Elisabeth Ehler *Editor*

Cardiac Cytoarchitecture

How to Maintain a Working Heart

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Cardiac Cytoarchitecture: Why Bother?

The heart is the first functional organ in mammals. Its contractile work is needed to pump blood throughout the body to supply peripheral tissues with oxygen and nutrients. Any impediment of heart function dramatically affects the quality of life, and loss of function is incompatible with life. The bulk of the heart mass is made up of cardiomyocytes, which occupy about 90 % of the left ventricular tissue (Reiss et al. 1996). However, counting individual cells leads to a different picture with only 15 % of cells having cardiomyocyte identity in the murine heart (Soonpaa et al. 1996). Therefore, while the muscle mass is made up by cross-striated cardiomyocytes, they are outnumbered by non-muscle cells that make up the blood vessels and also the interstitial cells, the fibroblasts. This remarkable arrangement means that in case of loss of cardiomyocytes due to necrosis following limited blood supply, as it happens, for example, during myocardial infarction, the majority of remaining cells in a particular area of the heart are actually non-muscle cells, such as fibroblasts. They will replace the necrotic area by fibrotic tissue, which has obvious deleterious consequences on overall heart function. An additional complication in this scenario is that in contrast to skeletal muscle, there is numerous population of stem cells such as satellite cells in cardiac muscle that could regenerate a damaged area. Cells with stem cell-like properties were identified by several research groups in the heart (Laugwitz et al. 2005; Oh et al. 2003; Urbanek et al. 2005), but their numbers appear to be too scarce to result in robust regeneration. Adult cardiomyocytes are terminally differentiated and may respond to an insult with polyploidy but not with cell division. It is assumed that one of the reasons why they cannot be induced to divide is their complex cytoarchitecture. They have a rod-like shape and are completely filled with myofibrils and mitochondria (Fig. 1; for schematic drawing, see Fig. 2a). In a way, they resemble a salami sausage, with the red, meaty parts resembling the myofibrils and the interspersed white fat bits resembling the mitochondria. In order to undergo cell division, this tightly packed arrangement would have to be broken. Embryonic cardiomyocytes, which still possess the ability to divide, have a polygonal shape and much more loosely packed myofibrils, which have to be disassembled for the cells to undergo cytokinesis (Ahuja et al. 2004). This procedure is probably too costly in a fully

differentiated adult cardiomyocyte, which is why the expression of cytokinetic marker proteins is induced in cardiomyopathy (Ahuja et al. 2007), but no cell division takes place.

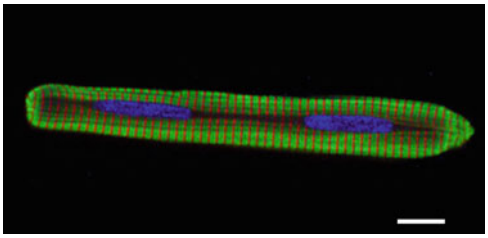

Fig. 1 Confocal micrograph of a freshly isolated individual mouse cardiomyocyte. Myofibrils are tightly packed in a paracrystalline fashion in cardiomyocytes. The myofibrils were immunostained for the proteins sarcomeric alpha-actinin (*red signal*, delineating the Z-disc of the sarcomere) and MyBP-C (*green signal*, the doublets show the A-band of the sarcomere). The nuclei are shown in *blue* (DAPI staining). Scale bar equals 10 μm

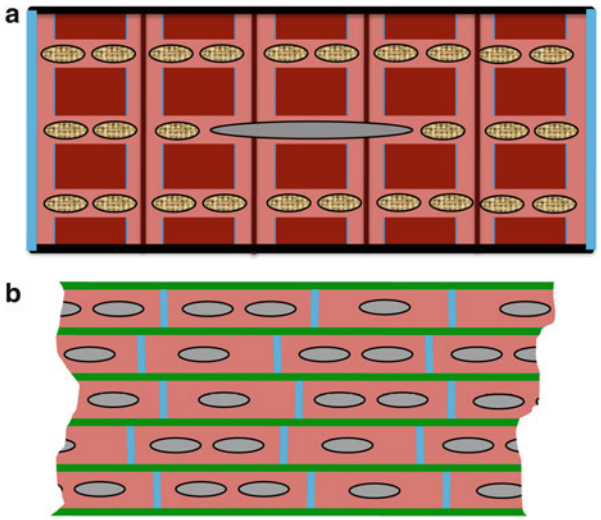

Fig. 2 Schematic depiction of an isolated adult cardiomyocyte (**a**) and of the arrangement of cardiomyocytes (**b**). (**a**) The myofibrils are shown in *red* (*darkest red* is Z-disc, *dark red* is A-band), the mitochondria are in *brown*, the nucleus in *grey* and the intercalated disc, the specialised type of cell–cell contact, is shown in *light blue*. (**b**) The intercalated discs are shown in *light blue*, and the costameres, which are the specialised contacts that cardiomyocytes make laterally to the extracellular matrix that ensheathes them, are shown in *green*

Not only are cardiomyocytes themselves characterised by this very rigid shape, but also the way they are assembled into functional heart tissue is very organised.

For example, in a healthy adult heart, the direct contacts between neighbouring cardiomyocytes are restricted to their bipolar ends in a specific kind of cell–cell contact structure, the intercalated disc (Fig. 2b). No direct cell–cell contacts are found at the lateral borders of the cardiomyocytes (Perriard et al. 2003). There, the cardiomyocytes are ensheathed in extracellular matrix, which is mainly composed of collagens I and III with some laminin and collagen IV. The myofibrils are mechanically anchored to the surrounding extracellular matrix via a specific cell–matrix contact called the costamere (Pardo et al. 1983), which is a rib-like ring along the cardiomyocytes at the level of the Z-disc of the sarcomere and which provides the link to the ECM.

Why is this peculiar and so highly organised three-dimensional arrangement of the cardiomyocytes so important for the function of heart tissue? The segregation of cell–cell contacts at the ends and cell–matrix contacts at the sides ensures that the electrical propagation of the stimulus to contract happens in a directed fashion. The gap junctions, which make up the ion channels that transfer the electrical signal, are only at the ends of the cells, while their sides are ensheathed in insulating material, similar to an electrical cable. The heart can be seen as composed of tightly packed electrical cables that are arranged in a complex but predictable course to a three-dimensional mesh with helical and circular components (Smerup et al. 2009). This tightly regulated geometry leads to a controlled contraction of the ventricle and an efficient expulsion of blood in the healthy heart. Too much lateral leakage of the electrical signal would interfere with this process. In cardiomyopathy this tissue composition is compromised, leading to often potentially fatal arrhythmias.

The fact that heart tissue is made up in an extremely regular fashion by very rigidly shaped cells is the biggest problem in all attempts to replace fibrotic tissue by contractile cardiomyocytes. Ideally the same complicated cytoarchitecture should be achieved since otherwise a successful outcome is endangered by potentially life-threatening arrhythmias.

As mentioned above, adult cardiomyocytes do not divide and the numbers of residing cardiac stem cells are limited and disputed. While adult zebrafish can regenerate their heart fully, if the tip of the ventricle is cut off (Poss et al. 2002), this ability is lost in mammals in the first week after birth (Porrello et al. 2011). Interestingly this failure to regenerate is tightly correlated with the arrest in dividing activity in the cardiomyocytes (Soonpaa et al. 1996). Apart from the activation of the stem cell population, there are two major options that can be envisaged to replenish damaged heart tissue, either triggering proliferative behaviour in resident adult cardiomyocytes or introducing replacement cells that integrate with the endogenous tissue and help with the contractile work. For both cases, full function will only be achieved if the rules of cardiac cytoarchitecture and cardiomyocyte integration are adhered to. So far the results from most human trials that attempted to repopulate scar tissue after a myocardial infarction with several different (usually multipotent) cell types were hugely disappointing after initial success in small rodent models (for recent critical reviews, see Behfar et al. 2014; Pavo et al. 2014; Rosen et al. 2014). This is partially due to the abysmal survival rates of injected cells, suggesting that the scar area is an extremely hostile environment.

Any residual beneficial effects are usually attributed to paracrine effects of the injected cells by inducing vascularisation or loosening fibrotic mass. A recent study in a primate model that tried to counteract loss of cells by injecting billions of them in a more differentiated state (Chong et al. 2014) reported regeneration and functional integration with the host myocardium but also had to admit that arrhythmias occurred in every single experimental animal. This suggests that not only is it crucial to identify a cell population that can differentiate to a cardiomyocyte and is able to survive in the scar tissue environment but also that the functional integration of any introduced cell with the remaining host myocardium is absolutely required for successful therapy. Tissue engineering approaches that use repopulated cardiac extracellular matrix (Ott et al. 2008) or differentiated cardiomyocytes that are organised to sheets (Kawamura et al. 2012) or contractile rings (Zimmermann et al. 2006) solve the problem of organising the transplanted cell population initially to a cardiac tissue-like arrangement and ensure survival of the cells, but should ideally also result in functional mechanical and electrical integration with the host tissue to be more than a helpful "patch".

In order to determine the "gold standard" for cells or tissue-like assemblies of cells to be used in regenerative therapy in the heart, we first have to establish the base line as far as cardiomyocyte structure and integration to a fully functional tissue are concerned. The aim of this book was therefore to collect a number of articles that look at cardiac cytoarchitecture from slightly different angles and that describe changes that are seen at the cellular level in heart disease (Fig. 3). The first chapter (Pluess and Ehler) sets the stage by discussing the general phenotype of a healthy adult cardiomyocyte in more detail. This "ideal" cardiac cytoarchitecture is then compared with the phenotype that is seen in cardiomyocytes in the embryonic heart and the changes in cytoarchitecture that are seen in cardiomyopathy. The second chapter discusses how cardiac cytoarchitecture is shaped by the environment that the cardiomyocyte finds itself in and which effects mechanical challenges have on structure and thus function (Knight, Grosberg and McCain). The specialised type of cell–cell contact between cardiomyocytes, the intercalated disc, and its role in cardiomyocyte growth in the adult and how it is affected in cardiomyopathy are described in Chap. 3 (Bennett). The following five chapters deal with different parts of the myofibrils and discuss their molecular composition, regulation, signalling roles and changes in disease. Henderson and Gregorio (Chap. 4) look at how thin (actin) filaments are assembled and which proteins regulate their stability and define their length. Chapter 5 deals with thin filament function and how it is regulated (Vetter, Thompson and Metzger). Krüger discusses the elastic filament protein titin and the effect that posttranslational modifications have on its function (Chap. 6). Agarkova and Ehler describe the M-band in the middle of the sarcomere and its role in dealing with active mechanical stretch during the contractile cycle (Chap. 7). Chapter 8 extends the molecular players of the sarcomeres beyond the stalwarts actin and myosin and discusses signalling roles of recently discovered sarcomeric proteins (Frauen, Frank and Frey). The architecture of the nuclear cytoskeleton and its link to the cytoplasm is covered by Brayson and Shanahan in Chap. 9, and in Chap. 10 light is shed on energy

metabolism in the heart including the mitochondria (Pelosse, Tokarska-Schlattner and Schlattner). Chapter 11 focuses on familial hypertrophy cardiomyopathy and discusses some of the potential functional consequences of mutations in—mainly—sarcomeric proteins (Cahill and Gehmlich). To round it all up, the final Chap. 12 (Blondelle and Lange) focuses on garbage disposal in the cardiomyocytes, which is of utmost importance in a terminally differentiated cell and which is carried out and regulated in an intricate fashion.

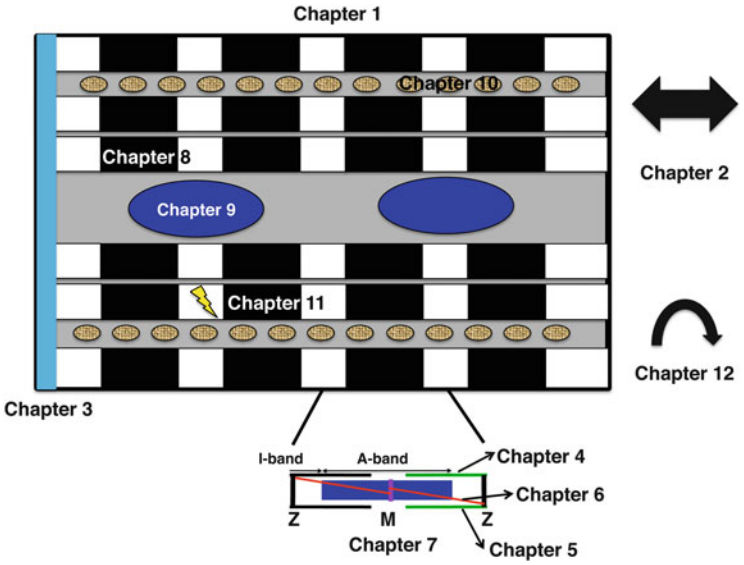

Fig. 3 Schematic depiction of an isolated adult cardiomyocyte and illustration of the topics the different chapters of this book deal with. The myofibrils are shown in *black* (A-bands) and *white* (I-bands), the nuclei are shown in *blue*, the mitochondria are shown in *brown* and the intercalated disc is highlighted at the *left end* in *light blue*. Below the cell is a schematic depiction of a sarcomere that shows the titin filaments in *red*, the thick filaments in *blue*, the thin filaments in *green* and the M-band in *purple*

Together all these chapters give an insight into the life and structure of a cardiomyocyte. They tell us how cell biological research can contribute to a better understanding of a disease phenotype, since the changes that are seen at a cellular level in the cardiomyocytes in heart disease can explain to a certain extent the compromised function that is observed at the tissue and organ level.

Acknowledgements

I would like to thank all the authors in this book for their exciting contributions. It was a pleasure to interact with all of them and I deeply appreciate their—mostly—enthusiastic response to my invitation to contribute a review to this compilation. A particular mission of mine was to ensure a more balanced representation of female to male authors than is seen usually, and this book is proof that there is a fantastic pool of female scientists, who are perfectly able to write reviews. In addition, I am grateful to Springer Publishing for their patience. The writing of review articles for a book is not necessarily top priority for researchers these days, and the submission date of the collated articles was therefore significantly later than initially anticipated.

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London, UK Elisabeth Ehler

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Contents

Chapter 1
Cardiac Cytoarchitecture in Health and Disease

Marlene Pluess and Elisabeth Ehler

Abstract Cardiomyocytes are distinguished by a particularly regularly arranged cytoskeleton. Both the myofibrils, which perform the contractile work of the heart, and the intercalated discs, a special type of cell–cell contact that serves for mechanical and electrochemical connection between individual cardiomyocytes, are multiprotein complexes that must be assembled in a regular fashion during development to guarantee a fully functional heart. In heart disease such as hypertrophic cardiomyopathy, dilated cardiomyopathy or arrhythmogenic right ventricular cardiomyopathy, these structures can be compromised in their composition and thus function. The aim of this chapter is to discuss how cardiac cytoarchitecture is established during development and how it is altered in disease.

Cardiomyocytes, which make up the contractile tissue of the heart, are characterised by a particularly regular cytoarchitecture that ensures that they deliver the maximum in contractile force from sliding actin and myosin molecules. They are arranged into a three-dimensional tissue in a way that guarantees a minimum of leakage of the signal that leads to a coordinated contraction throughout the heart chamber. This complex cytoarchitecture is mainly defined by the organisation of cytoskeletal proteins to multiprotein complexes such as, e.g., the myofibrils or the intercalated discs. This regular arrangement has fascinated researchers for decades and many studies were carried out over to years to investigate the assembly and maintenance of these structures either in cultured cardiomyocytes or in the animal in situ.

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1.1 Cardiomyocytes During Development

The heart is the first functional organ in developing mouse and chick embryos. It starts beating at embryonic day 9 in mouse and Hamburger–Hamilton stage 10 in chick and failure to function results in early embryonic lethality (Van Vliet et al. 2012). In the zebrafish embryo, the somites differentiate before the heart and also defects in heart function can be survived a bit longer due to the possibility of nutrient diffusion (Bakkers 2011). Remarkably this means that while the embryonic heart already has to carry out a certain amount of work, it is still being reshaped into a four-chambered organ in mammals and it also has to grow in size by a process called hyperplasia as the embryo grows (Rana et al. 2013). In contrast to other tissues, where new supply of cells comes from a less differentiated precursor population, all cardiomyocytes in the embryonic heart appear to possess functional myofibrils and can contract. Analysis for classical components of the myofibrils such as sarcomeric alpha-actinin, sarcomeric myosin heavy chain, titin or myomesin reveals no difference in sarcomeric protein expression throughout an embryonic ventricle (Ehler et al. 1999), suggesting that there is no numerous population of less differentiated cardiomyocyte precursor cells. However, it appears that the dynamic processes of contraction and cell division are not compatible with each other. Therefore, in order to allow for hyperplastic growth, the cardiomyocytes disassemble their myofibrils, undergo cell division and reassemble their myofibrils immediately after cytokinesis, while always remaining integrated in a tissue surrounded by beating neighbouring cardiomyocytes (Ahuja et al. 2004). Around postnatal day 4 in the mouse, cell division ceases and from then onwards the heart grows by increase of the size of the individual cardiomyocytes, a process called hypertrophy (Leu et al. 2001). Interestingly, this time of cessation of divisional activity of cardiomyocytes overlaps exactly with the time when successful repair of injury in the mouse heart cedes to happen (Porrello et al. 2011). If the tip of a mouse heart ventricle is cut off at postnatal day 0, the heart recovers fine and the missing tissue is replaced by neighbouring cardiomyocytes that are stimulated to undergo cell division following the injury. At postnatal day 7 this recovery is no longer possible and a similar scar is produced as in an injured adult heart, for example following myocardial infarction (Porrello et al. 2011). Ability to divide and to regenerate therefore seems to be tightly coupled and it is of huge interest to elucidate which signalling pathways need to be triggered to allow for regeneration in case of injury. At the same time, this arrest in cytokinetic activity in cardiomyocytes means that there are no “heart cancers”, at least not any caused by hyperplasia of adult ventricular cardiomyocytes.

Why are embryonic cardiomyocytes able to undergo cell division and why does this activity stop after birth? The answer seems to lie once more in their cytoarchitecture. Embryonic cardiomyocytes are polygonal and have cell–cell contacts over their entire surface (Hirschy et al. 2006); for schematic drawing see Fig. 1.1a. Almost half of their cellular volume is taken up by the nucleus, with myofibrils only making up a relatively sparse meshwork that is in close proximity to the plasma membrane. At the late fetal stage the cardiomyocytes start to grow in length, the

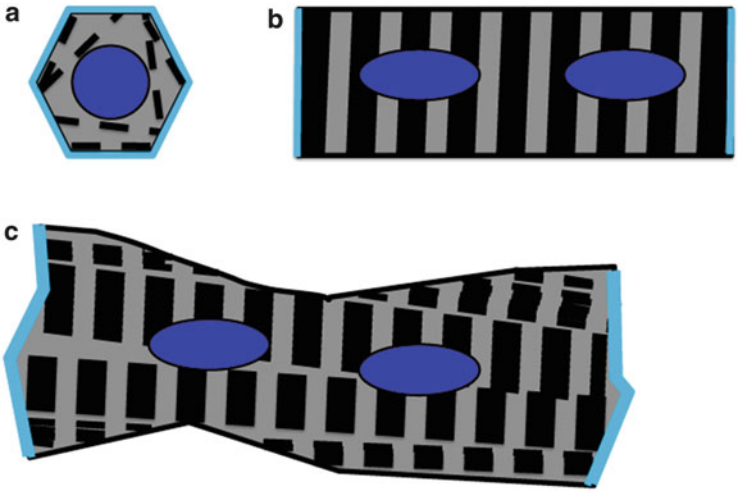

Fig. 1.1 Cardiac cytoarchitecture changes during development and in disease. Schematic representations of an embryonic cardiomyocyte (**a**) showing its polygonal shape, an adult cardiomyocyte (**b**), which is *rod-shaped* and a cardiomyocyte from a DCM heart (**c**), which is characterised by a more irregular shape. The myofibrils are shown as *black striations*; the nuclei are shown in *blue*. While the embryonic cardiomyocytes make direct cell–cell contact all around their surface (*turquoise signal*), these become restricted to the bipolar ends of mature cardiomyocytes postnatally. DCM is characterised by an increased membrane convolution and elevated expression of actin-anchoring proteins, which is depicted as a thickening of the *turquoise line*

myofibrils get better aligned and the cell–cell contacts become restricted to the bipolar ends around birth (Perriard et al. 2003; Hirschy et al. 2006). In contrast, postnatal cardiomyocytes are tightly packed with myofibrils separated by regular rows of mitochondria (Fig. 1.1b). It may be much easier to disassemble a limited, less well-integrated set of myofibrils than the almost paracrystalline arrangement found in postnatal cardiomyocytes to allow the cells to divide. The cellular elongation and increased packing with myofibrils is accompanied by a specialisation of the plasma membrane organisation which also happens immediately after birth. The cardiomyocytes start to organise the triad system, consisting of plasma membrane invaginations, called T-tubules, which are flanked by sarcoplasmic reticulum cisterns and which are in the differentiated state located above the Z-discs (Franzini-Armstrong et al. 2005). This may be also more complicated to disassemble and to re-establish than the simpler plasma membrane of the embryonic cardiomyocyte. Another impediment for successful cell division may be that the size of a hypertrophying cardiomyocyte becomes a challenge for successful assembly of the mitotic spindle. It has been proposed that for example in cultured cells, rounding of cells in mitosis is a prerequisite to limit the distances to which centrosomally nucleated microtubules have to grow to efficiently capture chromosomes (Lancaster and Baum 2014). Dividing embryonic cardiomyocytes in culture do not round up and also in the embryonic heart in situ they remain more or less polygonal (Ahuja et al. 2004). Still, the postnatal increase in size may go beyond what is possible in

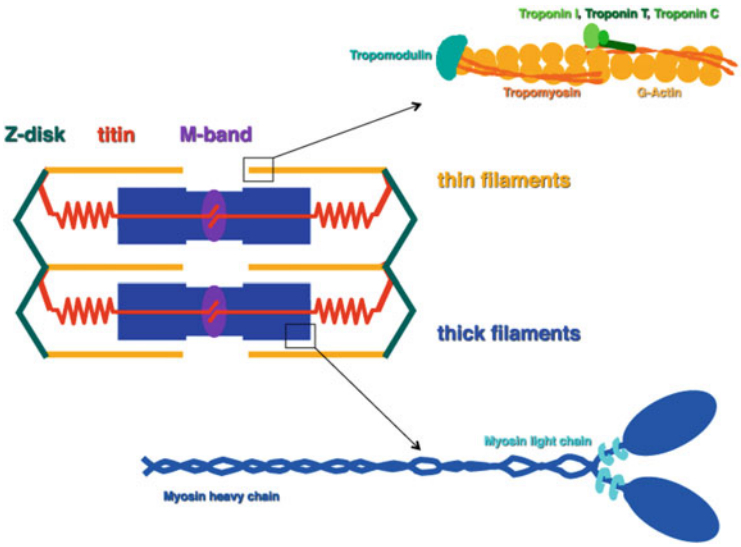

Fig. 1.2 Simplified schematic representation of a sarcomere, the basic unit of a myofibril. A sarcomere is defined as the region between two Z-discs, where the thin (actin) filaments are anchored. The M-band is a structure in the very middle of the sarcomere and serves for the integration of the titin filaments with the bipolar myosin (thick) filaments. An enlarged scheme of the pointed end of a thin filament is shown in the *top right*, depicting the actin subunits and their associated proteins tropomyosin and the troponins. The *bottom right* shows a myosin molecule, which is composed of two myosin heavy chains and four myosin light chains. Myosin binding protein-C is associated with a subset of the myosin molecules but was omitted from this scheme for reasons of clarity

microtubular growth with the given set of microtubule-associated proteins that are expressed in cardiomyocytes. The general organisation of the microtubule cytoskeleton in cardiomyocytes also becomes more complex postnatally with microtubules forming a meshwork that runs in parallel to the myofibrils but also consists of transverse struts in rod-shaped adult cardiomyocytes (Ehler and Perriard 2000).

How are the different cytoskeletal multiprotein complexes assembled in the heart? As mentioned above, cross-striated myofibrils characterise the first beating cardiomyocytes. The basic unit of a myofibril is a sarcomere, which is defined as the region between two neighbouring Z-discs (Fig. 1.2). The actin-containing thin filaments are anchored at the Z-disc, mainly via a protein called alpha-actinin (Ribeiro Ede et al. 2014). The thick filaments, which consist of myosin and associated proteins, are found in the middle of the sarcomere, where they are anchored to the elastic filament system composed of titin in a structure called the M-band. During myofibrillogenesis the first organised complexes that can be detected consist of concentrations of sarcomeric alpha-actinin and the N-terminus of titin, which are organised along filamentous actin with a regular distance from each other in close proximity to the plasma membrane (Sparrow and Schöck 2009). Study of the process of myofibrillogenesis in cultured cardiomyocytes has identified several precursor structures such as IZI brushes or premyofibrils (for review see

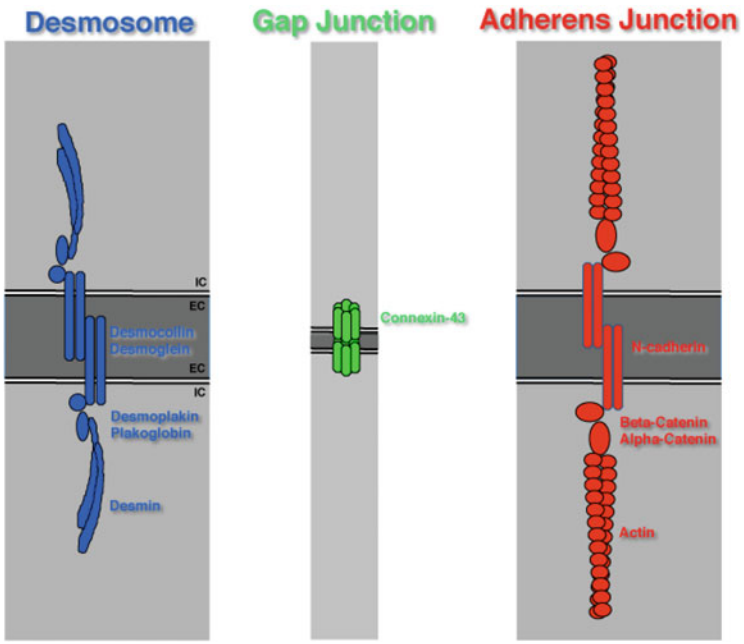

Fig. 1.3 Simplified schematic representation of the different contact structures at the intercalated disc. Desmosomes, gap junctions and adherens junctions are depicted. Desmosomes link up to the intermediate filament cytoskeleton, which is composed of desmin in muscle cells, while adherens junctions link up to actin filaments (myofibrils). This scheme shows a minimalist version of these structures and many associated proteins were omitted for reasons of clarity. *IC* intracellular, *EC* extracellular

Sanger et al. 2005). However, non-muscle myosin, which defines a premyofibril (Rhee et al. 1994), is not seen in between the alpha-actinin–titin complexes in the embryonic heart in situ and also the distance between them is wider than the distance observed in premyofibrils or stress fibre-like structures (Ehler et al. 1999). This is in contrast to myofibrillogenesis in skeletal muscle of the embryo, where two distinct populations of assembling myofibrils can clearly be distinguished in zebrafish somites (Sanger et al. 2009). Also sarcomeric protein expression during heart development is not switched on sequentially like it is observed for skeletal muscle (Ehler et al. 1999; Fürst et al. 1989); therefore the process of differentiation and thus myofibrillogenesis in the embryonic heart in situ may be too rapid to allow or require precursor structures (Ehler and Gautel 2008).

The assembly of the specialised type of cell–cell contact, the intercalated discs, is only achieved postnatally in the heart. The intercalated discs are classically defined as having three types of contact (Forbes and Sperelakis 1985): adherens junctions that anchor actin filaments (or in the case of muscle cells the myofibrils), desmosomes that anchor the intermediate filaments (desmin) and gap junctions, which are composed of connexin-43 channels and mediate intercellular communication (Fig. 1.3). Embryonic cardiomyocytes have all three types of cell–cell

contact distributed all around their plasma membrane and restriction of contact sites to the intercalated disc proper only happens around birth (Hirschy et al. 2006). Establishment of mature gap junctions happens even later than that (Angst et al. 1997). Recently the concept of a separate existence of adherens junctions and desmosomes at the intercalated disc has been challenged and a novel type of contact was proposed, the *area composita*. This is based on the observation that in the heart there is no strict segregation between the distribution of classical adherens junction and desmosomal proteins and that a mixed composition of these junctions is predominant (Franke et al. 2006).

