4 Results

Titin’s M-line region has been proposed to be critical for the structure of the sarcomere and its maintenance during force generation. Titin also acts as a blueprint for sarcomere assembly by providing binding sites for structural and signaling proteins. To distinguish a role in sarcomere assembly from a role in stabilizing preexisting sarcomeres and to address potential non-muscle functions, the conditional M-line Titin knockout (Gotthardt et al., 2003) was converted into a constitutive knockout using germline recombination. Homozygous deletion of Titin’s M-line exon 1 and 2 will be referred to as M-line knockout. The conditional Titin M-line knockout already indicated a critical role of M-line Titin in maintenance of sarcomere structure and function in the adult skeletal and cardiac muscle (Gotthardt et al., 2003). Thus, excision of Titin’s M-line exon 1 and 2 using Cre recombinase under the control of the protamine promoter was expected to generate an early phenotype.

The first chapter will characterize the constitutive knockout histologically and morphologically. The physiological relevance of Titin’s M-line region, its kinase domain and interacting proteins will be focused in the second chapter. The expression of Titin and its binding proteins in wildtype and knockout animals was investigated by cell culture, immunostaining, ultrastructural, apoptosis, and RT-PCR studies. The analysis of the M-line knockout will reveal whether sarcomere assembly and expression of muscle proteins or rather stabilizing preexisting sarcomeres depend on Titin’s M-line. Thus, it will help to better understand the molecular mechanism of myofibrillogenesis.
4.1 Phenotype of the Titin M-line knockout model

The Cre-lox recombination system was used to excise Titin’s M-line exons 1 and 2 in striated muscle cells conditionally (Gotthardt et al., 2003). These animals were viable but showed muscle weakness, myopathy, and died at 5 weeks of age. The conditional knockout approach generated adult animals to study Titin’s function in the mature heart and skeletal muscle. Nevertheless, the expression kinetics of the cre recombinase transgene precluded the analysis of Titin’s role in sarcomere assembly during embryonic development. To distinguish a role in sarcomere assembly from a role in stabilizing preexisting sarcomeres and to address potential non-muscle functions, the conditional knockout model was converted into a constitutive knockout using germline recombination.

4.1.1 Generating a constitutive mouse model

The conditional mouse model lacking Titin’s M-line region (Gotthardt et al., 2003) was converted into a constitutive mouse model. Therefore, the transgenic mice with loxP sites flanking Titin’s M-line exons 1 and 2 (Fig. 4.1, A) were crossed with transgenic mice expressing Cre under the control of the protamine promoter (The Jackson Laboratory) (Fig. 4.1, B, lane 1 and 2).

Figure 4.1: Strategy to generate a constitutive mouse model. (A) Outline of the exon/intron structure of Titin’s M-line region and location of the genotyping primers (PL1, 2, and 4). The loxP sites flanked MEx1 and 2. The allele was designated Recf and the deletion was mediated by the Cre recombinase. (B) PCR-based genotyping of a protamine-Cre transgenic mouse (1) and Recf mouse (2) that were mated to obtain double heterozygotes (3). After germline recombination, offspring contained only the recombined allele (4).
After recombination the truncated Titin allele was transmitted through the germline and caused expression of a Titin protein deficient in M-line exon 1 and 2. Male double heterozygotes (Fig. 4.1, B, lane 3) were backcrossed with 129/SvEms-+Ter?/J to obtain a colony of heterozygous knockouts devoid of the cre transgene (Fig. 4.1, B, lane 4). The heterozygous offspring was healthy and fertile. However, matings between heterozygotes produced no viable homozygous Titin M-line deficient mice, implying that the knockout embryos died during embryogenesis. For genotyping template DNA was prepared from yolk sac or tail and recombination of the Titin locus was monitored by PCR using the PL1 and PL4 primers (Fig. 4.1, A). Lox and wildtype loci were typed using primers PL1 and PL2. All primers have been described previously (Gotthardt et al., 2003).

In order to characterize the development of knockout embryos and determine the time of death, timed matings were set up between heterozygous animals. Timed matings required the presence of sperm following overnight matings. The morning of detection of a vaginal plug was regarded as day 0.5 post conception (E0.5). Pregnant mice were sacrificed according to German animal protection law. Embryos were dissected at various stages of gestation. The age was determined according to their number of somites. PCR genotyping of yolk sac DNA revealed the presence of homozygous knockout embryos that fit to a Mendelian ratio until embryonic day 11.5 (Fig. 4.2, A and B). From E12.0, the number of knockout embryos declined to zero. Genotyping embryonic tissue from resorption bodies identified the homozygous deletion of Titin’s M-line.

![Figure 4.2: PCR analysis of a litter derived from a heterozygous mating](image)

(A) PCR-based analysis of embryos at E9.5 confirmed early embryonic survival of homozygous knockouts (5, 7, 10) and a Mendelian distribution of genotypes within one litter. (B) PCR genotyping confirmed the expected Mendelian ratio that was consistent up to E11.5 of embryogenesis. However, there was no homozygous knockout during late development.
4.1.2 Titin antibodies, riboprobes, and amplicons

Titin extends through half the sarcomere from the Z-disc to the M-band being more than 1 \( \mu \text{m} \) long. Investigating full length Titin it was necessary to focus on different regions (Fig. 4.3). The expression levels of Titin isoforms were monitored by quantitative RT-PCR. To distinguish between isoforms, primers and probes were designed to amplify RNA of distinct regions: exon 1 and 2 within the Z-disc, the N2B region, and exon 6 within the M-line region (see black bars in Fig. 4.3).

