in adjacent non-muscle cells (**B**). In LGMD-1C muscle fibers, note the vacuolated structures that are penetrated by lanthanum and show a lanthanum-positive membrane (**C**, **arrowsheads**). Some of these structures revealed continuity with the cell membrane (**C, D**, **arrows**). Furthermore, some vacuolated structures were associated with honeycomb structures, typical of abnormal proliferation of the T-tubule system (**D**, **arrowheads**). **A:** Original magnification, ×7,000; **B:** Original magnification, ×12,500; **C:** Original magnification, ×7,000; **D:** Original magnification, ×10,000.

sential for the formation and organization of caveolae at the cell surface in human muscle.

During the process of caveolae formation, caveolin undergoes two stages of self-association or oligomerization. First, shortly after caveolin synthesis, caveolin oligomerizes in the endoplasmic reticulum to form homo-oligomers of 300 to 350 kd, each containing approximately 14 to 16 individual caveolin monomers. Second, at a later stage, these caveolin homo-oligomers can interact with each other to form clusters of caveolin oligomers that are approximately 25 to 50 nm in diameter. Thus, through the interaction of caveolin with itself and the caveolin-mediated selection of endogenous lipid components, a caveolae-sized vesicle is generated.[6–8]

We demonstrated that heterozygous mutations in CAV3 gene are associated with a severe caveolin-3 deficiency at the cell surface.[15] The molecular mechanisms underlying this deficiency have not yet been elucidated. A possible mechanism is that the heterozygous mutations exert a dominant-negative effect that induces rapid degradation of both the wild-type and mutant caveolin-3 proteins. Consistent with this hypothesis, we investigated the phenotypic behavior of these caveolin-3 mutations using heterologous expression in NIH 3T3 cells. The results show that LGMD-1C mutants of caveolin-3 form unstable high molecular mass aggregates of caveolin-3, which are retained within the Golgi complex and are not targeted to the plasma membrane.[21] These data provide a molecular explanation for why caveolin-3 levels are down-regulated in patients with LGMD-1C.

Caveolin-3 deficient mutant mice, designed to disrupt exon 2 of the caveolin-3 gene, show a reduction of caveolae at the plasma membrane.[22] Interestingly, the pathogenetic mechanisms leading to the impairment of caveolae formation at the cell surface seem to be different in the mutant mice as compared with humans. In fact, heterozygous mutant mice do not behave in a dominant negative fashion and only homozygous mutants reveal a severe impairment of caveolae formation. Furthermore, no apparent muscle degeneration was observed in these mutants.[22] In contrast, here we demonstrate that heterozygous mutations of the CAV-3 gene, involving the caveolin scaffolding and membrane spanning domains, exert in a significant down-regulation of caveolin-3 protein, leading to a severe impairment of the formation of caveolae at the cell membrane of muscle fibers from LGMD-1C patients.

Electron microscopic studies have shown that caveolae-like structures are associated with forming T-tubules at an early developmental stage.[23] Several morphological studies have demonstrated that caveolae initially form tubular structures that later associate to develop into a larger three-dimensional tubular network.[23,24] Furthermore, it has been recently reported that caveolin-3 transiently associates with T-tubules during development and may be involved in the early development of the T-tubule system in muscle.[16,25,26] In mature muscle fibers, however, caveolin-3 is no longer detectable within the T-tubule system, but is highly concentrated in sarcolemmal caveolae.[25] These results suggest that a functional relationship may exist between caveolin-3 expression, caveolae formation, and T-tubule biogenesis.

We have recently reported that caveolin-3 null mice, lacking caveolin-3 protein expression and sarcolemmal caveolae membranes, show T-tubule abnormalities in muscle fibers.[27] Thus, we examined here the possible consequences of caveolin-3 deficiency on the formation of T-tubule domains in caveolin-3 deficient muscle fibers of LGMD-1C patients. We found the presence of abnormal large vacuolated and membranous structures in the subsarcolemmal area, associated with an abnormal pro-

liferation of T-tubule like structures. These alterations were independent from the age of the patients, varying from 10 to 45 years, and were more severe than those we observed in muscle from caveolin-3 null mice.

In conclusion, our data suggest that LGMD-1C muscle fibers with severe caveolin-3 deficiency show an impairment of caveolae formation at the cell surface and a disorganization of the T-system openings at the subsarcolemmal level. Because the T-system plays an important role in muscle contraction and relaxation by regulating the distribution of the Ca^{2+} ions, this alteration may explain, at least in part, the pathogenesis of muscle weakness in LGMD-1C patients.

It has been recently reported that mutations in the CAV3 gene may cause the mechanical hyperirritability of skeletal muscle seen in rippling muscle disease.[28] However, the pathogenetic mechanism(s) underlying this disorder still remain to be elucidated. Further studies will be necessary to evaluate if the alterations we find in LGMD1C muscle fibers are also present in other clinical phenotypes due to *CAV3* mutations, such as isolated hyperCKemia and rippling muscle disease.

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