5 Discussion

Titin, the largest protein known so far, is composed of different functional units that enable Titin to fulfill various functions. Due to its size, it has been difficult to study Titin's signaling and structural functions. Although multiple Titin deficient animals and cell lines have been generated, it has not been possible to address the role of Titin's M-line in early cardiac development. This work presents a novel animal model to investigate Titin in sarcomere assembly of cardiac muscle cells. The tissue-specific Titin M-line knockout was converted into a constitutive knockout. Germline recombination deleted Titin's M-line exon 1 and 2 but maintained both the N- and C-terminal epitopes to follow Titin in sarcomere assembly and address potential non-muscle functions.
The objective of this work was to understand the functions of Titin's M-line in embryonic development, which does not only involve differential expression of Titin isoforms, but also of Titin binding proteins.

5.1 Knockout technology to understand Titin

The introduction of defined modifications at a genomic level by gene targeting has become a widely used technique. It allows to design mice lacking single gene products or exhibiting changed regulatory properties to study the physiological functions of genes. The possibility of homologous recombination in the mammalian genome was studied in the eighties (Smithies et al., 1985). Parallel it was demonstrated that pluripotent murine stem cell lines have the capacity to contribute to the germline tissue of mice, even after extended periods in culture. Mutations introduced into these cell lines are transmitted to the offspring (Robertson et al., 1986). Thus, knockout technology developed to a suitable tool to study functions of genes *in vivo*.
The human Titin protein is encoded by 363 exons, has a size of about 3 MDa, and spans half the sarcomere from the Z-disc to the M-band. Because of its various domains, multiple animal models and cell lines have been investigated (Person et al., 2000; Xu et al., 2002; Garvey et al., 2002; Miller et al., 2003a; Gotthardt et al., 2003). However, data on the function of Titin's M-line region and its binding proteins in the embryonic sarcomere are

not available, yet.
The deletion of M-line exon 1 and 2 was expected to provide insights into structural and regulatory roles of Titin's M-line. MEx1 encodes for a 200 kDa M-line portion of the Titin molecule. This region is highly conserved between species and contains a kinase domain. It also provides several binding sites for structural and signaling proteins that help Titin maintaining the filament lattice of the sarcomere (Gautel et al., 1995; Lange et al., 2002; Mayans et al., 1998; McElhinny et al., 2002, 2004), convert mechanical to biochemical signals (Lange et al., 2005b), and are involved in signaling cascades contributing to hypertrophy (Miller et al., 2003b; Bodine et al., 2001).
In summary, knockout technology is the best technique to study Titin's M-line regions *in vivo*. So far, there are only data on cultured myoblasts with a similar Titin M-line deficiency available. This makes the constitutive knockout model unique and enables to address Titin's role in early cardiac development.

5.2 Non-muscle functions of Titin and its M-line

The function of Titin has been extensively described in striated muscle tissue. Additionally, Titin was shown to be a component of non-muscle cells such as the intestinal epithelial cells of the brush border where it also plays a structural role (Eilertsen and Keller, 1992). That Titin also has non-muscle functions was evaluated by its localization to chromosomes suggesting a role for chromosome structure, elasticity, and cell division (Machado et al., 1998; Keller et al., 2000). Moreover, Titin was recently identified to interact with the nuclear filament Lamin A being essential for nuclear architecture (Zastrow et al., 2006). Also data on mutations of Titin's homologue D-Titin identified in drosophila, showed to cause embryonic muscle disorganization as well as severely disrupted chromosome structure (Machado and Andrew, 2000). Nevertheless, Titin's physiological role in non-muscle cells of vertebrates is still poorly understood and remains to be addressed. Neither mutant zebrafish nor mutant mouse models described before displayed any non-muscle phenotypes. Non-muscle tissue showed a phenotype only as a consequence or secondary effect of impaired cardiac and skeletal muscle function (Garvey et al., 2002; May et al., 2004; Xu et al., 2002).
Titin M-line knockout embryos developed normally until mid-gestation. This required cell division and differentiation into specific cell types following morphogenesis of fully functional organs such as the heart. Thus, an essential cellular function of Titin's M-line region in cell cycle and other basic cellular processes in muscle as well as in non-muscle cells can be excluded.
In the developing mouse embryo Titin is first found in the early heart tube at E8.25 (Schaart et al., 1989). But if Titin is expressed in non-muscle cells and has non-muscle functions as proposed in Drosophila, transcripts of Titin should also be detectable during embryonic

development. However, expression of Titin in non-muscle cells, tissues, or organs such as the epithelia cells, mesenchyme, and brain was not observed. Titin expression was highest in the myocardium of the common ventricular chamber. Lower expression was observed in the wall of the common atrial chamber and the muscle progenitor cells of the somites. The embryonic lethality precluded the analysis of Titin's expression and function in the gastro-intestinal tract of adult animals.
Cell culture studies also addressed the non-muscle function of Titin's M-line. Primary cultures that were derived from wildtype and knockout embryos were composed mainly of murine embryonic fibroblasts (MEFs). These are non-muscle cells of the connective tissue. MEFs are commonly used as feeder cells for embryonic stem cells. However, mitosis and growth of wildtype and knockout MEFs were not impaired. Moreover, cells derived from dissected embryos were not pure embryonic murine fibroblasts. There was also a number of undifferentiated cells derived from internal organs of dissected embryos. Under the given conditions these cells started to differentiate into a variety of cells. Among these were also cardiomyocytes which could easily be identified by their ability to contract.
In summary, Titin was expressed in cardiac muscle as well as in muscle progenitor cells but not in non-muscle tissue of knockout and wildtype embryos. Cell division and differentiation of muscle and non-muscle cells to functional tissues and organs exclude Titin's M-line to have a critical non-muscle function. Embryonic lethality of knockout animals precluded studies on tissues and organs that have been described to express Titin. Thus, the function of Titin's M-line in non-muscle tissue such as intestinal epithelial cells could be addressed in respective tissue specific knockout animals.