![Figure 4.3: Location of antibodies, riboprobes, and amplicons.](image)

The schema shows the cardiac specific N2B isoform (red, Ig domains and unique sequences; black, kinase; yellow, PEVK; blue, N2B; white, FN3/Ig domain). Red bars indicate the location of riboprobes and black bars show the region that was amplified by RT-PCR. Antibodies are mapped at the top.

To localize Titin expression by *in situ* hybridization two regions were chosen. An antisense probe recognized RNA expression of the 3’ region of the kinase domain (referred to as prekinase). The second antisense probe hybridized to the kinase domain (referred to as kinase) confirming the recombination event of the loxP sites (see red bars). In order to follow incorporation of Titin into the sarcomere by immunostaining the anti-Titin N2B region (Trombitas et al., 1999) and anti-M8/M9 (Centner et al., 2000) antibodies were applied (see flipped Y in Fig. 4.3).

4.1.3 Knockout embryos expressed the truncated Titin protein

The targeted deletion of Titin’s M-line exon 1 and 2 was confirmed by vertical agarose gel electrophoresis (VAGE) of wildtype, heterozygous, and knockout heart lysates. The electrophoretic separation of high-molecular-weight proteins (> 500 kDa) using polyacrylamide is difficult because gels with a sufficient pore size for adequate protein mobility are mechanically unstable (Warren et al., 2003). The 1% VAGE system gels were stained with SYPRO® Ruby that demonstrated greater sensitivity than Coomassie™ blue stained gels (Lopez et al., 2000). The knockout protein was expected to be truncated by about 200 kDa. Truncated Titin proteins of the predicted sizes were expressed in heterozygous and knockout embryos and in heterozygous adult mice (Fig. 4.4). Since embryonic Titin is expressed as a larger isoform that prevent separation, differences in migration were more prevalent in the T2 Titin isoform. T2 Titin is the degradation product of the native T1 Titin. This
4.1 Phenotype of the Titin M-line knockout model

degradation occurs during sample preparation for Titin agarose gel electrophoresis. The reduced size of T2 facilitated a separation. However, N2BA Titin is the largest isoform and has a size of 3.7 MDa. It is predominately expressed during embryonic development but rapidly disappears after birth and is replaced by a small N2B isoform (3.0 MDa) (Lahmers et al., 2004; Opitz et al., 2004). This N2B Titin isoform with a size of 3.0 MDa is expressed in the heart of adult mice. Thus, the separation of full length and truncated Titin protein expressed in adult tissue was more prevalent in its native form (Fig. 4.4). Interestingly, protein expression of the truncated Titin found in heterozygous embryo hearts was slightly increased compared with adult heterozygotes. Homozygous adults could not be obtained.

![Figure 4.4: Titin protein expression in embryonic and adult hearts. SDS-agarose gels of E9.5 wildtype (WT), heterozygous (Het), and knockout hearts (KO) and the lysates of adult heterozygous (Het) and wildtype ventricles (WT). Truncated Titin protein of the predicted size was expressed in knockouts and heterozygotes. In embryonic tissue differences in migration were more prevalent for the degradation product of the native T1 Titin designated as T2. Truncated Titin (Rec) was more stable in the embryo compared with adult heterozygotes. Homozygous adult hearts could not be obtained.]

4.1.4 Expression of a truncated and a full length Titin transcript

*In situ* hybridization is a technique that allows to specify expression of mRNA and DNA of a certain gene in its native location. Titin expression was first monitored with a probe that recognized the region 3’ of the kinase domain. This region was not deleted in knockout embryos (referred to as prekinase in Fig. 4.1). Whole mount hybridization revealed that the expression of Titin was restricted to the ventricle and atrium of the embryonic heart (Fig. 4.5, a and b). Somites contain muscle progenitor cells that also expressed Titin. To confirm the targeted deletion of Titin’s M-line exon 1 and 2 on transcript level, an riboprobe recognizing the kinase domain was generated (referred as kinase in Fig. 4.1). There was no Titin M-line Exon 1 and 2 expression detectable in the heart and somites of the knockout embryo (Fig. 4.5, d).
The wildtype embryo expressed the full length transcript in the heart and somites. Calling attention was the reduced body size of knockout embryos compared to their wildtype littermates.

![Figure 4.5: In situ analysis of Titin gene expression.](image)

**Figure 4.5: In situ analysis of Titin gene expression.** The expression pattern of the targeted Titin allele was determined by in situ hybridization of E9.5 embryos using a riboprobe directed against the kinase region (kinase in c and d). Full length Titin was detected with a probe that recognized RNA transcripts of a region 3' of the kinase domain (prekinase in a and b). Titin was expressed in the heart and somites in all controls, while the kinase probe did not produce a signal in knockout animals (d). Size bar: 0.5 mm.

### 4.1.5 Titin M-line deficiency caused impaired development

The phenotype of knockout embryos and the morphology of their hearts from E9.0 until E11.0 was monitored to characterize the order of events that caused cardiac dysfunction. At E9.0 when the embryos had just turned, knockout as well as wildtype embryos showed a normal overall appearance and a regular body size. Knockout and wildtype embryos showed proper development of the heart tube that was functionally active (arrows in Fig. 4.6, A, a and b). Ten days after fertilization the knockout embryo appeared still normal referring to the ratio of heart to body size. The functionality of the heart was indicated by the blood that was distributed throughout the vasculature (arrows in Fig. 4.6, A, c and d). However, knockout embryos were considerably smaller in size and paler in the dorsal trunk region than their wildtype littermates (c and d). From E10.0 knockout embryos failed to thrive. They were strongly reduced in size and a functionally active heart was absent (Fig. 4.6, A, f).
4.1 Phenotype of the Titin M-line knockout model

