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Review

The physiological role of cardiac cytoskeleton and its alterations in heart failure [☆]



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ABSTRACT

Cardiac muscle cells are equipped with specialized biochemical machineries for the rapid generation of force and movement central to the work generated by the heart. During each heart beat cardiac muscle cells perceive and experience changes in length and load, which reflect one of the fundamental principles of physiology known as the Frank–Starling law of the heart. Cardiac muscle cells are unique mechanical stretch sensors that allow the heart to increase cardiac output, and adjust it to new physiological and pathological situations. In the present review we discuss the mechano-sensory role of the cytoskeletal proteins with respect to their tight interaction with the sarcolemma and extracellular matrix. The role of contractile thick and thin filament proteins, the elastic protein titin, and their anchorage at the Z-disc and M-band, with associated proteins are reviewed in physiologic and pathologic conditions leading to heart failure. This article is part of a Special Issue entitled: Reciprocal influences between cell cytoskeleton and membrane channels, receptors and transporters. Guest Editor: Jean Claude Hervé

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1. Introduction

1.1. Heart failure

Heart failure (HF) affects over 15 million people in Europe and continues to increase, representing a major cause of hospitalization and death [1]. HF is a condition that is characterized and clinically defined by the inability of the heart to sufficiently supply normal blood perfusion to organs and tissues as the end-point of several physiological, genetic and environmental abnormalities. Coronary heart disease accounts for ~70% of total clinical HF manifestations [2]. Other main causes of HF are hypertension (~10%), cardiomyopathies (~10%) and factors such as drug abuse, toxins and endocrine abnormalities (~10%) [1]. About half of the patients show near normal contractile function and often hypertrophied heart (HF with preserved ejection fraction; HFpEF) with abnormal diastolic function, which contrasts with the other half that present contractile dysfunction and a dilated heart (HF with reduced ejection fraction, HFrEF) and impaired diastolic function [1,3].

1.2. Cardiac remodeling and contractile dysfunction

During each heart beat cardiac muscle cells (cardiomyocytes) undergo changes in length and load. Diastole reflects the ability of the heart muscle to relax and fill with blood (preload). During the diastolic phase, cardiomyocytes are elongated or stretched. During systole, contraction and ejection of blood is forced against the arterial resistance/pressure (afterload) as a result of shortening of cardiomyocytes. Sustained wall stress as occurs during pathological conditions such as volume- and/or pressure-overload, ultimately reshapes cardiomyocytes and the extracellular matrix (ECM) resulting in remodeling of the wall geometry of the heart [4]. Eccentric remodeling of the heart is associated with replication of sarcomeres in series and lengthening of cardiomyocytes in order to accommodate a large increase in end-diastolic volume (EDV; volume-overload) and maintain LVEF [4–6]. The cellular alterations decrease wall thickness and dilate the heart, and eventually impair systolic function and reduce LVEF [4,5]. Concentric remodeling of the heart is associated with replication of sarcomeres in parallel and cardiomyocyte thickening, and is the direct result of sustained pressure increase [6]. Concentric remodeling is associated with incomplete LV relaxation and increased filling pressures, i.e. increased LV end-diastolic pressure [4,5].

Cytoskeletal changes are both a cause and consequence of contractile dysfunction and cardiac remodeling in HF patients. In this review we will discuss the major changes in function and

structure of cytoskeletal components of cardiomyocytes which occur during HF.

2. The ultrastructure of cardiac muscle

2.1. Contractile and cytoskeletal components

Cardiac muscle fibers are composed of myofibrils that contain the contractile components of striated muscle, responsible for the conversion of chemical energy into mechanical energy, in order to perform work and generate force. Running parallel along the axis of the cell, myofibrils are defined by a homogeneous succession of transverse stripes, containing repeating individual units called sarcomeres (Fig. 1, upper image). The sarcomeres can be subdivided in two main components based on their specific characteristics: 1) the contractile proteins that govern muscle contraction and relaxation and 2) the structural scaffolding or cytoskeletal proteins. Myofibril contraction is governed by the thin actin and thick myosin filament proteins that interact (cross-bridge) to generate force [7]. Force production and muscle shortening ensues as the collective sum of all “activated” tension-generating cross-bridges. Regulation of this interaction is dependent on the amount of available Ca^{2+} and ATP as well as the thin filament regulatory troponin–tropomyosin complex that binds to actin and regulates cross-bridge interaction [8,9]. The cytoskeleton forms the scaffold of cardiomyocytes as it regulates cell shape, provides mechanical integrity and resistance, and stabilizes the sarcomeric proteins. Importantly this structural framework mediates biomechanical and biochemical signaling, both inwards and outwards the cell, that thereby alters gene expression, post-translational modulation and protein synthesis, directly remodeling the myocardium [10,11].

2.2. The role of contractile proteins in cardiac muscle function

Myofibril activation and contractility depends on the interaction between the thin actin and thick myosin filament. This interaction is initiated upon electrical activation of cardiomyocytes and the resulting increase in intracellular $[\text{Ca}^{2+}]$. It has been suggested that the myofibrils oscillate between three biochemical equilibrium transitions, reflecting different interactions of actin and myosin termed the blocked (B-state), closed (C-state) and open (M-state) states of thin filament regulation [8,9]. In the B-state Ca^{2+} is not bound to cardiac troponin C (cTnC) and tropomyosin sterically blocks the myosin-binding sites on actin. The B-state equilibrium is associated to a weakly bound state of cross-bridges (i.e. weakly-bound cross-bridges). In the C-state Ca^{2+}

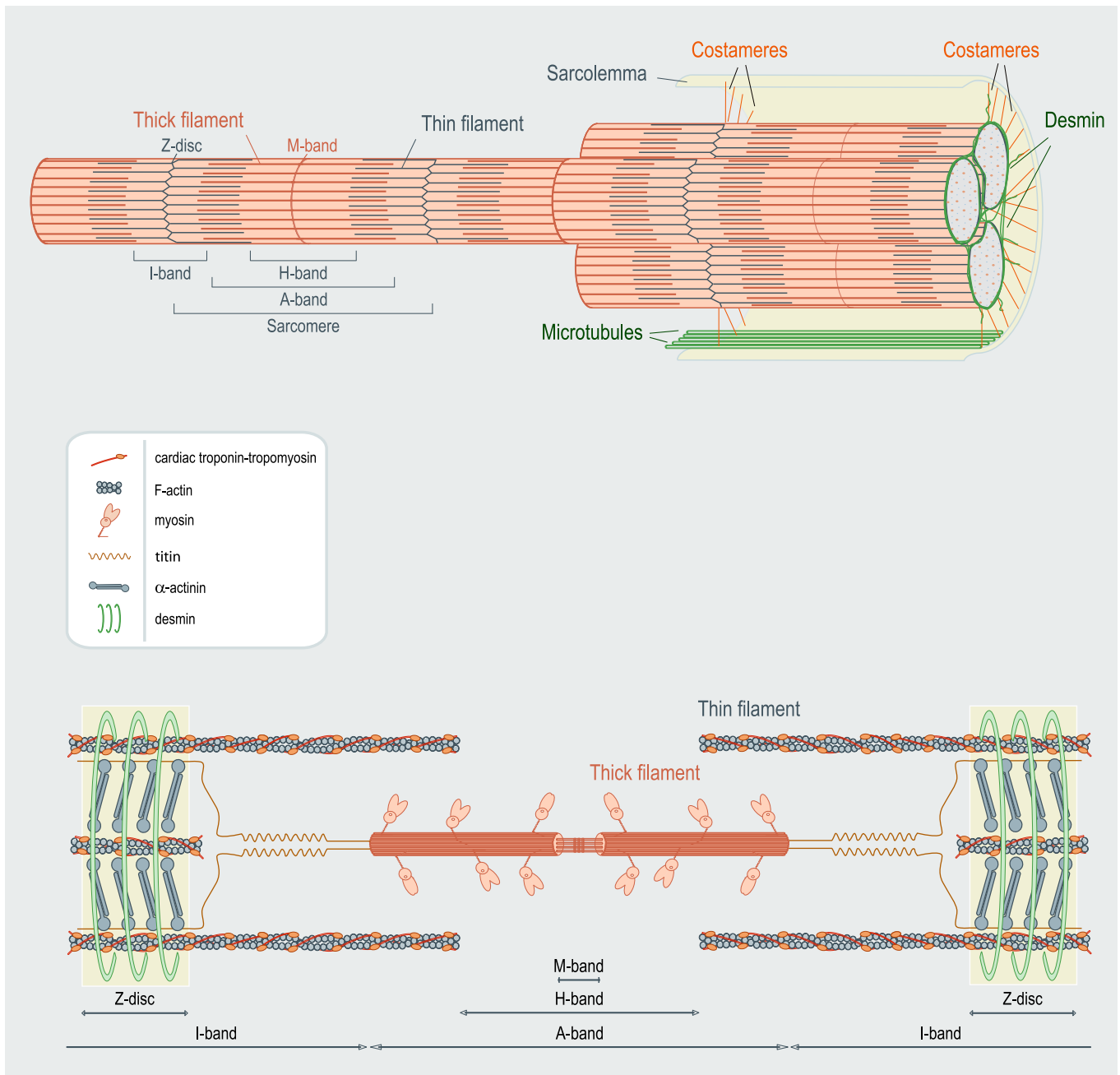


Fig. 1. Schematic drawing of the anatomy of cardiac muscle. (Upper image) Illustration of a group of myofibrils connected to the sarcolemma via the costameric network. Note the striated pattern and separation of each individual sarcomere from one Z-disc to the other. Various sub-regions are observed that are based on their lighter or darker appearance when viewed under the light microscope. (Bottom image) Illustration of an individual sarcomere. Note the formation of distinct band regions.

binds to cTnC, which changes conformation of the troponin complex and results in a $\sim 25^\circ$ movement of tropomyosin on the thin filament, thereby exposing most of the myosin-binding sites on actin [9,12]. However in the C-state the myofilament is not yet activated as non-tension-generating cross-bridges are still weakly-bound to actin (i.e. intermediate thin-filament activation). The third and last state, the M-state, involves the strong binding of tension-generating cross-bridges that induce an extra $\sim 10^\circ$ movement of tropomyosin on the actin filament, resulting in myofilament contraction and force development [9,12].

Apart from beat-to-beat changes in intracellular $[\text{Ca}^{2+}]$, muscle length and protein phosphorylation are important regulators of myofilament contractile properties.

2.3. Length-dependent activation of the heart

The ability of the heart to adjust the force of its contraction (stroke volume) in response to changes in ventricular filling (end-diastolic volume) forms the basis of the Frank–Starling relation. Ventricular filling sets the relation between sarcomere length and tension development and determines the degree of muscle shortening, and thereby regulates ventricular contraction and ejection [13,14]. At the ultrastructural level (cardiomyocyte) an increase in muscle fiber length as a result of increased filling during diastole, enhances the maximal force generating capacity and Ca^{2+} -sensitivity of myofilaments, leading to increased force development during ventricular contraction [13,14]. In other words there is a direct relation between sarcomere

length and myofilament sensitivity to Ca^{2+} ions, i.e. more force is generated at a given concentration of Ca^{2+} as sarcomere length is increased. The term 'myofilament length-dependent activation' is used to describe the length-dependent properties of the myofilaments [13].

3. Sarcomeric dysfunction: from post-translational, to inherited pathologies and Ca^{2+} -mishandling

3.1. β -adrenergic receptor pathway and protein kinase A-mediated phosphorylation

Intracellular Ca^{2+} -levels are regulated by activation of the β -adrenergic receptor pathway. Upon stimulation of the β -adrenergic receptors by circulating catecholamines, cyclic AMP levels are elevated, resulting in activation of the downstream kinase, protein kinase A (PKA) [15]. PKA regulates positive inotropic, lusitropic and chronotropic responses via phosphorylation of proteins involved in Ca^{2+} -handling and the myofilament proteins cardiac troponin I (cTnI) [16,17], cardiac myosin-binding protein C (cMyBP-C) [18,19] and titin [20]. Multiple roles have been assigned to increased PKA-phosphorylation of myofilament proteins, such as reduced myofilament Ca^{2+} -sensitivity, increased length-dependent activation and enhanced cross-bridge cycling kinetics via phosphorylation of cTnI and cMyBP-C [21–25].

