

# 1 Introduction

## 1.1 The heart and cardiac muscle cells

The heart is a hollow muscular organ that pumps blood through the vessels by repeated, rhythmic contractions. In this process deoxygenated blood from the body enters the right atrium through two great veins, the superior and the inferior vena cava. The blood then passes through the tricuspid valve to the right ventricle. The right ventricle pumps the deoxygenated blood to the lungs through the pulmonary artery. In the lungs gaseous exchange takes place and the blood releases carbon dioxide into the lung cavity and picks up oxygen. The oxygenated blood then flows through pulmonary veins to the left atrium. From the left atrium this newly oxygenated blood passes through the mitral valve to enter the left ventricle. The left ventricle then pumps the blood through the aorta to the entire body. The left ventricle is much more muscular than the right ventricle as it has to pump blood through the entire body, which involves exerting a considerable force to overcome the vascular pressure. As the right ventricle needs to pump blood only to the lungs, it requires less muscle tissue. The contractility of the heart is provided by cardiac muscle cells which can work continuously without fatigue. The heart wall is made of three distinct layers: the epicardium, myocardium, and endocardium. The outer layer is the epicardium which is composed of flattened epithelial cells and connective tissue. Beneath the epicardium is the much thicker myocardium that is made up of cardiac muscle cells. The endocardium consists of flattened epithelial cells and connective tissue which lines the chambers of the heart.

### 1.1.1 Cardiac development in the mouse

The heart is the first organ during embryogenesis that is functionally active by the formation of cardiac muscle cells within the myocardium. The cardiac muscle cells

are autonomous, stimulating their own contraction without a peripheral neural impulse.

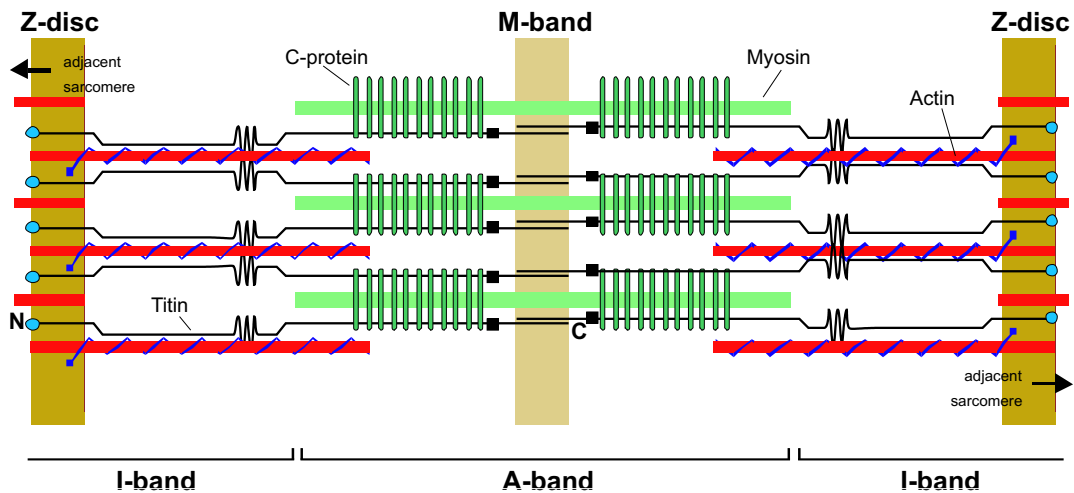
The first evidence of a developing heart can be seen in embryos with 3-5 pairs of somites that corresponds to an age of E7.5 after fertilization. The cardiogenic plate differentiate towards the end of the developmental age E8.0 to form a wide broad tubular mass and shows initially irregular contractions. The outflow tract directly leads into the first branchial arch artery. By E8.5 the heart is S-shaped and can be divided into a common atrial and ventricular chamber. Embryos with 11-13 pairs of somites (E9.0) contain a heart that beats powerfully and regularly. The myocardial wall of the common ventricular chamber extents and the degree of trabeculation increases in this period. When embryos at E9.5 are maintained under optimal culture conditions *in vitro*, the heart rate is about 100-110 beats/min. Except for the initiation of the septation of the common atrial chamber there are no fundamental changes until E10.5. At E11.5 first membranous components of the interventricular septum are formed. Form E12.0 the atrial septation is progressing apace and first evidence of interventricular septation of the common ventricular chamber can be observed. At E12.5 the separation of the outlet of the left ventricle (aorta) and right ventricle (pulmonary trunk) is completed. By day E15.0 all components of the heart are formed and have achieved their definite prenatal configuration (Kaufmann, 1992).

### 1.1.2 Muscle cells

There are 400 muscles in the human body that account for about one third of the human body weight. Muscle cells can be classified into three general types: skeletal, cardiac, and smooth muscle cells. Skeletal muscle cells form myofibrils and they in turn fuse to muscle fibers that are attached to the skeleton. These cells are striated, contract via nerve stimulation and contain in contrast to cardiac muscle cells more than one nucleus. Cardiomyocytes are also striated muscle cells but only found within the myocardium of the heart. Smooth muscle cells are non-striated muscle cells that are spindle shaped. They contain thick and thin filaments that slide against each other to produce contraction. The contraction of smooth muscle cells tend to be slower than that of striated muscle cells and can be sustained for long periods. Smooth muscle cells are found within the walls of hollow organs such as blood vessels, the uterus, and the gastrointestinal tract. Like cardiac cells they also have only one nucleus.

