Human Molecular Genetics, 2008, Vol. 17, No. 21 ***3271–3280***
doi:10.1093/hmg/ddn223
Advance Access published on August 1, 2008

Novel role of calpain-3 in the triad-associated protein complex regulating calcium release in skeletal muscle

Irina Kramerova[1], Elena Kudryashova[1], Benjamin Wu[1], Coen Ottenheijm[2], Henk Granzier[2] and Melissa J. Spencer[1,*]

[1]Department of Neurology, David Geffen School of Medicine, University of California, Los Angeles, CA 90095, USA and [2]Department of Molecular and Cellular Biology, University of Arizona, Tucson, AZ 85724, USA

Received June 17, 2008; Revised and Accepted July 30, 2008

Calpain-3 (CAPN3) is a non-lysosomal cysteine protease that is necessary for normal muscle function, as mutations in CAPN3 result in an autosomal recessive form of limb girdle muscular dystrophy type 2A. To elucidate the biological roles of CAPN3 in skeletal muscle, we performed a search for potential substrates and interacting partners. By yeast-two-hybrid analysis we identified the glycolytic enzyme aldolase A (AldoA) as a binding partner of CAPN3. In co-expression studies CAPN3 degraded AldoA; however, no accumulation of AldoA was observed in total extracts from CAPN3-deficient muscles suggesting that AldoA is not an *in vivo* substrate of CAPN3. Instead, we found CAPN3 to be necessary for recruitment of AldoA to one specific location, namely the triads, which are structural components of muscle responsible for calcium transport and excitation–contraction coupling. Both aldolase and CAPN3 are present in the triad-enriched fraction and are able to interact with ryanodine receptors (RyR) that form major calcium release channels. Levels of triad-associated AldoA and RyR were decreased in CAPN3-deficient muscles compared with wild-type. Consistent with these observations we found calcium release to be significantly reduced in fibers from CAPN3-deficient muscles. Together, these data suggest that CAPN3 is necessary for the structural integrity of the triad-associated protein complex and that impairment of calcium transport is a phenotypic feature of CAPN3-deficient muscle.

INTRODUCTION

Limb girdle muscular dystrophy type 2A (LGMD2A) caused by mutations in the non-lysosomal cysteine protease calpain-3 (CAPN3), is one of the most frequently occurring forms of LGMD, a disease characterized by high genetic and clinical variability (1). It is often assumed that enzymatic activity of CAPN3 is essential for its physiological function. However, pathogenic mutations are not concentrated only in the catalytic domain of CAPN3 but are spread along the entire length of the protein (1). In a recent study, it was estimated that about one-third of the LGMD2A biopsies had normal levels of CAPN3 proteolytic activity suggesting that CAPN3 serves other physiological roles besides that of a protease (2). It has been demonstrated that at least some mutations which do not affect proteolytic activity, decrease the ability of CAPN3 to interact with titin, an established CAPN3-binding partner (3). The anchorage to titin occurs at two places, at the N2-line and the M-line regions of titin (4,5). Interestingly, mutations at both of these regions of titin cause muscle disease in humans and mice (6–8).

In order to further elucidate physiological roles of CAPN3 and gain insight into mechanisms of LGMD2A, we used several approaches to identify additional substrates and binding partners, and validated the candidates in CAPN3-deficient (C3KO) mice described previously (3). As a result of these assays, in the present study we identified the metabolic enzyme aldolase A (AldoA) as a binding partner of CAPN3. This isoform of aldolase is expressed only in skeletal muscle and erythrocytes (9). Aldolase catalyzes the conversion of fructose-1,6-bisphosphate into glyceraldehyde-3-phosphate and dihydroxyacetone phosphate in glycolysis. Glycolytic enzymes are abundant, ubiquitous proteins present in all types of cells;

*To whom correspondence should be addressed at: Department of Neurology, David Geffen School of Medicine, University of California, 635 Charles Young Dr, NRB, Rm. 401, Los Angeles, CA 90095, USA. Tel: +1 3107945225; Fax: +1 3102061998; Email: mspencer@mednet.ucla.edu

however, in muscle a fraction of these enzymes has been shown to be associated with specialized subcellular structures called triads (10). Triads are the anatomical structures where the T-tubules (which are sarcolemmal invaginations), and the terminal cisternae of the sarcoplasmic reticulum (SR) interface. At this site, a close contact is made between voltage-sensitive calcium channels (dihydropyridine receptors, DHPR) in the T-tubule, and calcium release channels (ryanodine receptors, RyR) in the SR that allows for coupling neural excitation with calcium release and contraction in muscle (11) (see Fig. 3A for the schematic of triad structure). Association of glycolytic enzymes with triads may provide compartmentalized adenosine triphosphate (ATP) production necessary for calcium uptake into the SR after excitation (12). However, glycolysis also modulates energy-independent calcium release during excitation (13); moreover, some glycolytic enzymes physically interact with calcium channels present in the T-tubule and SR membranes. AldoA, in particular, interacts with the RyR and can modulate RyR activity *in vitro* (14). Interestingly, mutations in AldoA cause muscle disease, however, mechanisms of the disease are not known (15,16).

It is usually assumed that, through its interaction with titin, CAPN3 is associated with the sarcomere in skeletal muscle. However, we found previously that in myogenic cells there is a pool of CAPN3 associated with the membrane (17). In the present investigation we show that in mature adult muscle there is also a pool of membrane-associated CAPN3 which links to triads. Loss of CAPN3 *in vivo* results in decreased recruitment of AldoA and RyR to triads and leads to reduced calcium release during muscle activation. Both proteins were shown to be binding partners of CAPN3. Thus, these studies identify a novel cellular function for CAPN3 in calcium transport and demonstrate that in this context CAPN3 serves a structural, rather than a proteolytic, role. The data also suggest that calcium transport abnormalities may be one of the mechanisms that lead to dystrophy in CAPN3-deficient muscles.

RESULTS

Identification of AldoA as a binding partner for CAPN3

To investigate the biological functions of CAPN3, we searched for new binding partners of CAPN3 using the Cytotrap (Stratagene) yeast-two-hybrid screening assay that detects protein–protein interactions in the cytoplasm. Several proteins were identified, including the M-line fragment of titin (4), as well as a novel CAPN3 partner, the glycolytic enzyme AldoA. In order to confirm the interaction between CAPN3 and AldoA, the proteolytically inactive mutant of CAPN3-C129S (18) and AldoA were expressed as recombinant fusion proteins with glutathione-S-transferase (GST) and calmodulin-binding peptide, respectively. A solid-phase binding assay using GST as a negative control was performed, and the interaction between the two proteins was confirmed (Fig. 1A).

To determine if AldoA is a substrate for CAPN3 proteolytic activity, insect cells were transfected with equal amounts of baculoviral vector, carrying AldoA cDNA, either alone or together with vectors expressing active CAPN3 or inactive CAPN3-C129S. Figure 1B shows that the concentration of AldoA was significantly reduced if it was co-expressed with active CAPN3. Thus, CAPN3 is able to degrade AldoA when the two proteins are co-expressed in a heterologous system. However, when total extracts from C3KO and wild-type (WT) muscles were blotted for AldoA, no difference in its concentration was found (Fig. 1C) suggesting that either AldoA is not an *in vivo* substrate of CAPN3, or CAPN3 regulates AldoA at a specific subcellular location and these changes are not detectable in total extracts. Previously we showed that this was the case for CAPN3's regulation of β-catenin at the cell membrane (but not the cytoplasm) during myogenesis (17).