1.2 Cardiomyocytes in Disease

Apart from loss of cardiac tissue as for example by ischemia as caused by a myocardial infarction, two major forms of cardiomyopathy can be defined, hypertrophic cardiomyopathy (HCM) and dilated cardiomyopathy (DCM). Another type of cardiomyopathy, arrhythmogenic right ventricular cardiomyopathy (ARVC), is less frequent, but also associated with changes in cytoarchitecture (Basso et al. 2012). As we accumulate more data from next generation sequencing, the frequent hereditary component of these diseases becomes more evident and is no longer restricted to huge families. On a macroscopic basis HCM is defined as a thickening of the ventricular and/or septal wall and thus a reduction in chamber size and blood volume at filling (Seidman and Seidman 2001). DCM is characterised by a ballooning heart with an increased diameter of the ventricle (Seidman and Seidman 2001). As sudden cardiac death, especially in young athletes, can be the first sign of a HCM or an ARVC, more knowledge on the genetic cause of these diseases and screening for known variations can be potentially life-saving (Basso et al. 2012). Classically HCM is defined as being caused by mutation in proteins that constitute the myofibrils, while DCM is often associated by mutated cytoskeletal proteins (Chien 1999). The most frequent mutations that cause HCM are seen in sarcomeric myosin heavy chain and truncations of myosin binding protein-C (Frey et al. 2012), while truncating mutations in titin were initially seen in DCM (Gerull et al. 2002) and recently proposed to be among the main causes for DCM (Herman et al. 2012). However, the more sequence data are accumulated the more obvious it becomes that these simple associations are not correct (for recent review of disease causing mutations see McNally et al. 2013) and that for example proteins such as MLP and AMPK, which are not integral myofibrillar proteins, can be mutated in HCM, while mutations in classical myofibrillar proteins such as myosin can also lead to DCM. Truncating mutations in titin were also found to be associated with all types of cardiomyopathies (McNally et al. 2013). A more clear-cut link seems to exist at present for ARVC, which has been named the disease of the desmosome, a special type of cell–cell contact that links the intermediate filament cytoskeleton to the plasma membrane and is seen as mainly playing a structural role (Rickelt and Pieperhoff 2012). However, also for ARVC the situation will become more

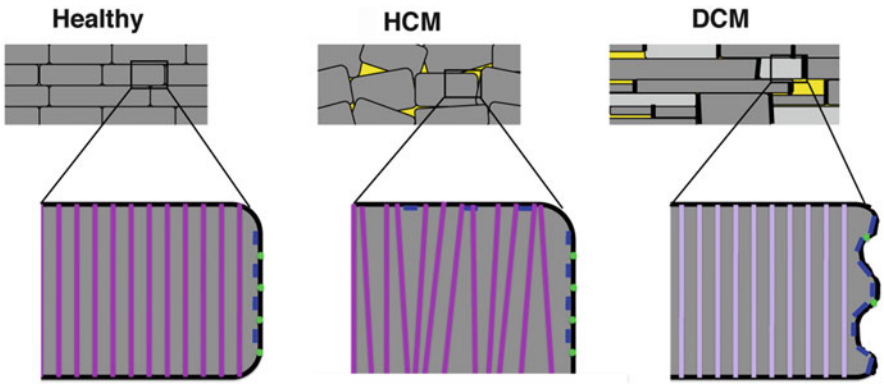

Fig. 1.4 Hypertrophic cardiomyopathy (HCM) is characterised mainly by myocyte disarray while dilated cardiomyopathy (DCM) is characterised by a general loss of size control, alterations in intercalated disc composition and by a switch to the expression of more elastic isoforms of the structural proteins titin and myomesin. Schematic representation of heart tissue from control, HCM or DCM patients. Apart from areas of cell loss and fibrosis (*yellow*), there is usually no dramatic myocyte disarray seen in DCM, which is in contrast to HCM. The cardiomyocytes display more variability in their sizes and can have more irregular shapes. At the cellular level, HCM is characterised by myofibril disarray and lateral cell–cell contacts (shown in *blue*), while DCM shows a relatively mild myofibrillar phenotype with just changes in isoform expression (*light purple* representing EH-myomesin containing M-bands), but has its major alterations in intercalated disc phenotype and composition. Gap junction expression (*green dots*) is reduced in DCM in the mouse

complex the more next generation sequencing (NGS) data are available and already now a link between the developing disease and Wnt signalling, which is usually active mainly in early embryonic heart development, is proposed (Asimaki et al. 2009; Swope et al. 2013).

At the level of the cardiomyocyte, HCM and DCM present quite different phenotypes (Fig. 1.4). HCM is usually already evident by Hematoxylin and Eosin staining and presents as myofibrillar disarray (Frey et al. 2012). The cardiomyocytes have a larger diameter and there is also a change in the arrangement of the tissue, with cell–cell contacts as characterised by the adhesion junction protein beta-catenin also being found at the sides of the cells. Apart from obvious regions of necrosis, DCM tissue can appear relatively unharmed at the histological and ultrastructural level and it required the careful analysis of the first mouse model for this disease, the MLP knockout mouse (Arber et al. 1997), to get a grip on the alterations that happen at the cellular level in this disease (Ehler et al. 2001). Freshly isolated cardiomyocytes from MLP knockout hearts are characterised by a more irregular shape compared to healthy hearts (see Fig. 1.1c), but there is no consistent cellular elongation, which might explain the ballooning ventricles but rather a general loss of size control (Leu et al. 2001). Similar observations were also made in other mouse models for DCM and even in samples from human DCM patients, suggesting that this may be a more general phenomenon of the disease (Hirschy et al. 2010; Pluess et al. 2015). With the obvious consequences that this

has on the three-dimensional arrangement of heart tissue, it may provide some of the explanations for the alterations that are seen in the ways the cells make contact with their extracellular matrix. For example, the costameres become disorganised in the MLP knockout mouse and also the way the extracellular matrix is distributed over the cardiomyocytes changes in cardiomyopathy (Ehler 2010; Ehler et al. 2001; Hoskins et al. 2010). This probably reflects a loss in membrane organisation especially as far as triads are concerned and was also reported by ion conductance microscopy on chronic heart failure samples (Nikolaev et al. 2010). While the organisation and composition of the myofibrils is much less affected in DCM compared to HCM and there is for example no consistent re-expression of embryonic isoforms of the contractile proteins actin and myosin (MacLellan and Schneider 2000), there is a shift towards the expression of more compliant isoforms of elastic proteins that are needed to maintain a working sarcomere such as longer versions of titin and the EH-myomesin isoform (Makarenko et al. 2004; Schoenauer et al. 2011; Pluess et al. 2015). The major changes that are observed in the subcellular organisation of the cardiomyocytes in DCM happen at the intercalated disc. We consistently see an upregulation in the expression of actin-anchoring proteins such as adherens junction components (beta-catenin, cadherin) and also vinculin and N-RAP (Ehler et al. 2001; Pluess et al. 2015). This is potentially a response to an increased convolution of the plasma membrane and is also accompanied by a more intense signal for filamentous actin. Whether the elevated levels of an actin polymerising factor, the formin FHOD1, which are observed at the intercalated disc in DCM cause this increase in actin remains to be demonstrated (Dwyer et al. 2014). In mouse models for DCM this altered adherens junction composition is accompanied by a reduction in the numbers of gap junctions that are detected, suggesting impaired intercellular communication (Ehler et al. 2001). This observation can only be reproduced in a subset of the human patient material, indicating a difference between mouse and human phenotype and a potential compensatory effect by differential medication of human patients.

1.3 How to Study Cardiac Cytoarchitecture

A multitude of studies have been carried out that investigate and compare the subcellular localisation of a variety of cytoskeletal proteins either in cardiomyocytes in culture or on heart sections using immunofluorescence with antibodies that specifically recognise a particular protein. Especially when combined with confocal microscopy, these investigations have told us a lot about the subcellular distribution of proteins that make up the cardiac cytoarchitecture during development and in disease. However, the researcher wants to go beyond these descriptive studies and to explore the role of different components in a more functional way. There the problem starts, because the ideal model system to study cardiac cytoarchitecture does not exist and what is available to us comes all with advantages and disadvantages (for a list comparing different systems see Table 1.1). Over the years many laboratories have

Table 1.1 Model systems to study cardiac cytoarchitecture

Type	Advantages	Disadvantages
NRC (neonatal rat cardiomyocytes)	Robust, accessible for drugs and microscopy, beat spontaneously	Primary culture with limited life span, two-dimensional, artefacts due to culture conditions
NMC (neonatal mouse cardiomyocytes)	Beating, accessible for drugs and microscopy, can isolate from transgenic or knockout mice, beat spontaneously	Primary culture with limited life span, two-dimensional, artefacts due to culture conditions, much more fragile than NRC
ARC (adult rat cardiomyocytes, freshly isolated)	Robust, represent three-dimensional architecture from cells in situ, accessible for drugs and microscopy	Only very short time culture possible (unless dedifferentiation—redifferentiation is induced by high serum conditions)
AMC (adult mouse cardiomyocytes, freshly isolated)	Can isolate from transgenic or knockout mice, represent three-dimensional architecture from cells in situ, accessible for drugs and microscopy	Only very short time culture possible
Differentiated ES (embryonic stem cells; mouse or human)	From cell lines, human possible, accessible for drugs and microscopy, 3D in embryoid bodies	Huge variability in differentiation extent between different laboratories, immature as far as contractile parameters and sarcomere composition are concerned
CM (cardiomyocytes) from human iPSC (inducible pluripotent stem cells)	Available for purchase, accessible for drugs and microscopy, beat spontaneously, human system	Expensive, immature as far as contractile parameters and sarcomere composition are concerned
HL-1 (mouse atrial)	Cell line, can be propagated indefinitely, express cardiac transcription factor set	Phenotype similar to very early embryonic cardiomyocytes, only limited myofibrillogenesis
H9c2 (rat embryonic)	Cell line, available for purchase, can be propagated indefinitely	Are myoblasts and will fuse to form multinucleated myotubes, i.e. closer to a skeletal muscle model system
Chick (embryo)	Relatively easy to manipulate experimentally (in egg), well-characterised embryology	Only useful for early embryonic studies, genetic manipulation not well established, yet
Zebrafish (embryo)	Very easy to manipulate experimentally, well-characterised embryology, several manipulation techniques (morpholino, CRISPR, TALEN)	Partial duplication of genome means more redundance, off-target effects from morpholinos common, two-chambered heart
Mouse (adult)	Transgenic or knockout models, can study effect of physiology, experimental intervention (TAC)	Expensive maintenance, does not always reflect human phenotype
Human (patient material)	Closest to the “target”, i.e. human cardiomyopathy	Difficult to come by, heterogeneity in tissue preservation, medication, genetics, control material often compromised, usually no intervention possible

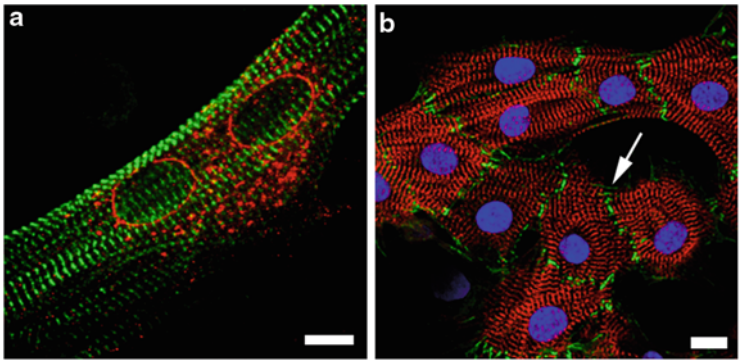

Fig. 1.5 Neonatal rat cardiomyocytes (NRC) in culture express ANF (atrial natriuretic factor) and can have a slightly abnormal organisation of their cell–cell contacts. (**a**) NRC stained in *green* for myomesin and in *red* for ANF. ANF granula can be seen around the nucleus and dispersed through the cytoplasm in about half of the cells at baseline and become even more enhanced by exposure to hypertrophic stimuli such as phenylephrine to the culture medium. (**b**) NRC stained for myomesin in *red*, for beta-catenin in *green* and for the nuclei in *blue*. The *arrow points* at an intercalated disc-like structure, where the myofibrils are not terminally inserted along its length but run in parallel along part of it. Scale bars are 10 μm

used cultured cardiomyocytes for their analyses, which have the advantage that they retain some of their differentiated characteristics, for example they usually continue to beat rhythmically, but the obvious disadvantage that these cells are taken out of their environment and placed into a plastic dish in culture medium. This makes them extremely amenable for interference by addition of drugs and for microscopy, but also means that it cannot be excluded that some of the observations that are made are a culture artefact. Especially in the response to growth factors and also in the way myofibrils are assembled there seem to be dramatic differences between two-dimensional as in the petri dish and three-dimensional arrangements (Armstrong et al. 2000; Ehler et al. 1999). Another potential pitfall is that the stalwart system of cardiomyocytes in vitro are neonatal rat cardiomyocytes (NRC), which means that they are taken from an animal at a time when full maturity is not reached, yet. It is therefore entirely possible that the responsiveness to stimuli will be different compared to adult cardiomyocytes and their cytoarchitecture is definitely distinct (Rothen-Rutishauser et al. 1998). A lot of isoforms and proteins that are specific for either the embryonic or the adult heart are in the process of being switched around birth; therefore embryonic markers such as ANF expression and EH-myomesin isoform expression are still maintained at least in a subset of the NRC (Fig. 1.5a), while markers of sarcomere maturity such as the expression of telethonin or M-protein are not yet present in all the cells. In addition, a beating frequency in the hundreds per minute makes them dramatically different from the beating rate that would be expected from a human cardiomyocyte. Cardiomyocytes that were differentiated from human inducible pluripotent stem cells may become the method of choice for in vitro studies in the future, but are at the moment characterised by their costliness and also immature features as far as their physiology and cytoarchitecture

is concerned, which makes them similar to differentiated human embryonic stem cells (Földes et al. 2014). Another inherent problem with cultured cardiomyocytes whether rodent or human is that their cytoarchitecture is different compared to in situ, especially as far as the cell–cell contacts are concerned. While in the postnatal heart cell–cell contacts would only be found where myofibrils terminate, they can also be situated laterally to myofibrils in cultured cardiomyocytes (see arrow in Fig. 1.5b). This means that the terminology intercalated disc-like structure would be more appropriate in this situation since while they contain all the major components, their ultrastructural architecture will be different to an intercalated disc in situ. Freshly isolated cardiomyocytes from adult mouse or rat hearts can only be obtained by retrograde perfusion of the tissue with collagenase using a Langendorff apparatus and are characterised by a cytoarchitecture that is as close to the tissue as possible. However, they only have a limited life span to do experiments on them, which is even shorter for the ones from mouse than for the ones from the rat heart. The dedifferentiation process starts immediately and for example the specialised plasma membrane organisation is lost, with slightly different dynamics between rat and mouse (Pavlovic et al. 2010). A possibility to maintain the characteristic subcellular distribution of proteins such as connexin-43 a bit longer may be to assemble the adult cardiomyocytes to three-dimensional spheres using gravity (Kelm et al. 2006).

Unfortunately no cell line exists at present that would truly reflect the cytoarchitecture of cardiomyocytes. HL-1 cells, which were initially isolated from a mouse atrial tumour (Claycomb et al. 1998), do express a good set of cardiac differentiation markers. However, their morphology in culture resembles very early embryonic cardiomyocytes and they grow in an epitheloid way with tight cell–cell contacts. Myofibrillogenesis can be observed, but is often stalled at the premyofibril stage (Hirschy et al. 2010). H9c2 cells (Kimes and Brandt 1976), while used by several laboratories, tend to fuse to multinucleated myotubes and are hence closer in their characteristics to skeletal than to heart muscle cells.

Three different vertebrate model systems were used over the years to study heart development and disease, the chick, the zebrafish and the mouse. The chick has the obvious advantage of easily accessible embryos and a long-standing tradition of classical developmental biology (Hamburger and Hamilton 1951), but would not be the model of choice beyond the embryonic stage. The zebrafish is ideal as far as its experimental accessibility and as far as imaging are concerned, but has the disadvantage of having a two-chambered heart and of a partial duplication of its genome. While the former may not be so much of a problem as far as physiology is concerned (Jensen et al. 2013), the latter can severely hamper progress, since a lack of a phenotype following interference may be due to redundancy (Bakkers 2011). However, recent techniques such as CRISPR and TALEN that make it easier to create genetically modified zebrafish and seem to have less off-target effects than the morpholino knockdown approach that was used classically (Auer and Del Bene 2014) will definitely see an expansion in work on adult zebrafish. The mouse is often the model of choice, especially for transgenic or knockout models, but has the disadvantage that from its physiology, especially as far as the heart rate is concerned, it is a far way from human and also that as a system its sensitivity for example

to disease causing mutations appears to be often different. Mutations that cause hypertrophic cardiomyopathy in human in the heterozygous state only lead to a pronounced phenotype in the mouse when homozygous or when accompanied by experimental or lifestyle manipulation (e.g. Geisterfer Lowrance et al. 1996). Whether this is due to the different life span, distinct physiology or epigenetic regulation is not quite clear at the moment. Ultimately, while model systems in vitro and in vivo are essential to establish new concepts and hypotheses, all observations and conclusions will have to be substantiated by research on samples from human patients.

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Chapter 2
In Vitro Tools for Quantifying Structure–Function Relationships in Cardiac Myocyte Cells and Tissues

Meghan B. Knight, Anna Grosberg, and Megan L. McCain

Abstract The heart is a dynamic, electrically activated, chemically sensitive, mechanical pump with a regular rhythm that must operate without interruption for decades. The function of the heart is an emergent property of highly organized structures that span multiple spatial scales (Fig. 2.1). On the organ level, the heart is divided into four chambers. Two of these chambers, the ventricles, are thick-walled muscular chambers that are particularly constructed to work as pressure pumps and contract in a twisting manner to efficiently squeeze blood from the chamber. The walls of the ventricle comprise layers of two-dimensional sheets of laminar cardiac tissue. The tissue itself consists of highly aligned, elongated, cylindrical cardiac myocytes. Cardiac myocytes are spanned by parallel bundles of myofibrils, which consist of repeating sarcomere units. Sarcomeres are nanoscale structures composed of thick myosin filaments and thin actin filaments that slide past each other and shorten the sarcomere in response to an action potential. Because all sarcomeres within a cell are aligned, and all cells in a tissue are aligned, the amount of uniaxial force generated by the tissue as a whole is maximized due to its multi-scale organization. To achieve synchronous contraction, myocytes couple together via specialized cell–cell junctions, known as intercalated discs, which provide both mechanical adhesion and rapid electrical communication. Thus, the pumping function of the heart, which is multiple centimeters in diameter, is dependent on spatial organization that spans all the way down to the nanoscale. In this chapter, we will describe the role of the structure of single cardiac myocytes, cell–cell junctions, and multicellular tissues in the function of the healthy heart and how these

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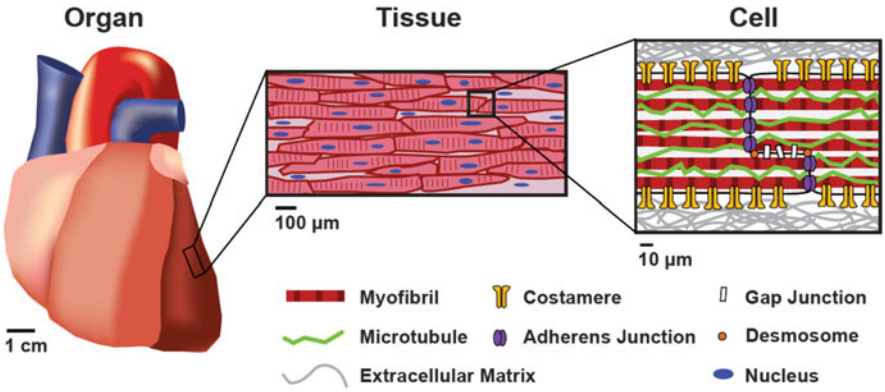

Fig. 2.1 The multi-scale structure of the heart. The function of the heart is dependent on its hierarchical architecture, which spans organ, tissue, cellular, and molecular levels

structure–function relationships become disrupted in disease. We will focus on studies that have used in vitro tools to mimic different architectures observed in developing, healthy, and diseased hearts and make functional readouts in a controlled setting.

2.1 Structure–Function Relationships in Single Cardiac Myocytes

The heart consists of millions of individual cardiac myocytes, rhythmically contracting in synchrony to pump blood through the vasculature for the lifetime of the organism. As described in this section, the contractile function of each individual cardiac myocyte is dependent on several intracellular and extracellular factors, including myofibril architecture, myocyte geometry, and the chemical and mechanical properties of the extracellular matrix, each of which can be individually investigated in vitro.

2.1.1 Myofibrillogenesis: Self-Assembly of the Contractile Apparatus

Cardiac myocytes contract because they are densely packed with myofibrils, which consist of repeating sarcomere units that span the long axis of the cell. Each sarcomere consists of interdigitating actin and myosin filaments that slide past each other to shorten the cell and generate contractile forces that sum together to

induce organ-level pumping (Boateng and Goldspink 2008). Sarcomeres and myofibrils are highly ordered structures that self-assemble during embryonic and postnatal development by processes that are not well understood, limiting our ability to understand sarcomere dysfunction in disease or replicate mature myofibrillogenesis in engineered cardiac cells and tissues for regenerative medicine.

Much of our knowledge about myofibrillogenesis originates from in situ imaging of developing embryo hearts. Although these studies can reveal how myofibrillar proteins interact and organize in living embryonic hearts (Ehler et al. 1999; Hirschy et al. 2006), tissues inside embryos are difficult to image in real time and thus must be fixed and sectioned, providing only "frozen snapshots" of developmental processes. Thus, in vitro systems are valuable because cells in a dish are more accessible to imaging, allowing us to capture dynamic data about how proteins interact, assemble, and function as myocytes rebuild their cytoskeleton in culture (Sanger et al. 1986; Hilenski et al. 1991; Rhee et al. 1994; Dabiri et al. 1997; Du et al. 2003, 2008). For example, in vitro studies have shown that contractile forces generated by myofibrils are important for maintaining their mature structure (Sharp et al. 1993; Simpson et al. 1993, 1996), indicative of positive feedback between sarcomere maturation and contractility. In silico models have also been developed to test hypotheses about force-dependent myofibril assembly that are impossible or very challenging to test in living systems. A combination of in vitro experiments with computational modeling has shown that myofibril organization is guided by three important phenomena: (1) The geometry of the extracellular matrix available for the cell to bind to its surroundings controls the architecture of its myofibrils, which can serve to organize multiple cells, (2) the amount of force generated by a myofibril increases as it becomes longer, which in turn stabilizes it and promotes its further maturation, and (3) myofibrils couple together laterally to form mutually aligned, parallel bundles of fibers that can maximize contractile forces generated by the cell as a whole (Grosberg et al. 2011b). This type of synergy between in situ, in vitro, and in silico models furthers our understanding of how the highly specialized sarcomeres and myofibrils responsible for myocyte contractility form in the heart.

Understanding myofibrillogenesis is becoming especially important because we now have the technology to differentiate cardiac myocytes from embryonic and induced pluripotent stem cells and progenitor cells (Zhang et al. 2009; Lundy et al. 2013). However, these myocytes often lack the mature striations that are characteristic of primary differentiated cardiac myocytes and, as a consequence, generate significantly lower contractile forces (Domian et al. 2009; Feinberg et al. 2013; Sheehy et al. 2014b), which limits their utility for cell therapy or regenerative medicine. Thus, determining and applying the factors that regulate the structural maturation of myofibrils will be important for directing the maturation of stem cell-derived cardiac myocyte cells and tissues with functional properties comparable to their native counterparts.

2.1.2 Myocyte Shape Remodeling in Development and Disease

Cardiac myocytes in the healthy adult heart are cylindrical, elongated, and densely packed with aligned myofibrils. The rectangular shape of cardiac myocytes emerges over the course of pre- and postnatal development (Hirschy et al. 2006) due to the influence of both intrinsic and extrinsic mechanical forces, such as contractility (Auman et al. 2007) and hemodynamic pressure (Hove et al. 2003). Myocyte shape remodeling is observed in many cardiac diseases, which impacts the morphology of the entire ventricle and its capacity to fill and pump blood. For example, excessive pressure in the ventricle due to factors such as high blood pressure or aortic valve stenosis causes the ventricle to become thicker and hypertrophied (Grossman et al. 1975). This thickening of the ventricle is at least partially attributed to decreases in the length:width aspect ratio of individual cardiac myocytes, which is approximately 7:1 in healthy hearts and decreases to about 4:1 in concentric hypertrophy (Gerdes 2002). In response to volume overload, the ventricle becomes thinner and dilated, known as eccentric hypertrophy (Grossman et al. 1975), and myocyte aspect ratio increases to about 10:1 (Gerdes 2002). Thus, both concentric and eccentric hypertrophy are associated with distinct forms of cellular remodeling that impact gross organ morphology and contribute to contractile dysfunction.

Although we know that distinct changes in myocyte geometry are associated with different cardiac diseases, determining the direct functional implications of cell shape remodeling is challenging because many other parameters are also changing in these diseases. Thus, in vitro systems are useful for isolating the effects of cell shape from other confounding factors. However, cardiac myocytes isolated from the hearts of animals, such as neonatal rats, lose their native architecture in culture and instead adopt arbitrary shapes that are not physiologically relevant (Bray et al. 2008). To control cell shape in culture, extracellular matrix proteins, such as fibronectin, can be micropatterned onto culture substrates with a spatial resolution down to 1 μm using photolithography and microfabrication techniques (Chen et al. 1997). Cardiac myocytes seeded onto these substrates then adhere and spread only on the micropatterned regions of the substrate (Geisse et al. 2009). Using this technique, cardiac myocytes with length:width aspect ratios matching those found in health and disease have been engineered in culture (Fig. 2.2a, b). These studies have revealed that sarcomere organization is regulated by myocyte shape, where myocytes with moderate to high aspect ratios have higher global sarcomere alignment than those with lower aspect ratios (Bray et al. 2008). Thus, optimizing sarcomere alignment is one important consequence of the rectangular shape that cardiac myocytes adopt in the healthy heart.

Another advantage of in vitro systems is that structure–function relationships on the cellular level can be quantified in a controlled setting with techniques such as traction force microscopy (TFM). This method is used to calculate contractile forces generated by cells cultured on compliant hydrogels by tracking the

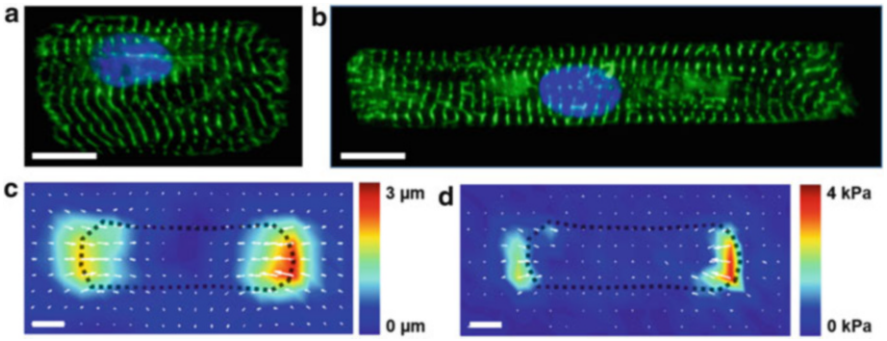

Fig. 2.2 Quantifying structure–function relationships in single cardiac myocytes. The shape of neonatal rat ventricular myocytes can be controlled by seeding cells on micropatterned islands of fibronectin. These myocytes have 2:1 (**a**) and 7:1 (**b**) length:width aspect ratios to mimic hypertrophied and healthy myocytes, respectively. *Green*: alpha-actinin, *blue*: nuclei. Traction force microscopy can be used to calculate peak systolic displacement (**c**) and stress (**d**) in contracting cardiac myocytes cultured on compliant hydrogels. Bars: 10 μm

movement of fluorescent beads embedded in the gels (Fig. 2.2c, d) (Butler et al. 2002). TFM studies of micropatterned cardiac myocytes on gels with physiological elasticity have revealed that myocyte shape regulates contractile stress generation in a non-monotonic fashion, with 7:1 aspect ratios generating more peak systolic stress than either lower or higher aspect ratios (Kuo et al. 2012). Thus, the in vivo aspect ratio of myocytes in the healthy heart (7:1) is likely important for maximizing the contractile output of the heart. Calcium cycling was also been found to be regulated by myocyte shape (Kuo et al. 2012), indicating that myocyte shape can impact other functional behaviors such as electrophysiology. Thus, the shape of cardiac myocytes is an important regulator of myocyte phenotype in health and disease.

2.1.3 Extracellular Matrix Regulation of Cardiac Myocyte Structure and Function

Cardiac myocytes are surrounded by an extracellular matrix network with a defined composition that remodels in development (Corda et al. 2000; Williams et al. 2014) and disease (Jane-Lise et al. 2000; Berk et al. 2007). In fetal hearts, the matrix is dominated by fibronectin (Samuel et al. 1994; Farhadian et al. 1995), but over the course of development, fibronectin expression decreases and collagen becomes the primary extracellular matrix protein by adulthood (Borg et al. 1982). Interestingly, many cardiac diseases are associated with reexpression of fibronectin (Farhadian et al. 1995; Ulrich et al. 1997; Konstandin et al. 2013), suggesting that this protein has a role in both developmental and pathological remodeling. Adding to the complexity, signaling from the extracellular matrix is dependent on myocytes

expressing the appropriate integrin receptors, which are transmembrane protein complexes that couple specific extracellular matrix proteins to the cytoskeleton (Humphries 2000). Cardiac myocytes express different integrin subtypes throughout development, health, and disease (Terracio et al. 1991; Carver et al. 1994; Nawata et al. 1999; Ross and Borg 2001; Parker and Ingber 2007), which in turn dictates the sensitivity of cardiac myocytes to the evolving composition of the extracellular matrix.