### 1.1.3 The Sarcomere

The skeletal as well as the cardiac muscle cell is composed of bundles of filaments called myofibrils. They reach throughout the cell and attach to the cell surface membrane at each end. The basic contractile unit of the striated muscle myofibril in vertebrates is the sarcomere. The sarcomere is a structure of filaments that gives skeletal and cardiac muscles their striated appearance. A sarcomere is defined as the segment between two neighboring Z-discs (Fig. 1.1). In electron micrographs of cross striated muscles the Z-disc appears as a series of dark lines. Surrounding the Z-disc is the region of the I-band. It is named after its isotropic properties under a polarizing microscope. Next to the I-band is the A-band with anisotropic properties. Within the A-band is a paler region designated as M-band.



**Figure 1.1: Schema of a skeletal muscle sarcomere.** A sarcomeric unit is delimited by the Z-disc located in the center of the I-band. The I-band contains the thin filament system that anchor within the Z-disc. The A-band comprises the thick filament that interacts via the C-protein with Titin. The central region is the M-band where Titin molecules overlap. Titin proteins span half the sarcomere from the M-band to the Z-disc (adapted from (Gregorio et al., 1999)).

Actin filaments, also called thin filaments are the major component of the I-band and extend into the A-band. They are formed of F-Actin that in turn is composed of helically polymerized G-Actin. Myosin forms the thick filament system. It extends throughout the A-band and is thought to overlap in the M-band (Fig. 1.1). The Myosin filament system is composed of a bundle of Myosin proteins. They are hexamers that consist of two identical heavy chains and 4 light chains.

The giant protein Titin extends from the Z-disc of the sarcomere, where it binds to the thin filament system, to the M-band, where it is thought to interact with the thick filament (Furst et al., 1988). The Titin molecule is the largest protein found and is also designated as third filament system. It provides binding sites for numerous proteins and plays an important role as sarcomeric ruler and as blueprint for the assembly of the sarcomere. Actin filaments and Titin molecules are cross-linked in the Z-disc via the Z-disc protein  $\alpha$ -Actinin. The M-band protein Myomesin cross-links the thick filament system and Titin's carboxy-terminus (C-terminus). The interaction between Actin and Myosin filaments in the A-band of the sarcomere is responsible for the muscle contraction.

### 1.1.4 Contraction of muscle cells

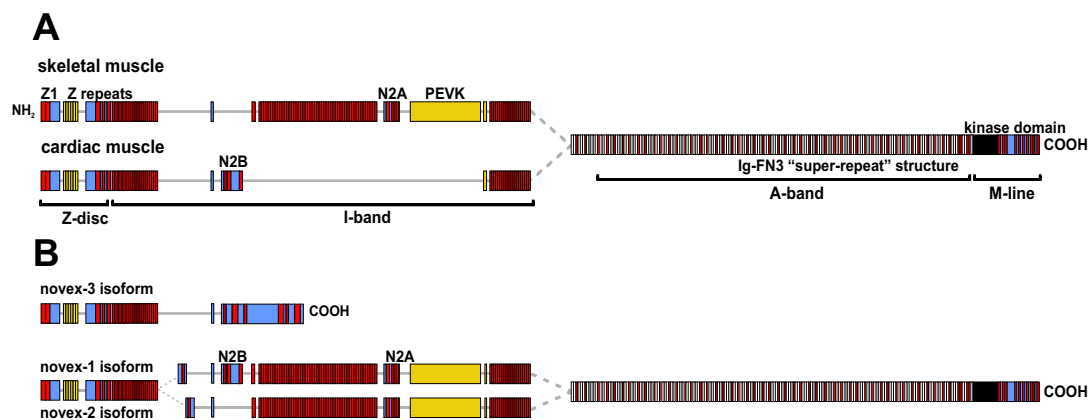
The contraction of striated muscle cells has been described in the sliding filament model. It is a complex and energy consuming process. In contrast to cardiac muscle cells the contraction of skeletal muscle cells is controlled by the nervous system and thus activated by the action potential that arrives at the axon of a motor neuron. Ion channels for Sodium and Potassium open. Because the cell membrane is more permeable for Sodium, it becomes more positively charged, triggering an action potential. The action potential on the muscle fiber causes the sarcoplasmic reticulum to release Calcium into the sarcomere. The protein Tropomyosin covers the Myosin binding sites of the Actin molecules. To allow the muscle cell to contract, Tropomyosin must be moved to uncover the binding sites. Calcium ions bind to Troponin molecules and alter the structure of the Tropomyosin, forcing it to reveal the cross bridge binding site on Actin. ADP is bound to Myosin and interacts with the newly uncovered binding sites on the thin filament. It then releases ADP and delivers a power stroke in which the Actin filaments are pulled along Myosin towards the center of the sarcomere. ATP binds to Myosin, forcing it to change conformation and break the Actin-Myosin bond, causing Myosin to assume its ready state. Myosin then hydrolyzes ATP to ADP and returns to a ready state. Muscle contraction ends when Calcium ions are pumped back out of the sarcomere into the sarcoplasmic reticulum. When Calcium is no longer present on the thin filament, Tropomyosin changes conformation back to its previous state to block the binding sites again. The action potential that triggers the heartbeat is generated within the heart itself and drives contraction of the heart from cell to cell through gap junctions.

## 1.2 Titin

About 25 years ago, a promising candidate emerged that could potentially explain poorly understood physiological properties of vertebrate myofibrils. Connectin was described 1977 as an elastic protein of the muscle (Maruyama et al., 1977). In 1979 electrophoretic analysis of protein components of striated muscle cells identified a molecule with a megadalton size. This protein was named “Titin” (Wang et al., 1979) and found to be identical with Connectin. The name was chosen after the “titans” that were a race of powerful deities that ruled during the legendary Golden Age in the Greek mythology.