5.3 Characterizing the severity of the phenotype

Knockout embryos were lethal by E11.5. Dead embryos were rapidly and completely resorbed in the uterus. However, the time of death and the onset of the cardiac phenotype could not exactly be determined. The first evidence of a delayed development such as reduced body size and a decreased number of somites could be observed in most knockout embryos by E10.0. Nevertheless, a few affected embryos were still indistinguishable from their wildtype littermates. To determine the time of death, the number of wildtype, heterozygous, and knockout embryos with a beating heart was counted at three stages of development. The results revealed a delay in the development of the phenotype between knockout embryos. At E9.5 many knockout embryos had a contracting heart. The number of embryos lacking a contracting heart increased rapidly by E10.5. At E11.5 no knockout embryo was viable. This suggests that the beginning of the disassembly of the sarcomere that caused incapability of the cardiomyocytes to contract was different between knockout embryos. A possible explanation for the delayed development of the phenotype is the variation of individual embryonic stages within one litter. In the developing rat this was

shown before. The number of somites of embryos from a single litter deviated by 13% (Landacre and Amstutz, 1929). Also in mice it was shown that the individual embryonic stages within one litter may differ by nearly one day (Thiel et al., 1993). This suggests, that in growth and differentiation of a particular structure when compared with a standard such as age or number of somites, a wide range of variation might be encountered.
In contrast to an unprecise time when the phenotype started to develop, the progression of sarcomere disassembly that caused cardiac dysfunction and lethality was comparable in all knockout embryos. Histological analysis showed that an impaired overall development was always accompanied with a reduced thickness of the myocardium and less trabeculation of the heart. On ultrastructural level the phenotype could by identified even earlier. Although electron micrographs did not show a major difference between knockout and wildtype myofibrils until E10.5, determining the size of wildtype and knockout sarcomeres revealed that the knockout sarcomeres were already significantly decreased in size from E9.0.
To summarize, a variability in the time when the phenotype started to develop was observed between embryos of one litter. In contrast, the process leading to impaired cardiac function and lethality was comparable between knockout embryos. Similar findings could be observed in Tropomodulin 1 (Tmod1) null mice. Tmod1 is an Actin capping protein that regulates Actin dynamics and filament length in muscle and non-muscle cells (Fischer and Fowler, 2003). Knockout mice showed a phenotype similar to that described for Titin's M-line knockouts. The embryonic development was normal up to E9.0. Thick and thin filaments assembled even in the absence of Tmod1 but myofibrils failed to mature causing impaired development of the myocardium, heart defect, and lethality by E10.5. Analyzing the phenotype they also observed a variability in the onset and severity of the phenotype (Fritz-Six et al., 2003).

5.3.1 Apoptosis as an effect of cardiac dysfunction

Apoptosis and necrosis are two major processes by which cells die. Apoptosis is the tightly regulated programmed cell death whereas necrosis normally results from a severe cellular injury, infection, cancer, or inflammation and generates a inflammatory response (Blumig, 1954; Sealy and Lyons, 1949). Unlike in apoptosis, cells that die by necrosis release harmful chemicals that damage other cells. So far, there are only data on Titin triggering necrosis. It was shown that an increase in Calpain-3 activity caused the proteolysis of Titin's PEVK region accompanied by myofilament instability and cell death by necrosis. Thus, it was hypothesized that degradation of Titin is an initial step leading to both myofibrillar disarray and cardiomyocyte necrosis (Lim et al., 2004).
Titin M-line knockout embryos showed a normal gross morphological development until mid-gestation but failed to thrive followed by embryonic lethality at E11.5. TUNEL-