Because both the composition of the extracellular matrix and myocyte integrin expression remodel through cardiac development and disease, these components together are thought to regulate the phenotype of cardiac myocytes. To investigate this, isolated cardiac myocytes have been cultured on substrates coated with different matrix proteins, revealing that matrix composition regulates processes such as signaling (Bullard et al. 2005) and myofibrillogenesis (Hilenski et al. 1991). Proliferation of neonatal rat cardiac myocytes is highest on extracellular matrix isolated from fetal hearts, which is rich in fibronectin, compared to adult matrix, which is primarily collagen (Williams et al. 2014). Adult cardiac myocytes, however, survive only on collagen and laminin, but not fibronectin (Lundgren et al. 1985), consistent with the finding that β-1 integrin, which attaches to fibronectin, is highly expressed in developing (Hornberger et al. 2000) but not adult hearts (Carver et al. 1994). Interestingly, myocytes isolated from rat hearts subjected to volume overload initially show increased adhesion to matrix proteins followed by decreased adhesion (Stewart et al. 2014), indicating that integrin expression and thus interactions with the matrix are dynamic during disease progression. The extracellular matrix can also be used as a cue to differentiate stem cells into cardiac myocytes, as matrices mimicking the native heart have been shown to promote cardiac differentiation of embryonic (Baharvand et al. 2005; Duan et al. 2011) and bone marrow-derived (Sreejit and Verma 2013) stem cells. Thus, the protein composition of the extracellular matrix is an important signal in both cardiac development and disease.

In addition to protein composition, the elasticity of the extracellular matrix also remodels during physiological and pathological growth. In the embryo, the elastic modulus of the heart is approximately 1 kPa and increases to approximately 10 kPa in the adult heart (Engler et al. 2008; Majkut et al. 2013). Many cardiac diseases are associated with fibrosis, which increases the elastic modulus of the tissue to above 50 kPa (Berry et al. 2006; Chaturvedi et al. 2010). To determine how the mechanical properties of the heart regulate myocyte structure and function, investigators have cultured neonatal rat ventricular myocytes on hydrogels with tunable elastic moduli, such as polyacrylamide hydrogels, that have mechanical properties similar to native heart tissue. These studies have shown that sarcomerogenesis and beat rate are optimal at approximately 10 kPa (Engler et al. 2008). At lower elastic moduli, myofibrils do not have enough resistance to generate force and mature (Bajaj et al. 2010). At higher elastic moduli, myocytes contain more stress fibers and less striations (Jacot et al. 2008; Forte et al. 2012). Other functional parameters, such as calcium cycling (Jacot et al. 2008; Galie et al. 2013), are also sensitive to matrix elasticity and are reportedly optimal at an elastic modulus matching the

healthy heart. Thus, the mechanical properties of the extracellular matrix play an important role in cardiac myocyte maturation in development and dysfunction in disease.

In concentric hypertrophy, cardiac myocyte aspect ratio decreases (Gerdes 1992) and extracellular matrix elastic modulus increases due to fibrosis (Ho et al. 2010). As described above, both of these factors impact cardiac myocyte structure and function, making it difficult to determine their respective roles in pathogenesis. An advantage of in vitro systems is that we can decouple these two factors by controlling myocyte shape with micropatterning and matrix elasticity with tunable polyacrylamide hydrogels. Using these two techniques, studies have shown that myocytes with healthy length:width aspect ratios (~7:1) generate the most contractile work on gels with healthy elastic moduli (13 kPa), while myocytes with hypertrophied length:width aspect ratios (~2:1) generate the most contractile work on gels with fibrotic elastic moduli (90 kPa) (McCain et al. 2014c). Thus, myocyte shape remodeling in concentric hypertrophy could be the result of myocytes "tuning" their aspect ratio to generate the maximum amount of contractile work for a given matrix elasticity.

2.2 Structure–Function Relationships at the Intercalated Disc

Cardiac tissue functions as an electromechanical syncytium because individual cardiac myocytes are tightly coupled mechanically and electrically by highly specialized cell–cell junctions known as intercalated discs. Intercalated discs consist of adherens junctions, desmosomes, and gap junctions that localize primarily to the short ends of adult cardiac myocytes in ventricular tissue. However, the factors that regulate the assembly, structure, and function of intercalated discs remain mostly elusive, and thus in vitro systems have been useful tools for studying these processes.

2.2.1 Co-development of the Intercalated Disc with the Contractile Apparatus

In situ studies have shown how cell–cell junctions develop relative to other cellular structures, such as the cytoskeleton. In embryonic hearts, myocytes are proliferating and mostly round, myofibrils are immature, and cell–matrix and cell–cell adhesions are uniformly distributed around the cell membrane (Hirschy et al. 2006). In neonatal hearts, myocytes begin to elongate and myofibril striations become more apparent, but both cell–matrix and cell–cell adhesions remain randomly localized around all cell borders (Wu et al. 1999, 2002; Hirschy et al. 2006). By the time the

heart reaches full maturity, myofibrils are highly striated and aligned and cell–cell adhesions are localized primarily to the short ends of the myocytes at intercalated disc structures (Angst et al. 1997; Hirschy et al. 2006). Due to the co-development of the contractile apparatus with intercalated discs, one hypothesis is that myofibril development, and thus contractility, might be an important regulator of intercalated disc assembly.

To investigate how mechanical forces regulate cell–cell junction maturation, in vitro systems are used because they allow investigators to probe dynamic structure–function relationships in living cells and tissues. Studies using pairs of noncardiac cells, such as endothelial cells, cultured on compliant hydrogels or flexible microposts have shown that the size of cell–cell junctions is regulated by cytoskeletal tension (Liu et al. 2010; Maruthamuthu et al. 2011). Similar cell pair systems been used to characterize relationships between contractile forces and cell–cell junction formation in beating cardiac myocytes (McCain et al. 2012b). Early in culture, myocyte pairs contain sarcomeres and contract, but much of the contractile force is transmitted to the extracellular matrix adjacent to the cell–cell interface, suggestive of weak mechanical coupling between cells. At this stage of growth, myocytes are also not completely synchronous, indicative of weak electrical cell–cell coupling. Later in culture, the magnitude of contractile force increases and, importantly, force is mostly transmitted across the cell–cell junction instead of to the extracellular matrix. Myocytes also become synchronous, indicating formation of a functional mechanical and electrical syncytium (McCain et al. 2012b). This study shows that, early in tissue morphogenesis, myocytes are primarily adhered to the extracellular matrix, but gradually lose their adhesions to the matrix while increasing adhesion to each other as time progresses. During this process, myofibrils are also actively contracting and maturing, suggesting that adhesion remodeling in the heart is dynamic and occurs even while myocytes are shortening and actively pulling against the cell–cell junction. Studies have also shown that cell–cell junction protein increases in cardiac myocytes exposed to cyclic stretch (Zhuang et al. 2000; Salameh et al. 2010), emphasizing the mechanosensitivity of the intercalated disc. Further studies are needed to better understand the role of mechanical forces in intercalated disc assembly and maturation in developing cardiac tissues.

2.2.2 *Potential Role of the Extracellular Matrix in Pathological Intercalated Disc Remodeling*

Many cardiac diseases are associated with both cell–cell junction remodeling (Matsushita et al. 1999; Severs 2002; Cabo et al. 2006), which can be arrhythmogenic, and increased fibrosis, which stiffens the myocardium (Doering et al. 1988; Berry et al. 2006; Chaturvedi et al. 2010). Crosstalk between cell–matrix and cell–cell adhesions has been observed in many cell types (Chen

et al. 2004; Kim and Asthagiri 2011; Maruthamuthu et al. 2011), suggesting that increased stiffness secondary to fibrosis could induce pathological remodeling of cell–cell adhesions in the heart. To test this, pairs of cardiac myocytes have been cultured on polyacrylamide gels with elastic moduli matching that of a healthy heart and a fibrotic heart (McCain et al. 2012b). On gels mimicking healthy myocardium, myocyte pairs formed mature cell–cell junctions with essentially seamless mechanical communication between cells. Relatively low amounts of force were transmitted to the extracellular matrix. Conversely, on gels mimicking fibrosis, myocytes retained cell–matrix adhesions adjacent to the cell–cell interface and thus a fraction of contractile force was transmitted to the matrix instead of the neighboring cell (McCain et al. 2012b), which potentially disrupts mechanoelectrical coupling between cells. Thus, mechanical properties of the extracellular matrix are potentially propagated to the intercalated disc and could influence cell–cell coupling in healthy and fibrotic hearts.

2.2.3 Engineering Gap Junctions

Gap junction channels facilitate electrical coupling between cardiac myocytes. A single channel consists of two transmembrane connexon hemichannels from neighboring cells, each of which consists of six connexin (Cx) proteins arranged in a hexamer (Noorman et al. 2009). Three Cx isoforms are primarily expressed in the heart: Cx40, Cx43, and Cx45, each of which has its own unique conductance and localization in the heart (Kanter et al. 1992; van Veen et al. 2001). For example, atrial gap junctions consist of Cx40 and Cx43 with small amounts of Cx45. Gap junctions in the ventricle consist mostly of Cx43 with small amounts of Cx45 (Vozzi et al. 1999). Many cardiac diseases are associated with gap junction remodeling, which is thought to underlie arrhythmogenesis by locally changing conductance and propagation velocity (Dupont et al. 2001; Kostin et al. 2003; Yamada et al. 2003; Li et al. 2006; Severs et al. 2006). Thus, determining the factors that regulate the structural and functional properties of gap junctions is important for understanding electrical dysfunction in cardiac disease.

In vitro studies are useful for characterizing the stepwise assembly of protein complexes at the intercalated disc because dissociated cardiac myocytes gradually reform cell–cell junctions in culture, which can be easily imaged (Fig. 2.3a) (Atherton et al. 1986; Hertig et al. 1996; Zuppinger et al. 2000). These studies have shown that adherens junctions and desmosomes, which provide mechanical cell–cell adhesion, form first in developing cardiac tissues. After mechanical adhesion is established, gap junctions form to electrically and chemically couple myocytes together (Kostin et al. 1999; Geisler et al. 2010), indicating a hierarchical relationship where mechanical adhesions precede electrical connections.

Dual voltage clamp is a technique for measuring the electrical conductance between two cultured cells by inserting patch clamp electrodes into each cell, applying a voltage pulse to one cell, measuring current from the second cell, and

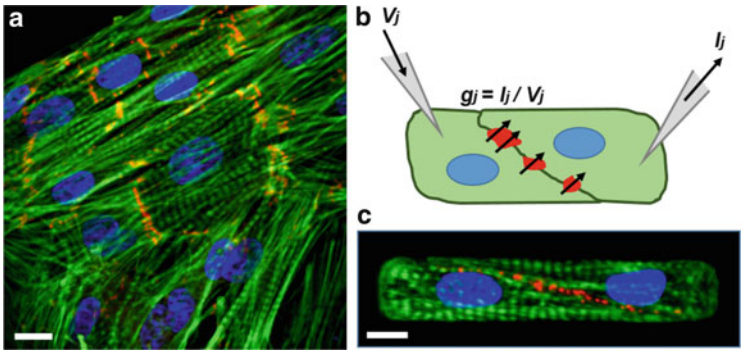

Fig. 2.3 Quantifying structure–function relationships at the intercalated disc. (**a**) Neonatal rat ventricular myocytes rebuild cell–cell adhesions in culture, providing an easily accessible model system for characterizing intercalated disc assembly. *Green*: actin, *red*: β-catenin, *blue*: nuclei. (**b**) Dual voltage clamp is used to quantify the electrical conductance of gap junction channels in pairs of cardiac myocytes by applying a voltage (V_j) to one cell, measuring current (I_j) from the second cell, and calculating conductance (g_j). (**c**) Electrical conductance can then be correlated to gap junction density with immunostaining. *Green*: actin, *red*: Cx43, *blue*: nuclei

calculating conductance with Ohm's law (Fig. 2.3b) (Van Rijen et al. 1998). This technique has traditionally been used on cells randomly plated in culture with little control over the morphology of the cells or the cell–cell junction. By instead culturing myocytes on microcontact printed islands of fibronectin, the size and shape of myocyte pairs can be regulated, as well as cell–cell junction morphology (Pedrotty et al. 2008). For example, neonatal rat ventricular myocytes on micropatterned islands form pairs with highly consistent junction morphologies (Fig. 2.3c), which reduces the variability in functional measurements by controlling for cell–cell contact (McCain et al. 2012a). These studies have shown that electrical conductance between myocytes is affected by the shape of the myocytes (McCain et al. 2012a), which could imply that myocyte shape remodeling in disease could affect gap junction density.

To determine the functional properties of individual gap junction isoforms, investigators have engineered pairs and strands of cardiac myocytes from transgenic Cx knockout mice (McCain et al. 2014b), which have revealed that Cx43 is the primary facilitator of electrical coupling between ventricular myocytes (Beauchamp et al. 2004). The heterogeneous expression of gap junction channels observed in certain pathologies has also been modeled by mixing together Cx43 knockout myocytes with wild-type myocytes in culture (Beauchamp et al. 2012). These studies have reported a significant reduction in Cx43 gap junction formation and electrical coupling between Cx43 knockout and wild-type myocytes on the two-cell level. However, on the tissue level, electrical signals still propagate relatively normally by meandering around the knockout myocytes and finding conduction pathways through wild-type myocytes (Beauchamp et al. 2012). This study highlights the importance of a multi-scale approach because a pathological

phenotype, in this case, electrical dysfunction, was apparent on one spatial scale but not another.

Myocytes from Cx knockout mice have also been engineered in culture to determine the function of different gap junction isoforms in the atria. Interestingly, knocking out Cx43 in atrial myocytes decreases propagation velocity, but knocking out Cx40 increases propagation velocity. Knocking out Cx43 also decreases Cx40 expression, but knocking out Cx40 increases Cx43 expression (Beauchamp et al. 2006). Complementary patch clamp studies have shown that Cx43 ablation in atrial myocytes affects the function of sodium ion channels (Desplantez et al. 2012), showing how Cx expression also impacts other protein complexes within the cell. Collectively, these observations indicate that Cx isoforms interact not only with each other but also with ion channels, highlighting the complexity between Cx expression levels and the electrophysiological properties of the tissue.

2.3 Structure–Function Relationships in Multicellular Cardiac Tissues

The electrical and mechanical function of multicellular cardiac tissues is highly dependent on the structure of the tissue. In recent decades, new experimental techniques have been developed to study tissue-level interactions between structure and function in vitro. These experiments have revealed that mimicking the anisotropic structure of healthy heart tissue is essential to fully recapitulating a functioning myocardium, which is a complex and challenging task.

2.3.1 Organization and Structure In Vivo and In Vitro

Structural remodeling of cardiac tissue is evident in many heart diseases, which contributes to functional declines. Two common types of remodeling are seen in the heart: hypertrophy and dilation. Hypertrophy is characterized by thickening of the ventricle walls associated with a disorganization of myocardial tissue. Dilation is characterized by a thinning of the ventricle walls and is also associated with changes in myocyte structure (Ahmad et al. 2005; Ho 2009). Significant regional anatomical remodeling, such as sarcomere disorganization and fibroblast infiltration, can be seen in failing hearts when compared to healthy hearts, further supporting the concept that the structural organization of the heart at multiple scales is important to proper cardiac function (Matsushita et al. 1999; Helm et al. 2006).

To determine how the microscopic organization of cardiac tissue affects its physiological function, several techniques have been developed to control the architecture of cardiac tissues in vitro. One technique is microcontact printing,

where myocytes are seeded onto elastic polymer substrates micropatterned with extracellular matrix proteins, such as fibronectin, which induces them to grow along the protein pattern (Tan et al. 2004; Grosberg et al. 2012). A second approach to patterning is to use external topographical cues. Studies have shown that cardiac myocytes organize anisotropically on PDMS scaffolds with repeating ridged features (Kim et al. 2010; Chung et al. 2011) or microgrooved hydrogel substrates (Agarwal et al. 2013a; McCain et al. 2014a). To mimic the multi-scale nature of the heart, other research has produced topographical cues with features that range from micro- to nanoscale by using wrinkled shrink-wrap films as culture substrates (Chen et al. 2011). A final technique to guide organization of cardiac tissue is mechanical stretching. In vitro, cardiac myocytes seeded onto thin PDMS substrates that are cyclically stretched for at least 24 h orient along the direction of stretch (Matsuda et al. 2004; McCain et al. 2013). Thus, several techniques can be used to mimic the architecture of healthy or diseased cardiac tissues in vitro, allowing investigators to study how tissue structure regulates function in a controlled setting.

Although 2D monolayers are helpful for understanding the function of sheets of heart tissue, engineering more complex three-dimensional (3D) tissues is also important for revealing mechanisms of cardiac disease. However, appropriate 3D scaffolds must provide structural and physiological characteristics that mimic that of the heart in vivo. Vascularized cardiac tissue constructs formed by seeding synthetic polylactic acid/poly(lactic-*co*-glycolic acid) (PLLA/PLGA) porous scaffolds with cardiac myocytes derived from human embryonic stem cells (hESC), endothelial cells, and embryonic fibroblasts have been shown to produce electrophysiological functions similar to those seen in vivo, but do not recreate the anisotropic architecture of native cardiac tissue (Caspi et al. 2007). Rotary jet spun scaffolds of nanofibers have been promising 3D constructs with anisotropic features that promote alignment of cardiac myocytes (Badrossamay et al. 2014), but there is no evidence that these fibers can promote maturation or vascularization. Other studies have shown that electrically pacing neonatal rat cardiac myocytes cultured onto collagen hydrogels produces electrically mature myocytes aligned in the direction of the field lines (Radisic et al. 2004). However, the dimensions of these constructs are currently limited to the depth of the diffusion of oxygen, although adding fluidic channels to the scaffold has been implemented to improve perfusion (Radisic et al. 2008). Thus, although synthetic scaffolds are promising, we still lack the technology to engineer 3D cardiac tissues with proper structural and functional properties.

Recently, decellularized hearts have been used as native cardiac ECM scaffolds, which are perfused and repopulated with cardiac myocytes. Decellularized ECM is advantageous because it maintains all of the necessary structural cues of the native heart. Eight days after reperfusing decellularized hearts with cardiac myocytes, the constructs generate weak pumping function with the aid of electrical stimulation (Ott et al. 2008). Though decellularization followed by repopulation is promising, repopulated hearts are insufficiently mature and revascularization is not possible with current methods. Decellularized mouse hearts have also been repopulated with human iPS-derived cardiac myocytes, demonstrating the potential for

decellularized cadaver hearts to serve as scaffolds for a patient's own cells (Lu et al. 2013). This method would reduce the need for immune suppression after organ transplant. Thus, although engineered 3D cardiac tissues are an attractive "quick fix" to many problems such as heart disease or heart transplant availability, we still do not have the tools to accurately replicate the physiological and mechanical functions of the natural heart.

2.3.2 Tissue Organization and Electrophysiology

In order to efficiently pump blood, the heart relies on a complex network of electrical signals that activates myocytes to contract and relax in a synchronous, pulsatile pattern (Feher 2012). The electrical signals of the heart can be traced in vivo via electrocardiography (ECG) (Guyton and Hall 2000). During infarct, reperfusion, or arrhythmia, the electrical activity of the heart becomes abnormal, which can be detected by ECGs (Zimetbaum and Josephson 2003). Electrophysiological changes have also been observed in diseased hearts with remodeled cardiac tissue. For example, studies have shown that left ventricular hypertrophy, which is characterized by enlarged myocytes, results in lower conduction velocities (Carey et al. 2001). The electrical activity of living hearts can be measured ex vivo using Langendorff preparations, where explanted hearts are immediately perfused with nutrient-rich solutions so that their physiology is preserved outside of the animal's body. Because Langendorff preparation allows the heart to continue functioning, the electrical properties of the heart can be studied using techniques such as optical mapping (Antz et al. 1998; Efimov et al. 2004; Hucker et al. 2005). Optical mapping entails loading a tissue with a voltage-sensitive dye, which changes its emission properties based on the membrane potential of the tissue. Thus, action potential propagation can be tracked using cameras equipped for high-speed fluorescent imaging. For example, optical mapping of Langendorff preparations has been used to show changes in electrophysiology with respect to molecular changes in the tissue (Morley et al. 1999).

Electrical activity within engineered 2D cardiac monolayers can also be visualized with optical mapping by tracking the fluorescence of voltage-sensitive dyes loaded in tissues that are mounted on a microscope (Fast and Kleber 1993; Bursac et al. 2002). In addition, calcium-sensitive dyes can be used to detect calcium transients within cardiac myocytes and their contributions to atypical beating (Sirenko et al. 2013). Using these methods, it has been shown, in vitro, that electrophysiological abnormalities depend on tissue organization. For example, cardiac tissue alignment has a significant effect on the electrophysiological properties of the heart, such as action potential duration, conduction velocities, and calcium transients (Fast et al. 1996; Bursac et al. 2004; Kim et al. 2010; Chung et al. 2011; Feinberg et al. 2012). Importantly, aligned tissues have a higher longitudinal conduction velocity, which is likely because action potentials propagate more rapidly through cytoplasm than across cell–cell junctions (Spach

et al. 2004). These studies indicate that cardiac tissue architecture and electrophysiology are tightly coupled.

2.3.3 Tissue Organization and Contractility

Cardiac contractility is vital to the function of the cardiovascular system. The contraction of the heart can be analyzed at various scales ranging from the cellular scale to the full organ scale (Humphrey 2010). Changes in heart contractility are usually associated with poor pumping function, which are signs of a diseased heart (Guyton and Hall 2000). Ejection fraction is a measure of the volumetric fraction of blood leaving the heart with each contraction and is typically 55 % or greater during systole in a normal healthy heart (Wackers et al. 1979). The contraction during systole is characterized by a 14 % shortening of the myofibers accompanied by a significant thickening of the heart wall (Chen et al. 2005). Additionally, pressure changes within the chambers themselves act as secondary driving forces to transport blood into and out of the chambers, which aids contractility.

Heart disease is highly correlated with changes in contractility, as measured by changes in ejection fraction. For example, hypertrophy, characterized by an enlarged heart, is associated with a decrease in ejection fraction (Feild et al. 1973). Using the Langendorff preparations previously mentioned, it has been shown that ischemic hearts have much higher diastolic pressures than healthy, non-ischemic hearts (Mirica et al. 2009). To begin to understand what factors are contributing to poor function of the diseased heart, studies that elucidate these elements on a smaller scale must first be conducted.

Several methods have been developed to measure the contractility of cardiac tissues in vitro. In one method, micron-sized flexible PDMS posts are molded from a silicon wafer and seeded with cardiac myocytes, which displace the posts as they contract. By measuring the displacement of the posts with a high-speed camera, contractile forces generated by the tissue can be calculated (Boudou et al. 2012; Thavandiran et al. 2013; van Spreeuwel et al. 2014). Another assay, known as muscular thin films (MTFs), has also been developed to measure the diastolic and peak systolic stresses of monolayers of engineered cardiac tissues (Feinberg et al. 2007, 2012; Grosberg et al. 2011a; McCain et al. 2013). MTFs consist of thin films of PDMS cut into cantilevers and temporarily adhered to a glass coverslip by a temperature-sensitive polymer. The PDMS is micropatterned with fibronectin and seeded with cardiac myocytes to form anisotropic cardiac tissues. After the tissues are cultured for several days, the MTFs are carefully peeled from the glass coverslip, which causes the temperature-sensitive polymer to release the MTF cantilevers from the glass coverslip. MTFs are then paced by electrical stimulation to contract at a specific frequency, and MTF curvature is analyzed to determine the stress produced by the cardiac tissue. The “heart on a chip” technology combines multiple MTFs onto one coverslip to increase throughput (Grosberg et al. 2011a; Agarwal et al. 2013b). “Heart on a chip” can also be combined with a range of other

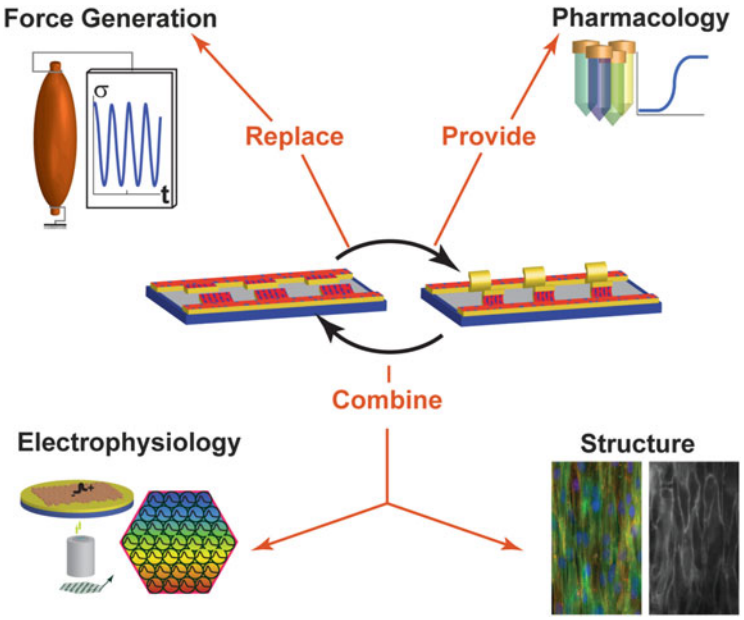

Fig. 2.4 Overview of "heart on a chip" technology (Grosberg et al. 2011a)—it replaces the classical force generation experiments measured with a force gauge from a muscle strip; it can be combined with optical mapping experiments to study electrophysiology and immunostaining techniques to study tissue structure; the technology provides an efficient way to test cardiotoxicity in vitro

techniques, such as optical mapping, immunostaining, and drug dose response studies (Fig. 2.4), to correlate multiple structural and functional parameters. With MTFs and "heart on a chip," it has been shown that the peak systolic stress produced by a tissue is highly dependent on the alignment of the sarcomeres within the tissue (Grosberg et al. 2011a; Feinberg et al. 2012; McCain et al. 2013). This suggests that organization is a key factor to reproducing viable, functional cardiac tissue in vitro, and loss of organization is likely one of the key factors contributing to poor function of diseased hearts. Thus, analyzing both structural and functional properties of engineered cardiac tissues is essential for understanding disease progression in the heart.

2.3.4 Stem Cell-Derived Cardiac Tissues: Potential Therapeutic Applications

Many of the body's tissues and organs are able to repair themselves after injury due to cell proliferation and/or the presence of stem cells that can differentiate and repopulate the site of injury (Fausto 2004; Yin et al. 2013). However, the adult heart

has very limited regenerative potential because mature cardiac myocytes are essentially post-mitotic (Mollova et al. 2013), and there is an extremely limited number of cardiac myocyte progenitor cells (Anversa et al. 2006). Thus, after an infarct, the portion of myocardium that dies is replaced with scar tissue, which greatly impairs the function of the heart (Guyton and Hall 2000). For these reasons, stem cells and stem cell-derived cardiac myocytes are being actively pursued as therapeutic options to repair and regenerate injured myocardium.

One strategy for repairing infarcted myocardium is to inject bone marrow-derived mesenchymal stem cells directly into damaged areas of the heart, with the goal that they will differentiate into cardiac myocytes due to environmental cues. This strategy does promote cardiac regeneration and reduce fibrosis, but only partially recovers the function of the infarcted heart (Amado et al. 2005). One major problem with this approach is the retention of stem cells at the transplantation site is very low, as the majority of cells die within the first few days after transplantation (Robey et al. 2008). Additionally, there are severe risk factors involved, including the potential for tumor formation or the potential for stem cells to differentiate into cells other than myocytes (Herberts et al. 2011). Injections of human embryonic stem cell-derived cardiac myocytes have been shown to re-muscularize infarcted primate hearts, although with a high risk of arrhythmias (Chong et al. 2014). Thus, using stem cells and stem cell-derived cardiac myocytes to repair damaged myocardium is promising but still poses tremendous challenges.

Drug testing is another potential application for stem cell-derived cardiac myocytes. Drug failure due to cardiotoxicity is a major burden on the pharmaceutical industry (Ferri et al. 2013) and is partially attributed to the industry's use of animal models or overly simplified cell culture systems for drug testing, both of which have limited relevance to native cardiac tissue. Thus, a source of fully functional human cardiac myocytes derived from stem cells would resolve problems such as species differences in the behavior of in vitro cardiac models (Braam et al. 2010; Lu et al. 2013). However, while stem cells can be differentiated into cardiac myocytes, these cells are very immature and there is evidence that they retain some noncardiac myocyte gene expression (Zhang et al. 2009; Robertson et al. 2013). Thus, to utilize stem cell-derived cardiac myocytes for drug studies, it is essential to ensure that their in vitro function mimics in vivo behavior, which can be achieved using the "heart on a chip" assay described above (Grosberg et al. 2011a). For example, comparing primary neonatal cardiac myocytes to cardiac myocytes derived from either embryonic or induced pluripotent stem cells (iPSCs) has shown functional deficiencies in both populations of stem cell-derived cardiac myocytes (Sheehy et al. 2014a). Quantifying the function of stem cell-derived cardiac myocytes as differentiation protocols improve will help evolve these cells into a usable population of cells useable for predictive drug testing.

In vitro models of cardiac tissue engineered with iPSC-derived cardiac myocytes also have significant potential for personalized medicine. Because iPSCs are typically reprogrammed from patient skin cells or blood cells, iPSC-derived cardiac myocytes have the same genetic background as the patient from which they were isolated and thus they can be expected to respond to drugs similar to the patients'

native cardiac myocytes (Zhang et al. 2009; Kim et al. 2013). By combining the "heart on a chip" assay with iPSC-derived cardiac myocytes from patients with genetic diseases, such as inherited cardiomyopathies, researchers can study disease mechanisms in a human-relevant model system and rapidly screen the functional effects of therapies on a patient-by-patient basis (Wang et al. 2014). Thus, in vitro tools are essential for evaluating stem cell-derived cardiac myocytes and their functional responses to drugs.

