Removal of the calpain 3 protease reverses the myopathology in a mouse model for titinopathies

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The dominant tibial muscular dystrophy (TMD) and recessive limb-girdle muscular dystrophy 2J are allelic disorders caused by mutations in the C-terminus of titin, a giant sarcomeric protein. Both clinical presentations were initially identified in a large Finnish family and linked to a founder mutation (FINmaj). To further understand the physiopathology of these two diseases, we generated a mouse model carrying the FINmaj mutation. In heterozygous mice, dystrophic myopathology appears late at 9 months of age in few distal muscles. In homozygous (HO) mice, the first signs appear in the Soleus at 1 month of age and extend to most muscles at 6 months of age. Interestingly, the heart is also severely affected in HO mice. The mutation leads to the loss of the very C-terminal end of titin and to a secondary deficiency of calpain 3, a partner of titin. By crossing the FINmaj model with a calpain 3-deficient model, the TMD phenotype was corrected, demonstrating a participation of calpain 3 in the pathogenesis of this disease.

INTRODUCTION

The giant protein titin is one of the most abundant proteins of the striated muscle (1). The titin protein is a ~1 μm long molecule that spans half the length of a sarcomere, from the N-terminal Z-disc region to the C-terminal M-line region (2). Mutations in the titin gene (TTN) induce a spectrum of severe cardiac and skeletal muscle disorders (3). So far, four different phenotypes are associated with mutations in the titin C-terminus: hereditary myopathy with early respiratory failure (4), early-onset myopathy with fatal cardiomyopathy (EOMFC) (5), tibial muscular dystrophy (TMD) (6) and limb-girdle muscular dystrophy type 2J (LGMD2J) (6). Interestingly, the last two phenotypes were initially described as both segregating in the same large Finnish family (7). They were found to be due to a unique complex mutation in the last coding exon of titin. This mutation, referred to as the FINmaj mutation, leads to the replacement of the four amino acids EITW by VKEK in the M-line portion of titin (6). TMD arises when the mutation is present on one allele and presents as an autosomal dominant late-onset distal myopathy with weakness and atrophy of the anterior compartment muscles of the lower leg (8). LGMD2J, a far more severe phenotype, arises when the mutation is present on both alleles and presents as an autosomal recessive early-onset proximal muscular dystrophy (7,9,10). The TMD prevalence was reported to be >1 in 10 000 in Finland, with almost 10% of the patients presenting phenotypes differing from the classical TMD presentation (10). In addition, six other mutations in the C-terminal titin, both missense and nonsense, found in unrelated French, Belgian, Spanish and Italian families, also lead to TMD/LGMD2J phenotypes (11–14).

Titin is the largest known protein with a mass varying between 3 and 4 MDa, depending on the isoform (3,15). It has a multimodular structure formed mostly by immunoglobulin (Ig) and fibronectin-like domains, interspersed with

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several unique sequences, including the N2A/B, PEVK regions and a serine/threonine kinase domain (titin kinase, TK) near the C-terminus (3). The region of titin which is anchored in the M-line of the sarcomere extends from the TK domain up to the C-terminus and is divided into 10 different Ig-fold domains (M1–M10) and seven titin-specific domains (is1–is7; Fig. 1A) (3). This region is encoded by the last six coding exons (Mex1–Mex6), Mex5, which encodes the is7 domain, is the only exon in this region known to be subject to alternative splicing, leading to a variable proportion of is7+ or is7− titin isoforms depending on the muscle (16,17).

Titin is primarily known as a structural protein crucial for sarcomere assembly, structure, elasticity and integrity but it has been thought to orchestrate several signalling pathways (3). Through its various partners, the M-line region participates in various functions: (1) the translation of mechanical signals into modulation of serum-response factor-dependent gene expression is performed by a molecular cascade initiated by the autoinhibitory kinase of the TK domain, through its binding partner Next-to-BRCA1 (Nbr1) (4); (2) the precise alignment of thick filaments is ensured by cross-linking of the M4 domain of M-line titin with myomesin and M-protein, stabilizing the sarcomere during muscle contraction (18); (3) several metabolic enzymes such as creatine kinase are anchored in the M4 region of titin by the titin partner four and a half LIM domain-2 (FHL2) and participate in ATP production within this energy-demanding region (19) and (4) finally, an important role in the excitation/contraction mechanism seems to be carried out by obscurin, a titin M10 partner four and a half LIM domain-2 (FHL2) and participate in ATP production within this energy-demanding region (19).

Interestingly, mutations in another M-line partner, calpain 3, are responsible for a muscle disease, LGMD2A (23). Calpain 3, a multi-substrate calcium-dependent cysteine protease whose function is not yet fully understood, binds titin at several points, including is7 (24). Titin has been suggested to stabilize calpain 3 from autolytic degradation, although it was analysed only for the N2A region of titin (25,26). Moreover, the calpain 3 level was demonstrated to be reduced in LGMD2J and in some TMD patients (27), suggesting that this protease might play a role in the pathophysiology of TMD/LGMD2J.

RESULTS

A partial embryonic lethality is observed at N3 but, once born, the HE and HO FINmaj animals have a normal lifespan and exhibit a normal growth

To obtain a model that would enable us to investigate the pathological mechanisms of TMD and LGMD2J, we introduced the FINmaj mutation in the murine genome by a knock-in strategy. The Mex 6 sequence GAA ATA ACA TGG of the Tit gene was replaced by GTG AAA GAA AAAA in order to lead to the modification of the four amino acids EITW to VKEK of the FINmaj mutation (for details, see Materials and Methods). The targeting vector encompasses Mex2–Mex6 and carries a neomycin (neo) cassette in its 3′ region and two flanking homologous recombination arms: a short one 2.8 kb upstream of the mutation and a longer one 3.6 kb downstream of the neo resistance gene (Fig. 1B). After electroporation of the construction and subsequent introduction of G418-resistant embryonic stem (ES) clones in blastocysts, chimeric animals were generated and mated with Cre-transgenic mice in order to obtain germline transmission and excision of the neo cassette. After backcrosses on a C57BL/6 background, HE N3 mice were interbred to generate HE (FINmaj/WT), HO (FINmaj/FINmaj) and wild-type (WT) mice. PCR genotyping (Fig. 1C, left) and sequencing (Fig. 1C, right) confirmed the introduction of the mutation in the knock-in animals.