positive cells were detected at E9.5 in the heart and body in both knockout and wildtype embryos. These apoptotic cells entered the regulated cellular self-destruction that functions in the normal control of development and tissue homeostasis (Wyllie et al., 1980). In contrast, at E10.5 the apoptosis assay revealed an abundance of cells undergoing programmed cell death in various tissues of the knockout embryo. However, the myocardium of knockout hearts did not show an increased number of apoptotic cells suggesting that apoptosis occurred independent of the expression of Titin. The programmed cell death is rather a consequence of impaired blood supply of tissue and organs. Cardiac dysfunction that was accompanied by an impaired cardiovascular system was triggered by the disassembly of the sarcomere.
In summary, the findings indicated that the knockout embryos developed normally with a regulated cellular self-destruction by apoptosis until mid-gestation. Apoptosis did not cause myofibrillar dysfunction since apoptotic cells were observed at a time when the knockout phenotype was strongly reflected. An increased number of cells with apoptotic nuclei in the abdominal region triggered by impaired blood supply of the periphery supported this hypothesis.
Studies on primary knockout cells underlined a secondary effect of cardiac dysfunction and impaired blood supply of tissues and organs. Primary cultures were derived from E8.5, E9.5, and E10.5 embryos by enzymatical digestion in order to cleave cell-cell contacts obtaining a culture of mixed cell population. The viability of cells was determined by trypan blue staining whereas this method does not distinguish between apoptosis and necrosis. However, dissected knockout embryos provided 5 times more dead cells compared to wildtype embryos at a time when the knockout phenotype started to develop. In contrast, more than 90% of knockout cells derived from E8.5 embryos were viable and correlated with wildtype data. The strong increase of dead cells at E9.5 indicated a restriction of the cardiovascular system even before the phenotype of the knockouts developed. The force of heart contractions has not been considered so far, suggesting that due to an progress of sarcomere impairment, the muscle contraction was present but less effective. Blood was not sufficiently distributed and tissues and cells started to enter the process of apoptosis. The dissection of embryos at a low temperature and enzymatical treatment could have enhanced the chance of apoptosis. However, the degradation of Titin could not be correlated with cardiomyocyte necrosis since SDS-agarose gel electrophoresis demonstrated that the wildtype as well as the truncated Titin protein showed degradation but rather due to processing.

5.4 Titin isoforms during embryogenesis

The expression study of Titin isoforms was limited to cardiac cells since primary muscle fibres appear at about E11.0 to E14.0 in the mouse limbs. Progenitor cells originate in

the somites (Ontell and Kozeka, 1984). Since knockout embryos were lethal by E11.5 the analysis of skeletal muscle cells was precluded.
During embryonic development the large cardiac specific N2BA Titin isoform with a size of 3.7 MDa is expressed. It disappears in the heart after birth and is replaced by the small N2B isoform (3.0 MDa). The adult Titin N2B isoforms are lacking various internal exons of the Ig domains and PEVK regions. The shift by about 700 kDa ensures higher passive stiffness (Opitz et al., 2004; Lahmers et al., 2004). Agarose gel electrophoresis revealed that Titin often migrated as two closely-spaced bands designated as T1 and T2 (Warren et al., 2003). These findings have been confirmed by analyzing knockout, wildtype, and heterozygote embryonic Titin and wildtype and heterozygote Titin expressed in the adult heart. In the embryonic heart the large N2BA Titin isoform was expressed. Thus, differences in migration of truncated and full length Titin were more prevalent in the T2 Titin. The shorter N2B Titin was expressed in the adult heart. The reduced size enabled the separation of truncated and full length Titin in its native form. However, on protein level there was no differential expression of Titin's isoform in the absence of Titin's M-line observed.
Additional expression studies of three distinct Titin regions at different developmental stages were performed by RT-PCR. Correlating Titin's Z-disc and M-line expression determined the amount of full length versus a putative novex-3 Titin isoform. The novex-3 isoform, which contains a unique I-band Titin exon and encodes a truncated 625 kDa protein, does not include the M-line and the elastic N2B region (Bang et al., 2001). So far, the expression level of the novex-3 isoform was only characterized for cardiac and skeletal muscle of adult rat, mouse, bovine, and human (Bang et al., 2001). However, increased embryonic Titin M-line RNA levels indicated a higher ratio of full length Titin compared to the truncated novex-3 isoform in the developing embryo at E9.5. There was also reduced expression of the heart specific Titin N2B region that has not been observed before. It could imply altered elastic properties of the Titin filament system. The higher ratio of full length and truncated Titin was maintained throughout the embryonic development. However, before birth a balanced ratio of Titin's Z-disc and M-line transcripts was observed. This implied an increased expression of shorter Titin proteins which seem to improve the order of contractile filaments and therefore the efficiency of sarcomere contraction.
The expression pattern of Titin isoforms was not changed in the absence of Titin's M-line at E9.5. Thus, the truncation by 200 kDa was not compensated by an increased expression of shorter isoforms.

5.5 Titin in sarcomere assembly

Impaired myofibril assembly and cardiac dysfunction caused lethality of knockout embryos. Until mid-gestation heterozygote matings delivered offspring at an expected Mendelian

ratio but viable homozygous knockout mice were not born. Morphology and cardiac development in knockout embryos appeared normal until E9.5. A beating heart in knockouts showed that cardiac progenitor cells differentiated into cardiomyocytes that contain myofibrils. Thus, Titin's M-line region seemed to be dispensable for the initial steps of myofibrillogenesis when premyofibrils fuse to nascent myofibrils.

Sarcomere assembly has been shown to be different in skeletal muscle and cardiac muscle cells. In developing skeletal C2C12 myotubes the integration of Titin's C-terminus into the M-band appeared almost simultaneously with the formation of Z-discs (Kontrogianni-Konstantopoulos et al., 2005). In contrast, data on embryonic chicken hearts showed a delayed integration of Titin's C-terminus into the M-band (Dabiri et al., 1997; Ehler et al., 1999). However, the importance of Titin's M-line for sarcomere assembly was shown by cultured myoblasts that expressed a truncated Titin protein lacking its kinase domain and downstream sequences. Impaired sarcomere assembly in these cells suggested that Titin's M-line is important in organizing myofibrils (Miller et al., 2003a). Nevertheless, the capability of mutated myoblasts to contract was not discussed.

