CAPN3-mediated processing of C-terminal titin replaced by pathological cleavage in titinopathy

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Abstract
Mutations in the extreme C-terminus of titin (TTN), situated in the sarcomeric M-band, cause tibial muscular dystrophy (TMD) and limb-girdle muscular dystrophy 2J (LGMD2J). The mutations ultimately cause a loss of C-terminal titin, including a binding site for the protease calpain 3 (CAPN3), and lead to a secondary CAPN3 deficiency in LGMD2J muscle. CAPN3 has been previously shown to bind C-terminal titin and to use it as a substrate in vitro. Interestingly, mutations in CAPN3 underlie limb-girdle muscular dystrophy 2A (LGMD2A). Here, we aimed to clarify the relationship of CAPN3 and M-band titin in normal and pathological muscle. In vitro analyses identified several CAPN3 cleavage sites in C-terminal titin that were defined by protein sequencing. Furthermore, cleavage products were detected in normal muscle extracts by western blotting and in situ by immunofluorescence microscopy. The TMD/LGMD2J mutation FINmaj proved to alter this processing in vitro, while binding of CAPN3 to mutant titin was preserved. Unexpectedly, the pathological loss of M-band titin due to TMD/LGMD2J mutations was found to be independent of CAPN3, whereas the involvement of ubiquitous calpains is likely. We conclude that proteolytic processing of C-terminal titin by CAPN3 may have an important role in normal muscle, and that this process is disrupted in LGMD2A and in TMD/LGMD2J due to CAPN3 deficiency and to the loss of C-terminal titin, respectively.

Introduction
Titin (TTN), forming the third filament system in the myofibrils, has structural, mechanical and regulatory functions in skeletal and cardiac muscle [recently reviewed in (1)]. The gigantic protein is mostly comprised of repeated immunoglobulin (Ig)-like and fibronectin 3-like domains, and spans over the half-sarcomere. The N-terminal ends of titin molecules are anchored to the Z-disc, while the C-terminal ∼200 kDa are embedded in the M-band in the middle of the sarcomere (Fig. 1). The M-band part of titin contains a kinase domain and 10 Ig-like domains (M1 to M10), interspersed by seven unique sequence regions (is1 to is7). The functions of M-band titin are poorly understood, and few of the reported protein interactions have been thoroughly characterized on a functional level.

One of the known interaction partners for M-band titin is calpain 3 (CAPN3), a member of calpains, non-lysosomal Ca2+-activated cysteine proteases (2). CAPN3 is predominantly

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expressed in skeletal muscle, where it localizes variably to the sarcomeric Z-disc, I-band and M-band regions (3–5). The activity of CAPN3 is highly regulated, and most of the enzyme pool in muscle is in the inactive state. Activation proceeds through an autolytic mechanism that removes an internal autoinhibitory propeptide from the active site (5). Although a number of proteins have been identified as potential substrates for CAPN3 in vitro, the physiological substrates and functions remain largely unknown. Based on its identified substrates and myofibrillar association, CAPN3 is thought to function mainly in cytoskeletal and sarcomeric remodeling (6,7), but it has also been implicated in other processes such as development, metabolism and apoptosis (8–11). In addition, recent studies have indicated that CAPN3 has structural functions independent of its proteolytic activity (12,13).

Interactions between titin and CAPN3 have been described in the I-band and M-band (Fig. 1). In the M-band, CAPN3 binds the unique sequence region is7, encoded by the penultimate TTN exon Mex5 (14). Alternative splicing of Mex5 leads to a variable expression of is7+ and is7− titin isoforms in different muscles (15), hence affecting the number of available CAPN3 binding sites. However, the physiological importance of this phenomenon has not been addressed. Coexpression experiments have demonstrated that M-band titin can act as a substrate for CAPN3 in vitro (5,16), but the cleavage events have not been characterized in detail, and their physiological relevance is unknown.

Besides their interaction, another feature M-band titin and CAPN3 in common is their involvement in hereditary muscle diseases. Mutations in the very C-terminal part of titin underlie two distinct disease phenotypes, titubal muscular dystrophy (TMD), a late-onset distal myopathy primarily affecting the anterior compartment muscles of the lower leg, and limb-girdle muscular dystrophy 2J (LGMD2J), a severe early-onset limb-girdle muscular dystrophy (17–19). All TMD/LGMD2J patients described so far harbor at least one missense or truncating mutation in one of the two last exons of TTN, Mex5 and Mex6, encoding the is7 region and the M10 domain respectively (17,20–24) (Fig. 1). TMD is typically seen in patients carrying a single heterozygous mutation, whereas patients with two mutated alleles typically develop LGMD2J (17,18) (Fig. 1). The most frequent TMD/LGMD2J mutation ‘FINmaj’, widespread in Finland, is an in-frame deletion-insertion of 11 bp in Mex6, leading to a substitution of four amino acids (EVTW > VKEK) in the M10 domain (17). FINmaj, and most of the other TMD-causing missense changes, have been shown to disrupt the Ig fold of the M10 domain and to abolish its interactions with obscurin (OBSCN) and obscurin-like 1 (OBSL1) (25–27). Mutations in CAPN3, on the other hand, lead to the autosomal recessive LGMD2A (28). Patients with LGMD2A exhibit progressive atrophy and dystrophy of the muscles in the pelvic and scapular girdles, while the distal anterior lower leg muscles are relatively spared.

