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Muscle atrophy in Titin M-line deficient mice

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Abstract

We investigated the response to deletion of the titin M-line region in striated muscle, using a titin knockout model and a range of techniques that include histology, *in situ* hybridization, electron microscopy, and 2D gel analysis. We found that the loss of titin's kinase domain and binding sites for myomesin and MURF-1 causes structural changes in the sarcomere that proceed from the M-line to the Z-disc and ultimately result in disassembly of the sarcomere. Disassembly goes along with central localization of nuclei (a hallmark for muscular dystrophy), up-regulation of heat-shock proteins, and induction of proteasome activity. While fiber type composition does not change in soleus and extensor digitorum longus muscle, fiber size is reduced. Animals die from complications of muscle atrophy at five weeks of age. In addition to the structural importance of the titin M-line region in any striated muscle, our data show how differences in M-line composition between heart and skeletal muscle affect sarcomere stability and function.

Introduction

Titin alias connectin is a filamentous protein with a mass of 3.0–3.7 MDa, depending on the isoform type (Labeit and Kolmerer, 1995; Freiburg *et al.*, 2000), that forms a separate myofilament system in both skeletal and cardiac muscle. Titin is, after myosin and actin, the third most abundant muscle protein (Wang, 1984) and forms a continuous elastic scaffold with multiple binding sites for sarcomeric and signaling proteins (Obermann *et al.*, 1996; Gregorio *et al.*, 1998; Young *et al.*, 1998).

Close to its C-terminal M-line region, the titin polypeptide contains a kinase domain with homology to the catalytic serine/threonine kinase domain of smooth muscle myosin light chain kinase (Olson *et al.*, 1990), as well as the kinase domain of the giant invertebrate muscle proteins twitchin (Benian *et al.*, 1989), projectin (Ayme-Southgate *et al.*, 1995), and stretchin-MLCK (Champagne *et al.*, 2000). *In vitro* studies with a mutant kinase domain have provided evidence that telethonin, alias T-cap, is a titin kinase substrate in embryonic muscle (Mayans *et al.*, 1998).

The ubiquitin ligase MURF-1 binds N-terminal to the titin kinase region (repeats A168/A169) (Centner *et al.*, 2001) and has been shown to be involved in muscle atrophy (Bodine *et al.*, 2001). Through heterodimerization with the microtubule binding protein MURF-3 and the transcription factor GMEB-1 it is involved in differentiation and signal transduction (Spencer *et al.*, 2000; Centner *et al.*, 2001; McElhinny *et al.*, 2002). A second protein binding site deleted in the titin kinase region knockout is the Ig-domain M4, which binds myomesin, a structural protein that confers stability to the M-line region during contraction (Obermann *et al.*, 1997). Thus, the titin M-line region integrates signaling and structural functions.

To study the titin kinase region, we have generated an inducible knockout of titin's M-line Exons 1 and 2 (MEx1 and 2) using the cre-lox recombination system (Gotthardt *et al.*, 2003). Embryonic lethality as a result of early cardiac depletion of the titin kinase region was circumvented by using the muscle creatine kinase promoter to drive expression of the Cre recombinase (MCKcre). Analysis of the cardiac phenotype revealed progressive sarcomeric disassembly with death at 5 weeks of age.

Here we investigated the skeletal muscle phenotype with progressive atrophy. With deletion of the titin kinase region, skeletal muscle displays sarcomeric disassembly with loss of striation, centralized nuclei, and, in contrast to cardiac muscle, an absence of M-line widening.

Materials and methods

Animal model

Titin kinase region knockout mice have been described previously (Gotthardt *et al.*, 2003). In brief, the

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MCKcre transgenic animals (Bruning *et al*., 1998) were used to target deletion of the titin kinase region (M-line exons 1 and 2) to striated muscle cells. Animals heterozygous for the MCKcre allele and homozygous for the floxed titin kinase region (MCKcre$^+$ Ti MEx1 + 2$^{lox/lox}$) are referred to as knockout (KO) animals. The MCKcre negative homozygous floxed titin kinase region animals (Ti MEx1 + 2$^{lox/lox}$) contain intronic lox sites and express wildtype titin protein. They are indistinguishable from wildtype animals and were used as controls (WT). Breedings (Ti MEx1 + 2$^{lox/lox}$ × MCKcre$^+$ Ti MEx1 + 2$^{lox/lox}$) resulted in KO and control making up 50% each of the offspring.

All animal experiments were carried out under protocols approved by the Washington State University Institutional Review Committee for animal use and followed the NIH Guidelines, ''Using Animals in Intramural Research''.

SDS-agarose electrophoresis

Vertical SDS agarose gel electrophoresis (VAGE) was used to separate titin isoforms (Warren *et al*., 2003b).

In situ hybridization

In situ hybridization was performed with α-^{33}P labeled probes directed against sequences shown in Figure 2A) as described previously (Trommsdorff *et al*., 1999).

Fiber typing

Fibers types were determined using 8-μm serial cryosections of triceps surae and quadriceps muscles that were immuno-stained with a monoclonal antibody that recognizes MHC type I isoform (NOQ7.5.4D, 1:16,000; Sigma, St. Louis, MO), and MHC type IIa (SC-71, 1:1200; generously provided by Dr. S. Schiaffino, Padva, Italy) as described elsewhere (Chin *et al*., 1998).

