Conditional Expression of Mutant M-line Titins Results in Cardiomyopathy with Altered Sarcomere Structure*

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Michael Gotthardt‡‡§, Robert E. Hammer¶, Norbert Hübner¶, Jan Monti†, Christian C. Witt***, Mark McNabb‡‡§, James A. Richardson‡‡§, Henk Granzier‡‡§, Siegfried Labeit***, and Joachim Herz‡‡¶¶

From the Departments of ¶¶Molecular Genetics, ¶¶Biochemistry, and ¶¶Pathology, University of Texas, Southwestern Medical Center, Dallas, Texas 75390, Klinische Genetik von Herz Kreislauf-Erkrankungen, Max-Delbrück-Center for Molecular Medicine (MDC), D-13122 Berlin-Buch, Germany, the **Institut für Anästhesiologie und Operative Intensivmedizin, Universitätshilsklinikum D-68131 Mannheim, Germany, and the ***Department of Veterinary and Comparative Anatomy, Pharmacology, and Physiology, Washington State University, Pullman, Washington 99164

Titin is a giant protein responsible for muscle elasticity and provides a scaffold for several sarcomeric proteins, including the novel titin-binding protein in MURF-1, which binds near the titin M-line region. Another unique feature of titin is the presence of a serine/threonine kinase-like domain at the edge of the M-line region of the sarcomere, for which no physiological catalytic function has yet been shown. To investigate the role(s) of the titin M-line segment, we have conditionally deleted the exons MEx1 and MEx2 (encoding the kinase domain plus flanking sequences) at different stages of embryonic development. Our data demonstrate an important role for MEx1 and MEx2 in early cardiac development (embryonic lethality) as well as postnatally when disruption of M-line titin leads to muscle weakness and death at ~5 weeks of age. Myopathic changes include pale M-lines devoid of MURF-1, and gradual sarcomeric disassembly. The animal model presented here indicates a critical role for the M-line region of titin in maintaining the structural integrity of the sarcomere.

Titin is the third myofilament of vertebrate striated muscles and spans the half-sarcomere by integrating into the Z-disc and M-line through its amino and carboxyl terminus, respectively (1–6). Titin consists of subdomains that perform distinct functions (7, 8). Close to its carboxyl-terminal M-line region, titin contains a kinase domain that shares homology with the catalytic serine/threonine kinase domain of smooth muscle myosin light chain kinase (MLCK), and the invertebrate muscle protein twitchin, projectin, and stretchin-MLCK and Ce titin (9–14). Although a variety of different functions have been hypothesized for the titin kinase domain, its physiological substrate(s) remain unknown. Based on its crystal structure, Mayans et al. proposed that the titin kinase is under strong auto-inhibitory control with a dual activation mechanism that controls kinase activity (15). In vitro phosphorylation experiments using a constitutively active kinase fragment suggested that the kinase domain phosphorylates the sarcomeric protein telethonin/T-cap, a Z-line-associated protein. More recently, Centner et al. searched for proteins that specifically interact with the titin kinase region to identify potential titin substrate or regulator molecules (16). This demonstrated that the novel RING finger protein MURF-1 binds to the A168/A169 Fn3/Ig repeats of titin, which locate amino-terminal to the titin kinase domain at the periphery of the M-line lattice. MURF-1 together with its close homologues MURF-2 and MURF-3 form a distinct subgroup of the known RING finger proteins that has been implicated in the control of muscle degeneration, microtubule stabilization, M-line function, and transcriptional regulation (17, 18). Within the MURF family, only MURF-1 interacts with the A168/A169 Fn3/Ig repeats of titin, and it has been suggested that MURF-1 may participate in the regulation of titin kinase activity (16).

Recently, titin has been identified as the gene mutated in an autosomal dominant form of dilated cardiomyopathy (19, 20). The mutation results in a truncated titin, and patients that express the truncated protein suffer from heart failure of variable age of onset in the absence of clinically detectable skeletal muscle disease. In contrast, mutations within the carboxyl-terminal region of titin cause the distal type skeletal muscular dystrophy TMD without affecting the heart (21). Because these mutations occur in a constitutively expressed region of titin it is unclear why some of these mutations cause a disease specific to the heart muscle, whereas other mutations cause a skeletal-muscle-specific myopathy.