### 1.2.1 Molecular structure

Full length Titin has a size of 3.7 MDa and is the largest protein known so far (Labeit et al., 1992; Labeit and Kolmerer, 1995). The gene locus was mapped to chromosome 2q in humans (Pelin et al., 1997) and gives rise to a transcript of up to 100 kilobases. In the mature sarcomere Titin has a length of  $>1\mu\text{m}$  (Nave et al., 1989). It is composed of up to 297 copies of immunoglobulin (Ig) repeats and fibronectin type III (FN3) domains that account for about 90% of Titin’s mass (Fig. 1.2, A) (Improta et al., 1998; Muhle-Goll et al., 2001).



**Figure 1.2: Domain structure of skeletal and cardiac muscle Titin.** (A) Composition of skeletal and cardiac Titin. Ig domains (red), unique sequences (blue), and FN3 domains (white) set up the structure. The I-band contains the PEVK region (yellow) and in the cardiac muscle the N2B segment. The A-band is composed of Ig-FN3 repeats and Titin’s M-line contains the kinase domain. (B) The novex-1 and 2 isoforms contain unique I-band exons N-terminal of the N2B region. Novex-3 lacks the N2B region and all downstream domains. All isoforms are expressed in skeletal and cardiac muscle (adapted from (Bang et al., 2001)).

Titin extends from the Z-disc to the M-band. The Z-disc of skeletal and cardiac muscle sarcomere is one of the most densely packed cellular structures. Titin's Z-disc region consists of two Ig-like domains, referred to as Z1 and Z2. X-ray crystallography recently showed, that this region is assembled into an antiparallel (2:1) sandwich complex by the Z-disc ligand T-cap (Zou et al., 2006). Titin's I-band region is composed of Z-repeats, Ig-domains, unique sequences, and a so called PEVK region. This segment is rich in P (proline), E (glutamate), V (valine), and K (lysine). Depending on the isoform between 0.8 -1.5 MDa of Titin account for the I-band region and provide Titin with its elastic properties (Itoh et al., 1988). The A-band is characterized by an Ig-FN3 super-repeat structure (Fig. 1.2, A) that account for about 2 MDa of the relatively stiff C-terminus of Titin (Labeit and Kolmerer, 1995). Titin's C-terminal A-band region integrates into the M-band and is called Titin's M-line region. This region is encoded by 6 exons, of which M-line exon 5 is alternatively spliced (Kolmerer et al., 1996). Titin's kinase domain is encoded within M-line exon 1 (Labeit and Kolmerer, 1995).

### 1.2.2 Titin isoforms

There are three main Titin isoforms. The N2A isoform is expressed in skeletal muscle (Fig. 1.2, A). Titin's N2B and the larger N2BA Titin isoforms are exclusively in cardiac muscle whereas the N2BA isoform is predominantly expressed during embryogenesis. All isoforms are differentially expressed and thus modulate passive muscle stiffness (Cazorla et al., 2000).

There are 3 distinct domains in Titin's Z-disc and I-band region that determine its elastic property. The tandem-Ig, the PEVK, and N2B region are the spring elements of Titin. The elasticity can be distinguished between tissues according to the numbers of tandemly arranged Ig domains. The elastic vertebrate striated soleus muscle expresses 90 tandem-Ig repeats, whereas the heart only expresses 37 of these domains (Labeit and Kolmerer, 1995). The central localized PEVK region forms another motif that is differentially expressed. The shortest PEVK segment is found in the heart, the stiffest striated muscle. In skeletal muscle cells the PEVK region is much longer (Labeit and Kolmerer, 1995). The region N-terminal of the PEVK segment is also differentially expressed. Skeletal muscle sarcomeres express the N2A isoform, whose length varies in different tissue types. Cardiac cells express N2B and N2BA isoforms, the latter of which contain structural elements from both the N2A and N2B isoforms. They vary in length in their proximal Ig segments and PEVK domain. The expression of different levels of N2B and N2BA Titin likely contributes

to the elastic diversity of atrial and ventricular myofibrils (Freiburg et al., 2000). Additional isoforms are generated by alternative splicing. The Z-disc region was not only shown to be differentially expressed in skeletal and cardiac muscle but also the expression of diverse numbers of the Z-repeat copies (a sequence of 45-residue repeats within the Z-disc) is regulated during embryonic development (Sorimachi et al., 1997).

Additionally, three unique I-band Titin exons (named novex-1 to -3) were identified in human skeletal and cardiac muscle and represent other isoforms (Fig. 1.2, B). Novex-3 functions as an alternative C-terminus of Titin. It forms a complex with Obscurin a giant sarcomeric protein composed of adhesion modules and signaling domains that is likely to participate in myofibrillar signaling (Bang et al., 2001). Novex-3 has a size of about 700 kDa and spans from the Ig domain Z1 to the novel exon 3. The novex-1 and novex- 2 isoforms contain unique exons that are located N-terminal of the N2B region (Bang et al., 2001). Their function is unknown so far.

### 1.2.3 Functions of Titin

A single Titin molecule extends from the Z-disc to the M-band. According to its size and location within the sarcomere Titin fulfills several functions in the striated muscle: it directs assembly of contractile filaments and provides elasticity by its serial spring elements. Based on the presence of a kinase domain and phosphorylation sites Titin acts presumably in myofibrillar signaling (Centner et al., 2001; Gregorio et al., 1999; Labeit and Kolmerer, 1995; Trinick, 1996).

Titin has been proposed to serve as a protein ruler of the thick filament, keeping the Myosin filament centered within the sarcomere during force generation (Horowitz et al., 1986). Furthermore, the  $\alpha$ -Actinin binding sites at Titin's C-terminus cross-linking Titin and thin filaments, are required for the structural integrity of the sarcomere (Gregorio et al., 1998). Titin acts as a molecular spring due to the elastic properties of the Ig repeats and PEVK region within the I-band (Labeit and Kolmerer, 1995). These regions provide elasticity depending on the physiological demands of the tissue where they are expressed. Finally, Titin's kinase domain that is located at Titin's C-terminus has been proposed to convert mechanical to biochemical signals by interacting with signal transduction proteins. In summary, Titin plays a role in muscle assembly, force transmission, maintenance of resting tension, and signal transduction (Furst et al., 1988; Labeit and Kolmerer, 1995).