2D gel electrophoresis

Quadriceps from 30-day-old mice were dissected and immediately frozen in liquid N_2. Tissue powder was resuspended in 2D-lysis buffer (8 M urea, 2 M thiourea, 4% w/v CHAPS, 10 mM DTT and 1 mM PMSF). After sonication protein lysates were centrifuged for 30 min at 4°C and 50,000 rpm. Isoelectric focusing was performed with IPG strips (Amersham Biosciences, Uppsala, Sweden; 18 cm, pH 3–10L) according to the manufacturer's instructions, with the MultiphorII unit (Amersham Biosciences) employing the following voltage profiles: linear increase from 0 to 300 V for 30 min, 300 V for 30 min, increase from 300 to 500 V for 1 h, 500 V for 1 h, linear increase from 500 to 3500 V for 1.5 h, and a final phase of 3500 V for 4.5 h. After equilibration of the gels strips with DTT and iodoacetamide the second dimension was performed in polyacrylamide gels of 12.5% T and 2.6% C. The gels were then stained with silver nitrate (Bloom and Goodpasture, 1976) or with Colloidal Coomassie G 250 (Amersham). The gel images were analyzed with the software Delta2D (Decodon) as described previously (Bernhardt *et al*., 1999). Coomassie stained proteins were identified by MALDI/MS peptide mass fingerprinting at the Institute of Microbiology, EMAU, Greifswald, Germany (Eymann *et al*., 2004).

Results

Histology

The morphological correlate of muscle weakness in titin KO mice is the disassembly of the sarcomere, which is revealed at the light microscopy level. Fibers that lack titin's kinase region do not maintain their striated appearance (Figure 1A, arrows). The myopathic changes in skeletal muscle at 4 weeks of age range from ripples or vertices in striation to overt myopathy with loss of striation and centralized nuclei (Figure 1A b, c). Fibers are reduced in size when compared to control animals, in all skeletal muscle types investigated (quadriceps, diaphragm, and triceps surae). Overall, the diaphragm is less affected (as judged by a relatively normal striation pattern and peripherally located nuclei; Figure 1A e, f) than triceps surae and quadriceps muscle. The low magnification overview of KO triceps surae muscle shows a reduced and uneven fiber diameter with the majority of the nuclei centralized (Figure 1B).

Ultrastructure

Electron microscopy of KO animals revealed areas of intact sarcomeres as well as sarcomeres in various stages of disassembly. Unlike in the sarcomeres of titin deficient hearts, we could not detect progressive M-line widening with increased levels of mutant titin (Gotthardt *et al*., 2003). Three sarcomeres at different levels of disassembly show the progression of the structural rearrangements (Figure 1C, white arrow). While the left sarcomere (line 1) is largely intact, the right sarcomere (line 3) shows a pale M-line, and the central sarcomere (line 2) displays complete disassembly of the M-line. Major disassembly with filaments at various angles is present at the left. Only some residual Z-discs of various shapes (black areas) remain.

Correlation of titin kinase expression and myopathy

Expression of titin and proper splicing after the deletion of MEx1 and MEx2 is documented by RT-PCR and sequencing of the splice junction (data not shown) and *in situ* hybridization with probes that detect the region 5′, within, and 3′ of the floxed M-line exons (Figure 2A). In wildtype muscle all three exons are