To investigate the physiological function(s) of the titin kinase domain, we have used a conditional knockout approach to selectively delete the M-line exons MEx1 (which encodes the kinase domain) and MEx2 (to maintain the reading frame) in heart and skeletal muscle. When exons MEx1/MEx2 are excised early in embryonic development (using the α-MHC promoter) mice die in utero. In contrast, excision during late embryonic development (using the MCK promoter) allows the mice to survive but causes development of a progressive myopathy, resulting in death at 5 weeks of age. Ultrastructural analysis indicates that sarcomeres incorporate the mutant MURF-1 protein.
tитins but subsequently disassemble. Gene expression profiling data suggest that a family of molecules involved in myocyte signaling become dysregulated after deletion of MEx1/MEx2, including members of the ankyrin repeat family. This raises the possibility that MEx1/MEx2 are involved in myofibrillar signaling and in maintaining the structural integrity of the contracting sarcomere.

EXPERIMENTAL PROCEDURES

**Targeted Deletion of the Titin Kinase Domain and M-line Titin**—A targeting construct was assembled by standard procedures from a mouse genomic BAC clone (bacterial artificial chromosome library MGS1 from mouse ES cells; Genome Systems/Incyte Genomics) spanning the 3’ region of the mouse titin gene (Fig. 1a). To preserve the open reading frame we flanked exon MEx1, which contains the kinase domain, and the neighboring downstream exon MEx2 with loxP sites. Briefly, a PCR-based strategy was used to introduce a neomycin expression cassette flanked by loxP- and FRT-sites into the intron 3’ of exon MEx2 (for nomenclature of M-line titin exons see Ref. 22). An additional loxP site was placed in the intron 5’ of MEx1. The targeting vector was verified by sequencing.

Homologous recombination with this targeting vector occurred in 10% of all G418-resistant ES cell colonies. ~30% of the clones also contained the loxP site 5’ to MEx1. These clones were used to derive chimeric animals as described previously (23). The intronic neomycin cassette has the potential to affect the phenotype of knockout animals (24). Therefore, we mated heterozygous animals that contained the altered titin locus to transgenic mice that expressed the Flp recombinase in their germline (25). Offspring from this mating in which the neomycin cassette had been removed by Flp-mediated excision was used to generate a colony of homozygous mice that contained the targeted allele.

**Genotyping (PCR and Southern Blotting)**—Genomic DNA from embryonic and postnatal mouse tails or yolk sacs was genotyped as described (Fig. 1 and Refs. 26, 27). Primers were designed to detect homozygous animals, presence of the 5’ loxP site, and Flp- and Cre-mediated recombination (Fig. 1a). For Southern genotyping, genomic DNA was digested with HindIII and probed with a SmaI fragment 3’ of exon MEx2 (Fig. 1a) according to standard procedures (26).

**Animal Procedures (Breeding and Maintenance)**—Timed matings and harvesting of embryos were performed as follows: female mice were superovulated using 10 IU intraperitoneal pregnant mare’s serum (Sigma), followed 48 h later by 10 IU intraperitoneal human chorionic gonadotropin (Sigma) and mating to corresponding males. The morning of detection of a vaginal plug was regarded as day 0.5 postconception.

![Figure 1](image-url)