One of our early findings leading to this study was that western blotting of normal control muscle extracts with C-terminal titin antibodies consistently revealed a number of small fragments in the range of 10–100 kDa, suggesting that C-terminal titin normally undergoes specific proteolytic processing. These bands totally disappear in muscle samples of LGMD2J patients and FINmaj knock-in (FINmaj KI) mice, homozygous for the FINmaj mutation, and are reduced by ∼50% in the heterozygous state (21,29), suggesting that TMD/LGMD2J mutations may lead to a loss of C-terminal titin. This is supported by immunofluorescence (IF) microscopy studies, showing a loss of M-band titin epitopes beyond the mutated domains in LGMD2J and homozygous FINmaj KI muscles (17,29). Interestingly, LGMD2J and FINmaj KI muscles also show a secondary CAPN3 deficiency (29,30), reflecting the loss of the M-band CAPN3 binding site. Moreover, muscle pathology in heterozygous FINmaj KI mice is ameliorated by a heterozygous CAPN3 knockout (C3KO) suggesting that CAPN3 is involved in the pathogenesis of C-terminal titinopathy (29).

In this study, we addressed the relationship of M-band titin and CAPN3 in normal muscle and in the context of TMD/LGMD2J diseases. We demonstrate that CAPN3 cleaves C-terminal titin in vivo in normal murine and human muscles, and present exact cleavage sites determined in vitro. We show that while the TMD/LGMD2J-causing titin mutations do not directly affect the interaction between CAPN3 and mutant titin, they can alter the cleavage pattern of titin in vitro. Finally, we show that the pathological cleavage induced by the FINmaj mutation, leading
to loss of C-terminal titin, is not caused by CAPN3 but depends on other proteases, likely the ubiquitous calpains.

Results

Cleavage of C-terminal titin by CAPN3 in vivo

The C-terminal part of titin harbors a binding site for CAPN3, and coexpression experiments in cultured cells have previously suggested that this region of titin can be a target substrate for CAPN3 (5, 16). This encouraged us to characterize in more detail the C-terminal titin fragments detected in normal muscle samples (21) and to investigate if they could be generated by CAPN3. For this purpose, we analyzed muscle extracts from normal and LGMD2A human biopsies (Supplemental Material, Table S1) using an antibody directed against the M10 domain. In samples prepared from healthy human biopsies, we detected four prominent fragments at ∼45 kDa (denoted p45), ∼18 kDa (p18), ∼15 kDa (p15) and ∼13 kDa (p13). These bands, most remarkably p45, were severely reduced in CAPN3-deficient LGMD2A biopsies, supporting the idea that CAPN3 is involved in generating these titin fragments (Fig. 2A).

Analysis of mouse samples gave similar results (Fig. 2B). Bands resembling in size the human fragments were present in samples from wild-type mice, whereas they were highly reduced in muscles from CAPN3-deficient (C3KO) animals. Samples from the FINmaj KI mouse model, previously demonstrated to lack C-terminal titin staining on western blot (WB) (Fig. 2B). IF microscopy of C3KO muscles showed a normal M9 domain staining in the M-band, indicating that the absence of CAPN3 does not disrupt titin C-terminus. Scale bar: 10 μm.

Prediction and characterization of CAPN3 cleavage sites in C-terminal titin

The CAPN3 recognition motif [LIMV]X(n-3)[LIMV]X(n-2)[LIMV][DE], defined by three hydrophobic and one acidic key residues, was recently identified from known CAPN3 substrates (31). Sequence analysis of C-terminal human titin (domains M1–M10) revealed three matches to this motif, ‘LYVKD’ (31), ‘VKWVLYNGVE’ and ‘MGISMTQLE’, situated in M1, M8 and is7, respectively (Fig. 3A). Allowing alanine in the second key position—a change compatible with motif functionality (31)—produced an additional match, ‘VKKSAATLSE’ in the is6 region (Fig. 3A). The positions of the putative cleavage sites present in is6 and is7 were in agreement with the molecular weights of the p45 and p13 titin fragments observed in western blotting of muscle samples. Mouse titin also harbored recognition motifs at the corresponding positions in is6 (‘VKRSAAASLE’) and is7 (‘MGKSSMTQLE’) (Fig. 3A). Interestingly, these two motifs were found to be widely conserved in mammals, suggesting that they may be functionally important (Supplementary Material, Fig. S1).

As purification of the titin fragments generated in vivo was not feasible due to their low abundance, we chose to model titin proteolysis in cell culture experiments where C-terminal titin constructs were coexpressed in 293T cells with active wild-type CAPN3 or the inactive C129S mutant, followed by western blotting of proteolytic fragments. To determine the exact cleavage sites, fragments were purified from large-scale cultures using immobilized metal affinity chromatography (IMAC) and immunoprecipitation, and analyzed by N-terminal sequencing.

When coexpressed with active CAPN3, C-terminally V5/His6-tagged constructs spanning the region is6-M10 of the human titin is7+ and is7− isoforms (is6-M10-V5 is7+ and is7− wt) gave
rise to patterns of C-terminal bands that were absent or very weak in samples expressing inactive CAPN3 (Fig. 3B, band C). As the major fragments were found to react with both the V5 and His6 antibodies (not shown), and hence contain the entire C-terminal tag of 4.9 kDa, they will be hereafter referred to by the approximate molecular weights of the titin moieties. The most prominent bands of the is7+ construct corresponded to titin fragments of $\sim$45, $\sim$20, $\sim$19 and $\sim$13 kDa (Fig. 3B), being in good agreement with bands observed in muscle extracts. Protein sequencing placed the cleavage site of the 45 kDa fragment in the is6 region (\ldots TSLE|KSIV\ldots), whereas the 13 kDa fragment was mapped in is7 (\ldots SFMG|ISNM\ldots). Interestingly, these cleavage sites correlate well with the conserved CAPN3 recognition motifs: the N-terminus of the 45 kDa fragment immediately follows the

