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Review

Molecular and cellular basis of calpainopathy (limb girdle muscular dystrophy type 2A)

Irina Kramerova [a], Jacques S. Beckmann [b], Melissa J. Spencer [a,*]

[a] *Departments of Neurology and Pediatrics and UCLA Duchenne Muscular Dystrophy Research Center, University of California, Los Angeles, Neuroscience Research Building, 635 Young Dr. South, Los Angeles, CA 90095-7334, USA*

[b] *Department of Medical Genetics, Faculty of Medicine, University of Lausanne, Lausanne, Switzerland*

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Abstract

Limb girdle muscular dystrophy type 2A results from mutations in the gene encoding the calpain 3 protease. Mutations in this disease are inherited in an autosomal recessive fashion and result in progressive proximal skeletal muscle wasting but no cardiac abnormalities. Calpain 3 has been shown to proteolytically cleave a wide variety of cytoskeletal and myofibrillar proteins and to act upstream of the ubiquitin-proteasome pathway. In this review, we summarize the known biochemical and physiological features of calpain 3 and hypothesize why mutations result in disease.

Keywords: Calpain; Dystrophy; Muscle; Dysferlin; Titin

Most autosomal recessive limb girdle muscular dystrophies (LGMD) are due to mutations in genes encoding structural proteins that make up the cytoskeletal scaffold of muscle. These LGMDs are slowly progressive and involve wasting in the proximal shoulder and pelvic girdle muscles. The observation that mutations in proteins with similar cellular functions produce overlapping clinical phenotypes suggested a common pathophysiological pathway for all the limb girdle muscular dystrophies [1]. Thus, when researchers discovered that mutations in a protease called calpain 3 (CAPN3 or p94) caused LGMD type 2A, it suggested that in spite of the similar clinical phenotype, pathogenesis in these disease entities might differ [2]. The mutation in *CAPN3* was the first identified muscular dystrophy mutation found to occur in an enzyme rather than in a structural protein. Since then, hundreds of pathogenic mutations in the *CAPN3* gene have been described. Of interest is the fact that numerous mutations lead to loss of calpain 3 protein, indicating that lack of this protease leads to pathogenic consequences. Early speculations about the function of CAPN3 suggested that it might modulate members of the dystrophin–glycoprotein complex (DGC), since all previously identified dystrophies had been shown to have some relationship to these proteins. The possible link to the DGC remains, however, cryptic. While indirect relationships between CAPN3 and the DGC have been described, such as CAPN3 cleavage of the sarcoglycan binding protein filamin C [3], the preponderance of the data that have been generated since that time suggest that CAPN3 is more likely to be involved in disassembly of the sarcomere and muscle cytoskeleton to allow for proper protein turnover during muscle remodeling [4,5]. In this review, we will describe the known, biochemical characteristics of calpain 3 and features of calpainopathy. With this information in mind, we will attempt to provide a cohesive explanation for the biological function of calpain 3 and hypothesize why mutations in *CAPN3* might be pathogenic.

1. Genetics and etiology of LGMD2A

The genetic study of the autosomal recessive form of limb girdle muscular dystrophy (AR-LGMD) was prompted by the uncovering of a cluster of LGMD patients in the southern part of the isle of La Réunion. They all presented symptoms fitting the classical description of LGMD (a term introduced in 1954 by

* Corresponding author. Tel.: +1 310 794 5225; fax: +1 310 206 1998.
E-mail address: mspencer@mednet.ucla.edu (M.J. Spencer).

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Walton and Nattras), provided a century earlier by the German neurologist Wilhelm Heinrich Erb [6,7]. Furthermore, these patients belonged to a set of inter-related families, thus facilitating the genetic quest for the etiologic gene. This study led to the initial mapping of the disease locus to chromosome 15q [8], thereby providing an unambiguous demonstration for the existence of this challenged and controversial clinical entity [9,10]. The mapping assignment was soon confirmed on other ethnic isolates. The inclusion of these large consanguineous pedigrees allowed researchers to refine the incriminated interval. These efforts eventually led, after a long and painstaking 5 years search involving linkage mapping, physical mapping [11,12], and association studies [13,14], to the identification of etiologic mutations. All identified mutations fell inside a previously known, yet totally unsuspected gene, *CAPN3*, encoding the calpain 3 protease [15]. Initial descriptions of this gene reported that in humans its 24 exons span close to 53 kb of genomic DNA, leading to a transcript of 3.5 kb. Additional alternatively spliced exons and promoter sequences were subsequently described, contributing to the complexity of this gene's products and confounding analysis of its function [16,17].

As these studies progressed, it soon became clear that the LGMDs formed a genetically heterogeneous entity, involving at least ten recessive forms and three dominant ones, referred to collectively as LGMD2 and LGMD1, respectively [18,19]. A minority of LGMD patients is still unaccounted for by any of these genes, thus suggesting the involvement of additional loci. *CAPN3* mutations account for the largest fraction of the AR-LGMDs, varying regionally from 10 to 50% and reaching as high as 80% in some ethnic populations [20]. Genetic heterogeneity was also documented in isolated ethnic populations such as the inter-related northern and southern Indiana Old Order Amish populations in which families with either calpainopathies or beta-sarcoglycanopathies can be found [21,22].

2. Mutations in the CAPN3 gene

Ten years later, there are now over 280 documented distinct pathogenic *CAPN3* mutations and a smaller number of apparently neutral polymorphic or unclassified variants [23] (Table 1). These mutations were documented in patients covering wide geographic areas. All types of mutations have been found, from gene inactivating mutations such as point mutations leading to premature stop codons, splice sites or frameshift mutations or even larger insertion/deletion type of mutations to missense mutations that affect the primary sequence of the protein, resulting often [20,24] (but not always) [24,25] in loss of calpain 3′s proteolytic activity. With the exception of founder effects in isolated populations, there are no major predominant mutations and in most instances, mutation screening still leads to the discovery of new mutations [26–28]. As many as three out of four newly examined *CAPN3* alleles present with new mutations. Nevertheless, some mutations are encountered more frequently than others. For example, c.550delA and c.2362_2363delinsTCATCT are present in more than 144 and 143 (respectively) of the 1204 individuals,

Table 1
Distribution of CAPN3 mutations by exon and mutation type

Exon number	Exon length bp	Variants		Types of pathogenic mutations				
		Non-pathogenic[a]	Pathogenic	Missense	Stop	Frameshift	Splice	In frame deletion
1	309	11	29	19	0	8	0	2
2	70	1	6	3	1	2	0	0
3	119	6	17	12	1	1	2	1
4	134	4	25	16	1	2	4	2
5	169	4	29	22	2	3	1	1
6	144	4	8	2	1	3	2	0
7	84	4	11	6	2	1	2	0
8	86	1	14	8	2	1	3	0
9	78	0	3	3	0	0	0	0
10	161	4	21	15	1	4	1	0
11	170	3	21	15	0	4	2	0
12	12	3	0	0	0	0	0	0
13	209	5	22	15	1	3	3	0
14	37	3	0	0	0	0	0	0
15	18	2	6	0	0	3	2	1
16	114	3	8	3	0	5	0	0
17	78	0	7	2	0	3	1	1
18	58	2	8	1	0	2	4	1
19	65	1	9	7	0	1	0	1
20	59	1	5	3	1	2	0	0
21	78	1	17	7	3	3	4	0
22	117	5	11	5	0	4	1	1
23	59	2	1	1	0	0	0	0
24	27	3	3	0	0	0	3	0
Total	2465	72	284	165	16	55	36	12

[a] Non-pathogenic or unclassified variant.

mostly calpain-deficient patients, listed in the Leiden muscular dystrophy database (http://www.dmd.nl), possibly reflecting common ancestral founder mutations. This is particularly striking in inbred populations, (e.g. the predominant c.2362_2363delinsTCATCT encountered in patients from Basque origin), as often indicated by the fact that they also share a common haplotype around this mutation. Interestingly, there remains a small but sizeable fraction of patients, for which only one mutant allele of the *CAPN3* gene is identified [29]. This raises the possibility that additional mutations may reside in parts of the gene that were not scanned (for example in remote regulatory regions) or that these patients are hemizygous due to a large cryptic deletion in this region.