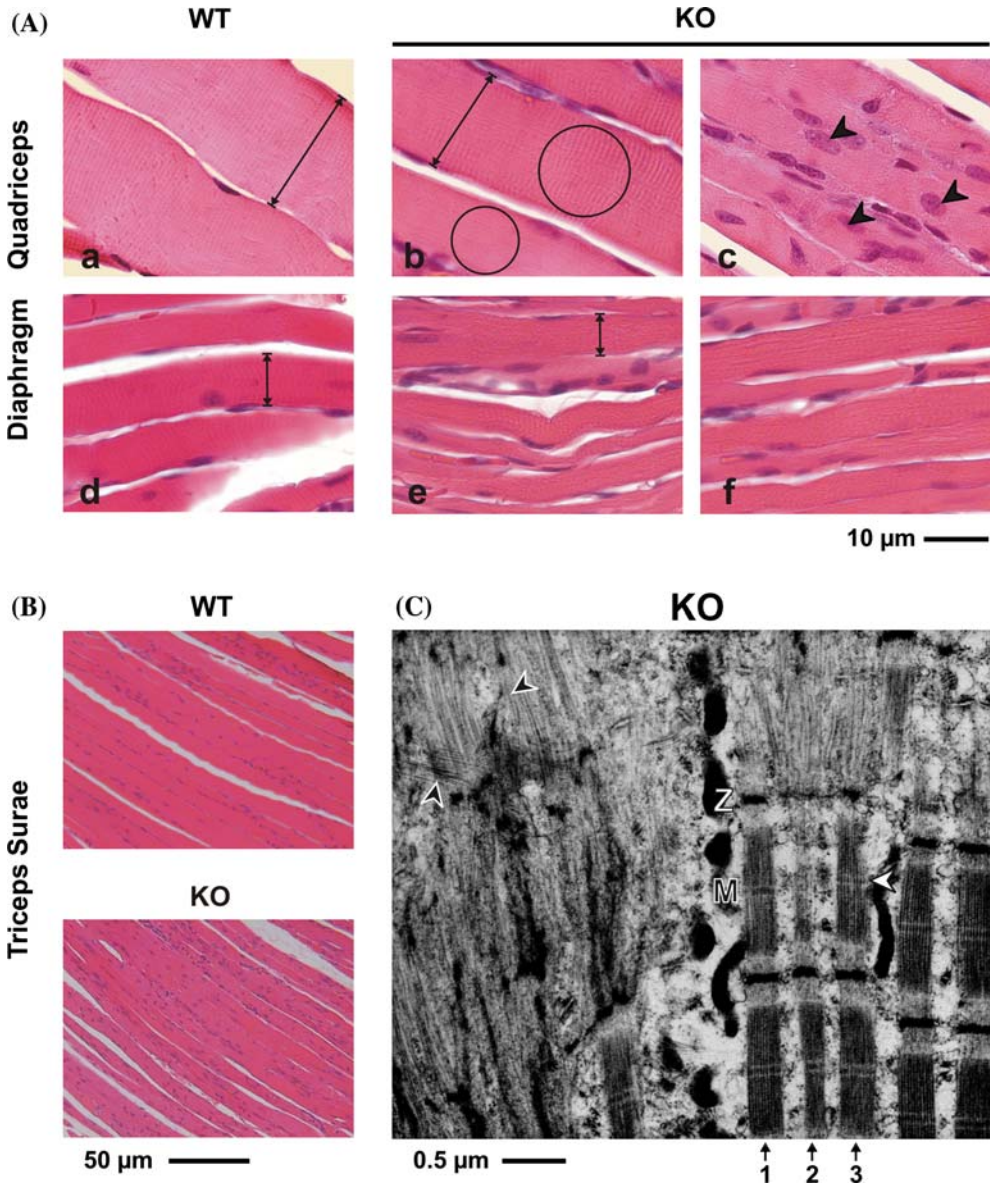

Fig. 1. Morphology of titin deficient striated muscle. (A) Hematoxylin and eosin staining of longitudinal sections from striated muscle (quadriceps a,b,c; diaphragm d,e,f). Knockout quadriceps at 4 weeks of age displays various stages of disease from atrophy with reduced fiber diameter (b vs. a and d vs. e – arrows) to overt myopathy with centralized nuclei (c, arrows). Disassembly of sarcomeres is revealed by loss of striation in individual muscle fiber segments (small circle, compared to striation in the unaffected area in the large circle). The diaphragm shows signs of atrophy, but overall a less severe phenotype than quadriceps muscle. (B) Lower magnification of triceps surae muscle shows the extend of myopathy reflected in the distribution of nuclei (peripheral vs. centralized). (C) Ultrastructure of knockout quadriceps. Sarcomere disassembly progresses from M-line (M) to Z-disc (Z) and ends in complete disarray with intersecting filaments (black arrows). Three lines of sarcomeres at various stages of disassembly are labeled 1–3.

expressed (Figure 2B). In knockout muscle only nuclei that are not recombined produce titin mRNA containing MEx1 (white dots in Figure 2B f). The Mex-1/2 flanking exons (pre-kinase and post-kinase) are expressed in knockout animals indicating proper splicing of the kinase region deficient mRNA. Compared to the wildtype sections, knockouts show increased levels of titin transcripts (compare Figure 2B a and e / c and g).

Titin expression

At the protein level, recombination is >95% complete in skeletal muscle of animals of 4 weeks of age (Figure 3). Different splice isoforms are present in skeletal muscle of WT animals (Figure 3A, left). In KO animals all isoforms are truncated (as suggested by their increased mobility) revealing that splicing does not seem to be affected in the kinase deficient animals.

Fiber typing

We used transversal sections of soleus and extensor digitorum longus (EDL) muscle as examples of muscles that are mainly composed of type 2 fibers