**Fig. 1. Generation of tissue-specific knockout mice.** Analysis was done on tail DNA. a, protein structure of the region designated for excision and outline of the targeting vector including restriction sites used for linearization and genotyping (HindIII, H; NotI, N). Exons are indicated in white, 357–363 above refers to the numbers of the titin exons in the human gene sequence (42); the probe for Southern blot as a black box, the neomycin cassette is depicted in gray. LoxP and FRT sites are represented by filled and empty triangles, respectively. The binding sites for MURF-1 and p94 are indicated with filled arrows. Different mutant alleles after targeting and successive recombination with the Cre and Flp recombinase are shown. Immunoglobulin domain, ig; fibronectin 3 domain, fn3. The location of primers used in genotyping is indicated on the targeted allele. b, Southern blot analysis of titin knockout animals after digestion with HindIII. T is the fragment produced by the targeted allele, WT by the wild-type allele. c, PCR genotyping of a litter derived from heterozygous parents containing the targeted allele (T) using primers PL1 and PL2. d, PCR-based verification of Flp/FRT recombination. Offspring from a Flp transgenic father and a heterozygous-targeted titin mouse (T/+). e, PCR-based verification of Cre/loxP recombination. Offspring from a Cre deleter mouse (CAGcre) mated with homozygous floxed titin mice. CRE, PCR product corresponding to the Cre transgene; REC (primers PL1 and PL4), recombinant titin fragment.
Embryos and resorbtion bodies were harvested at E15. Transgenic mice were generously provided by Susan Dymecki (Flp deleter) (25), Jun-Ichi Miyazaki (CAGcre) (28), Michael Schneider (MHCcre) (29), and Ronald Kuhn (MCKcre) (30). ROSA-26 β-galactosidase reporter mice were obtained from the Jackson Laboratories (www.jax.org). All animal experiments were carried out under protocols approved by the University of Texas Southwestern Institutional Review Committee for animal use and followed the National Institutes of Health guidelines, “Using Animals in Intramural Research.”

In Situ Hybridization—Templates to generate in situ hybridization probes were amplified from genomic DNA and included the kinase region (kin) and the exon MEx5 3′ of the kinase domain, respectively. PCR fragments were subcloned using the TA cloning kit (Invitrogen), sequenced, linearized with HindIII (kin) or BamHI (MEx5), and transcribed with T7 polymerase using the Ambion transcription kit. Immunostaining was performed using the “Stain for β-Galactosidase Expression” (Speciality Media (www.specialitymedia.com)) according to the manufacturer’s instructions.

Immunoelectron Microscopy—Skinned heart muscle strips were stretched, fixed, immunolabeled with an antibody directed against the titin M-line region (see immunoblot analysis, Ref. 16) or against the main immunogenic region of titin (32), embedded, and processed for immunoelectron microscopy as described previously (33).

PAGE and Immunoblot Analysis—Cells and tissues designated for protein preparation were snap-frozen, pulverized, and quickly solubilized with 9 volumes of hot 1.1× solubilization buffer (50 mM Tris-Cl, 2% SDS, 10% glycerol, 80 mM dithiothreitol, 30 μg/ml pyronin Y, pH 6.8 at 25 °C) (34). Titin protein gels and Western blots were performed and analyzed as described (35) using rabbit polyclonal antibodies specific to Z1/Z2, A169/A170 (MEx1), M7/M8/M9 (MEx3 + 4) (32), CARP, and ankrd2 (36, 40).

RESULTS

Generation and Characterization of Titin Kinase-deficient Mice—To allow conditional tissue-specific excision of the titin kinase region we have used homologous recombination in ES cells to introduce loxP and FRT recombination sites into introns flanking the kinase (Fig. 1a). To maintain an uninterrupted open reading frame, exon MEx1, which encodes the kinase domain, and the neighboring downstream exon MEx2 were both designated to be deleted. Introduction of loxP sites into the gene did not interfere with normal mRNA transcription and had no discernable phenotypic effect in mice that were homozygous for the floxed allele. The targeting frequency was 1:10 with one-third of the targeting events including the lox site 3′ of MEx1 (data not shown). Chimeric mice derived from targeted ES cells were mated to Fp transgenic mice to remove the intronic neomycin expression cassette and to avoid any potential adverse physiological effects (24). Offspring in which the neomycin cassette had been deleted was used to derive a colony of homozygous mice (RECf/+/+) that only contained the loxP sequences in the two introns flanking MEx1 and MEx2 and one residual FRT site in the 3′ intron (Fig. 1a).