To check whether the mutation affects titin expression, Mex2–3-specific quantitative RT–PCR (qRT–PCR) was performed on muscles of 4-month-old mice. Titin mRNA levels were similar in the three populations tested, i.e. in WT, HE and HO mice, as seen in Tibialis Anterior (TA) and heart muscle (Fig. 1D, left). Similar results were obtained using Gastrocnemius (GA), Soleus (SOL), Extensor Digitorum Longus (EDL) and Psoas (PSO) (data not shown). The respective expression of each allele (WT and FINmaj) was determined in WT, HE and HO TA and heart by FINmaj and WT allele-specific qRT–PCR. Consistent with the genotype, 100% of the transcripts were of the WT genotype in WT mice, 100% are of the FINmaj genotype in HO mice and 50% are of each genotype in HE mice, reflecting the stability of the mutated transcript (Fig. 1D, right panel). Immunostaining using a titin antibody whose epitope is situated outside the mutated region (2Q1063 N2A antibody, US Biological) confirmed the sarcomeric presence of the titin protein in every condition in TA muscles (Fig. 1E). These results indicate that the introduction of the FINmaj mutation does not influence either the overall expression level or the integration of mutant titin within the sarcomeres.

A severe transmission ratio distortion was observed with this mixed background. Among the 198 offspring engendered from HE crosses, 93 mice had a WT genotype, 93 mice had a HE genotype and 12 had a HO genotype, displaying a statistically significant deviation (P < 0.01) from the Mendelian transmission (Table 1). Considering that the number of WT mice obtained in HE breeding (93 animals) should correspond to 25% of the total of expected pups, 372 animals should have been born (instead of 198). Therefore, as only 93 and 12 animals were obtained for HE and HO animals, respectively, the real distribution of heterozygotes and homozygotes is 25 and 3.2%
Figure 1. Construction and molecular characterization of a transgenic mouse model reproducing the human FINmaj mutation. (A) Top: Representation of the C-terminal extremity of titin messenger with the last six exons of titin (Mex1–Mex6). Bottom: Modular structure of M-line titin protein showing the locations of the domains, of the antibodies used herein and of the FINmaj mutation causing TMD and LGMD2J. (B) Schematic representation of the strategy used to construct the FINmaj mouse model. A 2.2 kb fragment encompassing exons Mex2–Mex6 was used to introduce the FINmaj mutation in Mex6. Two fragments of 2.8 and 3.6 kb were amplified by PCR to construct the two homology arms. (C) Left: WT, HE and HO mice genotyping. Specific PCR was used to amplify WT and mutant alleles on tail DNA, generating fragments of 414 and 502 bp, respectively. Right: Sequencing of the mutated region confirms the GAA ATA ACA TGG > GTG AAA GAA AAA replacement. (D) Left: Titin mRNA levels in WT, HE and HO animals. qRT–PCR was performed on the TA with Mex2–3-specific primers and probe and results were normalized by P0 (n = 4). No difference in titin expression is observed in TA and heart. Right: Quantification of WT allele and mutated FINmaj allele in WT, HE and HO TA and heart by qRT–PCR using Mex6 WT and Mex6 FINmaj primers and probe and normalized by P0 (n = 4). As expected, about 50% of each allele is present in HE mice in each muscle. AU, arbitrary unit. (E) Titin localization within the myofibre. Immunostaining of N2A-titin domain was carried out on TA longitudinal sections of WT, HE and HO mice and shows no differences between the three phenotypes. Scale bar = 25 µm.
of fibres containing centrally located nuclei at 9 months of age showed that the TA and the SOL are the most affected muscles in HE and HO animals, respectively (Fig. 2B). Among all the muscles tested, the HO SOL is the only one presenting developmental myosin heavy chain (dMHC) positive fibres as a marker of necrotizing injury (in 9-month-old animals; Supplementary Material, Fig. S2A) and secondary lymphocytic infiltrates (detected at 4 and 9 months of age by anti-CD11b staining; Supplementary Material, Fig. S2B).

Slow-twitch fibres were quantified using slow MHC (sMHC) staining on SOL, TA and PSO in 9-month-old mice and did not display any significant difference in the proportion of fibre types in any genotype (data not shown). Furthermore, neither HE nor HO animals presented noticeable increase in sarcolemmal membrane permeability as assessed by intraperitoneal injection of Evans blue dye (EBD), even if the animals were excised before the analysis (data not shown).

**Electron microscopy examination reveals the integrity of sarcomere structure**

Both light and electron microscopy studies on human TA muscle biopsy of TMD patients have previously evidenced the presence of rimmed vacuoles within these muscles (28). Since light microscopic study of transgenic mice did not show rimmed vacuolar change, electron microscopy was performed on TA and SOL muscles of both HE and HO mice at 9 months of age. No autophagic vacuoles were detected in any of the muscles examined. Importantly, as in human patients, the overall sarcomeric structure is preserved in all HE and HO muscles studied (Fig. 2Cb–f). In HO muscles, the only remarkable feature is the presence of a higher number of vacuoles in the I-band and I/A-band junction than in WT (twice as much), probably representing enlarged sarcotubular structures (Fig. 2Cc, h and i).