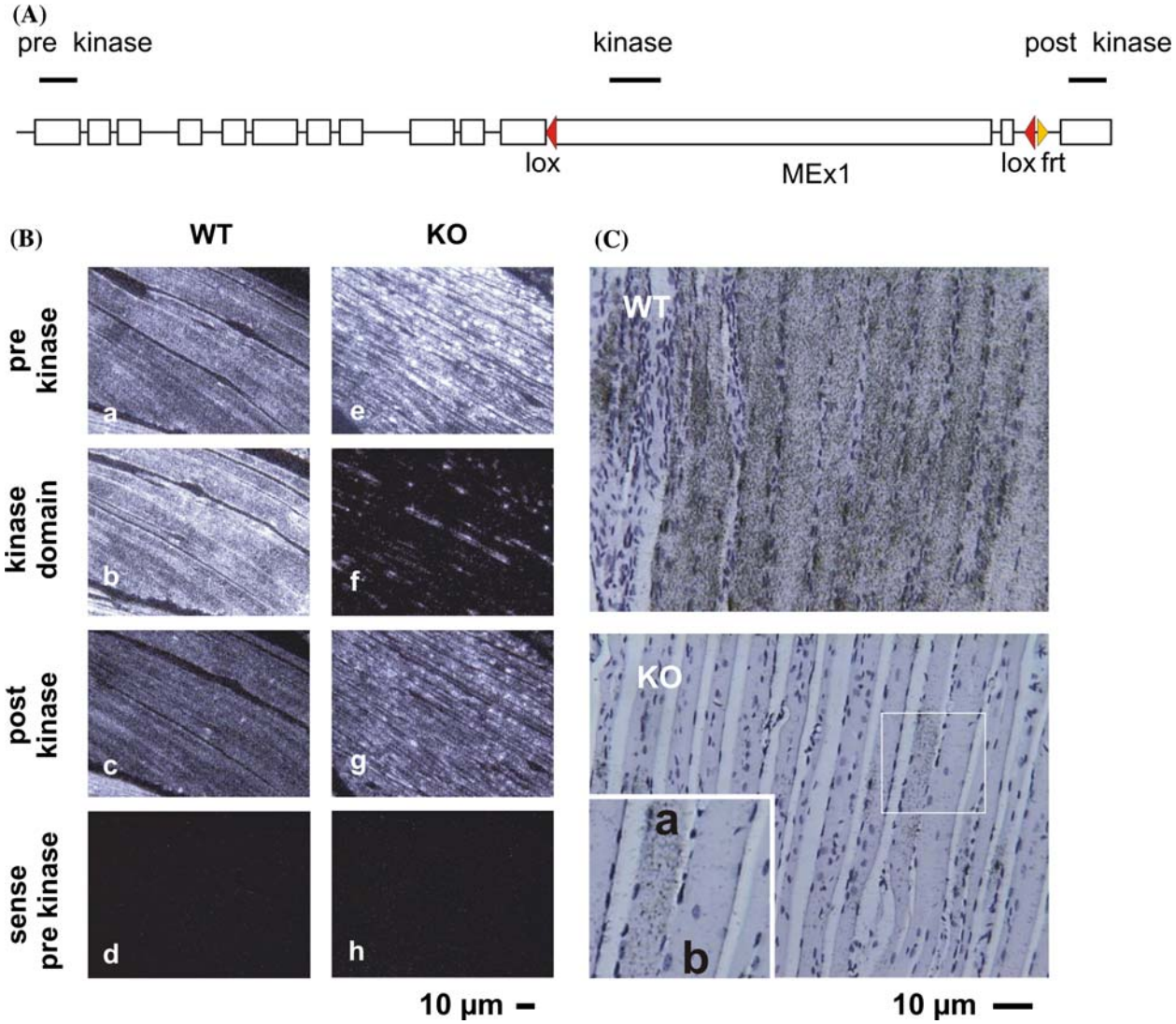

Fig. 2. Deletion of the titin kinase region. (A) Location of *in situ* probes before, in, and after the kinase domain, which is contained in M-line exon 1 (MEx1). (B) *In situ* hybridization (dark field) with S35 labeled antisense RNA probes demonstrated that in skeletal muscle the titin kinase region is deleted after cre-lox recombination (f), while the flanking exons (pre- and post-kinase) are expressed at increased levels compared with the wildtype (a vs. e and c vs. g). Only few unrecombined nuclei remain in the knockout (white spots in (f). (C) *In situ* hybridization – bright field. Fibers that still express the kinase domain (a) show normal localization of nuclei, while fibers that lack the kinase domain contain centralized nuclei, a sign for myopathy and regeneration.

(EDL) and type 1 and type 2 fibers (soleus muscle). With progressive loss of the titin kinase region, there is no obvious change in fiber composition but fiber size is generally reduced in the knockout animals (Figure 4).

Biochemical changes

After 30 days, muscle weakness is accompanied by altered expression of heat-shock proteins, components of the proteasome, and cytoskeletal proteins. Three knockout animals and three controls were compared by 2D gel analysis (Figure 5). Immobilin Dry Strips (Amersham biosciences) pH 3–10 were used for isoelectric focusing to separate ~600 proteins, of which 177 were identified by mass spectrometry. For comparison of knockout and wildtype tissue we used silver staining because of its higher sensitivity compared to Coomassie staining. Differentially regulated proteins were pseudocolored using the Decodon Delta2D software (blue – wildtype; orange – knockout; black – no change). We found consistent upregulation of the heat-shock proteins alpha-B crystalline, HSP27 (HSPB1) and cvHSP (HSPB7). The expression of proteasome subunits alpha type 1 and beta type 7 is another late change in development of the knockout phenotype visible at 30 days of age. These changes in protein expression have been associated with denerva-

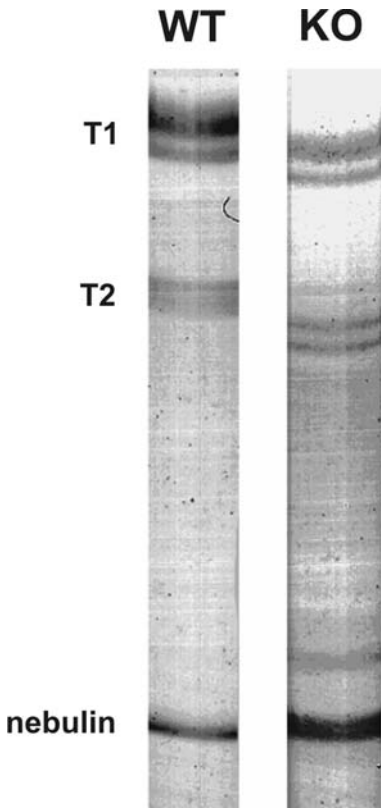

Fig. 3. SDS agarose gel electrophoresis of control and knockout muscle. Excision of the titin kinase region results in expression of titin with reduced mobility. In quadriceps of 29-day-old animals recombination is almost complete. Both full length titin (T1) and its degradation product (T2) show increased mobility in the knockout. The double band for T1 and T2, respectively represents different isoforms. After 4 weeks, wildtype protein is almost completely replaced by kinase deficient titin in the knockout animals.

tion, starvation, glucocorticoid, and disuse atrophy (Medina *et al.*, 1995; Auclair *et al.*, 1997; Stevenson *et al.*, 2003).

Discussion

Titin deficiency

The analysis of titin *in vivo* has mainly relied on differences in titin isoform expression between species, between muscle types, in embryonic development vs. adult tissue, and in health vs. disease (Trombitas *et al.*, 2000; Neagoe *et al.*, 2003; Warren *et al.*, 2003a; Lahmers *et al.*, 2004; Nagueh *et al.*, 2004). Added to this has the availability of naturally occurring mutants such as the MDM mouse (muscular dystrophy with myositis), mutants derived from genetic screens in drosophila or zebrafish, and the recently identified families with cardiomyopathy and muscular dystrophy due to titin deficiency (Machado and Andrew, 2000; van der Ven *et al.*, 2000; Garvey *et al.*, 2002; Gerull *et al.*, 2002; Hackman *et al.*, 2002; Xu *et al.*, 2002). The resulting phenotypes affect skeletal muscle alone (Tibial muscular dystrophy TMD; MDM mice), exclusively the heart (zebrafish mutants Pickwick$^{m171,\ m242,\ m740,\ m186}$, patients with cardiomyopathy due to titin mutations), or both (Pick^{mVO62H} mutation in zebrafish). Except for mutations affecting heart specific exons, such as the zebrafish Pickm^{171} mutant with a T to G transversion in the N2B exon it has been difficult to predict which type of muscle would be affected.