CAPN3 is necessary for proper localization of AldoA

In order to test the hypothesis that CAPN3 regulates AldoA at a specific subcellular location, we first used immunohistochemistry of frozen sections of muscle to verify sites of AldoA accumulation. It was shown previously that CAPN3 immunostaining appears as a striated pattern, presumably corresponding to two sites of CAPN3–titin interaction at the N2-line and M-line (the schematic structure of the sarcomere is shown in Fig. 2A) (4,5,19). Based on previous biochemical observations showing that AldoA can interact with the RyR (14), AldoA was expected to be concentrated at the triads. As shown in Figure 2A, there are two triads per sarcomere located at either side of the Z-disk near the N2-line epitope of titin. Immunostaining showed that AldoA is concentrated at both sides of the Z-disk and co-localizes with the N2-line epitope of titin (Fig. 2B). Because the distance between two adjacent fluorescent bands is very short for light microscopy, the staining is more often visualized as a single broad band (as in Fig. 2C). Thus, AldoA immunolocalization data are in agreement with previously published results based on biochemical association of AldoA with triad components (14). To see if localization of AldoA is affected by the absence of CAPN3, C3KO muscles were stained with anti-AldoA antibody. This experiment showed that the striated pattern of AldoA immunostaining was lost in the absence of CAPN3 (Fig. 2D). To verify that the structural integrity of the sarcomere was intact in the tissue sections, the muscles were co-immunostained for the Z-disk protein α-actinin (Fig. 2D, left panels). These results demonstrated that CAPN3 is required for proper localization of AldoA.

Based on previous studies that AldoA is associated with membranous structures, triads, and the observation that AldoA is mislocalized in C3KO muscles, we sought to biochemically verify the subcellular localization of CAPN3 in mature muscle. Previous studies that showed an interaction between CAPN3 and titin have led to the assumption that CAPN3 would be primarily associated with myofibrils. Following biochemical fractionation of total muscle extracts, it was observed that CAPN3 is not only concentrated in the myofibrillar fraction but that a significant amount of CAPN3 is also present in the membrane and cytosol fractions (Fig. 2E). Thus, immunolocalization and subcellular fractionation studies suggest that CAPN3 and AldoA may co-localize at the triads, and that CAPN3 is necessary for proper localization of AldoA.

Concentration of triad-associated AldoA is decreased in C3KO muscles

To further confirm that CAPN3 and AldoA are co-localized at the triads and that CAPN3 is necessary for the recruitment of

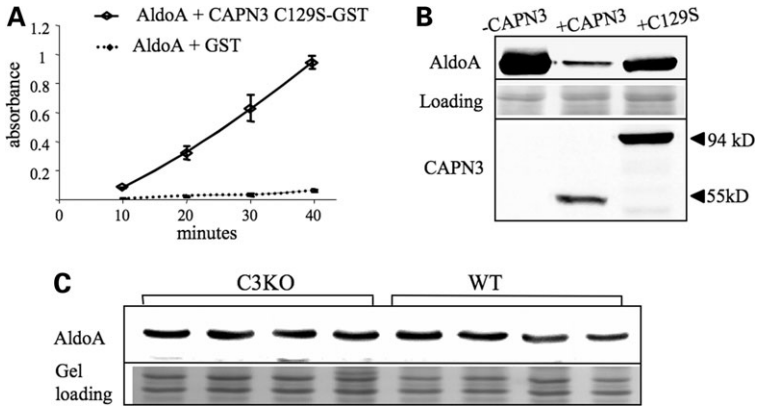

Figure 1. Interaction between CAPN3 and AldoA: (**A**) CAPN3 binds AldoA in a solid-phase protein interaction assay. CAPN3-C129S inactive mutant was expressed as a GST-fusion protein. In the ELISA-based binding assay, GST was used as a negative control and anti-GST antibody was used to detect the interaction between AldoA and CAPN3; (**B**) AldoA is a substrate for CAPN3 in co-expression experiments. Equal amounts of AldoA-expressing baculoviral constructs were used to infect insect cells alone and together with active CAPN3, or its inactive C129S mutant as a negative control. AldoA was greatly reduced in cells expressing active CAPN3. The lower panel shows an identical western blot stained for CAPN3 to ensure its expression and activity. Proteolytically active CAPN3 undergoes autolysis to produce a 55 kDa band while inactive C129S runs as a 94 kDa band; (**C**) AldoA concentration is not different between WT and C3KO in total muscle extracts. Western blot was performed using four diaphragm muscle extracts for each genotype.

AldoA to traids, we isolated microsomes from C3KO and WT muscles and subjected them to sucrose gradient centrifugation to obtain triad-enriched fractions (20). The schematic structure of the triad is shown in Figure 3A. Western blot analysis with triad-specific antibodies revealed that the triads were primarily present in fraction-2 (Fig. 3B). Fraction-2 contained the DHPR (a voltage-sensitive Ca^{2+} channel present in the T-tubules), the RyR (a calcium release channel present in the membrane of the terminal cisternae of the SR), and β-calmodulin kinase II, previously shown to be associated with triads (21,22). Importantly, both CAPN3 and AldoA were concentrated in the triad-enriched fraction of WT muscles. In agreement with the immunolocalization studies, a decreased concentration of AldoA was observed in fraction-2 of C3KO muscle. Levels of another glycolytic enzyme, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), were equal in triad fractions from C3KO and WT muscles (Fig. 3B) thereby showing that the reduction is specific to AldoA. If AldoA were a CAPN3 substrate, one would expect to see accumulation of AldoA in the absence of CAPN3. However, western blot analysis and immunostaining showed an unexpected decrease of AldoA in triads from C3KO muscles. These data substantiate the hypothesis that in this context, CAPN3 acts not as a protease but rather as a structural protein that is necessary for proper localization of AldoA to triads.

Concentration of RyR is decreased in C3KO muscles

Examination of triad-containing fractions by western blotting (Fig. 3B) revealed that AldoA was not the only protein reduced in C3KO triads. The levels of other proteins associated with the terminal cisternae of the SR, such as RyR and β-calmodulin kinase II (but not the T-tubule component DHPR) were also reduced in triad fraction isolated from the pooled C3KO muscles compared with WT muscles (Fig. 3B). In order to further investigate if the absence of CAPN3 and reductions in AldoA affect the RyR in C3KO muscle, we stained longitudinal sections and membrane fractions of C3KO and WT muscles with anti-RyR antibody. Figure 4A shows that, unlike AldoA, the expected striated pattern of RyR staining was well preserved in C3KO muscles. However, the concentration of the RyR in the membrane fractions isolated from C3KO muscles was decreased compared with WT (Fig. 4B) further confirming our earlier observation (Fig. 3B). To rule out the possibility that the decreased RyR concentration was caused by reduced transcription of the RyR gene, we performed real-time RT–PCR. As shown on Figure 4C, no difference was found in the expression level of RyR gene in C3KO versus WT muscles. Thus, these data suggest that the observed decrease in concentration of the RyR in C3KO muscle was not due to a change in transcription level of the corresponding gene and could be due to abnormal triad protein complex stability in C3KO muscle.