Figure 4.6: Development and histological analysis. (A) Wildtype and knockout embryos at E9.0 (a and b) displayed normal morphological and cardiac development. The knockout embryo at E10.0 (d) appeared normal including proper cardiac growth but was small for age. At E11.0 embryonic and cardiac development of the knockout was delayed with reduced body size, reduced number of somites, and pericardial hemorrhage (f, arrow). (B) Histological analysis showed normal development of the common ventricular chamber at E9.0 (a and b). At E10.0 the common atrial chamber in knockout and wildtype embryos was formed (c and d). Trabeculation was first reduced in the knockout heart at E10.0 (d). At E11.0 the trabeculation was absent and the common atrial and ventricular chambers were disrupted, while the myocardium showed destructed areas. A, common atrial chamber; V, common ventricular chamber; LA, early left atrium; RA, early right atrium; M, myocardium; T, trabeculation. Size bar: 0.5 mm.

The developmental delay and the severity of the phenotype was reflected in a decreased number of somites. Blood leaked out passively and had accumulated in the pericardial and peritoneal cavity in knockout embryos (arrow in f). A consequence of cardiac atrophy was the instability of the ventricular wall. By E12.0, all knockout embryos were dead and the process of resorption was initiated.

There was no difference observed between wildtype and heterozygous embryos at any stage of development. For further analysis only homozygous wildtype animals were used as controls.
4.1.5.1 Malformation of the knockout heart

Histology is the microscopic study of tissue. Hematoxylin and eosin are among the most commonly used stains. Knockout and wildtype embryonic hearts of different stages of development were analyzed histologically on transversal paraffin sections (Fig. 4.6, B). At E9.0 the left ventricle and myocardium of wildtype and knockout hearts developed normally (a and b). Until E10.0 the hearts of wildtype and knockout embryos showed a normal size of the common ventricular and atrial chamber. However, there was evidence for an impaired cardiac development of the knockout heart. It showed an irregular ventricular wall thickness, an abnormal thin myocardium, and lacked well-defined trabeculation (d). At E11.0 the knockout heart (f) was misshaped and failed to grow in size. Atrophy resulted in instability of the ventricular wall with pericardial effusion (arrowheads). The heart lacked defined trabeculation and there was a complete absence of cellularization within the atrioventricular canal. In contrast, in the wildtype heart the septation of the common atrial chamber was initiated, the myocardium increased in size, and the common ventricle chamber was fully trabeculated.

4.1.5.2 Impaired myocardial development

The wall of the common ventricular chamber consists of the endocardium, the myocardium, and the pericardium. This composition is already present during early embryonic development. To follow the development of the myocardium in knockout hearts, the ventricular wall was stained with haematoxylin and eosin and analyzed at a high magnification. The myocardial wall of the common ventricular chamber developed normal in wildtype and knockout hearts at E9.0 (Fig. 4.7, a and b). At this stage the myocardium consisted of

![Figure 4.7: Impaired development of the myocardium.](image)

The wall of the common ventricular chamber of knockout and wildtype embryos developed normally until E9.0 (a and b). Unlike the wildtype (c) the knockout hearts (d) showed a thinner myocardium at E10.0. The myocardium of the wildtype increased rapidly in size at E11.0 (e) whereas the ventricular wall of the knockout heart failed to grow (compare blue bars in e and f). Size bar: 0.5 mm.
only two cell layers. However, the myocardium at E10.0 appeared in the knockout heart (d) much thinner and was still formed of two cell layers compared to more than 4 cell layers in wildtype hearts (compare blue bars). Moreover, the pericardium of knockout hearts was disconnected at certain regions. Until E11.0 the myocardium of the wildtype heart extended rapidly. The knockout myocardium failed to increase in size (blue bars in e and f). The pericardium was formed by a compact cell layer in wildtype (arrowhead in e) in contrast to a fragile pericardium in the knockout heart (arrowhead in f). Additionally, the H&E staining of the cytoplasm and nuclei of knockout cardiac cells from E10.0 was much paler compared to a distinct separation of blue stained nuclei from violet cytoplasm in wildtype cells.

4.1.6 Knockout embryos were lethal by E11.5

Mouse embryos first show a beating heart at E8.0 after the heart tubes fuse. The heart rate usually ranges from about 60 beats/min at E8.5 to about 120 beats/min at E10.5 as determined by Doppler ultrasonography (Gui et al., 1996). To define the effect of Titin M-line deficiency on embryonic heart function and to specify the stage of development when the heart of the knockout embryos failed to contract, wildtype, heterozygous, and knockout embryos were dissected at different stages of development and the heart rates were monitored (Fig. 4.8).

Figure 4.8: Analysis of the cardiac contractility. Knockout embryos reached midgestation and developed a contracting heart. Unlike wildtype (WT) and heterozygous animals (Het), the hearts of Titin M-line deficient animals (KO) were more fragile and fail to contract autonomously at increasing stage of development. At E11.5 there was no knockout embryo with a contracting heart. (Numbers within the columns indicate dissected embryos.)
The hearts of wildtype and more than 60% of knockout embryos at E9.5 showed rhythmic contractions. At this stage of development there was no obvious difference of contraction rate and intensity between wildtype, heterozygous, and knockout embryos. However, the contraction rates were below the published values since the embryos were not monitored in utero, but dissected from the uterus and placenta. The number of knockout embryos with a beating heart decreased by about 50% at E10.5. Almost all wildtype and heterozygous embryos showed a strong and steadily contraction of the heart. At E11.5 none knockout embryo with a heart that was functionally active could be observed. There was again no difference between heterozygous and wildtype embryos.