Figure 3. CAPN3 cleavage sites in C-terminal titin. (A) C-terminal titin was analyzed for the CAPN3 recognition motif \{LIMV\}[X\{3,4\}][LIMV\}[X\{2\}][LIMV\}DE], and a variant with alanine in the second key position. Motif matches are highlighted, with key residues in orange, and approximate distances from titin C-terminus shown in kilodaltons (kDa). Arrowheads indicate cleavage sites experimentally determined for human titin. (B-D) C-terminal titin constructs were coexpressed in 293T cells with inactive (−) or active (+) HA-CAPN3, and analyzed by western blotting (WB) using an antibody against the C-terminal V5 tag. Human constructs and major proteolytic fragments are depicted next to each WB panel, with N-terminal sequences and estimated or calculated molecular weights (tag excluded). White and black arrowheads represent the estimated and experimentally determined cleavage sites, respectively. (B) The human is6-M10-V5 is7+ construct produced major fragments corresponding to 45, 20, 19 and 13 kDa of titin, and sequencing identified two cleavage sites. The murine construct produced a similar but not identical fragment pattern. (C and D) The major fragment from human and murine is6-M10-V5 is7+ constructs, corresponding to 33 kDa of titin, shared its N-terminus with the 45 kDa is7+ fragment. Another slightly smaller fragment suggested a cleavage near the is6/M8 boundary; this was confirmed with the human is6-M8-V5 construct. (E) The M10-1 antibody revealed co-migrating fragments in untagged human is6-M10 is7+ coexpressed with active HA-CAPN3, and in human muscle extract. Dashed lines indicate likely corresponding fragments between is6-M10-V5 is7+ (repeated from B) and is6-M10 is7+.
is6 motif (VKKSAATSLE), while that of the 13 kDa fragment lies within the is7 motif (MGJISMTQNL).

For the is6-M10-V5 is7− construct, the major cleavage product corresponded to ~35 kDa of titin, and protein sequencing showed it to arise from the same cleavage site as the 45 kDa is7+ fragment (Fig. 3C). A band migrating just below the ~35 kDa product was detected in some experiments, suggesting another cleavage at a nearby site. Using an is6-M8-V5 construct that allowed better separation of the fragments, this additional cleavage site was confirmed and, based on the molecular weights of the fragments, placed near the boundary of is6 and M8 (Fig. 3D).

For comparison, we also analyzed murine titin constructs covering the same amino acid regions as the human is6-M10 constructs (is6-M10-V5 is7− and is7+ Mmu). They produced fragment patterns similar to the corresponding human constructs, albeit with some differences evident in the smaller bands (Fig. 3B and C).

Importantly, the untagged human construct is6-M10 is7+ co-expressed in 293T cells with active CAPN3 gave rise to a fragment pattern nearly identical to that seen in muscle extracts as revealed by the M10 antibody (Fig. 3E), supporting that our cell culture model can recapitulate the proteolytic events taking place in muscle.

**Neo-epitope antibody confirms titin cleavage at the is6 site in situ**

To investigate whether the experimentally determined cleavage sites are relevant in vivo, we decided to raise a neo-epitope antibody against one of the identified cleavage products. The amino terminus of the 45 kDa fragment (NH2-KSIHVHE) was chosen as the target, since the residues are identical in human and murine fragments, and the antibody could hence be utilized in both species. The resulting rabbit polyclonal antibody was named p45-neo. In WB testing, the antibody fraction affinity-purified with the immunogenic peptide showed strong reactivity towards extracts of cells coexpressing C-terminal titin constructs with active CAPN3, while samples with inactive CAPN3 showed little signal (Fig. 4A). This indicated that the p45-neo antibody preferentially recognized the cleaved N-terminus, with minimal binding to the uncleaved sequence. Specificity of p45-neo for the cleavage product gained further support from IF studies of 293T cells expressing titin with inactive or active CAPN3 constructs (Fig. 4B). Depletion of the antibodies recognizing the uncleaved sequence was also attempted to further increase the specificity towards the cleavage products, but this resulted in a dramatic loss of binding affinity, and the non-depleted fraction was therefore utilized in the experiments.

The p45-neo antibody was then used for probing WBs of human and mouse muscle samples (Fig. 4C and D). The antibody recognized in both species a band of ~45 kDa, co-migrating with the previously detected p45 fragment, as well as smaller bands likely resulting from further cleavages of p45. These were absent in muscle samples from LGMD2J patients (Fig. 4C) and homozygous FINmaj KI mice (Fig. 4D), consistent with the pathological truncation of C-terminal titin due to a homozygous FINmaj mutation. These results demonstrate that titin cleavage at the experimentally identified is6 site indeed occurs in normal muscle and that the p45 fragment is a product of this cleavage event.

Since the p45 fragment detected in WBs seemed to be at least partly generated during sample preparation, we set out to demonstrate the presence of the cleaved fragment in intact muscles by IF staining with p45-neo. A clear M-band label could be detected in several murine skeletal-muscle samples fixed with paraformaldehyde (PFA) perfusion or immersion prior to flash freezing, supporting the notion that proteolytic cleavage at the p45 site can indeed occur in vivo (Fig. 4E).

**The TMD/LGMD2J mutations do not affect CAPN3 binding but alter titin cleavages in vitro**

The dramatic secondary reduction of CAPN3 previously observed in LGMD2J and FINmaj KI muscles suggested that the TMD/LGMD2J-causing mutations in C-terminal titin can destabilize CAPN3 (29,30). We were therefore interested in the effects of these mutations on the relationship of titin and CAPN3. First, we used a yeast two-hybrid assay to investigate whether three of the previously reported mutations: the so-called 'Belgian' p.I35947N, ‘French’ p.L35956P (numbering based on NP_001254479.2) and FINmaj, affected the binding of CAPN3 to titin. When tested against a CAPN3 bait construct, wild-type and mutant titin prey constructs covering the region is7−M10 supported yeast growth on a selective medium equally well, suggesting that the TMD/LGMD2J mutations do not directly affect the ability of CAPN3 to bind C-terminal titin in vitro (Fig. 5A).