Myopathy in the titin kinase region mutants

Knockout technology has provided a means to target mutations to specific regions in the genome and generate additional model systems to study titin *in vivo* and in tissue culture cells (Gotthardt *et al.*, 2003; Miller *et al.*, 2003). To circumvent embryonic lethality, we have used the cre-lox recombination system to study titin's M-line region, which combines structural and

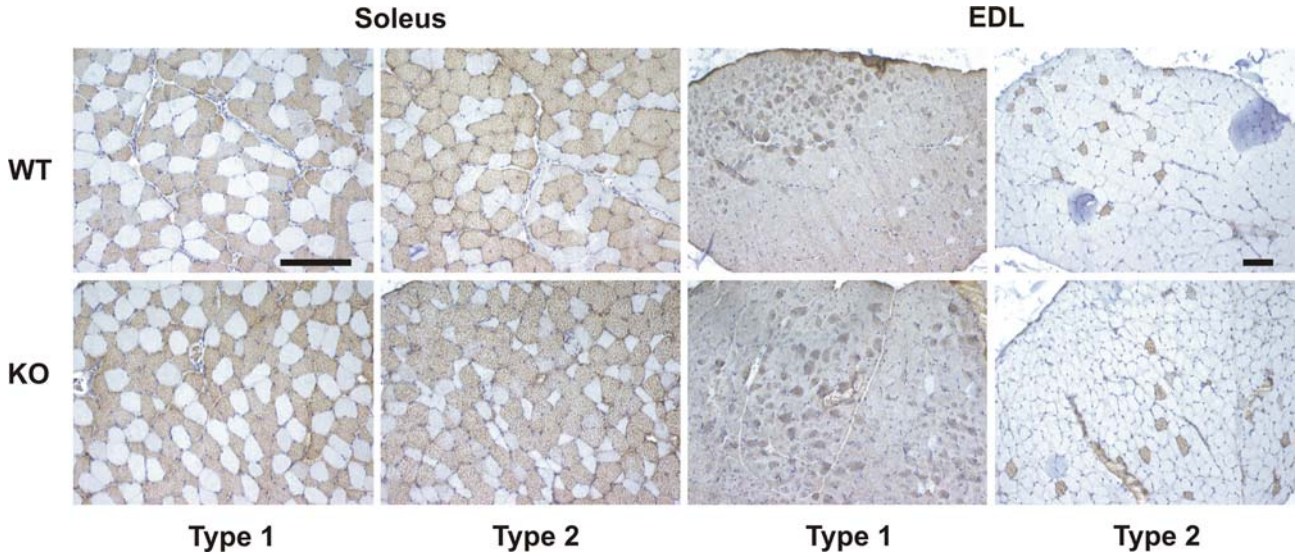

Fig. 4. Fiber typing. Immunostaining of striated muscle titin kinase region deficient mice and cre negative control mice at 4 weeks of age. Staining of soleus and extensor digitorum longus (EDL) muscles shows a reduced fiber size in the knockout animals, but essentially no differences in fiber composition between control and knockout mice. Size bar corresponds to 200 μm.

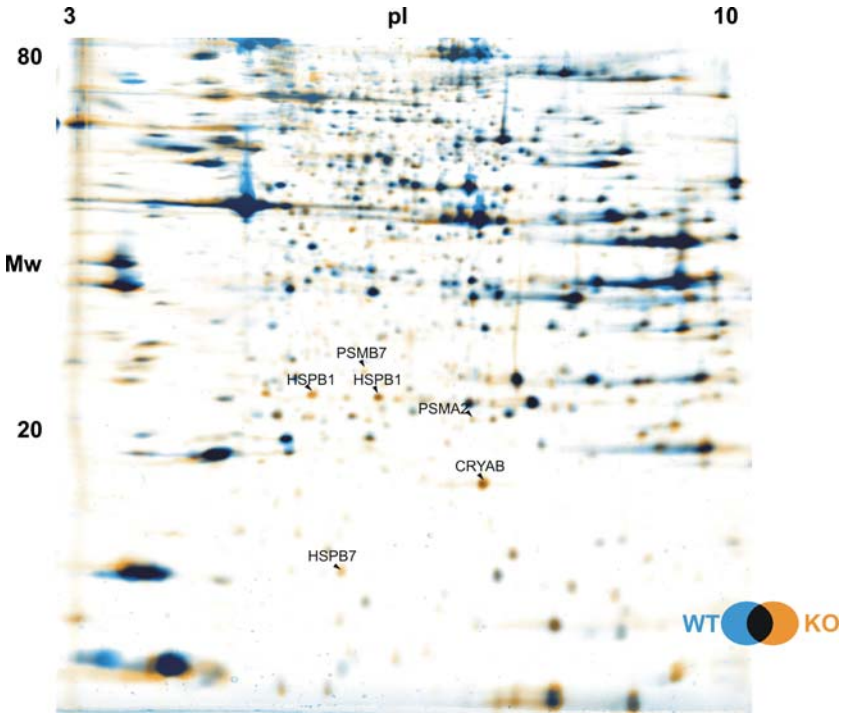

Fig. 5. Heat-shock response and signs of atrophy in the titin deficient muscle. Representative 2D gel ($n = 6$) comparing the wildtype (blue) and knockout (orange) protein expression. Among the identified proteins upregulated in the knockout are heat-shock proteins (HSPB1, HSPB7, alpha-B crystalline [CRYAB]), and proteasome subunits alpha type 2 and beta type 7 (PSMA2, PSMB7).

signaling functions. We have not been able to separate the two in the adult organism, since tissue specific knockout technology is confined to exon/intron boundaries. Thus, we cannot exclusively target the kinase region but produce a combined phenotype with deletion of signaling as well as adaptor domains.

Recombination of the kinase region advances over time so that wildtype titin is replaced by mutant titin. By *in situ* hybridization and sequencing of the splice junction we have verified that the MEx1 and -2 deficient mRNA is spliced properly, maintaining the M-line region, and that recombination is virtually complete at 4 weeks of age. The resulting myopathy with loss of striation in the histological sections and centralized nuclei in affected fibers leads to atrophy and death before the animals reach sexual maturity.