4.1.7 Early cardiac morphogenesis was normal in knockout embryos

Cell growth and differentiation can take place in cell culture. In contrast, morphogenesis can only be observed in vivo. It deals with the shape of tissue or organs and the position of various specialized cell types. The study of morphogenesis involves the processes that control the organized spatial distribution of cells that arises during the embryonic development. For the cardiac morphogenesis, the transcription factor Nkx2.5 is well-characterized. Nkx2.5 is a cardiac homeobox protein that is essential for normal heart morphogenesis, myogenesis, and function (Lints et al., 1993). Furthermore, its gene is a component of a genetic pathway required for myogenic specialization of the ventricles (Lyons et al., 1995). A link between Nkx2.5 and Titin was found in mdm mice. Affymetrix analysis showed that nine genes belonging to the CARP-mediated Nkx2.5 pathway were dysregulated (Witt et al., 2004).

Figure 4.9: Expression of Nkx2.5. In situ hybridization of E9.5 knockout and wildtype embryos showed expression of Nkx2.5 in both the common ventricular (open arrowheads) and atrial (filled arrowheads) chamber. There was no difference between knockout and wildtype animals. Size bar: 0.5 mm.
Morphogenesis of knockout compared to wildtype heart tube at early embryonic development was analyzed by whole mount \textit{in situ} hybridization using Nkx2.5 as RNA riboprobe. At embryonic day 9.5 the heart tube had already turned and the common ventricular and atrial chamber were distinguishable. There was no difference between knockout and wildtype observed. Nkx2.5 was expressed in the atrium and ventricle of the heart (Fig. 4.9) suggesting no function of Titin’s M-line for heart morphogenesis until mid-gestation.

### 4.1.8 Expression of Titin isoforms in the heart was not affected

There are distinct Titin isoforms that are generated by alternative splicing depending on the tissue and stage of development. In the embryonic heart the N2BA Titin isoform with a size of 3.7 MDa is predominantly expressed (Opitz et al., 2004). To evaluate if Titin expression is affected in the absence of its M-line or whether the deletion is compensated by alternative splicing, quantitative RT-PCR was performed. The transcript levels of the different isoforms, generated in the I- and A-band were monitored at early embryonic development. Regions located within the Z-disc, the N2B region, and MEx6 (M-line) were chosen for analysis (see localization in Fig. 4.3). In order to obtain sufficient amounts of total RNA from knockout and wildtype embryos and to reduce variability, dissected hearts of multiple E9.5 litters were pooled. All Titin regions were expressed at similar amounts in wildtype and knockout hearts (Fig. 4.10).

![Figure 4.10: Expression of Titin isoforms.](image)

\textbf{Figure 4.10: Expression of Titin isoforms.} Transcript levels of Titin’s Z-disc (Z1/2), I-band (N2B), and A-band (M-line) in E9.5 wildtype and knockout hearts were monitored by RT-PCR. Ct values were normalized to the endogenous control 18S RNA and fold changes were determined relative to adult heart transcripts. There was no difference between knockout and wildtype transcripts and overall mRNA levels varied from 7\% to 25\% of adult levels.
Comparing to adult expression profile, Titin’s Z1/2 region was expressed at about 12%, the N2B region was reduced to about 7%, and Titin’s M-line transcript levels ranged at about 25%.

### 4.1.9 Titin has no essential non-muscle function

Titin has been suggested to be a component of non-muscle cells and to fulfill non-muscle functions. In order to elucidate Titin’s role in non-muscle cells, primary cells were prepared from knockout and wildtype embryonic tissue. At E9.5 embryos were dissected, internal organs removed, and isolated cells cultivated in feeder medium. Wildtype cells attached overnight to the culture plate and started to divide and differentiate (Fig. 4.11, A). In contrast, knockout cells that were plated at the same amount attached to a lesser extend. After 5 days in culture wildtype cells grew confluent. The number of cells rapidly increased indicated by shifting pH values after incubating the culture overnight. The medium contained phenolred as indicator that turned yellow when pH values changed. Knockout cells grew slower but were able to divide. However, they did not reach the confluence of wildtype cells. Wildtype primary cells formed a tight network after 8 days. The number of knockout cells was judged by eye and determined as about 10 times less compared to knockout cells.

Depending on the tissue dissected, primary cells can differentiate to various cell types including cardiomyocytes. These cells could easily be recognized by rhythmical contraction. They mainly formed cell clusters that beaten simultaneously. In cell cultures from both knockouts and wildtypes, beating cardiomyocytes were observed. There was no difference of contraction rate and intensity between wildtype and knockout. Concerning the shape and size, knockout cardiomyocytes were indistinguishable from wildtype cells. However, the number of cells forming those cell clusters was reduced. Cultures were monitored for 2 more weeks but there were no additional changes.

Dead cells can be identified by trypan blue staining. Viable cells exclude trypan blue, while dead cells stain blue due to trypan blue uptake. However, this assay does not distinguish between apoptotic and necrotic cells. The viability of cells was investigated for cells isolated from wildtype, heterozygous, and knockout embryos at E8.5, E9.5, and E10.5 (Fig. 4.11, B).