### 1.2.4 Non-muscle Titin

Titin was originally discovered and extensively described in striated muscle tissue. Nevertheless, Titin was also identified to be a component of various non-muscle tissues such as the cytoskeleton of the intestinal epithelial cell brush border (Eilertsen and Keller, 1992). Titin has been observed to localize to the nucleus and to condensed chromatids of human and drosophila cells. This suggests that Titin may participate in mitosis (Machado et al., 1998) and has non-muscle functions. Analyzing molecular forces of condensed chromatin even revealed elastic properties similar to those found in Titin (Houchmandzadeh and Dimitrov, 1999). New evidence of additional non-muscle functions derived from a yeast-two hybrid screen. Titin's C-terminus (is6 and is7 of Titin's M-line) was shown to interact with Lamin A (Zastrow et al., 2006). Lamins are intermediate filament proteins in the nucleus determining nuclear size, shape, mechanical integrity, and positioning of nuclear pores (Dahl et al., 2004). Moreover, there are additional *in vitro* data available that localize Titin to the mitotic spindle machinery providing a molecular basis for chromosome structure, elasticity, and cell division (Machado et al., 1998; Wernyj et al., 2001). This hypothesis was supported by a study of D-Titin identified in drosophila. Mutations in D-Titin show to cause chromosome undercondensation, chromosome breakage, loss of diploidy, and premature sister chromatid separation (Machado and Andrew, 2000).

### 1.2.5 Interaction of Titin with structural and signaling proteins

The Titin protein provides binding sites for sarcomeric proteins that contribute to a precise assembly of myofibrillar proteins *in vivo*. These protein binding sites are not randomly distributed along the Titin filament but instead seem restricted to certain areas at and near the Z-disc region, in the central I-band region, and in the M-line region of the molecule.

*Titin's Z-disc region.* Co-localization studies, two-hybrid interaction screens, and pull-down assays have demonstrated that Titin's Ig domains Z1 and Z2 interact with the N-terminal region of T-cap (Titin-cap, telethonin) (Gregorio et al., 1998). X-ray crystallography revealed that T-cap assembles Titin into an antiparallel (2:1) sandwich complex at the Z-disc (Zou et al., 2006). The physiological importance of the interaction has been supported by linking mutations within Titin's Z-disc to a subset of human dilated cardiomyopathy (Knoll et al., 2002). Mutations in the gene encoding T-cap were found to cause Limb-girdle muscular dystrophy type 2G (Moreira et al., 2000). Interestingly, T-cap has also been described to be a substrate



of Titin's kinase domain during myofibrillogenesis (Mayans et al., 1998). The most N-terminal region of Titin contains binding sites for  $\alpha$ -Actinin (Ohtsuka et al., 1997a,b) and for the C-terminal region of Obscurin (Bang et al., 2001). These binding sites comprises two specific Ig-like domains of Titin. Obscurin is a giant multidomain muscle protein (approximately 800 kDa) that has been shown to co-assemble with Titin during myofibrillogenesis (Young et al., 2001). It was demonstrated to be indispensable for positioning of contractile proteins, for the structural integration, and stabilization of myofibrils in cardiac cells and plays also a role in hypertrophic response (Borisov et al., 2005). The interaction of Actin with Titin's Z-disc region helps to anchor the Titin molecule at its amino-terminus (N-terminus) and maintain a high stiffness of relaxed skeletal and cardiac myofibrils (Linke et al., 1997).

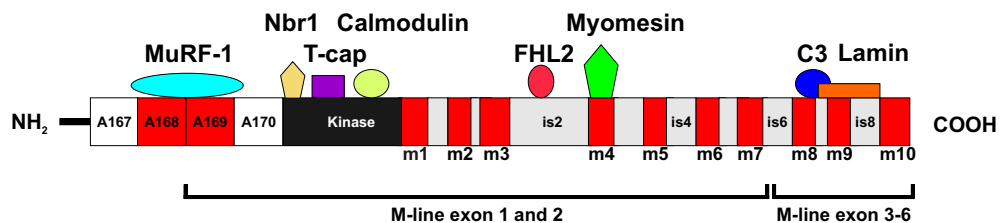
*Titin's A- and I-band region.* In the A-band, Titin forms a complex with Myosin's C-terminus (Wang et al., 1992) and with the C-protein (Myosin-binding protein C, MyBP-C) (Freiburg and Gautel, 1996). The C-protein binds to Titin's C zone of the A-band that consists of a series of 11x11-Ig and FN3 domain super-repeats (Labeit et al., 1992; Labeit and Kolmerer, 1995). The interaction of Titin with the muscle-specific Calpain-3 protease (p94, C3) is localized at two distinct regions, the central I-band region and Titin's C-terminus (Sorimachi et al., 1995). A deletion of Titin's Ig repeats (exons I81 to I83) that bind to Calpain-3 in mouse was reported to cause muscular dystrophy with myositis (mdm) (Garvey et al., 2002).

*Titin's M-line region.* The most C-terminal region of Titin is designated as Titin's M-line region and is encoded by 6 exons. It has a size of more than 200 kDa and contains also a kinase domain encoded within M-line exon 1. The mouse model that was investigated in this work is M-line exon 1 and 2 deficient. This region contains several protein binding sites and will therefore be discussed in a separate chapter (see 1.3.1).