**SOL muscle mechanical function is impaired in HO mice**

Considering the histopathological changes observed in skeletal muscles of both HE and HO animals, we analysed the functional status of their muscles. *In vivo*, no differences were evidenced on the global force of 4- or 9-month-old animals, as measured by escape test or in an open-field actimeter (data not shown). *In vitro*, the specific force generated after tetanic stimulation was measured in the EDL and SOL of 4- and 9-month-old male and female mice. EDL force remains normal in HE and HO mice, consistent with the fact that this muscle is histologically unaffected in both models (data not shown). Inversely, the SOL-specific force is significantly reduced in HO animals, with a loss of 32 and 40% at 4 and 9 months of age, respectively (Fig. 3). The half-relaxation time after isometric tetanic contraction was normal in both HE and HO animals (Supplementary Material, Table S1).

Considering the role of titin in passive tension and muscle elasticity, we also performed an experiment to evaluate the stiffness profiles of the EDL and SOL muscles. Isolated muscles were subjected to a passive stretch protocol where the muscles were submitted to three of ramp-and-hold extensions. The peak incremental stiffness and the steady incremental stiffness were normal compared with WT muscles (Supplementary Material, Table S1).
The HO mice displays dilated cardiomyopathy with heart muscle fibrosis and left ventricular dysfunction

The impact on the heart of the presence of the FINmaj mutation was analysed. First, cardiac histology was examined at 4 and 9 months of age in each genotype. The HPS-stained cross-sections of HE hearts always show normal features, whereas HO hearts display a marked pathology noticeable as early as 4 months of age, as shown by the large areas of damaged tissue (Fig. 4A). Consistently, Sirius red staining...
revealed extensive regions of interstitial fibrosis in hearts of HO mice (Fig. 4B). The histological aspect and quantification of collagen areas coloured by Sirius red showed that myocardial alterations increase with age in HO mice (Fig. 4A–C). The expression pattern of myomesin is similar in HO and in WT, indicating that the titin binding region for myomesin is conserved in HO muscles (Fig. 5C). In the heart, cardiac sections of WT and HO immunostained with M10 or myomesin antibodies revealed a similar disappearance of the M10 staining, while the myomesin staining is unaffected (Fig. 5D).

**In HO muscles, obscurin staining at the M-band is modified**

Immunostaining using antibodies directed against obscurin has previously demonstrated that obscurin loses its sharply defined M-band localization in LGMD2J biopsies (6,12). To check whether the mouse model reproduces this feature, analyses using various titin M-line antibodies were performed on skeletal and cardiac muscles of WT, HE and HO mice. In western blot, the intensity of the titin bands is markedly reduced in HE mice and completely lost in HO animals for both TA and cardiac muscles (Fig. 5A). The same results were obtained in WT and HO SOL, EDL, TA, PSO and QUA muscles (Supplementary Material, Fig. S4A). To check whether the staining is, as expected from the results obtained in human patients, specifically lost downstream of M4, antibodies directed against the A169/170 domain (Mex1) and M9 (T51) were used for co-immunostaining on TA of WT and HO mice (Fig. 5B). M9-specific staining totally disappears in HO mice TA, whereas A169/170 staining is unaffected by the presence of the mutation in the HO state, confirming that the destabilization of titin starts downstream of the A169/170 domains. Co-immunostaining with antibodies directed against M8/9 domain and α-actinin shows the same pattern and confirms that the loss of signal truly concerns the M-line (Supplementary Material, Fig. S4B). In an attempt to localize more precisely the initiation site of this destabilization, we performed immunostaining with an antibody recognizing myomesin, a titin partner reported to interact at M4 (29).

**The FINmaj mutation leads to a loss of the C-terminal extremity of titin in skeletal and cardiac muscles**

Immunostaining experiments have previously demonstrated that the titin protein is lost downstream of M4 in human LGMD2J muscles biopsies (6,12). To check whether the mouse model reproduces this feature, analyses using various titin M-line antibodies were performed on skeletal and cardiac muscles of WT, HE and HO mice. Western blot, the intensity of the titin bands is markedly reduced in HE mice and completely lost in HO animals for both TA and cardiac muscles (Fig. 5A). The same results were obtained in WT and HO SOL, EDL, TA, PSO and QUA muscles (Supplementary Material, Fig. S4A). To check whether the staining is, as expected from the results obtained in human patients, specifically lost downstream of M4, antibodies directed against the A169/170 domain (Mex1) and M9 (T51) were used for co-immunostaining on TA of WT and HO mice (Fig. 5B). M9-specific staining totally disappears in HO mice TA, whereas A169/170 staining is unaffected by the presence of the mutation in the HO state, confirming that the destabilization of titin starts downstream of the A169/170 domains. Co-immunostaining with antibodies directed against M8/9 domain and α-actinin shows the same pattern and confirms that the loss of signal truly concerns the M-line (Supplementary Material, Fig. S4B). In an attempt to localize more precisely the initiation site of this destabilization, we performed immunostaining using an antibody recognizing myomesin, a titin partner reported to interact at M4 (29).

**The FINmaj mutation leads to a secondary reduction in calpain 3 in both HE and HO mice**

Another defect previously observed in human patients is a secondary deficiency in calpain 3 (27). Calpain 3 expression and activity in WT, HE and HO muscles were therefore measured simultaneously using a test previously developed in our...
The results show that calpain 3 expression is reduced in HO TA muscles (Fig. 7A, left and middle panels, \( P < 0.01 \)), although some decrease is observed occasionally in some HE muscles. Nonetheless, calpain 3 enzymatic-specific activity normalized to the quantity of protein is unchanged in presence of the titin mutation, indicating that the FINmaj mutation has no consequences on the functionality of the remaining calpain 3 molecules (Fig. 7A, right). Calpain 3 qRT–PCR was carried out on RNA extracts of muscles of the three genotypes. No difference in calpain 3 mRNA expression was ever observed (Fig. 7B), which indicates that the reduction in calpain 3 expression results from the destabilization of the protein. This destabilization is possibly due to an autolytic activation and instability caused by the loss of the stabilizing interaction with titin.