At E8.5 the number of dead cells did not differ between wildtype, heterozygotes, and knockouts. About 10% to 13% of the total cell number were designated as dead. In contrast at E9.5 there was a significant increase of dead cells prepared from knockout embryos. About 50% of isolated primary cells were dead compared to 10% dead cells in wildtype tissue. Surprisingly, at E10.5 the overall number of dead cells increased in wildtype and heterozygotes to about 16% whereas the number of dead cells isolated from knockout was reduced to 10%. This difference was shown to be significant.
4.2 Molecular mechanism

Figure 4.11: Primary cells of knockout and wildtype embryos. (A) Primary culture contained a mixed population of cells and were derived by dissection of E9.5 wildtype and knockout embryos. (B) Trypan blue staining showed the viability of wildtype, heterozygous, and knockout cells isolated from embryos at E8.5, E9.5, and E10.5. **, P < 0.01; *, P < 0.05 compared with wildtype. (Numbers within the columns indicate dissected embryos.) Size bar: 0.2 mm.

In summary, wildtype as well as knockout cells derived from E9.5 whole mount embryos were able to grow and divide. Both cultures contained cells differentiating into spontaneously beating cardiomyocytes. These findings suggested no essential non-muscle function for Titin’s M-line. Interestingly the fact that 5 times more cells isolated from knockout embryos were dead at a time when the phenotype started to develop.

4.2 Molecular mechanism

Titin has been linked to sarcomere assembly and diverse functions through its elastic, adaptor, and signaling domains. It acts as a blueprint for sarcomeric and signaling proteins and is involved in signal transduction. Titin has been proposed to induce hypertrophy and convert mechanical stress into biochemical signal.

So far, the morphological and histological analysis revealed that Titin M-line deficiency
resulted in impaired cardiac development and embryonic lethality by E11.5. The next chapter will focus on the physiological relevance of Titin’s M-line region, its kinase domain and interacting proteins.

4.2.1 Apoptosis as a consequence of cardiac dysfunction

The reduced cardiac wall thickness and asystole first detected from E9.5, together with the failure to thrive required the investigation of apoptosis as potential mechanism or as secondary effect to cardiac dysfunction. Apoptosis is the regulated cellular self-destruction that is a prerequisite for embryonic development and tissue homeostasis (Wyllie et al., 1980). It constitutes a basic mechanism that accounts for many morphogenetic and histogenetic events during normal and abnormal development of embryonic organs and tissues (Prindull, 1995).

The TUNEL assay was applied to cryosections of E9.5 and E10.5 wildtype and knockout embryos. All sections were counterstained with the sarcomeric α-Actinin to distinguish the cardiomyocytes of the heart from the body and determine the localization of apoptotic cells. At E9.5, when the first genotype dependent difference of size was observed, the number of cells with apoptotic nuclei was comparable between knockout and wildtype embryos. Apoptotic cells were present in the heart as well as in the abdominal region (Fig. 4.12, a,b and e,f, respectively).

![Figure 4.12: Apoptosis assay.](image)

Wildtype and knockout embryos were investigated for apoptosis by a TUNEL assay. Tissue was counterstained with α-Actinin to label cardiomyocytes (red, top lane). At E9.5 apoptotic cells (green) were present in both knockout and wildtype animals at comparable levels in the heart (a, b) and abdominal region (e, f). The number of cells with condensed chromatin increased in knockout animals at E10.5 but apoptosis was not restricted to cardiac ventricles. Apoptotic nuclei were observed predominantly in the abdominal region (d vs h). Size bar: 100 µm.
4.2 Molecular mechanism

However, at E10.5 apoptosis was increased in knockout embryos, in particular in the abdominal region (compare g and h). Wildtype cardiomyocytes and cells of the abdominal region did not show an increased number of apoptotic nuclei compared to E9.5. Since non-muscle knockout cells divided and differentiated normally before cardiac function was affected, the increase in apoptosis from E10.5 was a secondary consequence of embryonic lethality and did not imply a non-muscle function.

4.2.2 Impaired sarcomere assembly in knockout embryos

In the mouse, cardiac contractions start at embryonic day 8.0 in the mouse, even before myofibrillogenesis is completed and sarcomeres are fully matured. Electron microscopy allows the analysis of sarcomere assembly on the ultrastructural level. It even enables to follow the incorporation of thick and thin filaments into the sarcomere which leads to the ability of the cardiomyocytes to contract. To investigate the mechanism that triggered cardiac dysfunction electron micrographs were generated from knockout and wildtype cardiomyocytes at E9.0 to E11.0.

The electron micrographs at E9.0 confirmed normal sarcomere organization in knockout and wildtype cardiomyocytes (Fig. 4.13, A, a and b). Premyofibrils have fused to nascent myofibrils which were able to contract. The dark regions within the myofibrils were composed of Z-bodies which fused to form Z-discs. Between neighboring Z-discs is the M-band located. It could be observed as pale region. From embryonic day 9.5 knockout sarcomeres did not reveal any alteration in the structure but rather failed to grow laterally (quantification in Fig. 4.13, B). The difference of sarcomere thickness between knockout and wildtype was shown to be statistically significant. However, Z-disc and M-band structures were maintained through E10.0 in the knockout sarcomere (Fig. 4.13, A, f).

Higher magnification showed slight variations in M-band alignment (arrowhead in Fig. 4.13, C, a and b). The width of the Z-discs (c and d) indicated mechanical strain that affected both knockout and wildtype sarcomeres. From E10.0 to E11.0 knockout sarcomeres disassembled and only a few filaments remained in disarray within the cytoplasm (Fig. 4.13, A, f,h,j).