Defects in the response of myofilaments to Ca^{2+} , sarcomere length and kinase-mediated phosphorylation have been described in cardiac disease and may contribute to impaired cardiac function. Defects in sarcomere contractile properties in HF will be briefly discussed (see Ref. [26]). A more thorough discussion will be given on contractile dysfunction of sarcomeres in inherited hypertrophic cardiomyopathy.

3.2. Sarcomere dysfunction in cardiac disease

Previous studies have shown that disease-related changes in protein phosphorylation via deficits in the β -adrenergic receptor pathway underlie changes in functional properties of the contractile apparatus. Defects in PKA-mediated protein phosphorylation are known to impair Ca^{2+} -handling and contractile function of the myofilaments [27–29]. Low phosphorylation of cTnI [23,25], cMyBP-C [21,28,30] and titin [20,31] have been associated with high myofilament Ca^{2+} -sensitivity and high passive stiffness of cardiac myocytes and may be important modifiers of disease progression. While post-translational modifications, such as phosphorylation of contractile proteins may be secondary in response to cardiac disease, mutations in genes encoding contractile cardiac proteins have been identified as direct cause of genetically inherited cardiomyopathies.

3.3. Mutations in sarcomere genes as a cause of hypertrophic cardiomyopathy

Defective proteins as a result of gene mutations encoding sarcomere proteins directly impair regulation of muscle contraction and lead to manifest hypertrophy [32]. Hypertrophic cardiomyopathy (HCM) reflects the pathological phenotype associated with sarcomere gene mutations and is the most common cause of sudden death in young people [32,33]. Mutations in genes encoding the thick filament proteins myosin heavy chain (MyHC) and cMyBP-C, and the thin filament proteins account for ~98% of all HCM mutations reported thus far [34]. Recent studies in human [35,36] and mice [37] demonstrated early signs of cardiac dysfunction even before a hypertrophic phenotype is observed. In addition, acute cardiac arrest occurs in young mutation carriers without evident cardiac remodeling [38]. Several possibilities might account for the encompassing remodeling and progression to HF including altered Ca^{2+} -handling and impaired β -adrenergic receptor signaling, which may be the direct or indirect result of defective sarcomeric contractile proteins.

3.4. Altered myofilament Ca^{2+} -handling in HCM

As indicated above, regulation of intracellular Ca^{2+} levels is central to contraction and relaxation of cardiomyocytes. In this process, the myofilaments represent a major intracellular buffer for Ca^{2+} and any perturbation may provide a substrate for cardiac arrhythmias. Both familial and non-familial forms of HF in human and animals are associated with prolonged action potentials, sustained intracellular Ca^{2+} accumulation and sudden cardiac death [27,39]. Alterations in Ca^{2+} -handling and subsequent perturbations in action potentials and the occurrence of arrhythmias have been recently documented in a transgenic HCM mouse model harboring mutations in the gene (*TNNT2*) encoding the thin filament protein cardiac troponin T (cTnT). Altered intracellular Ca^{2+} -handling was linked to sensitization of myofilaments to Ca^{2+} [27]. Studies in transgenic animal models with thick and thin filament mutations and in vitro studies using thin filament mutant proteins indicate that elevated myofilament Ca^{2+} -sensitivity is central in HCM development [32,37,40,41]. Accordingly, we recently observed high myofilament Ca^{2+} -sensitivity in cardiac samples from patients with manifest obstructive HCM [28]. Apart from the negative effect on intracellular Ca^{2+} -handling, myofilament Ca^{2+} -sensitization has been associated with impaired cardiac relaxation. One of the early clinical defects observed in human mutation carriers without hypertrophy is impaired diastolic function [35,36]. In accordance, transgenic mice with HCM mutations that show an increased Ca^{2+} -sensitivity, exhibit impairment of myocardial relaxation [37,42–44]. The combination of human and animal data provides evidence that high myofilament Ca^{2+} -sensitivity increases the intracellular Ca^{2+} buffering capacity of myofilaments, via mutation-induced mechanisms, resulting in cardiac arrhythmias and compromising myocardial relaxation.

High myofilament Ca^{2+} -sensitivity was also observed in end-stage HF patients and has been ascribed to phosphorylation deficits, which are most likely secondary to cardiac disease development [23]. Our recent study in human HCM with mutations in the gene (*MYBPC3*) encoding cMyBP-C showed that elevated Ca^{2+} -sensitivity was associated with reduced phosphorylation of the PKA-targets, cTnI and cMyBP-C [28]. As PKA-mediated phosphorylation of cTnI decreases myofilament Ca^{2+} -sensitivity, the high myofilament Ca^{2+} -sensitivity in HCM may be explained by low phosphorylation of PKA target proteins. Indeed, exogenous administration of PKA reduced myofilament Ca^{2+} -sensitivity to control levels [28]. It remains to be investigated if myofilament Ca^{2+} -sensitivity is high in HCM with mutations in the other contractile proteins and whether the observed functional deficits are caused by the specific mutant contractile protein or a consequence of secondary disease-related post-translation protein modifications.

3.5. Low phosphorylation of PKA targets in HCM

Low phosphorylation of PKA target proteins and high myofilament Ca^{2+} -sensitivity, as found in human HCM [28,45,46], may be due to impairments of the β -adrenergic signaling cascade. A blunted response to isoproterenol, a β -adrenoreceptor agonist, has been reported in transgenic mice harboring *TPM1* and *MYH7* mutations [47,48]. Similar findings have been reported in mice expressing the troponin T (cTnT) gene (*TNNT2*) mutation I79N, which showed high myofilament Ca^{2+} -sensitivity and a limited inotropic response to isoprenaline [29]. In the I79N muscle fibers exogenous treatment with PKA was sufficient to decrease myofilament Ca^{2+} -sensitivity to control levels [29]. These studies thus indicate that mutations in genes encoding myofilament contractile proteins potentially impair the β -adrenergic signaling cascade of cardiomyocytes. Several possibilities (receptor down-regulation and desensitization) that are known from HF development may underlie the impaired response to β -adrenergic receptor stimulation in HCM [49]. Receptor down-regulation is well documented in non-familial forms of HF [50,51] and it has been reported in HCM patients [52]. However, reduced

β -adrenergic receptor density does not appear general to all HCM cases, as both receptor density and affinity were unaltered in the *MYH7* R403Q model compared to wild-type mice [47]. Instead, up-regulation and increased activity of β -ARK1 (β -adrenergic receptor kinase 1) was found in the latter study. β -ARK1 uncouples β -adrenergic receptors from its downstream targets via phosphorylation and thereby reduces the responsiveness to catecholamines [53]. More recently, it has been demonstrated that cTnT is an A-kinase anchoring protein (AKAP), as it tethers PKA-regulatory subunits to the myofilaments [54]. AKAPs tether inactive PKA complexes at specific cellular locations, exposing the kinase to isolated cAMP gradients, allowing activation of the PKA-catalytic subunit [55]. It is tempting to speculate that mutation-induced structural alterations of the troponin complex alters the AKAP action of cTnT to activate PKA upon an increase in free cAMP levels and thereby inhibit PKA activity and its subsequent functional effects.

3.6. Impaired length-dependent activation in HCM

Both a depressed [56–58] or preserved [59–61] Frank–Starling relation have been reported in HF studies in human and animals. The study of HCM provides an important mechanistic asset to understand how the sarcomere “senses” length alterations and translates these length changes into myofilament Ca^{2+} -activation. It is known that sarcomere length changes and myofilament Ca^{2+} -activation are tightly modulated via the thin and thick filaments [13]. It follows that genetically-induced abnormalities in these structural proteins should directly impact the function and dynamics of the sarcomere.

A reduced Frank–Starling reserve has been observed in HCM patients and transgenic HCM mouse models with severe left ventricle hypertrophy [62,63]. Studies on length-dependent activation of cardiac muscle in human HCM are scarce. In human HCM with mutations in the *MYBPC3* gene, we recently reported impairment of length-dependent activation which was restored after exogenous administration with PKA [28]. Data on length-dependent activation in transgenic mouse models harboring HCM mutations have been conflicting and reported either reduced or preserved length-dependent activation [21,64–66]. Findings in mouse models are sometimes difficult to extrapolate to human due to differences in MyHC background: the α -MyHC isoform is predominant in mouse ventricles, whereas β -MyHC is the predominant form in the human adult ventricle. A recent study by Ford and colleagues [66] in mice expressing the *TNNT2* R92L mutation in the physiological (mouse-like) α -MyHC background demonstrated preserved length-dependent Ca^{2+} -activation. Interestingly, length-dependent activation was impaired in mice harboring the R92L mutation in a human-like β -MyHC background [66]. These studies emphasize the importance of sarcomere studies in cardiac samples from human HCM patients.

3.7. Filament geometry as a center of disease development

There is evidence to suggest that mutations disrupt protein–protein interactions in the thin filament which result in abnormal thin filament Ca^{2+} -activation. The group of Geeves [67] emphasized the importance of the troponin complex in stabilization of the B-state. In particular, the N-terminal region of cTnT, which interacts with tropomyosin [68], is able to maintain the thin filament in the B-state in complete absence of other troponin subunits [69]. The terminal ends of cTnT and cTnI which interact with tropomyosin and actin have been implicated in stabilization of the B-state [70,71]. Murakami and colleagues [72] showed that the C-terminal region (human residues 137–210) of TnI that contains the second actin-binding site (mobile domain; residues 164–210), docks the troponin–tropomyosin complex into the outer domain of actin at low cytoplasmic $[\text{Ca}^{2+}]$. When intracellular $[\text{Ca}^{2+}]$ rises both the first actin-binding site (inhibitory region; residues 137–148) and the mobile domain of TnI are “pulled away” by TnC and detach from actin, allowing tropomyosin movement on the

thin filament and exposure of myosin-binding sites. Because of the central roles of cTnT and cTnI in the transition from the B-state to the C-state, it is possible that mutation-induced irregularities in protein–protein interactions translate into thin filament abnormalities. Of particular interest, ~86% of cTnI HCM-causing mutations are distributed in the C-terminal region [73]. Similarly, ~75% of cTnT HCM-causing mutations are found in the TNT1 (residues 1–182) region and adjacent residues, a region that confers increased affinity of the troponin–tropomyosin interaction, which regulates tropomyosin movement and cooperative Ca^{2+} -activation of the thin filament [74,75]. Indeed, disruption of the B-state has been suggested in HCM-causing troponin mutations that were associated with increased Ca^{2+} -affinity of reconstituted cardiac thin filaments [70,71]. Molecular dynamics simulation of the thin filament, supports that HCM-causing mutations in the *TNNT2* gene reduce cTnT–tropomyosin interactions and changes cooperative Ca^{2+} -activation of the thin filament [76]. Disruption of protein–protein interactions and resulting abnormalities, may be propagated via tropomyosin over a distance that spans the entire thin filament [32,74,76].

Previous findings suggest that myosin is not involved in the formation of the first two equilibrium states (B- and C-states), but is crucial to the M-state (myosin-induced), as strong-binding of tension-generating cross-bridges are required for thin filament activation and force production [9,12]. Diverse β -MyHC HCM-causing mutations are localized in the myosin S1 domain, responsible for actin-binding [32,77]. Mouse models and in vitro studies of the β -MyHC R403Q mutation demonstrated increased actin-sliding velocities, Ca^{2+} -sensitivity and ATPase activity [32], suggesting that the deleterious effects of *MYH7* mutations are mediated via perturbations of the M-state. A recent study by Farman and colleagues [78] highlights the essential role of myosin heads' orientation that precede thin filament activation for proper lattice spacing and length-dependent activation. Thus altered myosin head orientation as a result of mutations may structurally and biochemically impair formation of the M-state and affect length-dependent activation.

In summary, disruption of protein–protein interactions accompanied by reduced troponin–tropomyosin affinity may result in aberrant filament geometry and underlie the pathogenesis of HCM. Impaired myocardial relaxation precedes development of hypertrophy in HCM-carriers [35–37], indicating that sarcomere mutations are the initial trigger of the disease. Elevated myofilament Ca^{2+} -sensitivity and possibly increased end-diastolic $[\text{Ca}^{2+}]$ [27,79], may reduce the capacity of the human heart to fully relax and develop force, reducing the Frank–Starling reserve which is observed in HCM pathology [29,62,63].