Then, to investigate if the TMD/LGMD2J mutations affect the CAPN3-mediated proteolytic cleavages of C-terminal titin, we expressed wild-type and mutant titin constructs together with active or inactive CAPN3 in cell cultures. Interestingly, when coexpressed with active CAPN3, titin constructs containing the FINmaj mutation produced altered fragment patterns compared with wild-type titin. The mutant is7− constructs showed a relative reduction of the 19 kDa fragment, disappearance of the 13 kDa fragment and the appearance of a novel band corresponding to ~8 kDa of titin (Fig. 5B). Likewise in is7+ constructs, a 8 kDa fragment was observed in FINmaj mutant samples (Fig. 5C). N-terminal sequencing of this fragment, isolated from cells coexpressing is6-M10-V5 is7− FINmaj with active CAPN3, identified the cleavage site (...AFTG|EPTP...) in the M10 domain, just N-terminally from the FINmaj mutation. Of note, this cleavage site did not coincide with a predicted CAPN3 recognition motif.

**The pathological cleavage of C-terminal titin due to the FINmaj mutation is not CAPN3-dependent**

A total absence of C-terminal titin has been observed both in human and murine muscles homozygous for the FINmaj mutation (17,24,29). This pathological loss of mutant titin was shown to involve at least the ultimate C-terminal domains M9-M10, while the domains A169-A170 at the A-band/M-band boundary are preserved. To investigate the extent of this titin truncation in more detail, we performed IF studies on human control and LGMD2J muscle with additional antibodies against C-terminal titin (Fig. 6A). The polyclonal antibody ‘3460’ against is6 (32), and the novel polyclonal ‘is5-1’ raised against a peptide in the is5 region, both lost their normal M-band localization in LGMD2J muscle. Instead, these antibodies showed variable Z-disc reactivity, which could indicate that some products of the pathological cleavage remain in the cytoplasm and may have affinity to the Z-disc. The monoclonal antibody ‘T41’ recognizing the is4 region (33) did label the M-bands in LGMD2J muscle, although the staining appeared less defined than in control muscle, with regions of clearly reduced M-band signal. These findings place one pathological cleavage point between the T41 epitope in is4 and the is5-1 epitope in the C-terminal part of is5, and hence N-terminally from the identified CAPN3 cleavage sites. The variable T41 results suggest that additional cleavages take place in is4 N-terminally from the epitope.
The ability of the FINmaj mutation to modify the way CAPN3 cleaves titin in vitro prompted us to investigate if the pathological disappearance of C-terminal titin due to TMD/LGMD2J mutations in vivo could be due to constitutive cleavage by CAPN3. To test this hypothesis, we generated double homozygous mice by crossing FINmaj KI with C3KO mice, and analyzed the presence of C-terminal titin by western blotting and IF microscopy. Staining for M-band domains was negative on both WB of the cytoskeletal fraction (Fig. 6B), and on IF of longitudinal muscle sections (Fig. 6C), indicating that the loss of C-terminal titin seen with the FINmaj mutation is not a consequence of a CAPN3 cleavage. At the same time, C3KO mice showed preserved M-band titin staining in IF (Fig. 6C), confirming that the absence of CAPN3 per se does not lead to loss of C-terminal titin.

As CAPN3 proved to be dispensable for the pathological loss of mutant titin, we investigated the possible involvement of ubiquitous calpains (CAPN1 or \( \mu \)-calpain and CAPN2 or m-calpain). These proteases have indistinguishable substrate specificities but differ in their calcium requirements (34). First, the protein sequence of C-terminal titin was analyzed for putative calpain cleavage sites with two bioinformatic tools, GPS-CCD (35) and the preference matrix of Tompa et al. (36). Both methods predicted many of the ‘is’ regions to contain good calpain substrate sequences (Fig. 7A). Interestingly, the CAPN3 cleavage site...
inactive (and FINmaj mutant human titin constructs were coexpressed in 293T cells with CAPN3 that the mutations do not disrupt the binding of CAPN3 to C-terminal titin.

To study the ability of ubiquitous calpains to cleave C-terminal titin in the region affected by pathological cleavage in TMD/LGMD2J, several cleavage products were mapped in the is7 region, and positive staining with the p45-neo antibody demonstrated that this corresponded to the previously identified CAPN3 cleavage site (Fig. 7B). Mutant constructs showed a prominent C-terminal fragment corresponding to ~8 kDa of titin, and comparison with CAPN3-coexpression samples suggested it to be identical to the fragment produced by CAPN3 from the mutant constructs at the (...AFTGEPFT...) site (Fig. 7D). Another, smaller fragment was also produced by CAPN2 specifically from FINmaj constructs. This corresponded to ~5 kDa of titin, placing the cleavage site in the middle of the M10 domain. Extra bands likely reflecting these two mutant-specific cleavages were also seen in the p45-neo staining (Fig. 7B). Interestingly, the FINmaj constructs also seemed to produce an increased amount of larger fragments of ~25 kDa, suggesting that the mutation-induced changes in titin proteolysis may not be limited to the mutated M10 domain.