2.4 Conclusions

As described in this chapter, the pumping function of the heart is highly dependent on its tissue, cellular, and molecular organization, as well as gene and protein expression (Ahmad et al. 2005; Chung et al. 2011; McCain et al. 2013). Because structure, function, and gene expression are interconnected, it is difficult to decouple these factors from each other using only in vivo systems. Thus, developing and utilizing in vitro models that can replicate and decipher these interactions at the molecular, single cell, and/or tissue level will not only help understand how the heart as an organ functions in health and disease but also lead to the development of biomimetic cardiac tissues that can be used for disease modeling and drug screening. Furthermore, recapitulating cardiac tissue in vitro can lead to the development of new therapies such as heart patches or even engineered heart transplants for cardiac diseases such as heart failure, which currently have extremely limited therapeutic options.

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Chapter 3
The Intercalated Disc: A Focal Point for Sarcomere Growth and Disease

Pauline M. Bennett

Abstract Heart muscle cells are glued together end to end by the intercalated disc (ID), a complex junction fulfilling many functions; it transduces the forces of contraction and transmits electrical signals from one cell to the next; it mechanically holds the cells together and is the site of other signalling pathways including those involved in calcium homeostasis. This chapter aims to describe the functional roles of the ID with respect to the observed structure. Particular emphasis is given to the relationship between myofibrils and the ID, and the evidence for cell growth and sarcomere addition at the ID is presented. Because of its complex nature, it is not surprising that the ID has been implicated in a number of heart diseases and malfunctions. Changes in its structure and composition associated with these heart problems are described with special regard to the role of the ID in dilated cardiomyopathy.

3.1 Introduction

Heart muscle cells are glued together end to end by the intercalated disc (ID), a complex junction fulfilling many functions, transducing the force and transmitting electrical signals from one cell to the next, mechanically holding the cells together, and the site of other signalling pathways including those involved in calcium homeostasis. The ID is also thought to be where sarcomere addition and growth occurs. It is no wonder then that it is implicated in a number of heart diseases and malfunctions. This chapter aims to describe the structural components of the ID and present the evidence for cell growth and sarcomere addition. Further, changes in the structure and composition of the ID associated with some heart problems, mainly dilated cardiomyopathy (DCM), will be described.

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3.2 Postnatal Heart Growth

3.2.1 Proliferation and Hypertrophy

The development of the heart during gestation and very early growth is not a subject of this chapter. For information about the development from the heart field, the formation of the chambers, and the differentiation and organisation of cells that occurs before birth, see reviews (Gregorio and Antin 2000; Miquerol and Kelly 2013).

Of interest to us here is how the heart increases in size after the general morphology has been established. Heart weight increases with body weight. In the rat there is a ~35-fold increase in heart weight in the first year after birth (Sasaki et al. 1968). This increase initially comes about by proliferation (hyperplasia) and subsequently cell growth (hypertrophy).

Hyperplasia As well as the contractile cells, the cardiomyocytes, the heart contains a significant number of other interstitial cell types, such as fibroblasts and capillary wall smooth muscle cells. All types proliferate greatly during embryogenesis. After birth all continue to divide but to a different extent. The interstitial cells increase in number throughout life approximately in line with heart weight (Sasaki et al. 1968). In the rat there is a 20-fold increase over the first year. The myocytes, however, multiply only a few fold with most of this increase occurring over the first few weeks. Significantly, after this stage many of the myocytes lose their proliferative capacity. Most become binucleate suggesting that karyokinesis has occurred but the final splitting of the cell has not been achieved. In rat this change occurs mostly in the first 12 days by which time 90 % of the myocytes are binucleate (Li et al. 1996). The binucleate cells in the 12-week-old rat are approximately twice the size of the mononucleate cells (Bishop et al. 1979). In larger animals binucleation appears to be a slower process; in the neonatal pig the number of multinucleate cells is only 67 % by 2 weeks of age (Beinlich et al. 1995). In mammals the regenerative power of the heart after maturity is small (Bergmann et al. 2009; Porrello et al. 2011). On the other hand, birds and cold blooded animals retain a significant possibility for regeneration. Chicks post hatch have a slow change in the ratio of mono/binucleate myocytes. Even at 42 days only 55 % of the cells are multinucleate, and even then they appear to retain their ability to divide (Li et al. 1997).

Hypertrophy Following neonatal hyperplasia the increase in heart weight with age is mainly due to hypertrophy of the cardiomyocytes (Sasaki et al. 1968; Beinlich et al. 1995; Li et al. 1996, 1997). Even before birth it is clear that during hyperplasia there is significant concurrent hypertrophy of the myocytes. Figure 3.1 shows the measured width and length of mouse cardiomyocytes from embryonic day 8.5 to 30 weeks after birth (data from Leu et al. 2001; Hirschy et al. 2006). It can be seen that the increase in length of the cells is fast and continuous from an early age, changing abruptly at about 2 weeks after birth. The rate of growth on average during this early stage is ~2 μm/day. After this there is a slow and continuous

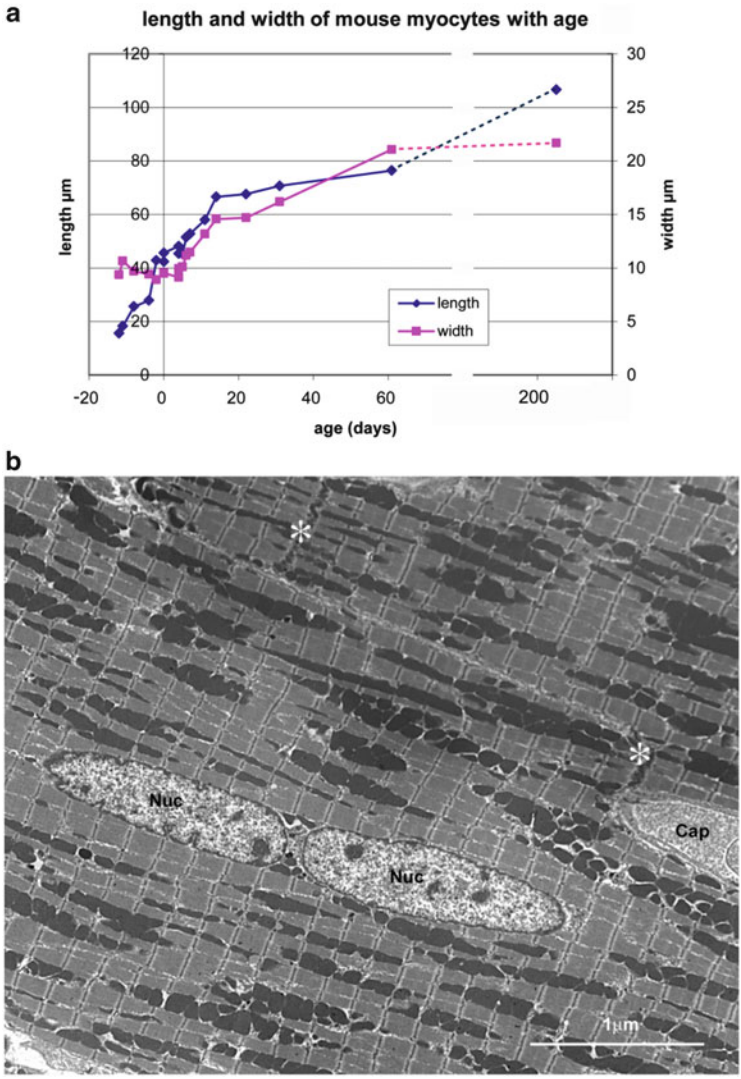

Fig. 3.1 (**a**) Changes of length and width of mouse cardiomyocytes during growth. Birth day set as 0. Data from embryonic day 8.5 (−11.5) to 4 days after birth taken from Hirschy et al. (2006). Data from birth to 205 days taken from Leu et al. (2001). Data from RV and LV were averaged. Plotted width is average of cell thickness and width data. (**b**) Electron micrograph from mouse heart showing part of a binucleate (*nuc*) cardiomyocyte and its connections through the intercalated disc (*asterisk*) to two other cells. A capillary (*cap*) is also indicated

increase with age averaging a few micrometres a week. The width however has a different behaviour. It is very steady at about 10 µm throughout gestation and even for a few days after birth before it takes off and increases rapidly for 2 weeks and subsequently increases at a slower rate before levelling off at ~20 µm at maturity

(~2–3 months). It is noteworthy that in the chick this increase in width is less, an observation that may relate to the continued ability of these cells to divide (Li et al. 1997).

The final size of cardiomyocytes in adult mammals is relatively similar from mouse to man. It is difficult to compare exactly because it depends on many factors such as age, nutrition and exercise, but a length of ~150 μm, and a width of 20 μm is common (see, for example, Bishop et al. 1979; Smith and Bishop 1985; Gerdes et al. 1992; Leu et al. 2001). Alterations in these dimensions occur in diseased hearts as will be seen later.

3.2.2 Morphology of Cardiomyocytes During Growth

There are significant changes in the morphology of cardiomyocytes during different phases of growth. At first (e.g. embryonic day 8.5 in mice) the cells are small and relatively round; a few myofibrils, which are not well aligned, surround the nucleus (Hirschy et al. 2006). Gradually, the cells elongate with a concomitant lengthening and improved alignment of the myofibrils. At birth the cardiomyocytes are long narrow elliptical cells ~50 μm in length and 10 μm in width. After birth, continued elongation and a spate of lateral growth bring the cells in a few weeks to their adult appearance, some 50 % longer and wider. They are mostly by now binucleate cylindrical cells with stepped ends (Fig. 3.1b). The ends overlap and interdigitate with several other cells (Figs. 3.1b and 3.2a). During subsequent hypertrophic growth, the structure is consolidated (Fig. 3.2c). How and where this growth, illustrated diagrammatically in Fig. 3.2b, occurs and is maintained is the major subject of this chapter.

3.2.3 Cardiomyocyte Connections

Even at the earliest stage the myocytes are connected to each other through cadherin-rich structures, adhering junctions, found over most of their surface (Hirschy et al. 2006). The early myofibrils are attached through these junctions to the plasma membrane, and the spontaneous contraction of the cells which occurs very early in heart development sets up a pattern of forces which leads to the reorganisation and alignment of the myofibrils and the changes in morphology of the cells. The importance of these structures in heart development is demonstrated by studies such as that of the N-cadherin knockout mouse where the mouse dies in mid-gestation when the heart cells fail to adhere (Radice et al. 1997). In the early heart, the tessellated organisation of the almost round cells means that they interact with several others. As the cells elongate connections through their ends with several neighbouring cells are maintained (Figs. 3.1b and 3.2a). As the cells

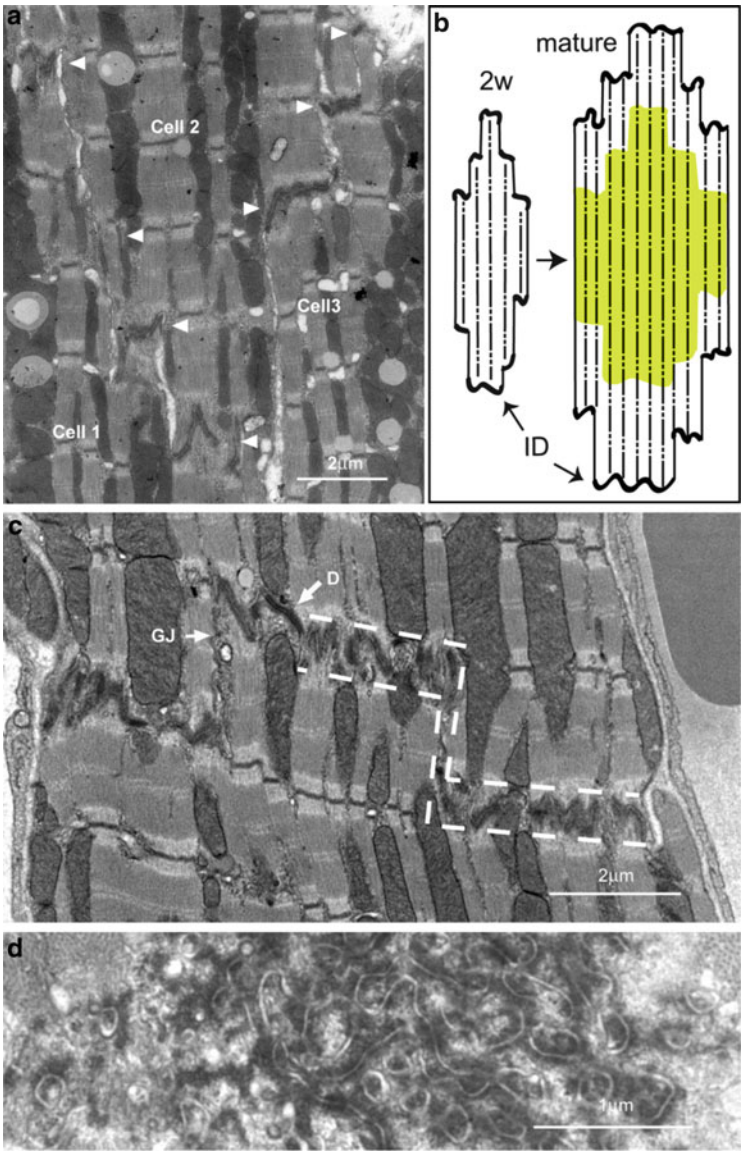

Fig. 3.2 (**a**) Electron micrograph of 2-week-old mouse heart showing stepped ID between three cardiomyocytes. At this age the treads of the steps (*arrowheads*) are narrow. (**b**) Diagram to show growth of cardiomyocytes from 2 week cells at the end of the hyperplastic, neonatal, stage with small lateral ID steps to longer adult cells (2+ months) with established wide ID steps. (**c**) Electron micrograph of a longitudinal section of 6 month mouse papillary muscle illustrating the increase in lateral width of the ID treads compared to (**a**). *Dotted lines* show outline of the area defined as the ID. (**d**) Transverse section through an ID tread showing the circular profile of the membrane folds. *D* desmosome, *GJ* gap junction

lengthen, the cadherin-rich junctions apparently move towards the ends of the cells (Fig. 3.2b). As the width of the cell increases these connections become wider and more substantial, and the stepped intercalated disc as it is normally described is created (Fig. 3.2b, c).

3.3 Myofibril Formation and Growth

3.3.1 Early Formation of Myofibrils

There are a number of theories about how myofibrils are formed initially (see Sanger et al. 2005). Much of the work has been carried out on cultured cell lines or isolated neonatal cells. In such myocytes or myotubes, the observations have led to the idea of premyofibrils or stress fibre-like structures which contain sarcomeric proteins but only later become full length mature sarcomeres. These in vitro studies use essentially a two-dimensional system rather than a three-dimensional one. In three-dimensional or in vivo systems, there is little evidence for these intermediate sarcomeric structures (Ehler et al. 1999, 2004). There is, however, in agreement that some kind of scaffold of Z-disc and M-band proteins is laid down first and thick and thin filaments are then incorporated. Increase in length of the myofibrils in the in vitro systems appears to be by the addition of new sarcomeres at their ends.

In early heart, in vivo, only short myofibrils with a mature sarcomere length are seen (Hirschy et al. 2006). The question arises, 'Where are new sarcomeres added?' The evidence suggests that, at least in the mature heart, they are added at the ends of the fibrils near the intercalated disc.

3.3.2 Evidence for Fibril Growth at the Ends of Myocytes

Evidence for the growth of muscle cells occurring at their ends is reviewed in (Russell et al. 2010). In skeletal muscle, growth occurs at the ends of the fibres near the myotendinous junction (MTJ). In early work Williams and Goldspink (1971) labelled young rapidly growing skeletal muscle with tritiated adrenaline to follow where actin is deposited and found it accumulated at the ends of the fibres. This was supported by the observation in the electron microscope of copious ribosomes in these areas and bundles of thick filaments (no Z-discs) and narrow myofibrils at the periphery of the muscle. Dix and Eisenberg (1990) using mRNA detection of slow myosin heavy chain on stretched stimulated TA muscle in the rabbit which normally expresses the fast isoform, found a large accumulation of the slow isoform at the MTJ. In addition, in contrast to the control muscle where the regular sarcomeric structure is present up to the edge of the MTJ, in stretched muscle, bundles of

filaments and thin myofibrils in a sea of new T-tubules, ribosomes and mitochondria were seen at the MTJ.

There is a constant turnover of sarcomeric proteins in muscle fibres (Willis et al. 2009). However, evidence for formation of new sarcomeres within the body of the fibre is seen only after eccentric exercise. Here, local changes in the distribution of desmin and Z-disc proteins such as α-actinin are seen and have been attributed to myofibril remodelling after damage inflicted by sudden large transient stretches (Yu et al. 2004). Such repair sites are not observed in control muscle. Similar repair or addition of sarcomeres has been seen in cardiomyocytes as a result of a major 10 % stretch (Yu and Russell 2005). In this case the stretch was permanent so the cells had to generate a number of additional sarcomeres in order to normalise the sarcomere length. The rate at which this occurred was about one sarcomere (2 μm) per hour much greater than normal even in early heart growth (see Sect. 2.1). In addition to fibril remodelling, the stretch also generated substantial changes in the morphology of the ID suggesting the involvement of this region in growth. This observation is supported by recent evidence that there are significant changes at the ID correlated with growth brought about by volume overload in the rabbit heart (Yoshida et al. 2010). In addition, evidence for sarcomere addition at the ID has been seen in both normal heart and those suffering from dilated cardiomyopathy (DCM) (Wilson et al. 2014). In order to understand this, and indeed the more extensive role of the ID, we have to look more closely at its structure and its relationship with the myofibril.

3.4 Structure of the Intercalated Disc

3.4.1 General Organisation

What exactly do we mean by the intercalated disc? The main body of the cardiomyocyte comprises the myofibrils interspersed with columns of mitochondria extending from one end of the cell to the other (Fig. 3.1b). These are held, together with the sarcoplasmic reticulum and t-tubules, within a cytoskeletal network of proteins such as desmin, the whole being anchored to the extracellular matrix (ECM) at the lateral plasma membrane by the costameres (Granger and Lazarides 1978; Craig and Pardo 1983; Pardo et al. 1983) (Fig. 3.2c). The ID is seen in the light microscope as a high density junction between the end of one heart muscle cell and the next. With the advent of electron microscopy, it became clear that it was a complicated structure with regions of highly folded membrane of variable amplitude (compare Fig. 3.2a, c). Here, we adopt the definition of the ID given by Forbes and Sperelakis (1985). It includes the membrane folds and their axial connections as well as the material within the folds delineated by the dotted lines in Fig. 3.2c.

The structure of the ID has been beautifully described in several excellent electron microscope investigations (see, for example, Fawcett and McNutt 1969;

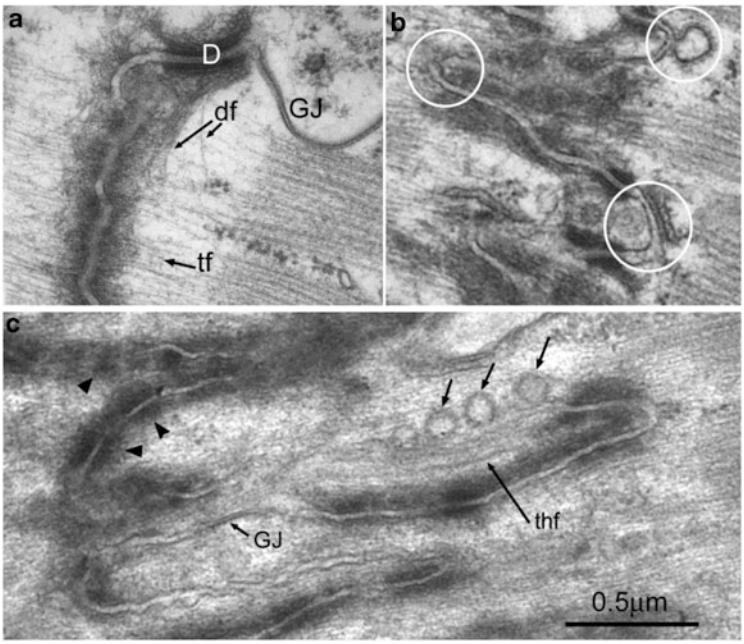

Fig. 3.3 Electron micrographs of longitudinal sections through mouse heart showing ID folds of different amplitudes. (**a**) Thin filaments (*tf*) run into dense protein plaque at the ID membrane. Desmin filaments (*df*) can be seen leaving a desmosome (*D*). *GJ* gap junction. (**b**) ID of intermediate amplitude. *Circles* show the tops of the folds which are devoid of CJ plaque which is associated with the sloping sides of the folds. One of the *circles* rings a fold with a junctional SR vesicle closely opposed to the ID membrane. Another shows a coated vesicle apparently combining with it. (**c**) ID of extreme length shows long regions of uncoated membrane some of which is associated with vesicles. *Arrowheads* show regions where CJ plaque is fragmenting. *GJ* small gap junction, *thf* thick filament within a long fold

Forbes and Sperelakis 1985). The general form of the ID is a stepped structure. The treads of the steps are lateral stretches containing folded membrane (Fig. 3.2). In transverse section the folds (or peaks) are seen as circular sections so that the ID tread membrane can be thought of as having a structure like an egg box (Fig. 3.2d). Within the folds, the thin filaments from the terminal sarcomeres of the myofibrils run to the membrane (Fig. 3.3a). Since the myofibrils take up approximately two-thirds of the cross-sectional area of the cell, much of this membrane is associated with actin adhering junctions. However, since the mitochondria occupy typically most of the other third of the cell a significant portion of the ID structure is available for other functional regions such as desmosomes, signalling domains and possible some that relate to mitochondria. The risers of the ID steps are axial and usually have a length of one or more sarcomere lengths. Often in the mouse these steps contain gap junctions (Fig. 3.2c). It is worth noting that the close association of the membrane of the neighbouring cells at the ID means that there is little or no ECM here.

3.4.2 ID Membrane: Domains and Connections

Desmosomes Desmosomes are very dense junctions, often found between cardiomyocytes where the mitochondria columns end (Figs. 3.2c and 3.3a). The role of the desmosome is to weld the plasma membranes of two cells together. In addition, they attach through desmoplakin to the main intermediate filament in muscle, desmin. In Fig. 3.3a, the desmin filaments are seen leaving a desmosome (see also Forbes and Sperelakis 1985). They run across the fibril and appear to weave their way across the tops of the ID peaks. As well as being found at high concentration at the ID, these intermediate filaments are a cytoskeletal component at the costameres, where the lateral PM is attached to the Z-disc, and wind their way at the Z-disc level around the fibrils across the cell (Granger and Lazarides 1978). This distribution can be seen by immunofluorescence in Fig. 3.4. It is clear that at the ID desmin collocates closely, although not exactly, with the adhering junction protein cadherin (Fig. 3.4a). The principle proteins found in cardiomyocyte desmosomes are shown in Fig. 3.5. The arrangement of the proteins within the structure has been characterised by North et al. (1999). The desmosomal cadherins, desmoglein2 and desmocollin2, are transmembrane glycoproteins which link cells through their N-terminal extracellular domains. This binding is partly calcium dependent and a high level of extracellular calcium is required for strong binding and physiological function (Dusek et al. 2007; Garrod and Chidgey 2008). The intracellular C-terminals of the cadherins bind through the armadillo proteins plakoglobin and plakophilin to desmoplakin which in turn binds to desmin filaments.

Gap Junctions At gap junctions the membranes are closely apposed, bound together by pore proteins (Fig. 3.3a). Hexamers of the protein, connexin, align with those in the other cell creating connexons. These form a tightly packed array which allows ions and small molecules to pass across the membrane transmitting and propagating the electrical signal that keeps the heart beating regularly. In adult heart the main isoform is connexin43 (Fig. 3.5). In mice these junctions are generally found on the axial steps of the ID membrane as seen in Fig. 3.2c although there are small patches embedded in the ID folds (Fig. 3.3c). There is little overlap between gap junctions on the risers and the other junctions (Pinder et al. 2012; Wilson et al. 2014). This separation can be seen in Fig. 3.4b where the location of connexin43 is compared to desmin by immunofluorescence. Further, it has been shown that gap junctions relocate to the ends of the cells during development with a slower time course than the adhering junctions and desmosomes (Angst et al. 1997). There is thought to be a buffer zone around the gap junction patches rich in the tight junction protein, zonula occludens-1 (ZO1) (Hunter et al. 2005). However, it is otherwise unclear what locates the gap junctions in their place. For a review of connexin interactions, see (Herve et al. 2007).

Composite (Adhering) Junctions At high magnification in the electron microscope, the path of the thin filaments from the terminal sarcomere into the ID folds is clear (Fig. 3.3a). They feed into the dark protein-rich plaque at the adhering junction

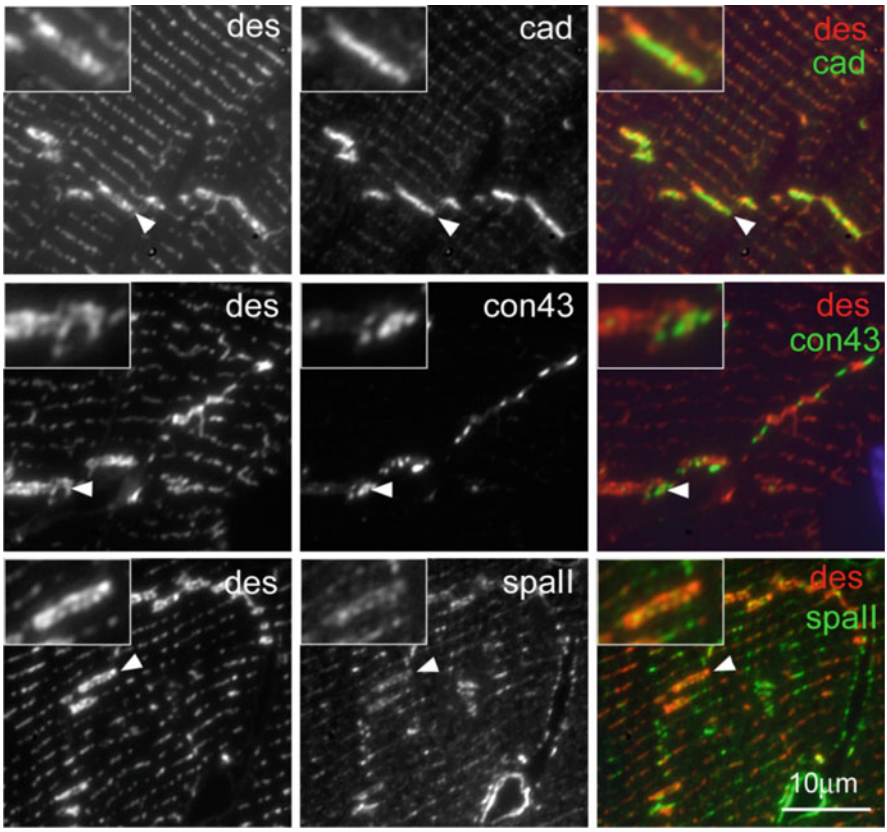

Fig. 3.4 Immunofluorescence of desmin and other ID proteins, N-cadherin, connexin43 and aIIspectrin in thin cryosections of mouse papillary muscle. (**a**) desmin (*des*, *red*) is strongly present in the ID (*arrow*) and at the Z-disc level in the rest of the cell. Pan cadherin (*cad*, *green*) localises closely but not exactly with it at the ID. (**b**) Connexin43 (*con43*, *green*) does not colocalise with desmin since it is found mostly in the axial risers of the ID and not the lateral treads. (**c**) αII spectrin (*spaII*, *green*) is seen at the ID treads and the Z-disc level but does not colocalise with desmin in either place. Inserts show at 2× magnification the ID indicated in the figure

(Forbes and Sperelakis 1985). The orientation of the thin filaments is such that it is their barbed ends that are at the membrane (Yamaguchi et al. 1988). The junctional protein is found on the sloping sides of the folds so that the actin filaments come in at an oblique angle to the membrane (Fig. 3.6). This geometry applies shear stress as opposed to strain at the membrane which has been shown to give more strength to the junction (Tidball 1983).