4. Myofibrillar cytoskeleton

4.1. Cytoskeletal dynamics and structure

The sarcomeric cytoskeleton represents the main structural scaffold of myocytes essential in the regulation of cell shape and mechanical integrity, providing the uniform transmission of tension along myofibrils. Additionally, the cytoskeleton allows an integrative connection to the extracellular milieu and thereby enables bidirectional signaling, allowing the muscle to monitor and quickly respond to biomechanical stress. For example, during conditions of sustained stress, the cytoskeletal Z-disc senses biomechanical load signals upstream to cardiomyocytes, such as mechanical overload, resulting in the activation of focal adhesion molecules at the sarcolemma. In turn these signals converge in the activation of downstream regulators, including kinases, phosphatases, and transcriptional modulators, which activate fetal gene programs and remodel the heart [80,81].

The sarcomeric cytoskeleton can be divided in two main groups: 1) the “true” cytoskeleton proteins including actin, tubulin and desmin [82] that structure and hold myofibrils in place and 2) the myofibrillar cytoskeleton which integrates three major structural constituents:

a) a pair of structures termed Z-disc, derived from the German *Zwischenscheibe*; meaning “intermediate disc”, that transversely delineates each side of the sarcomere, involved in the mechanical linkage of thin filaments, uniform transmission of force and connection of myofibrils to the sarcolemma and ECM; b) the elastic component titin, which connects the Z-disc to the M-band, and runs along the thick filament and parallel to the thin filament. Titin provides sarcomere flexibility and stability, and plays a major role both in passive shortening and mechanical signaling of striated muscle; and c) the M-band at the sarcomere center, from the German *Mittelscheibe* meaning “central disc”, involved in the organization and stabilization of the thick filaments (Fig. 1, bottom image).

4.2. Muscle disorders

4.2.1. Myofibrillar myopathies

Myofibrillar myopathies (MFM) are a group of skeletal muscular dystrophies histologically defined by primary degeneration of myofibrils, enhanced fibrotic material, in addition to necrotic and regenerative fibers (for extensive recent reviews on the topic see Refs. [83,84]). The majority of MFM patients exhibit symptoms of slow progressive muscle weakness that in some cases is accompanied by development of cardiomyopathies [85]. Ultrastructural analysis reveals that the MFM pathology characteristically involves abnormalities at the Z-disc. Myofibril disintegration initiates at the Z-disc with accumulation of myofibrillar degraded products and unusual local deposition of myofibrillar proteins [83,85]. These depositions include proteins such as desmin, α B-crystallin, γ -Filamin, myotilin, ZASP, FHL1 and others (discussed below) [85,86]. A small cohort of 80 patients with MFM revealed that about 43% had a disease-associated mutation [85] and with the growing improvements in genotyping and high resolution ultrastructural techniques many more genes are being recognized, such as recently the FHL1 (four-and-a-half LIM domain) protein [86]. In accordance, mutation-induced MFM are separately recognized dependent on the associated gene as desminopathy (desmin), α B-crystallinopathy (α B-crystallin), filaminopathy (filamin), myotilinopathy (myotilin), zaspopathy (ZASP) and FHL1opathy (FHL1) [85,86].

4.2.2. Cardiomyopathies

Evidence indicates that at the time MFM affected-patients are diagnosed, some are accompanied by development of cardiomyopathies (e.g. prolonged QT interval, intermittent atrial fibrillation, conduction block, abnormal depolarization) [87]. Mutations in desmin [88], α B-crystallin [89], γ -filamin [90], ZASP [91], all have been associated as causes of dilated cardiomyopathy (DCM) [92–94]. DCM is characterized by cardiac dilation and systolic dysfunction and is either acquired (e.g. enteroviral infection; see below) or has a familial origin. It is estimated that approximately 1:3000 individuals are affected worldwide, accounting the familial form up to 20–50% of all DCM cases [95,96]. Opposite to HCM-disease causing mutations that are mostly restricted to the contractile apparatus, DCM-causing mutations are identified at the sarcomeric apparatus and the Z-disc [97]. DCM-causing mutations have been shown to disrupt the cytoskeletal integrity and linkage to the sarcomeric machinery, and interfere with force generation and transmission, and energy production. A growing body of evidence implicate mutations in the gene that encodes titin (*TTN*) as a common cause of DCM (occurring in about 25% of idiopathic DCM cases) [98–100]. Because titin spans the entire sarcomere structure connecting the cytoskeleton and the sarcomeric apparatus (discussed further below), mutation-induced alterations decrease its binding affinity to Z-disc elements and truncated forms of titin are found to lack important binding sites for thick filament proteins [98–100]. Recently, titin mutations have also been associated with arrhythmogenic right ventricular cardiomyopathy (ARVC), thus supporting the central role of titin in the heart [101]. Abnormal mechanical stretch-based responses due to

diminish affinity of cytoskeletal elements with accompanying weak attachment of the cytoskeleton with the sarcolemma and the ECM are all common disorders observed in DCM-disease [94,102,103].

4.3. Cytoskeletal proteins and cardiac disease

4.3.1. Actin

Initially termed as myosin B in 1941 [104], actin was thought to represent a second form of myosin when extracted and purified from muscle. Myosin B as later called actomyosin, represented the polymerized form of the two most abundant proteins found in striated muscle, i.e. myosin and actin [105]. In striated muscle two isoforms are predominantly expressed: α -skeletal actin (*ACTA1*; >95% present in adult skeletal muscle) and α -cardiac actin (*ACTC1*; >80% in the adult heart) that share near 99% homology [106]. Actin assembles into a polymerized strand called the filamentous actin (F-actin) that serves as a rigid cytoskeleton platform for the binding of troponin-tropomyosin, the thin filament. Spanning the I-band, the opposing fast growing (barbed) ends of actin filaments are “capped”, i.e. blocked, and anchored by a protein called CapZ [107] to the Z-disc, in opposition to the slow growing (pointed) filament ends that are capped in the A-band by tropomodulin (Fig. 2) [108].

While striated muscle actins have a central role in thin filament Ca^{2+} -activation of force, several studies suggest that additional cellular pools of actin monomers exist, which are unrelated to actomyosin interactions (and thus the contractile apparatus), and are part of the cellular cytoskeleton, such as cytoplasmic actin isoforms (γ -cytoplasmic actin, *ACTG1*; herein referred to as actin-based cytoskeleton) [109]. Also termed γ -smooth muscle actin, they compose the major isoactin present in visceral organs [106]. Their *in vitro* separation has been however technically challenging in striated muscle, because over 99% of total actin is α -actin [109,110]. With the development of high-affinity antibodies to recognize γ -cytoplasmic actin it was shown in chicken skeletal muscle that they are absent from the thin filament apparatus, but are internal constituents of the Z-disc, surround the mitochondria and are found near the sarcolemma [109,110]. In addition, it was observed that γ -cytoplasmic actin also co-localizes with subsarcolemmal proteins, such as dystrophin, involved in ECM-membrane linkage (i.e. the costameres, see below; Fig. 2) in both rabbit [111] and mice [112,113] skeletal muscles.

Additionally, because actin interacts with a multitude of interacting partners, i.e. actin-binding proteins, which include capping proteins involved in actin assembly/disassembly and anchoring proteins such as α -actinin [114] and γ -filamin [115], it is not surprising that mutation-induced actin abnormalities cause cardiomyopathies [93,116,117]. The majority of actin-null mice do not survive (~60%) or die within 2 weeks after birth [118], supporting the essential cytoskeletal role of actin in striated muscle.

4.3.2. Tubulin

Together with actin, microtubules constitute the major component of the cardiomyocyte cytoskeleton. Microtubules consist of $\alpha\beta$ -tubulin heterodimers, which assemble into microtubule polymers [119]. Together with intermediate filaments (e.g. desmin), microtubules are involved in the assembly of myofilaments during myogenesis and adult myofilament maintenance [10,82]. Polymerized tubulin accounts for a small portion of total tubulin (~30%), while free non-polymerized tubulin accounts for ~70% of cytosolic tubulin [120,121]. Microtubules are organized around the nucleus and in the longitudinal direction of the myofibril space close to mitochondria and along the plasma membrane, where they are suggested to act as a dynamic transport system [122,123].

There is evidence to suggest that microtubules modulate cardiomyocyte signaling and may have an important role in disease conditions, such as cardiac hypertrophy and HF where the heart is subjected to sustained high loads [124]. Cooper and colleagues

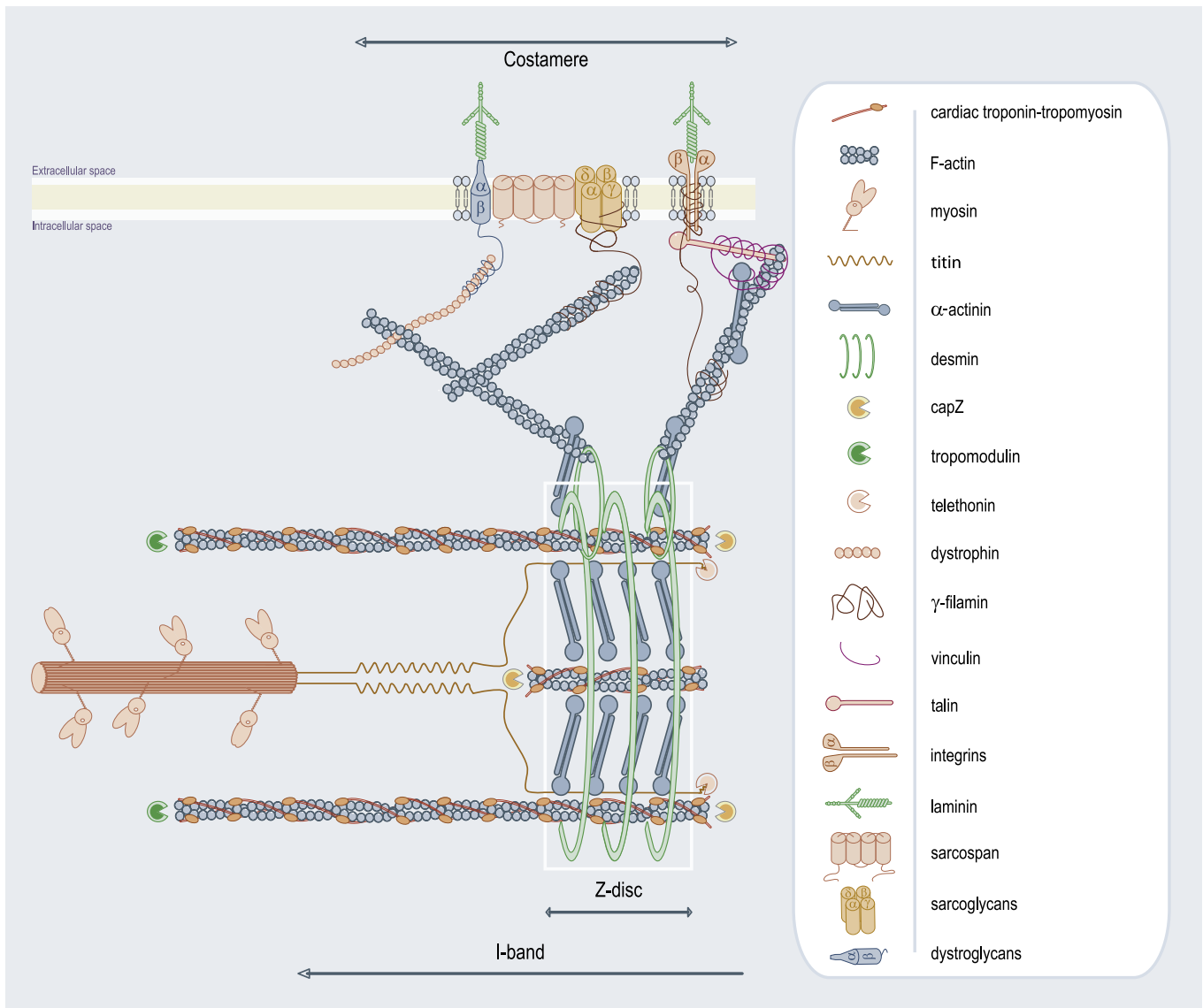


Fig. 2. Schematic drawing of Z-disc and associated structures. Note the connection of the Z-disc to the sarcolemma and ECM (laminin).