In addition to genetic heterogeneity in LGMD2A, unsuspected allelic heterogeneity was also encountered in small inbred isolates, which were a priori considered to represent homogeneous genetic isolates, each presumably reflecting a unique founder mutation [13,21,30]. Based on genealogical studies, the Réunion island LGMD2A pedigrees were thought to form a megafamily related through multiple consanguineous links that trace back to a single common ancestor who was one of the first settlers of this island in the 17th century. Hence, the present day families were expected to represent a clinically and genetically homogeneous set. Their study eventually led to the unsuspected discovery of at least six distinct segregating *CAPN3* mutations [30,31]. Similar observations were reported for the Basque LGMD2A community [32]. This extensive and unexpected allelic heterogeneity created a puzzle, referred to as the Réunion paradox. Different explanations were provided to account for this apparent paradox invoking digenic [33] or monogenic [34,35] inheritance.

The *CAPN3* mutations span almost the entire length of the *CAPN3* gene, though this distribution is inhomogeneous (Fig. 1 or Table 1) sparing only one small exon (exon 12) on the one hand, while showing a grouping of mutations in a subset of exons, on the other hand. When the number of missense mutations is plotted per scanning window of 100 bp, instead of per exon (given that the latter vary widely in size), a clustering of mutations can be observed (Fig. 1 and [36]). Such standardization reveals a non-uniform distribution of mutations, with clusters of missense mutations apparent around domains IIa, IIb and III while a cluster of preserved regions or cold spots is apparent in the IS1 and IS2 domains. Many of these missense mutations have been modeled onto the crystal structure of CAPN3 and from this analysis it could be deduced that these mutations affect enzyme activity, primarily through thorough disruption of domain movement necessary for formation of the catalytic triad at the active site

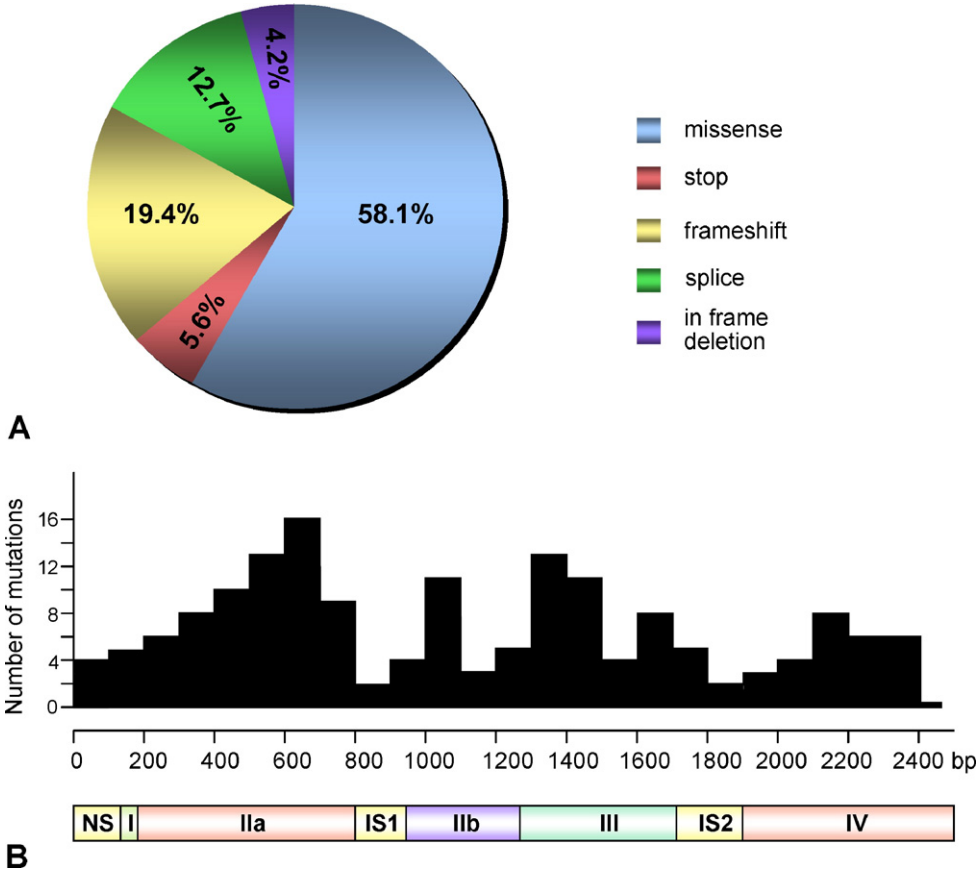

Fig. 1. CAPN3 mutations are predominantly missense mutations that occur in domains II and III. (A) A pie chart is shown in which the distribution of *CAPN3* mutations by type is represented. Missense mutations make up the largest type of *CAPN3* mutation. (B) Distribution of missense mutations along the length of the *CAPN3* coding region and protein domains. Nucleotides were clustered into 100 bp intervals (1–100, 101–200, etc.) for the purpose of determining if any clustering of mutations was evident. This figure demonstrates that clusters can be observed in domains IIa, IIb and III.

[36]. However, some mutations preserve catalytic activity. Of these latter missense mutations that have been directly investigated, it has been determined that several affect the ability of CAPN3 to bind to titin (the relationship between CAPN3 and titin will be discussed elsewhere in this article) [24,25]. It is likely that additional mutations that impair CAPN3/substrate interactions will be identified, although these mutations are yet to be recognized. Hence, loss of proteolytic activity and loss of titin anchorage are the two ways in which *CAPN3* mutations might be pathogenic.

Besides genetic variability, there is also frequent irregularity in the severity of the clinical phenotype of LGMD2A, leading to the suggestion that the genetic makeup of the patients or second mutations might play an important role in determining the symptoms, rate of progression and magnitude of the disease (e.g. modifier alleles) [37,38]. For example, it was observed that in two siblings with calpain deficiency one manifested the disease as a metabolic myopathy while the other as an advanced LGMD [38]. The report of LGMD patients homozygous for null-type mutations demonstrated the pathogenic nature of loss of this activity. However, attempts to derive genotype–phenotype correlations are difficult, due to the extensive heterogeneity of *CAPN3* mutations; therefore, these studies are mostly restricted to the study of patients carrying two gene inactivation alleles or to patients belonging to large consanguineous families (which could be homozygous for a missense mutation). In general, it has been deduced that carriers of two inactivating *CAPN3* alleles tend to be more severely affected with the disease and to display symptoms of LGMD2A at an earlier age than carriers of missense mutations.

3. Clinical phenotype of LGMD2A

The onset of LGMD2A is usually in the second decade of life, although it has been documented to occur as early as age 2.5 and as late as age 49 [20,29,39–41]. Serum CK levels vary from slightly elevated to as high as 50 times normal. A proportion of the LGMD2A patients have severe contractures [42]. The course of the disease is slow but progressive, leading to loss of ambulation by approximately 1 or 2 decades after diagnosis. Scapular winging and hip abductor sparing are two common features with the primary muscles involved being the glutei and hip adductors (Fig. 2). The clinical phenotype has been shown to be highly variable, even in affected siblings, especially with regard to calf hypertrophy [37]. In an MRI study of 7 patients with LGMD2A by Muntoni and colleagues, the predominantly affected muscles in ambulatory patients were in the posterior compartment of the thigh and included the adductors and semimembranous muscles [42]. Patients who had reduced ambulation also showed involvement of the posterior/lateral muscles of the thigh with relative sparing of the sartorius and gracilis. In the calf, all patients showed signs of disease in the soleus and medial head of the gastrocnemius with sparing of the lateral head. In the upper body, the biceps was strongly affected with relative sparing of the triceps. There are no reports of cardiac or facial involvement in this disorder, a feature that stands in great contrast with other forms of LGMD,

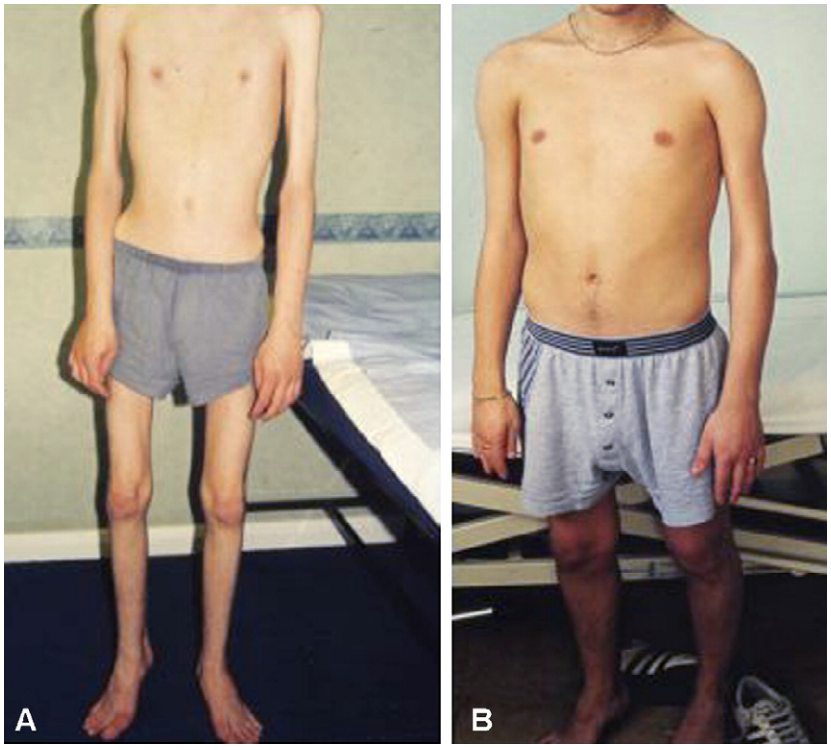

Fig. 2. CAPN3 mutations cause severe muscular dystrophy. Panels A and B show pictures of two patients with LGMD2A. Severe atrophy is apparent in all muscles of the patient in panel A. The patient in panel B shows atrophy of the biceps muscle. Both patients stand on their toes due to contracture of the Achilles tendon.