## 1.3 Titin's M-line region and its integration into the M-band

An important element for structural organization of Myosin filaments is the M-band. It was shown to align the nascent thick filament during myofibrillogenesis, to maintain the thick filament lattice (Knappeis and Carlsen, 1968), and to help Titin to keep the Myosin filament centered (Agarkova et al., 2003). In electron micrographs of the skeletal sarcomere the M-band appears as a series of parallel lines that can be designated as M-bridges M6', M4', M1, M4, and M6. The M4'/M4 lines are present

in all muscle cells, while the density of M1 and M6'/M6 bridges correlates with the physiological demands of different muscle types (Edman et al., 1988; Sjoström and Squire, 1977). According to observations on isolated filaments, Myomesin and the M-protein bind to the central zone of Myosin filaments (Bahler et al., 1985) and to the C-terminus of Titin (Nave et al., 1989), indicating participation in the sarcomeric cytoskeleton. The 250 kDa C-terminal region of Titin enter the M-band. Titin is tightly bound to the thick filament except in the central M-band. Neighboring Titin molecules are oriented in an antiparallel and staggered fashion (Obermann et al., 1996) that is crucial to compensate force imbalance and restoring order in the relaxed sarcomere. Titin's C-terminus is designated as Titin's M-line region. It is composed of 10 repetitive Ig domains termed as m1-m10 (Fig. 1.3).



**Figure 1.3: Titin's M-line region and localization of its interacting proteins.** The C-terminal region of Titin reveals Ig (red), FN3 (white), and Ser/Thr kinase (black) domains. Titin's M-line is composed of 10 Ig domains (m1-m10) and unique sequences (light grey). There are 6 exons that are designated as MEx1-6. Interacting proteins are arranged at their binding sites (modified from (Centner et al., 2001; Obermann et al., 1997)).

They are separated by intervening sequence stretches of varying length (is1 - is8). Six exons cover the M-band region (MEx1 - MEx6) of which MEx5 is alternatively spliced (Kolmerer et al., 1996).

### 1.3.1 Proteins of the M-band

The arrangement and maintenance of the M-band require the interaction of several proteins with each other. There are structural and signaling proteins such as Myomesin, MuRF-1, Calpain-3, FHL2, and Lamin that are found in the nucleus and bind to Titin. They link cytoskeletal architecture and muscle gene expression and even propose a non-muscle function for Titin.

Myomesin is a structural protein that is composed of Ig and FN3 domains. It localizes to the M4' and M4 lines of the M-band (Obermann et al., 1996). Myomesin

binds to Myosin and to the m4 domain of Titin that is located within Titin's M-line (Fig. 1.3). It forms antiparallel dimers (Obermann et al., 1996) and acts with its Ig and FN3 domains as a molecular spring. The EH-fragment is included in the major isoform of Myomesin and is expressed in the embryonic heart (Myomesin-EH) (Grove et al., 1985). The EH-fragment contains a serine and proline-rich insertion and has elastic properties analogous to the PEVK region of Titin (Schoenauer et al., 2005). The length of Myomesin and Titin isoforms correlates with muscle fiber types and the physiological demands. The long Myomesin-EH isoform is coexpressed with the long Titin isoform in slow fibers (Agarkova et al., 2000). In cardiac muscle, there is a dramatic transition from the longest to the shortest Titin splice isoform during development (Lahmers et al., 2004; Opitz et al., 2004). This correlates perfectly with the switch from the long Myomesin-EH to the short Myomesin isoform after birth (Agarkova et al., 2000). The coordinated length decrease in both filament systems could explain the developmental changes in contractility and sarcomeric proteins from embryonic to adult stage. A shorter connecting filament system seems to improve the order of contractile filaments and therefore the efficiency of sarcomere contraction (Siedner et al., 2003).

Yeast two-hybrid screens revealed the muscle-specific RING finger protein-1 (MuRF-1) as a M-line binding partner of Titin (Centner et al., 2001). MuRF-1 is implicated in diverse cellular functions including signaling, ubiquitination, and the stability of the sarcomeric M-band region (Bodine et al., 2001; McElhinny et al., 2002). MuRF-1 binds to Titin's Ig domains A168-169 N-terminal of Titin's kinase domain. The Titin binding site on MuRF-1 was mapped to its central region (Centner et al., 2001). Recent studies have revealed an important physiological role for MuRF-1 in muscle. Expression was upregulated in models of skeletal muscle atrophy. Moreover, MuRF-1 knockout mice were resistant to skeletal muscle atrophy consistent with the findings that MuRF-1 regulates muscle protein degradation (Bodine et al., 2001). The interaction of MuRF-1 with Titin in chick cardiac myocytes was shown to maintain the stability of the filament structure (McElhinny et al., 2002). MuRF-1 is also a nuclear component that interacts with transcription factors and other nuclear components (Dai and Liew, 2001). Consequently, MuRF-1 may be a dynamic protein that links nuclear functions, myofibril signaling pathways, and sarcomeric stability.

Titin's M-line and its I-band region contain a binding site for muscle-specific Calpain-3 (p94, C3). At Titin's M-line it binds to the intervening sequence 7 (is7) (Sorimachi et al., 1995). The Titin binding site on Calpain-3 has been mapped to its is2 domain, a unique sequence that also harbors a nuclear localization signal. This suggests that Calpain-3 may link nuclear functions with sarcomeric components (Sorimachi et al.,

1995). Calpain-3 is a cysteine protease with autolytic activity and loss of function was shown to result in limb girdle muscular dystrophy type 2A (LGMD2A) (Richard et al., 1995). Thus, the proteolytic activity of Calpain-3 is critical for normal skeletal muscle function but it is unknown so far, whether Titin is a substrate of Calpain-3. The four and a half LIM-only protein FHL2 belongs to the LIM domain protein family. FHL2 is characterized by the possession of a N-terminal half LIM domain followed by four complete LIM domains. They consist of double zinc finger structures that enable FHL2 to mediate protein-protein interactions. It also targets metabolic enzymes to Titin's N2B and is2 regions, suggesting that it acts as a specific adaptor protein to couple metabolic enzymes to sites of high energy consumption (Lange et al., 2002).