As we found in this research, AldoA is a binding partner of CAPN3, but AldoA was also shown previously to interact with the RyR (14), and thus may provide a link between CAPN3 and RyR. CAPN3 may also directly interact with RyR. In any case, one would expect CAPN3 and RyR to be co-purified from the same protein complex. To check this possibility we first attempted to co-immunoprecipitate CAPN3 and RyR from the membrane fraction of WT muscle. Unfortunately, at the end of the experiment essentially all CAPN3 was autolysed to an ∼55 kDa product which co-migrated exactly with the anti-RyR antibodies that were used for immunoprecipitation, thus making it impossible to detect CAPN3 (data not shown). As an alternative approach we expressed CAPN3 as a GST fusion protein, and performed a GST pull-down assay using muscle membrane fractions as a source of RyR. As shown in Figure 4D (left upper panel), RyR binds to CAPN3-GST-containing beads with much higher affinity than to control GST-containing beads even though the concentration of CAPN-GST bound to the beads was lower than GST alone (Fig. 4D, left lower panel). Thus, our results demonstrate that CAPN3 co-fractionates together with triad components (Fig. 3B) and was able to interact with AldoA and RyR *in vitro* (Figs 1A and 4D). Moreover, CAPN3 is necessary for proper recruitment of these proteins to the triads since their

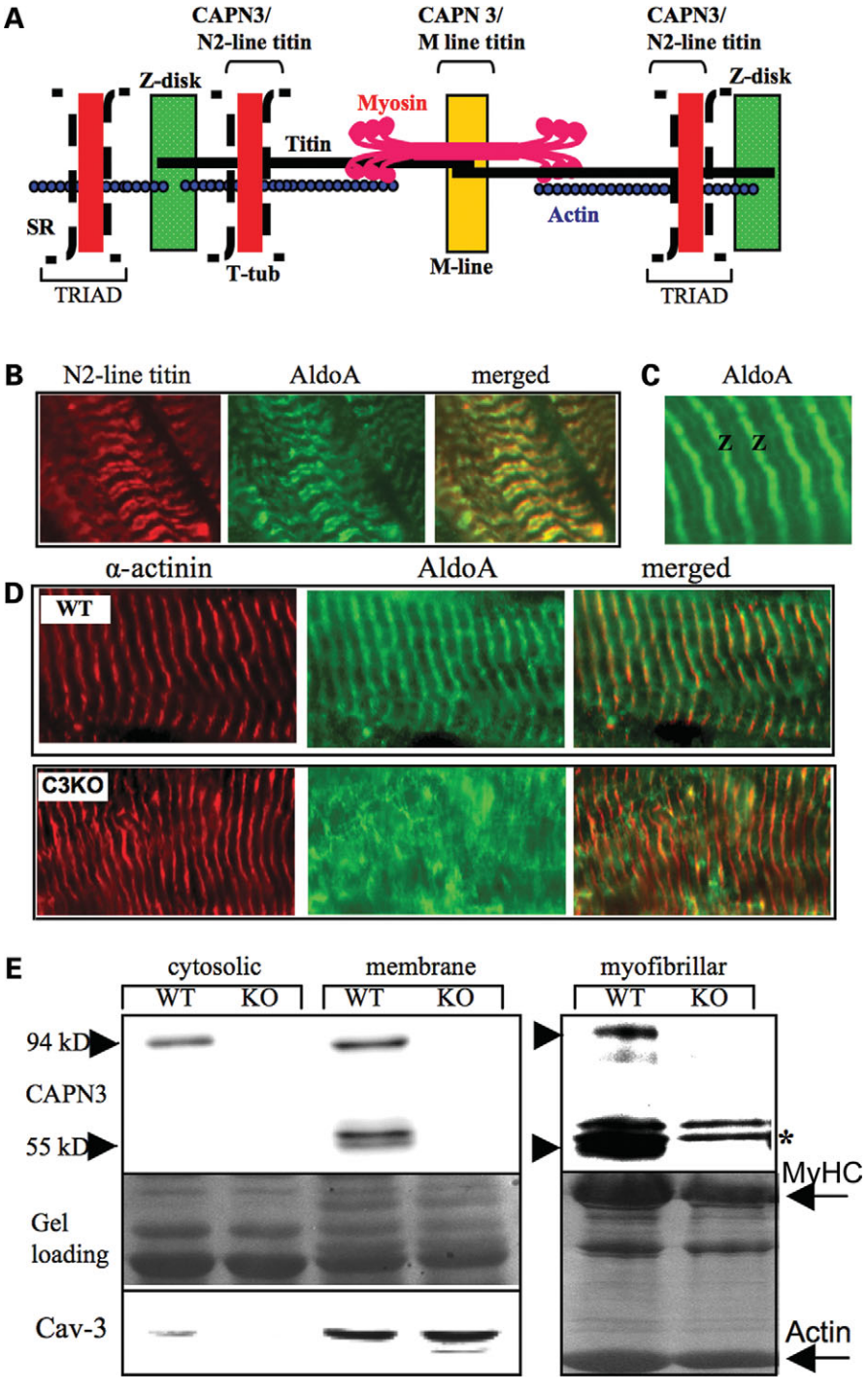

Figure 2. CAPN3 is necessary for proper subcellular localization of AldoA: (**A**) Schematic representation of sarcomere structure. A sarcomere spans between two adjacent Z-disks. Triads are located at both sides of the Z-disk (two triads per sarcomere). CAPN3 was shown to interact with two fragments of titin that are located at the N2-line and M-line of the sarcomere; (**B, C**) Immunostaining of WT muscles showed accumulation of AldoA at both sides of the Z-disk which is consistent with its association with triads. Longitudinal sections of tibialis anterior muscles from WT mice were co-immunostained with anti-AldoA and anti-N2-line titin antibodies. The latter was used because the N2-line and triads have a similar location adjacent to the Z-disk. The distance between two adjacent bands located at the N2-line/triad is difficult to resolve by light microscopy and very often two adjacent bands coincide to produce a broad band in the vicinity of the Z-disk as can be seen in panel (C); (**D**) Mislocalization of AldoA in C3KO muscles. Co-immunostaining of longitudinal sections of WT and C3KO muscles with anti-AldoA and anti-α-actinin (marker of Z-disk) antibodies showed loss of the normal striated pattern of AldoA immunostaining in C3KO muscles; (**E**) CAPN3 is present in membrane fractions of WT muscle. Cytosolic, membrane and myofibrillar fractions from WT and C3KO muscles were stained for CAPN3 in order to determine the subcellular localization of CAPN3. To ensure proper fractionation, an identical blot was stained with antibodies to the membrane protein caveolin-3. Asterisk shows non-specific bands recognized by anti-CAPN3 antibody in myofibrillar extracts; arrows identify MyHC (myosin heavy chain) and actin, the major constituents of myofibrils. Semi-quantitative analyses of western blots showed that the ratio of CAPN3 to the protein content in each fraction was 0.113 for myofibrillar, 0.069 for cytosolic and 0.208 for membrane fraction.