nor of any intellectual impairment. The lack of cardiac pathology likely stems from the fact that CAPN3 is not detectable in cardiac tissue although the mRNA is expressed in the heart during development [17,43]. Thus, there is selective skeletal muscle involvement in LGMD2A, a feature that may aid in the determination of whether a given patient should be tested for *CAPN3* mutations.

4. Histopathology

Biopsies from patients with LGMD show a typically dystrophic appearance that consists of necrosis, regeneration, fiber diameter variability, myofibrillar disorganization, and fibrosis [40,41,44]. Abundant and disorganized mitochondria have been observed in two studies in which electron microscopy was performed [40,41]. These mitochondria are likely the basis for the lobulated fibers that have been repeatedly reported in human and mouse studies [25,41,45]. Large cohorts of patient biopsies have been examined from countries such as Japan [41], Italy [20], the Czech Republic [44] and Brazil [46] and these studies all reported the same dystrophic picture mentioned above; however, there are differences between these groups of biopsies that are probably attributable to the ethnic make-up of the subjects in a given group. For example, in a study of 21 biopsies from Japanese LGMD2A patients, the authors observed fragmentation of nuclei and a predominance of type I atrophic fibers [41]. These fragmented nuclei may have derived from apoptotic cells in the later stages of the disease or they could be due to generalized necrosis. In contrast, Hermanova et al. described 14 cases of Czech LGMD2A biopsies in which some patients showed a type I fiber predominance while others had atrophic type II fibers along with hypertrophic type I fibers [44]. In this latter study, the atrophic type II fibers suggested a neurogenic lesion because of the severe atrophy observed. In an Italian cohort, the observance of subsarcolemmal glycogen accumulation was reported [20]. Thus, there are features that are common to all LGMD2A biopsies (necrosis, regeneration, mitochondrial disorganization) but also additional features (fiber type specific atrophy, glycogen accumulation) that may or may not present, depending on the genetic make-up of the patients being examined.

Zatz and colleagues examined biopsies from patients from a large consanguineous family (that were homozygous for the null type R110X mutation) in which biopsies were available from 4 affected patients and 3 family members who carried the same mutation but were essentially asymptomatic (with the exception of elevated CK) [46]. While the affected patients had a typically dystrophic phenotype, the three other pre-clinical biopsies looked essentially normal with the exception of one focal area of necrosis in one fascicle. A fourth asymptomatic individual was identified carrying a P82L mutation and she also showed the same picture of normal looking fibers and isolated necrosis. The authors concluded that the early disease features observed in these pre-clinical biopsies suggest that LGMD2A pathogenesis is distinctive from other dystrophies. This conclusion is presumably based on the infrequent detection of necrotic areas in the LGMD2A biopsies, a finding that is in contrast to what is frequently observed in muscles from other asymptomatic LGMD patients.

5. CAPN3 and the ubiquitous calpains

CAPN3 (also known as p94) is a member of the calpain family of intracellular soluble cysteine proteases, a large clan of calcium dependent enzymes (for review see [47]) (Fig. 3). Genome sequencing projects have revealed the presence of calpain genes in various organisms from yeast and fungi to *C. elegans*, *Drosophila* and mammals. At least 13 mouse and 14 human family members have been identified, some of which are ubiquitous and others which are tissue specific. Two of these family members, called calpains 1 and 2, expressed by most mammalian tissues, are the best characterized. The ubiquitous calpains exist as heterodimers consisting of a large 80 kDa, catalytic subunit and a small 28 kDa, regulatory subunit that are non-covalently associated through their C terminal EF hand domains [47,48]. All of the protease activity is contained in the large subunit while the small subunit appears to be vital for correct folding, regulation and stability of the large subunit.

Examination of the structure of the ubiquitous calpains has allowed for the designation of four defined domains in the large catalytic subunit and two domains in the regulatory subunit (Figs. 3 and 4). The domains of the large subunit include a propeptide domain (domain I), a catalytic domain containing the three active site residues (domain II), a third domain containing a calcium and phospholipid binding C2 domain (domain III) and a fourth calmodulin-like Ca^{2+} binding domain containing 5 EF hand sequences (domain IV). The small subunit consists of domain V, which is glycine and proline rich, and domain VI, which is similar to domain IV with 5 EF hand regions. Additional calcium binding sites exist in domain II that play a role in calcium dependent activation [49].

CAPN3 differs from the ubiquitous calpains in several ways. First, while calpains 1 and 2 are ubiquitously expressed isoforms, *CAPN3* transcripts are primarily found in skeletal muscle. The predominant 3.5 kb long mRNA for CAPN3 is expressed at least ten fold higher in muscle than the mRNAs for the conventional calpains [15]. Second, CAPN3 does not interact with a small subunit; instead, it appears to homodimerize through its PEF domains (Fig. 4) [50,51]. Third, while most of the CAPN3 molecule has approximately 50% homology with the large subunit of conventional calpains, CAPN3 has three additional insertion sequences that are found (1) at the N terminus (47 amino acids called NS); (2) within domain II (48 amino acids called IS1); and (3) between domains III and IV (77 amino acids called IS2) [52]. A nuclear localization signal is present near IS2, but the nuclear localization of CAPN3 has not been concretely confirmed.

There are no sequences that are paralogous to the IS1 and IS2 genomic sequences in the Genbank database. It is hypothesized that they either confer a tissue specific function or that they regulate CAPN3 since these sequences distinguish CAPN3 from the other known calpain isoforms. Yeast two-hybrid mapping [53] has suggested that the IS2 domain may mediate the interaction between CAPN3 and titin (please

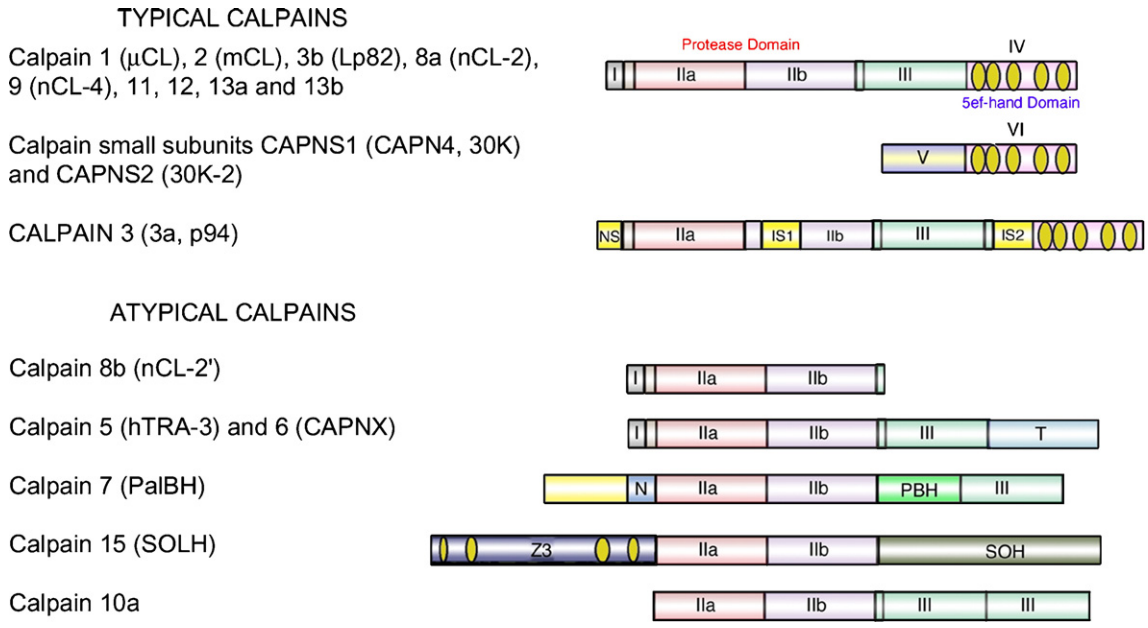

Fig. 3. Calpain family of proteases. Mammalian calpain family members are shown, grouped into "typical" and "atypical" calpains. The ubiquitous (conventional) calpains exist as two subunit heterodimers of a small and large subunit. CAPN3 is shown below the conventional calpains. The NS, IS1 and IS2 regions are shown in yellow. EF hand regions are shown as yellow ovals. Domain numbers are indicated by roman numerals. Atypical calpains are shown as a group in the bottom part of the figure. These calpains lack one or more of the four domains. Note the conservation of the protease domain (domains IIa and IIb) between all calpain family members. Domains IIa and IIb are also referred to as domain I and II by some investigators (see Figs. 4 and 5).