Our IF findings on LGMD2j muscle placed the pathological cleavage of mutant titin in the region is4-M6-is5. To look at CAPN2 cleavages in this region, samples of CAPN2-digested M4-M10-V5 is7− constructs were double stained with the is4 antibody T41 and the is6 antibody 5460 (Fig. 7E). This revealed a ladder of fragments of 30–40 kDa, positive for both antibodies and hence spanning from is4 to is6. As the V5 staining of C-terminal fragments did not indicate multiple cleavage sites in or around is6, these fragments are likely to result from a common C-terminal cleavage at the ‘p45’ site and a series of closely spaced cuts in is4, N-terminally from the T41 epitope. This was in accordance with the prediction of the Tompa preference matrix and indicated that ubiquitous calpains can indeed cleave titin in the region affected by pathological cleavage in TMD/LGMD2j.

Discussion

Following previous in vitro observations that C-terminal titin is a substrate of CAPN3 (5,16), we characterized in this study CAPN3-mediated proteolytic processing of C-terminal titin in the sarcomeric M-band. The identified cleavages take place in the is6 and is7 regions of titin and lead to the production of small C-terminal fragments, with the major N-terminal part of titin still anchored in the sarcomere. The initial C-terminal cleavage product is likely to be the p45 fragment that is rapidly further cleaved into smaller fragments with certain steady state levels on WBs. In our in vitro studies, both is7+ and is7− isoforms were cleaved by CAPN3 in a coexpression setup. In muscle extracts, however, the fragments observed with M10-1 and p45-neo stainings seemed to originate exclusively from is7+ titin, as judged from their sizes. This suggests that in the context of muscle, the action of CAPN3 which binds to the titin is7 region, could be limited to an in cis cleavage of the same titin molecule.

Biological presence and importance of the identified titin processing is supported by the conservation of CAPN3 target sequences and, more importantly, detection of the cleaved p45 N-terminus in situ. Further support for the relevance of the
Cleavages in vivo comes from the recent observation of a C-terminal titin fragment, likely corresponding to the p13 fragment identified in this study, in the urine of patients with various myopathies (37). Since the regulation of CAPN3 activity in vivo is still poorly understood, it is not possible to predict which conditions trigger the physiological processing of C-terminal titin. Activation of CAPN3 requires Ca\(^{2+}\) in a submicromolar concentration (38), and is facilitated by calmodulin (39). Accordingly, CAPN3 can be activated by a prolonged increase in cytoplasmic [Ca\(^{2+}\)] (40), and activation has been detected in vivo following eccentric exercise (41,42). Calcium may hence be one factor regulating the physiological titin cleavage, but additional stimuli and regulatory mechanisms could be involved.

Titin processing could potentially serve a number of functions. First, it could facilitate local remodeling of the sarcomere, for example, to modify the stiffness of the M-band lattice, or to mediate the turnover of titin or other M-band components. This would be in line with the demonstrated role of CAPN3 in sarcomere remodeling (43). Another possibility is that the normal physiological cleavage would modulate the function of proteins interacting with C-terminal titin. The known binding partners include obviously CAPN3 itself, interacting with the is7 region (14).
Binding to titin has been suggested to regulate the activity of CAPN3 by protecting it from autolytic activation (44–46), and experimental support for such a stabilizing effect exists for I-band but not for M-band titin (47). Remarkably, the severe secondary CAPN3 deficiency seen in WB in LGMD2J and homozygous FINmaj KI muscles (29,30) indicates that the loss of the binding site in the M-band due to the pathological titin cleavage disrupts the regulation of CAPN3 in the entire myofiber, and suggests that the M-band constitutes a key regulatory spot for CAPN3. Another partner whose function could possibly be modified is myospryn (CMYA5), which binds the titin region M9–is7–M10 (48). The biological function of this interaction is currently unknown but again it may play a role in controlling the CAPN3-mediated proteolysis. The direct interaction of myospryn and CAPN3, and the ability of CAPN3 to cleave myospryn in vitro (48) are in line with the possibility that the titin-myospryn complex is regulated by CAPN3-mediated proteolysis and vice versa that the complex regulates CAPN3. In addition to titin and CAPN3, myospryn binds protein kinase A and calcineurin, likely regulating their local activity (49,50). Interestingly, another A-kinase anchoring protein, the intermediate filament protein α-synemin, has recently been reported as an interacting partner of titin.

Figure 7. Ubiquitous calpains and pathological titin cleavage. (A) Prediction of calpain cleavages. C-terminal titin was analyzed with the Tompa preference matrix and GPS-CCD for putative calpain target regions. Both methods gave high scores for the M-band intervening sequences (is). The Tompa matrix predicted especially high scores in is4, the likely site of pathological cleavage(s) in TMD/LGMD2J. Red and blue graph segments represent the Ig domains M1–M10 and the intervening regions is1–is7, respectively. (B) Cleavage of C-terminal titin by CAPN2 in vitro. Titin M4-M10-V5 constructs (is7+ and is7−, wild-type and FINmaj) were incubated with or without CAPN2 for the indicated times, and analyzed by double staining with V5 and p45-neo antibodies. Arrowheads show the full-length constructs (black), the is7+ and is7− forms of p45 (white), and fragments induced by the FINmaj mutation (red). (C and D) Wild-type (wt) and FINmaj (FM) is7+ and is7− titin constructs were digested with CAPN2 (C2) for 1.5 min (C) or 10 min (D), or coexpressed with active (C3) or inactive (−) CAPN3. Comparison of C-terminal fragments indicated that CAPN2 can cleave titin at the same sites as CAPN3, and at several unique sites (C). (E) Wild-type and FINmaj is7− titin constructs were digested (+) or not (−) with CAPN2 for 60 min. A ladder of fragments at 30–40 kDa, positive for both T41 and 5460 antibodies in double staining, suggested that several CAPN2 cleavages occur in is4.
M10 (51). The ternary complex of obscurin/OBSL1 and myomesin with the titin M10 domain (52) could also be regulated by titin cleavage, as obscurin shows some mislocalization in LGMD2J and in the FINmaj KI mouse (29,52). Interestingly, obscurin interacts with the small ankyrins Ank1.5 and Ank1.9, which link this complex to the sarcoplasmic reticulum (53,54). The processing of C-terminal titin could potentially have a role in local calcium regulation and hence again possibly on the regulation of calpain activation. These interactions suggest that C-terminal titin may constitute a signaling hub, whose functions could be modulated by titin cleavage.