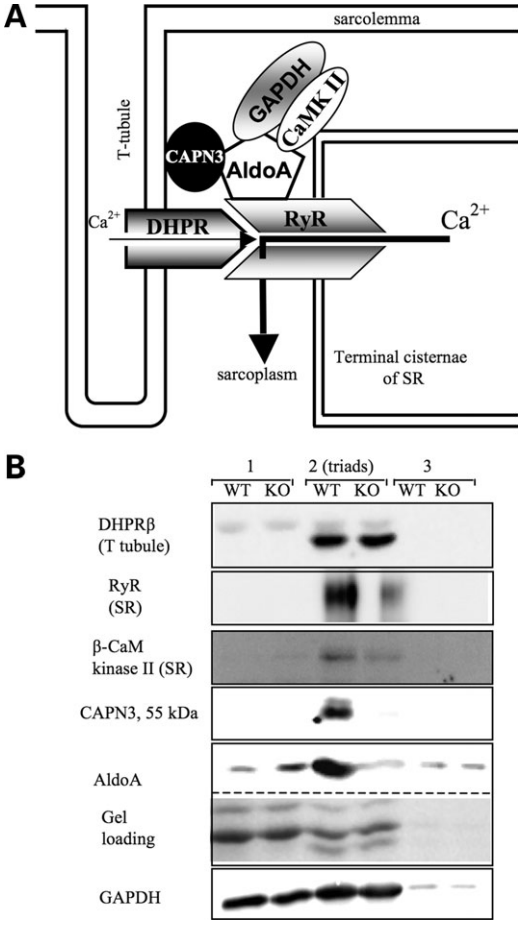

Figure 3. CAPN3 is necessary for recruitment of AldoA to triads: (**A**) Schematic representation of the interface between the T-tubule and SR in triads. A large number of proteins are located at this interface (only proteins that are important for this study are shown). Many of them interact with DHPR (a voltage sensor and Ca^{2+} channel at the T-tubule) and RyR (Ca^{2+} release channel at the SR membrane). Glycolytic enzymes are known to be associated with the SR and interact with Ca^{2+} receptors. The CaMKII (Ca^{2+}/calmodulin-dependent protein kinase II) is also recruited to the triads where it assembles with the glycolytic enzyme complex (21,22); (**B**) AldoA and CAPN3 co-localize in the triad fractions and CAPN3 is necessary for recruitment of AldoA to these fractions. After sucrose gradient centrifugation fractions were collected and analyzed by western blotting using antibodies against triad components shown in (A). Fraction-2 was identified as a triad-enriched fraction because it contained T-tubule components (β-subunit of DHPR) as well as terminal SR proteins (RyR and CaMKII). CAPN3 also was detected in this fraction from WT muscles. AldoA was significantly reduced in fraction-2 that was isolated from C3KO muscles (the gel loading control for this blot is shown below the antibody staining). This reduction is specific for AldoA since another glycolytic enzyme GAPDH was equally represented in both WT and C3KO fractions.

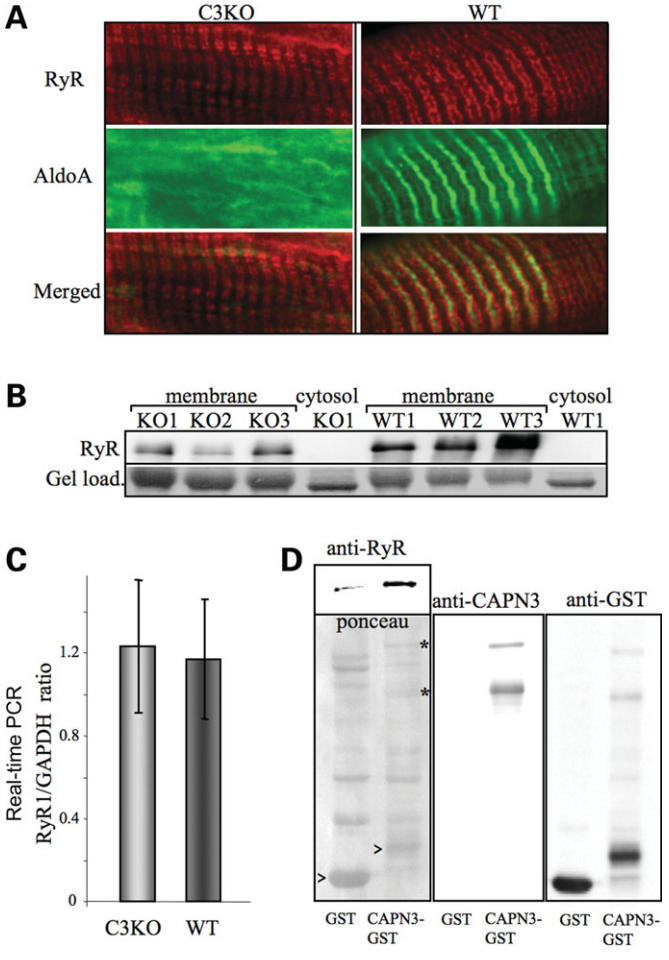

Figure 4. Concentration of RyR is decreased in C3KO muscles: (**A**) RyR localization was not altered in C3KO muscles. Co-immunostaining of C3KO and WT muscles with anti-RyR and anti-AldoA revealed that, unlike AldoA staining, the striated pattern of RyR immunostaining was retained in C3KO muscles; (**B**) Levels of RyR in membrane fractions from C3KO muscles were decreased compared with WT muscles. Membrane fractions from C3KO and WT mice were used for immunoblotting and staining with anti-RyR antibody. Corresponding cytosolic fractions were used as a control to ensure that fractionation was carried out successfully; (**C**) The transcription level of RyR gene does not vary between C3KO and WT muscles. Real-time PCR was performed using cDNAs isolated from three C3KO and three WT muscles. Data from each sample were normalized by dividing the quantity of target gene cDNA (RyR) by the quantity of a house-keeping gene cDNA (GAPDH) to correct for variability in the individual samples; (**D**) GST pull-down assay showing that CAPN3 interacts with the RyR. CAPN3-GST or GST alone were bound to glutathione sepharose and were incubated with muscle membrane extracts. Upper left panel shows that RyR binds to CAPN3-GST with much higher affinity than to GST even though a much higher concentration of GST protein (identified by open arrowheads) than CAPN3-GST (asterisks) was bound to the beads (left lower panel, ponceau staining). Middle and right panel show immunostaining of eluates with anti-CAPN3 and anti-GST antibodies, respectively.

concentrations in the triad were significantly reduced in CAPN3-deficient muscles. Taken together, the data suggest that CAPN3 acts as a structural component of the triad-associated protein complex and is important for complex formation and/or stability.

Calcium release is decreased in C3KO muscle fibers

RyRs form calcium channels that allow calcium release from the SR upon muscle activation. A reduced number of RyRs at the triads may lead to decreased calcium release in C3KO muscles. Recent studies have shown that glycolysis also modulates calcium release even though this process is not energy-dependent (13). On the other hand, association of glycolytic enzymes with the SR may be important for compartmentalization of energy production for ATP-dependent calcium re-uptake. To address the question of whether decreased triad-associated AldoA, and RyR affects calcium transport,

we performed calcium imaging studies in isolated fibers from C3KO ($n = 28$) and WT ($n = 24$) muscles. A significant decrease in calcium release but not in the rate of calcium uptake was observed in fibers isolated from C3KO muscles (Fig. 5). Thus, lack of CAPN3 affects the structural integrity of triad-associated protein complex that includes RyR and AldoA, and impairment of this complex leads to decrease in calcium release upon muscle activation. Taken together, our results identify a new role of CAPN3, particularly membrane-associated pool of CAPN3, in the maintenance of a protein complex regulating calcium release in skeletal muscle.