**Crossing this model with a calpain 3-deficient model rescues the number of siblings and attenuates the myopathy in HE**

Considering the possibility that the secondary reduction in calpain 3 was due to an increased turnover and therefore an
Transthoracic echocardiography of 11-month-old WT, HE and HO mice was performed. LVEDD (mm), LV end-systolic diameter (LVESD, mm), IVS wall thickness (mm) and LVPW wall thickness (mm) were measured at diastole and systole stages. LV volume (LVvol, μl) is calculated at diastole as LVVol_d = [(7/2.4 + LVEDD)] × LVEDD^2 × 1000 and at systole as LVVol_s = [(7/2.4 + LVESD)] × LVESD^2 × 1000. LV mass divided by the total mouse weight (LVm/w; where w is the total weight of the mouse) was obtained after calculation of LVm = 1.055 × (LVd_d + LVEDD + LVpw_d) – (LVd_d)^2, where 1.055 is the specific gravity of the myocardium. LV fractional shortening (FS, %) and LV ejection fraction (EF, %) were calculated using the following equations: FS (%) = (LVEDD – LVESD)/LVEDD × 100 and EF (%) = (LVVol_d – LVVol_s)/LVVol_s × 100 as measures of systolic function. Posterior wall thickening (PWth) was calculated from the above dimensions using: (LVPW_s – LVPW_d)/LVPW_d. Cardiac output was calculated as follows: CO (ml/min) = (LVVol_d – LVVol_s) × HR × 0.14. Velocity of posterior wall (VelPW, mm/s) is measured using the tissue doppler tool of the echocardiograph. Only the HO mice show dilated cardiomyopathy as characterized by increased LV dimensions with no modification of septum and wall thicknesses. A systolic dysfunction of the heart was also noticed since LV fractional shortening and ejection fraction were significantly lower in HO than in WT. Values in bold are significantly different from the WT values. *Reflects a significance of P < 0.05.

Table 2. Echocardiographic assessment of WT, HE and HO mice

<table>
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<th>WT</th>
<th>HE</th>
<th>HO</th>
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<tr>
<td>LVEDD</td>
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<td>3.72 ± 0.02</td>
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<td>LVESD</td>
<td>2.69 ± 0.15</td>
<td>2.34 ± 0.31</td>
<td>3.09 ± 0.42</td>
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<td>IVS_d</td>
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<td>1.28 ± 0.07</td>
<td>1.19 ± 0.11</td>
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<td>LVPW_d</td>
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<td>0.96 ± 0.11</td>
<td>0.82 ± 0.04</td>
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<td>LVPW_s</td>
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<td>CO (ml/min)</td>
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<td>VelPW_s (mm/s)</td>
<td>21.47 ± 2.17</td>
<td>25.73 ± 10.77</td>
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**DISCUSSION**

The observation that distinct phenotypes, one dominant, late onset and distal (TMD) and the other recessive, early onset and proximal (LGMD2J) arise depending on the presence of an identical Mex6 mutation in one or two alleles is particularly intriguing. To further understand the physiopathology of these two diseases, we generated a knock-in mouse model carrying the FINmaj mutation and analysed the phenotypes of HE and HO animals. Interestingly, this model shows, at the level of the skeletal muscle, a myopathology with a high similarity to the human situation (Table 4).

The classical TMD presentation is mild, with clinical symptoms occurring after the age of 35 years. The anterior compartment of the lower legs is predominantly affected, the initial atrophy of the TA extending later on to the long toe extensor muscles and hamstrings. Occasionally, QUA, Peroneal, SOL or GA muscles can also be involved (10). HE animals present a late-onset muscle pathology with a high selectivity of impairment affecting TA, BF and QUA from 9 months of age, though no significant functional impairment is detected. It should be noted that we never observed any alterations of the foreleg muscles, consistent with the absence of upper limb impairment in humans. The involvement of the TA muscle both in human TMD and the HE mouse is of interest. Regarding the conventional fibre-type categories, the TA in human is a slow fibre muscle, whereas the mouse is of interest. Regarding the conventional fibre-type categories, the TA in human TMD and the HE mouse is of interest. The observation that distinct phenotypes, one dominant, late onset and distal (TMD) and the other recessive, early onset and proximal (LGMD2J) arise depending on the presence of an identical Mex6 mutation in one or two alleles is particularly intriguing. To further understand the physiopathology of these two diseases, we generated a knock-in mouse model carrying the FINmaj mutation and analysed the phenotypes of HE and HO animals. Interestingly, this model shows, at the level of the skeletal muscle, a myopathology with a high similarity to the human situation (Table 4).