All animals analyzed were 4 weeks of age, contain one allele of the rosa26–lacZ reporter construct in heart and large arteries of the lung (Fig. 2, a–f). Deletion of the kinase domain from cardiac titin during development (by the MHCcre transgene) led to early embryonic lethality. Resorption bodies from knockout animals

The Cardiac Titin Kinase Domain Is Essential for Embryonic Development—Deletion of the kinase domain from cardiac titin led to early embryonic lethality. Resorption bodies from knockout animals
were isolated at embryonic day 15 (Fig. 3a). No homozygous offspring was obtained, indicating a critical role for titin in heart development.

**FIG. 3. Phenotypes of the heart and striated muscle-specific titin kinase knockout mice.** a, litter of embryos at E15 with two resorption bodies out of 10 offspring from a MHCcre+ RECF+/− x RECF+/+ mating. b, at 4 weeks, the striated muscle-specific knockout mouse (right) is smaller than its littermate (left), does not groom, and cannot open its eyes. c, PCR genotyping of animals shown in a. The resorption bodies (8 and 9) contain the Cre transgene (Cre) and are homozygous for the RECF allele. C, wild-type control; d, weight curve of the striated muscle-specific knockout animals. Wild-type (MCKcre−, n = 9) and heterozygous animals (MCKcre+ RECF+/−, n = 3) show normal weight gain, while knockout animals (MCKcre+ RECF+/+, n = 5) fail to thrive and deteriorate at 4 weeks of age.

**FIG. 4. Histological comparison of MCKcre RECF+/+ knockout mice.** a, light microscopy of hematoxylin- and eosin-stained cardiac sections reveals reduced ventricular size in knockout animals but no apparent pathology. b, in situ hybridization confirms that the titin kinase domain message is reduced in cardiac ventricles. While exon MEx5, located 3’ of the kinase domain, is expressed at similar levels in wild-type and knockout animals (panels C and D; G and H at higher magnification), the mRNA level for the exon encoding the kinase domain is significantly reduced in kinase knockout mice (panel A compared with B; G and F at higher magnification). Higher magnification reveals that individual nuclei still express titin mRNA containing the kinase region (panel F).

A Syndrome of Severe Muscular Weakness in the Titin Kinase-deficient Knockout Animals—In contrast to the MHCcre animals expressing the Cre recombinase from the early stages during development, the skeletal muscle-specific MCKcre transgene allowed generation of live-born mice that expressed the recombinase and that were homozygous for the floxed allele at the expected Mendelian ratios (data not shown). These animals, in which deletion of the titin kinase domain in skeletal muscle was virtually quantitative (see below), developed a severe syndrome characterized by progressive muscle weakness, which affected their posture and gait and caused ptosis of the eyelids (movie available at titin.mdc-berlin.de/titinmovie.html; Fig. 3b). Failure to thrive was usually apparent at 3 weeks of age, with progression of the phenotype until death at ~5 weeks of age (Fig. 3d). During autopsies, the heart of titin kinase-deficient animals appeared small but in proportion when compared with body size (results not shown). Light microscopy did not show evidence for necrosis or myositis (Fig. 4a).

To verify that this phenotype was due to the selective loss of the targeted titin M-line segment and not caused by aberrant splicing of the transcript, which might introduce additional defects in the carboxyl-terminal end of the molecule, we confirmed by *in situ* hybridization that exons 3′ of the deleted kinase region were transcribed at normal levels (Fig. 4b). Furthermore, reverse transcribed-PCR across the new splice junction confirmed removal of MEx1/MEx2 and maintenance of the open reading frame when joining A170 to MEx3 (data not shown). *In situ* hybridization demonstrated that by 4 weeks of age, wild-type titin message was found in only a few isolated fibers (Fig. 4b, panel F), whereas mutant titin protein had almost completely replaced full-length titin in heart muscle (Fig. 5, b and c). Western blots with MEx1- and MEx3/MEx4-specific antibodies and quantification by densitometry demonstrated that >80% of the expressed titin was deficient in its kinase region. The presence of the carboxyl terminus in the recombinant titin protein was confirmed by Western blotting with antibodies directed against the kinase domain or against epitopes located at the amino- and carboxyl terminus of titin (Fig. 5, a and d).