[125,126] put forward the idea that the microtubule network plays an important role during the transition from adaptive to maladaptive hypertrophy. The authors [125,126] showed that cardiac function is inversely correlated with microtubule densification and likely potentiates and contributes to HF. Following pressure-overload and wall stress increments, there was a substantial increase of tubulin and microtubule network density in maladaptive hypertrophied hearts. In contrast, tubulin and microtubule density were normal in adaptive hypertrophy with normal contractile function and wall stress, suggesting that microtubule density is associated to contractile defects. Colchicine (which causes microtubule depolymerization) normalized the contractile function in the dysfunctional heart, whereas treatment of normal cardiomyocytes with taxol (to hyperpolymerize the microtubules) caused the characteristic contractile dysfunction of pressure-overload [125,126]. Increased tubulin production was also observed in animal models with dilated hearts after supraventricular tachycardia [127] and monocrotaline-induced pressure-overload (pulmonary hypertension) [128] associated with increased wall stress, and in failing human myocardium caused by DCM [129]. Together these findings indicate that mechanical forces caused by pressure-overload and tachycardia initiate tubulin up-regulation and microtubule assembly,

potentiating cardiac dysfunction and HF. In support, passive stretch and contractile activity have been shown to increase tubulin content in neonatal cardiomyocytes [130] and to result in cardiomyocyte hypertrophy [131].

It has been suggested that the microtubule network density increases to compensate for the abnormal increase in wall stress by imposing a viscous load [124,125]. A less compliant cardiac ventricle associated with increased passive viscoelastic components may normalize wall stress as it would restrict sarcomere shortening. The Cooper hypothesis is nonetheless controversial. Although the authors [125] provided evidence that microtubules play both a role in the viscous (up to 4-fold increase) and elastic (about 2-fold) properties of pressure-overload animals by the use of magnetic twisting cytometry, their role is challenged by the involvement of titin. It has been demonstrated that the contribution of titin within physiological levels, i.e. 1.8 to 2.2 μm , accounts for ~80% of total viscoelasticity (far greater than collagen) and that the contribution of the microtubules is very modest (about 10%) [132,133]. Additionally, actomyosin contributions to diastolic stress has been recently shown to be about 30%, which would form a greater viscoelastic element than the tubulin-based viscosity [134]. As will be discussed below, the putative role of microtubules

during disease conditions may be associated with their ability to interact with sarcolemmal proteins, such as dystrophin [135,136] (possibly involved in the stabilization of the microtubule network) and integrins [131] (involved in stress or pressure-overload responses), and thereby play a role in the response to stress stimuli induced by pressure-overload (Fig. 3).

4.3.3. Desmin

Desmin is a muscle-specific cytoskeletal intermediate filament that makes up the transverse network which maintains the myofibril apparatus in register and connects the nuclear membrane, Z-disc and sarcolemma (Figs. 1 and 2) [123,137]. In cardiomyocytes, desmin is transversely distributed along the myofibril space, where it associates with the outer portion of the Z-disc and with the intercalated disc (cell–cell junction) [123]. In pressure-overload animal models, desmin is longitudinally distributed between two displaced Z-discs, associated with loss of the myofibril register pattern and disorganization of the sarcomeric structure [123]. This finding suggests that during pressure-

overload hypertrophy, desmin forms a physical link with newly formed sarcomeres, holding and maintaining myofibrils in register, so that force is normally propagated across myofibrils [123]. Atrophy and facial muscle weakness as well as cardiomyopathies are observed in desminopathy patients, making it one of the major intermediate filament disorders in human that is associated with mutations in desmin [138]. Desminopathies are associated with cardiac arrhythmias and restrictive HF [139], idiopathic DCM [88] and ARVC [140,141]. Desmin-null mice have severe disruption of sarcomere architecture, associated with loss of lateral displacement and impaired myofibril anchorage to the sarcolemma and cell adhesion, indicative for the essential role of desmin in maintenance of the sarcomere integrity [142,143]. In failing human myocardium caused by DCM, where the sarcomeric architecture is misaligned, desmin is up-regulated possibly in an attempt to maintain the functional integrity of myofibrils [129]. Mitochondrial and nuclear mislocalization and loss of organization, with increased intercellular space, is also found in mouse lacking desmin, suggesting that it also holds subcellular organelles in place [144].

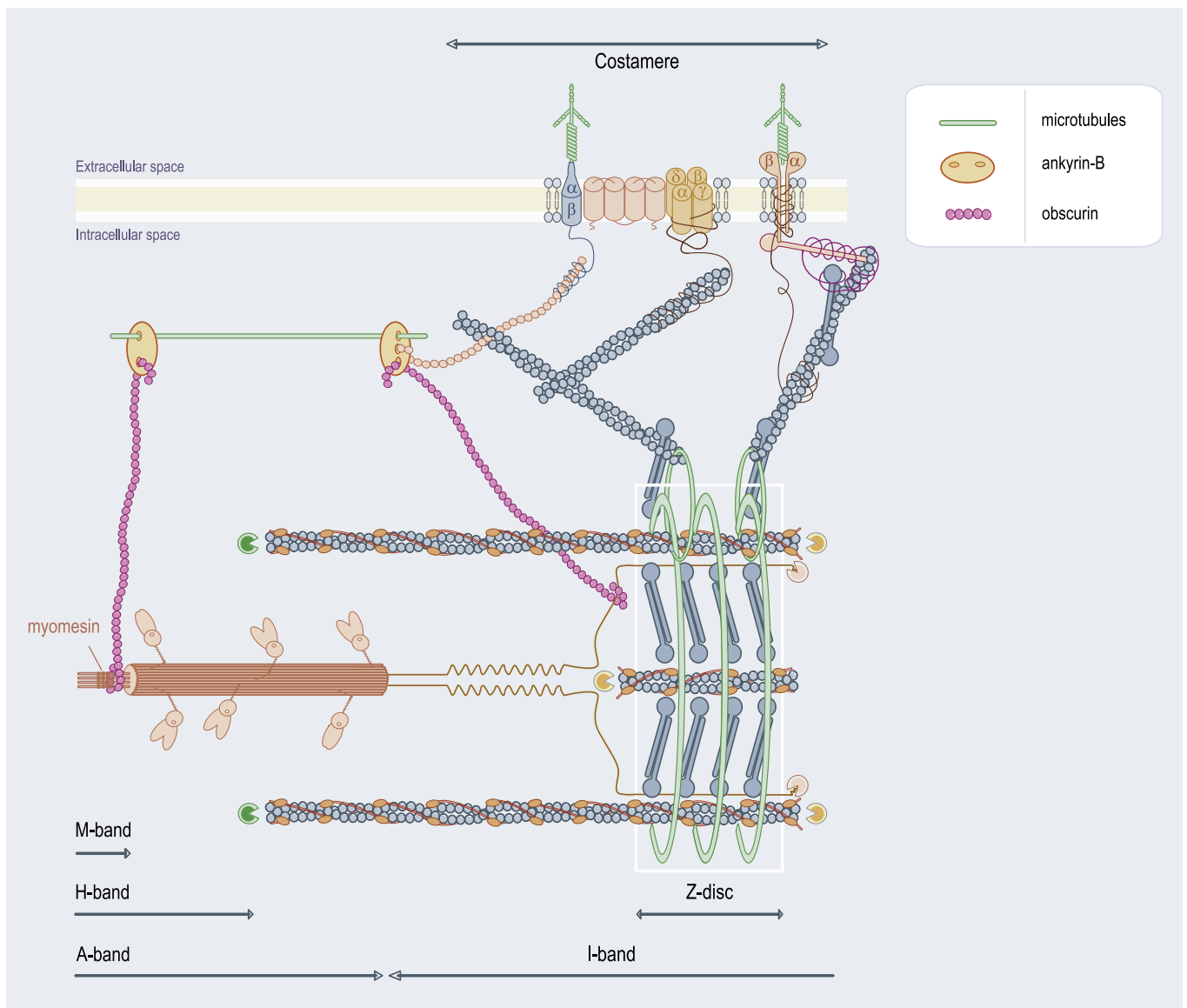


Fig. 3. Schematic drawing of Z-disc and M-band components. Note the connection of M-band and Z-disc components, possibly involving the microtubule network with obscurin and ankyrin-B. It deserves to be mentioned that the intermolecular interactions between obscurin, ankyrin-B, the microtubules and dystrophin have only been identified in skeletal muscle and so, whether this complex is also structured in cardiac muscle remains to be determined. For complete legend check Fig. 2.

Desmin-null mice have also been reported to develop cardiomyocyte hypertrophy and chamber dilatation with systolic dysfunction and HF [144].

Desmin-related myopathies have also been associated with α B-crystallin [83]. α B-crystallin belongs to the family of small heat-shock proteins that localize to the Z-disc (I-band) and interact with desmin, possibly involved in intermediate filament assembly and protection from stress [145,146]. Missense mutations (R120G) in the gene encoding α B-crystallin (*CRYAB*) are linked with desminopathy and are characterized by myofibrillar accumulation of dense desmin filaments [147]. Transgenic mice overexpressing wild-type α B-crystallin do not show any signs of myofibrillar or cardiac myopathy which contrasts with mice expressing the missense mutation R120G that exhibit desmin-related cardiomyopathy with myofibril misalignment and 100% lethality during initial adulthood [148]. As recently reviewed [83,85], multiple α B-crystallin associated-mutations have been identified, which supports the categorization of α B-crystallin associated-myopathies as a subgroup of MFM, i.e. α B-crystallinopathy. A newly-identified α B-crystallin mutation (R157H) has been associated with DCM in humans that is unrelated to α B-crystallinopathy [89]. It was previously shown that α B-crystallin binds to the I-band region of titin (N2B region, but not the PEVK; see below), which possibly confers cardioprotection to titin upon stress [149,150]. The α B-crystallin R157H mutant was found to have decreased affinity with the cardiac specific N2B domain of titin in familial DCM, suggesting that α B-crystallins may be involved in the onset of DCM [89].

In summary, the cytoskeleton is a complex network of proteins involved in assembly and maintenance of sarcomeres and myofibrils. F-actin assembles thin filament proteins and governs contraction and relaxation, and provides the scaffold of all the cellular content via an F-actin-based cytoskeleton. Interfilaments, such as desmin, hold myofibrils structural components in registration across the fibers, giving rise to the striated muscle pattern, and stabilize subcellular organelles. The interfilament network is capable to physically link newly formed sarcomeres and hold myofibrils in register to maintain the uniform transmission of force. Although the role of the microtubule network remains unclear and less likely to have a mechanical role during cardiac hypertrophy, it is likely that their contribution is linked with sarcolemmal proteins during biomechanical stress (discussed below).

As will be further discussed the sarcomeric machinery, in particular the Z-disc, links external stimuli to the intracellular environment of cardiomyocytes via interaction with membrane-associated proteins of the costameric network.

4.4. The Z-disc

Not just a static complex that both provides the physical separation between each sarcomere and stabilizes the propagation of force along and laterally to neighboring sarcomeres, the Z-disc is able to monitor and sense biomechanical stress. This unique property confers the myofibrils the ability to quickly adapt to different load conditions. The lateral connections of the Z-disc with the sarcolemma and ECM allow bidirectional communication. Although a precise division of all proteins at or near the Z-disc is extremely challenging, we make a simplified subdivision taking into consideration their main functions and locations. Three main groups can be classified in terms of: 1) formation of the structural scaffold of the Z-disc. This group includes structural proteins mainly located at the Z-disc, which directly interact with actin and may provide a link of the Z-disc to transmembrane proteins. These structural proteins include α -actinin, CapZ, filamins, myotilin and members of the nebulin family; 2) transmembrane complexes that are adjacent to the Z-disc and interact with actin and/or actin-binding proteins, thereby connecting the Z-disc to the sarcolemma and ECM. These include the costameres, which can be sub-divided into two major protein complexes that give

physical stability and regulate biomechanical signaling either from the ECM into the Z-disc or vice versa, such as the dystrophin-glycoprotein complex and the integrin–vinculin–talin complex [151]; and 3) Z-disc associated proteins involved in strengthening of the cytoskeleton-sarcolemma grid via interaction with actin-binding proteins and the costameres, involved in signaling. These associated proteins include FATZ (filamin-, actinin-, and telethonin-binding protein of the Z-disc) and PDZ-LIM and LIM-only proteins. A representative picture of some Z-disc components is presented in Fig. 2.

Countless components of the Z-disc have been extensively reviewed in recent years and although the vast majority have been linked to cardiac dysfunction and development of cardiomyopathies [11,152], their thorough and detailed discussion is outside the scope of the present review. Instead, the main constituents and their associations will be presented below.