In summary, nascent myofibrils in knockout cardiomyocytes assembled and formed Z-discs and M-bands. However, myofibrils did not grow laterally and mature. Thus, the Titin M-line region could have an effect on the assembly of the sarcomere. It might be involved in regulating lateral growth, maturation, and maintenance of the sarcomere.
Figure 4.13: Ultrastructural analysis of cardiac sarcomere maturation. (A) Sarcomeres assembled in wildtype and knockout cardiomyocytes until E9.5. Lateral growth was impaired from E10.0 in knockout embryos followed by sarcomere disassembly (f,h). At E11.0 all sarcomeric structures were dissolved (j). (B) Sarcomere diameter was quantified for >100 fibers in 2 embryos per group. The lateral growth of knockout sarcomeres (KO) lagged behind the increase in wildtype sarcomere width. The loss of sarcomere structure precluded analysis of E10.5 and E11.0 knockout sarcomeres. **, P < 0.01 compared with knockout. (C) M-band and Z-disc structures at day E10.0 did not differ significantly between knockout and wildtype sarcomere. (M, M-band; Z, Z-disc; Mi, mitochondrion; N, nucleus; L, lipid droplets). Size bars are indicated.
4.2 Molecular mechanism

4.2.3 Knockout Titin integrated into the I-band but not in the M-band

Although knockout and wildtype sarcomeres were indistinguishable at the ultrastructural level early in cardiac development, alteration in the molecular composition of sarcomeric proteins might have impaired their stability. Titin molecules from adjacent sarcomeres overlap at the Z-disc and at the M-band to form a continuous filament system (Gregorio et al., 1998; Obermann et al., 1996; Young et al., 2001). The thin filaments are cross-linked via α-Actinin whereas Myomesin forms a complex with Titin and the thick filament in the M-band. The integration of Titin into the sarcomere was monitored using antibodies directed against Titin’s I-band region (N2B) and its C-terminus (M8/M9).

![Image of immunostaining results](image-url)

**Figure 4.14:** Integration of Titin and α-Actinin into the sarcomere. Immunostaining with antibodies directed against α-Actinin, the Titin N2B (A), and the M-line region (B). (A) Titin was expressed and incorporated into the Z-disc of the sarcomere in wildtype and knockout animals. The N2B region is located proximally to the Z-disc and thus N2B and α-Actinin partially co-localize at E9.5 in wildtype and knockout (yellow). Upon disassembly co-localization could still be observed at E10.5 (open arrow). In areas of disassembly, where α-Actinin localized in spotted aggregates (filled arrows), the N2B region was distributed diffusely. (B) Titin’s M-line region (M8/M9) was not integrated into the M-band of the sarcomere in knockout cells. Wildtype sarcomeres showed the expected striated pattern with alternating Z-disc and Titin M-line epitopes. (C) Western blot analysis showed the presence of the M8/M9 epitope in the truncated protein of a E15.5 embryo. Size bar: 5 µm.
4 Results

Anti-α-Actinin is well-characterized and was used as a marker for the Z-disc (Ehler et al., 1999). Localization studies were performed on cryosections of wildtype and knockout E9.5 and E10.5 hearts (Fig. 4.14, A and B). At E9.5 the Z-disc protein α-Actinin assembled organized in a striated pattern in knockout and wildtype myofibrils. The heart specific Titin N2B region, which is located C-terminal of the Z-disc region, partially co-localized with α-Actinin in knockout and wildtype (Fig. 4.14, A). This suggested that Titin’s N-terminus integrated into the I-band independent of the presence of Titin’s M-line region. However, upon disassembly in knockout sarcomeres, Z-discs distributed from regular striation (open arrows in Fig. 4.14, A) to random patches of variable sizes (filled arrows) at E10.5. In these areas Titin’s N2B epitope did not co-localize with α-Actinin. Unlike Titin’s N-terminus Titin’s M-line region was not integrated into the developing sarcomere in knockout cardiomyocytes (Fig. 4.14, B). This was shown by a diffuse localization of Titin’s M8/M9 region in the knockout embryo at E9.5 as well as at E10.5. However, in wildtype cardiomyocytes the expression of Titin’s M-line was detected alternating with the Z-disc. Both mislocalization of the Titin M8/M9 epitope as well as failure to incorporate Titin’s M8/M9 domain into the M-band could explain the diffuse background staining in the knockout hearts. Hence, western blot analysis of embryonic hearts from heterozygous animals were used to confirm proper expression of the truncated M-line region (Fig. 4.14, C). The M8/M9 epitope was detected in a heterozygous heart of an E15.5 embryo. This indicated partial integration of the truncated Titin protein into the sarcomere.

In summary, Titin’s N-terminus integrated into the I-band whereas Titin’s C-terminus failed to be incorporated into the M-band. Titin was partially integrated but if Titin was only connected to the Z-disc or extended over the A/I junction remains to be addressed.

4.2.4 Localization of the M-band protein Myomesin

The M-band of the sarcomere is a highly complex structure which contributes to the assembly and maintenance of the thick filament lattice. Multiple protein binding sites were identified on the Titin molecule, but so far Myomesin is the only structural protein binding to Titin’s M-line region. The binding site is the Ig domain m4 (Obermann et al., 1996), a region that is encoded by M-line exon 1 and thus deleted in the knockout. The Myomesin-EH (embryonic heart) isoform is predominantly expressed in embryogenesis. In adult Myomesin-EH is expressed only in little amounts (Fig. 4.15). Therefore, immunofluorescence staining using an antibody against Myomesin-EH was used to follow the incorporation of Myomesin into the sarcomere of E9.5 and E10.5 cardiomyocytes. The periodic Myomesin staining in wildtype and knockout cardiomyocytes at E9.5 indicated that even in the absence of Titin’s M-line Myomesin was incorporated into the sarcomere (Fig. 4.15).
4.2 Molecular mechanism

Figure 4.15: M-band assembly in the absence of Titin’s M-line region. Although Titin was not incorporated into the M-band, Myomesin (Myom1 EH) localized properly between Z-discs as shown by staining with α-Actinin at E9.5. Upon disassembly at E10.5 Myomesin staining was more diffuse. Size bar: 5 µm.