Sarcomere disassembly

Our titin M-line deficient animals assemble intact sarcomeres, which disintegrate upon deletion of the kinase region. In contrast to the heart, quadriceps muscle does not show widening of the M-line region with progressive replacement of wildtype with mutant titin (Gotthardt *et al*., 2003). This can be attributed either to differences in mechanical stress with the continuous beating heart vs. the less frequently used skeletal muscle or to differences in M-line structure: in contrast to skeletal muscle, cardiac muscle expresses the LIM-protein DRAL/FHL-2, which is not only involved in cardiac signal transduction (Purcell *et al*., 2004), but also coordinates metabolic enzymes at the M-line region (Lange *et al*., 2002) and is involved in myocyte differentiation (Martin *et al*., 2002).

Structural differences in skeletal muscle M-lines exist between fiber types, which are classified as type I (oxidative, slow) and type II (glycolytic, fast) and vary in metabolism, fatique, and force generation (Thomason and Booth, 1989; Olson and Williams, 2000). The EH-splice isoform of the M-line protein myomesin is expressed almost exclusively in type I fibers and the embryonic heart (Agarkova *et al*., 2004) while all M-bands in type II fibers contain M-protein (Grove *et al*., 1989). Skeletal muscle can switch fiber type i.e. in response to changes in motor nerve activity or calcineurin signaling (Schneider *et al*., 1999; Olson and Williams, 2000). To determine if the composition of the sarcomeric M-line affects the phenotype, we investigated if different fiber types would show altered phenotypical changes. In the titin kinase region knockout animals, there is atrophy in type I as well as in type II muscle fibers and we do not see major changes in fiber type (Figure 4). The uneven fiber diameter in atrophic muscle may reflect the kinetics of recombination, which results in different levels of deficient titin protein around individual nuclei.

In skeletal muscles disassembly of the sarcomere seems to proceed from M-line to Z-disc, which is maintained even in areas with loss of thick filament and disarray of thin filament structures (as seen in

Figure 1C). Thus, disassembly is a local event and depends on the titin M-line, which covers signaling and structural functions. The underlying mechanism might involve the titin binding protein myomesin, a structural M-line protein (Obermann *et al*., 1997), the titin kinase region, implied in the regulation of sarcomere assembly (Mayans *et al*., 1998), or the titin binding protein MURF-1, a ubiquitin ligase involved in atrophy (Dehoux *et al*., 2003).

Myomesin integrates titin into the sarcomeric M-line. The reduced stability of the M-line might be secondary to loss of the myomesin binding site, aggravated by mechanical strain. The relevance of the titin kinase domain for the myopathy phenotype is still unclear. While it has been implied in sarcomere assembly through its possible substrate T-cap/telethonin (Mayans *et al*., 1998), the titin kinase function could so far not be confirmed *in vivo*.

Heat-shock response and protein degradation after excision of the titin M-line

To investigate the mechanism underlying the myopathy phenotype in our M-line deficient animals, we used 2D-PAGE to investigate changes in protein expression.

The most striking changes were upregulation of heat-shock proteins such as alpha-B crystalline and HSPB1/HSPB7 as well as proteasome subunits. These features have been described in various atrophy models ranging from denervation, and disuse atrophy to starvation and glucocorticoid treatment (Medina *et al*., 1995; Auclair *et al*., 1997; Stevenson *et al*., 2003). In denervation atrophy abundant disused proteins are ubiquitinated resulting in both increased degradation (proteasomal activity) and aggregation with induction of heat-shock proteins (Kato *et al*., 2002). In our knockout animals changes in titin's M-line region would cause destabilization of the sarcomere and failure to properly assembly newly synthesized sarcomeric proteins. The fate of these proteins would be aggregation with induction of heat-shock proteins or degradation with increased proteasome activity.

It is tempting to speculate, that the mislocalization of the ubiquitin ligase MURF-1 in our knockout animals (Gotthardt *et al*., 2003) could contribute to the development of the phenotype. With deletion of M-line exons 1 and 2, the MURF-1 binding site N-terminal to the kinase region is excised resulting in mislocalization of MURF-1 as demonstrated by electron microscopy (Gotthardt *et al*., 2003). The increased level of "free" MURF-1 might result in increased ubiquitination, activation of the heat-shock response, and increased protein degradation.

In summary, multiple molecular changes in the M-line titin deficient animals affect the development and progression of the myopathy phenotype. These include decreased M-line stability, altered signal transduction, as well as increased protein degradation. Future experiments will have to dissect the respective contribution of structural and signaling functions to understand how sarcomere assembly, maintenance, and degradation are regulated.

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References

Agarkova I, Schoenauer R, Ehler E, Carlsson L, Carlsson E, Thornell LE and Perriard JC (2004) The molecular composition of the sarcomeric M-band correlates with muscle fiber type. *Eur J Cell Biol* **83:** 193–204.

Auclair D, Garrel DR, Chaouki ZA and Ferland LH (1997) Activation of the ubiquitin pathway in rat skeletal muscle by catabolic doses of glucocorticoids. *Am J Physiol* **272:** C1007–C1016.

Ayme-Southgate A, Southgate R, Saide J, Benian GM and Pardue ML (1995) Both synchronous and asynchronous muscle isoforms of projectin (the Drosophila bent locus product) contain functional kinase domains. *J Cell Biol* **128:** 393–403.

Benian GM, Kiff JE, Neckelmann N, Moerman DG and Waterston RH (1989) Sequence of an unusually large protein implicated in regulation of myosin activity in *C. elegans*. *Nature* **342:** 45–50.