4.4.1. Z-disc structural scaffolding

4.4.1.1. α -actinin. The main component of the Z-disc is α -actinin, which accounts for ~20% of total Z-disc proteins [153]. In the human heart, only one of the four isoforms (α -actinin-2; *ACTN2*) is expressed and mutations in this gene have been associated with both HCM and DCM [154–156]. Alpha-actinin cross-links and connects the ends of F-actin [114,157] and provides the direct physical connection between the actin cytoskeleton and transmembrane receptors, thereby maintaining the integrity of the Z-disc structure. Aside from its physical role, α -actinin governs the activity of diverse signaling pathways via direct interactions with several binding partners. α -actinin is known to interact with many Z-disc and membrane-associated proteins including CapZ [107], myotilin [158], N-RAP [159], vinculin [160], integrins [161], PDZ-LIM domain proteins [162–165], zyxin [166], titin [167] and FATZ [168]. Given its multiple associating binding partners, it suggests that α -actinin may play a determinant role in mediating and integrating mechano-connections at the Z-disc, as indicated by the diverse clinical presentations of α -actinin-associated diseases [154–156].

4.4.1.2. CapZ. α -actinin strengthens the anchorage of F-actin to the Z-disc via an actin capping protein, called CapZ [107]. CapZ binds to the barbed ends of F-actin and blocks, i.e. “caps”, either actin depolymerization and polymerization (prevents either the loss or addition of actin monomers) [107,169]. CapZ is arranged in a dimer association of α - and β -subunit isoforms [170]. In cardiomyocytes, two β -subunit isoforms are expressed with different cellular locations. β_1 -subunits are primarily located at the Z-disc responsible for anchorage of F-actin, whereas β_2 -isoforms are present in intercalated discs [171,172]. Over-expression of β_2 -isoform in transgenic mice is associated with development of HCM as a result of disruption of the sarcomeric architecture, while β_1 -isoform over-expression results in altered morphology of the intercalated disc [172]. In agreement, loss of function of β_1 -isoforms may lead to a diminished number of attached thin filaments to the Z-disc and consequent myofibrillar disarray [172].

4.4.1.3. γ -Filamin. Filamins are a family of cytoskeletal actin-binding proteins involved in the organization of actin filaments in bundle networks. In humans, three different filamin isoforms exist, of which γ -filamin (also known as filamin C or filamin-2) is the only isoform expressed in striated muscle [115]. In cardiac muscle, filamin is located both at the Z-disc and sarcolemma [173], where it interacts with a variety of transmembrane receptors including members of the costameric network, such as the sarcoglycan complex [174] and the focal adhesion β_1 -integrin [175]. γ -Filamin also interacts with other actin-binding proteins including myotilin [176], N-RAP [159], and the multiprotein complex FATZ [168]. Mutations in the gene encoding γ -filamin (*FLNC*), i.e. filaminopathy, are characterized by myofibrillar disorganization and abnormal accumulation/aggregation of myofibrillar proteins

including filamin, desmin, myotilin, dystrophin and sarcoglycans and exhibit progressive muscle weakness [177,178]. In addition, filamin mutations have also been linked to DCM [90].

Together these studies highlight the dual role of filamin: it provides structure and stability to the Z-disc via its interaction with actin and links membrane-related signaling via transmembrane proteins. In addition, filamin plays a role in early embryonic events, where it has been associated with early myotube formation [179].

4.4.1.4. Myotilin. Myotilin is a protein expressed in both cardiac and skeletal muscle that interacts at the Z-disc with α -actinin, and is present along the sarcolemmal membrane [158]. It binds to F-actin and is able to promote F-actin cross-linking, either alone or in association with α -actinin, and thereby involved in Z-disc integrity [180]. In addition, myotilin interacts with FATZ [175] and γ -filamin [176]. It has been suggested that myotilin plays an important role in stabilization and anchorage of Z-disc elements, and possibly is required for proper Z-disc organization. Mutations in the myotilin gene (*MYOT*) were first found by Hauser and colleagues [181] whom identified the disease-causing mutation associated with limb-girdle muscular dystrophy 1A (LGMD1A) in patients. Mutations in myotilin are known causes of muscular dystrophies, i.e. myotilinopathy, which are associated to Z-disc abnormalities, as well as cardiomyopathies, thus supporting its role in Z-disc stabilization and integrity [181,182]. In addition, it has been recently demonstrated that myotilin interacts at the Z-disc with ZASP/Cypher, a member of the enigma subfamily, ALP (α -actinin associated LIM protein), involved in Z-disc stability, and also binds titin at the Z-disc [176,183,184]. Interestingly, Ca^{2+} /calmodulin-dependent protein kinase (CaMKII) was shown to phosphorylate myotilin, which appears to strengthen the binding of myotilin to ZASP/Cypher [183]. Myotilin-null mouse have structural preserved Z-discs with normal sarcomeric and sarcolemmal integrity, and do not show morphological abnormalities of neonatal and adult hearts [185]. This finding suggests that myotilin either does not play a role in embryonic development and basal maintenance of the heart or that other proteins may compensate for its absence, such as the titin-interacting protein telethonin, which was found to be upregulated [185].

4.4.1.5. The nebulin family members. The nebulin family of actin-binding proteins comprises three proteins that are specific of striated muscle: nebulin, nebullette and N-RAP (nebulin-related anchoring protein) and two additional proteins with ubiquitous tissue expression: *lasp-1* and *lasp-2* (LIM and SH3 protein-1 and -2, respectively) [186].

Nebulin is a giant actin-binding protein that makes up 3% of the myofibrillar content of skeletal muscles [187]. Because it spans the entire thin filament with its C-terminal region anchored to the Z-disc (Mw 600–800 kDa), nebulin has been regarded as a “protein-ruler” of skeletal muscle thin filaments [187,188]. Nebulin-deficient mice have on average shorter thin filament lengths that are associated with reduced force production [188,189]. Recent evidence, however, demonstrates that nebulin is far from being just a strict length “ruler”. In accordance, nebulin is suggested to be part of a multiprotein complex that laterally links myofibrils at the Z-disc and regulates its structure (i.e. Z-disc width), essential for lateral transmission of force [190]. Additionally, there is evidence to support that nebulin regulates muscle contraction via regulation of cross-bridge cycling kinetics and Ca^{2+} -sensitivity of force (for an extensive recent review on the topic see reference [190]). Mutations in the nebulin gene are associated with nemaline myopathy (NM) [191], a non-progressive type of neuromuscular disorder characterized at the ultrastructural level by the presence of rod-like bodies (i.e. nemaline rods) with disrupted sarcomeric structure and widened Z-discs [192]. Clinically, the hallmark of NM-patients is muscle weakness and it is estimated that approximately 1:50,000 individuals are affected worldwide making it the most common non-dystrophic congenital myopathy [192]. Mutations in several thin filaments genes including skeletal muscle *ACTA1*, *TPM2*

and *TPM3*, and *TNNT1* have been associated with NM, while mutations in the nebulin gene (*NEB*) represent the most common cause of autosomal recessive forms of NM [191].

Nebulette, the myofibrillar homolog protein of skeletal nebulin, is a small isoform (Mw 107 kDa) specific of cardiac muscle that it is located at the Z-disc [193,194]. In contrast to nebulin that spans the entire thin filament of skeletal muscle, nebullette is predicted to only extend 25% of the thin filament length from the Z-disc region [193,194]. Because nebullette does not span the entire cardiac thin filament length, it is unlikely to serve as a protein-ruler. However, over-expression of nebullette's C-terminal linker region (anchored within the Z-disc) is associated with loss of endogenous nebullette and a decrease in thin filament length [195], which suggests that nebullette is involved in a mechanism that specifies and stabilizes thin filament lengths.

The central nebulin-like repeat domains of nebulin/nebullette interact with several thin filament proteins including actin, troponin and tropomyosin [196,197]. Nebullette is required to maintain tropomyosin interaction with the thin filament [198], such that it increases the affinity of the troponin–tropomyosin assembly to F-actin, suggesting a possible role in the assembly and regulation of thin filament transitions. In addition, the C-terminal linker region of nebulin/nebullette interacts with interfilaments and Z-disc associated proteins, such as desmin [199], α -actinin [193,200], CapZ [201], myopalladin [202], zyxin [203], titin [201,204], ZASP and γ -filamin/filamin C [205]. Nebullette mutations are associated with DCM in patients, and transgenic mice die before birth or develop DCM with disrupted I-band and/or Z-disc structure [206]. Down-regulation of contractile proteins including cTnI, cTnT and tropomyosin was observed in some nebullette DCM-causing mutations, in addition to down-regulation of α -actinin, nebullette and desmin, and cleavage of γ -filamin/filamin C [206]. One can speculate that lack of (functional) nebullette would impair thin filament length and reduce the amount of expressed cardiac troponin subunits. Finally, nebullette has been demonstrated to be present during early stages of cardiogenesis along F-actin, suggesting a possible role during early formation of the cytoarchitecture of the sarcomere [207]. Indeed, upon mechanical stretch in cardiomyoblasts, nebullette's distribution shifts from a uniform perinuclear localization to the whole cytosol along with F-actin, suggesting that nebullette may function as a mechano-sensor during myogenesis to maintain the structural and functional integrity of cardiomyocytes [206].

N-RAP is a large actin-binding protein of the nebulin family (Mw 196 kDa) that has been implicated in the assembly of myofibrils during myofibrillogenesis in both cardiac and skeletal muscle [186,208]. In accordance, evidence indicates that N-RAP's early localization is in Z-disc precursors where it shifts to the myotendinous junctions and intercalated discs of matured skeletal and cardiac muscle, respectively [186]. N-RAP also binds to α -actinin and filamin, possibly assisting the assembly of F-actin and promoting the regulation of Z-disc assembly during early stages of development [159,208]. Specific N-RAP protein knock-down with RNA interference in cultured embryonic cardiomyocytes demonstrated that N-RAP is required for α -actinin organization and proper formation and assembly of myofibrils [209].

The other two nebulin protein members, *lasp-1* and *lasp-2*, comprise the smallest members of the nebulin family (Mws 37 kDa and 34 kDa, respectively), and have multiple tissue expression patterns [186]. *Lasp-1* is ubiquitously expressed in almost all tissues although expression is relatively low in the heart and skeletal muscle [210]. Its role in striated muscle is largely unknown. *Lasp-2* is a splice-variant of nebullette (also termed LIM-nebullette) that is present in muscle and non-muscle tissue [203]. Opposite to *lasp-1*, *lasp-2* has been recently associated with early development of myofibril structures of chicken embryos and thus seems to play a role during myofibrillogenesis [211]. In accordance, *lasp-2* is found in nascent Z-discs, where it interacts with both actin and α -actinin, but also localizes at the focal adhesions and intercalated discs of cultured cardiomyocytes [211]. According to Ziesenis and colleagues [211] *lasp-2* may promote the stabilization or

anchorage of the thin filaments to the Z-disc in addition to the tethering of myofibrils to the sarcolemma at the focal adhesions or intercalated disc areas.

Taken together these findings suggest that members of the nebulin family may be involved in myofibril assembly with maintenance of the Z-disc structure and thin filaments maturation, myofibrils attachment to the sarcolemma, in addition to Ca^{2+} -activation of the thin filament.

4.4.2. Membrane-associated proteins, the costamere

Responsible for the physical communication between the Z-disc, the sarcolemma and the ECM, the costameres surround the circumference of cardiomyocytes (Figs. 1 and 2) [109,212]. Lateral transmission of force by the costamere is thought to maintain a homogenous sarcomere length of contracting and resting cells, thereby withstanding high forces and minimizing stress [213,214]. The stability exerted by the costamere may in part be due to its direct interaction with the ECM [215]. It is generally assumed that the biomechanical role exerted by the costamere is bidirectional, as it either transmits signals from the extracellular environment to the intracellular milieu and vice versa [151,214]. The costamere is known to be involved in physiological or pathological growth of cardiomyocytes as a result of mechanical loads [216,217].

The costamere can be subdivided in two main protein complexes, the dystrophin–glycoprotein and the integrin–vinculin–talin complexes, which provide physical stability and integrity to the costamere, and have an important role in biomechanical signaling in response to stress stimuli [151].