The main protein components of the ID plaque are those of adherens junctions (AJ) and desmosomes (Fig. 3.5) (Borrmann et al. 2006; Franke et al. 2006). AJs are similar in organisation to desmosomes. The transmembrane protein, N-cadherin, is bound to the armadillo proteins, p120, β-catenin and α-catenin. This complex is

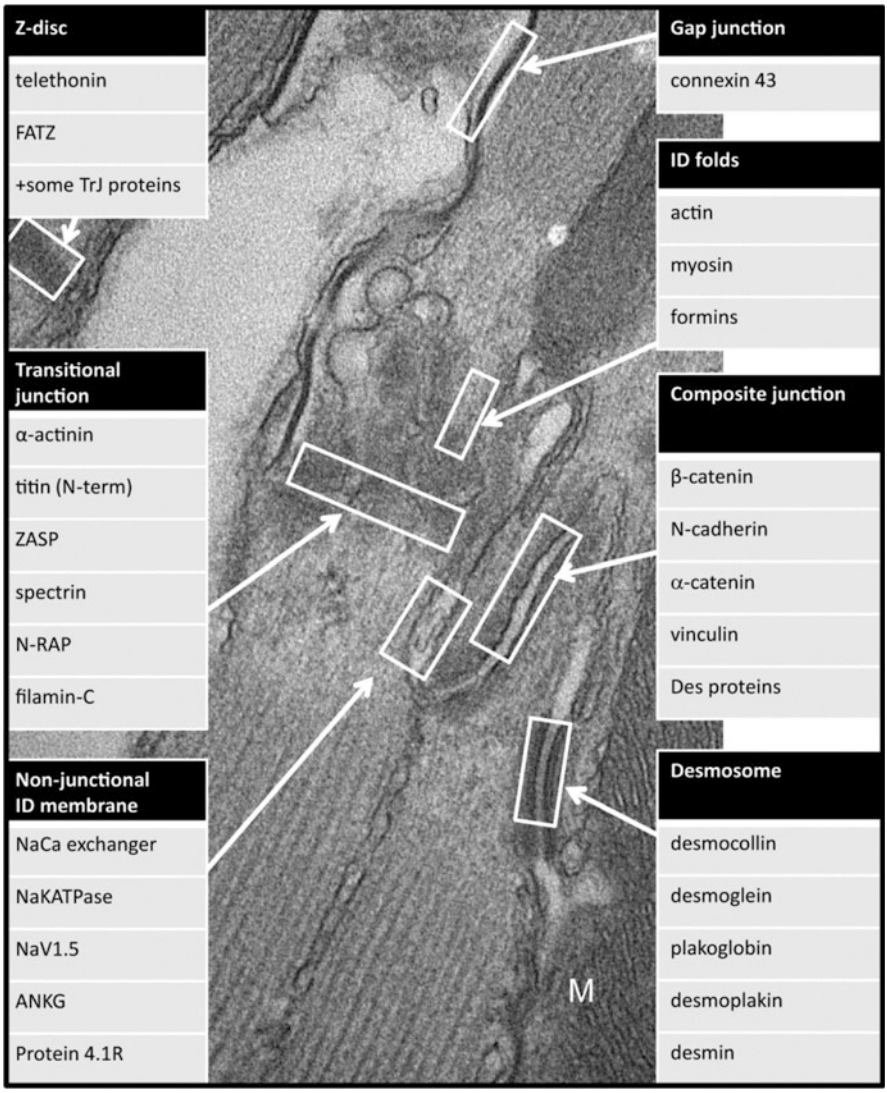

Fig. 3.5 Electron microscope image showing the different regions within the ID and the proteins associated with them

connected through vinculin, an actin binding protein, to the thin filaments. It seems that in the very early embryonic heart, desmosomes and adherens junctions are separate but by birth they are intimately combined (Pieperhoff and Franke 2007). The resulting junctions have been called areae compositae (Borrmann et al. 2006) or hybrid AJs (Li and Radice 2010). Here they will be called composite junctions (CJ). It is not clear what the functions of the desmosomal proteins are in the CJ. There are apparently obvious desmosomes where desmin filaments attach as

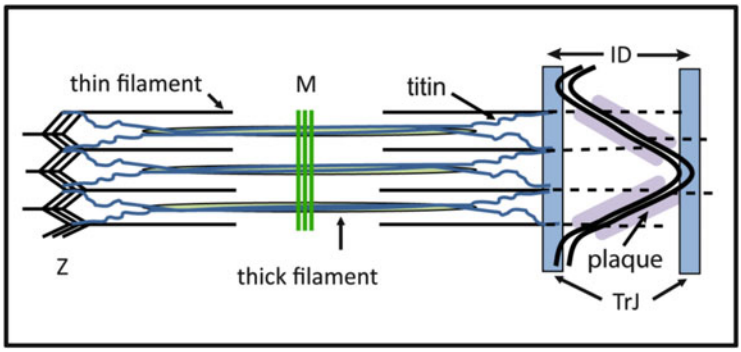

Fig. 3.6 Diagram showing the arrangement of the thin filaments in the intercalated disc and the transitional junction. Thin filaments run from the last half sarcomere into the CJ plaque at the ID membrane. At a position equivalent to the Z-disc, a region rich in Z-disc proteins including N-terminal titin is found. This position is equivalent axially to that of the tops of folds of the ID membrane. The membrane tops and the 'Z-disc' region is defined as the transitional junction (*TrJ*)

described above, but it is not obvious that these filaments penetrate the dense plaque of the CJ and bind. The distribution of desmin with respect to N-cadherin at the ID seen in Fig. 3.4a suggests that there is not a strong correlation between the two. Possibly, the close association of AJ and desmosome gives rise to a stronger junction that is necessary to support the high tensions found in contracting heart. Of the proteins involved, one, plakoglobin (γ-catenin), is known to operate in both types of junction and can replace β-catenin in the adherens junctions (Zhurinsky et al. 2000). Another desmosomal protein, plakophilin-2, has been shown to interact with a specific region of αT-catenin, its adhesion modulation domain (Goossens et al. 2007). These two interactions might be the means of knitting the adherens and desmosomal proteins into a specialised domain.

Non-junctional Membrane Apart from the structural junctions described above, there are a number of 'bare' or unspecialised membrane regions in the ID (see Forbes and Sperelakis 1985). These probably comprise different types of domains that may play a number of different roles. Among these areas are the tops of the folds (Fig. 3.3b) and part of the axial risers devoid of gap junctions (Fig. 3.5). In addition, extra membrane is generated when big amplitude ID folds are formed (Fig. 3.3c). Estigoy et al. (2009) have identified some 200 proteins at the ID using immunohistochemical data on the Human Protein Atlas (HPA) website, ExPASy (website) protein binding data and published papers on IDs. A large number of these are not obviously associated with the junctional domains already described.

Two primary cytoskeletal complexes are known to underlay and support membranes. One based on dystrophin and the other on spectrin. Both are found at the lateral plasma membrane in the cardiomyocyte, but only spectrin is seen at the ID (Stevenson et al. 2005). Several components of the spectrin complex have been located there, including αII spectrin, βII spectrin, ankyrin-G and proteins 4.1R and

4.1G (Mohler et al. 2004; Bennett et al. 2006; Pinder et al. 2012). The distribution of two of these at least, αII spectrin and 4.1R, does not overlap with β-catenin or connexin43, in support of their presence on the non-junctional membrane (Bennett et al. 2006; Bennett 2012; Pinder et al. 2012; Wilson et al. 2014). 4.1R is a multivalent protein stabilising the actin/spectrin interaction and interacting through its FERM domain with membrane signalling proteins (Baines et al. 2014). Ankyrin-G also links spectrin with membrane bound signalling proteins and has a pivotal role in locating the sodium voltage channel $NaV_{1.5}$ at the ID (Mohler et al. 2004).

3.4.3 The ID as Z-Disc: The Transitional Junction

Tension Transducer Myofibrils reach to the edge of the ID but there is no Z-disc density at the ID side of last sarcomere. Since myofibrils in neighbouring cells connect through the ID, it has often been said that the ID acts as a 'Z-disc'. However, the Z-disc has many functions and two of these, at least, are spatially separated at the ID. A principle role is to connect the thin filaments from one sarcomere to the next and to transmit the tension along the myofibril. The CJ with its dense protein complement fulfils this role. The protein complex present, including vinculin and α-catenin, are involved in tightly binding the thin filament to the membrane and use cadherins to transduce the tension across the cell membranes to the next myofibril (Fig. 3.6).

Titin Anchor and Passive Tension A second role of the Z-disc is to anchor titin molecules. The giant titin molecule runs from the Z-disc, through the I-band, along the thick filament in the A-band ending in the M-band (Fig. 3.6) (Kontrogianni-Konstantopoulos et al. 2009). I-band titin carries the passive tension in the sarcomere and thus maintains the ordered arrangement of filaments (Horowits 1992). This is effective because the I-band thin filaments are all the same length. However, the thin filaments from the terminal half sarcomere near the ID are all different lengths since they insert in the membrane at different points on the slope of the fold. If titin from the terminal half sarcomere was inserted at the membrane end of the thin filaments, each molecule would exert a different passive tension on the thick filaments and there would be disorder in the A-band. However, it is clear that the ordered arrangement of the sarcomeres is maintained right to the edge of the ID. Furthermore, the sarcomeric thin filament proteins tropomyosin and troponin finish at this point and are not found in the ID folds. This led to the definition of a transitional junction (TrJ), a region at the margin between myofibril and ID that behaves like a Z-disc with respect to titin although it has no Z-disc density (Fig. 3.6) (Bennett et al. 2006).

The Transitional Junction The proteins associated with the TrJ have a characteristic distribution. They show themselves as a doublet about the ID with an axial separation corresponding to the amplitude of the ID folds. Several sarcomeric Z-disc proteins are to be found at this position. These include N-terminal titin

(Fig. 3.6), α-actinin and ZASP [(cypher/oracle; Faulkner et al. 1999; Zhou et al. 1999; Passier et al. 2000) Bennett et al. 2006; Bennett 2012]. How they maintain their location is unknown. Compared to the Z-disc there is a lack of mass at the TrJ. This is partly due to the absence of overlapping thin filaments of the opposite polarity. In addition, some Z-disc proteins are absent (Bennett 2012; Wilson et al. 2014). The two known to be missing are telethonin (T-cap) which binds to the N-terminal Ig domains of two titin molecules (Valle et al. 1997; Gregorio et al. 1998) and FATZ (calsarcin/myozenin) which is a ZASP/titin binding protein (Faulkner et al. 2000; Takada et al. 2001; Frey and Olson 2002). Their location at the Z-disc may rely on the antiparallel arrangement of thin filaments.

Other, non-Z disc, proteins exhibit the same doublet distribution across the ID. N-RAP is a nebulin-like repeat protein with five nebulin super repeats suggesting it binds to actin/tropomyosin filaments (Luo et al. 1997; Zhang et al. 2001). It may replace the Z-disc protein, nebulette, at the TrJ. Another protein with the doublet spacing is αII spectrin which has been localised at the tops (peaks) of the ID folds (Fig. 3.4c) (Bennett et al. 2006; Wilson et al. 2014). This suggests that there is a membranous region associated with the TrJ and that there is a close structural and functional connection between them. A protein that may link between the spectrin-rich membrane folds and the myofibrillar TrJ is filamin C which exhibits the doublet distribution (van der Ven et al. 2000). Filamin C is known to bind between membrane proteins, sarcoglycans, present at the ID and the ID proteins, N-RAP and Xin (van der Ven et al. 2006).

The ID Thin Filaments Within the ID folds themselves, there is evidence that the actin isoform in the thin filaments is not cardiac α-actin (Bennett et al. 2006) but possibly β-actin (Benz et al. 2013). Other proteins have been associated with these filaments in the ID (Fig. 3.6). One is formin, the protein that aids barbed end linear actin polymerisation (Randall and Ehler 2013); the muscle specific FHOD3 is strongly represented at the ID (Iskratsch et al. 2010), and the ubiquitously expressed FHOD1 has been shown to interact with α-catenin (Kobielak et al. 2004). The ID formins may therefore be located at the CJ and involved in the elongation of thin filaments there. MENA/VASP also has an important role in actin polymerisation. It interacts with αII spectrin and has been found in the ID (Eigenthaler et al. 2003; Benz et al. 2013). Non-muscle myosin II is also located in the ID (Takeda et al. 2000).

3.5 The ID/Transitional Junction in Longitudinal Growth

3.5.1 Variation of ID Amplitude

We have seen that the increase in length of cardiomyocytes is likely to occur at the end of the cells, at the ID. An interesting feature of the ID is that the amplitude of

the membrane folds varies (Figs. 3.2 and 3.3). Measurements of its amplitude show that it can vary from 20 nm to 2 μm with an average of 0.5 μm (Wilson et al. 2014). A myocytes 100 μm in length could therefore grow by about 1–2 % simply by increasing the ID amplitude. The heart grows as a result of local pressures to pump more blood. This is likely to be a small incremental effect over time so the ability of the cells to slowly change their length will be important. Equally so will be the ability to reverse the process when demands on the heart are reduced (Frenzel et al. 1988).

Increase in the ID amplitude involves both the elongation of the thin filaments and an increase in the surface area of the ID membrane. As described above, proteins that aid thin filament length extension, formins and MENA/VASP, are present in the ID. As the thin filaments extend the membrane also extends. In a normal muscle the ID amplitude is on average ~0.5 μm. A crude estimate suggests that the area of the ID membrane at this amplitude is about 1.6× that at its smallest amplitude. An increase in amplitude to 2 μm involves a ~5-fold increase in membrane area. Perhaps not surprisingly, high amplitude IDs are often the site of multiple vesicles (Fig. 3.3c). Both coated vesicles and calveolae have been previously described at the ID (Fawcett and McNutt 1969; McNutt and Fawcett 1969).

At higher amplitudes, there is a difference in the appearance of the CJ regions. At low amplitude the CJ covers essentially all the membrane slopes (Fig. 3.3a). As the amplitude increases, the CJ seems to fragment and smaller lengths of plaque are seen (Fig. 3.3b, c). This is perhaps not surprising since there are a fixed number of thin filaments coming from a sarcomere. We might assume that a single thin filament will require a given amount of plaque complex for a firm attachment to the membrane. Consequently, a smaller proportion of the membrane of high amplitude IDs will be needed for this function. As a result of the plaque breakup, there is now a significant amount of 'bare' membrane which could lead to an increase in signalling domains.

3.5.2 Sarcomere Insertion

It is clear that there is a natural limit to the amplitude of the ID folds. They rarely grow longer than 2 μm. This corresponds to one sarcomere length and suggests that at this point, a new sarcomere could be inserted. Evidence for this comes from the work of Yoshida et al. (2010). Working with a model of volume overload by arterio/venous fistula in rabbits, they showed that there was hypertrophic growth and that the myocytes grew every 2 days by two sarcomeres. In the course of this time there was change in the ID morphology. In particular, starting from a normal ID amplitude the ID grew more convoluted until it reached a size of ~2 μm. It then returned to its original size. During this time it went through several morphological stages one of which the authors attributed to the insertion of a sarcomere on each side of ID. On removal of the overload the process reversed.

Wilson et al. (2014) have looked for evidence for this happening during normal growth. Since the normal rate of growth in the mouse heart is something like one sarcomere a week and it has been shown that sarcomeres can be inserted in neonatal rat cardiomyocytes at the rate of 1 per hour, it might be a rare event to capture in normal growth. In high amplitude folds in IDs, sarcomeres are seen occasionally (Fig. 3.7b). Furthermore, it was sometimes possible to see isolated A-bands, bundles of thick filaments, or single filaments within these folds (Figs. 3.3c and 3.7a). Interestingly there was no Z-disc associated with these partial structures. They were reminiscent of the nascent filament assemblies described at the ends of skeletal muscle fibres near the MTJ in young rapidly growing animals or after stretch (Williams and Goldspink 1971; Dix and Eisenberg 1990).

Since the long folds are symmetrical with respect to the neighbouring cells the insertion of sarcomeres on both sides of the ID are possible. But it is not clear that both fibrils that meet at the ID will grow.

3.5.3 Mechanism of Sarcomere Addition

These observations suggest a possible mechanism of ID growth and sarcomere addition in the ID (Fig. 3.7c) (Wilson et al. 2014). The TrJ is already a proto Z-disc containing many of the proteins necessary for Z-disc formation. In addition, there is continual turnover of sarcomeric proteins throughout the myocyte so the materials would be available (Willis et al. 2009). When the ID amplitude reaches a length of ~2 μm, thick filaments begin to appear in the ID fold. The proteins could be made in the ID region since ribosomes are found here or brought in the vesicles observed in the long ID folds. Alternatively, thick filaments could be transported in from elsewhere along microtubules as previously reported (Pizon et al. 2002). It certainly seems that the thick filaments are organised into bundles at the ID. This would involve the cooperation of M-line proteins, titin, myomesin and obscurin (Van der Ven et al. 1999). The new Z-disc based on the TrJ would require severing the old thin filaments at the TrJ and production of a new set of thin filaments of opposite polarity. Horowits and his colleagues have shown that N-RAP is involved in Z-disc formation (Manisastry et al. 2009). They suggest that dimers of N-RAP could lead to antiparallel organisation of thin filaments at nascent Z-discs (Crawford and Horowits 2011). The presence of N-RAP at the TrJ could therefore facilitate the formation of a new Z-disc there. As the Z-disc matures N-RAP apparently dissociates presumably to make way for nebulette together with the other Z-disc proteins, telethonin and FATZ. Telethonin, in particular, is known to be one of the last proteins to appear at the maturing Z-disc.

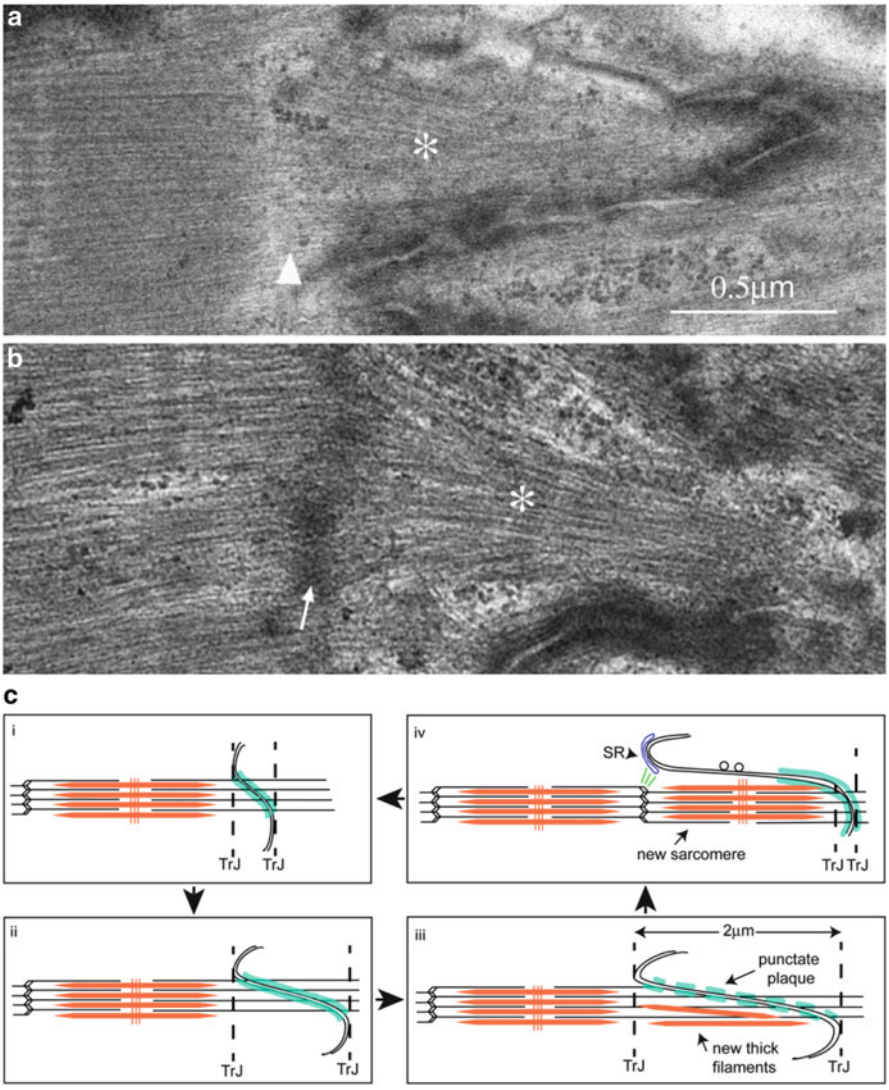

Fig. 3.7 Evidence for sarcomere insertion at the ID. (**a**) In mouse heart an A-band (*asterisk*) is seen within the fold of an ID but there is no Z-disc (*arrowhead*) separating it from its neighbouring sarcomere. (**b**) The insertion of a sarcomere (*asterisk*) in a fold in the ID complete with a Z-disc (*arrowed*) in human heart. (**c**) Diagram to show stages of new sarcomere formation in the heart. (i) ID with small amplitude. (ii) ID folds increase in amplitude to allow cell growth and CJ plaque (shown in *turquoise*) becomes punctate (iii). (iii) At 2 µm, thick filaments and (iv) whole sarcomeres are incorporated within a membrane fold. Vesicles are found on long stretches of uncoated membrane. The new Z-disc is associated with the SR at the top of the fold that may bud off to become a t-tubule. A new TrJ is established at the end of the new sarcomere. Parts of figure taken from Wilson et al. (2014) with permission

3.5.4 SR Extension and T-Tubule Addition

Inserted sarcomeres are surrounded by long tongues of membrane which would need to break up and be reabsorbed (Yoshida et al. 2010). It has been seen that peripheral junctional SR is often present at the top of the ID folds (Fig. 3.3b). Also, there are membrane associated proteins such as spectrin and myofibril/membrane bridging proteins such as filamin which are also found within the myocyte at T-tubule/Z-disc locations (Kostin et al. 1998). Possibly the tops of the ID folds together with the associated SR are involved in the formation of new t-tubules and SR at the new Z-disc (Fig. 3.7c).

The mechanism of sarcomere insertion and cell growth described here is simplistic and takes no heed of the myriad of factors that must control this process. In particular, what is the signal that indicates that a new sarcomere should be formed? There is now a considerable literature describing the continuous turnover of sarcomeric proteins and the dynamic processes that influence muscle structure (Willis et al. 2009). Much thought is being given to how the forces generated within cells are continuously monitored by, for example, the complex structures of the Z-disc and M-line, and how the organisation is modified as a result (Gautel 2011). Undoubtedly, similar mechanisms will apply in the ID. The forces that act on the ID membrane and the junctional proteins will change as the membrane folds get longer leading to protein modifications, such as phosphorylation. In addition, the increase in non-junctional membrane will support an increase in non-junctional membrane proteins and signalling complexes, changing the balance of influences in this region which may accelerate sarcomerigenesis.

3.5.5 Lateral Growth of the ID

Little is known of the mechanism whereby the width of myocytes increases. In in vitro observations on NRCs, new fibrils seem to form at the plasma membrane suggesting that cells grow at their lateral edges. However, adult cardiomyocytes tend to overlap with several cells at their ends so that lateral growth in one cell will correspond to central growth of another. In this case the cells may expand laterally by increases in width of the myofibrils themselves. In the heart the myofibrils are not uniform in thickness from one end of the cell to the other as skeletal muscle fibrils tend to be. Rather they anastomose; they branch and recombine throughout the cell. This might allow for easier changes in width.

The idea of lateral myofibrillar growth within a cell agrees with the observations on the size of ID steps. In young cells the lateral width of ID treads is small (Fig. 3.2a) whereas in wider adult cells the tread width is much greater (Fig. 3.2b, c) (Wilson et al. 2014).

3.6 The ID in Disease

Vascular disease of the heart resulting in heart attack and subsequent ischemia leads to remodelling and structural changes in cardiomyocytes in the vicinity of the lesion, but cells remote from the injury are not affected (Smith et al. 1991). Such remodelling includes repositioning of gap junctions to a more lateral position. This appears to relate to the propensity of the heart to revert to a more flexible neonatal state when stressed. Another example of this is the re-expression of the embryonic form of the M-band protein, EH-myomesin, in dilated cardiomyopathy (DCM) (Schoenauer et al. 2011). Heart diseases other than ischemia, such as hypertrophic cardiomyopathy (HCM), DCM, afibrillar right ventricle cardiomyopathy (AVRC) and Brugada syndrome, have a more global effect on the structure and/or the operation of the cardiomyocytes and their organisation. These may relate to the ID. Of the large number of proteins that have been associated with the ID, many are structural or signalling molecules. Others have enzymatic or regulatory roles that may have an indirect effect on the ID function. For many of these proteins, there is evidence that mutations or changes in expression lead to disease (Supplementary data Estigoy et al. 2009). The symptoms of many heart diseases overlap, for example, arrhythmias, hypertrophy, dilation and fibrosis, and mutations in the same protein can lead to different phenotypes. However, general categories of disease and their relationship to ID domains are outlined below and in Table 3.1.

3.6.1 Hypertrophic Cardiomyopathy

Hypertrophy of the heart may be a benign and beneficial effect of exercise, especially in elite sportsmen (Maron and Pelliccia 2006). However, other factors, both genetic and physical, can contribute to chronic concentric hypertrophy and lead to congestive heart failure (Table 3.1). In this disease the ventricular wall thickens, growing into the ventricular chamber and reducing the volume of blood that can be pumped at each stroke during systole. The cause of the disease can be pressure overload due, for example, to aortic restriction. Alternatively, it arises with age due to a genetic cause. Where the mutation has been identified it has been found often to be associated with the sarcomeric proteins of the myofibrils (Maron and Maron 2013). Two major contributors are myosin binding protein-C and myosin.

The size of the cardiomyocytes increases in hypertrophy. Observations point to increased cross-sectional area but little change in length (Gerdes 2002). The ID is not usually implicated but in some cases changes are observed. As in ischemia the gap junctions have been observed to relocate to a more lateral position on the cell surface, moving away from the desmoplakin-rich ID (Sepp et al. 1996). In obstructive hypertrophy, a loss of vinculin at the ID but not at the Z-disk level has been noted (Vasile et al. 2006). In the golden hampster model of hypertrophy, a mutation

Table 3.1 Heart disease and effects on the ID

Disease	Structural changes at ID	General cause	Known protein mutations/loss
HCM Concentric hypertrophy, congestive heart failure	No obvious changes Movement of gap junctions[a]	Pressure overload (during systole); mutations or loss in sarcomeric proteins	Mutations/insufficiency in titin, MyBP-C, myosin[b]
DCM Excentric hypertrophy, ventrical wall thinning, fibrosis	Bigger amplitude folds, loss of risers and gap junctions, one sided sarcomere addition and disordered mitochondrial organisation[c]	Volume overload (during diastole); mutations of AJ proteins Increase in AJ proteins[d]	Loss of MLP,[e] vinculin[f] β-catenin over-expression[g] Mutations in vinculin, β-catenin and α-catenin[h]
ZASPopathies	DCM and ventricular non-compaction		Mutations in ZASP[i]
AVRC Atrial fibrillation, calcification and fatty fibrosis	Order maintained, loss of gap junctions and desmosomes[j]	Loss/mutation of desmodomal proteins[h]	Mutations in desmosomal cadherins, desmoplakin, plakoglobin[k]
Desminopathies	Breakdown of end-to-end connections, ID disorder[l]	Loss/mutation of desmin Desmin accumulations	Desmin ko[l], mutations in C-terminal dimerisation domain[m]
Brugada syndrome Channelopathies	Not known if changes occur	Mutation in Ca^{2+} channel proteins and membrane association	$NaV_{1.5}$, ankyrin-G[n] 4.1R[o]

References: [a]Sepp et al. (1996); [b]Maron and Maron (2013); [c]Wilson et al. (2014); [d]Perriard et al. (2003); [e]Arber et al. (1997); [f]Zemljic-Harpf et al. (2007); [g]Hirschy et al. (2010); [h]Sheikh et al. (2009); [i]Sheikh et al. (2007); [j]Basso et al. (2006); [k]Lahtinen et al. (2011), Gehmlich et al. (2012); [l]Thornell et al. (1997), Milner et al. (1996); [m]Dagvadorj et al. (2004), Goldfarb et al. (2004); [n]Mohler et al. (2004); [o]Stagg et al. (2008)

in γ-sarcoglycans results in a general loss of sarcoglycans and changes of morphology of the ID accompanied by an increase of β-catenin (Masuelli et al. 2003).

3.6.2 Dilated Cardiomyopathy and the Adherens Junction

Hypertrophy is often an initial phase in dilation and heart failure. In this case however the cause is eccentric hypertrophy where the ventricular chamber increases in diameter. Subsequently, the ventricular wall thins and loses the ability to expel sufficient blood. There are extensive morphological changes in the ventricular walls including significant fibrosis. In addition, the cardiomyocytes exhibit myofibril disorder, mitochondrial reorganisation and alterations in ID morphology.

DCM can be considered a disease of diastole resulting from volume overload. Too great a volume of blood in the ventricular cavities will extend the relaxed cells beyond their resting sarcomere length which would be a signal for increased longitudinal growth. Indeed, observations of cell size in DCM support this idea and increased length and width are generally seen (Beltrami et al. 1995; Gerdes and Capasso 1995; Poole-Wilson 1995). There has been some debate as to whether the increase in length could account for the thinning of the ventricular walls (Gerdes 2002). An alternative idea is that the cells can slip past each other (Beltrami et al. 1995). In the case of one mouse model for DCM, a knockout of muscle LIM protein (MLP), there is no overall increase in the average cell length (Leu et al. 2001), so some kind of slippage is indicated. It seems likely that the fibrotic growth between groups of cells would allow this, so that, in general, both growth and slippage may occur.

Morphological Changes in the ID The disordered morphology of the ID in DCM together with protein changes (see below) have prompted Perriard and colleagues to suggest that DCM is a disease of the ID (Perriard et al. 2003). In the light microscope the ID appears thicker and more broken up and the isolated cardiomyocytes have brush-like ends instead of the sharply stepped outline of normal cells. In the electron microscope the ID folds are more exaggerated, and greater interdigitation of neighbouring cells is apparent (Thornell et al. 1997; Ehler et al. 2001; Ferreira-Cornwell et al. 2002).

In a few cases these changes have been looked at in more detail, in particular, in the MLP knockout mouse and the mouse expressing constitutively active β-catenin (cΔex3) (Wilson et al. 2014). In many areas the cells seem normal with the order of the myofibrils more or less maintained (Fig. 3.8). In these areas the measurements of the ID fold amplitude show that although the range stays the same as controls (0.2–2 μm), the average increases significantly with many large amplitude IDs and more indications of sarcomere insertion. Elsewhere there are signs of a loss of control at the ends of the cells. The ID steps become less sharp, and there is a slide from one level to the next. As a result the gap junctions are less clearly associated with the risers and only small areas incorporated into the folded regions are seen. In addition there are places where extra sarcomeres are inserted across part of a cell (Fig. 3.8a). The result of these changes is that the myofibrils in the neighbouring myocytes are not axially aligned and the tension exerted is not strictly longitudinal, but spread laterally to give forces leading to branched cells (Ehler et al. 2001). Further evidence that the disease affects the growth at the end of the cells is found in regions close to the ID where sometimes a proliferation of sarcomeres without clear Z-discs occurs giving a streaming appearance accentuated by long strands of ID membrane (Fig. 3.8b). This may only affect one cell, its neighbour looking normal. These observations are generally supported by images of IDs in human DCM samples (Wilson et al. 2014).