Yet another intriguing possibility is that the titin fragments could have a signaling function. By shuttling to other locations in the myofiber such as the Z-disc or myonuclei, the fragment(s) released from the M-band could transmit information on the sarcomere status to intracellular signaling pathways. Alternatively, the fragments could have a role in paracrine signaling. This option is supported by the recent finding that subfragments of M-band titin (domains A170 and M4), as well as myomesin and myosin-binding protein C, can induce the expression of certain insulin-like growth factor 1 isoforms in cultured myoblasts by binding to cell-surface receptors (55). Fragments of myofibrillar proteins released upon muscle damage were proposed to promote muscle regeneration by stimulating myoblast proliferation and/or differentiation (55,56). The observation of the p13 fragment in urine of patients may also support this hypothesis.

In the titinopathies TMD/LGMD2J, the physiological titin processing is altered. The FINmaj mutation renders the M10 domain susceptible to inappropriate calpain cleavage as shown by in vitro studies, and a large part of titin C-terminus is observed to be absent in vivo. Interestingly, our IF analysis indicated that the loss of C-terminal titin originates from a pathological cleavage in the is4–is5 regions, therefore N-terminally of the identified CAPN3 cleavages. By crossing the FINmaj KI mouse with CAPN3 KO, we excluded CAPN3 as responsible for this abnormal event. Instead, the ubiquitous calpains could be involved, as we found that CAPN2 is able to cleave titin in vitro in the pathological cleavage region. Another candidate protease would be MMP2 that was previously shown to cleave I-band titin upon cardiac ischemia-reperfusion injury, and to show weak M-band localization in addition to its major Z-disc targeting (57). However, bioinformatic analyses were not reported to show putative MMP2 cleavage sites in M-band titin (57).

In contrast to the physiological cleavage that is likely to be tightly regulated at specific conditions, the pathological cleavage probably occurs soon after the incorporation of titin to the sarcomere, leading to a functional loss of the titin C-terminus. The molecular mechanism by which mutations in the M10 domain induce the pathological cleavage of titin more N-terminally warrants further investigation. Nevertheless, the 50% decrease in C-terminal titin fragments in FINmaj-heterozygous muscle (21,29) implies that the pathological cleavage is triggered by an intrinsic property of the mutant protein rather than local hyperactivation of proteases per se. The absence of C-terminal titin does not seem to be pathogenic in itself, since titin is similarly cleaved in both the affected and unaffected muscles of FINmaj KI mice (29). Furthermore, based on analysis of one post mortem sample, pathological cleavage comparable with skeletal muscle seems to take place also in the LGMD2J heart without clinically manifest cardiomyopathy (A.V. and B.U., unpublished observation). Hence, the pathomechanism of TMD/LGMD2J likely depends on a skeletal-muscle specific function or interaction of the cleaved region such as CAPN3 whose expression level is at least two orders of magnitude higher in skeletal muscle compared with heart (58).

Indeed, the rescue of the myopathy phenotype in the FINmaj KI/C3KO double heterozygous mice suggests that dysregulated activity of CAPN3 plays a downstream pathogenic role in TMD/LGMD2J (29). However, other functions or binding partners of C-terminal titin may also contribute to the pathomechanism, at least in the FINmaj-homozygous situation since in addition to the above-discussed partners of the extreme C-terminus of titin, the pathological cleavage would affect any currently unknown protein interactions involving the titin region is4–is6, between the pathological and physiological cleavage sites.

The recent years have revealed a surprising complexity of titin mutations in human disorders of skeletal and cardiac muscles (59–61). Of particular interest in the context of this discussion are the other diseases where truncating mutations have been identified in the M-band titin: dilated cardiomyopathy (61) and autosomal recessive multi-minicore disease with heart disease (AR MmD-HD), a disease spectrum that includes the patients originally described as early-onset myopathy with fatal cardiomyopathy or Salih myopathy (59,62). Some of the truncations associated with these diseases are similar in extent to the pathological cleavage seen in TMD/LGMD2J, yet there is considerable variation in terms of clinical phenotype, age of onset and mode of inheritance (59,61,62). Explanations for these intriguing differences are still missing, and due to the scarcity of reported cases, the mechanisms can only be speculated. One factor that may modify the presentation of the mutations is nonsense-mediated decay (NMD). In TMD, where the pathological cleavage occurs posttranslationally, the truncation likely affects ∼50% of the titin protein incorporated in the sarcomeres. In contrast, in AR MmD-HD heterozygotes, a partial degradation of mutant transcripts by NMD could lead to preferential expression of the wild-type protein, keeping the proportion of dysfunctional titin below a pathogenic threshold. On the other hand, in patients homozygous for truncations, extensive NMD could impair the cellular capacity for titin biosynthesis. Indeed, in line with the role of NMD in modifying the phenotypes, some titin truncations with recessive presentation seem to lead to NMD (63), while transcripts harboring dominantly presenting truncations may escape degradation (61). Moreover, it cannot be excluded that other variants in titin or in interacting partners, or use of cryptic splice sites may participate in modifying the molecular and clinical outputs. The parallels and differences between these diseases are very interesting to note, and uncovering the reasons behind them will be crucial for understanding the genetics and biology of titin and the human titinopathies.