4.4.2.1. Dystrophin–glycoprotein complex. The dystrophin–glycoprotein complex is responsible for providing the physical association between the ECM and F-actin and includes subsarcolemmal proteins, such as dystroglycan, dystrophin, sarcoglycans and sarcospan [151]. A complex of dystroglycan proteins binds the basal lamina protein (laminin) of the ECM to dystrophin, that via actin–dystrophin interactions anchors and stabilizes the dystrophin–glycoprotein complex to the cytoskeleton [111,113,218]. Mutations in dystrophin, known to cause either Duchenne's or Becker's muscular dystrophy, are involved in the development of DCM, and are associated with loss of sarcolemmal integrity [219–221]. Additionally, mutations in an actin domain known to interact with dystrophin have been found in DCM [93]. Not just confined to familial forms, DCM has been shown to be acquired by enteroviral infection. Badorff and colleagues [222] demonstrated that the enterovirus protease 2A cleaves dystrophin and contributes to an acquired form of DCM. Additionally, it has been suggested that dystrophin links the costamere to the cytoskeleton, possibly directly organizing and/or stabilizing the microtubule network [135]. In a similar way, Randazzo and colleagues [136] suggested that the microtubule network affects the localization of dystrophin at the sarcolemma, under the modulation of the giant protein obscurin (Fig. 3, discussed further below). Together these findings support a linkage of microtubules with the costameric network and the ability of the microtubules to respond to stress. Moreover, the stabilization of dystrophin–glycoprotein complex at the membrane is strengthened by sarcoglycans–sarcospan interactions [223]. Sarcoglycans are required for maintenance of the membrane integrity [223] and mutations that reduce their expression, without affecting the dystrophin–glycoprotein complex, cause muscular dystrophies [224,225]. Of interest, an up-regulation of the γ -filamin/filamin-2 was observed in patients with mutations in both sarcoglycan and dystrophin [174]. It appears that γ -filamin/filamin C over-expression is the required compensatory response to balance low expression of sarcoglycan and dystrophin in order to maintain mechanical stress resistance [174]. Altogether these findings support that disruption of the dystrophin–glycoprotein complex is associated with a reduction of the sarcolemma's stability and integrity, weakening the attachment of the ECM to the cytoskeleton.

4.4.2.2. Integrin–vinculin–talin complex. The second complex group of transmembrane proteins comprises the integrin–vinculin–talin complex that is responsible for bidirectional transduction of biomechanical signals in response to stress or pressure-overload [226]. Vinculin was the first protein to be found at the costameres [212] and it is involved in the mechanical linkage of the actin cytoskeleton to the sarcolemma [160]. Talin is able to interact with F-actin, vinculin and integrins, allowing the formation of a integrin–vinculin–talin multicomplex [151,227–229]. It has been demonstrated that vinculin and its isoform variants are responsible for normal mechanical stress responses of the heart [230]. Transgenic heterozygous knock-out mice with reduced protein levels of vinculin and its splice variant metavinculin showed misalignment of α -actinin and abnormal sarcomeric architecture accompanied by cardiac dysfunction and increased mortality after pressure-overload [230]. Mutations in metavinculin have been recently associated to DCM in humans and result in disruption of the transmission of force at the intercalated discs via abnormal actin filament organization leading to irregular intercalated discs and sarcomere structure [231,232]. Together these findings support the role of vinculin in the formation of a multicomplex at the costamere, providing a stable connection of the myofibril to the sarcolemma, whereby cardiomyocytes are able to respond to stress stimuli following pressure-overload.

Central to the integrin–vinculin–talin complex and mechano-transduction of signals are integrins, regulators of cellular adhesion and mechano-transduction. Integrins are the interface between the intra- and the extracellular network, and are responsible for mediating bidirectional signaling [226]. Via their extracellular domain, integrins bind to various ligands present in the ECM (e.g. fibronectin, vitronectin, collagen, laminin), and make a bridge to the actin cytoskeleton via talin and vinculin [151,233]. It has been suggested that cardiac remodeling after myocardial infarction is initiated at the intercalated disc of the damaged area via translocation of ECM and integrin constituents [234]. Up-regulation of integrins is observed during mechanical stress and as a result it has been suggested that integrins govern central mechanical stress responses via strengthening of the cytoskeleton–matrix association [235,236]. Upon mechanical overload, the stress response is propagated from the ECM to integrins, promoting expression of fetal and growth genes leading to hypertrophy [235,237,238]. In human failing myocardium caused by DCM, vinculin is up-regulated (in combination with tubulin and desmin) possibly via integrin–ECM interactions to maintain the physical integrity of the cytoskeleton [239]. In addition, one can hypothesize that integrins may associate with the microtubule network. β_1 -integrin blockade suppressed polymerization of microtubules and hypertrophy, in neonatal cardiomyocytes, mimicking the effects of colchicine in the inhibition of the stretch-based response [131]. This finding suggests a possible association between microtubules and integrins, which together with dystrophin–microtubule interactions, may represent a hot-spot of microtubules organization at the costameres, allowing the cytoskeleton to remodel following mechanical stress.

4.4.3. Z-disc associated-proteins involved in structure and signaling

4.4.3.1. Myopalladin. Myopalladin (Mw 145 kDa) was identified by Bang and colleagues [202] using a yeast two-hybrid approach when searching for potential protein candidates involved in tethering of nebulin/nebulin to the Z-disc. Because of its high homology to palladin, a protein present in stress fibers of non-muscle cells [240], the authors named it myopalladin [202]. Together with myotilin, myopalladin belongs to a family of cytoskeletal proteins that regulate actin organization, Z-disc assembly and morphogenesis [241]. Myopalladin is a Z-disc binding protein that is expressed in striated muscle, where it localizes to the Z-disc, the I-band and the nucleus [202]. At the Z-disc, myopalladin interacts with several proteins including α -actinin, titin and nebulin/

nebullette, where it is suggested to provide maintenance of sarcomeric structure, since over-expression of its N-terminal region leads to Z-disc disruption and sarcomere disassembly [202]. In addition, in the I-band myopalladin interacts with cardiac ankyrin repeat protein (CARP), which is involved in cardiogenesis [242], suggesting a putative link between the Z-disc and cardiac gene expression regulation [202]. In agreement, myopalladin mutations are known to cause either DCM or HCM in humans, which are associated with disturbed myofibrillogenesis and sarcomere/Z-disc assembly [243–245].

4.4.3.2. FATZ (filamin-, actinin-, and telethonin-binding protein of the Z-disc). FATZ, the abbreviation for γ -filamin, α -actinin, telethonin-binding protein of the Z-disc, is a sarcomeric protein family implicated in the assembly and stabilization of the Z-disc in striated muscle [168]. Independently, two additional laboratories identified FATZ in a yeast two-hybrid assay, although different terms were given: calsarcin (for calcineurin-associated sarcomeric protein) [246] and myozenin (a γ -Filamin and α -actinin interacting protein) [247].

The FATZ family is comprised of 3 members, FATZ-1 (calsarcin-2 and myozenin-1), FATZ-2 (calsarcin-1 and myozenin-2) and FATZ-3 (calsarcin-3 and myozenin-3), that interact with the Ca^{2+} /calmodulin protein phosphatase, calcineurin, tethering it to the Z-disc [168,248]. Calcineurin is a known Ca^{2+} -activated phosphatase that is involved in the expression of hypertrophic genes [249,250]. FATZ-1 and FATZ-2 are expressed during embryonic development of cardiac and skeletal muscle. FATZ-1 is restricted to adult fast skeletal muscle, while FATZ-2 is mainly expressed in adult cardiac and slow skeletal muscle [246]. FATZ-3 is exclusively expressed in fast skeletal muscle [248]. In addition, FATZ members interact with several other proteins, including ZASP/Cypher/Oracle [184,248] (members of the PDZ–LIM–protein family), ALP [183], and myotilin [175], indicating a potential role as structural scaffold. The role of FATZ and regulation of calcineurin has been evaluated using transgenic mice lacking FATZ-2 [251]. In FATZ-2-null mice calcineurin activity was up-regulated, suggesting that functional FATZ-2 represses calcineurin activation. Mutations in the FATZ-2 gene (*MYOZ2*) have been identified as a novel gene for HCM [252]. However, after screening a large cohort of over 400 HCM patients no mutation was found which led the authors to conclude that *MYOZ2* mutations are an uncommon cause of HCM [253]. Overall, studies in human and mice models suggest that FATZ-2 may have a modulating role in development of hypertrophy.

4.4.3.3. PDZ–LIM proteins. PDZ–LIM proteins are characterized by a PDZ and one or more LIM domains, which via their interaction with α -actinin constitute one of the biggest family members of Z-disc associated proteins [162–165]. The term PDZ is the abbreviation for the first three identified PDZ proteins ‘postsynaptic density 95, discs large, and zonula occludens-1’ and consist of protein domains that play an active role in targeting and assembly of protein complexes [254]. LIM domains are the acronym for the first three identified LIM-domains ‘LIN-11, Isl1m, and MEC-3’, which mediate protein–protein interactions [255].

As recently reviewed [256,257] 10 genes in mammals share both a PDZ domain and one or more LIM domains and have been subdivided based on their structure and phylogenetic similarities: 4 genes of the ALP subfamily (ALP, CLIM1/CLP36, Mystique and RIL), 3 genes of the Enigma subfamily (Enigma, Enigma Homolog and ZASP/Cypher/Oracle), 2 genes of LIM kinases (LIMK1 and LIMK2) and the LIM only 7 (*LMO7*) gene. LIMK1, LIMK2 and *LMO7* have been found in the developing and adult heart, and although in non-muscle cells they are involved in microtubule stability and F-actin polymerization, and possibly cell–cell junction integrity, their roles remain to be determined in the heart [256].

4.4.3.4. ALP (α -actinin associated LIM protein). Four protein members have been classified as belonging to the ALP (α -actinin associated LIM protein) subfamily and each consist of an N-terminal PDZ domain and

a C-terminal LIM domain. All interact with α -actinin however solely ALP and CLP36 are known to be present in the heart [257]. While CLP36 (in rats) and the human homologue CLIM1 are highly abundant in heart muscle [258,259], their function remains to be determined.

ALP was the first member of this subfamily to be described and it was shown to bind and co-localize with α -actinin at the Z-disc via its PDZ domain [260]. In striated muscle, ALP is expressed in high levels in skeletal muscle and low levels in the heart [260], although it predominates in the right ventricle [261]. ALP-deficient mice present right ventricular chamber dilation and dysfunction, which led the authors to suggest that ALP is essential for the embryonic development of the right ventricle of mice possibly assisting the embryo to high biomechanical load [261]. In accordance, ALP would enhance the ability of α -actinin to cross-link actin filaments, thus ensuring a greater mechanical protection [261]. In addition, over-expression of ALP in a transgenic mouse model revealed that ALP colocalized with α -actinin and β -catenin at the intercalated disc [261]. Interestingly, β -catenin is also able to bind to α -actinin [262], indicative for an ALP– α -actinin– β -catenin multiprotein complex. β -catenin accumulation at the intercalated discs is associated with cell–cell junction disruption and development of hypertrophy [263]. ALP-deficient not solely present right ventricular chamber dilation [261], but also exhibit a compromise ability to trigger hypertrophic growth [264]. It is possible that the inability to bind to α -actinin disrupts the formation of a stable complex of ALP– α -actinin– β -catenin, resulting in a defective physical connection with the actin cytoskeleton.

4.4.3.5. Enigma, enigma homolog and ZASP/Cypher/Oracle. Enigma consists of the typical N-terminal PDZ domain and 3 C-terminal LIM domains [265]. Enigma has been shown to bind to actin [266] and together with APS (adaptor protein with PH and SH2 domains) has been shown to bind F-actin and regulate actin–cytoskeleton reorganization in non-muscle cells [267]. In the sarcomere, Enigma is found anchored to the Z-disc (~80%), with the rest localized in the I- and A-bands [268]. Interestingly via its PDZ domain, Enigma binds to the C-terminal region of skeletal β -tropomyosin at the boundary of the I-band and Z-disc, indicative for a regulatory role in cardiac thin filament assembly at the Z-disc [268]. Despite the interesting putative functions of Enigma association to cytoskeleton assembly and transduction of signals, at present the role of Enigma in the heart remains to be determined.