see below for more details about this interaction). Molecular modeling used in conjunction with biochemical studies, has predicted that the IS1 region blocks the active site of the molecule until it is autolytically cleaved to allow substrate access [54]. Modeling studies have also placed these sequences towards the outside of the molecule, suggesting that they might participate in protein–protein interactions [36].

Alternatively spliced isoforms of *CAPN3* (that lack NS, IS1 or IS2 or both) are expressed in several other tissues including rodent lens (Lp82) [55], retina (Rt88) [56] and cornea (Cn94) [57]. Interestingly, the *CAPN3* transcript alternatively splices out IS1 and IS2 containing exons during skeletal muscle development [17]. Following birth, these *CAPN3* specific sequences are retained and are only re-expressed during muscle regeneration [17], suggesting that they serve a developmental role. Support for this hypothesis comes from transgenic studies in which transgenic over expression of the IS1 deleted form of CAPN3 in adult skeletal muscle leads to developmentally immature muscles [58].

The alternatively spliced isoforms lacking IS1 or IS2 are proteolytically active and appear to be more stable than full-length CAPN3 [3,58]. This increased stability may relate to an increased requirement for calcium to achieve activation [59]. The loss of the IS1 sequence removes two of the three autolytic sites in the molecule [50,54], the absence of which also contributes to the stabilization [3,5,17,58,59]. So far, no differences in substrate specificities have been identified between the isoforms however, the inhibitor E64 differentially inhibits full length CAPN3 compared to the alternatively spliced isoforms in lens or peripheral blood cells, suggesting that substrate binding may differ between these species that express or lack the IS1 and IS2 domains [60]. Collectively, these studies suggest that the IS1 and IS2 insertions may be important for determining substrate specificity, regulation of activity and calcium dependence of CAPN3 and its activation. The differential splicing of *CAPN3* in development, post-natal and regenerating muscles suggests that this gene's products may serve different roles in developing vs. adult muscle.

A few other cell types have been shown to express CAPN3 but the significance of the protein in these tissues is yet to be investigated. CAPN3 has been detected in peripheral blood mononuclear cells [61], astrocytes in the brain [62] and in myeloid precursor cells [63]. Interestingly, a recent paper described a cohort of patients that were previously diagnosed with idiopathic eosinophilia and it was observed that 6 of these individuals had mutations in *CAPN3*. At this time, it is not clear if the myositis is derived from primary pathogenesis of the muscle or the myeloid cells; however, this observation extends the spectrum of diseases that involve *CAPN3* mutations. In addition, the identification of a monogenic disorder that shows tremendous variability in phenotypic consequences is a theme that is being observed repeatedly in the area of neuromuscular disorders.

6. Activation of CAPN3

The structure of calcium-free calpain 2 (a conventional calpain) was obtained in 1999. In these studies, the authors showed that the large subunit consists of four subdomains occupying one general oval plane connected to an N-terminal anchor peptide [64]. The small subunit also resides in the same oval plane. While the model of four subdomains fit with the previously predicted structure that had been based on the

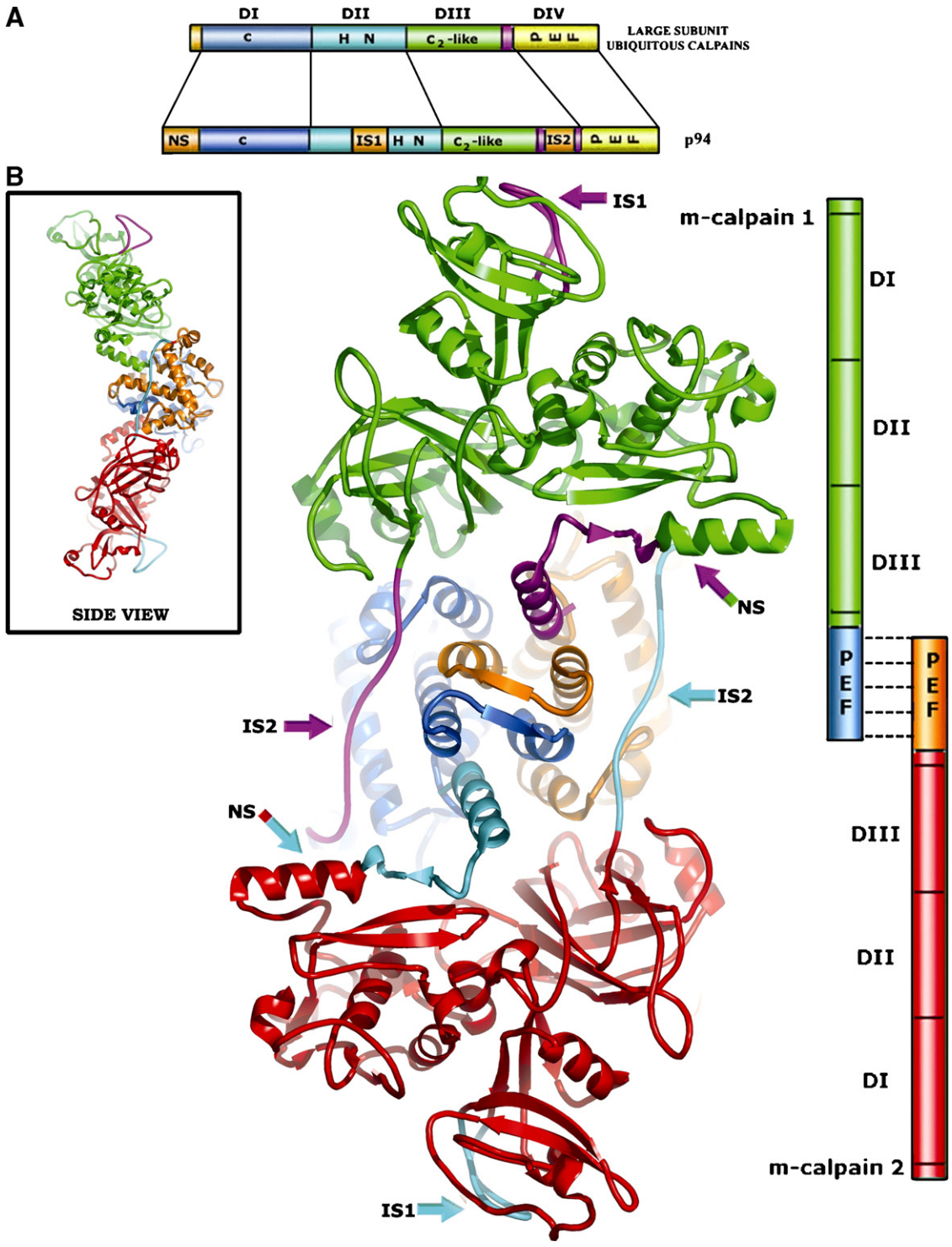

Fig. 4. Crystal structure of CAPN3 shown as a homodimer. Shown in panel A is a diagram of the four domains of the large subunit of m-calpain (top) and CAPN3 (bottom). The three additional sequences of NS, IS1 and IS2 are shown in yellow. Shown in panel B is a graphical representation of the crystal structure of two CAPN3 molecules homodimerizing through their C termini. NS, IS1 and IS2 regions are indicated by arrows. To the right of the crystal structure is a bar figure that represents a side view, demonstrating how the two CAPN3 molecules interact through the five EF hand domains. Figure taken from Ravulapalli et al. [51]. Please note that domains I and II in this figure correspond to domains IIa and IIb in Fig. 3.

primary sequence [65,66], the structural studies further showed that the protease domain, can be subdivided into subdomains I and II. Domains I and II contain the three catalytically active residues that form a triad in the cleft between them. In general, calpains 1 and 2 are inactive in the absence of calcium due to the large distance (10.5 Å) between these residues (cysteine 105, histidine 262 and asparagine 286 in calpain 2) that make up the catalytic triad [49,67]. Activation of calpains occurs in two

steps. First, binding of calcium to domains III, IV and VI helps to break the electrostatic salt links between domains I and IIa and between the acidic residues in domain III with the lysine residues in subdomain IIb [68]. Calcium binding also occurs in the active site to allow for it to properly form the catalytic triad between Cys, His and Asn [49]. To form a catalytically active site, the 10.5 Å distance between the catalytic residues must be decreased to 3.7 Å [49,64,69]. Thus Ca^{2+} binding to numerous sites along the molecule in conjunction with cleavage of the N terminal peptide allows for the release of constraints and proper alignment of the active site residues.