The distribution of mitochondria is also affected in DCM. In the desmin null mouse heart, clumping of the mitochondria is accompanied by loss of function (Milner et al. 2000). Loss of mitochondrial function has also been reported in the

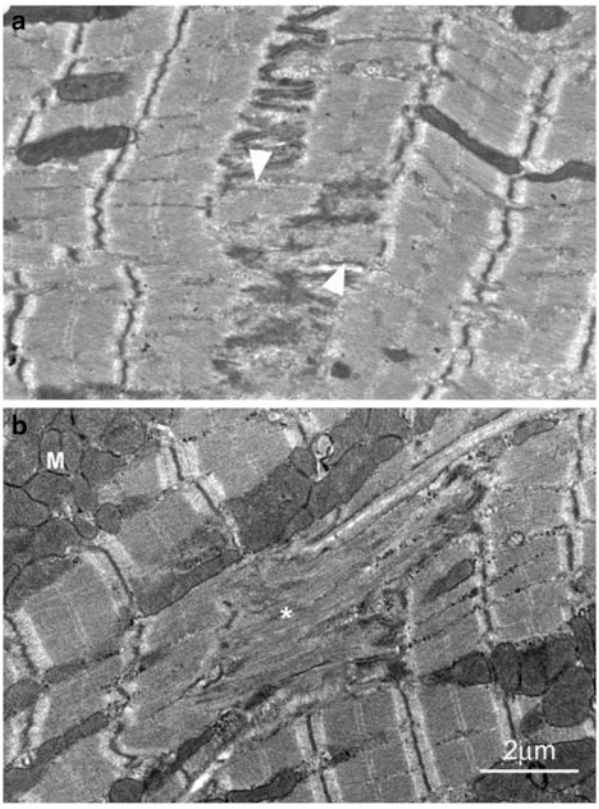

Fig. 3.8 Electron micrographs of mouse heart from MLP KO mice exhibiting DCM. (**a**) An ID with large amplitude folds. In addition, extra sarcomeres are inserted over part of the width of the myocyte (*arrowheads*). Note also the lack of mitochondria within a sarcomere of the ID. (**b**) Region showing loss of regular sarcomere structure near the ID (*asterisk*) in one myocyte surrounded by well-ordered regions. *M*, a clump of mitochondria. (**b**) taken with permission from Wilson et al. (2014)

MLP null mouse heart (van den Bosch et al. 2005; Wilding et al. 2006). In these hearts mitochondria are less regularly sized compared to controls, and the columns between the myofibrils are not so uniform. In addition, there are subsarcolemmal clumps (Fig. 3.8b). Even in cells that at first impression look 'normal', there is a subtle difference in the mitochondria distribution. They are not so numerous close to the ID folds as in the body of the cell (Wilson et al. 2014).

This loss of synchrony in mitochondria placement with sarcomere growth may be related to faults in the mechanism of t-tubule addition. It has been shown that there is loss in the regularity of disposition, as well as amount, of t-tubules in cardiomyocytes from failing hearts, including DCM (Lyon et al. 2009). This results in defects in calcium signalling and loss of function.

Protein Changes in DCM Although the cause of DCM may be a physical factor such as chronic mitral regurgitation, in many cases it is genetic. Significantly, mutations have been identified in several AJ proteins (Estigoy et al. 2009). Mutations in vinculin, β-catenin and α-T-catenin have been identified in idiopathic and familial DCM and the results supported by mouse models (Table 3.1) (see Sheikh et al. (2009) for a discussion).

A further observation is that in some cases DCM has been associated with changes in the expressed level of ID proteins. In particular, several AJ proteins are increased in the MLP KO mouse (Ehler et al. 2001). This is also the case for the AJ protein, β-catenin, in mice overexpressing tropomodulin (Ehler et al. 2001) and a conditional knockout for αE catenin (Sheikh et al. 2006). In the case of human IDCM, β-catenin and N-cadherin also appear to be upregulated (Perriard et al. 2003; Wilson et al. 2014). Interestingly, little change has been observed in the level of desmosomal proteins suggesting the possibility that the correct balance of AJ and desmosomal components in the CJ is necessary for correct function.

The level of the transitional junction protein, N-RAP, is also increased in DCM specimens (Ehler et al. 2001; Perriard et al. 2003). Indeed, it is the first protein to show an upregulation during heart development in the MLP KO mouse compared to controls, and it is suggested that it could be used as a marker for DCM (Ehler et al. 2001).

Another TrJ protein, ZASP, has also proved to have a major involvement in heart disease and has spawned the term 'Zaspologies' (Sheikh et al. 2007). Eliminating ZASP is embryonically lethal in mouse. Mutations in the human protein lead to non-compaction in the LV (INLVM) and DCM (Vatta et al. 2003; Xing et al. 2006). One mutation in its third LIM domain increases the affinity of ZASP for phospho-kinase C (Zhou et al. 1999). This interaction is thought to have a regulatory role in sarcomerogenesis (Mansour et al. 2004).

3.6.3 Arrhythmic Right Ventricle Cardiomyopathy and the Desmosome

Though the demosomal proteins in DCM appear to be unaffected, there is a disease, arrhythmic right ventricle cardiomyopathy (ARVC), where changes in desmosomal proteins occur. Here the ventricular myocytes, most obviously those of the right ventricle, are targeted and replaced by fibrotic and adipose tissue. This results in dilation, tachychardia and sudden death. Mutation in or loss of several desmosomal proteins have been found to lead to this disease (Table 3.1) (Sheikh et al. 2009). Structural studies on human ARVC samples show no loss of order at the ID but a reduction in the number of desmosomes and gap junctions (Basso et al. 2006). Mutations in desmosomal cadherins, desmoplakin and plakoglobin result in a depletion of plakoglobin at the ID (Lahtinen et al. 2011; Gehmlich et al. 2012). Clearly the reduced function of desmosomes would weaken the links at the ID between neighbouring cells and the loss of desmosomal proteins particularly plakoglobin will change the character of the CJs (Li and Radice 2010). Furthermore, it will affect the disposition of desmin filaments and their role in maintaining the integrity of the myocyte.

Several human heart diseases have been related to mutations in desmin. Symptoms of these so-called desminopathies affect many organs but in the heart lead to

dilation and arrhythmias. In some cases the mutations lead to accumulation of desmin (Arbustini et al. 2006). Other mutations are C-terminal and prevent dimerisation or disrupt formation of intermediate filaments (Kaminska et al. 2004; Bar et al. 2007). The desmin null mouse is viable but has a tendency to die during exercise (Capetanaki et al. 1997; Capetanaki and Milner 1998). The heart exhibits calcification and compromised systolic function (Milner et al. 1999). The myofibrils near the ID are disordered and the ID structure is disrupted with few CJs and gaps between the cells (Thornell et al. 1997). Clearly, the absence of desmin filaments reaching as they do throughout the myocyte will have a profound effect on the ability of the cell to transmit and absorb mechanical stress.

3.6.4 Gap Junction Disorders

The heart is very dependent for coordinated function on the transfer of the action potential from cell to cell via the connexin43 connexons in the gap junctions. Mutation or loss of connexin43 leads, as expected, to arrhythmias and atrial fibrilation (Severs et al. 2004). Many of the DCM phenotypes, including those described above, which result in AJ and desmosomal protein loss also lead to a downregulation of connexin43 and a reorganisation of the gap junctions (Ehler et al. 2001; Wilson et al. 2014).

3.6.5 Brugada Syndrome and Non-junctional Domain Cardiomyopathies

So far we have concentrated on the intercellular junctions at the ID and the diseases that result in mutations of the proteins involved. The cytoskeletal complex that fills in the gaps is that associated with spectrin. One of those proteins is ankyrin-G whose presence is important for the localisation of the sodium voltage channel $NaV_{1.5}$ (SCN5A) at the plasma membrane, in t-tubules and at the ID (Mohler et al. 2004). Mohler and colleagues found that a mutation in $NaV_{1.5}$ which prevents it from binding to ankyrin-G and localising correctly leads to changes in the excitation pattern of the heart. There is a long QT period which has been associated with sudden cardiac death, in particular, Brugada syndrome. A recent study of channelopathies related to sudden cardiac death has associated over 50 % of the cases with variations in the SCN5A channel (Wang et al. 2014).

Ankyrin-G is a multifunctional protein able to bring together a number of membrane proteins (Bennett and Healy 2009). Protein 4.1R, another spectrin binding protein at the ID, has a similar functionality and could work in collaboration with ankyrin-G in maintaining and regulating many of the non-junctional proteins in a signalling network (Baines et al. 2014). For example, the sodium calcium exchanger and Na^+K^+ATPase also have a presence at the ID and both

partially overlap with 4.1R there (Pinder et al. 2012). Furthermore, a loss of one of the 4.1R heart isoforms in mouse leads to bradycardia and a reduction in the sodium calcium exchanger current density (Stagg et al. 2008). These observations indicate that the spectrin associated cytoskeleton is involved in maintaining both structure and calcium homeostasis in the heart, in particular at the ID.

3.7 Conclusion

In this chapter the cytoskeletal elements underlying the functional domains found at the ID have been described. The domains have been considered separately so as to emphasise the multiple functions carried out by the ID. The heart is not a fixed structure. It must continually adjust to demands, both of increase and loss of capacity. The intercalated disc, the region where one cell interacts directly with its neighbours physically, electrically and chemically, is where changes in these signals will be first detected and compensatory actions undertaken. The response of the domains and their cytoskeletal elements to these signals is complex. The interrelationship between them has not been tackled here. It is only now starting to be unravelled.

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Chapter 4
Dynamics of Actin in the Heart: Defining Thin Filament Length

Christine A. Henderson and Carol C. Gregorio

Abstract Cardiac sarcomeres are composed of overlapping actin-thin filaments and myosin-thick filaments. Efficient muscle contraction is dependent on the proper overlap of thick and thin filaments; therefore filament lengths are tightly regulated. The actin-thin filament is a polar structure with a distinct barbed end (at the Z-disc) and pointed end (at the M-line). Capping proteins bind to and regulate thin filament lengths at the ends of the filament. For instance, tropomodulin 1 (Tmod1) caps (blocks the incorporation and dissociation of G-actin) the pointed end of the thin filament, while CapZ caps the barbed end. Leiomodin 2 (Lmod2), a close family member of Tmod1, also binds to the pointed end but it elongates thin filaments. Lmod2 and Tmod1 compete to bind to the pointed end, which fine tunes thin filament length. While capping/elongation proteins play a critical role in length regulation, other proteins have been proposed to contribute. For instance, tropomyosin plays a vital role in stabilizing the thin filament. In addition, cyclase-associated protein 2 (CAP2) has been proposed to regulate thin filament length by severing filamentous actin and sequestering globular actin. Actin-monomer-binding proteins may also work in conjunction with capping proteins to regulate length. Due to the critical role these proteins play in maintaining lengths, it is not surprising that alterations or mutations in many of these proteins result in the development of human cardiomyopathies. Current research is focused on dissecting the role that alterations in thin filament length have in both normal heart function and the development of disease.

4.1 Introduction

Actin is one of the most abundant and ubiquitous proteins in eukaryotic cells. As a key cytoskeletal component, actin plays a significant role in many important cellular processes including regulation of cell shape, cell division and cytokinesis,

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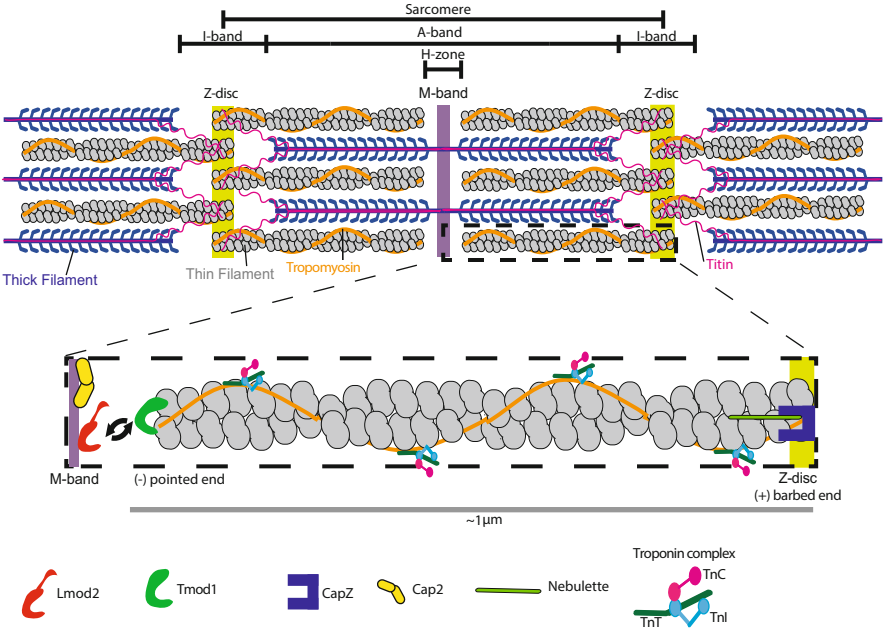

Fig. 4.1 Cardiac Sarcomere. The sarcomere is the basic contractile unit of muscle. The lateral boundaries of a sarcomere are defined by protein-dense Z-discs. The I-band is the region surrounding the Z-disc in which actin-containing thin filaments do not overlap with the myosin-containing thick filaments. The A-band comprises the region extending the entire length of the thick filaments, while the H-zone is the region in the center of the sarcomere, devoid of thin filaments. The enlarged region depicts the actin-thin filament. CapZ caps the barbed end of the thin filament, while Tmod1 caps the pointed end. Lmod2 binds to the thin filament pointed end and likely competes with Tmod1 to fine-tune thin filament length. Cap2 binds in the region of the M-band in adult myofibrils. One tropomyosin (*orange*) associates with every seven actins and a troponin complex. Nebulette localizes to the Z-disc

adhesion, establishment and maintenance of cell junctions, as well as cell motility. Frequently, these activities are coordinated by extensive interactions of actin with cell membranes. The dynamic properties of the actin cytoskeleton are regulated by a plethora of identified actin-binding proteins, which allow actin to assemble and dissemble into numerous conformations including bundles and networks. The presence of actin cytoskeletal-based assemblies is vital in many diverse cell types, from the stereocilia in the cochlea necessary for hearing to the actin cytoskeleton in neurons vital to learning and memory. In striated muscle (cardiac and skeletal) actin is a core component of the contractile machinery. Actin comprises an impressive 20 % of the total mass of striated muscle with the majority organized into the sarcomeres, the individual contractile units (Fig. 4.1).

In order for actin to function efficiently in muscle contraction, it must be organized within the sarcomere to the level of single molecules. The sarcomere is composed of numerous structural and regulatory proteins, with the three prominent

cardiac filaments consisting primarily of actin (thin filaments), myosin (thick filaments), and titin. The lateral boundaries of the sarcomere are defined by protein-dense structures known as Z-discs. Actin-thin filaments are precisely organized in striated muscle with the filament barbed ends inserted in the Z-disc and their pointed ends extending out to the center of the sarcomere (M-line) where they overlap with myosin (motor protein)-containing thick filaments. The terminology of barbed and pointed end came from the arrowhead morphology of myosin S1 fragments bound to actin filaments in vitro. Titin is the largest protein known in humans. One titin molecule spans half of a sarcomere and functions as a molecular spring that provides the passive tension in muscle.

The numerous contractile and regulatory proteins that constitute the sarcomere are precisely orchestrated to generate the force necessary for muscle contraction. In fact, approximately 60 years ago the sliding filament theory of muscle contraction was first proposed (Huxley and Niedergerke 1954; Huxley and Hanson 1954; Huxley 1985), which states that myosin remains stationary in the sarcomere and during contraction the thin filament slides past myosin to generate tension. Remarkably, Huxley's sliding filament theory was proposed prior to the discovery of most of the regulatory molecules involved in muscle contraction, and while it has been refined over the years, this model is the basis for current models of muscle contraction (reviewed in Cooke 2004; Szent-Györgyi 2004; Williams 2011).

4.2 Major Components of the Thin Filament

The thin filament, while composed of a core of filamentous actin (F-actin), also contains many regulatory proteins that are necessary for efficient contraction. Sarcomeric F-actin polymerizes into a helical filament with one tropomyosin coil–coil associating with every seven actin molecules. Associated with each tropomyosin coil–coil is a troponin (Tn) complex, which consists of TnI (inhibitory), TnT (tropomyosin-binding), and TnC (calcium-binding) (Fig. 4.1). The regulation of contraction is controlled by calcium and the tropomyosin/troponin complexes (reviewed in Kobayashi and Solaro 2005). These regulatory thin filament proteins are also hotspots for numerous mutations that have been linked to both dilated and hypertrophic cardiomyopathies (for review, see Tardiff 2011).

4.3 Thin Filament Length Regulation

Strict regulation of uniform thin filament lengths in striated muscle is necessary for proper muscle contraction. Interestingly, thin filament lengths vary based on species (Burkholder and Lieber 2001) and even between different types of muscles (Granzier et al. 1991; Kruger et al. 1991). In addition, thin filament lengths can vary between different regions of the heart. For instance, the stiffer papillary muscle has

a narrower range of thin filament lengths when compared to the less stiff atria muscles (Robinson and Winegrad 1977), indicating thin filament lengths may be fine-tuned based on the requirements (e.g., load) of specific muscle types. In cardiac muscle, thin filament lengths have a broader range of lengths when compared to skeletal muscle (Page and Huxley 1963; Robinson and Winegrad 1977; Littlefield and Fowler 2002; Burgoyne et al. 2008). The implications of a broader range of lengths in cardiac muscle is an interesting, yet unexplored, field of research.

Thin filaments are not static structures. Uniform thin filament lengths are maintained even with continuous dynamic exchange of actin monomers at the ends of the filament. Based on the 3–10 day half-life of the major components of the thin filament (actin, troponin complex, and tropomyosin) (Martin 1981), it has been estimated that actin monomers can move in and out of the cardiac thin filament an average of over 100 times in their lifetime (reviewed in Littlefield and Fowler 1998). A class of molecules known as capping proteins contributes to the maintenance of thin filament lengths. In vitro experiments have demonstrated that actin monomers are strongly inhibited from exchanging into the thin filament when the filaments ends are bound by capping proteins. The two primary striated muscle capping proteins that have been identified are tropomodulin 1 (Tmod1) and CapZ, which bind to the pointed and barbed end of the thin filament, respectively (Casella et al. 1986; Weber et al. 1994). Remarkably, although in live cardiomyocytes, CapZ and Tmod1 are transiently bound to the thin filament, allowing monomer incorporation at both pointed and barbed ends, the lengths of the filaments are not altered (Littlefield et al. 2001). Therefore, the dynamics of capping proteins are critical in determining the length of thin filaments.

4.4 Capping Proteins: Tmod and CapZ

Tmod1 plays a key role in regulating and stabilizing thin filament pointed ends by blocking monomeric, globular (G-actin) actin incorporation and dissociation in vitro (Weber et al. 1994). The Tmod family of proteins contain four isoforms encoded by separate genes (Tmod1-4), with Tmod1 being the primary isoform in the heart (Fowler 1996). Tmod1 is expressed in multiple tissues and was originally described in red blood cells as a tropomyosin-binding partner (Fowler 1987). In striated muscle, Tmod1 binds to thin filament pointed ends via its two actin-binding domains and two tropomyosin-binding domains (Babcock and Fowler 1994; Weber et al. 1994; Kostyukova et al. 2000, 2001, 2006; Fowler et al. 2003). Tmod1 is necessary for embryonic development, demonstrated by genetically engineered Tmod1 null mice dying at E10.5; at death the embryos displayed numerous defects including failure of the heart to loop and aborted cardiac myofibrillogenesis (Chu et al. 2003; Fritz-Six et al. 2003). The heart was the primary defect in the Tmod1 deficient mice as cardiac-specific expression of Tmod1 rescued the embryonic lethality (McKeown et al. 2008).

Further insights into the role of Tmod1 in cardiac muscle were demonstrated by experiments that showed that when barbed-end incorporation of fluorescent rhodamine-actin was blocked (via CapZ), thin filament lengths were not changed while overexpression of GFP-tagged Tmod1 resulted in shorter thin filament lengths (Littlefield et al. 2001). Additionally, in isolated live cardiomyocytes, a higher ratio of rhodamine-actin incorporated into the pointed end compared to the barbed end. Together these results suggest that the pointed end was more dynamic than the barbed end. The consequences of altering thin filament lengths in vivo are not well studied and is an exciting area of active research.

As described above, thin filament lengths are highly dependent on the cellular levels of Tmod1. Other examples include, in neonatal rat cardiomyocytes, overexpression of Tmod1 resulted in shorter actin filament lengths and myofibril degeneration, while knockdown of Tmod1 resulted in longer actin filaments (Sussman et al. 1998a). Altering the levels of Tmod1 might change the exchange and/or dynamics of Tmod1 at the pointed end. For example, an increase in Tmod1 protein expression may change it from a dynamic to a stable cap, which could potentially explain the observed reduction in thin filament lengths upon overexpression. Additionally, when the interaction of Tmod1 with tropomyosin was blocked in isolated chick cardiomyocytes (via an anti-Tmod1 antibody), actin filament elongation occurred and cell beating was compromised (Gregorio et al. 1995), indicating loss of thin filament length control had a detrimental effect on contractile function.

Within the Tmod family are the leiomodin (Lmod) proteins. While the Tmod family has been studied for over 25 years (Fowler 1987), the Lmod proteins were identified over a decade later (Conley et al. 2001). Lmod has three isoforms encoded by separate genes: smooth muscle (Lmod1), cardiac/skeletal (Lmod2), and fetal/skeletal (Lmod3) (Conley et al. 2001). Lmod and Tmod proteins are approximately 40 % identical at the protein level (Chereau et al. 2008). Lmods differ from Tmods in that they do not contain a second tropomyosin-binding domain and have a C-terminal extension that, importantly, contains a third actin-binding site [Wiskott–Aldrich syndrome protein 2 (WH2) domain (reviewed in Yamashiro et al. 2012)]. Due to the presence of a third actin-binding site, Lmod2 has potent nucleation abilities in vitro (Chereau et al. 2008). Surprisingly, although similar in domain structure to Tmod1, currently there is no evidence that Lmod2 "caps" actin-thin filament pointed ends (Tsukada et al. 2010). However, when the unique C-terminal extension of Lmod2 is removed, actin filament capping activity is detected, indicating that the C-terminus of Lmod2 acts to blunt its ability to cap the pointed end (Tsukada et al. 2010). The functional properties of Lmod2 in vivo have not been reported.

While Lmod2 is the most well-studied Lmod isoform, little is known about the role of Lmod2 in actin filament assembly and regulation. However, like Tmod1, Lmod2 has been shown to bind to the pointed end of thin filaments in primary cardiomyocytes (Tsukada et al. 2010). Manipulation of Lmod2 levels also alters thin filament lengths. In fact, Lmod2 was the first mammalian pointed end elongation factor identified, as overexpression of Lmod2 displaced Tmod1 and resulted in

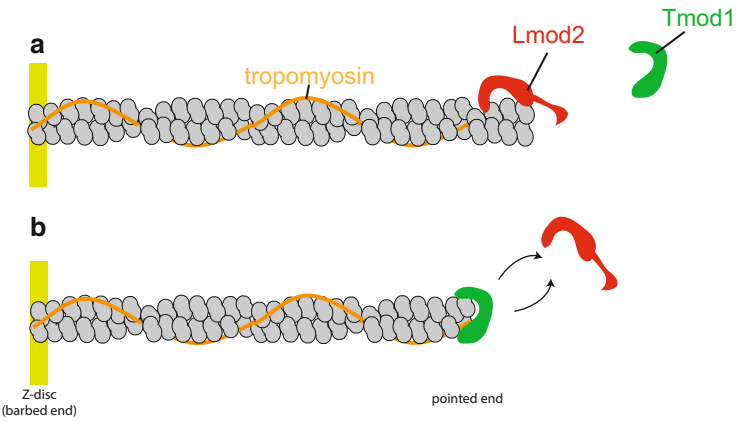

Fig. 4.2 Model for thin filament length regulation at the pointed end. Lmod2 and Tmod1 compete to bind to the thin filament pointed end and function to fine-tune thin filament length. (**a**) When Lmod2 is bound to the pointed end, Tmod1 cannot bind and the thin filament elongates. (**b**) Conversely, when Tmod1 binds to the pointed end, Lmod2 cannot bind and lengths are restricted. Lmod2 and Tmod1 exchange from the pointed end to maintain proper thin filament length. Figure modified from Tsukada et al. (2010)

longer thin filaments (Tsukada et al. 2010). In *Drosophila*, SALS (sarcomere length short), similar but not homologous to Lmod2, has a WH2-binding domain as well as a proline-rich domain and is localized to the pointed end. Like Lmod2, overexpression of SALS elongated thin filaments, while knockdown shortened thin filament lengths (Bai et al. 2007).

One current model is that Lmod2 and Tmod1 have an antagonistic relationship in which they compete for binding to the pointed end, thus contributing to the fine-tuning of thin filament lengths (Fig. 4.2). When Lmod2 is present at the pointed end, Tmod1 cannot bind and thin filaments elongate. Alternatively, when Tmod1 is present on the pointed end, Lmod2 cannot bind and lengths are reduced (Tsukada et al. 2010). Another model proposes that there are two populations of actin filaments: more stable core filaments associated with Tmod1 and more dynamic filaments associated with Lmod2 (Skwarek-Maruszewska et al. 2010; Tsukada et al. 2010). Further investigation is necessary to determine how Tmod and Lmod coordinate to regulate thin filament length.

Lmod2 binds to mature myofibrils and localization is dependent on the contractile state of the cardiomyocytes and a dynamic actin monomer population. Treatment of cardiomyocytes with blebbistatin (a myosin II inhibitor that reduces contractility) resulted in the loss of Lmod2 localization at the pointed end. Cardiomyocytes treated with Latrunculin B, which binds actin monomers and prevents their polymerization, also resulted in loss of pointed-end localization of Lmod2. Interestingly, Tmod1 localization was unaffected by blebbistatin and Latrunculin B treatment. These experiments suggest that the localization of Lmod2 (but not Tmod1) requires an actin monomer pool capable of polymerization (Skwarek-Maruszewska et al. 2010). Lmod2 likely plays an important role in the

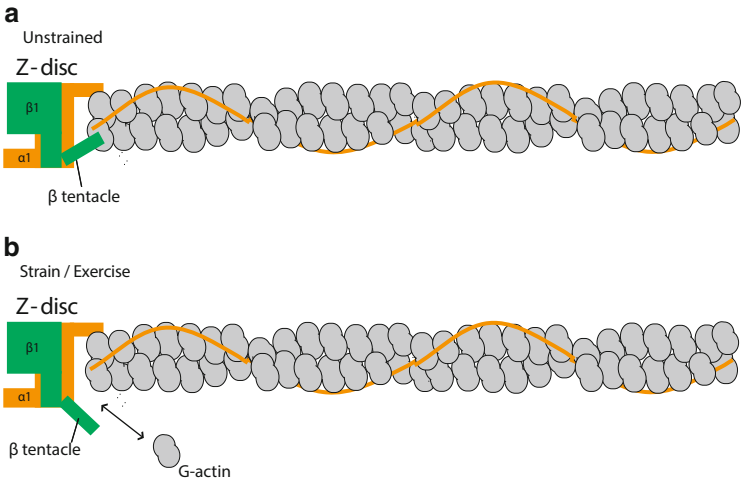

Fig. 4.3 Model of how CapZ interacts with actin at the Z-disc. (**a**) When unstrained, CapZ caps the barbed end of the thin filament. The CapZ α1 and β1 tentacles each bind to one actin, which decreases the off rate of actin (less dynamic). (**b**) Following cyclic strain (mimics exercise), CapZ β1 tentacle undergoes a putative structural change that results in increased actin dynamics. This figure was modified from Lin et al. (2013)

maintenance of thin filament lengths. However, functional studies of the physiological role of Lmod2 in vivo are necessary.

CapZ (also known as "capping protein") binds to the barbed end of thin filaments in striated muscle Z-discs and stabilizes the filaments (Isenberg et al. 1980; Casella et al. 1987; Caldwell et al. 1989). Capping protein is a heterodimeric protein composed of α and β subunits (Isenberg et al. 1980). Vertebrates have three α subunits encoded by three different genes and three β subunits that result from alternate splicing of a single gene (Hurst et al. 1998; Hart et al. 2000). Capping protein isoforms display tissue-specific distributions (Yamashita et al. 2003). The isoform α1β1 is the primary isoform that localizes to the Z-disc and is referred to as CapZ (Casella et al. 1987). CapZ is a highly dynamic protein which binds transiently to the thin filament and regulates polymerization and depolymerization of thin filaments (Wear et al. 2003).

The C-terminal extension of each CapZ α and β subunit (tentacles) interacts with one terminal actin molecule (Fig. 4.3a) (Yamashita et al. 2003). Binding of the β-tentacle to actin is critical for capping, as mutations in the β-tentacle increased actin dynamics while mutations in the α-tentacle did not significantly affect actin dynamics (Barron-Casella et al. 1995; Wear et al. 2003; Kim et al. 2010; Lin et al. 2013). In order for actin exchange to occur, the CapZ α-tentacle dissociates, with only the β-tentacle still attached (Yamashita et al. 2003).