Lately a two-hybrid screen identified the interaction of Titin's C-terminus (is6 and is7) with the Ig-fold domain of Lamin A and B (Zastrow et al., 2006). They are intermediate filament proteins that provide structural and mechanical support for the nucleus (Dahl et al., 2004). The interaction of Titin with Lamin B was shown to be important for nuclear architecture since overexpression of Titin in HeLa cells caused misshaped nuclei and gaps in the Lamin B network (Zastrow et al., 2006).

### 1.3.2 Titin's kinase domain

Titin's kinase domain is encoded within M-line exon 1. It is a serine/threonine kinase that belongs to the Myosin-light-chain kinase (MLCK) family (Heierhorst et al., 1994). The kinase domain is composed of a catalytic domain and downstream a regulatory tail (Gautel et al., 1995). Analysis of the crystal structure revealed that the activation of the kinase is based on a dual mechanism, phosphorylation of a tyrosine residue inhibiting the active site and binding of Calcium/Calmodulin to the regulatory tail (Mayans et al., 1998). Titin's kinase domain has also been suggested to phosphorylate the Z-disc binding protein T-cap (Fig. 1.3). This was shown in differentiating myocytes during myofibrillogenesis implying that the kinase is activated when sarcomeres assemble (Mayans et al., 1998). However, the physiological function of the kinase and its activation *in vivo* have not been fully clarified.

#### 1.3.2.1 Mechanically induced kinase activation

Sarcomere assembly is a process accompanied by the sequential expression of structural and signaling proteins, which ultimately support the formation of mature myofibrils. The sarcomere is maintained and constantly remodelled to adapt to changes

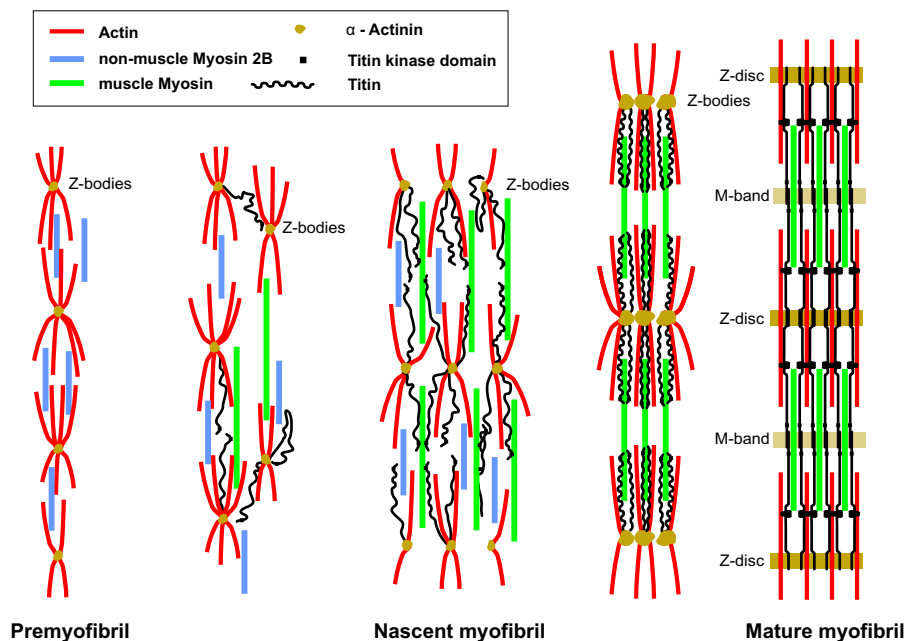
in mechanical load by providing specific attachment sites for numerous proteins. The conversion of mechanical stress into biochemical signal in a muscle cell requires a force sensor and transmitter. A suitable candidate is Titin's kinase domain. Force-probe molecular dynamic simulations were performed to study Titin's autoinhibited kinase. The regulatory tail unfolds and subsequent rearrangement of the autoinhibitory regulatory tail. This leads to the exposure of the active site, as it is required for Titin kinase activity (Grater et al., 2005).

Interaction studies revealed that kinase domain associated protein localization is mechanically modulated and regulates muscle gene expression. Nbr1 (neighbor of Brca1 gene 1) is a zinc-finger protein. It was recently identified to bind to the semi-opened catalytic site of the kinase domain (Lange et al., 2005b). Additional yeast-two hybrid screens showed that the zinc-finger related protein Sqstm1 (Sequestosome1, p62) acts as a ligand of Nbr1 but being a poorer substrate of the kinase as Nbr1 and T-cap. However, Sqstm1 is involved in several kinase signaling pathways (Geetha and Wooten, 2002). It might channel kinase signaling into muscle-specific responses by its interaction with MuRF-2 via the ubiquitin-associated domain (Lange et al., 2005b). MuRF-2 belongs to the muscle-specific RING finger protein family. Recent *in vitro* data suggest nuclear localization of MuRF-2 upon mechanical arrest. It dissociates from Sqstm1 and acts as a ligand of the transactivation domain of the serum response factor (SRF). This interaction causes reduction of nuclear SRF and repression of transcription of muscle genes (Lange et al., 2005b). Structural features of MuRF-2 were identified in MuRF-2 deficient myocytes. They display a defective M-band arrangement and a delayed myofibrillogenesis (McElhinny et al., 2004). Furthermore, MuRF-2 was shown to translocate to the nucleus under atrophic conditions (Pizon et al., 2002) and might thus be involved in an additional signaling cascade from the sarcomere to the nucleus.