Enigma Homolog protein (ENH) was first identified after a yeast two-hybrid screening of putative interacting proteins with protein kinase C (PKC) [269]. Via its PDZ domain ENH interacts with α -actinin at the Z-disc of cardiac and skeletal tissue [270,271]. In addition, it has been shown that ENH interacts with protein kinase D (PKD) and L-type Ca^{2+} -channels in rat cardiomyocytes [272]. In humans, four ENH isoforms have been identified, of which ENH1 is ubiquitously expressed and the other three isoforms are limited to the adult heart [269,271]. Yamazaki and colleagues [273] showed that ENH1 is highly expressed in the embryonic and neonatal rat hearts, while it is down-regulated and replaced by ENH3 and ENH4 in the adult heart. Over-expression of ENH1 in neonatal cardiomyocytes was associated with expression of hypertrophic markers, while ENH4 over-expression prevented those alterations. Of particular interest, hypertrophy induced by aortic constriction resulted in ENH1 upregulation, in contrast to down-regulation of ENH4 [273]. As recently proposed [274] ENH1 up-regulation following mechanical stretch may anchor PKC and/or PKD via its LIM domains, since ENH3 and/or ENH4 are unable to interact and activate these kinases (they lack LIM domains). In turn, PKC-induced phosphorylation would initiate transcription of hypertrophic growth genes [274]. Transgenic mice lacking ENH develop DCM following mechanical overload, which supports the role of ENH as potential mediator of hypertrophic responses [275].

ZASP [162]/Cypher [164]/Oracle [276] was independently identified by three different groups and was found to detain an important role in

the maintenance of sarcomere structure. ZASP (Z-band alternatively spliced PDZ motif protein) is the human ortholog member and was shown to localize at the Z-disc, where it interacts through its PDZ domain with α -actinin [162,164] and nebulin [205]. Cypher also interacts with myotilin and FATZ-2, although the role of these alterations remains unclear [184]. Cypher-null mice have early postnatal death with disorganized Z-discs and DCM development [184,277]. It has been suggested that Cypher is not required for striated muscle development or Z-disc embryonic assembly, but instead is required for adult Z-disc maintenance during muscle contraction [184,277]. Mutations in the ZASP gene have been identified in patients with DCM [91,278] and HCM [279]. In addition, zaspopathy-causing MFM have also been found and are characterized by late onset with muscle weakness [280].

ZASP/Cypher also interacts with PKC via its LIM-domains, which suggests that it couples PKC-mediated signaling to the cytoskeleton [164]. Of particular interest, PKC ϵ over-expression enhances its co-localization with membrane proteins, such as RACK (receptors for activated C kinase), resulting in cardiac hypertrophy, while inhibition of this interaction is observed in DCM [281–283].

Altogether these studies support the notion that these proteins serve dual roles in the myofilaments as both cytoskeleton adaptor proteins and signaling proteins. In agreement, via their LIM domains they recruit signaling proteins, such as PKC, and via their PDZ domain interact with cytoskeletal proteins including α -actinin, tropomyosin and nebulin.

4.4.3.6. LIM-only proteins. LIM-only proteins are Z-disc associated proteins that consist of LIM domains and are involved in cytoskeletal organization and integrity. In the present review, we will focus on two of these members: zyxin [284] and muscle LIM protein (MLP) [285].

Zyxin is suggested to be both involved in cytoskeleton organization, where it anchors to the Z-disc by interacting with α -actinin [166], nebulin [203], MLP [286] and myopodin [287], and is involved in intracellular signaling between the nucleus and the costamere [288,289]. cGMP-dependent nuclear translocation of zyxin after ANP (atrial natriuretic peptide) receptor stimulation of cardiomyocytes, results in zyxin–Akt interaction and potentiates cell survival, indicative of its anti-apoptotic effects [290]. Activation of cGMP/PKG (protein kinase G) has proven essential in survival signaling during ischemia–reperfusion [291], which is possibly partly associated with the role of zyxin-induced survival of cardiomyocytes.

MLP is specifically expressed in striated muscle and it is suggested to be involved in myogenesis and stretch signaling responses [92,285,292]. Several binding partners have been demonstrated to interact with MLP including α -actinin and N-RAP [293], zyxin [286], telethonin [294], ILK [295], calcineurin [296], myoD [297] and β -spectrin [298]. The precise location and function of MLP remains however controversial. It has been reported that MLP is either flanking [92] or located at the Z-disc (bound to telethonin, see below), suggesting that MLP is a cardiac-stretch sensor [294]. MLP-causing mutations associated with HCM result in decrease MLP binding to both N-RAP and α -actinin [293], which may argue for Z-disc location during early steps of myofibril assembly. In addition, MLP-null mice develop severe DCM with major defects in cytoskeletal organization [92], associated with decreased passive tension compared to wild-type mice [294] and increase N-RAP expression [299], possibly to maintain normal myofibril assembly. On the other hand, other laboratories however failed to detect MLP at the Z-disc in both neonatal and adult cardiac tissue. Recent evidence indicates that MLP rather has a subcellular localization including the nucleus, the costamere and the cytosol, making it less likely that MLP works as a direct sarcomeric-stress sensor [292,298,300,301]. Instead, MLP may act as a signaling transducer in mechano-sensory cascades [292,302]. In failing human hearts, due to

ischemic cardiomyopathy and DCM, MLP expression was reduced [303]. Mutations in the MLP gene have been linked to either HCM [301] or DCM [156], which highlights the complexity of MLP-derived effects in disease progression.

In summary, the Z-disc comprises the lateral boundaries of the sarcomere, responsible for the anchorage of the thin filament and maintains the stable propagation of force during contraction. Traditionally it is believed that the generated forces of cross-bridges within each sarcomere are transmitted longitudinally to the adjacent sarcomere along the myofibril, although evidence suggests it may only account to ~20–30% of total force transmitted [215]. This indicates that the major propagation of force follows a lateral pathway across the sarcolemma, which possibly involves the costameres. The costameres make up the lateral connections of the Z-disc with the sarcolemma and ECM, thus providing a homogeneous transmission of force between neighboring sarcomeres and ensure greater mechanical resistance. The costameres are responsible for the physiological and pathological adaptations of cardiomyocytes accompanying mechanical load. Dystrophin–glycoprotein and integrin–vinculin–talin complexes are central to preserve sarcolemmal and ECM integrity via integration and transduction of biomechanical signals. Abnormalities caused by mutations in these elements may restrict their capacity to monitor stress, weakening attachment of the ECM to the cytoskeleton, disrupting Z-disc alignment and transmission of force, as observed in DCM and muscular dystrophies. Nonetheless, as shown by full knock-out animal models of several of these components, the Z-disc tries to compensate for the loss of some of its members. Transduction of biomechanical signals is accomplished via anchorage at the Z-disc of specialized cytoskeletal proteins (e.g. FATZ, ENH, ZASP and zyxin) that monitor and sense mechanical stress. These are able to recruit and interact with protein phosphatases and/or kinases, and regulate cardiac remodeling. Altogether these findings support a very dynamic Z-disc structure, capable of integrating and transducing biomechanical signals, and adapt cardiomyocytes during physiological and pathological conditions.

4.5. The third filament: titin

4.5.1. Titin assembly

Involved in the development and organization of the sarcomere, titin [304] (or connectin [305]) has been considered central in the heart's adaptation to biomechanical stress. Titin is a giant protein with a molecular weight ranging from 3.0 to 3.8 MDa that extends from the Z-disc to the M-band [306,307]. In the Z-disc, the N-terminal ends overlap adjacent titin polypeptides and are capped by the cap protein telethonin (also known as T-cap) [308,309]. Telethonin has been detected in early myotube formation in differentiating myocytes where it is phosphorylated by the titin-kinase [309,310]. Additionally, the N-terminal insertion of titin in the Z-disc interacts with actin [311] and possibly also α -actinin [312,313] via a 45 amino acid repeat region (i.e. Z-repeats) suggested to provide a mechanism of Z-disc assembly of variable thickness as a result of alternative splicing [314]. The I-band region of titin is the extensible region and consists of three elastic components that act as a spring element: 1) tandem immunoglobulin (Ig)-like domain regions, with proximal (near Z-disc) and distal (near I–A regions) segments, 2) the PEVK sequence-region rich in proline (P), glutamic acid (E), valine (V) and lysine (K) and 3) the N2B and N2BA elements (both isoforms contain N2B segments, but only the N2BA isoform contains an additional N2A element) [315]. The A-band region of C-terminal ends of titin is inextensible, as they interact with thick filament and associated proteins, such as myosin and cMyBP-C [307,316,317]. In the M-band, titin molecules from opposing half-sarcomeres intersect, where they are interconnected by M-band proteins, thereby forming a continuous filament from the M-band towards the Z-disc [318,319].

4.5.2. Titin stretch-based passive tension

Under resting conditions, striated muscle resists muscle lengthening by producing passive tension in response to stretch. In the heart, titin accounts for approximately 80% of total passive tension between physiological operating sarcomere lengths (i.e. 1.8 to 2.2 μm). Interestingly, over-stretching cardiac muscle above 2.2 μm , where the contribution of collagen is greater, the titin stretch-based passive tension remains high, indicative of the central role of titin to respond to stretch [132,133]. Passive (resting) tension results from the extensible I-band spring segment of titin that elongates as sarcomere length increases. The tandem Ig-like segments are the first to extend, followed by the PEVK segment and lastly, the elongation of the N2B segment [318,319]. The flexibility and stretch-based passive tension of the titin spring elements can be regulated by two major mechanisms: a fast “acute” modulation by 1) post-translational modifications via protein phosphorylation and a 2) “chronic” isoform shift due to alternative splicing of the I-band encoding region of the titin (*TTN*) gene gives rise to different isoforms.

Titin-dependent phosphorylation by protein kinases has been demonstrated to regulate cardiomyocyte stiffness. In accordance, PKA and PKG phosphorylate titin at the N2B unique sequence (N2Bus) and result in decreased cardiomyocytes stiffness [20,320–322], whereas PKC α phosphorylates titin at the PEVK region which increases stiffness [323,324]. In cardiomyocytes from HFpEF patients high stiffness has been detected, which was corrected upon PKA administration [325]. The high passive stiffness in HFpEF was related to a titin phosphorylation deficit [31]. More recently, reduced cGMP/PKG activity has been found in HFpEF patients and a large HFpEF animal model [326–328]. Exogenous administration of PKG was able to lower the high titin-based stiffness [326–328]. In addition both the N2Bus and PEVK can be phosphorylated by CaMKII δ , which has been associated with a reduction in passive stiffness [329,330]. Both activity and expression of CaMKII were found to be higher in end-stage failing human hearts than in non-failing donors [330].

In addition to post-translational modifications of titin, alternative splicing [331]. The small isoform (~3.0 MDa) of titin is termed N2B and contains a unique sequence (N2Bus) characteristic of cardiac titin [331]. In addition to N2B, the I-band region of titin also contains the N2A element, producing a larger titin termed N2BA, resulting in a longer PEVK segment and additional Ig-like domain regions (~3.3–3.5 MDa), turning it into a more compliant titin [318,331]. During heart development and neonatal stages, the isoforms contain additional spring elements in both tandem Ig-like domain regions and PEVK regions, which result in an even greater titin isoform (~3.6–3.8 MDa) [318,332]. In the adult human heart, both N2B and N2BA isoforms are expressed, although the population of the smaller N2B spring element predominates. To this extent, the isoform content ratio (N2BA:N2B) determines the amount of passive tension, such that the smaller this ratio, the greater cardiac stiffness is generated [333–335]. An increased N2BA:N2B ratio is observed in DCM patients resulting in reduced cardiomyocyte stiffness [334,336]. Together, these studies support the central role of titin-isoform shift and phosphorylation in modulation of cardiomyocytes stiffness in HF patients.

4.5.3. Titin, a regulator of length-dependent activation

Titin is not just solely a passive spring element, as emerging evidence indicates it may also play a role in the development of active tension and serve as a length-dependent sensor. In accordance, a correlation between enhanced length-dependent activation and higher levels of passive tension has been reported [337–339]. It is suggested that titin-based passive tension potentiates cross-bridge formation via reduction of lattice spacing upon stretch [338,339]. A recent study supports this idea, as reduced myofilament force development and cycling rates, and impaired length-dependent activation [340] were found in a rat that harbors a homozygous autosomal mutation that expresses a giant titin isoform (N2BA-G, ~3.9 MDa) [341]. Altogether, these findings indicate that titin affects the contractile

apparatus by affecting cross-bridge cycling kinetics, possibly regulating myofilament length-dependent activation.

4.5.4. Titin, an integrator of the mechano-sensory machinery

Titin also mediates mechano-sensory events in response to stress. A variety of titin binding-partners have been reported to adapt to sustained pressure-overload and activate hypertrophic signaling responses. It is beyond the scope of this review to detail a thorough discussion of these pathways and therefore, we refer the reader to extensive reviews on the topic (see Refs. [318,319,342]).