It was localized alternating with Z-discs as demonstrated by co-staining with the Z-disc protein α-Actinin. However, in knockout cardiomyocytes Myomesin staining was more diffuse. This might be due to the deletion of Myomesin’s binding site and the incapability of Titin’s C-terminus to integrate into the M-band. Progressing disassembly of the sarcomere at E10.5 changed the staining pattern. Myomesin localized in wide patches to the M-band in knockout cardiomyocytes. In wildtype cardiomyocytes Myomesin still localized to the M-band alternating with α-Actinin. Clearly visible in wildtype cells was the lateral growth of the myofibrils demonstrated by a longer staining pattern for α-Actinin. Although Myomesin’s staining in knockout cells was diffuse, it localized to the M-band suggesting that Titin’s M-line was not essential for proper M-band assembly. Unspecific signals of Myomesin at the M-band were rather a consequence of sarcomere disassembly.

To investigate if Myomesin was differentially regulated in the absence of Titin’s M-line, its expression in wildtype and knockout hearts was analyzed by western blotting. At E9.5 before the phenotype of the knockout embryo was prominent and Myomesin was still incorporated into the M-band, expression of Myomesin was slightly reduced in the knockout (Fig. 4.16). Thus, the diffuse expression of Myomesin in cardiomyocytes did not correspond to an increased expression.

Figure 4.16: Expression of Titin binding proteins. Western blot analysis showed a slight reduction of Myomesin expression in E9.5 knockout hearts. MuRF-1 was expressed at comparable levels in knockout and wildtype E9.5 hearts. Actin was applied as loading control.
The ubiquitin ligase MuRF-1 binds just upstream of the kinase domain, thus its binding site was also deleted. Differentially expression of MuRF-1 was controlled by western blot analysis of protein lysates from wildtype and knockout hearts. The expression of MuRF-1 in knockout hearts was weak but not changed in response to the loss of Titin’s M-line.

4.2.5 Sqstm1 localized to the I-band

Sqstm1 has been shown to be involved in conversion of mechanical to biochemical signal by contributing to a signaling cascade upon mechanical arrest. It has been localized to Titin’s kinase domain where it interacts with Nbr1 (Lange et al., 2005b). So far, no studies concentrated on the expression of Sqstm1 in cardiac muscle cells during embryogenesis. Immunostaining on wildtype embryonic heart revealed that Sqstm1 was not expressed during embryonic development (Fig. 4.17, A). This was confirmed by western blot analysis showing that Sqstm1 is not expressed before E12.5 (Fig. 4.17, B). However, in situ localization studies on adult skeletal muscle cells showed that Sqstm1 was expressed at the I-band. Sqstm1 concentrated periodically between Titin’s C-terminus as shown by co-staining with anti-M8/M9 recognizing Titin’s M-line region.

![Figure 4.17: Expression of Sqstm1.](image)

Figure 4.17: Expression of Sqstm1. Localization (A) and expression (B) studies of Sqstm1. (A) Immunostaining on cryosections of a E9.5 heart and an adult skeletal muscle from quadriceps. Sqstm1 was not detected at E9.5 but localized in adult to the I-band as shown by co-staining with the Titin antibody recognizing M8/M9 epitope at the sarcomeric M-band. (B) Western blot confirmed expression of Sqstm1 during late embryonic development.

4.2.6 Expression of Titin and its M-line binding proteins remained constantly low during embryogenesis

In both the conditional Titin knockout animals (Gotthardt et al., 2003) and the constitutive knockout described here, Titin M-line deficient sarcomeres disassembled. Nevertheless, the structure was preserved better in the adult than in the developing sarcomere, with Titin’s M-line integrated properly, even in the absence of Titin’s kinase region. To elucidate potential candidates among Titin binding proteins that play a role in assembly and maintenance of the sarcomere during myofibrillogenesis, expression analysis by RT-PCR was performed.
Dissected hearts (E9.5, E12.5, E15.5, E18.5, and adult) of different litters were pooled, total RNA isolated, transcribed, and examined in triplicates. mRNA expression levels of both Titin and its binding proteins at various stages of development were monitored. The threshold values (Ct) of the RT-PCR analysis were normalized to the housekeeping gene 18S RNA.

Overall mRNA levels of Titin and its binding proteins were reduced in embryonic development compared to adult levels but increased by up to two orders of magnitude from E9.5 to adulthood (Fig. 4.18).

**Figure 4.18:** RNA and protein expression of Titin and sarcomeric proteins. Quantitative RT-PCR analysis of Titin (A) and its M-line binding proteins (B) demonstrated that expression increased by up to two orders of magnitude from early cardiac development to adulthood. The threshold values (Ct) were normalized to the housekeeping gene 18S RNA. (A) Titin’s cardiac specific N2B region was barely expressed in the embryonic heart at E9.5, whereas the Z-disc and M-line transcripts were expressed at 10% and 30% of adult level, respectively. However, all expression levels increased in embryonic development. (B) Except for Calmodulin and MuRF-2, transcript levels of most binding proteins were less than 20% of adult levels at E9.5, which includes the proposed kinase substrates T-cap, Sqstm1, and Nbr1. (C) Most of Titin’s M-line binding proteins could be detected by western blot during late embryonic development, when cardiac pathology in knockout animals was already present. Notable exceptions were MuRF-1, Myomesin-EH, and Calmodulin which were constantly expressed throughout embryonic development. Calm, Calmodulin; FHL2, four and a half lim domains 2; Nbr1, neighbor of Brec1 gene 1; Sqstm1, Sequestosome1; MuRF, muscle-specific RING finger protein; T-cap, Titin cap.
In early development (E9.5) reduced expression of the heart specific elastic Titin N2B region was observed. Differences in Z-disc and M-line levels indicated a higher ratio of full length Titin compared to truncated isoforms in the developing embryo. This ratio was maintained through embryonic development until E18.5. Prenatally, full length versus truncated isoforms reached equal amounts. However, overall transcript levels increased during embryogenesis but did not reach expression levels as observed in adult hearts (Fig. 4.18, A). This suggested that the expression of Titin and its isoforms reaches highest levels not after birth but rather during the first weeks of life.