Information regarding the activation mechanism of CAPN3 has been derived from modeling studies that have predicted the structure of normal and mutated CAPN3 as well as traditional biochemical studies [36,54,59,70]. Early analysis of CAPN3 showed that it rapidly autolyzed if it was expressed in vitro, or following homogenization of muscle tissue [50,71]. These studies also showed that deletion of the IS1 or IS2 domains increased CAPN3's half-life and it has been speculated that this increase in stability relates to a reduction in calcium sensitivity [71]. Follow up studies in which CAPN3 was expressed in insect cells allowed for some traditional biochemical characterization of the enzyme to be carried out. In these early studies it was determined that full length CAPN3 was calcium dependent, but required much less calcium than the conventional isoforms [72]. While the authors could express a small amount of soluble and active CAPN3, the levels of expression were not sufficiently high to allow for biochemical analysis.

The catalytic core domains of all calpains are similar and the critical residues for Ca^{2+} binding are well conserved, suggesting that Ca^{2+}-dependent activation may be a common feature for all calpains including CAPN3. Recently, Davies and colleagues utilized a truncated form of CAPN3 that only consists of domains I and II (referred to as p94I–II) [54,70,73]. This truncated form of the enzyme is stable in EDTA but autolytically cleaves in the presence of calcium, thus enabling the use of p94I–II to facilitate understanding of the biochemical properties of CAPN3 activation. Together, with molecular modeling based on the known crystal structure of calpain I (domains I/II), these studies have demonstrated that intramolecular and intermolecular autolytic cleavage occurs in two sites of NS and at least two sites in IS1. IS1 acts like an internal propeptide, removal of which must occur in order to make the active site available to hydrolyze exogenous substrates. NS does not have a similar function because activation of a NS deletion mutant is indistinguishable from that of the full-length p94I–II [54]. It has also been shown in these studies that the two autolytically produced fragments, both of which contain critical catalytic residues, remain associated non-covalently in the presence of Ca^{2+}. Based on their observations, the authors proposed a two-stage model of activation of the proteolytic core of CAPN3 shown in Fig. 5. According to this model, upon Ca^{2+} binding, the CAPN3 proteolytic core undergoes a conformational change similar to the ubiquitous μ- and m-calpains [49,70]. This change allows for release of structural constraints such as salt bridges, and formation of the catalytic triad that makes up the active site. The low calcium requirement of CAPN3 might be due to the

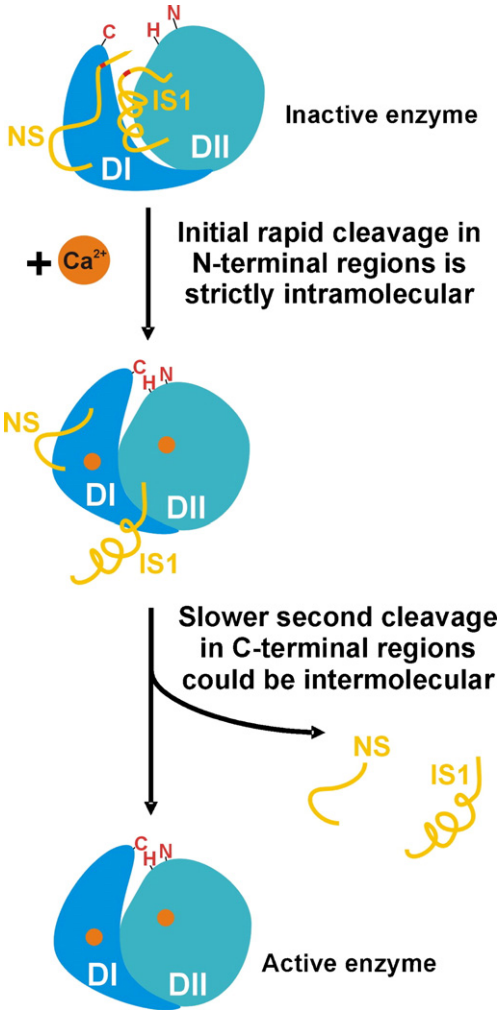

Fig. 5. Model of the activation mechanism of CAPN3. Shown is a graphic representation of proposed activation mechanism of CAPN3 from Garcia Diaz et al. [70]. In this model, Davies and colleagues propose that activation happens in two steps involving intra and intermolecular cleavages. In step 1, calcium binds, allowing domain movement and formation of the catalytic triad (cysteine, histidine and asparagine). This active site formation is followed by intramolecular cleavages at the N terminal region of both NS and IS1. In step 2, intermolecular cleavage occurs at the C terminal regions of NS and IS1. The latter step allows for the enzyme to be fully active.

presence of the IS1 and IS2 insertions that may add flexibility to the molecular structure, thus facilitating formation of the active site [59]. Incidentally, several studies have shown that deletion mutants of the CAPN3 proteolytic core (p94I–II) lacking IS1 [54] or full length CAPN3 lacking IS1 [3,17] are also fully active in the presence of Ca^{2+}.

Due to the unstable nature of CAPN3, only a limited amount of information about the calcium requirement of full-length CAPN3 is available. While calpain 2 is known to require millimolar concentrations and calpain 1 is known to require micromolar concentrations, CAPN3 appeared to require nanomolar concentrations in early studies [72]. However, there is no agreement about the specific Ca^{2+} requirement for CAPN3 activity in the literature. For example, it was shown by one group that CAPN3 underwent rapid autolysis even in the presence of EDTA [50] but another group reported that CAPN3

was stable in the presence of EGTA [72]. Studies of the catalytic core fragment of CAPN3 showed that the full-length p94I–II and its deletion mutant lacking NS and IS1 require Ca^{2+} for activity. Unlike studies using the recombinantly expressed protein, p94I–II had half maximal activity values of 150 μM $CaCl_2$ for p94I–II and 90 μM $CaCl_2$ for the IS1-deletion mutant [70]. However, it was observed in these studies that slow accumulation of autoproteolytic fragments was detected even at substoichiometric levels of Ca^{2+} and in the presence of EDTA.

7. CAPN3 in physiological processes

The exact mechanisms involved in the pathogenesis of LGMD2A are still unknown. Since CAPN3 is a protease with a potentially broad range of substrates, there is a high probability that it is involved in regulating multiple physiological processes. This diverse role for CAPN3 means that its absence or mutations in CAPN3 can affect many pathways in muscle cells. Indeed, studies using cell culture, human biopsies and mouse models have revealed a potential involvement of CAPN3 in the regulation of processes as diverse as apoptosis, muscle cell differentiation, sarcomere formation, muscle remodeling and regulation of the cytoskeleton. Below we discuss current knowledge of the role of CAPN3 in these processes and its possible contribution to the pathogenesis of LGMD2A.