**Structural Analysis of Sarcomeres with Mutant Titins—**Electron microscopy of RECF+/+ control animals revealed normal sarcomeres with well resolved M-line bridges (see arrow in Fig. 6a, panel A). Myocytes of 4-week-old KO animals (MCKcre RECF+/+) contained regions with sarcomeres with relatively normal structure (>50% of the area of the cells) as well as
irregular regions with sarcomeres in various stages of disarray. Sarcomeres of KO animals had typically widened and pale M-lines that were devoid of M-line bridges (arrows in Fig. 6a, panels B and C). When sarcomeres were labeled with anti-M7-M9 (MEx3/MEx4) M-line epitopes were obtained that were ~100 nm apart in control animals (Fig. 6b, panel A), but in the KO animals this distance had significantly increased (Fig. 6b, panel B, and 6c). Interestingly, the epitope separation appeared to correlate with the degree of disassembly of the sarcomere (see Fig. 6c, myofibril with ends labeled with arrows). We also used an antibody against MURF-1 and found M-line labeling in control animals (Fig. 6d, panel A) but absence of M-line labeling in KO animals (Fig. 6d, panel B). Because in vitro studies have shown that MURF-1 binds to the A169/A170 domains that are encoded by MEx1 (16), absence of MURF-1 supports the notion that MEx1 has been excised from sarcomeres in KO animals, whereas labeling with M7-M9 indicates that the titin carboxyl terminus is present.

Fig. 5. Characterization of the truncated titin protein and up-regulation of CARP expression. a, location of the epitopes recognized by the anti-titin antibodies used for Western blotting. Below the protein domain structure, Z-disc and M-line domains are magnified to show antibody binding sites (Y) as well as binding sites for MURF-1, myomesin, and calpain 3 (C3). b, SDS-PAGE analysis of myocardium reveals that in knockout animals (KO) titin has a higher mobility than in littermate controls (C), consistent with quantitative excision of the titin kinase domain. c, magnification of the upper region of the gel shown in b. Note that there is no visual difference in the total amount of titin protein in KO compared with C. d, Western blot analysis of titin knockout (KO) and littermate control (C) animals with antibodies directed against epitopes amino-terminal, carboxyl-terminal, and within the kinase domain. The titin kinase domain is removed in knockout animals, while the carboxyl-terminal M-line region is still produced. Densitometry reveals that the titin kinase region is reduced in the knockout heart by a factor of 5.5 compared with the carboxyl-terminal region M7-M9. India ink stain, Ink; Z-line titin, Z1-Z2; titin kinase domain, kinase; M-line titin, M7-M9. e, Western blot analysis of titin knockout (KO) and littermate control animals (C) animals demonstrates the up-regulation of CARP and ankrd2 in knockout animals, consistent with their regulation on transcription level (data not shown).

DISCUSSION

Rationale for the Animal Model—Impaired titin function most likely cannot be substituted by another gene, since no other gene coding for a protein of similar size and complexity is known. Therefore, we expected that mice after conventional gene targeting of the titin locus would die during early embryonic development, precluding studies of the role of titin in muscle. Accordingly we introduced an intragenic deletion in a specific region of titin using a conditional gene-targeting strategy. We anticipated that the deletion of the MEx1 exon might provide insights into structural and regulatory roles of titin because MEx1 codes for the 200-kDa M-line portion of titin, which is highly conserved between species and contains a serine/threonine kinase domain and a binding site for the RING finger protein MURF-1.

We addressed the biological roles of the titin kinase in the heart and in skeletal muscle at different stages of embryonic and postnatal development by using transgenic animals that
express Cre recombinase under control of the well characterized muscle-specific promoters α-MHC and MCK. These transgenic animals used to derive the MEx1–2 knockouts have been described by others (29, 30) but have not been compared side by side. We confirmed their tissue distribution (Fig. 2) with more generalized muscular expression of the MCK promoter. In rat, expression of MCK begins at embryonic day 17, rises to 40% of maximum activity by birth, and plateaus at day 10 of life (37). We hypothesized that the delayed activity of the MCKcre system may allow homozygous mice to survive through early postnatal stages, whereas Cre expression driven by the α-MHC promoter may lead to early developmental recombination with potential embryonic and perinatal effects (38). These expectations were supported by the stronger β-galactosidase staining in α-MHCcre hearts versus hearts from MCKcre animals (Fig. 2, a and f) and by the early embryonic lethality of the α-MHCre RECf/+ mice.