Various Titin binding proteins have been proposed to act as a substrate for Titin’s kinase domain or contribute to sarcomere assembly. Of these T-cap, Sqstm1, and Nbr1 were expressed less than 20% of adult levels at E9.5 (Fig. 4.18, B). An exception was MuRF-2 that was shown to interact with Sqstm1 and Nbr1. Calmodulin is a Ca\(^{2+}\)-binding protein that is involved in activation of the active site of Titin’s kinase domain (Mayans et al., 1998). MuRF-2 and Calmodulin transcript levels reached about 60% of adult level at E9.5, whereas MuRF-2 increased to 280% of adult level until E18.5. All other transcripts remained low during development.

Western blot analysis of Titin binding proteins revealed constantly high expression of MuRF-1 and Myomesin-EH during embryogenesis (Fig. 4.18, C). MuRF-2, FHL2, and T-cap were detected from E15.5 whereas Calmodulin was weakly expressed at E9.5 but increased slightly during development. Actin was applied to confirm equal loading. Combining RNA and protein data (Fig. 4.18, B and C), Calmodulin, MuRF-1, MuRF-2, and Myomesin were the only proteins expressed in significant amounts at the time when the knockout phenotype developed.

4.2.7 Expression of sarcomeric proteins and those involved in hypertrophy did not lead to disassembly

The sarcomeric phenotype can be explained by structural features of M-line Titin. However, additional changes in signal transduction or secondary effects could precipitate or modify the phenotype. Additionally, the adult conditional Titin M-line knockout elicited a hypertrophic signaling response including an increased activity of the mitogen-activated protein (MAP) kinase pathway. The MAP kinase pathway has also been implicated in hypertrophy as well as regulating serial versus parallel assembly of the sarcomere (Nicol et al., 2001) and was therefore required to be investigated. Expression of proteins involved in hypertrophic signal transduction as well as Titin binding proteins in wildtype and knockout embryos were compared to the expression in the adult heart. Total RNA was isolated from wildtype and knockout E9.5 hearts that were pooled from different litters. Ct values were normalized to endogenous 18S RNA and compared to adult transcript levels.
4.2 Molecular mechanism

Figure 4.19: Secondary changes in response to kinase region deficiency. RT-PCR expression analysis of wildtype and knockout total RNA from E9.5 hearts. The loss of Titin’s M-line region did not lead to compensatory upregulation of Titin’s binding proteins (A) and no significant reduction of hypertrophy markers (B) as verified in two pools of 6 embryos each per genotype. ANP, atrial natriuretic peptide; MAPKAP, Mitogen-activated protein Kinase Activating Protein; Mef2C, Myocyte enhancer factor 2; MEK5, mitogen-activated protein kinase kinase 5; TGF-β, Transforming Growth Factor Receptor β.

Unexpectedly, neither Titin’s binding proteins nor one of the hypertrophic response proteins were remarkably upregulated in response to the elimination of Titin’s M-line to compensate for the loss of functional sarcomeres (Fig. 4.19, A and B). Moreover, except for Calmodulin and MuRF-2, transcript levels of Titin’s binding proteins were low comparing to adult heart (A). Furthermore, the hypertrophic gene response accompanying the adult kinase region knockout phenotype was not elicited in the embryonic heart (B). MAPKAP and MEK5 were found to be expressed at about 30% to 40% of adult levels. The transcript levels of ANP, Mef2C, and TGF-β were rather comparable to levels expressed in adults.
4.3 Summary of results

The phenotype of the Titin M-line knockout mouse model

- The constitutive Titin M-line knockout was generated by germline recombination. The Titin protein was truncated by 200 kDa at its C-terminus.

- Cardiac and morphological development of homozygous Titin M-line deficient mice were normal until embryonic day 9.5.

- From E9.5 cardiac growth was delayed, followed by disassembly of the sarcomere, inability of the heart to contract, and lethality by E11.5.

- Homozygous knockout embryos did not display any non-muscle phenotype apart from defects secondary to impaired cardiac function.

Molecular mechanism

- Apoptosis was shown to be a secondary effect of cardiac dysfunction.

- Sarcomeres assembled initially in knockout cardiomyocytes but failed to grow in width.

- Titin’s N-terminus integrated into the I-band but its C-terminus failed to incorporate into the M-band in knockout cardiomyocytes. Thus, Titin failed to form a continuous filament system in the absence of its M-line region.

- In knockout cardiomyocytes Myomesin localized to the M-band even in the absence of its binding site to Titin.

- Titin binding proteins MuRF-1, MuRF-2, Myomesin, and Calmodulin were expressed in significant amounts at the time when the knockout phenotype developed.

- Signaling molecules, such as the in vitro substrates of Titin’s kinase T-cap, Sqstm1, and Nbr1 were expressed at low levels during development and did not have a compensatory effect in knockout embryos.

- The hypertrophic gene response accompanying the adult conditional knockout phenotype was not elicited in the embryonic knockout heart.