Materials and Methods

In vivo experiments

Mice were housed in a barrier facility with 14 h light, 10 h dark cycles, and provided food and water ad libitum.

Mice from the 129SvPasIco and C57Bl/6 strains were purchased from Charles River Laboratories (Les Oncins, France). The construction and genotyping of CAPN3-deficient (B6-Capn3tm2.Jcis; “C3KO”) and FINmaj KI (B6-Ttnm2Gnt; ‘FINmaj KI’) mice were previously described (29,64,65). The mouse model homozygous for both the FINmaj mutation and a CAPN3 deficiency was obtained by crossing heterozygous female FINmaj KI mice with male C3KO mice. Genotyping for the FINmaj and CAPN3 mutations was performed on tail DNA as previously described (29,64).

For histological and molecular analysis of mouse tissues, specimens were collected immediately after sacrifice, snap frozen in liquid-nitrogen-cooled isopentane and stored at −80°C.
Perfusion-fixed wild-type murine muscle, used in IF staining with the p45-neo antibody, was obtained from another study. The mouse was perfused with 0.16 mg/ml sodium heparin in PBS for 10 min, and with 4% PFA in PBS for 10 min. After dissection, muscles were cryoprotected with glue in PBS (10% 1 h, 20% 1 h, 30% a/n) and frozen in liquid-nitrogen-cooled isopentane.

### Human muscle samples

Muscle biopsies were obtained after informed consent from four healthy controls, three LGMD2A patients and one LGMD2j patient (Supplemental Material, Table S1). One additional sample (LGMD2j-1) was obtained in autopsy of an LGMD2j patient (Supplemental Material, Table S1).

### IF stainings and microscopy

For IF studies, frozen skeletal muscles were cut into cryosections of 8–10 µm. The sections were fixed with 4% PFA in PBS for 10 min. Sections were then permeabilized with 0.2% Triton X-100 in PBS, and blocked with 5% BSA for 30 min. The sections were incubated with primary antibodies in 1% BSA for 2 h at RT or overnight at +4°C, and then with goat anti-mouse or donkey anti-rabbit secondary antibodies conjugated with Alexa Fluor 488 or 564 dyes (dilution 1: 500; Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA) for 1 h at RT. Images were acquired with a LSM 780 confocal microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) using a 63× oil objective. For IF studies on cultured cells, samples were fixed 15 min with 4% PFA/PBS, stained as above, and imaged on an Axioplan 2 epifluorescence microscope (Carl Zeiss) using a 20× objective.

### Plasmid constructs for mammalian expression

The human wild-type and FINmaj mutant titin constructs is6-M10-V5 is7+ and is7−, is6-M8-V5, and untagged is6-M10 is7+ in pEF6-V5/His TOPO have been described previously (48). For making the new titin constructs, inserts were polymerase chain reaction (PCR)-amplified from human or mouse muscle cDNA, adenylation and ligated into pEF6-V5/His TOPO (Invitrogen, Life Technologies) by TOPO TA cloning. The human wild-type and FINmaj M4-M10-V5 is7+ and is7− constructs spanned from A32992 to C-terminus in NP_001254479.2. The murine is6-M10-V5 is7+ and is7− constructs spanned the range from 145 442 to C-terminus in NP_001254479.2.

### Antibodies

The primary antibodies utilized in the study are listed in Supplemental Material, Table S2. The ‘p45-neo’ rabbit polyclonal neo-epitope antibodies, recognizing the novel N-terminus generated by CAPN3 cleavage in titin is6, were produced at BioGenes GmbH (Berlin, Germany). Two rabbits were immunized with the synthetic peptide NH2-KSIVHEEC (Innovagen, Lund, Sweden) coupled to Limulus polyphemus hemocyanin (LPH) through the C-terminal cysteine residue. Specific antibodies were affinity-purified from the antisera with the immunogen peptide, and a part of the affinity-purified fractions was further depleted using an affinity column containing the uncleaved peptide AATSLEKSVHEEC. The resulting antibody fractions were tested in WB, using extracts of 293T cells coexpressing titin is6-M10-V5 constructs with active or inactive (C129S) HA-CAPN3 as positive and negative control samples, respectively.

The rabbit polyclonal antibody ‘is5-1’ against the is5 region of human titin was produced at BioGenes GmbH. Two rabbits were immunized with the synthetic peptide CTETVTKTEKPAP coupled to LPH, and specific antibodies were affinity-purified from the antisera with the immunogen peptide.

### Protein extraction from muscle samples

Cytoskeletal and soluble proteins were extracted from human and murine muscle samples using the ProteoExtract Subcellular Proteome Extraction Kit (Calbiochem, Merck KGaA, Darmstadt, Germany). The extraction was performed according to the manufacturer's instructions, except that the final pellet containing the cytoskeletal proteins was mixed with a solution composed of 7 M urea, 2 M thiourea, 1% Triton X-100, 1% ASB-14, 2% CHAPS and 2% SDS, and incubated 15 min at 60°C. After centrifugation (10 min at 10 000g, 4°C), the cytoskeletal proteins were recovered in the supernatant.

### Cell culture experiments

Human 293T cells (ATCC, Manassas, VA, USA) were cultured at 37°C, 5% CO2, in DMEM supplemented with 10% FCS, l-glutamine and penicillin/streptomycin. Transfections were performed using FuGENE 6 (Promega, Madison, WI, USA) according to the manufacturer's instructions.