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LGMD2J patients have a severe muscular dystrophy, with early onset in the first to third decade and impairment of all proximal muscles (9). Consistently, HO animals present the first dystrophic signs early, at 1 month of age in the SOL that extends later on to other muscles (TA, PSO, QUAD,
Figure 5. Titin M-line disruption in muscle and heart. (A) Cytoskeletal proteins prepared using the S-PEK kit were separated by SDS–PAGE and analysed by western blot. Membranes were probed with M10-1 antibody, specific for the titin M10 domain (12). Under these experimental conditions, western blots of the cytoskeletal fraction using the M10 antibody revealed specific titin bands as seen in Supplementary Material, Figure S4. The intensity of C-terminal titin bands is reduced by half in HE mice and completely lost in HO animals in skeletal muscle (left panel). The same results were obtained with cardiac samples (right panel). Alpha-actin is used to normalize the quantity of proteins. (B) The co-immunostaining of a longitudinal section of TA with antibodies directed against A169/170 and M9 domains show that the signal is completely lost downstream of the M9 C-terminal region of titin in HO mice. Scale bar = 50 μm. (C) Myomesin staining of longitudinal sections of TA is normal in HO muscle, indicating that the loss of the C-terminal M-line region starts downstream of the M4 domain. Scale bar = 50 μm. (D) M10 and myomesin stainings reveals the same loss of the titin C-terminus in HO mice downstream of the M4 domain on heart sections. Scale bar = 50 μm.
Figure 6. Obscurin mislocalization at the M-band in HO animals. (A) Colocalization at the M-band of obscurin (green) and myomesin (red) in confocal analysis of 2-month-old WT, HE and HO PSO muscle sections. The results show that obscurin localization appears unaffected by the titin mutation. Scale bar = 30 μm. (B) Image analysis of the width of obscurin staining compared with myomesin. Left: Superimposition of pixel-intensity curves across four sarcomeres for obscurin (green) and myomesin (red) in WT, HE and HO mice. X-axis: nm and Y-axis: pixel intensity. Right: Plots of obscurin width and myomesin width staining in WT, HE and HO muscles show that obscurin is found in broader stripes in the M-band in comparison to WT and HE animals, whereas myomesin remains constant (**P < 0.01).
GA, GLU and BF), but spares the EDL and DIA. The murine SOL contains a larger proportion of slow myosin than most other mouse muscles, with a proportion similar to that of human proximal muscles (34). Unfortunately, because the examination of LGMD2J patients occurred too late in the course of the pathology, the first muscles affected are not known and no parallel can be put forward between the two species so far. Finally, relative sparing of DIA in the murine model is not surprising, since respiratory failure occurs very late in human LGMD2J patients, after the total loss of all limb muscle activity (13).

Cardiomyopathy has not been diagnosed in TMD patients (28) and the absence of cardiac phenotype in HE mice is consistent with these human data. Concerning LGMD2J, of the originally described five Finnish patients, they all died at ages ranging between 52 and 65 years, four of respiratory failure and one of myocardial infarction. One of these patients underwent echocardiography at the age of 55 years with no indication of cardiomyopathy. However, an autopsy of another patient who died at the age of 64 showed that his heart presented mild LV hypertrophy (13). Considering these observations, mild subclinical cardiomyopathy may exist in LGMD2J but severe presentation is evidently not a systematic feature. The situation is more clear-cut concerning the HO mice, since they all present a dilated cardiomyopathy phenotype apparent at both histological and echocardiographic levels. This phenotype presents as extensive myocardial fibrosis starting from 4 months of age and LV dilation with impaired systolic function at 11 months of age. Considering these observations, a strict cardiac follow-up of patients affected with a LGMD2J could be necessary and should help clarify the issue of cardiac involvement.

Overall, it seems that C-terminal part of titin encompasses functions of importance for the homeostasis and the integrity of the striated muscle and that are conserved in between human and mouse species. In view of its characteristics, the FINmaj model seems perfectly relevant for studies towards understanding these particular titin functions, as well as the downstream mechanisms leading to TMD or LGMD2J. Since the mutated FINmaj titin is incorporated into the sarcomere, the corresponding titin domain is apparently dispensable for sarcomere assembly during development, in contrast to what was observed with the titin deleted of the TK domain (35). However, partial embryonic lethality starting from E12 was observed both for HE and HO animals. It should be noted that primary and retrospective examination of the large original Finnish family did not reveal any indications of abnormal offspring proportions (9). We pursued a backcross on a C57BL/6 background and crossed N9 animals to obtain HO mice. Interestingly, the ratio obtained on 44 pups is congruent with a Mendelian transmission. These preliminary data indicate that there is a modifying factor expressed at the embryonic stage in the 129 murine genome that is deleterious for FINmaj embryos. Understanding the cause of the lethality would require further investigations and could

Figure 7. Calpain 3 in FINmaj model. (A) Assessment of both calpain 3 expression and activity in TA muscles. A V5-tagged inactive form of calpain 3 (used as a substrate) was pre-incubated with the muscle proteins extracted from WT, HE and HO animals and calpain 3-specific western blot was carried out. For the analysis of calpain 3 expression, the quantification of calpain 3 was performed using a specific antibody and normalized to α-actin (left panel). The quantity of calpain 3 is approximately three times lower in HO than in WT mice (middle panel, P < 0.05). Similar results were observed in SOL, EDL, QUA and BF muscles. The activity of calpain 3 is calculated using the quantification of the remaining calpain 3 substrate (detected using an antibody recognizing the V5-tag) normalized by the total quantity of calpain 3 (right panel). Calpain 3 is at least as active in HO as it is in WT. (B) Calpain 3-specific qRT–PCR. No significant difference in calpain 3 transcript expression was observed between the three phenotypes, indicating that the decreased expression of the calpain 3 protein is due to the destabilization of the protein and not due to impaired transcription.
It has been shown, in a forced yeast two-hybrid assay, that myofibrillar apparatus to the sarcomeric reticulum (3,22,30). The cause of this instability is probably related to the loss of anchoring of critical structural proteins such as myomesin. Ultimately, it may be used to document the disruption of the buffering inhibitory domain and that calpain 3 protein level is reduced, it is possible that the presence of the mutation in the vicinity of the calpain 3-binding site causes a major conformation change (6,37), leading to the disruption of the buffering inhibitory regulation of the proteolytic activity of calpain 3. As a result, calpain 3 activity would heighten, leading to an increased turn-over of the protein, as reflected by a decrease in its steady-state level, and an excess of cleavage of its substrates. It is indeed possible that the disappearance of the titin C-terminus would be one of the consequences. Interestingly, although mostly expressed in skeletal muscle, calpain 3 is weakly expressed in heart, which is consistent with the possibility that calpain 3 is the protease responsible for this cleavage (38,39). Nevertheless, since the non-affected EDL muscles present as the other muscles the loss of M-band titin, it seems that it is not this event per se that is pathogenic. The pathophysiological mechanism may then be related to aberrant proteolysis of other substrates and/ or loss of anchoring of C-terminal titin partners that would be deleterious only in specific muscles. This mechanism should be present in both TMD and LGMD2J. The difficulty in obtaining HO/capn3−/− has prevented us to evaluate at late stage whether the phenotype of the HO is reduced, at least in the TA and therefore to confirm this hypothesis.