Accordingly, putative mechano-sensory pathways involving titin include telethonin and several telethonin-interacting partners. It has been demonstrated that telethonin binds at the Z-disc and recruits MLP [294]. Resting non-stimulated cardiomyocytes present cytosolic accumulation of MLP, which are translocated to the nucleus after hypertrophic stimulation [343]. In addition, MLP elevation is observed after myocardial infarction, indicative of its putative role during cardiac remodeling [344]. MLP also binds to calcineurin [296] and over-expression of calcineurin triggers cardiac hypertrophy, while its inhibition suppresses development of hypertrophy [345]. Of particular interest, telethonin-deficient mice have normal heart development and function [346], but show progressive loss of t-tubules and dys-synchronous Ca²⁺-transients that worsens with age [347]. Following pressure-overload increments, telethonin-deficient mice develop HF which was partly associated with cardiomyocyte death by apoptosis [346] and further loss of t-tubules [347]. In accordance, Knöll and colleagues [346] observed that telethonin and p53 (tumor suppressor protein) co-localize in the nucleus following biomechanical stress, resulting in p53's transcription repression. Mice lacking telethonin are not able to repress p53 function and thus, have an increased p53 expression and elevated apoptosis following pressure-overload with progression to HF [346]. Together, these findings support that telethonin and its telethonin-interacting partners, may be involved in the mediation of cardiac remodeling and hypertrophy growth responses, in addition to maintain normal Ca²⁺-transients associated with normal t-tubule structure. Additional support for this view can be drawn from telethonin-causing mutations. Telethonin-mutations can either cause HCM or DCM, which has been associated with either increased interaction of telethonin with titin and FATZ-2, or decreased binding of telethonin with MLP, titin and FATZ-2, respectively [348]. One can speculate from these results that increased binding of telethonin with its interacting-partners would potentiate the hypertrophic growth response, in contrast to its decrease binding associated with a deficit to trigger the hypertrophic signaling and development of a dilated phenotype.

In addition, a link has been described between titin and members of the four-and-a-half LIM domains (FHL) family at the I-band and M-band, possibly involved in biomechanical stretch responses [349,350]. Sheikh and colleagues [350] demonstrated that FHL1 interacts with members of the mitogen-activated protein kinases (MAPK) cascade (i.e. Raf1, MEK1/2 and ERK2) that are localized with the N2B element of titin. Interestingly, FHL1 deficient mice have a blunted hypertrophic response with preserved cardiac function [350]. In accordance, deletion of the PEVK segment is associated with reduced N2BA:N2B ratio and up-regulation of FHL1/2, and development of cardiac hypertrophy [351], in contrast to cardiac atrophy with decreased FHL2 expression resulting from deletion of the N2B element [352]. Together, these studies indicate that FHL plays an essential role in biomechanical stress signaling via the N2B element of titin, possibly by negative regulation of MAPK/hypertrophic signaling cascades. In agreement, it was shown in FHL1-deficient mouse that the N2B element is a target of ERK2 phosphorylation, which is believed to increase muscle compliance by lowering titin-based cardiac stiffness [353]. These findings suggest that FHL1 and ERK2 are part of a stretch-sensor complex in combination with the N2B element of titin, in which they possibly exert dual roles following biomechanical

stress: 1) recruit MAPK-mediated effectors triggering development of cardiac hypertrophy and 2) mask phosphorylation sites on the N2B element, such as ERK-mediated phosphorylation, maintaining baseline cardiac stiffness [353].

4.6. The M-band

4.6.1. M-band proteins

Located in the center of the A-band, the M-band provides the regular packing of the thick filaments and allows the uniform distribution of tension along the filament lattice structure. It has been suggested that the M-band prevents sarcomere displacement by promoting symmetric shortening of both sarcomere halves during each contraction cycle, maintaining the alignment of the A-band [354,355]. In addition to the C-terminus of titin and myosin tails, three protein family members have been identified at the M-band: myomesin [356] (*MYOM1*); ubiquitously expressed in all striated muscles), M-protein [357] (*MYOM2*; expressed in adult heart and fast-twitch skeletal muscle) and myomesin 3 (*MYOM3*; expressed in human adult heart [358] (but not in mouse) and skeletal muscle) [359]. Both myomesin and M-protein are suggested to interact with titin and myosin [360–362], and the newly identified myomesin 3 shares the predicted-binding regions with M-protein to bind both myosin and titin [359]. Myomesin exerts an analogous role to α -actinin, as it forms anti-parallel dimers that link the thick filaments [363]. Mutations in myomesin that result in abnormal dimerization of the protein have been linked to HCM in humans [364], indicative of its putative role in sarcomere stability. During embryonic development another myomesin isoform is expressed (almost absent in the adult heart) via alternative splicing of the myomesin gene (*MYOM1*), termed embryonic heart (EH)-myomesin, resulting in the inclusion of 100 amino residues [354,355]. Accordingly, it has been shown that the EH-segment may serve as a molecular spring in the M-band, working in a similar way to the PEVK region of titin, protecting and preventing against extreme stretching and rupture [365]. Since EH-myomesin is primarily expressed during embryonic development with reduced postnatal expression and M-protein is only expressed after birth, it indicates that differential amounts of these M-band proteins may adjust the mechanical requirements of the M-band to different working load conditions. One can speculate that during HF situations, where up-regulation of fetal proteins are observed, the mechanical rigidity of the M-band would be varied by shifting from stiffer proteins (M-protein) to fetal more compliant isoforms (EH-myomesin). In agreement, a recent study using MLP-deficient animals and human DCM patients observed a marked up-regulation of EH-myomesin (41-fold) compared to both controls and HCM patients [358]. In the MLP-deficient animals, M-protein is down-regulated and expression of myomesin 3 is observed [358]. As already mentioned, increased N2BA:N2B ratio is observed in DCM patients resulting in reduced cardiomyocyte stiffness and much greater compliant titin isoforms [334,336]. Together, these findings suggest a dual adaptation of the cytoskeleton (i.e. M-band and titin) to adapt during DCM development and support the dynamic role exerted by the M-band.

4.6.2. M-band proteins and associated binding-partners

Several binding-partners have been shown to localize at the M-band, the most pronounced interaction being muscle-type creatine kinase (MM-CK) with myomesin and M-protein [366]. CK catalyzes the re-phosphorylation of ADP to ATP by using phosphocreatine as a substrate, thereby preventing excessive accumulation of ADP and replenishing the available ATP essential for cross-bridge cycling [367]. In addition, other metabolic enzymes have been demonstrated to localize at the M-band, such as adenylate kinase (involved in the conversion of ATP to cAMP) and phosphofruktokinase (responsible for the regulation of glucose levels) via interaction with FHL-2 [349]. Altogether

these studies indicate a hot-spot for metabolic enzymes co-localization within the sarcomere. Since FHL-2 interacts with the M-band region of titin [349] and CK with myomesin and M-protein, the M-band seems to represent a target for enzymes involved in energy consumption of cardiac muscle.

As mentioned above, FHL members are suggested to modulate stretch responses via interaction with integrins [368], which suggests that the M-band may be able to mediate mechanical stress responses. Following mechanical stress a link has been proposed between the titin-kinase domain (located in the M-band region of titin) and the MURF pathway involved in myocardial hypertrophic signaling, such that it could serve as a mechano-sensory complex to trigger cardiac hypertrophy [319]. A novel M-band component, termed myomasp (myosin-interacting M-band-associated stress-responsive protein)/LRRC39, has been recently discovered and suggested to be involved in mechano-stretch sensing [369]. Of particular interest, myomasp interacts with myosin, and myomasp-null mice have a dramatically reduced expression of *MYH7* and *MYOM1* and *MYOM2* genes with up-regulation of *BNP* (a marker for hypertrophic signaling). Disturbed M-band architecture associated with low force generation was observed in myomasp-deleted engineered hearts, and animal models with aortic constriction showed a dramatic down-regulation of myomasp. It suggests that myomasp is a negative regulator of stretch-sensitive genes and a modulator of the molecular composition of the M-band [369]. Moreover, it has been shown that both titin and myomesin interact with obscurin (Z-disc titin is also known to interact with obscurin), which appears essential for M-band assembly [370]. Obscurin is a giant protein expressed in striated muscle (Mw ~800 KDa) that is suggested to modulate myofibril and sarcoplasmic reticulum (SR) organization and assembly [371–373]. In accordance, obscurin interacts with ankyrin-1 at the M-band (involved in sarcolemma-cytoskeleton interactions) and establishes obscurin–SR interactions [371]. Additionally, it has been demonstrated that ankyrins function as adaptors for the localization of dystrophin and dystroglycans at the costameres [374]. Recent work has demonstrated a reduced localization of dystrophin (but not dystroglycans) associated with reduced exercise tolerance, muscle strength and sarcolemma fragility in obscurin-null mice [136]. Of particular interest, wild-type animals presented a predominant localization of ankyrin-B at the M-band that was lost in obscurin-deficient mice, indicative for the putative role of obscurin in recruiting ankyrin-B [136]. Ablation of obscurin impaired the normal assemblies of the microtubule network to the sarcolemma. Based on this observation the authors hypothesized that upon M-band disruption, the microtubule network at the subsarcolemmal region collapses, and thereby alters the localization of dystrophin at the Z-disc. This study thus suggests that obscurin is essential for ankyrin-B dependent localization of dystrophin to the sarcolemma. A representative picture of these interactions (proposed by Randazzo and colleagues [136]) is presented in Fig. 3. It deserves however to be mentioned that the intermolecular interactions between obscurin, ankyrin-B, the microtubules and dystrophin reported [136] have only been identified in skeletal muscle, and thus the existence of such interactions remain to be determined in cardiac muscle and potential associated cardiac pathologies.

5. Discussion

Cardiomyocytes are efficient biochemical machineries specialized in the rapid generation of force and movement, central to the work generated by the heart. Thick myosin filaments, powered by ATPase activity, interdigitate with thin actin filaments, resulting in the repetitive cycle of continuous attachment, sliding and detachment, known as the cross-bridge cycle. Central to this system is the delicate balance of intracellular Ca^{2+} ions, mediated by a mechanism known as excitation–contraction coupling, that bind to cTnC and promote the steric Ca^{2+} -regulation of troponin–tropomyosin movement

and exposure of myosin-binding sites. Cardiomyopathies as in the case of HCM-causing mutations of thick and thin filament proteins account for ~98% of all cases and are associated with alterations in Ca^{2+} -handling, actin-sliding velocities and ATPase activity, emphasizing the essential role of these proteins in the contractile activity of the heart.

Mechanical stability of the thin filament proteins is ensured by its anchorage to multiple-cytoskeletal proteins found at the Z-disc, which contact the lateral boundaries of the sarcomere and provide the stable propagation of force during contraction. The cross-linker α -actinin forms a dynamic web capable of integrating multiple protein complexes, and strengthens and aligns the Z-disc. The costameres form the lateral connections of the Z-disc with the sarcolemma and ECM, thus ensuring greater mechanical resistance. Mediated by γ -filamin, F-actin bundle networks interact with both dystrophin-glycoprotein and integrin-vinculin-talin complexes at the sarcolemma, which make up a continuation to the ECM. DCM-causing mutations and muscular dystrophies are associated with weakened attachment of the ECM to the cytoskeleton, disruption of Z-disc alignment and reduced propagation of force. Inability of the costameres to monitor and sense stress, thus limit their capacity to modulate and activate stress-signaling pathways. In accordance, DCM has been associated with increases in both dystrophin-glycoprotein protein synthesis [375] and vinculin, but also in tubulin and desmin [239]. The increased expression of cytoskeletal proteins possibly reflects compensatory responses to preserve the impaired management of contractile stress.

Not solely important for the generation of active tension, cardiac muscle is composed of spring elements like titin that importantly align the A-band during cardiac relaxation and together with the surrounding connective tissue (ECM) resist excessive stretching, which is known to cause severe myocardial damage [376–378]. Titin is the main passive stretch modulator within physiological limits. Acute-responses via post-translational modifications of titin or long-term switches of titin's isoforms modulate the ability of the spring elements of titin to respond to stretch and adapt both systolic and diastolic function of HF patients. This dynamic function of titin and Z-disc components in modulating signaling pathways emphasize their role as stress sensors. Recent evidence suggests that the M-band is also able to respond to stretch, which indicates that all structures of a myofibril are able to manage stress and quickly adapt their function to altered physiological and pathological situations.

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