8. CAPN3 and NFκB-mediated apoptosis

The hypothesis that loss of CAPN3 proteolytic activity leads to myonuclear apoptosis was based on the histological identification of TUNEL-positive myonuclei in biopsies from patients with LGMD2A [74]. In this study, the observation was made that biopsies from LGMD2A showed an approximately 100 fold higher frequency of myonuclear apoptosis than that found in biopsies from other muscular dystrophy patients, suggesting that apoptosis may be a specific pathogenic feature of LGMD2A; yet overall, the fraction of apoptotic myonuclei was still very small [74]. Interestingly, in this study, apoptotic myonuclei were detected in morphologically normal fibers that were found in patches of variable size. In the same study, IkBα, a negative regulator of NFκB, was found to accumulate in apoptotic myonuclei, while NFκB was found to accumulate under the sarcolemmal membrane. These findings led to the hypothesis that IkBα is a substrate for CAPN3 and that in the absence of proteolytically active CAPN3, IkBα concentration increases. This accumulation of IkB would lead to stabilization of the IkBα/NFκB complex in the cytoplasm and inhibition of NFκB signaling, leading to the loss of NFκB-mediated expression of survival genes. To confirm this hypothesis, IkBα was shown to be a substrate for CAPN3 when the two proteins were co-expressed in insect cells [74]. However, if this hypothesis were correct, one would expect to observe accumulation of IkBα in protein extracts from LGMD2A biopsies, which was not shown. Instead, an accumulation of NFκB was seen in 2 LGMD2A patients (out of 8 total). While these studies are intriguing, the NFκB inhibition hypothesis contradicts recent data obtained using in vivo mouse models of NFκB activation and repression [75]. In those studies, NFκB inhibition due to transgenic expression of an IkB mutant resistant to degradation had no effect on muscle mass or pathology, whereas NFκB activation, achieved through enhanced degradation of IkB, caused severe muscle wasting. Thus, while apoptosis may be a mechanism occurring in LGMD2A, it is neither clear that loss of NFκB signaling is the basis for its occurrence nor whether this apoptosis is a primary or an ancillary feature of the pathophysiological process of LGMD2A.

Two mouse models of LGMD2A have been generated, and both of them have been used to test the question of apoptosis in this disease [25,76]. One mouse model of LGMD2A carried an in-frame deletion of 63 amino acids at the catalytic domain of CAPN3, that most likely eliminated proteolytic activity but preserved other properties of CAPN3 such as binding to titin [76]. In this study, the authors reported the observation that apoptotic nuclei were a pathological feature of calpainopathy; however, because double staining for a sarcolemmal marker was not performed in this investigation, it was unclear if the apoptotic nuclei were located inside or outside of the myofibers. The frequency of occurrence of observed apoptotic nuclei was also not reported. Studies on the second model of *CAPN3*-null mice also revealed the presence of apoptotic nuclei, but demonstrated that they were not apoptotic myonuclei but rather, apoptotic nuclei from infiltrating immune cells [25]. In this case, double staining for apoptotic nuclei in conjunction with staining for a sarcolemmal marker clearly showed that apoptotic nuclei were located outside of the myofibers and were co-stained with an immune cell marker [25]. This contradiction may indicate that apoptosis is not a primary mechanism of LGMD2A but that it is a secondary event in the pathogenic process. Since the *CAPN3*-deficient mouse models have a rather mild phenotype compared to LGMD2A patients, it is possible that apoptotic myonuclei, being one of the late manifestations of the disease, may not be readily detectable in mice.

9. Role of CAPN3 in regulation of the cytoskeleton

Several cytoskeletal proteins have been shown to be substrates for CAPN3 in various in vitro assays [3,5]. Among those, probably, the most interesting is filamin C, a muscle-specific isoform of filamin that interacts with actin, myotilin, γ- and δ- sarcoglycans, and was hypothesized to provide a signaling link between the sarcolemma and Z disk of myofibrils [77–79]. Recently, a novel type of autosomal dominant myofibrillar myopathy was described that is associated with a mutation in the filamin C gene [80]. In this study, a pathogenic mutation occurred in the C-terminal region of filamin C that prevented dimerization of the protein [80]. Interestingly, this form of myofibrillar myopathy showed clinical signs of LGMD and cytoplasmic aggregates that contained not only filamin C but other sarcolemmal and Z disk proteins [80]. CAPN3 was shown to interact with and cleave filamin C at the C-terminal region in both in vitro transcription–translation assays and in co-expression

experiments. Moreover, this cleavage may regulate the interaction of filamin C with the sarcoglycans, since cleavage of filamin C inhibited its ability to interact with γ- and δ-sarcoglycans [3]. The ability of CAPN3 to cleave filamin was confirmed in independent co-expression experiments [5]; however, no evidence was provided in any of these studies that filamin 3 serves as a physiological substrate for CAPN3 in vivo using *CAPN3* knockout mice.

A number of other cytoskeletal proteins have been identified as potential substrates for CAPN3 such as talin, vinexin and ezrin [5]. Taveau et al., 2003 investigated the relationship between the CAPN3 isoforms and the cytoskeleton in a cell culture system [5]. In these studies it was shown that overexpression of full length CAPN3 in cultured cells caused cell rounding associated with disorganization of the actin cytoskeleton and focal adhesions. CAPN3 mutant forms with either deletion or mutation of the internal cleavage sites (IS1 region) were not able to recapitulate this phenotype, lending support for the idea that cleavage of IS1 is important for proteolytic activity [5]. At the same time, an alternatively spliced isoform of CAPN3 carrying an IS1 deletion was able to cleave a number of cytoskeletal proteins with approximately the same efficiency as full-length CAPN3 [5]. Even though these studies demonstrated that CAPN3 cleaves many cytoskeletal proteins in vitro, future studies are necessary to confirm that any are bona fide in vivo substrates before drawing any conclusion about the role of CAPN3 in regulation of the cytoskeleton.

10. CAPN3 and dysferlin

Dysferlin is a member of the ferlin family of transmembrane proteins shown to be involved in vesicle trafficking and membrane repair (for review see Bansal and Campbell, 2004 [81]). Mutations in dysferlin cause three clinically distinct autosomal recessive muscle diseases, LGMD2B, Miyoshi myopathy and distal anterior compartment myopathy [82–84]. Dysferlin knock-out mice have been generated and it was demonstrated using these mice that in the absence of dysferlin, vesicles that provide an additional membrane to reseal the disrupted sarcolemma accumulate at the site of repair but don't fuse, suggesting a role for dysferlin in muscle membrane fusion [85]. Intriguingly, a secondary reduction of CAPN3 was reported in at least some patients with LGMD2B and Miyoshi myopathy [86]. Reciprocally decreased sarcolemmal staining of dysferlin was occasionally found in patients with calpainopathy [87]. Recently it was shown that dysferlin and CAPN3 biochemically interact in co-immunoprecipitation assays [88]. Thus, the reciprocal loss of CAPN3 and dysferlin in LGMD2A and LGMD2B biopsies and the finding of a biochemical interaction between these proteins leads to the suggestion that dysferlin functions to stabilize CAPN3 at the membrane and visa versa. Alternatively, CAPN3 may play a role in dysferlin-mediated plasma membrane repair. Others have hypothesized that CAPN3 might function in membrane repair by regulating annexins A1 and A2, proteins that interact with dysferlin [89]. If CAPN3 were important in dysferlin's ability to function in membrane repair, one would expect to find disrupted plasma membrane repair in *CAPN3*-deficient mouse models. The issue of membrane instability in calpainopathy has been investigated using both available mouse models. Richard et al. reported a significant loss of membrane integrity in *CAPN3*-deficient mice, even without exercise [76]. However, follow up studies by the same group with the same mice did not reveal any membrane alterations, even after downhill and treadmill training [90]. We also did not find any indication of significant membrane damage in *CAPN3* knock-out mice after they were challenged with downhill running exercise for 4 days [4]. Thus, the question of the possible involvement of CAPN3 in processes mediated by dysferlin remains to be answered. There is a possibility that while membrane integrity is not affected by loss of calpain, membrane repair may be jeopardized. Studies specifically addressing this issue need to be undertaken.

11. CAPN3 and sarcomeric proteins

The localization of CAPN3 in skeletal muscle has been unclear. While CAPN3 has been localized at the N2-line [91], Z disc, M-line, costameres, myotendinous junctions and nuclei [5,74,91] in various reports, none of these studies have been confirmed using CAPN3-null muscles as a negative control. Yeast two hybrid assays have identified two regions of titin that bind to CAPN3 called the N2 line and the M line [53,92]. Recently, those CAPN3–titin interactions were confirmed at the protein level [25]. Therefore, an important site for CAPN3 anchorage in the cell is on the muscle-specific protein titin at the N2 and M line regions. Future studies will be necessary to confirm if alternative cellular sites of localization exist.

Titin (also called connectin) is a giant cytoskeletal protein that spans half of the sarcomere and is important for both sarcomere assembly and function [93]. During myofibrillogenesis, titin acts as a molecular ruler that controls alignment of actin and myosin filaments and acts as a scaffold for other sarcomeric proteins. In mature muscle, titin forms a third myofilament system that provides the muscle with elasticity and passive tension [94]. The elastic properties of titin are achieved primarily through folding and unfolding of a unique proline-rich PEVK region located just after the N2 line region in the sarcomere [93,95,96]. Another unique sequence in the titin molecule is the kinase domain located at the C-terminal end (near the M-line of the sarcomere). Recent studies have demonstrated that titin serves as a molecular sensor of mechanical stretch that activates this kinase domain and subsequently regulates gene expression [97].