In conclusion, this new mouse model reproduces with reasonable reliability clinical and molecular features of the human diseases and shows that the disruption of calpain 3 regulation seems to be a major factor in the pathological mechanism in HE animals. This abnormality, together with the anomaly of obscurin localization observed in HO animals provides the first clue to understand the basis of the pathology of obscurin localization.

As shown by the correction of phenotype obtained after crossing FINmaj mice with calpain 3-deficient mice, an overall disrupted calpain 3 regulation seems to be a major part of the mechanism, at least for TMD-related pathogenesis. Considering that titin staining is lost downstream of the M4 domain and that calpain 3 protein level is reduced, it is possible that the presence of the mutation in the vicinity of the calpain 3-binding site causes a major conformation change (6,37), leading to the disruption of the buffering inhibitory regulation of the proteolytic activity of calpain 3. As a result, calpain 3 activity would heighten, leading to an increased turn-over of the protein, as reflected by a decrease in its steady-state level, and an excess of cleavage of its substrates. It is indeed possible that the disappearance of the titin C-terminus would be one of the consequences. Interestingly, although mostly expressed in skeletal muscle, calpain 3 is weakly expressed in heart, which is consistent with the possibility that calpain 3 is the protease responsible for this cleavage (38,39). Nevertheless, since the non-affected EDL muscles present as the other muscles the loss of M-band titin, it seems that it is not this event per se that is pathogenic. The pathophysiological mechanism may then be related to aberrant proteolysis of other substrates and/or loss of anchoring of C-terminal titin partners that would be deleterious only in specific muscles. This mechanism should be present in both TMD and LGMD2J. The difficulty in obtaining HO/capn3−/− has prevented us to evaluate at late stage whether the phenotype of the HO is reduced, at least in the TA and therefore to confirm this hypothesis.

In conclusion, this new mouse model reproduces with reasonable reliability clinical and molecular features of the human diseases and shows that the disruption of calpain 3 regulation seems to be a major factor in the pathological mechanism in HE animals. This abnormality, together with the anomaly of obscurin localization observed in HO animals provides the first clue to understand the basis of the different phenotypic outcomes between mono- and biallelic titinopathy. Considering the human–murine similarity in phenotype and in molecular consequences, at both HE and HO levels, the FINmaj model will be valuable for enlarging our understanding of the function of the titin extreme C-terminus. In particular, it may be used to document the complex functional relationships between titin, calpain 3 and the other partners in the M-line of the sarcomere. Ultimately, this knowledge should help to define appropriate therapeutic strategies.
**MATERIALS AND METHODS**

**Mouse model construction and genotyping**

Construction of the targeting vector and generation of the murine knock-in model for the FINmaj titin mutation were performed at the ‘Mouse Clinic Institute’ (MCI, France). A 2.2 kb fragment encompassing exons Mex2–Mex6 of titin was amplified by PCR on 129S2/SvPas genomic DNA with modified primers to introduce the mutation GAAATAACA TGG GTGAAAGAAAAA in exon Mex6. The mutated fragment was subcloned in an MCI proprietary vector containing a loxed neo resistance cassette. Two fragments of 2.8 and 3.6 kb (corresponding to the 5' and 3' homology arms, respectively) were amplified by PCR on 129S2/SvPas genomic DNA and subcloned directly upstream and downstream of the construction in the previous plasmid to generate the final targeting construct. The plasmid sequence was verified by restriction digestion and all exons and exon–intron junctions were sequenced.

The linearized construct was electroporated into 129S2/SvPas mouse ES cells, and G418-resistant colonies were isolated and expanded. The sequences of targeted clones were validated by PCR using external primers and further confirmed by Southern blot with 5' and 3' external probes. The presence of the correctly targeted allele was confirmed in one positive ES clone. After a caryotyping, the transgenic ES clone was injected into C57BL/6J blastocysts which were re-implanted into foster mothers to generate chimeric mice. Germline transmission was obtained through the breeding of male chimeras with CMV-cre (cre recombinase under the control of the CMV promoter) transgenic females, which also permits the excision of the neo cassette. The Cre transgene was

**Figure 8.** Calpain 3 role in the molecular pathophysiological mechanism in HE animals. **Left:** Histology of (A) TA muscles for WT, HE/capn3+/− and HE/capn3+/− at 9 months of age. White arrows indicate some fibres with centrally located nuclei. The general histological aspect of HE/capn3+/− muscles is improved when compared with HE muscles. Scale bar = 50 μm. **(B)** Quantification of CNFs in TA, BF and QUA of 9-month-old animals. The centronucleation index of HE/capn3+/− muscles is similar to the one measured in WT mice, showing that the reduction of calpain 3 expression within the HE background ameliorates muscle histology (**P < 0.01). (C) Histological analysis of heart sections of HE and HE/capn3+/−. Representative HPS-stained sections of hearts at 9 months of age. The heart is not affected in either HE or in HE/capn3+/− mice. Scale bar = 200 μm.
Note that embryonic lethality was observed at N3 on a C57BL/6 backcross. Two mutant mice with calpain 3-deficient male mice (32). Genotyping for deficiency in calpain 3 was obtained by crossing of HE female the embryonic tissue. were determined by TTN PCR as above using DNA from removed under sterile conditions. The genotypes of embryos intact. The uterus was dissected and the embryos were E15.5. Pregnant mice were sacrificed by cervical dislocation, and/or embryo staging by comparison with established refer-
ences (40). The studies covered embryonic days E12.5 to
ctions of 380 and 480 bp, respectively.