For proteolysis of C-terminal titin with CAPN3 in cell culture, 293T cells were cotransfected with different pEF6 titin constructs and wild-type or C129S mutant HA-CAPN3 constructs in a 4:1 mass ratio. After 24 h, the cells were fixed for IF microscopy, or harvested for the preparation of cell lysates as follow. The cells were lysed in ice-cold RIPA buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) with 1 x HALT protease inhibitor cocktail (Pierce, Thermo Fisher Scientific) 15 min on ice, followed by centrifugation (15 min at 16 000g, 8°C) for pelleting the insoluble material. The supernatant was used for isolation of titin fragments as described below, or mixed with SDS sample buffer, heated 5 min at 95°C, and analyzed by western blotting. Alternatively, cells were directly scraped into pre-heated 2× SDS sample buffer, heated 5 min at 95°C and analyzed by western blotting.

For CAPN2 digestion experiments, 293T cells were transfected with M4-M10-V5/His6, titin constructs and harvested after 24 h. The cells were lysed in ice-cold lysis buffer (20 mM Tris–HCl, pH 7.4, 20 mM NaCl, 1% Triton X-100, 1× HALT without EDTA), and incubated on ice for 10 min. NaCl was added to the final concentration of 165 mM, after which lysates were incubated on ice for 5 min and centrifuged 15 min at 16 000g at 8°C. DTT (5 mM) and CaCl2 (10 mM) were added to the supernatants. After addition of 0.2% porcine CAPN2 (Calbiochem, product # 208715), the digestion reactions were incubated in room temperature. Samples were taken at given time points and immediately quenched with 4× Laemmli buffer (Bio-Rad, CA, USA) containing 10% 2-mercaptoethanol, heated for 5 min at 95°C and analyzed by western blotting. Control reactions were performed without a CAPN2 addition.

### Isolation and N-terminal sequencing of titin fragments

C-terminal protein fragments produced by coexpression of V5/His6-tagged titin constructs and active CAPN3 in 293T cells
were isolated from RIPA lysates by IMAC followed by immunoprecipitation. The fragments were first bound to EZView Red HIS-Select HC Nickel Affinity Gel (Sigma-Aldrich, St. Louis, MO, USA) for 90 min at +8°C, washed with ice-cold RIPA containing 5 mM imidazole and eluted into RIPA containing 250 mM imidazole. The eluted fragments were then bound to Anti-V5 Agarose Affinity Gel (Sigma-Aldrich), washed with ice-cold RIPA and eluted into non-reducing SDS sample buffer at 95°C. After addition of 2-mercaptoethanol to a final concentration of 5%, the eluates were heated 5 min at 95°C. The samples were separated by SDS-PAGE in 4–20% TGX gels, and transferred onto PVDF membranes. The bands of interest were cut out from coomassie-stained membranes and analyzed by Edman degradation on an Applied Biosystems Procise 494 HT instrument at the Proteomics unit, Institute of Biotechnology, University of Helsinki.

SDS-PAGE and western blotting

SDS-PAGE was performed in precast 4–20% TGX gels (Bio-Rad), 4–12% NuPAGE gels (Invitrogen) or self-cast discontinuous gels. After electrophoresis, proteins were transferred onto PVDF (for chemiluminescent detection or protein sequencing) or nitrocellulose membranes (for fluorescent detection). Immunostaining was performed with standard methods. Chemiluminescent detection was done with HRP-coupled secondary antibodies (Dako, Glostrup, Denmark, or Amersham Biosciences, Piscataway, NJ, USA) and Super Signal West Pico or Femto substrates (Pierce). Fluorescent detection was done using secondary antibodies with Alexa Fluor 680 (Molecular Probes, Life technologies) or IRDye 800 (LI-COR Biosciences, Lincoln, NE, USA) labels and an Odyssey infrared scanner (LI-COR).

Yeast two-hybrid studies

The AH109 (mat a) yeast strain was transformed with pGAD prey plasmid constructs covering the region is7–M10 of human titin in wild-type or different mutant forms, whereas the Y187 (mat a) strain was transformed with the pAS-CAPN3 bait construct, according to the manufacturer’s instructions (BD Biosciences, Franklin Lakes, NJ, USA). The prey and bait strains were mated for 36 h at 30°C on non-selective yeast-peptone-dextrose (YPD) medium (QBiogene, Morgan Irvine, CA, USA). Yeast colonies were then plated on selective histidine/leucine/tryptophan-dropout medium prepared by mixing 27 g/l of dropout (DOB) medium (Qbiogene) with 0.62 g/l of complete supplement mixture lacking histidine, leucine and tryptophan (Qbiogene), and cultured at 30°C. Activation of the HIS3 reporter gene was assessed after 5 days from the ability of yeast colonies to grow on the selective medium.

Cleavage site predictions

Previously published bioinformatics methods were used for analyzing C-terminal titin protein sequences for putative calpain cleavage sites. CAPN3 recognition motifs [LIMV]X(3,4) [LIMV]X(2)[LIMV][DE] and [LIMV]X(3,4)[LIMV]X(2)[LIMV][DE] (31) were identified using the ScanProsite software (http://prosite.expasy.org/scanprosite). Sites susceptible to cleavage by the ubiquitous calpains 1 and 2 were predicted using the GSD-CDF software (http://ccd.biocuckoo.org/) (35), and with Microsoft Excel using the preference matrix published by Tompa et al. (36).

Study approval

The study was approved by the Helsinki University Hospital IRB, and an additional written informed consent was received from participants for the muscle biopsies. All procedures on animals were performed in accordance with the directive of 24 November 1986 (86/609/EEC) of the Council of the European Communities and were approved by the Généthon ethics committee under the number CE-12-021.

Supplementary Material

Supplementary Material is available at HMG online.

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