Modest CapZ reduction in transgenic mice resulted in blunted protein kinase C (PKC)-mediated myofilament inhibition (Pyle et al. 2002, 2006). PKC inhibition is protective against the development of hypertrophy and CapZ reduction resulted in

increased baseline contractility and calcium sensitivity compared with wild-type mice (Gaikis et al. 2013), indicating CapZ may also be important in the hypertrophic response. In addition, modest CapZ reduction in mice is cardioprotective following ischemia-reperfusion injury (Yang and Pyle 2012). CapZ clearly has diverse biological functions, not only as a barbed-end capping protein but may also play a role in intracellular signaling and the cardiac hypertrophy response.

4.5 Stabilization of the Thin Filament

Tropomyosin is an α-helical protein that dimerizes in a head-to-tail fashion along every seven actin monomers and stabilizes the thin filament. Vertebrates have four tropomyosin genes (TPM1–TPM4) that generate numerous isoforms through alternative splicing: TPM1α is the predominant isoform in adult striated muscle (Schevzov et al. 2005; Dube et al. 2014). Tropomyosin is vital to cardiac function as mice deficient in α-tropomyosin are embryonic lethal at E9.5 (Blanchard et al. 1997). Thin filaments depolymerize more slowly when stabilized by tropomyosin (Broschat et al. 1989; Broschat 1990). Tropomyosin also stabilizes the thin filament by blocking depolymerization by the actin severing proteins cofilin (Nishida et al. 1984; Ono and Ono 2002) and gelsolin (Ishikawa et al. 1989; Khaitlina et al. 2013). In addition, the interaction of Tmod and tropomyosin enhances the efficiency of Tmod capping actin filaments in vitro (Weber et al. 1994, 1999; Gregorio and Fowler 1995). In other words, Tmod1 is a "leaky" cap in the absence of tropomyosin and can only partially inhibit polymerization and depolymerization (Weber et al. 1994, 1999; Kostyukova et al. 2005). In this regard, X-ray crystallography revealed a mechanism by which Tmod1 functions as a leaky cap. Specifically, one Tmod1 can bind to the thin filament pointed end through multiple distinct low affinity interactions with tropomyosin and actin; these weak interactions permit only part of Tmod1 to detach from the pointed end, allowing for actin exchange (Rao et al. 2014).

In skeletal muscle, the giant sarcomeric protein nebulin (~700–800 kDa) spans the length of the actin-thin filament, where it can interact with Tmod1 in the center of the sarcomere (McElhinny et al. 2001) and CapZ in the Z-disc (Wang and Wright 1988; Pappas et al. 2008). Nebulin knockout mice have shorter skeletal thin filament lengths and die within 2 weeks after birth (Bang et al. 2006; Witt et al. 2006; Gokhin et al. 2009). Nebulin was proposed to be a "molecular ruler" that specified thin filament lengths (for reviews, see McElhinny et al. 2005; Horowits 2006; Pappas et al. 2011). Notably, the molecular ruler hypothesis has been difficult to prove due to the large size of nebulin and its susceptibility to proteolysis. To directly test the ruler hypothesis, a shorter version of human nebulin called "mini-nebulin" was synthesized. Mini-nebulin contains all the unique C- and N-terminal regions of nebulin but has only 4 of nebulin's 22 super repeats (Pappas et al. 2010). Introduction of mini-nebulin into cultured primary skeletal myocytes

did not alter the lengths of the thin filaments. However, upon exposure to a depolymerization agent (Latrunculin A), thin filament lengths shortened to the length of mini-nebulin. This experiment supports the idea that nebulin plays an important role in stabilization of thin filaments and likely does not function as a strict ruler (Pappas et al. 2010). As such, current evidence suggests a two-segment model of thin filament length stabilization in skeletal muscle: (1) a core filament that is coated and stabilized by nebulin extending from the Z-disc to approximately 0.95 μm and (2) a nebulin-free region extending from 0.95 μm to the pointed end that is variable in length and regulated by Tmod1 via a nebulin-independent mechanism (reviewed in Gokhin and Fowler 2013).

While nebulin is highly abundant in skeletal muscle, it is a minor component in cardiac muscle (Kazmierski et al. 2003). Nebulin null mice present with no detectable cardiac dysfunction prior to premature death at 2 weeks old (Bang et al. 2006; Witt et al. 2006). Thus, the generation of a cardiac nebulin-specific knockout model in the future will assist in determining if nebulin has a role in thin filament length regulation in the heart.

A much smaller cardiac-specific nebulin-like protein, nebulette (encoded by a separate gene), localizes to the Z-disc (Moncman and Wang 1995; Millevoi et al. 1998). Nebulette is far less studied in comparison to nebulin. Nebulette also seems unlikely to act as a molecular ruler because it is only localized to the Z-disc, and it is too small to extend along the thin filament. Interestingly, however, knockdown of nebulette in cardiomyocytes resulted in shorter thin filament lengths (Moncman and Wang 2002), suggesting that the role of nebulette (like nebulin) in thin filament length regulation may include a stabilization mechanism. A nebulette knockout mouse has yet to be published.

4.6 Dynamic Equilibrium Between G-Actin and F-Actin

Another potential method of thin filament length control is the equilibrium between the pool of G-actin (globular actin) and F-actin (filamentous actin). Actively contracting sarcomeres have a high rate of actin turnover (Martin 1981). Therefore, proteins that bind or sequester G-actin likely contribute to thin filament length. Cyclase-associated proteins (CAPs), for example, are G-actin sequestering proteins. Vertebrates have two isoforms, CAP1 and CAP2. CAP1 is ubiquitously expressed and primarily functions in cell polarity and motility, while CAP2 is expressed in the brain, heart, skeletal muscle, and skin. CAP2 has a nuclear localization pattern during myofibrillogenesis, but is localized to the M-line (possibly binds to the actin thin filament pointed ends but this remains to be confirmed) in adult myofibrils (Peche et al. 2007). CAP2 is proposed to regulate thin filament stabilization and actin dynamics by severing F-actin and preventing polymerization by sequestering the G-actin pool (Fig. 4.4) (Peche et al. 2013). A direct link between CAP2 and filament length regulation has not yet been shown.

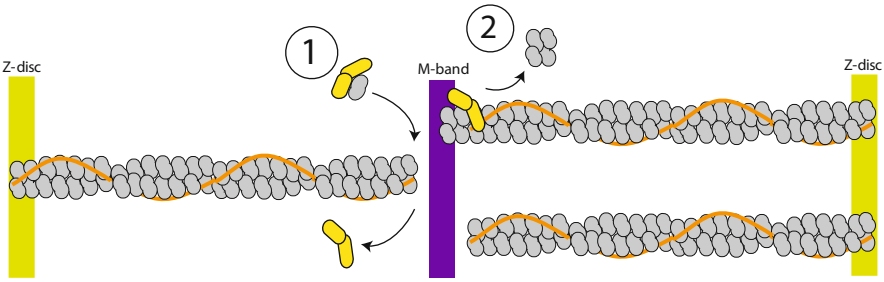

Fig. 4.4 Cap2 localizes to the region of M-band. The mechanisms by which Cap2 could maintain thin filament length is (1) to sequester and maintain a pool of G-actin for polymerization and/or (2) sever F-actin. Figure modified from Peche et al. (2013)

Actin-monomer-binding proteins likely work in conjunction with capping proteins to regulate thin filament length. In *Caenorhabditis elegans* striated muscle, Tmod1 has been shown to act synergistically with profilin (binds G-actin) and ADF/cofilin (severs F-actin) to prevent elongation of thin filaments. The dynamic exchange of Tmod1 allows free pointed ends to elongate; loss of Tmod1 at the pointed end leads to a transient tropomyosin-free pointed end. Data suggests that ADF/cofilin binds to the tropomyosin-free pointed end and severs the filament. Profilin can then bind to the depolymerized G-actin to prevent elongation of the thin filament (Yamashiro et al. 2008). While thin filament length could not be directly measured (due to the severe sarcomeric disorganization observed), it is postulated that the cooperation between Tmod1 capping with profilin and cofilin could be a mechanism to regulate thin filament length due to the clear role Tmod1 plays in length regulation. The potential role of proteins that bind or sever actin in thin filament length regulation has yet to be directly tested, but likely are important players in length regulation.

4.7 Links Between Alternations in Thin Filament Components and Myopathies

A notable link between thin filament length regulation in the heart and disease is that Tmod1 overexpression transgenic mice (TOT) develop dilated cardiomyopathy (DCM). Notably, TOT mice display shorter I-band length, which suggests shorter thin filament lengths [as was directly measured in isolated primary myocytes overexpressing Tmod1 (Sussman et al. 1998a, b)]. Although the Lmod2 gene is hypothesized to be involved in hypertrophic cardiomyopathy (HCM), due to its location near the HCM locus CMH6 (Conley et al. 2001), no studies to date have linked Lmod2 to the progression of cardiomyopathy.

Myopathies related to thin filament length have also been noted in skeletal muscle. Mutations in the giant sarcomeric protein nebulin result in nemaline

myopathy, and the muscle weakness associated with this disease has been linked to shorter thin filament lengths (Ottenheijm et al. 2010, 2013; Lawlor et al. 2011). The effect of thin filament length on contractile function has been studied in skeletal muscle (Granzier et al. 1991; Bang et al. 2006; Witt et al. 2006; Chandra et al. 2009; Gokhin et al. 2009; Ottenheijm et al. 2009), but it is not as well defined for cardiac muscle. For instance, nebulin knockout mice with shorter skeletal muscle thin filament lengths have reduced contractile function but the effect of nebulin knockout on heart contractile activity is not known (Bang et al. 2006; Witt et al. 2006; Gokhin et al. 2009).

Alteration in expression and localization of CapZ results in HCM. Cardiac-specific overexpression of the CapZ β2 isoform (which normally localizes to the intercalated disc) in mice inhibits expression and localization of the CapZ β1 isoform (Z-disc) and results in the development of HCM (Hart and Cooper 1999). From this study it was suggested that an important function of CapZ is to bind to the Z-disc and anchor actin filaments; loss of CapZ localization leads to myofibrillar disarray and HCM. Further evidence supporting the role of CapZ in HCM came from BAG3 (bcl-2-associated athanogene) knockout mice which show mislocalization of CapZ from the Z-disc resulting in myofibrillar degeneration and development of HCM (Hart and Cooper 1999). Both the BAG3 knockout mouse and CapZ β2 overexpression mouse illustrate the importance of CapZ in stabilization of the Z-disc and maintenance of thin filaments and reveal a role for a thin filament capping protein in HCM.

CapZ may also be an important factor in the remodeling associated with HCM. Treatment of isolated cardiomyocytes with endothelin-1 (ET1) or phenylephrine (PE), which induce cellular hypertrophy, decreases the affinity of CapZ for the barbed end. An exciting hypothesis based on this investigation is that increased exchange of CapZ may destabilize the Z-disc and facilitate insertion of new sarcomeres during hypertrophic remodeling through phosphatidylinositol-4,5-bisphosphate (PIP2) and protein kinase-C (PKC)-dependent pathways (Hartman et al. 2009). Following short, mechanical strain (to mimic exercise), CapZ capping is decreased and actin dynamics is increased in cardiomyocytes, which was proposed to be an adaptive hypertrophy response to add new sarcomeres (Fig. 4.3b) (Lin et al. 2013).

Nebulette has been proposed to be a DCM risk allele. A polymorphism in the actin-binding motif of nebulette (Asn654Lys) has been linked to non-familial DCM, and four nebulette polymorphisms (K60N, Q128R, G202R, and A592E) were found in patients with DCM and endocardial fibroelastosis (Arimura et al. 2000; Purevjav et al. 2010). Transgenic mice overexpressing mutant nebulette G202R and A592E develop DCM by 3 months of age when compared to wild-type nebulette overexpression. Mutant mice show defects in Z-disc mechanosensing (A592E) or force transmission and calcium sensitivity (G202R), while the K60N and Q128R nebulette mutations are embryonic lethal likely due to aborted myofibrillogenesis (Purevjav et al. 2010; Maiellaro-Rafferty et al. 2013). While knockdown of nebulette in cardiomyocytes results in shorter thin filament lengths (Moncman and Wang 2002), it is unknown if mutant nebulette results in alterations

of thin filament lengths, but it could, like the TOT mice, offer a link between a thin filament regulatory protein and the development of DCM.

Recently, a CAP2 knockout mouse was generated, and the loss of CAP2 leads to severe DCM. CAP2 is an M-line protein that inhibits actin polymerization and severs F-actin (Fig. 4.4), and loss of CAP2 was proposed to increase actin polymerization resulting in the loss of a clear M-line (Peche et al. 2013). The data suggests that ablation of CAP2 resulted in elongated thin filaments, though not directly measured. Further investigation is necessary to determine the precise role thin filament length regulation may play in the development of DCM or HCM.

4.8 Concluding Remarks

Regulating thin filament length is vital for efficient contractile function of striated muscle. Capping proteins have been identified that clearly have pivotal roles in thin filament length regulation biochemically in actin polymerization assays, as well as in isolated myocytes and in heart and skeletal muscle in vivo. However, little is known about how these proteins are regulated. In this regard, some data has been reported demonstrating the regulation of capping proteins by posttranslational modifications. For example, the kinase TRPM7 (transient receptor potential cation channel, subfamily M, member 7) phosphorylates Tmod1 and phosphorylation of Tmod1 residue T54 has been shown to blunt Tmod1's ability to cap the pointed end in vitro (Dorovkov et al. 2008; Bliss et al. 2014). In addition, CapZ dynamics are regulated by PKC and PIP2 (Hartman et al. 2009), and its dynamics are postulated to be modified by a yet unidentified posttranslational modification of the β-tentacle (Lin et al. 2013). In skeletal muscle, Lmod3 (a family member of Lmod2) stability is regulated by inhibition of ubiquitination by kelch-like family member 40 (KLHL40) (Garg et al. 2014). Identifying mechanisms for regulating capping function is an active area of research.

Numerous mutations in the sarcomere and cytoskeleton have been linked to both DCM and HCM (for reviews, see (Kho et al. 2012; Cahill et al. 2013; Garcia-Pavia et al. 2013). As described above, in genetic mouse models, perturbation of many of the proteins involved in thin filament length regulation (Tmod1, CapZ, nebulette) results in the development of cardiac myopathies. It is, however, currently unclear how alterations in thin filament lengths may be linked to the development of myopathies. Does the alteration in contraction and force generation as a result of altered filament lengths lead to myopathy? Does altering thin filament lengths affect thin filament stability resulting in degeneration of the sarcomere and consequently cardiomyopathy? An additional hypothesis, based on data from the transgenic CapZ β2 mouse, suggests that destabilization of the thin filament allows for the addition of new sarcomeres during hypertrophic remodeling. Deciphering the molecular and regulatory pathways that link alterations in thin filament lengths to cardiomyopathy is both compelling and clinically relevant.

Over 100 mutations have been identified in components of the thin filament that result in cardiomyopathy (for reviews, see Willott et al. 2010; Tardiff 2011; Spudich 2014). Thin filament protein mutations are responsible for 5–10 % of cases of both HCM and idiopathic DCM (Richard et al. 2003; Lakdawala et al. 2012). Identifying new disease-causing polymorphisms in thin filament length regulating proteins will provide crucial information on the in vivo function of these proteins and key clinical information. Of the proteins discussed in this article, polymorphisms in nebulette have been linked to DCM (Purevjav et al. 2010) and numerous tropomyosin and troponin polymorphisms are linked to both DCM and HCM (Willott et al. 2010; Tardiff 2011). One focus of future research will be to identify new disease-causing mutations. Regulation of the thin filament is vital to proper heart function, and any new information on thin filament regulatory proteins could be instrumental in the design of preclinical screening, therapy, and hopefully repair for heart disease.

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Chapter 5
Ca^{2+} Regulation of the Cardiac Thin Filament

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Abstract The physiological role of calcium in a cardiac myocyte is demonstrated in the coupling of excitation and contraction. Small extracellular calcium signals are amplified in the cardiac myocyte through calcium release from the sarcoplasmic reticulum. This amplified calcium signal is sensed by the thin filament heterotrimeric troponin complex. This is then transduced through a concerted series of inter- and intra-molecular conformational changes to ultimately allow the force generation required for the maintenance of normal cardiac pump function. This chapter seeks to delve into the biochemistry and biophysics that dictate the cellular physiology of the myofilaments.

5.1 Introduction

Myocardial ischemia and consequent myocyte death are significant deleterious outcomes of myocardial infarction. As noted in Starling and Visscher's seminal paper in 1927, the oxygen demand of the heart is directionally proportional to the work performed (Starling and Visscher 1927). When oxygen demand can no longer be met, there is a decline in the efficient generation of force. During the course of an ischemic event, there is a perturbation in the cardiac myocytes' biochemical milieu. At ischemia onset, oxygen tension declines and the myocyte undergoes an energetic switch from aerobic utilization of its preferred fatty acid metabolic substrate to an anaerobic glycolytic metabolism (Lopaschuk et al. 1992). As a consequence, there is an accumulation of anaerobic metabolites and significant acidification of the myoplasm. Penultimate to cell death, there is an uncoupling of Ca^{2+} homeostasis leading to a decline in force generation and a derangement of the Ca^{2+}-force relationship. Herein, we will provide an overview of the biophysics of the myocyte contractile cycle and discuss key alterations that can cause ischemic heart failure.

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5.2 Ca^{2+}-Induced Ca^{2+} Release

In the healthy cardiac myocyte, depolarization of the sarcolemma activates voltage gated L-type Ca^{2+} channels to increase their Ca^{2+} ion conductance. As Ca^{2+} ions begin to move down their concentration gradient into the cell, they bind to high affinity Ca^{2+}-binding sites on the nearby ryanodine receptors (RyRs) (Bers DM 2002). RyRs are the Ca^{2+} release channels of the sarcoplasmic reticulum (SR). RyRs display a biphasic response to Ca^{2+}; they are inactive at nanomolar concentrations of Ca^{2+}, activated by low micromolar concentrations, and again inactivated by higher concentrations of Ca^{2+} (Fill and Copello 2002; Smith et al. 1988). This initial Ca^{2+}-binding event is central to activation of RyRs via the process of Ca^{2+}-induced Ca^{2+} release. Thus, a rather small initial trans-sarcolemmal Ca^{2+} current serves as an elegant intracellular signal amplification process to facilitate a rapid rise in myoplasmic Ca^{2+}. This intracellular Ca^{2+} flux, which manifests in the plateau phase of the cardiac action potential, is responsible for the downstream activation of the thin-filament allosteric regulatory system described in detail below.

5.3 Sarcomere Function

When intracellular concentrations of Ca^{2+} rise following release from the SR, Ca^{2+} binds to the troponin complex on the thin filament. Binding of Ca^{2+} induces conformational changes within and distal to the complex. This causes the protein tropomyosin to move along actin and reveal specific binding sites for the motor protein myosin. Myosin binds to actin and upon ATP hydrolysis and product release, it performs a "power stroke" wherein it ratchets along actin to generate force-causing contraction/shortening of the myocyte (Rayment et al. 1993). When Ca^{2+} is removed from the myoplasm, the troponin complex again undergoes changes in its conformation that allows tropomyosin to once again occupy the myosin-binding site to induce relaxation of the myocyte.

5.4 Sarcomere Structure

With Ca^{2+} signal amplification occurring via the SR, the rise in intracellular-free Ca^{2+} leads to dynamic and highly ordered conformation changes in the sarcomere. In cardiac myocytes, the sarcomere is a highly regulated array of proteins in such tight stoichiometric control that the apparatus forms a nearly liquid crystalline arrangement (Fig. 5.1).

Several landmarks are readily observable to delineate the sarcomere. The Z line is a complex of structural proteins that physically delimit the sarcomere. From Z line to Z line, the sarcomere is composed of regularly spaced interdigitating thick and thin myofilaments. The thick filament is primarily composed of the molecular

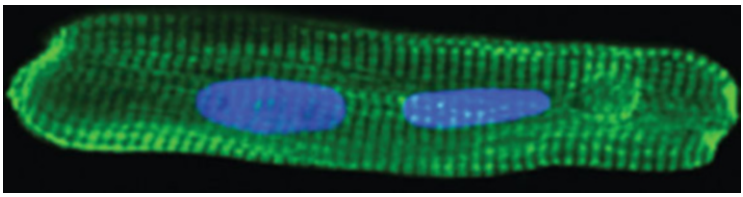

Fig. 5.1 An isolated cardiac myocyte in which sarcomeres are delineated by an antibody staining against α-actinin

motor protein myosin, and titin. Titin acts in part as a molecular ruler, stretching approximately half the length of the sarcomere, and provides elasticity (Granzier and Labeit 2004). Titin is anchored to the Z line via a protein complex including but not necessarily limited to telothenin and α-actinin. Myosin is a hexamer consisting of two heavy chains, two light chains, and two regulatory subunits. The hexamers are arrayed in bipolar coordination anchored to titin forming the thick filament. Myosin contains three distinct domains: the head, neck, and the tail. The head hydrolyzes ATP and directly interacts with the actin filament. The neck is a lever responsible for transducing force from the head domain. The neck domain additionally serves as a docking site for regulatory light chain proteins. The tail domain mediates the interaction with other myosin proteins in the thick filament. The myosin dense portion of the sarcomere is designated as the "A band," which derives its name "anisotropic" pertaining to its properties in polarized light (Huxley and Niedergerke 1954).

Interdigitating with thick myofilaments are the actin-containing thin myofilaments. The thin filaments can be observed in transmission electron microscopy and are denoted as the "I band" (for Isotropic, pertaining to its properties in polarized light) of the sarcomere. Actin is attached at its barbed end to the Z line via α-actinin. The pointed ends of the actin filaments contain capping proteins and can maintain dynamic treadmilling (Littlefield et al. 2001). The cross-bridge cycling of actin and myosin is regulated by the filamentous protein tropomyosin (Tm) and the Ca^{2+} regulated heterotrimeric troponin (Tn) complex. The ratio of these thin-filament proteins is tightly regulated at 7:1:1 (Actin:Tm:Tn) (Tobacman 1996).

At rest, the level of intracellular Ca^{2+} is insufficient to saturate Tn sites. Force can be generated by increasing the likelihood of myosin cross-bridges forming with actin by increasing intracellular Ca^{2+}, altering cross-bridge kinetics, or by increasing the Ca^{2+} sensitivity of the myofilament. At the myofilament level, contractile regulation is primarily regulated through a Ca^{2+} sensitive switch (Farah and Reinach 1995). As Ca^{2+} increases, regulatory proteins alter their interactions with other myofilament proteins and cause movements in the protein tropomyosin ultimately allowing for myosin heads to interact with the actin filament. Force is generated via the "power stroke." Myosin heads bind actin readily when not sterically hindered; hydrolysis of ATP and release of inorganic phosphate results in a ratcheting motion of the myosin along the actin filament that pulls the Z-discs

closer together. The binding of ATP to the catalytic domain of myosin allows for disengagement of the actin filament so the duty cycles can repeat (Warrick and Spudich 1987; Rayment et al. 1993; Ruppel and Spudich 1996).

5.5 Tropomyosin and the Steric Blocking Model

Tropomyosin is a ~40 nm long dimeric coiled-coil α-helical protein that polymerizes end to end to span the entire length of the thin filament (Xu et al. 1999). The tropomyosin dimer spans the length of seven actin molecules. Coiled-coil protein motifs are characterized by a typical seven residue helical repeat referred to as a "heptad." Through these heptad repeats, tropomyosin has multiple highly structured interactions with the actin filament. The prototypical heptad repeat features are labeled *abcdefg* where "*a*" and "*d*" are hydrophobic residues and "*e*" and "*g*" are charged residues (Lehman et al. 1995; Mason and Arndt 2004; Baruaa et al. 2011). Site "*d*" has an especially high propensity for being an alanine residue (35 %) (Brown et al. 2001). The regular spacing of nonpolar alanine residues allows for seven discrete actin recognition sites along the length of a single Tm dimer (Sousa et al. 2013). Mutations in these key residues can lead to significant cardiac disease (Michele et al. 1999).

Tropomyosin was first speculated to have a role in regulating contractile function nearly a half a century ago; since then, advances in biophysical techniques and microscopy have enabled high-resolution discernment of how Tm sterically modulates cross-bridge formation (Brown et al. 2005). Tropomyosin can assume at least three discrete structural states defined largely by biochemical observations: blocked, closed, and open. The "blocked state" can be observed in regulated thin filaments in the absence of Ca^{2+}. In the blocked state, myosin heads are unable to

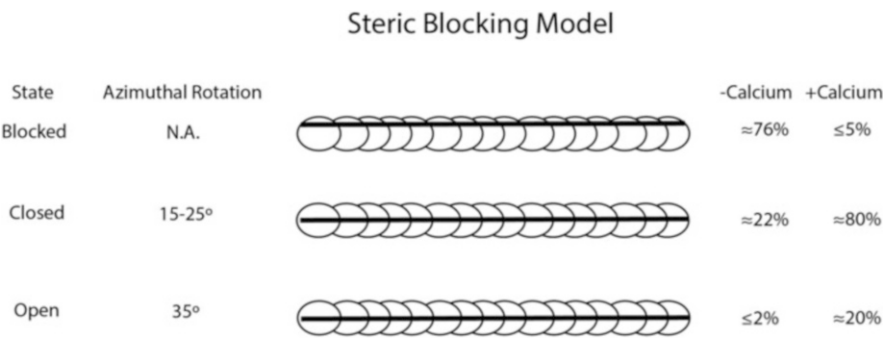

Fig. 5.2 The steric blocking model showing the relative rotational azimuthal movements of tropomyosin about the actin filament and the approximate percentage of populations occupying each biophysical state with and without calcium bound to troponin (right panel) (McKillop and Geeves 1993)

strongly bind to stereospecific sites on actin (Mckillop and Geeves 1993) (Fig. 5.2). With Ca^{2+}-binding troponin, Tm is capable of making an azimuthal rotation of 15–25° about the actin filament into the "closed state". This Ca^{2+}-bound state partially exposes myosin-binding sites on actin and allows for interaction between myosin heads and the thin filament. With the addition of strong myosin binding, Tm performs an additional movement along the actin filament to approximately 35° away from the Ca^{2+}-free blocked state to define the open state (Spudich et al. 1972; Parry and Squire 1973; Pirani et al. 2006; Poole et al. 2006). The exact nature of how Tm moves along the actin filament is still debated, whether it is a flexible rolling or a rigid sliding maneuver (Holthauzen et al. 2004; Li et al. 2010).

Critical to the position and movement of tropomyosin is the conformation of the heterotrimeric troponin complex. Tropomyosin is held in its blocked conformation primarily by the action of the N-terminal region of troponin T and by two regions of troponin I known as the "inhibitory region" and the "mobile domain." As Ca^{2+} binds to troponin C, the equilibrium shifts between populations in the steric blocking model. During diastole, low Ca^{2+} levels and weak myosin cross-bridges denote a dynamic equilibrium that rests predominantly in the blocked and closed states. During systole, the influx of Ca^{2+} and the positive feedback of strong myosin head attachment shift the equilibrium to chiefly the open state (Fig. 5.2).

5.6 Troponin: The Ca^{2+} Switch

Ebashi first discovered troponin in 1963 (Ebashi et al. 2008). At the time, it was recognized as "native tropomyosin," a tropomyosin-like protein that participated in the Ca^{2+} binding and relaxing action of muscle. In the over 50 years since its discovery, our understanding of troponin has grown from being another protein on the list of muscle regulators to being the master molecular switch complex that mediates the link between excitation and contraction (Ebashi 1963; Farah and Reinach 1995).

Troponin is a heterotrimeric complex of the proteins troponin I, C, and T. In striated muscle, the binding of Ca^{2+} to troponin regulates contraction (Farah and Reinach 1995). In the absence of troponin, muscle will contract independent of Ca^{2+} (Moss et al. 1986). The troponin complex is anchored to the thin filaments by multiple protein–protein interactions between TnI, TnT, Tm, and Actin. Troponin I is named for its inhibitory effects on actin/myosin ATPase; troponin C is named for its Ca^{2+}-mediated regulation of thin-filament conformational dynamics and troponin T for its role in anchoring the heterotrimeric complex to tropomyosin. Troponin functions to convert Ca^{2+} signaling into biophysical movements that dictate force generation. In 2003 the core domain of the troponin complex, which houses most of the regulatory function, was resolved to 2.6 Å resolution via X-Ray crystallography. This significant advance represented the first time the heterotrimeric troponin complex was visualized to atomic level resolution (Takeda et al. 2003).