Bernhardt J, Buttner K, Scharf C and Hecker M (1999) Dual channel imaging of two-dimensional electropherograms in *Bacillus subtilis*. *Electrophoresis* **20:** 2225–2240.

Bloom SE and Goodpasture C (1976) An improved technique for selective silver staining of nucleolar organizer regions in human chromosomes. *Hum Genet* **34:** 199–206.

Bodine SC, Latres E, Baumhueter S, Lai VK, Nunez L, Clarke BA, Poueymirou WT, Panaro FJ, Na E, Dharmarajan K, Pan ZQ, Valenzuela DM, DeChiara TM, Stitt TN, Yancopoulos GD and Glass DJ (2001) Identification of ubiquitin ligases required for skeletal muscle atrophy. *Science* **294:** 1704–1708.

Bruning JC, Michael MD, Winnay JN, Hayashi T, Horsch D, Accili D, Goodyear LJ and Kahn CR (1998) A muscle-specific insulin receptor knockout exhibits features of the metabolic syndrome of NIDDM without altering glucose tolerance. *Mol Cell* **2:** 559–569.

Centner T, Yano J, Kimura E, McElhinny AS, Pelin K, Witt CC, Bang ML, Trombitas K, Granzier H, Gregorio CC, Sorimachi H and Labeit S (2001) Identification of Muscle Specific Ring Finger Proteins as Potential Regulators of the Titin Kinase Domain. *J Mol Biol* **306:** 717–726.

Champagne MB, Edwards KA, Erickson HP and Kiehart DP (2000) Drosophila stretchin-MLCK is a novel member of the Titin/Myosin light chain kinase family. *J Mol Biol* **300:** 759–777.

Chin ER, Olson EN, Richardson JA, Yang Q, Humphries C, Shelton JM, Wu H, Zhu W, Bassel-Duby R and Williams RS (1998) A calcineurin-dependent transcriptional pathway controls skeletal muscle fiber type. *Genes Dev* **12:** 2499–2509.

Dehoux MJ, van Beneden RP, Fernandez-Celemin L, Lause PL and Thissen JP (2003) Induction of MafBx and Murf ubiquitin ligase mRNAs in rat skeletal muscle after LPS injection. *FEBS Lett* **544:** 214–217.

Eymann C, Dreisbach A, Albrecht D, Bernhardt J, Becher D, Gentner S, Tam LT , Buttner K, Buurman G, Scharf C, Venz S, Volker U

and Hecker M (2004) A comprehensive proteome map of growing *Bacillus subtilis* cells. *Proteomics*. **4:** 2849–2876.

Freiburg A, Trombitas K, Hell W, Cazorla O, Fougerousse F, Centner T, Kolmerer B, Witt C, Beckmann JS, Gregorio CC, Granzier H and Labeit S (2000) Series of exon-skipping events in the elastic spring region of titin as the structural basis for myofibrillar elastic diversity. *Circ Res* **86:** 1114–1121.

Garvey SM, Rajan C, Lerner AP, Frankel WN and Cox GA (2002) The muscular dystrophy with myositis (mdm) mouse mutation disrupts a skeletal muscle-specific domain of titin. *Genomics* **79:** 146–149.

Gerull B, Gramlich M, Atherton J, McNabb M, Trombitas K, Sasse-Klaassen S, Seidman JG, Seidman C, Granzier H, Labeit S, Frenneaux M and Thierfelder L (2002) Mutations of TTN, encoding the giant muscle filament titin, cause familial dilated cardiomyopathy. *Nat Genet* **30:** 201–204.

Gotthardt M, Hammer RE, Hubner N, Monti J, Witt CC, McNabb M, Richardson JA, Granzier H, Labeit S and Herz J (2003) Conditional expression of mutant M-line titins results in cardiomyopathy with altered sarcomere structure. *J Biol Chem* **278:** 6059–6065.

Gregorio CC, Trombitas K, Centner T, Kolmerer B, Stier G, Kunke K, Suzuki K, Obermayr F, Herrmann B, Granzier H, Sorimachi H and Labeit S (1998) The NH2 terminus of titin spans the Z-disc: its interaction with a novel 19-kD ligand (T-cap) is required for sarcomeric integrity. *J Cell Biol* **143:** 1013–1027.

Grove BK, Cerny L, Perriard JC, Eppenberger HM and Thornell LE (1989) Fiber type-specific distribution of M-band proteins in chicken muscle. *J Histochem Cytochem* **37:** 447–454.

Hackman P, Vihola A, Haravuori H, Marchand S, Sarparanta J, De Seze J, Labeit S, Witt C, Peltonen L, Richard I and Udd B (2002) Tibial muscular dystrophy is a titinopathy caused by mutations in TTN, the gene encoding the giant skeletal-muscle protein titin. *Am J Hum Genet* **71:** 492–500.

Kato K, Ito H, Kamei K, Iwamoto I and Inaguma Y (2002) Innervation-dependent phosphorylation and accumulation of alphaB-crystalline and Hsp27 as insoluble complexes in disused muscle. *FASEB J* **16:** 1432–1434.

Labeit S and Kolmerer B (1995) Titins: giant proteins in charge of muscle ultrastructure and elasticity. *Science* **270:** 293–296.

Lahmers S, Wu Y, Call DR, Labeit S and Granzier H (2004) Developmental control of titin isoform expression and passive stiffness in fetal and neonatal myocardium. *Circ Res* **94:** 505–513.

Lange S, Auerbach D, McLoughlin P, Perriard E, Schafer BW, Perriard JC and Ehler E (2002) Subcellular targeting of metabolic enzymes to titin in heart muscle may be mediated by DRAL/FHL-2. *J Cell Sci* **115:** 4925–4936.

Machado C and Andrew DJ (2000) D-Titin. A giant protein with dual roles in chromosomes and muscles. *J Cell Biol* **151:** 639–652.