The Titin M-line knockout model enabled to elucidate Titin's role in myofibrillogenesis *in vivo*. On ultrastructural level, wildtype as well as Titin M-line deficient myofibrils showed the presence of Z-discs and M-bands at E9.0. The integration of Titin into the sarcomere was addressed by localization studies on cryosections. Confocal images showed periodic staining of the heart specific Titin N2B region in the absence of Titin's M-line. Together with regularly spaced dots of sarcomeric α-Actinin this indicated correct I-band assembly in nascent myofibrils of knockout cells. This result contradict with the data from cultured myoblasts described before. They found that Titin's M-line and its kinase were important to form the structure of the Z-disc and I-band explaining their findings by a reduced kinase activity and a decreased phosphorylation of T-cap (Miller et al., 2003a).

Unlike Titin's N-terminal region, the M8/M9 epitope that was not excised by recombination did not show integration of Titin's C-terminus into the sarcomeric M-band. Thus, Titin failed to form a continuous elastic filament. Concluding one can say that the integration of Titin into the I-band does not require the M-line region whereas the incorporation of Titin into the M-band failed. Disassembly of the sarcomere could be triggered by Titin's failing to incorporate properly. Thus, Titin's C-termini from the opposite half sarcomere could not interact with each other resulting in an unstable M-band lattice. However, since M-line deficient myofibrils were able to contract one can hypothesize that the assembly of the I-band is more important than the formation of the M-band. Whether the I-band assembles earlier than the M-band and whether Titin's integration into the M-band was delayed as observed in chicken cardiomyocytes could not be confirmed. Wildtype cardiomyocytes showed proper localization of Titin at the Z-disc and M-band at the same stage of differentiation.

5.5.1 Titin's M-line and Myomesin in myofibrillogenesis

The M-band is an important element of the sarcomeric cytoskeleton that maintains the thick filament lattice. Myomesin is an essential component of the M-band and is expressed in all types of vertebrate striated muscle fibres. The major isoform in the embryonic heart is Myomesin-EH. It is expressed in the mature localization pattern as soon as the first myofibrils are assembled (Agarkova et al., 2000, 2004). The N-terminal and central part of Myomesin contain binding sites for Myosin and Titin forming a two-dimensional model of the M-band (Bahler et al., 1985; Nave et al., 1989; Obermann et al., 1996). Recent data showed that Myomesin can form antiparallel dimers via a binding site residing in its C-terminal domain 13. Thus, similar to α-Actinin in the Z-disc, the Myomesin dimers cross-link the contractile filaments in the M-band and have the ability to adapt to different stress requirements (Lange et al., 2005a). So far, there are no *in vivo* data on the interaction complex of Myosin, Titin, and Myomesin in early cardiac development. Following integration of Myomesin into the M-band dependent on Titin's M-line could however addressed this question. Although Myomesin's binding site was deleted in knockout embryos it was incorporated into the sarcomere. It localized properly between Z-discs as demonstrated by co-staining with the Z-disc protein α-Actinin. Nevertheless, in knockout animals Myomesin staining was more diffuse. This reflected the reduced number of binding sites in the absence of Titin's M-line, since Myomesin levels were not upregulated in knockout versus wildtype animals as shown by western blot analysis. Anyway, the data also demonstrated that myofibrils of knockout embryos were less well organized in the M-band than in the I-band. The failure to cross-link Myomesin and Titin in the M-band could have caused a higher mobility of the sarcomeric filaments and thus increased mechanical strain on the growing sarcomere, which ultimately led to disassembly. The data on cultured myoblasts that expressed a Titin protein lacking the kinase domain and its C-terminal region showed similar results. Despite the fact that a wildtype allele of the Titin gene was still expressed, heterozygous myoblasts showed impaired myofibrillogenesis, disorganization of Z-lines, and shorter myotubes (Miller et al., 2003a). In contrast, staining for Myomesin was less affected suggesting that the assembly of Myomesin into the M-band is an early event that does not depend on a full complement of Titin molecules in the A-band. Since only one allele was targeted there might be a compensation by full length Titin explaining localization of Myomesin to the M-band.
In summary, Myomesin did incorporate in the M-band of knockout sarcomeres and did not require the presence of Titin. Nevertheless, sarcomere disassembly demonstrated that a discontinuous Titin filament in the M-band was incompatible with maintenance of sarcomere structure under increased mechanical strain.

5.6 Titin's M-line in signal transduction

In recent years groups concentrated on a role of Titin as a biomechanical sensor and signaling molecule. Studies identified many Titin interacting proteins that were hitherto unknown components of the sarcomere. Some of these ligands do not only bind Titin but are also found in the nucleus, suggesting that they are part of interaction pathways between Titin and the nucleus. Their protein binding sites are not randomly distributed along the Titin filament but are restricted to certain regions such as in and near the Z-disc, in the central I-band region, and in the M-line region of the molecule. In cell culture experiments Titin's M-line region was shown to be important for sarcomere assembly and sarcomere integrity mediated by interacting with structural and signaling proteins. The deletion of these binding sites in Titin M-line deficient embryos addressed sarcomere organization *in vivo*.