DISCUSSION

In these studies we have described a novel role of CAPN3 in the protein complex that regulates calcium release in skeletal muscle. In summary, we showed that CAPN3 is present in triad-enriched membrane fractions and that it is necessary for proper recruitment of other components of the triads, namely the glycolytic enzyme AldoA and the calcium release channel RyR. In the absence of CAPN3, the concentration of both proteins in triad fractions is reduced. In agreement with these observations, a significant decrease in calcium release upon activation was found in isolated fibers from C3KO muscles, suggesting that CAPN3 is critically necessary to sustain the integrity of this complex in skeletal muscle. It is tempting to speculate that impairment of calcium transport may be one factor contributing to the clinical phenotype of LGMD2A. Decreased calcium release likely impairs the force generating capacity of the muscle; indeed LGMD2A patients are characterized by muscle weakness.

A surprising finding of this study is that in the context of the CAPN3 relationship with triad components, CAPN3 serves a structural role rather than a proteolytic one. We cannot exclude the possibility that cleavage of yet another unidentified component of triad-associated protein complex by CAPN3 may be required for AldoA and RyR association with triads, however, there are no data in the literature that would support this scenario. We demonstrated that CAPN3 is necessary for proper localization of a fraction of AldoA to triads, and in the absence of CAPN3, the concentration of AldoA at the triads is decreased. The concentration of the RyR is also decreased in C3KO muscles, which is a result that opposes the expected outcome if CAPN3 were acting as a protease, controlling turnover of these proteins. Even though it was suggested a long time ago, based on the biophysical properties of calpains that they may act as structural proteins (23), to the best of our knowledge this is the first report elucidating such a role. This situation may not be unique, however. Intriguing observations of reciprocal secondary reduction were reported for CAPN3 and dysferlin, a membrane protein, mutations in which cause LGMD type 2B (24,25). A direct interaction between CAPN3 and dysferlin was demonstrated in co-immunoprecipitation experiments (26). Although dysferlin was shown previously to play a role in membrane repair (27), no such role has been found for CAPN3. However, recent studies have also implicated dysferlin in T-tubule formation during myogenesis (28). Moreover, a fraction of dysferlin has been shown to be associated with

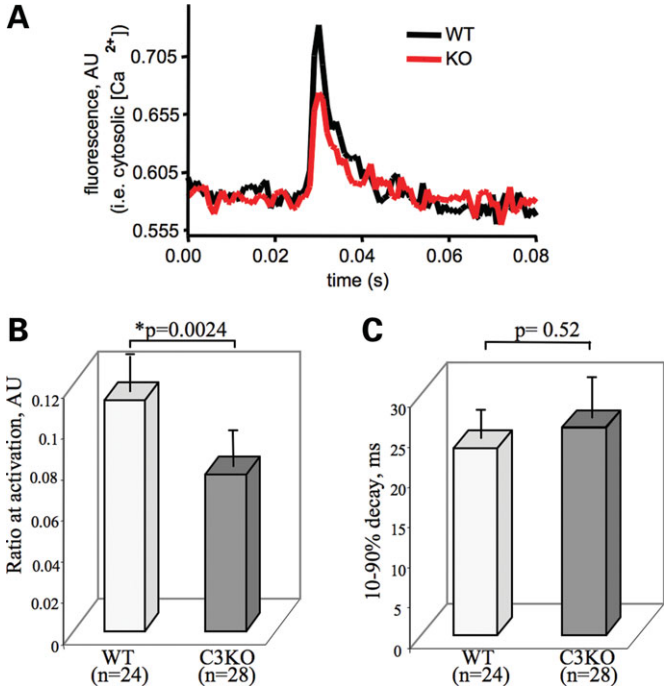

Figure 5. Ca^{2+} release is significantly reduced in C3KO muscles. Calcium imaging using Fura 2FF/AM fluorescent dye was performed in individual muscle fibers isolated from FDB. A total of 24 and 28 fibers were examined for WT and C3KO, respectively: (**A**) A typical Ca^{2+} transient recording from C3KO and WT FDB fibers. Note that the baseline Ca^{2+} level and speed of Ca^{2+} re-uptake are similar between C3KO and WT, whereas the amplitude of Ca^{2+} release is significantly reduced in the C3KO fiber; (**B**) The ratio of Ca^{2+}-bound to Ca^{2+}-unbound fluorescence at the peak that followed muscle activation, was used as a measure of Ca^{2+} release; (**C**) The time of fluorescence decay from 90 to 10% of its peak was used to measure Ca^{2+} uptake. The *P*-value was calculated using a two-tailed *t*-test.

T-tubule-enriched fractions from adult muscle, and to co-immunoprecipitate with DHPR (29). Thus, it is possible that both CAPN3 and dysferlin are components of the triad-associated complex and, based on a secondary reciprocal reduction in LGMD2A and LGMD2B patients, these two proteins may also relate as binding partners rather than as a protease and a substrate. Additional support for a structural role for CAPN3 comes from genetic and biochemical analysis of pathogenic CAPN3 mutations. LGMD2A is characterized by extreme genetic variability; >280 different pathogenic mutations have been identified to date, according to the Leiden Muscular Dystrophy database. The majority of these mutations (~60%) are missense mutations, and they are spread along the entire length of the gene rather than only being concentrated in regions responsible for proteolytic activity (1,30). Furthermore, it was shown that not all mutations affect proteolytic activity of CAPN3 (31). The most recent studies revealed that about one-third out of 79 tested LGMD2A biopsies showed a normal level of CAPN3 autolytic activity (2). All these data strongly support the hypothesis that CAPN3 not only functions as an intracellular protease, but also serves a structural role.

Since the discovery that mutations in CAPN3 cause LGMD2A, numerous attempts have been made to uncover molecular mechanisms of the disease, and yet these mechanisms

remain unclear. In studies from different laboratories, CAPN3 has been implicated in such diverse processes as apoptosis, cytoskeletal and sarcomere rearrangements, myogenesis and sarcomere formation (3,17,19,32–34). To add to this complexity we have described a new role for CAPN3 in the protein complex that regulates calcium release in skeletal muscle.

There are several plausible explanations for why CAPN3 might have so many different functions. CAPN3, like other members of the calpain family, does not have a consensus cleavage site and is able to cleave multiple substrates (35), potentially influencing many different cellular processes. Consistent with this idea it has been shown in several different studies that many proteins are cleaved by CAPN3 *in vitro* or when the proteins are co-expressed in heterologous cell cultures (3,17,19,36). The problem, however, is that very few potential substrates have been validated as bona fide *in vivo* substrates, using C3KO mice or cells, which is a necessary step to draw a conclusion about the physiological significance of any CAPN3–substrate relationship. In the current studies, we showed that CAPN3 can cleave AldoA upon co-expression of these two proteins in a heterologous system but we did not find any evidence that AldoA is a physiological substrate for CAPN3 proteolytic activity *in vivo*.

Besides having multiple substrates, CAPN3 has been shown to localize to different subcellular compartments. First, through its association with titin, CAPN3 is anchored to the sarcomere of mature skeletal muscle where it may regulate myofibrillar and cytoskeletal protein turnover (37). Secondly, in previous studies CAPN3 was detected in myonuclei by immunostaining, although no nuclear substrates have been identified (32). Previously, we showed that in primary myotubes, CAPN3 is present at the membrane and is necessary for regulation of the membrane-associated M-cadherin–β-catenin complex during myogenesis (17). Here, we demonstrate that in mature skeletal muscle, there is also a pool of membrane-associated CAPN3, specifically in triads (Figs 2E and 3). Thus, pools of CAPN3 are present in cytosolic, membrane, myofibrillar and possibly, nuclear fractions of mature skeletal muscles. It is reasonable to suggest that in different locations CAPN3 may have different substrate/binding partners and may serve different roles. Our finding that triad-associated CAPN3 is necessary for proper localization of other triad components and for normal calcium transport supports the notion that CAPN3 can perform numerous cellular roles, likely specified by its subcellular location.