Martin B, Schneider R, Janetzky S, Waibler Z, Pandur P, Kuhl M, Behrens J, von der MK, Starzinski-Powitz A and Wixler V (2002) The LIM-only protein FHL2 interacts with beta-catenin and promotes differentiation of mouse myoblasts. *J Cell Biol* **159:** 113–122.

Mayans O, van der Ven PF, Wilm M, Mues A, Young P, Furst DO, Wilmanns M and Gautel M (1998) Structural basis for activation of the titin kinase domain during myofibrillogenesis. *Nature* **395:** 863–869.

McElhinny AS, Kakinuma K, Sorimachi H, Labeit S and Gregorio CC (2002) Muscle-specific RING finger-1 interacts with titin to regulate sarcomeric M-line and thick filament structure and may have nuclear functions via its interaction with glucocorticoid modulatory element binding protein-1. *J Cell Biol* **157:** 125–136.

Medina R, Wing SS and Goldberg AL (1995) Increase in levels of polyubiquitin and proteasome mRNA in skeletal muscle during starvation and denervation atrophy. *Biochem J* **307:** 631–637.

Miller G, Musa H, Gautel M and Peckham M (2003) A targeted deletion of the C-terminal end of titin, including the titin kinase domain, impairs myofibrillogenesis. *J Cell Sci* **116:** 4811–4819.

Nagueh SF, Shah G, Wu Y, Torre-Amione G, King NM, Lahmers S, Witt CC, Becker K, Labeit S and Granzier HL (2004) Altered titin expression, myocardial stiffness, and left ventricular function in patients with dilated cardiomyopathy. *Circulation* **110:** 155–162.

Neagoe C, Opitz CA, Makarenko I and Linke WA (2003) Gigantic variety: expression patterns of titin isoforms in striated muscles and consequences for myofibrillar passive stiffness. *J Muscle Res Cell Motil* **24:** 175–189.

Obermann WM, Gautel M, Steiner F, van der Ven PF, Weber K and Furst DO (1996) The structure of the sarcomeric M band: localization of defined domains of myomesin, M-protein, and the 250-kD carboxy-terminal region of titin by immunoelectron microscopy. *J Cell Biol* **134:** 1441–1453.

Obermann WM, Gautel M, Weber K and Furst DO (1997) Molecular structure of the sarcomeric M band: mapping of titin and myosin binding domains in myomesin and the identification of a potential regulatory phosphorylation site in myomesin. *EMBO J* **16:** 211–220.

Olson EN and Williams RS (2000) Calcineurin signaling and muscle remodeling. *Cell* **101:** 689–692.

Olson NJ, Pearson RB, Needleman DS, Hurwitz MY, Kemp BE and Means AR (1990) Regulatory and structural motifs of chicken gizzard myosin light chain kinase. *Proc Natl Acad Sci USA* **87:** 2284–2288.

Purcell NH, Darwis D, Bueno OF, Muller JM, Schule R and Molkentin JD (2004) Extracellular signal-regulated kinase 2 interacts with and is negatively regulated by the LIM-only protein FHL2 in cardiomyocytes. *Mol Cell Biol* **24:** 1081–1095.

Schneider AG, Sultan KR and Pette D (1999) Muscle LIM protein: expressed in slow muscle and induced in fast muscle by enhanced contractile activity. *Am J Physiol* **276:** C900–C906.

Spencer JA, Eliazer S, Ilaria RLJ, Richardson JA and Olson EN (2000) Regulation of microtubule dynamics and myogenic differentiation by MURF, a striated muscle RING-finger protein. *J Cell Biol* **150:** 771–784.

Stevenson EJ, Giresi PG, Koncarevic A and Kandarian SC (2003) Global analysis of gene expression patterns during disuse atrophy in rat skeletal muscle. *J Physiol* **551:** 33–48.

Thomason DB and Booth FW (1989) Influence of performance on gene expression in skeletal muscle: effects of forced inactivity. *Adv Myochem* **2:** 79–82.

Trombitas K, Redkar A, Centner T, Wu Y, Labeit S and Granzier H (2000) Extensibility of isoforms of cardiac titin: variation in contour length of molecular subsegments provides a basis for cellular passive stiffness diversity. *Biophys J* **79:** 3226–3234.

Trommsdorff M, Gotthardt M, Hiesberger T, Shelton J, Stockinger W, Nimpf J, Hammer RE, Richardson JA and Herz J (1999) Reeler/disabled-like disruption of neuronal migration in knockout mice lacking the VLDL receptor and ApoE receptor 2. *Cell* **97:** 689–701.

van der Ven PF, Bartsch JW, Gautel M, Jockusch H and Furst DO (2000) A functional knock-out of titin results in defective myofibril assembly. *J Cell Sci* **113:** 1405–1414.

Wang K (1984) Cytoskeletal matrix in striated muscle: the role of titin, nebulin and intermediate filaments. *Adv Exp Med Biol* **170:** 285–305.

Warren CM, Jordan MC, Roos KP, Krzesinski PR and Greaser ML (2003a) Titin isoform expression in normal and hypertensive myocardium. *Cardiovasc Res* **59:** 86–94.

Warren CM, Krzesinski PR and Greaser ML (2003b) Vertical agarose gel electrophoresis and electroblotting of high-molecular-weight proteins. *Electrophoresis* **24:** 1695–1702.

Xu X, Meiler SE, Zhong TP, Mohideen M, Crossley DA, Burggren WW and Fishman MC (2002) Cardiomyopathy in zebrafish due to mutation in an alternatively spliced exon of titin. *Nat Genet* **30:** 205–209.

Young P, Ferguson C, Banuelos S and Gautel M (1998) Molecular structure of the sarcomeric Z-disk: two types of titin interactions lead to an asymmetrical sorting of alpha-actinin. *EMBO J* **17:** 1614–1624.