It has been proposed that titin might serve a stabilizing role for CAPN3 keeping it in an inactive state and preventing its rapid autolysis [98,99]. It is also possible that titin directly controls CAPN3 activity or access to its substrates. Support for the hypothesis that titin serves to stabilize CAPN3 from autoproteolytic degradation comes from several observations. First, CAPN3 is very stable in whole muscles until the muscle is subjected to homogenization, a process that might dislodge CAPN3 from its anchored position. Second, overexpression of wild type CAPN3 has a deleterious effect on cultured cells [5]

and yet overexpression of CAPN3 from a transgene does not produce any phenotype in adult differentiated muscles in vivo [58]. Finally, CAPN3 is not stable when expressed in vitro [71]. Taken together, these observations suggest that the absence of titin or disruption of the titin/CAPN3 interaction is deleterious for CAPN3 function.

Although a role for titin as a "molecular depot" for CAPN3 still needs to be proven in direct experiments, several recent observations lend support for this hypothesis. A titin mutation that leads to a severe muscular dystrophy was identified in the mdm mouse that carries a LINE insertion at the N2 line region of titin [98]. This insertion, causes mis-splicing of the titin mRNA, leading to a deletion near the N2 line and loss of the CAPN3/titin interaction in yeast two-hybrid assays [100]. Muscle extracts from mdm mice show a decreased concentration of CAPN3, lending support for the hypothesis that titin regulates CAPN3 stability [98]. Furthermore, patients with tibial muscular dystrophy, caused by a mutation in the M-line fragment of titin, also have a secondary CAPN3 deficiency [99]. Finally, some of the pathogenic CAPN3 mutations that are not predicted to affect proteolytic activity of CAPN3 showed a reduced binding affinity to titin in yeast two hybrid assays [24] and in direct protein–protein interaction assays [25]. Interestingly, a recently identified pathogenic LGMD2A mutation (Ser606Leu) was identified in the IS2 domain [101]. This mutation is unlikely to affect activation of the molecule and therefore, the mutation likely affects anchorage to titin. These studies suggest that the CAPN3/titin interaction is not only important but that loss of this interaction can have pathogenic consequences.

To address the question about the physiological role of the CAPN3–titin interaction in vivo, mouse models have been generated that either lack or overexpress CAPN3 in the mdm background [102]. As shown previously, overexpression of CAPN3 in the muscles of a wild type mouse is not toxic and does not produce any phenotype [58]. In contrast, overexpression of CAPN3 in mdm mice results in a worsening of the mdm phenotype [102]. These data strongly support the hypothesis that titin serves as an important buffer and regulator of CAPN3 activity. In wild type muscle, CAPN3 appears to be sufficiently sequestered on titin; however, in the mdm mouse, one of the two CAPN3 binding sites is absent. This loss of anchorage is clearly deleterious for muscle. Double mutants that lack CAPN3 on the mdm background, on the other hand, did not show any change in the severity or progression of the mdm phenotype suggesting that aberrant CAPN3 activity is not a primary mechanism of mdm pathology [102].

It is now known that defects in several proteins that have some association with titin, can cause muscular dystrophy (Fig. 6) [3,25,80,98,99,103–108]. This "titin macromolecular complex" (TMC) can therefore be considered to be a second protein complex in which any one mutation in a complex member is pathogenic. This idea has precedent in the dystrophin glycoprotein complex (DGC) [108,109,1996,110–116], a large protein complex that serves both structural and regulatory functions in muscle [117]. It has been shown that defects in any one of several members of the DGC or associated proteins can cause any of several muscular dystrophies that vary in severity and age of onset [118]. Investigations of the structure of the DGC in normal and DGC-mutant muscle has also provided convincing evidence that mutations in a single DGC member can have major effects on the ability of non-mutant DGC members to assemble and function [119]. Thus, all proteins in this complex subserve a common physiological function. If this functional and structural interdependence were also present in the TMC, it would help explain the common dystrophic consequences of defects in any of several titin complex members. Thus, understanding the relationship between titin and C3 and these other TMC members may be critical in uncovering the pathophysiological basis of several diseases and provide insight into basic questions about the myogenic process.

The possibility that titin binding to CAPN3 serves to modulate CAPN3 activity or put it in correct proximity to its substrates may have important implications for the structure and function of the TMC. CAPN3 may play an essential role in regulating the structure and assembly of the TMC by proteolytic modification of proteins that interact directly or indirectly with titin [93]. This potential role for CAPN3 in regulating the structure and function of the TMC is consistent with previous findings concerning the function of other calpain isoforms that can regulate cytoskeletal rearrangement and protein–protein interaction by limited proteolytic cleavage [120]. Baculoviral co-expression studies, in which different titin domains were co-expressed with CAPN3 showed specific cleavage of M line and PEVK regions of titin but not of N2 line of titin [25]. These studies demonstrated that titin can serve as a substrate for CAPN3 in regions adjacent to where it binds and lend support for the idea that succinct cleavage of titin by CAPN3 may allow for the exchange of proteins on the titin scaffold that is a necessary occurrence for remodeling during myofibrillogenesis and adaptation.

12. CAPN3 and sarcomere remodeling

Previous studies by our laboratories have shown that overexpression of the alternatively spliced isoform of *CAPN3* (minus exon 6) results in muscle that is developmentally immature [58]. This led to the hypothesis that full length CAPN3 may play a role in muscle maturation. To assess muscle maturation in the absence of CAPN3, primary cultures were generated from muscles of *CAPN3* knock out mice (C3KO). These cells have a defect in their ability to form sarcomeres [25]. To determine if the abnormal myofibrillogenesis observed in vitro might result in abnormal sarcomeres in vivo, electron microscopy of adult C3KO muscles was carried out [25]. These studies showed that the ultrastructure of C3KO sarcomeres in vivo was also abnormal. In these studies, otherwise healthy muscle fibers showed a misalignment of the edges of the A bands, which is the myosin-containing region of the sarcomere [25]. Thus, sarcomere formation and structure are abnormal in the absence of CAPN3. While it is evident that humans and mice lacking CAPN3 develop apparently normal and functional muscles, this occurrence may be due to compensating factors that are present in vivo but absent in vitro. The observation of

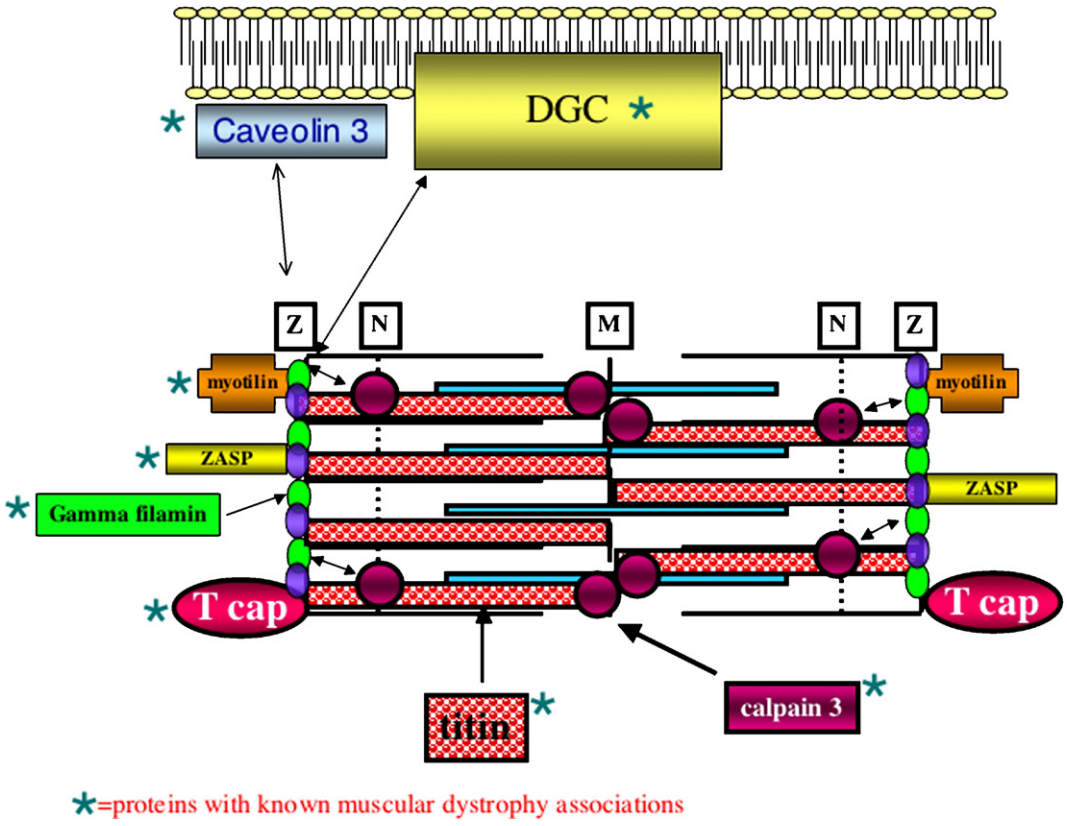

Fig. 6. Cartoon of a sarcomere and associated proteins that have mutations associated with muscular dystrophy. The localization of these proteins is shown. All proteins with a blue asterisk have mutations that result in muscular dystrophy.

abnormal sarcomere formation in C3KO myotubes and abnormal sarcomere ultrastructure in otherwise healthy muscle cells has led to the hypothesis that CAPN3 has a role in sarcomere remodeling processes in adult muscles.