Thus, it seems very possible that CAPN3 does not have a single cellular role but rather serves numerous roles. Therefore, a loss of WT CAPN3 protein can result in abnormalities in different physiological processes depending on the position of the pathogenic mutation. Based on these studies, we can suggest that some LGMD2A mutations may disrupt interaction of CAPN3 with triad proteins and thus affect calcium transport.

MATERIALS AND METHODS

Yeast-two-hybrid screening and protein–protein interaction assay

To identify new binding partners for CAPN3, the CytoTrap system (Stratagene) designed to search for protein–protein interactions in the cytoplasm, was used according to the manufacturer's instructions. Full-length proteolytically inactive C129S-CAPN3 was cloned in frame with human SOS protein and used as bait. A mouse skeletal muscle cDNA cloned into pMyr plasmid (Stratagene) was screened.

For the protein–protein interaction assay, AldoA was expressed with a calmodulin-binding peptide tag (pCAL-c expression vector) and purified using the Affinity Protein Expression and Purification System (Stratagene) as was instructed by the manufacturer. CAPN3-C129S was expressed as a GST-fusion protein in insect cells; recombinant GST was used as a negative control. The ELISA solid-phase assay system was used for binding experiments as was described previously in detail (3). Briefly, recombinant AldoA was used to coat the wells of ELISA plate (ImunoPlates with MaxiSorp Surface, Nunc); CAPN3-GST fusion protein or GST were added to the wells and incubated for 4 h at 4°C. After washes, bound proteins were detected with primary anti-GST antibody followed by secondary antibody conjugated with alkaline phosphatase. Chromogenic alkaline phosphatase substrate pNPP (KPL) was added to the wells and kinetics of color development was measured by absorbance at 405 nm.

Protein co-expression in insect cells

To test if AldoA is a substrate for CAPN3, both proteins were co-expressed in a baculovirus system according to the manufacturer's recommendations (BD Biosciences) as was described previously (3). Briefly, insect cells were plated at 50–70% confluence and infected with AldoA alone or together with active CAPN3 or its proteolytically inactive mutant C129S using high titer viral stocks. After 3 days, cells were collected and analyzed by western blot using anti-AldoA antibody to detect degradation or anti-CAPN3 12A2 antibody to ensure CAPN3 expression.

Tissue preparation, immunohistochemistry and western blot analyses

Tissue preparation, immunohistochemistry and western blot analyses were performed as described previously (3). Muscle tissues from C3KO or WT mice were used for all experiments. The following antibodies were used in these experiments: anti-AldoA (Chemicon), anti-CAPN3 12A2 (Novocastra), anti-N2-line titin antibody was a generous gift from Dr Siegfried Labeit, anti-caveolin-3 (Transduction Lab), anti-α-actinin (Sigma), anti-RyR (Affinity Bioreagent), anti-β-CaM kinase II (Zymed), anti-GAPDH (Chemicon), and anti-DHPR β-subunit were obtained from Developmental Studies Hybridoma Bank maintained by the University of Iowa, Department of Biological Sciences.

All experimental protocols and use of animals were conducted in accordance with the National Institute of Health Guide for Care and Use of Laboratory Animals and approved by the UCLA Institutional Animal Care and Use Committee.

Subcellular fractionation and isolation of triad-enriched fractions

Isolation of myofibrillar fractions was performed as described (38). For isolation of cytosolic and membrane fractions,

muscles were homogenized in detergent-free buffer (0.2 mM EDTA, 0.25 M sucrose and 10 mM Tris–HCl, pH 7.8) and subjected to series of centrifugations at 2000*g* × 8 min, 12 000*g* × 15 min and 130 000*g* × 60 min at 4°C. The first two centrifugations removed myofibrils, nuclei and mitochondria. After the last centrifugation, the supernatant contained cytosolic proteins, while the pellet was enriched in membranes.

Isolation of triad-enriched fractions was performed based on a previously described method of sucrose gradient centrifugation (20) with some modifications (39). Briefly, diaphragm, soleus and gastrocnemius muscles were collected from five mice of each genotype. Muscles were pooled and homogenized in 0.3 M sucrose, 20 mM Trizma-maleate buffer, pH 7.0, with protease inhibitor cocktail (Sigma). Homogenates were subjected to subsequent centrifugations at 2800*g* × 10 min, 8700*g* × 30 min and 78 600*g* × 60 min at 4°C. Pellets after the last centrifugation were homogenized in the same buffer and were loaded on 25–50% continuous sucrose gradients for centrifugation at 131 500*g* × 18 h at 4°C. After centrifugation, fractions were collected and analyzed by western blotting.

Real-time RT–PCR

Total RNA was isolated from diaphragms (three for each genotype) using Trizol reagent (Invitrogen) according to manufacturer's protocol. Genomic DNA contamination was removed by DNAase treatment for 30 min at 37°C. To produce cDNA, 2 μg of DNA-free RNA was used for first-strand cDNA synthesis with random hexamer primers and Superscript III reverse transcriptase (Invitrogen). The resulting cDNAs were used for PCR amplification of RyR cDNA fragment using the following primers: sense 5′-AGACAGAGCACACTGGTCAG-3′ (nucleotide position 15129 to 15148) and antisense 5′-AC AGTCTCCAGCAGGGAAGA-3′ (nucleotide position 15192 to 15211). Nucleotide positions are based on RyR1 mRNA sequence (accession number NM_009109). Parallel reactions were run with the same cDNA samples and GAPDH-specific primers: sense 5′-ACTCCACTCACGGCAAATTC-3′ (nucleotide position 193 to 212) and antisense 5′-TCTCCATGGTGGTGAAGACA-3′ (nucleotide position 344 to 363). Nucleotide positions are based on GAPDH mRNA sequence (accession number NM_008084). PCR amplification using these primers resulted in the generation of single bands. All real-time reactions were performed using IQ™ SYBR Green Supermix PCR reagent (Bio-Rad) and My IQ™ Single Color Real-time PCR Detection System (Bio-Rad). Optical System Software Version 1.0 (Bio-Rad) was used to analyze the results. Quantification utilized standard curves made from serial dilutions of control cDNA sample. Data from each sample were normalized by dividing the quantity of target gene cDNA (RyR) by the quantity of house-keeping gene cDNA (GAPDH) to correct for variability in the individual samples.

GST pull-down assay

CAPN3 cDNA was cloned into pGEX2T vector to express CAPN3-GST fusion protein in *Escherichia coli*. Cells expressing CAPN3-GST or GST alone were resuspended in Bug Buster HT Protein extraction reagent (EMD Biosciences) containing protease inhibitor cocktail (Sigma). Lysates were cleared by centrifugation and incubated with Glutathione Sepharose (GE Healthcare) for 4 h at 4°C with rotation. After incubation, beads were washed extensively with PBS. Membrane fractions were isolated from diaphragm muscles as described in a previous section of Materials and Methods. Pellet containing membrane-associated proteins was solubilized in 0.1 M NaCl, 1% CHAPS, 20 mM Tris–HCl, pH 7.5 and protease inhibitor cocktail (Sigma), and equal amounts of the extract were added to the CAPN3-GST or GST-carrying beads. After overnight incubation at 4°C, beads were washed with PBS. Proteins bound to GST-containing beads or CAPN-GST-containing beads were eluted with equal volumes of reducing sample buffer (80 mM Tris–HCl, pH 6.8, 0.1 M DTT, 2% SDS and 10% glycerol with protease inhibitors cocktail) and analyzed by western blotting.