## 1.4 Titin's implication in myofibrillogenesis

The heart is the first organ that differentiates and that is functionally active in the developing embryo. Cardiomyocytes synthesize sarcomeric proteins, which assemble into myofibrils in a process called myofibrillogenesis. A model for the assembly of mature myofibrils was described by studying embryonic cardiomyocytes *in vivo* (Dabiri et al., 1997). The first sarcomeric structure observed are the premyofibrils. They develop into nascent myofibrils and subsequently into mature myofibrils. Premyofibrils are the earliest arrangement in which  $\alpha$ -Actinin and Actin form punctuated

aggregates designated as Z-bodies. They are located near the spreading edge of the cell (Fig. 1.4). Non-muscle myosin IIB is found between Actin.



**Figure 1.4: Model for maturation of myofibrils.** In the premyofibril there is Actin, non-muscle Myosin, and  $\alpha$ -Actinin that form Z-bodies. Non-muscle Myosin IIB is replaced by muscle Myosin and Titin spreads from the Z-bodies towards the A-band in the nascent myofibril. Z-bodies fuse to Z-bands in the mature myofibril and Titin integrates into the M-band. Actin and Myosin align laterally (adapted from (Dabiri et al., 1997)).

The fusion of adjacent premyofibrils to form nascent myofibrils occurs at the level of the Z-bodies and is marked by lateral association. Titin's C-terminus is integrated into the Z-bodies where it interacts with  $\alpha$ -Actinin. Non-muscle Myosin IIB filaments are arranged in the region of the later emerging M-band. In the nascent myofibrils they are replaced by muscle Myosin filaments that align into the A-band. At this stage of development the sarcomeres begin to work even before the assembly process is completed. Nascent myofibrils appear to fuse laterally with one another to form the mature myofibrils. Thus, the Z-bodies fuse and form the Z-bands that grow in size. Titin molecules overlap and the arrangement of interacting proteins such as Myomesin and M-protein defines the M-band structure (Dabiri et al., 1997). Studying early embryonic chicken hearts a different model was proposed (Ehler et al., 1999). It showed that Titin is involved in the initial step of myofibrillogenesis. It describes the Z-body formation of  $\alpha$ -Actinin, Actin filaments, and Titin's N-termini

in the premyofibril. They also propose that Titin unfolds and therefore would function as a ruler to align the Z-bodies. The integration of Titin's C-terminus into the M-band was shown to be delayed (Ehler et al., 1999).

Recent work with developing C2C12 myotubes revealed that the M-band structure appears almost simultaneously with primitive Z-discs. Thus, the M-band self-assembles independently of the Z-disc. It was also shown that Obscurin is a component of the primitive M-band in skeletal muscle cells, and that the A-band assembles only after the M-band and Z-disc integrate into maturing sarcomeres (Kontrogianni-Konstantopoulos et al., 2005).

There are also data on the role of Titin's kinase domain and its surrounding region in sarcomere assembly available. Titin's kinase was shown to phosphorylate the Z-disc binding protein T-cap in differentiating myocytes during myofibrillogenesis suggesting that the kinase is activated when sarcomeres assemble (Mayans et al., 1998). Titin's M-line contains also multi-phosphorylation repeats (KSP motifs). *In vitro* phosphorylation assays detected high levels of Titin KSP phosphorylating kinases in developing but not in differentiated muscle. This suggests that Titin's C-terminal phosphorylation is regulated during differentiation, and that this may control the assembly of M-band proteins into regular structures during myogenesis (Gautel et al., 1993).

## 1.5 Titin knockout models and Titin's clinical relevance

Due to Titin's size it has been difficult to study its signaling and structural functions *in vivo*. Multiple Titin deficient animal models and cell lines have been generated. They revealed different structural and signaling functions of Titin and identified its possible role in muscular dystrophies and cardiomyopathies.

### 1.5.1 Cell culture models

Antisense oligonucleotides have been used to reduce expression of the Titin protein in cultured adult rat cardiomyocytes. They tested three antisense oligonucleotides, which were complementary to Titin mRNA. They annealed at the N- and C-terminal site of the N2B region and at the A/I-junction. All three antisense probes caused reduced Titin expression but sarcomere integrity, indicated by presence of  $\alpha$ -Actinin was not disturbed. Myosin incorporation into the sarcomere was impaired as shown

by diffuse Myosin labelling and a reduced area of regular Myosin cross-striation. These findings suggested that Titin acts as a template for Myosin integration and therefore as a prerequisite for sarcomerogenesis (Person et al., 2000).

To generate cultured myoblasts that express a truncated Titin protein a gene-targeting approach was used. The targeting vector was designed to delete the kinase region and its downstream domains. To obtain expression of only the truncated protein it would have been necessary to target both alleles. Although in that study only one allele was targeted a severe phenotype occurred. Myofibrillogenesis in these cells was impaired, accompanied by a disturbed organization of the sarcomere, and shorter myotubes. Next to that, they found a decreased expression of Myosin and a poorer organization of the M-band and Z-disc. These results suggest that the activity of the Titin kinase domain and downstream sequence are important in organizing myofibrils both at the M-band and the Z-disc early in myofibrillogenesis (Miller et al., 2003a).