5.6.1 Titin's kinase activity

The M-line region of Titin contains a conserved serine/threonine kinase domain. This domain and its substrate T-cap have been implied in the early stages of sarcomere assembly based on structural and *in vitro* data (Mayans et al., 1998). They demonstrated that there is an inhibition of the active site of the kinase which is removed by a dual-activation process. The phosphorylation of a tyrosine and binding of Calcium/Calmodulin to the regulatory tail. It remained to be addressed if these data can be confirmed in sarcomere assembly *in vivo*. Expression analysis of Calmodulin and T-cap in Titin M-line deficient embryos elucidated this question.

Calmodulin belongs to the main group of Calcium binding proteins. It was shown to be indispensable for activation of Titin's kinase activity (Mayans et al., 1998). Moreover, sarcomeric arrangement of Titin was also shown to depend on Ca^{2+} levels (Harris et al., 2005). This could not be shown since there was no significant compensatory effect of Calmodulin on transcript level in knockout embryos compared to wildtype. Although Calmodulin was expressed in significant amounts at the time when the knockout phenotype developed, it can be excluded as a potential candidate contributing to sarcomere assembly by activating Titin's kinase.

Activation of the Titin kinase and phosphorylation of T-cap in differentiating myocytes has been hypothesized to be involved in reorganization of the cytoskeleton during myofibrillogenesis (Mayans et al., 1998). The impaired organization of the Z-disc in myoblasts expressing a truncated Titin protein was explained by a reduced kinase activity that resulted in decreased phosphorylation of T-cap (Miller et al., 2003a). However, there is no suitable animal model supporting this hypothesis, yet. The data obtained when analyzing Titin M-line knockout embryos in this study argue against the proposed hypothesis. Not

only did sarcomeres form in the absence of Titin's kinase domain, but furthermore, the T-cap protein was not detectable in early sarcomere development by western blot. It was first expressed at E15.5 at a time when sarcomere already matured to form fully functional myofibrils and the knockout phenotype caused lethality. The earliest detection of the T-cap transcripts in mouse was shown to be at E10.5 when also fully assembled sarcomeres already exist (Gregorio et al., 1998). Moreover, patients deficient in T-cap develop Limb Girdle Muscular Dystrophy type 2G (Moreira et al., 2000). Since even homozygous patients do not show symptoms before 2 to 15 years of age, sarcomeres can assemble even in the absence of T-cap. The truncated T-cap protein expressed in these patients is not detected in the sarcomere - nevertheless, ultrastructure is maintained (Moreira et al., 2000). Combining data from the literature and data obtained in this study, it is unlikely that Titin signaling through T-cap is the basis for impaired sarcomere assembly.

5.6.2 Titin's kinase as a force sensor

The kinase domain has been proposed to act as a force sensor by converting mechanical stress into biochemical signals (Grater et al., 2005). The conversion has been shown to be mediated by the zing-finger protein Nbr1 (neighbor of Brca1 gene 1) that was shown to form a complex with Sqstm1 (Sequestosom1, p62). Both, Nbr1 and Sqstm1 were observed as *in vitro* substrates of Titin's kinase (Lange et al., 2005b). MuRF-2 another muscle-specific RING-finger protein in turn interacts with Sqstm1 and translocated from the M-band into the nucleus upon mechanical arrest. In the nucleus MuRF-2 bound to the serum response factor (SRF), transcriptional activity was repressed, and muscle protein expression was regulated (Lange et al., 2005b). There are additional *in vivo* data on skeletal muscle that show also mechanically induced re-localization of MuRF-2 to the nucleus followed by atrophy (Lange et al., 2005b). However, so far there are no data on the interaction complex of MuRF-2, Sqstm1, and Nbr1 in sarcomere assembly and increased mechanical strain in cardiac muscle cells available. Thus, proteins involved in this pathway were investigated for their expression in Titin M-line deficient embryos at E9.5 and in wildtype embryos throughout embryogenesis. There was no differential expression of Nbr1, Sqstm1, and MuRF-2 in the absence of Titin's M-line. Moreover, Nbr1 and Sqstm1 transcripts were found at low levels throughout the embryonic development. On protein level Sqstm1 could not be detected before E12.5, at a time when no viable knockout was present. In summary, the Titin kinase substrates Sqstm1 and Nbr1 were expressed only at low levels suggesting that they are of limited importance in cardiac myofibrillogenesis. Moreover, patients with a mutation in the Titin kinase domain that disrupted the binding site for Nbr1 and caused a hereditary muscle disease, did not exhibit a clinically dominant cardiac involvement (Lange et al., 2005b). Thus, the underlying molecular mechanism of Titin's kinase signaling activity might be different between cardiac and skeletal muscle.

There are *in vivo* data describing that the expression of MuRF-2 is restricted to cardiac tissues and appears to be downregulated during cardiac development (Centner et al., 2001; McElhinny et al., 2004). Data obtained in this study confirmed an increased expression of MuRF-2 on protein level from E15.5 whereas significant amounts of RNA appeared already at E9.5. However, the knockout embryos showed a cardiac dysfunction that was accompanied by mechanical arrest of myofibrils. It remains to be addressed if the conversion of mechanical to biochemical signal is the molecular mechanism that caused impaired sarcomere assembly since Nbr1 and Sqstm1 were not expressed. The role of Titin as a biomechanical sensor and signaling molecule in myofibrils in early cardiac cells could be different to skeletal and adult sarcomere assembly. There might be additional signaling molecules that disappear after birth and have not been identified yet. Moreover, the described signaling pathway was identified by *in vitro* studies and so far only confirmed *in vivo* in skeletal muscle cells (Lange et al., 2005b).