Functional Implications of the Titin Truncation—The expected mutant titin transcripts were detected by reverse transcribed-PCR in which the exons 357 (coding for A167/A168 located in the periphery of the M-line) and exon 360 (MEx3 coding for M8) were joined in-frame. Thus, at the protein level a 214-kDa segment coding for most of the M-line portion of titin was deleted, including the binding site for the RING finger protein MURF-1 and the serine-threonine kinase domain of titin. Consistent with this, when analyzing the myofibrillar proteins on low percentage protein gels, titin from the mutant mice showed an increased mobility on SDS gels. The levels of myofibrillar titin in the mutant and wild-type mice were comparable (Fig. 5b). Therefore, the mutant titin transcribed from the exon 358/359-deficient gene locus is correctly spliced, translated, and incorporated into myofibrils. The strategy applied here for the removal of the titin exons 358/359 is applicable for the deletion of other regions of interest in titin, while keeping the remainder of this giant molecule intact.

The most obvious phenotype of our mice is their progressive muscle wasting, leading to a pronounced weakness, apparent for example by ptosis of both eyes (Fig. 3b), inability to climb, and progressively impaired gait (compare at titin.mdc-berlin.de/titinmovie.html). Muscle weakness is evident at 2–3 weeks of age, before the mutant mice become dystrophic at around 4 weeks of age. This suggests that the muscle weakness it not a secondary effect of e.g. reduced cardiac output.

The sarcomeres containing mutant titin appeared to be unstable (Fig. 6). Within the same region of the myocyte, sarcomeric structure varied from relatively normal sarcomeres with pale M-lines devoid of M-bridges (Fig. 6b, panel B) to nearly fully disassembled sarcomeres (Fig. 6c, left). Interestingly, the C-terminal M7-M9 antibody labels two epitopes in the center of the A-band, separated by ~100 nm in the control but up to ~200 nm in the KO animals (Fig. 6c). The separation in control sarcomeres is consistent with a model in which titins from opposite half-sarcomeres overlap by ~100 nm within M-lines (39). The reduced size of mutant titin (~200 kDa) suggests that titin molecules do not overlap, but instead stop short of the center of the A-band. Absence of titin from the center of the
A-band, a region of the sarcomere under high systolic stress, is likely to compromise the mechanical strength of the sarcomere, and this may contribute to sarcomeric disassembly.

Changes in Muscle-specific Gene Expression in Kinase-deficient Animals—Our mouse model will allow future studies on the physiological role(s) of the titin kinase domain and its potential in vivo substrate(s). Interestingly, the potential candidate substrates for the titin kinase, Trapezelonin and MURF-1 (15, 16), were not significantly affected in our animal model as verified both by TagMan and Western blot analysis (data not shown). In contrast, transcripts for CARP (CARP-1) and its homolog ankrd2 (CARP-2) were both significantly up-regulated, and Western blotting with specific antibodies confirmed elevated CARP/ankrd2 protein levels in the titin kinase-deficient mice (Fig. 5e). CARP epitopes have been shown to co-localize with N2A titin and myopalladin epitopes during myocardial injury (20). CARP epitopes also co-localize with N2A titin and myopalladin epitopes during the early phases of this study and to Jim Stull for critical reading of the manuscript and suggestions.

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Conditional Expression of Mutant M-line Titins Results in Cardiomyopathy with Altered Sarcomere Structure

Michael Gotthardt, Robert E. Hammer, Norbert Hübner, Jan Monti, Christian C. Witt, Mark McNabb, James A. Richardson, Henk Granzier, Siegfried Labeit and Joachim Herz

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