For the analysis of embryonic lethality, timed matings were set-up between HE mice. The gestational age was dated by the appearance of the vaginal plug on the morning after mating and/or embryo staging by comparison with established refer-
ces (40). The studies covered embryonic days E12.5 to E15.5. Pregnant mice were sacrificed by cervical dislocation, and a caesarean section was performed to remove the uterus intact. The uterus was dissected and the embryos were removed under sterile conditions. The genotypes of embryos were determined by TTN PCR as above using DNA from the embryonic tissue.

The mouse model carrying both the FINmaj mutation and a deficiency in calpain 3 was obtained by crossing of HE female mice with calpain 3-deficient male mice (32). Genotyping for the FINmaj and calpain 3 mutations was performed on tail DNA, respectively, as described above and using primers for calpain 3 (forward primers: GW 255 and GW 257; reverse primer GW 259; Supplementary Material, Table S2). PCR amplification was performed using PCR ReadyMix™ (Sigma) for 30 cycles with annealing cycles at 59°C for 30 s. The resulting WT and mutant alleles generate PCR fragments of 414 and 502 bp, respectively.

Histology, immunohistochemistry and morphometry
Cryosections (8 or 10 mm thickness) were prepared from frozen skeletal and cardiac muscles. Transverse sections were processed for HPS or Sirius red histological staining. In some experiments, mice were injected intraperitoneally with EBD (1 mg/g of body weight) the day before sacrifice after a course of 30 min. EBD-positive fibres were revealed by fluorescence excitation at 633 nm and counted. Colorimetric immunostaining with laminin, dMHC, sMHC and CD11b were performed according to the ARK peroxidase kit protocol (DAKO) to assess the number and minimal diam-
eter of fibres, regenerative fibres, fibre types and inflammatory infiltrates, respectively. The antibodies used for these detections were: an anti-laminin polyclonal antibody (Progen, P-4417, dilution 1:1000), a monoclonal antibody specific for sMHC (Sigma, M-8421, dilution 1:1000), a monoclonal antib-
ody specific for dMHC (Novocastra, NCL-dMHC, dilution 1:1000) and a monoclonal mouse antibody produced against CD11b (BD Pharmingen, Mac-1α chain, 1:40). Digital images of stained sections were acquired with a CCD camera (Sony) and a motorized stage on a Nikon Eclipse E60 microscope. Images were analysed with Ellix (laminin), Cartograph (myosin and CD11b) or Histolab (Sirius Red) soft-
wares (Microvision, France).

Immunofluorescence detection of titin was performed on unfixed longitudinal TA and heart sections using the mouse monoclonal antibody: 2Q1063 (US Biological, dilution 1:10), M9 domain titin antibody (T51; a gift from Professor D. Furst; dilution 1:10), the rabbit polyclonal antibodies: A169-170 titin (Mex1; dilution 1:100) and M8/9 titin (a gift from Professor S. Labeit dilution 1:100), a mouse monoclonal antib-
ody alpha-actinin, (clone EA-53, Sigma-Aldrich; dilution 1:5000) and a rabbit polyclonal Mex6 antibody (Mex6.1; dilution 1:100 on boiled sections) that we generated by injection of the peptide NEFGSDSATVNINIRSMC from M10 domain (Agrobio, France).

Myomesin 1 and 2 and obscurin immunostaining was performed on PSO of 2-month-old mice sections fixed with pre-
chilled acetone for 5 min at −20°C and with 4% paraformalde-
hyde in phosphate-buffered saline (PBS) for 6 min. After neu-
ralization with 0.1 M glycine in PBS, sections were treated with 0.2% Triton X-100 in PBS. After washing with PBS, samples were blocked with 5% normal goat serum for 30 min. The sample was incubated with mouse M4 myomesin primary antibody (1:100; Developmental Studies Hybridoma Bank at The University of Iowa) or myomesin-2 (1/100; Santa Cruz Bio-
tchnology, H-65) and rabbit anti-obscurin (1:200; a gift from Professor M. Gautel) in 1% BSA for 1 h. Sections were then incubated for 30 min with goat anti-mouse or goat anti-rabbit antibodies conjugated with Alexa-488 or 594 dyes (dilution 1:1,000; Invitrogen). The sections were examined with a confocal microscope (TCS SP2.AOBS, Leica, Germany) using the 488 nm line of an argon laser (12% power range) and 633 nm (HeNe laser 25 mW, 40% Power).

All images were acquired in 1024 × 1024 format with a 63 × HCS PLAP0 objective. Image processing and analysis was done using Adobe Photoshop CS2 9.0.2 (Adobe Systems Inc., CA, USA), and ImageJ 1.38x (http://rsb.info.nih.gov/ij/). For the calculation of the obscurin/myomesin labelling width ratio, pixel-intensity curves transversely to sarcomeres were determined by Image J for both stains. Excel software was used to calculate the width of the staining in the second quartile of each oscillation, after adjustment of the baselines of both curves. Ninety sarcomeres for every phenotype (WT and HO) were

| Table 4. Similarires and differences between M-line titinopathies in human and mouse |
|---------------------------------|----------------|
| Human                          | Mouse         |
| One mutant allele              | Late dystrophic features | +  |
|                                | Few selective muscles involved | +  |
|                                | Embryonic lethality | −  |
|                                | Functional consequences | +  |
| Two mutant alleles             | Early disease manifestations | +  |
|                                | Severe         | +  |
|                                | Affect a larger number of muscles | +  |
|                                | Functional consequences | +  |
|                                | Myocardial alterations | −  |
|                                | Embryonic lethality | −  |
|                                | (at N3)        |     |

Note that embryonic lethality was observed at N3 on a C57BL/6 backcross.
counted. The difference in mean width between obscurin and myomesin was calculated and plotted.