Determination of Ca^{2+} transients in single muscle fibers

Flexor digitorum brevis (FDB) fibers were enzymatically dissociated as described previously (40). In short, FDB were incubated for 80 min at 37°C in mammalian Ringer solution containing 2 mg/ml collagenase Type II (Worthington CLS2). After incubation, muscles were washed with Ringer solution and gently separated from tendons using a pipette. The dissociated single muscle fibers were loaded with 4 μM ratiometric calcium dye Fura 2FF/AM (Teflabs) for 45 min at 20°C, transferred to the experimental chamber and allowed to adhere to the glass bottom. The experimental chamber was mounted on the stage of an inverted Olympus IX70 microscope equipped for fluorescence. A 360/380 nm excitation wavelength pair was used with a 510 nm emission filter (41). Fluorescence signals were collected from an area of ∼20 × 20 μm using a photomultiplier system (IonOptix). Intact fibers were activated with electrical field stimulation, and the change in the ratio of the fluorescence measured at 380 and 360 nm excitation was calculated to determine Ca^{2+} transients. The myosin inhibitor *N*-benzyl-p-toluene sulphonamide (BTS, 10 μM) was used to abolish force development (42) and prevent movement. The data were analyzed using Ionwizard software (Ionoptix).

FUNDING

This work was supported by National Institute of Health grants (RO1 AR048177 to M.J.S., R01 HL062881 to H.G.) and by Muscular Dystrophy Association grant (to M.J.S.).

ACKNOWLEDGEMENTS

Authors would like to thank Dr A.K. Peter and Dr R.H. Crosbie for their help with triad isolation experiments.

Conflict of Interest statement. There is no conflict of interest to report.

REFERENCES

1. Kramerova, I., Beckmann, J.S. and Spencer, M.J. (2007) Molecular and cellular basis of calpainopathy (limb girdle muscular dystrophy type 2A). *Biochim. Biophys. Acta*, **1772**, 128–144.