### **1.5.2 Animal models**

Different studies on animal models such as zebrafish, mouse, and drosophila investigated the function of Titin and its domains. A zebrafish model was generated by injection of morpholino antisense oligonucleotides. To disrupt gene function the cardiac-specific N2B exon was targeted. Analysis of mutant animals revealed that the cardiac contractility was reduced. This was accompanied by the reduction of systolic pressure and a thin myocardium. At 36 hours past fertilization (hpf) the thick and thin filaments assembled into nascent myofibrillar arrays but more rare than in wildtype cells. By 48 hpf, wildtype cardiac myocytes contained matured sarcomeres, but no such structures were noted in targeted heart cells. These data suggested that Titin is essential for sarcomere assembly and that it causes dilated cardiomyopathy (DCM) when absent (Xu et al., 2002).

Muscular dystrophy with myositis (mdm) is a naturally occurring recessive mouse mutation. It causes severe and progressive muscular degeneration and mice die at 2 months of age. The mutation was shown to result in a deletion of 83 amino acids (4 exons) and disrupt the N2A region of Titin. Titin lost its ability to bind Calpain-3 causing the mdm phenotype. For the first time it was demonstrated that a mutation in the Titin gene is associated with muscular dystrophy (Garvey et al., 2002).

The shrunken-head mutant was isolated in a genetic screen and the mutations were mapped to the Titin gene. Altered Titin expression resulted in an embryonic lethal cardiovascular phenotype. Titin was found to be required for the initiation of proper



heart contractions as well as for maintaining the correct overall shape and orientation of individual cardiomyocytes (May et al., 2004).

As described before Titin has been proposed to have also non-muscle functions. In *Drosophila melanogaster* the Titin gene encodes a 2 MDa protein designated as D-Titin. Mutations in D-Titin were shown to cause chromosome undercondensation, chromosome breakage, loss of diploidy, and premature sister chromatid separation. Additionally, D-Titin mutants have defects in myoblast fusion and muscle organization. The phenotypes of the D-Titin mutants suggest parallel roles for Titin in both muscle and chromosome structure and elasticity (Machado and Andrew, 2000).

### 1.5.3 Clinical relevance of Titin

Muscular dystrophies are a group of genetic and hereditary muscle diseases. They are characterized by progressive skeletal muscle weakness, defects in muscle proteins, and the death of muscle cells and tissue. Tibial muscular dystrophy (TMD) is an autosomal dominant late-onset distal myopathy characterized by weakness and atrophy of the anterior compartment of the lower leg. TMD was found in patients to be linked to a mutation within the Titin gene. A 11-bp deletion/insertion mutation in Titin's M-line exon 6 resulted in an amino acid change. This caused the specific loss of C-terminal Titin epitopes in the TMD muscle samples (Hackman et al., 2002).

Cardiomyopathies are diseases of the myocardium that are associated with cardiac dysfunction. Dilated cardiomyopathy (DCM), the most common form of cardiomyopathy, is characterized by an enlarged and weakened left ventricle. Hypertrophic cardiomyopathy (HCM) is a genetic disorder caused by various mutations in genes encoding sarcomeric proteins. In hearts of HCM patients the muscle is thickened, which can obstruct blood flow and prevent the heart from functioning properly. Mutations in the Titin gene have a primary clinical relevance as shown in HCM patients (Sato et al., 1999). A mutation close to Titin's Z-repeat leads in affected patients to an altered binding affinity to  $\alpha$ -Actinin.

A different family study demonstrated that a 2 bp insertion in the Titin exon 326 caused a frameshift and resulted in a 1.14 MDa truncated protein. This mutation has been linked to the autosomal dominant dilated cardiomyopathy (Gerull et al., 2002).

However, heart diseases can not only be caused by mutations within the Titin gene but affect Titin expression leading to another clinical relevance. Heart failure patients showed a reduced Titin/Myosin heavy chain ratio, expression of structurally different Titin isoforms (Morano et al., 1994) or even a Titin isoform switch in DCM

(Makarenko et al., 2004). Quantitative RT-PCR revealed a 47% decrease of total Titin mRNA levels in DCM patients but an increased average ratio of the N2BA to N2B isoform. Histological analysis showed a decreased number of myocytes but an increase of connective tissue in DCM hearts. This was associated with a reduced passive tension determined by force measurements on isolated cardiomyofibrils (Makarenko et al., 2004).

### **1.5.4 The conditional Titin M-line exon 1 and 2 knockout mouse**

Titin's M-line provides several binding sites for structural and signaling proteins. M-line exon 1 encodes for a region that contains a kinase domain. It shares homology with the catalytic serine/threonine kinase domain. Although a variety of different functions have been hypothesized for the Titin kinase domain, its physiological function remained unknown.

Generating a conditional Titin M-line knockout addressed the question what the role of M-line Titin is in the skeletal and cardiac sarcomere. The M-line exon 1 and 2 encoding the kinase domain plus adjacent sequences were flanked with loxP sites and conditionally deleted at different stages of embryonic development. Cre expression under the control of the muscle Creatine kinase promoter (MCK-Cre) caused a disruption of Titin's M-line in all striated muscle cells. Homozygous knockout mice were viable but showed postnatally muscle weakness and died at 5 weeks of age. Myopathic changes included pale M-bands that were devoid of MuRF-1 and gradual sarcomeric disassembly. Expression of the Cre recombinase under the control of the  $\alpha$ -Myosin heavy chain promoter ( $\alpha$ -MHC-Cre) resulted in excision of M-line Exon 1 and 2 in cardiac muscle cells during early embryonic development. The absence of Titin's M-line caused lethality of homozygous knockout embryos in utero. Thus, the study indicated a critical role for Titin's M-line region in sarcomere assembly and maintenance of preexisting sarcomeres (Gotthardt et al., 2003).

However, expression kinetics of the cre recombinase transgene precluded the analysis of Titin's role in sarcomere assembly during embryonic development, in non-muscle tissue, and for non-muscle functions. Thus, 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 was converted into a complete knockout using germline recombination.