The different molecular mechanism underlying sarcomere assembly during development and maintenance of preexisting sarcomeres in adulthood is depicted in Fig. 5.1. This model propose that the Titin kinase substrate T-cap and the signaling molecules FHL2, Sqstm1, SRF, and Nbr1 are dispensable for sarcomere assembly but become relevant for sarcomere maintenance after gestation. MuRF-1, MuRF-2, and Myomesin are suggested to be proteins necessary in early cardiac development.

In addition to its signaling role in myofibrillogenesis, MuRF-2 has been suggested to act as a cytoskeletal adaptor protein. MuRF-2 knock-down studies in skeletal myoblasts showed delayed myoblast fusion and impaired sarcomere assembly. Furthermore, contractile activity was also affected (McElhinny et al., 2004). The phenotype observed in Titin M-line deficient embryos was similar. However, if the phenotype can be explained by structural features of MuRF-2 or its signaling properties modulated via Titin-based mechanisms remains unclear.

In summary, there are different *in vitro* data showing activation of Titin's kinase by phosphorylation and Calcium/Calmodulin binding (Mayans et al., 1998) and by mechanical stimulation (Grater et al., 2005). This was shown to be important for sarcomere assembly and controlling muscle gene expression (Lange et al., 2005b; Mayans et al., 1998). For the first time, the Titin M-line knockout could show *in vivo* that activation of Titin's kinase is dispensable for initial sarcomere assembly. The data rather suggest that the active kinase is prevalent for maintenance of preexisting sarcomere and for conversion of signals to control muscle gene expression and protein turnover. Moreover, it remains to be addressed whether Titin's kinase is catalytically active in general since there are also data showing that kinases (guanylate kinase-associated protein) might lack enzymatic activity at all (Kim et al., 1997).