Electron microscopy

For electron microscopy, SOL and TA muscles were dissected and pre-fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (PB, pH 7.4) overnight at 4°C. Pieces of the sample were placed in the same fixative for a further 2 h, rinsed in PB and post-fixed in 2% osmium tetroxide. After washing with PBS, each sample was dehydrated in a graded series of ethanol baths and embedded in Epon resin (EMS LYNX Automated Tissue Processor). Semi-thin sections (1 μm) were cut and evaluated by light microscopy to define more accurately the field of observation. The tissues were then cross-sectioned at 80 nm thickness with a microtome (LEICA Ultracut R). The grids were stained with uranyl acetate and examined under a Philips Tecnai F20 transmission electron microscope.

In vitro and in vivo evaluation of muscle function

Animals were anesthetized by intraperitoneal injection of pentobarbital (100 mg/kg). The muscles were surgically excised and maintained in Krebs buffer. Measurements of isometric stiffness (N/m²) were calculated according to Anderson et al. (41). For the evaluation of stiffness, isolated muscles were subjected to a passive stretch protocol where the muscles were submitted to three ramp-and-hold extensions of 2.5 mm in amplitude. The peak incremental stiffness (N/m) and the steady incremental stiffness (N/m²) were calculated according to Anderson et al. (42). For the escape test, the mouse tail is attached to a force sensor and the force developed by the mice after pitching the tail is measured (43). The spontaneous global activity of mice was recorded during 6 h in an open field (LE 8811 IR motor activity monitor equipped with 16 horizontal infrared photocell beams; BIOSEB, France).

Echocardiogram

Conventional transthoracic echocardiographies were performed on mice placed on a heated, tilt platform and anaesthetized using 1% isoflurane. The heart rate (HR) was maintained constant during echocardiography. An echocardiography system (Vevo 770, VisualSonics, ON, Canada) with a 30 MHz scanhead was used for the investigation of the heart. In order to improve the quality of images, sweep speed, depth, focus and gain settings were optimized. B- and M-mode images were obtained from long-axis view at the level of the largest LV diameter for measurements of LVEDD (mm), LVESD (mm) (measures of LV dilation), inter-ventricular septal (IVS) wall thicknesses and posterior wall (LVPW) according to the rules defined by the convention of the American Society of Echocardiography (44).

Western blot and calpain 3 activity assay

Muscle tissue was weighed and homogenized using an Ultra-Turrax T8 (Ika, Germany) in a lysis buffer containing 20 mM of Tris–HCl pH 7.5, 150 mM NaCl, 2 mM EGTA and 0.1% Triton X-100 supplemented with Complete mini protease inhibitor cocktail (Roche) and 2 μM E64 (Sigma). In vitro calpain 3 activity assay was subsequently performed on these extracts as previously described (31). After SDS–PAGE performed on precast 4–12% acrylamide gradient Nu-PAGE gels (Invitrogen), reaction products were transferred onto a PVDF membrane for 1 h at 100 V in transfer buffer (0.2 M glycine, 25 mM Tris pH 8, 0.1% SDS, 20% ethanol). The efficacy of the transfer was verified by Ponceau red staining (0.2% Ponceau red/1% acetic acid). Analysis of calpain 3 protein level and cleavage activity was performed by probing membranes with a rabbit polyclonal anti-calpain 3 antibody directed against a part of the IS2 region (45) (dilution 1:150) and mouse monoclonal anti-alpha-actin (A4700, Sigma; dilution 1:500) for normalization. Membranes were incubated with anti-mouse and anti-rabbit secondary antibodies (1:10 000) coupled with IRDye® for the revelation by the infrared-scanner Odyssey (LI-COR Biosciences, NE, USA). Quantification of protein revealed by the Odyssey system was performed with the software Odyssey 2.1 (LI-COR Biosciences). The calpain 3 protein level was obtained by normalizing with the alpha-actin level and the activability of calpain 3 was calculated using the quantification of the remaining calpain 3 substrate normalized by the total quantity of calpain 3 (31).

For titin western blot, after Ultra-Turrax homogenization, proteins were extracted with the ProteoExtract® Subcellular Proteome Extraction Kit (S-PEK, Calbiochem, Germany). Western blots were performed as described above using 50 μg of proteins of the cytoskeletal fraction. Detection of titin bands was performed using the M10 antibody. Membranes were incubated with anti-rabbit secondary antibody (1:10 000) coupled with horseradish peroxidase (HRP; American Biosciences, NJ, USA). Revelation was performed with the HRP chemiluminescent substrate Super Signal West Pico kit (Pierce, IL, USA). Under these experimental conditions, western blots of the cytoskeletal fraction using M10 antibody revealed specific titin bands.

qRT–PCR

Total RNA was isolated from mouse muscles using Trizol reagent (GibcoBRL). One microgram of total RNA was used to synthesize cDNA using the SuperScript II first-strand synthesis kit (Invitrogen) and random oligonucleotides. Expression was monitored by a real-time qRT–PCR method using TaqMan probes and primers (Supplementary Material Table S2). The ubiquitous acidic ribosomal phosphoprotein (P0) was used to normalize the data across samples. P0 expression was monitored by SYBRGreen incorporation.

Statistical analysis

Individual means and distributions were compared using the Mann–Whitney and the Kolmogorov–Smirnov non-parametric tests, respectively. Differences were considered to be significant at $P < 0.05$ or $P < 0.01$. Differences in birth among the mice groups were tested using the $\chi^2$ test.
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