2. Milic, A., Daniele, N., Lochmuller, H., Mora, M., Comi, G.P., Moggio, M., Noulet, F., Walter, M.C., Morandi, L., Poupiot, J. *et al.* (2007) A third of LGMD2A biopsies have normal calpain 3 proteolytic activity as determined by an in vitro assay. *Neuromuscul. Disord.*, **17**, 148–156.
3. Kramerova, I., Kudryashova, E., Tidball, J.G. and Spencer, M.J. (2004) Null mutation of calpain 3 (p94) in mice causes abnormal sarcomere formation in vivo and in vitro. *Hum. Mol. Genet.*, **13**, 1373–1388.
4. Sorimachi, H., Kinbara, K., Kimura, S., Takahashi, M., Ishiura, S., Sasagawa, N., Sorimachi, N., Shimada, H., Tagawa, K., Maruyama, K. *et al.* (1995) Muscle-specific calpain, p94, responsible for limb girdle muscular dystrophy type 2A, associates with connectin through IS2, a p94-specific sequence. *J. Biol. Chem.*, **270**, 31158–31162.
5. Kinbara, K., Sorimachi, H., Ishiura, S. and Suzuki, K. (1997) Muscle-specific calpain, p94, interacts with the extreme C-terminal region of connectin, a unique region flanked by two immunoglobulin C2 motifs. *Arch. Biochem. Biophys.*, **342**, 99–107.
6. Garvey, S.M., Rajan, C., Lerner, A.P., Frankel, W.N. and Cox, G.A. (2002) The muscular dystrophy with myositis (mdm) mouse mutation disrupts a skeletal muscle-specific domain of titin. *Genomics*, **79**, 146–149.
7. Haravuori, H., Mäkelä-Bengs, P., Udd, B., Partanen, J., Pulkkinen, L., Somer, H. and Peltonen, L. (1998) Assignment of the tibial muscular dystrophy locus to chromosome 2q31. *Am. J. Hum. Genet.*, **62**, 620–626.
8. Udd, B., Vihola, A., Sarparanta, J., Richard, I. and Hackman, P. (2005) Titinopathies and extension of the M-line mutation phenotype beyond distal myopathy and LGMD2J. *Neurology*, **64**, 636–642.
9. Gamblin, S.J., Davies, G.J., Grimes, J.M., Jackson, R.M., Littlechild, J.A. and Watson, H.C. (1991) Activity and specificity of human aldolases. *J. Mol. Biol.*, **219**, 573–576.
10. Xu, K.Y., Zweier, J.L. and Becker, L.C. (1995) Functional coupling between glycolysis and sarcoplasmic reticulum Ca^{2+} transport. *Circ. Res.*, **77**, 88–97.
11. Franzini-Armstrong, C. (1994) The sarcoplasmic reticulum and the transverse tubules. In Engel, A.G. and Franzini-Armstrong, C. (eds), *Myology*. McGaw-Hill, Inc., New York, pp. 176–199.
12. Han, J.W., Thieleczek, R., Varsanyi, M. and Heilmeyer, L.M., Jr (1992) Compartmentalized ATP synthesis in skeletal muscle triads. *Biochemistry*, **31**, 377–384.
13. Kockskamper, J., Zima, A.V. and Blatter, L.A. (2005) Modulation of sarcoplasmic reticulum Ca^{2+} release by glycolysis in cat atrial myocytes. *J. Physiol.*, **564**, 697–714.
14. Seo, I.R., Moh, S.H., Lee, E.H., Meissner, G. and Kim do, H. (2006) Aldolase potentiates DIDS activation of the ryanodine receptor in rabbit skeletal sarcoplasmic reticulum. *Biochem. J.*, **399**, 325–333.
15. Yao, D.C., Tolan, D.R., Murray, M.F., Harris, D.J., Darras, B.T., Geva, A. and Neufeld, E.J. (2004) Hemolytic anemia and severe rhabdomyolysis caused by compound heterozygous mutations of the gene for erythrocyte/muscle isozyme of aldolase, ALDOA(Arg303X/Cys338Tyr). *Blood*, **103**, 2401–2403.
16. Kreuder, J., Borkhardt, A., Repp, R., Pekrun, A., Gottsche, B., Gottschalk, U., Reichmann, H., Schachenmayr, W., Schlegel, K. and Lampert, F. (1996) Brief report: inherited metabolic myopathy and hemolysis due to a mutation in aldolase A. *N. Engl. J. Med.*, **334**, 1100–1104.
17. Kramerova, I., Kudryashova, E., Wu, B. and Spencer, M.J. (2006) Regulation of the M-cadherin-beta-catenin complex by calpain 3 during terminal stages of myogenic differentiation. *Mol. Cell Biol.*, **26**, 8437–8447.
18. Sorimachi, H., Toyama-Sorimachi, N., Saido, T.C., Kawasaki, H., Sugita, H., Miyasaka, M., Arahata, K., Ishiura, S. and Suzuki, K. (1993) Muscle-specific calpain, p94, is degraded by autolysis immediately after translation, resulting in disappearance from muscle. *J. Biol. Chem.*, **268**, 10593–10605.
19. Taveau, M., Bourg, N., Sillon, G., Roudaut, C., Bartoli, M. and Richard, I. (2003) Calpain 3 is activated through autolysis within the active site and lyses sarcomeric and sarcolemmal components. *Mol. Cell Biol.*, **23**, 9127–9135.
20. Knudson, C.M. and Campbell, K.P. (1989) Albumin is a major protein component of transverse tubule vesicles isolated from skeletal muscle. *J. Biol. Chem.*, **264**, 10795–10798.
21. Bayer, K.U., Harbers, K. and Schulman, H. (1998) AlphaKAP is an anchoring protein for a novel CaM kinase II isoform in skeletal muscle. *EMBO J.*, **17**, 5598–5605.
22. Singh, P., Salih, M., Leddy, J.J. and Tuana, B.S. (2004) The muscle-specific calmodulin-dependent protein kinase assembles with the glycolytic enzyme complex at the sarcoplasmic reticulum and modulates the activity of glyceraldehyde-3-phosphate dehydrogenase in a Ca^{2+}/calmodulin-dependent manner. *J. Biol. Chem.*, **279**, 35176–35182.
23. Edmunds, T., Nagainis, P.A., Sathe, S.K., Thompson, V.F. and Goll, D.E. (1991) Comparison of the autolyzed and unautolyzed forms of mu- and m-calpain from bovine skeletal muscle. *Biochim. Biophys. Acta*, **1077**, 197–208.
24. Anderson, L.V.B., Harrison, R.M., Pogue, R., Vafiadaki, E., Pollitt, C., Davison, K., Moss, J.A., Keers, S., Pyle, A., Shaw, P.J. *et al.* (2000) Secondary reduction in calpain 3 expression in patients with limb girdle muscular dystrophy type 2B and Miyoshi myopathy (primary dysferlinopathies). *Neuromus. Dis.*, **10**, 553–559.
25. Chrobakova, T., Hermanova, M., Kroupova, I., Vondracek, P., Marikova, T., Mazanec, R., Zamecnik, J., Stanek, J., Havlova, M. and Fajkusova, L. (2004) Mutations in Czech LGMD2A patients revealed by analysis of calpain3 mRNA and their phenotypic outcome. *Neuromus. Dis.*, **14**, 659–665.
26. Huang, Y., Verheesen, P., Roussis, A., Frankhuizen, W., Ginjaar, I., Haldane, F., Laval, S., Anderson, L.V., Verrips, T., Frants, R.R. *et al.* (2005) Protein studies in dysferlinopathy patients using llama-derived antibody fragments selected by phage display. *Eur. J. Hum. Genet.*, **13**, 721–730.
27. Bansal, D. and Campbell, K.P. (2004) Dysferlin and the plasma membrane repair in muscular dystrophy. *Trends Cell Biol.*, **14**, 206–213.
28. Klinge, L., Laval, S., Keers, S., Haldane, F., Straub, V., Barresi, R. and Bushby, K. (2007) From T-tubule to sarcolemma: damage-induced dysferlin translocation in early myogenesis. *FASEB J.*, **21**, 1768–1776.
29. Ampong, B.N., Imamura, M., Matsumiya, T., Yoshida, M. and Takeda, S. (2005) Intracellular localization of dysferlin and its association with the dihydropyridine receptor. *Acta Myol.*, **24**, 134–144.
30. Jia, Z., Petrounevitch, V., Wong, A., Moldoveanu, T., Davies, P.L., Elce, J.S. and Beckmann, J.S. (2001) Mutations in calpain 3 associated with limb girdle muscular dystrophy: analysis by molecular modeling and by mutation in m-calpain. *Biophys. J.*, **80**, 2590–2596.
31. Ono, Y., Shimada, H., Sorimachi, H., Richard, I., Saido, T.C., Beckmann, J.S., Ishiura, S. and Suzuki, K. (1998) Functional defects of a muscle-specific calpain, p94, caused by mutations associated with limb-girdle muscular dystrophy type 2A. *J. Biol. Chem.*, **273**, 17073–17078.
32. Baghdiguian, S., Martin, M., Richard, I., Pons, F., Astier, C., Bourg, N., Hay, R.T., Chemaly, R., Halaby, G., Loiselet, J. *et al.* (1999) Calpain 3 deficiency is associated with myonuclear apoptosis and profound perturbation of the IkappaB alpha/NF-kappaB pathway in limb-girdle muscular dystrophy type 2A [published erratum appears in *Nat. Med.* (1999) **5**(7), 849]. *Nat. Med.*, **5**, 503–511.
33. Kramerova, I., Kudryashova, E., Venkatraman, G. and Spencer, M.J. (2005) Calpain 3 participates in sarcomere remodeling by acting upstream of the ubiquitin-proteasome pathway. *Hum. Mol. Genet.*, **14**, 2125–2134.
34. Benayoun, B., Baghdiguian, S., Lajmanovich, A., Bartoli, M., Daniele, N., Gicquel, E., Bourg, N., Raynaud, F., Pasquier, M.A., Suel, L. *et al.* (2008) NF-kappaB-dependent expression of the antiapoptotic factor c-FLIP is regulated by calpain 3, the protein involved in limb-girdle muscular dystrophy type 2A. *FASEB J.*, **22**, 1521–1529.
35. Goll, D.E., Thompson, V.F., Li, H., Wei, W. and Cong, J. (2003) The calpain system. *Physiol. Rev.*, **83**, 731–801.
36. Guyon, J.R., Kudryashova, E., Potts, A., Dalkilic, I., Brosius, M.A., Thompson, T.G., Beckmann, J.S., Kunkel, L.M. and Spencer, M.J. (2003) Calpain 3 cleaves filamin C and regulates its ability to interact with gamma- and delta-sarcoglycans. *Muscle Nerve*, **28**, 472–483.
37. Duguez, S., Bartoli, M. and Richard, I. (2006) Calpain 3: a key regulator of the sarcomere? *FEBS J.*, **273**, 3427–3436.
38. Knight, P.J. and Trinick, J.A. (1982) Preparation of myofibrils. *Methods Enzymol.*, **85**, 9–12.

39. Miller, G., Peter, A.K., Espinoza, E., Heighway, J. and Crosbie, R.H. (2006) Over-expression of microspan, a novel component of the sarcoplasmic reticulum, causes severe muscle pathology with triad abnormalities. *J. Muscle Res. Cell Motil.*, **27**, 545–558.
40. Capote, J., Bolanos, P., Schuhmeier, R.P., Melzer, W. and Caputo, C. (2005) Calcium transients in developing mouse skeletal muscle fibres. *J. Physiol.*, **564**, 451–464.
41. Westerblad, H. and Allen, D.G. (1991) Changes of myoplasmic calcium concentration during fatigue in single mouse muscle fibers. *J. Gen. Physiol.*, **98**, 615–635.
42. Pinniger, G.J., Bruton, J.D., Westerblad, H. and Ranatunga, K.W. (2005) Effects of a myosin-II inhibitor (*N*-benzyl-p-toluene sulphonamide, BTS) on contractile characteristics of intact fast-twitch mammalian muscle fibres. *J. Muscle Res. Cell Motil.*, **26**, 135–141.