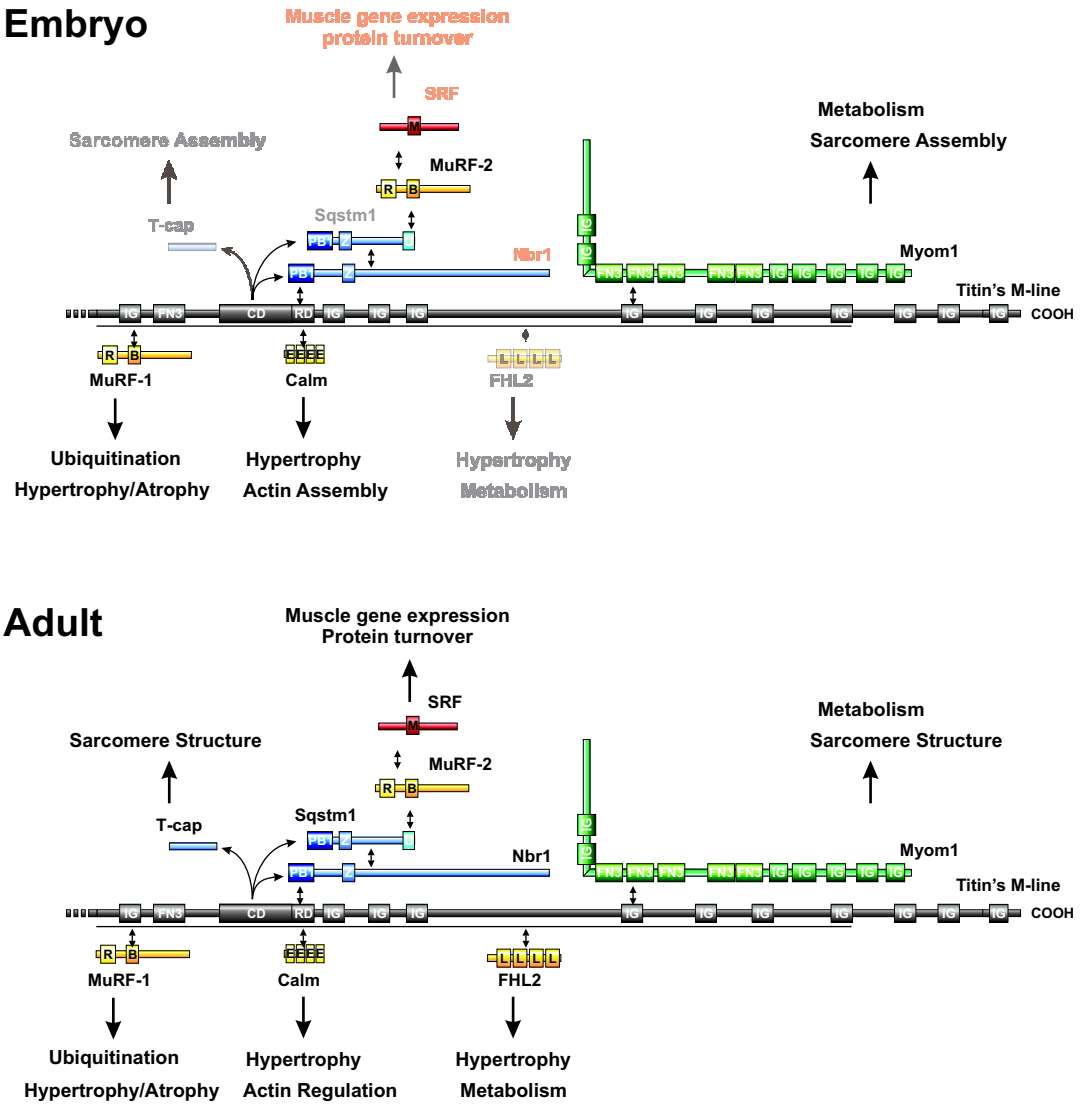

Figure 5.1: Signaling and structural functions of Titin's M-line region. The region deleted in Titin M-line deficient knockouts (underlined) contained binding sites for signaling proteins such as MuRF-1, MuRF-2, Nbr1, Sqstm1, Calm, FHL2, T-cap, and the structural protein Myomesin. Translucent symbols are proteins proposed to be dispensable for early cardiac development.

5.6.3 Signaling molecules in sarcomere assembly and signaling

As already discussed, Sqstm1 has been implicated in the conversion of mechanical to biochemical signals via Titin's kinase domain and MuRF-2 (Lange et al., 2005b). Analyzing expression of Sqstm1 during embryonic development revealed that Sqstm1 was detected first in the heart at E12.5. This explains the failed localization of Sqstm1 expression in wildtype cardiomyocytes at E9.5 and confirmed that Sqstm1 is dispensable for initial sarcomere assembly. However, data obtained from localization studies on skeletal muscle cells

showed that Sqstm1 did not localize to Titin's M-line as expected but to the I-band. This was shown by a striated staining with the Titin's M8/M9 epitope antibody. The result strongly indicated that signaling pathways required for sarcomere assembly and maintenance in cardiac muscle cells might be different to those observed in skeletal muscle cells. Thus, one can conclude that Titin and signaling molecules act as biomechanical sensor and transmitter depending on the developmental stage and tissue they are expressed in.
Downstream of Titin's kinase domain binds FHL2. It is a four and a half LIM-only protein. It mediates targeting of metabolic enzymes to Titin's is2 region (Fig. 1.2) located between the m3 and m4 Ig domains (Labeit and Kolmerer, 1995; Lange et al., 2002). In the mouse heart FHL2 was first detected at embryonic stage E12.5 but expression was upregulated prenatally (Lange et al., 2002). The generation and analysis of mice carrying a null mutation of the FHL2 gene showed that FHL2 is indeed highly expressed in the heart. However, its deficiency did not lead to lethality but normal cardiac function was observed (Chu et al., 2000). Expression studies on wildtype and Titin M-line deficient embryos confirmed these findings. FHL2 was expressed constantly low during embryonic development and was first detected at E15.5 by western blotting. Since this is a time when the knockout phenotype caused lethality one can exclude FHL2 as potential candidate to be involved in initial myofibrillogenesis and mediating signaling that might have triggered sarcomere disassembly.

5.7 Atrophy and hypertrophy in knockout embryos

Cardiomyopathy is a disease of the cardiac muscle. There is a variety of cardiomyopathies that are mostly accompanied by atrophy or hypertrophy of the cardiac muscle. Cardiac hypertrophy is the enlargement of the heart as a result of an increase in the size rather than the number of constituent cells. In contrast, cardiac atrophy is the decrease in size of the heart owing to disease, injury, or lack of use.

5.7.1 Atrophy regulated by MuRF-1

Atrophy is a phenotype that became prominent at E11.0 in the hearts of Titin M-line deficient embryos. It resulted in instability of the ventricular wall with pericardial effusion. Cardiac muscle atrophy refers to the loss of muscle tissue that is accompanied by a reduced size and strength of the heart. Muscle atrophy has been shown to be associated with the loss of cross striations of α-Actinin and Titin (Kawaguchi et al., 1997). Several candidates have been identified to function as molecular mediators of atrophy. Among these is MuRF-1, a ubiquitin ligase (Bodine et al., 2001) shown to bind *in vitro* to the Titin repeats A169/A170 that are adjacent to the Titin kinase domain (Centner et al., 2001).

The maintenance of muscle mass is controlled by a balance between protein synthesis and protein degradation pathways, which is thought to shift towards protein degradation during atrophy. The accelerated proteolysis via the ubiquitin-proteasome pathway can cause muscle atrophy (Jagoe and Goldberg, 2001). MuRF-1 is found in the nucleus (McElhinny et al., 2002) and regulates muscle atrophy likely through the degradation of muscle proteins by the proteasome pathway (Bodine et al., 2001). Thus, the interaction of MuRF-1 with Titin links myofibril signaling pathways with protein turnover (McElhinny et al., 2002). Although in Titin M-line knockout mice the binding site for MuRF-1 was deleted and their hearts suffered from atrophy MuRF-1 expression was not differentially regulated and a loss of cross striation was not observed. Thus, in this study it could not be confirmed that MuRF-1 expression was strongly induced during muscle atrophy. This result was not surprisingly since data obtained so far, were derived from skeletal muscle cells (Bodine et al., 2001; Lecker et al., 2004). The underlying signaling mechanism could be different in cardiac muscle cells as well as in early cardiac development. Therefore, it remains unclear if degradation of sarcomeric proteins in the cardiac muscle requires the proteolytic function of MuRF-1. However, since there was no binding of MuRF-1 with Titin in knockout embryos one can conclude that the interaction between myofibril signaling and muscle gene expression was impaired.