13. CAPN3 and post-natal muscle remodeling

In light of the abnormal sarcomeres observed in the C3KO mice and the previously known role for calpains in muscle protein turnover, the hindlimb suspension/reloading model was used to investigate the question of CAPN3's role in muscle remodeling [4]. In this procedure, mice are suspended by their tails for 10 days, a process that unloads the hindlimb muscles. During this period due to the lack of weight bearing, significant muscle mass loss occurs [121]. After the muscles are allowed to resume weight bearing (called "re-loading"), muscle mass is slowly regained over a period of 7–10 days. The advantage of using such a model is that muscle remodeling must occur if both the atrophic and growth phases are to occur normally. These studies demonstrated a reduction in the ability of C3KO muscles to adapt to changes in load bearing because null muscles did not change mass and cross sectional area as readily as controls. In particular, muscle growth was severely reduced in the knock out during reloading compared to controls. These findings suggest that CAPN3 plays an important role in removal and addition of myofibrillar proteins during muscle adaptation.

It is known that most muscle protein turnover is carried out by the proteasome; however, it is also known that the proteasome cannot degrade intact myofibrils [122,123]. Thus, it is likely that an upstream proteolytic pathway must be operating to release myofibrillar proteins from the sarcomere to target them for ubiquitination and proteasomal degradation. To determine if CAPN3 is acting upstream of the proteasome during muscle remodeling, mice that were undergoing remodeling were examined for overall ubiquitination levels [4]. It was shown that overall ubiquitination is reduced in reloaded muscles from C3KO compared to reloaded (weight bearing) controls supporting the hypothesis that CAPN3 acts upstream of the ubiquitin/proteasome pathway. This observation also suggests that CAPN3 must have multiple substrates because global reductions in ubiquitination could be detected by these methods.

14. Hypothetical mechanism by which loss of proteolytic capability might lead to cell dysfunction

The intracellular milieu provides conditions under which cellular proteins are continually damaged and then degraded by the proteasome. Processes such as oxidation can damage proteins, which can then become misfolded leading to protein aggregation and cellular toxicity (Fig. 7). Numerous examples exist in which loss of proteolytic capability via the ubiquitin/proteasome pathway leads to disease. Alzheimer's disease,

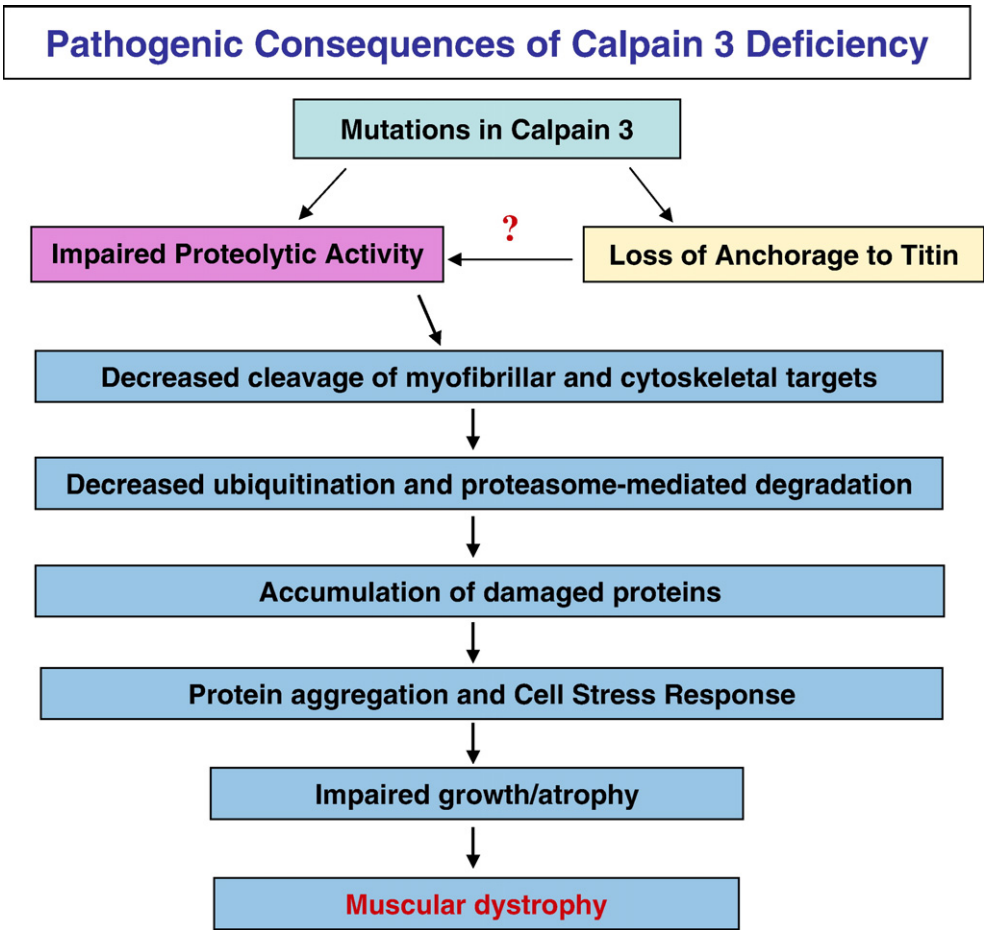

Fig. 7. Flow chart demonstrating the pathogenic consequences of CAPN3 deficiency. Mutations in CAPN3 can lead to loss of proteolytic activity or loss of titin anchorage. The latter is likely to reduce regulation of proteolytic activity or to remove CAPN3 from its substrates. Loss of proteolysis of substrates can cause accumulation of proteins that can subsequently become damaged and then aggregate. These aggregates can impair proper muscle function and impair growth, leading to muscular dystrophy.

juvenile recessive Parkinson's disease, Angelman's syndrome and several forms of cancer can result from mutations in proteins associated with the protein degradation machinery [124]. It is possible that accumulation of old and damaged or misfolded proteins in muscle tissue can also lead to toxicity and disease. These proteins form insoluble aggregates that recruit some components of the ubiquitination and proteasomal machinery as well as heat shock proteins of Hsp70 and small Hsp families (reviewed in [125]). Even though no morphologically visible protein aggregates have been observed in C3KO muscles, biochemical analysis showed accumulation of Hsp70 and Hsp25 in the insoluble fractions of C3KO muscles [4]. These results give support for the hypothesis that a reduced capacity for normal protein turnover in muscles deficient for CAPN3 can induce abnormal protein accumulation that leads to a cell stress response and toxicity. Fig. 7 shows a flow diagram in which this is described.

Involvement of CAPN3 in generalized proteolysis does not exclude the possibility that CAPN3 plays a role in controlling specific cellular pathways such as cytoskeletal rearrangements, sarcomere formation and remodeling or apoptosis as was suggested by numerous published studies [4,5,25,74,76]. Taking into account that CAPN3 may have many substrates, it is quite possible that LGMD2A develops from impairment of several cellular processes rather than a single one. In vitro studies have suggested that CAPN3 and ubiquitous calpains may have overlapping sets of substrates. The significance of this observation is that proteolytic activities of other calpains may be able to partly compensate for the absence of CAPN3. At the genetic level, it means that ubiquitous calpains may act as genetic modifiers. Taking into account these characteristics of CAPN3 (i.e. the pleiotropic nature of calpain mutations and the presence of functionally redundant calpains) one can explain the high variability in the phenotypic characteristics of the disease, even within a family. Future analysis of mouse models of LGMD2A and especially identification of in vivo substrates and interactive partners of CAPN3 are crucial for our understanding of LGMD2A pathogenesis and development of therapeutic approaches.

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