5.7.2 The MAPK pathway and hypertrophy

The mitogen-activated protein kinase (MAPK) pathway has been implicated in the induction of cardiac hypertrophy. It has also been implied in regulating serial versus parallel assembly of sarcomeres (Nicol et al., 2001). Mitogen-activated protein kinases represent key signaling molecules through which cells integrate a variety of extracellular stimuli and transduce intracellular signals. The extracellular signal-regulated kinase 5 (Erk5) is a mitogen-activated protein kinase. Deletion of Erk5 resulted in defective blood-vessel and cardiac development leading to embryonic lethality around E9.5 and E10.5 (Regan et al., 2002). Erk5 is activated via MEK5 (Chao et al., 1999). The myocyte enhancer factor 2C (Mef2C) was shown to be a substrate of activated Erk5 (Kato et al., 1995) and a cardiac transcription factor that is upregulated during cardiac hypertrophy (Kolodziejczyk et al., 1999). TGF-β is a cytokine that was shown to activate proteins of the MAPK pathway (Yamaguchi et al., 1995). It is induced during cardiac development (Millan et al., 1991) and upregulated in the myocardium that has undergone hypertrophy as a result of pressure overload (MacLellan et al., 1993). Another signaling protein is the MAP kinase-activated protein kinase 2 (MAPKAP) that has been shown to be involved in stress-activated signal transduction in the myocardium (Zu et al., 1997).
Data obtained in the adult conditional Titin M-line knockout showed increased expression of proteins involved in the map kinase signal transduction (Gotthardt et al., 2003). Titin

M-line knockout embryos were required to be investigated for the (MAPK) pathway and other signaling proteins since knockouts showed impaired lateral growth of myofibrils and cardiac atrophy. However, hypertrophic signaling response secondary to the cardiomyopathy phenotype could not be detected in Titin M-line deficient embryonic hearts using hypertrophy markers, including genes of the MAP kinase pathway. The mechanism underlying impaired lateral sarcomeric growth and initiation of atrophy in knockout myofibrils was independent of signal transduction by the mitogen-activated protein kinase (MAPK) pathway and other investigated signaling proteins.

5.8 Future directions

Analyzing Titin M-line deficient embryos helped to understand how Titin and its binding protein shape and regulate sarcomere assembly. It provided novel findings that are important and contribute to the characterization of cardiac myofibrillogenesis. However, data obtained in this study did not explain sufficiently if Titin's M-line is important for structural integrity by binding to Myomesin or whether its signaling properties via MuRF-1 and MuRF-2 are essential to function properly in early cardiac development.
Cell culture experiments with M-line deficient cardiomyocytes could be a future study to investigate structural aspects. The Flexcell® system allows stimulation of controlled mechanical strain and helps to investigate disassembly dependent on the presence of Titin's kinase. Since knockouts are embryonic lethal only little amounts of heart tissue are available. Thus, a pure culture of cardiomyocytes could not be obtained so far. Therefore, embryonic stem cells derived from blastocysts were generated. These cells are homozygous for the allele flanked with loxP sites at M-line exon 1 and 2. After transient transfection with Cre recombinase, homozygous ES-cells should be available. They can be differentiated to cardiomyocytes and used for further studies.
This work concentrated on the analysis of homozygous knockout embryos. However, previous studies also showed that targeting only one allele can also trigger impaired sarcomere assembly similar to that observed in this study (Miller et al., 2003a). Heterozygous Titin M-line deficient animals were viable, fertile, and did not show any obvious phenotype. However, since Titin's C-terminus was altered and could not integrate into the M-band one could focus on the compensatory effect of full length Titin. Data might help to understand the difference between Titin's M-line function in embryonic development, prenatal, and in adult. Moreover, adult mice are more eligible for applying force or mechanical arrest to skeletal muscle cells. Using denervation experiments or hind limb fixation using plaster cast could increase the understanding of Titin's kinase activation and signaling pathways that lead to muscle gene expression and protein turnover.
Until now, there are many signaling and structural proteins that have been shown to contribute to sarcomere assembly and maintenance of preexisting sarcomeres by interacting with Titin. However, differences in the adult and embryonic phenotypes of Titin M-line knockouts are likely related to altered molecular mechanisms that are necessary to build up and maintain a contractile sarcomere. Since the molecular mechanism of sarcomere assembly during embryonic development is still not fully understood, additional studies could focus on identifying new Titin binding proteins. The well established yeast two-hybrid approach would be a suitable technique as also shown by previous identifications of important interacting proteins.
Knockout technology is commonly used to study the physiological functions of genes *in vivo* and identify the genes that encode potential targets. So far, data suggest that Titin's M-line is important for sarcomere assembly and integrity. For the future it is necessary to

distinguish between effects based on the kinase region or those that are dependent on Titin interacting proteins. A knockout model lacking only the Titin's kinase but preserving the other binding sites such as for MuRF-1 and Myomesin would fulfill these requirements. Embryonic lethality of this model might exclude binding proteins of Titin's M-line as potential candidate and would raise the importance of the kinase and its substrates for early cardiac development.