5.7 Troponin C

Troponin C (TnC) is often described as a dumbbell shaped protein with two globular domains connected by a central linking helical domain. TnC is an approximately 18 kDa protein derived from two distinct genes (Fig. 5.3). TNNC1 encodes for the TnC isoform expressed in slow skeletal (ssTnC) and cardiac muscle (cTnC), whereas TNNC2 encodes for the fast skeletal isoform (fsTnC) found in fast twitch limb muscles. TnC belongs to the EF-hand domain family of proteins, characterized by their canonical helix-loop-helix divalent metal-binding sites. Each globular domain of troponin C contains two EF hand domains, from N-terminus to C-terminus the sites are numbered I, II, III, and IV (Herzberg and James 1985) (Fig. 5.3). Sites III and IV in the C-terminal globular domain are high affinity cation sites and constitutively bound to Ca^{2+}/Mg^{2+}; these are generally considered structural domains. In contrast, the N-terminal sites are regulatory. Despite 70 % sequence homology to the skeletal isoform, site I in the cardiac isoform is inactive due to seven key loop residue substitutions; subsequently, all cardiac Ca^{2+} responsiveness is conferred via site II (Van Eerd and Takahashi 1975). Troponin C can functionally be classified as a signaling protein, as binding of Ca^{2+} to site II causes dramatic conformational changes not only in troponin C but also the troponin

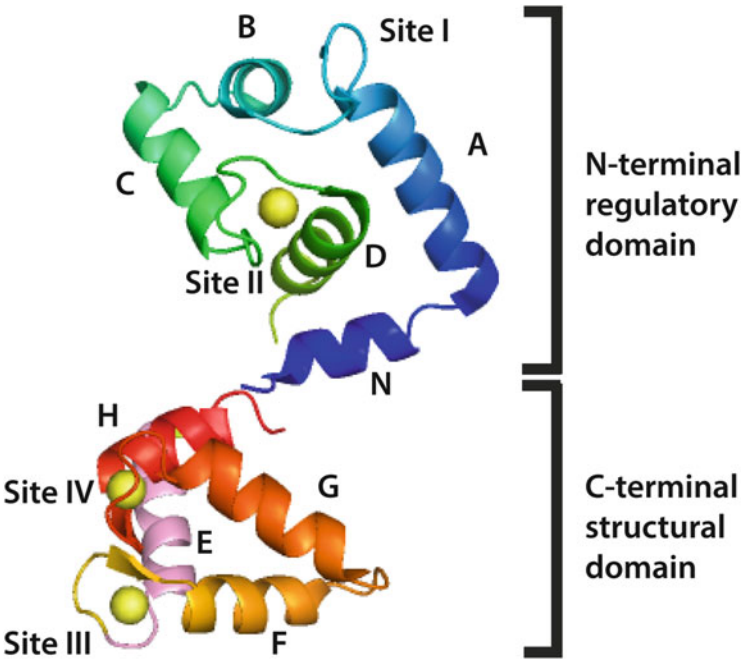

Fig. 5.3 Ribbon structure schematic of cTnC (PDB 1J1E) in the Ca^{2+}-saturated state with EF hands and critical helix domains labeled. In this structure, the central helix that links the DE helices is not resolved. Note that Site I is inactive in cTnC

complex and tropomyosin to activate the thin filament and ultimately generate force.

Much of the groundwork for understanding the conformational dynamics of cardiac troponin C is derived from the understanding of the fast skeletal isoform (fsTnC). X-ray crystallographic studies of the skeletal troponin from avian species suggest the elongated dumbbell shape of troponin C, where the two globular domains are connected by a central helix (Herzberg and James 1985; Satyshur et al. 1988). These crystal structures allowed a more intimate understanding of the conformational changes that occur upon Ca^{2+} binding to TnC (Strynadka et al. 1997). It was purported that binding of Ca^{2+} to the regulatory site would cause helices A and D of troponin C to move relative to helices B and C (see Figs. 5.2 and 5.3). This movement would expose hydrophobic residues of the N-terminal domain and allow for Ca^{2+}-dependent interaction with troponin I (Herzberg and James 1985; Sundaralingam et al. 1985; Mehler et al. 1991; Gagne et al. 1995). It is important to note that in the cardiac isoform, complete exposure of the hydrophobic residues requires troponin I in addition to Ca^{2+} (Gagne et al. 1995).

High affinity Ca^{2+}-binding sites III and IV ($K_{Ca} \sim 1.0 \pm 0.2 \times 10^7\ M^{-1}$) are sensitive to competitive binding to magnesium ($K_{Mg} \sim 3.0 \times 10^3\ M^{-1}$), unlike the lower affinity Ca^{2+}-binding site II ($K_{Ca} \sim 2.5 \pm 1.6 \times 10^5\ M^{-1}$). When TnC is complexed in either the dimeric complex with TnI or the native Tn complex, the Ca^{2+} affinities increase for both the non-regulatory and regulatory sites ($K_{Ca} \sim 3.2 \pm 1.2 \times 10^8\ M^{-1}$, and $K_{Ca} \sim 2.5 \pm 2.0 \times 10^6\ M^{-1}$, respectively) (Holroydes et al. 1980). This reflects the enhanced Ca^{2+} sensitivity of troponin C as thin-filament complexity increases.

Initially, it was thought that the rate of Ca^{2+} exchange with TnC was faster than the kinetics of contraction and relaxation in cardiac muscle. Kinetic studies of troponin C in isolation showed Ca^{2+} off rates between 590–995 s^{-1} at 4 °C and 1,000–1,263 s^{-1} at 15 °C using a variety of methodologies (Dong et al. 1996; Tikunova and Davis 2004). However, given the change in Ca^{2+} affinity at all sites when cTnC is in its native complex, it is unlikely that these rates reflect the true nature of Ca^{2+} dissociation in situ. Indeed, Ca^{2+} dissociation also slows as thin-filament complexity increases; with the addition of cTnI to cTnC dissociation of Ca^{2+} slows from >1,000 s^{-1} at 15 °C to 122 s^{-1} at 15 °C. With the addition of cTnT, Ca^{2+} dissociation slows further to 34 s^{-1} at 15 °C. It is worth noting that the addition of ssTnI to cTnC has an even more dramatic effect on Ca^{2+} dissociation lowering the off rate to 9.8 s^{-1} at 15 °C (Gomes et al. 2004). More recently, the introduction of a novel cysteine residue (T53C) for the covalent modification of fluorophores in the BC subdomain of the N-terminal globular domain of cTnC has allowed for detection of Ca^{2+} binding without perturbing biological function or reliance on a cytosolic Ca^{2+} reporter (e.g., BAPTA, Quin-2) (Davis et al. 2007). The use of this system has revealed similar off rates for the Tn complex at 15 °C (41.9 s^{-1}). Additionally, it has been shown that the addition of tropomyosin may further slow Ca^{2+} dissociation to 36 s^{-1}. Addition of actin to the Tn:Tm complex enhanced cooperativity ($n = 0.91$ vs. $n = 1.65$) of Ca^{2+} binding and increased Ca^{2+} dissociation to 105 s^{-1}. Introduction of myosin S1 to the system had drastic effects

on Ca^{2+} dissociation, slowing its off rate to 13 s^{-1} (Davis et al. 2007). Taken together this evidence suggests that unlike isolated cTnC in solution, Ca^{2+} dissociation from cTnC in the complex thin filament is compatible with rates of mechanical relaxation.

5.8 Troponin I

Troponin I (TnI) is an approximately 24 kDa protein encoded by three distinct genes. TNNI1 encodes for the slow skeletal isoform (ssTnI), TNNI2 encodes for the fast skeletal isoform (fsTnI), and TNNI3 encodes for the cardiac isoform (cTnI). Troponin I interacts with most other thin-filament proteins (TnC, TnT, Ac, and Tm). In this way, TnI acts as an adapter protein to facilitate the complex conformation dynamics of the thin filament. Here, cTnI is named for being the subunit of the troponin complex responsible for inhibition of actomyosin MgATPase. The immunological detection of cTnI has been used extensively in clinical settings for the specific determination of myocardial injury (Adams et al. 1993). Unlike cTnC, whose structure has been elucidated with high atomic resolution, the crystal structure of cTnI, free in solution, is not yet available. Despite this, cTnI has been the subject of extensive biochemical investigation and contains several noteworthy functional domains. The N-terminus of cTnI contains a 32 amino acid extension that is unique to the cardiac isoform. The C-terminal region contains several well-characterized regions important for the Ca^{2+}-dependent inhibition of myosin cross-bridge cycling. Residues 128–147 (excluding the initial methionine) contain the minimal peptide region responsible for inhibition of actomyosin ATPase activity; this region is typically referred to as the aptly named "inhibitory region" (Farah and Reinach 1995). This peptide is derived from the cyanogen bromide digest of the full length protein. From this region, 12 residues (136–147) alone can recapitulate troponin-mediated thin-filament activation/inhibition (Talbot and Hodges 1981; Lindhout and Sykes 2003). Residues 148–163 contain a region which binds to the N-terminal region of cTnC upon Ca^{2+} binding and facilitates in the full opening of the hydrophobic patch. Residues 167–210 contain a region referred to as the "mobile domain," with residues 168–188 containing a second actin-binding domain.

5.9 Troponin T

There are three distinct genes in the human genome that encode for troponin T. TNNT1 encodes for the slow skeletal isoform, TNNT2 encodes for the cardiac isoform, while TNNT3 encodes for the fast skeletal isoform. From each of these genes numerous isoforms are derived from various splice variants; in total there are at least 21 isoforms that have been recognized (UniProt 2014). There are four

different isoforms expressed in the heart, with three isoforms being expressed in the fetal heart (and potentially reexpressed in the failing heart as part of a fetal reprogramming) and one isoform in the adult heart (Anderson et al. 1991). The four cardiac isoforms are numbered cTnT1 through cTnT4 in order of decreasing molecular weight. Troponin T (TnT) is an approximately 35 kDa protein that anchors the heterotrimeric troponin complex to the thin filament via tropomyosin. Cardiac troponin T (cTnT) is homologous amongst species with the exception of a hypervariable N-terminal region (residues 1–79). TnT interacts strongly with troponin I through a region termed the "I-T arm" (residues 234–284). In addition to its role in anchoring troponin, TnT also interacts with all major components of the thin filament including tropomyosin which interacts with two distinct binding regions (residues 89–127 and 215–240) (Wei and Jin 2011). Through changes in fiber type specific isoform regulation, posttranslational modifications, and splice variants, troponin T is able to participate in the modulation of striated muscle contraction and relaxation.

5.10 Phosphorylation

β-adrenergic stimulation via activation of the sympathetic nervous system is a predominate mechanism by which the heart rapidly responds to stress and changes in circulatory demand. The heart is able to adapt to increased demand through the summed combination of inotropic, chronotropic, and lusitropic effects. β-adrenergic signaling is primarily thought to activate cAMP-activated protein kinase (PKA) which has numerous targets that modulate both Ca^{2+} entry and myofilament responsiveness to Ca^{2+} (Metzger and Westfall 2004). This response is achieved through the PKA-dependent phosphorylation of L-type Ca^{2+} channels, ryanodine receptors phospholamban, and cTnI. PKA-mediated phosphorylation increases Ca^{2+} currents through the L-type Ca^{2+} channels and ryanodine receptors (this is achieved secondary to SERCA disinhibition) (Yoshida et al. 1992). PKA-mediated phosphorylation of phospholamban reduces its ability to attenuate sarcoplasmic reticulum Ca^{2+} ATPase (SERCA) activity, thereby enhancing Ca^{2+} loading kinetics and increasing total SR Ca^{2+} content (MacLennan and Kranias 2003). However, this review will focus on the PKA-mediated effects on cTnI.

PKA phosphorylates cTnI at serine residues 23 and 24. Residues 23/24 are part of the unique 32 amino acid N-terminal extension found exclusively in the cardiac isoform of Troponin I (Zhang et al. 1995). Phosphorylation at these residues reduces Ca^{2+} sensitivity of the myofilaments; that is, it causes a rightward shift in the force-pCa relationship (Yasuda et al. 2007). Reduction of myofilament Ca^{2+} sensitivity and enhanced Ca^{2+} dissociation from cTnC result in accelerated relaxation of the myofilament (positive lusitropy) (Zhang et al. 1995; Yasuda et al. 2007).

The use of gene transfer and transgenic mice harboring mutations in key residues of cTnI has been essential in elucidating the mechanistic nature of these physiological phenomena. Chimeric TnI molecule structure–function studies were critical in resolving the key functional domains of TnI (Westfall et al. 2000, 2001). Here it was shown the C-terminal domain of TnI contains the key effector residues for affecting Ca^{2+} and pH sensitivity inherent in the TnI isoforms. Studies in live myocytes transduced with adenovirus expressing either the slow skeletal isoform of troponin I (ssTnI), which lacks the unique N-terminal extension of the cardiac isoform, or a chimera isoform with the N-terminus of ssTnI and the C-terminus of cTnI demonstrated definitively that the N-terminal extension of cTnI is responsible for mediating the Ca^{2+} desensitizing effects of PKA following β-adrenergic stimulation (Westfall et al. 2001). In 2002, generation of a transgenic mouse by Pi et al. expressing cTnI where residues S23/S24 were mutated to non-phosphorylatable alanines corroborated previous evidence that these tandem series in the N-terminal extension in particular were responsible for the β-adrenergic response (Pi 2002; Pi et al. 2003). Conversely, mice have also been generated which mimic constitutive phosphorylation at serines 23/24 by mutation to aspartate. These mice show enhanced relaxation even at high heart rates that could be normalized by use of β-adrenergic agonists (Takimoto et al. 2004). Perhaps the most convincing evidence that the lusitropic effects of PKA are primarily manifested through cTnI and not other targets comes from a series of experiments in transgenic mice harboring mutations in troponin I where serines 23/24 were replaced by phosphomimetic aspartate residues. In cardiac myocytes explanted from these mice, relaxation times were significantly faster at baseline compared to control mice (Yasuda et al. 2007). Additional treatment with β-adrenergic agonists only caused nominal increases in the relaxation speed, whereas the Ca^{2+} transient decay rate was comparable between both transgenic and control mice treated with β-adrenergic agonists (Yasuda et al. 2007). Taken together, this evidence suggests that the PKA-modulated lusitropy involves a key role of the myofilaments and in particular cTnI.

In addition to its lusitropic effects, PKA-mediated phosphorylation also increases the inotropic state of cardiac muscle. Part of this effect is mediated through increased Ca^{2+} loading of the sarcoplasmic reticulum subsequent to phospholamban phosphorylation thereby increasing SERCA activity. The end result from this is larger Ca^{2+} amplitudes thereby increasing myofilament activation. However, independent of this effect, phosphorylation of troponin I appears to enhance function. PKA treatment of skinned rat cardiac myocytes has shown an increase in maximal force and peak absolute power output during maximal Ca^{2+} activations; this increased power output may be explained by the increased cross-bridge cycling rate that occurs with β-adrenergic stimulation (Kentish et al. 2001). Additional support for the notion of PKA phosphorylation mediating positive inotropic effects via the myofilament comes from the observation that transgenic mice with the S23/24D mutation have increased systolic function at baseline and force-frequency response that is independent of the SR reconciled inotropic effects (Yasuda et al. 2007).

In addition to being a target of PKA, the thin filament also serves as substrate for protein kinase C (PKC). There are 11 identified isoforms of PKC that can be classified as conventional (α, β, β_{II}, γ), novel (δ, ε, η, θ), and atypical (ζ, λ, ι) (Steinberg 2012). In cardiac myocytes, PKC is typically activated downstream of G-protein coupled receptors (GPCRs) by the agonists: endothelin I, angiontensin II, norepinephrine, and epinephrine. Binding of agonists to GPCRs causes the activation of phospholipase C (PLC) which causes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) to membrane-bound diacyl glycerol (DAG) and inositol triphosphate (IP3). IP3 can cause the release of internal stores of Ca^{2+}, which along with DAG activate conventional PKCs (Steinberg 2012). Both negative and positive isotropic and lusitropic effects have been reported in cardiac mycoytes, so it is likely that there are isoform specific as well as both myofilament and non-myofilament responses to PKC activation (Westfall and Borton 2003). For this reason, we will focus on the role of PKC in phoshorylating troponin I.

Similar to studies with PKA, to discern the effects of PKC on one substrate versus another, the most effective methods have been using in vitro reconstituted skinned myocytes, acute gene transfer, and transgenic mouse models with constitutive phosphorylation mimetics. The use of PKC agonists has historically been confounded by off target effects when trying to assess the physiological consequences of thin-filament phosphorylation. Troponin I can be phosphorylated by PKC at sites S23/S24, S44/S46, and T144 (Noland et al. 1989). Phosphorylation of S44/46 causes a reduction in the maximal Ca^{2+}-activated force, MgATPase activity, maximal sliding velocity, and the cross-bridge cycling rate (McClellan et al. 1996). PKC phosphorylation of T144 results in a rightward shift in the pCa-filament sliding speed relationship. PKC-α preferentially phosphorylates sites S44/46. Phosphorylation of these serines is common among all PKC isoforms that target TnI except PKC-β which seems to preferentially phosphorylate S23/24 and T144 (Wang et al. 2006). Despite the differing accounts of the physiological role of PKC, there seems to be consensus in the literature that phosphorylation of troponin I at sites S44/46 reduces maximal Ca^{2+}-activated tension and T144 reduces Ca^{2+} sensitivity of sliding velocity which in concert may offer therapeutic targets in failing myocardium.

Troponin T has numerous putative PKC phosphorylation sites including T204, S208, T213, S285, and T294 (Noland 1996; Sumandea et al. 2009). It is apparent that cTnT is phosphorylated in vitro by various PKC isoforms; however, the studies investigating isoform specificity have returned conflicting results with some studies suggesting PKC-α and PKC-δ preferentially phosphorylated cTnI over cTnT whereas PKC-ζ was cTnT specific, while others reported the exact opposite relationship (Noland 1996; Pi 2002). Despite this, studies using phosphomimetics or reconstituted in vitro phosphorylated troponin T tend to have consensus that phosphorylation of troponin T results in reduced binding affinity of troponin T for tropomyosin resulting in a leftward shift in the pCa-actomyosin MgATPase relationship and decreases maximal tension (Noland and Kuo 1991, 1992).

In addition to posttranslational modification by the better-characterized PKA and PKC effects, troponin I also serves as substrate for several other kinases in cardiac myocytes. PKD has been shown to phosphorylate TnI at the same sites as PKA at Ser22/23 and causes a rightward shift in the tension-pCa relationship. The exact contribution by PKD in vivo is unknown, but it is believed to complement the modification of troponin I's phosphorylation state (Haworth et al. 2004; Cuello et al. 2007). Rho-associated protein kinase (ROCK) is also capable of posttranslational modification of the thin filament. ROCK treatment of myocytes causes an inhibition of tension and ATPase activity. Sites S23/24 and Thr144 of troponin I and Ser278/287 of troponin T are all substrate for ROCK (Vahebi et al. 2005). Although their exact physiological significance is still debated, ROCK kinases are known downstream agents of stress, hypertrophy, and heart failure signaling cascades. AngII has been used to cause hypertrophy in myocytes associated with activation of the RhoA pathway (Aoki et al. 1998). p21-activated kinase (PAK) is also capable of phosphorylating TnI and TnT as well as other sarcomeric proteins. PAK phosphorylation causes an increase in peak isometric force and Ca^{2+} sensitivity; however, its physiological significance remains elusive (Buscemi 2002).

5.11 Ischemic Insult and the Thin Filament

Ischemic heart disease is the leading cause of death worldwide accounting for as much as 12 % of all deaths (Finegold et al. 2013). Acute ischemia bouts are typically initiated by the disengagement of an atherosclerotic plaque into the coronary vasculature that begins a clotting cascade resulting in the partial or complete occlusion of the coronary vasculature (Davies and Thomas 1985). During bouts of ischemia, left ventricular pressures will rapidly drop due in part to the accompanying biochemical changes of the myocyte (Lee and Allen 1991). It is well established that ischemia causes biochemical changes in myocytes that can result in damage to organelles, namely the nuclei and mitochondria. As oxygen becomes depleted in the cardiac myocytes, metabolic changes from normal oxidative respiration to strictly anaerobic glycolysis cause increased production of protons lending to acidification. There is increasing evidence that ischemic insult causes modifications to the cardiac cellular machinery secondary to acidification.

Which proteins are modified, degraded, or lost may be in part dependent on the extent in which the tissue is ischemic (Rajabi et al. 2007). In a canine model, occlusion of the left descending coronary artery for 15 min resulted in disruption of the microtubule network that was reversible after an hour of reperfusion and could be halted with Ca^{2+} chelators suggesting a Ca^{2+}-dependent role in the disarrangement (Sato et al. 1993). α-actinin has also been shown to be highly susceptible to ischemia-induced degradation; α-actinin and troponin I were shown to be two of the more susceptible proteins to proteolysis subsequent to ischemic injury (Van Eyk et al. 1998). The alterations to α-actinin and troponin I may in part explain the observation of a time-dependent reduction in maximum force from skinned fiber

bundles. The extent and functional significance of these degradations is debated among groups; however, there is consensus that these changes lead to contractile dysfunction.

One of the better-studied proteins following ischemic injury is troponin. Troponin is routinely used diagnostically as a marker of myocardial damage due to its increased cardiac specificity compared to creatine kinase. An early study by Westfall implicates the degradation of troponin I and troponin T as cause for a Ca^{2+} desensitization in MgATPase assay (Westfall and Solaro 1992). Calpain has long been thought to be one of the primary agents causing proteolysis of troponin I. Calpains are Ca^{2+}-dependent and thought to be activated after reperfusion and Ca^{2+} overload. Calpains lack a consensus sequence for proteolysis but have been shown to cleave troponin I in vivo and in vitro at three unique sites (Kositprapa et al. 2000). Additionally, calpain is also capable of cleaving troponin T in its hypervariable N-terminal region during ischemia (Zhang et al. 2006). This calpain-mediated cleavage may be the cause of "myocardial stunning" after reperfusion following ischemia (Gao et al. 1997). Myocardial stunning is defined as the reversible abnormal contractile function of the myocardium following ischemia/reperfusion and may last up to several weeks following the initial ischemia insult (Bolli and Marbán 1999).

In addition to the cellular processes induced by ischemia and acidification that lead to contractile failure, there is evidence that acidification of the myoplasm has direct effects on Ca^{2+} activation. Acidification of the sarcomere causes reduced Ca^{2+} sensitivity; i.e., a greater amount of Ca^{2+} is required to achieve the same activation and force generation (Metzger and Moss 1987; Metzger and Westfall 2004; Nelson and Fitts 2014). This depression of Ca^{2+} sensitivity is highly sensitive to the TnI isoform expressed, with skeletal and embryonic isoforms being less sensitive than the adult cardiac isoform. The use of adenoviral gene transfer has revealed that expression of certain embryonic isoforms in the failing heart can mitigate the contractile dysfunction (Westfall et al. 1997, 2000; Herron et al. 2010).

A hallmark of diseased myocardium is the return to the fetal gene program (Rajabi et al. 2007). The fetal heart is well equipped with an arsenal of protein isoforms geared towards hemodynamic function in utero where hypoxia and carbohydrate metabolism are normal (Fisher et al. 1981). Returning to the fetal gene program can be viewed as a compensatory mechanism in failing myocardium; however, at what cost this compensation merits further investigation (Locher et al. 2009). In hearts explanted from patients with coronary artery disease, a form of chronic ischemia, there is evidence for isoform switching of titin; this is exemplified as the ratio of titin isoforms N2BA:N2B without any change in myosin heavy chain. N2BA is the predominant isoform in the fetal heart and is a more elastic isoform than the adult N2B (Neagoe 2002). Troponin T also undergoes isoform switching in the failing adult heart reverting to the embryonic isoform profile, where $cTnT_1$ and $cTnT_4$ are upregulated (Anderson et al. 1991). In the human heart, myosin heavy chain β (MHCβ) is the predominate isoform expressed during adult life although myosin heavy chain α (MHCα), the predominate fetal isoform, still represents approximately 10 % of the total MHC. During heart failure,

the ratio of MHCβ:MHCα increases as the level of MHCα is heavily repressed (Nakao et al. 1997; Reiser et al. 2001).

5.12 Histidine Modified Troponin I

Taking a cue from nature, reintroduction of other fetal isoforms may be therapeutically beneficial. The Ca^{2+} activation of troponin from skeletal muscle in particular has been shown to be more resilient to acidosis (Metzger et al. 1993). In mammals, the embryonic isoform of troponin I expressed in the heart is the same as the slow skeletal isoform (ssTnI/TNNI1). After birth, ssTnI expression in the heart irreversibly decreases independent of cTnI expression (Huang et al. 1999). Introduction of ssTnI into adult myocytes by acute gene transfer confers significant pH-insensitivity to the pCa-tension relationship (Westfall et al. 1997). Myocytes expressing ssTnI are resistant to Ca^{2+} desensitization by acidosis, and this has been corroborated in transgenic mice expressing ssTnI in lieu of cTnI; however, these mice also present with diastolic dysfunction (Wolska et al. 2001; Fentzke et al. 1999). Studies involving troponin I chimeras of the cardiac and slow skeletal isoforms revealed that the pH-insensitivity was derived from the C-terminal region of ssTnI (Westfall et al. 2000). Indeed, the pH-insensitivity that ssTnI confers can be explained through a single amino acid in ssTnI, His132. Introduction of a histidine at the cognate position in the cardiac isoform (A164H) recapitulates the pH-insensitivity in transgenic animals in vivo (Day et al. 2006). Histidine is an amino acid of particular interest due to its pKa being approximately 6.0; the protonation state of histidine residues can change with deviations in pH in the physiological range. Transgenic mice expressing cTnI A164H have normal heart rates, and the isolated myocytes retain a normal morphology and cytoarchitecture. They differ from wild-type mice most dramatically during ischemia mimetic conditions. In isolated transgenic hearts perfused with an ischemia mimetic solution, contractility was maintained relative to nontransgenic hearts especially in regards to maintenance of left ventricular developed pressures (LVDP) and left ventricular end diastolic pressures (LVEDP). Introduction of a histidine at A164 improves systolic and diastolic function and mitigates reperfusion-associated ventricular arrhythmias in isolated whole heart preparations (Day et al. 2006).

Computational analysis suggests that the switch region of cTnI interacts electrostatically with the regulatory region of cTnC significantly less relative to switch region of ssTnI (Palpant et al. 2010). This difference in affinity can at least partially be ascribed to a difference in how the switch regions of ssTnI and cTnI interact with cTnC. Indeed, molecular dynamics simulations and NMR experiments have shown that protonated H164 of cTnI (and H132 of sTnI) interacts with E19 of cTnC and results in increased affinity of cTnI for cTnC (Palpant et al. 2012; Pineda-Sanabria et al. 2013). Further studies revealed that the conformation adopted due to the H164-E19 electrostatic interaction decreases free energy of Ca^{2+} binding of cTnC (Thompson et al. 2014). These results in combination with the physiologic data

suggest a model of H164-E19 interaction resulting in a conformation that increases troponin function under acidic conditions thereby resulting in preservation of cellular and whole heart function in ischemic conditions.

This review has emphasized the complex nature of the allosteric protein machinery resident in the sarcomere that allows the heart to maintain its function in health and disease. The cardiac sarcomere contains an elegant network of intricate regulatory machinery and mechano-motors, working in a concerted fashion well over a billion times in an individual's lifetime. Advances in understanding the mechano-chemical nature of the sarcomere provide innumerable opportunities to target inherited and acquired cardiac myopathies with novel small molecule and gene therapies to correct cardiac muscle dysfunction in heart failure.

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Chapter 6
Posttranslational Modification of the Titin Springs: Dynamic Adaptation of Passive Sarcomere Stiffness

Martina Krüger

Abstract The giant sarcomeric protein titin has multiple important functions in cardiac muscle cells. Due to its gigantic size, its central position in the sarcomere, and its modular composition of elastic I-band domains, titin is a scaffold protein important for sarcomere assembly and serves as a molecular spring that defines myofilament distensibility. An increasing body of evidence suggests an important role for posttranslational modification of titin in modulating cardiomyocyte stiffness. This review provides an overview on the current knowledge of signaling pathways and kinases involved in phosphorylating I-band titin in healthy and diseased hearts and further highlights some recent findings that suggest posttranslational adaptation of titin stiffness in response to oxidative stress.

6.1 The Sarcomeric Protein Titin: Regulator of Myofilament Stiffness

With a molecular weight of 3–4 Megadalton (MDa), titin is the largest monomeric protein known to date and it spans an entire half sarcomere from the Z-disk to the M-band. The I-band part of titin contains several elastic elements that, according to the principle of an elastic spring, can be reversibly extended or shortened (Linke et al. 1996, 1999; Trombitas et al. 1998; Li et al. 2002; reviewed in Krüger and Linke 2009). In cardiomyocytes these elastic elements are the immunoglobulin-like domains (Ig domains), the N2-B unique sequence (N2-Bus), and the PEVK region, named so because of the high abundance of proline (P), glutamate (E), valin (V), and lysine (K) residues. Differential splicing of the titin gene provides the basis for numerous species- and muscle-specific titin isoforms with molecular weights ranging from 3.0 to 3.7 MDa. Most alternative splicing events occur in the I-band region

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of titin but to a smaller degree also affect the Z-disk and the M-band portion of the molecule (Bang et al. 2001; Krüger and Linke 2011).

In the A-band portion of titin, Ig- and fibronectin-type-3 domains (FN3 domains) are arranged in 7- and 11-super repeats that mediate the tight association of titin to myosin and myosin binding protein C (MyBP-C) (Bang et al. 2001; Tskhovrebova and Trinick 2004; Lange et al. 2006). The M-band portion of titin is characterized by several inserted sequences and the titin-kinase domain in the M-band periphery. Due to its exceptional length, its central position in the sarcomere, and its highly modular structure, titin has long been recognized as a scaffolding protein that probably serves as a molecular ruler for sarcomerogenesis and assists in the process of myofibrillar assembly (Tskhovrebova and Trinick 2010; Ehler and Gautel 2008). Multiple titin–protein interactions further involve titin in important signaling pathways, such as the regulation of protein quality control, mediation of hypertrophy-associated changes in gene expression, and mechanical stress signaling (Krüger and Linke 2011; Linke and Krüger 2010).

This review will focus on titin's functional role as the main source of passive sarcomere elasticity. Cardiac titin stiffness is largely defined by the composition of the titin isoforms in the sarcomere. Human heart expresses two main isoform types: the short and stiff N2B isoform (3.0 MDa) and longer and more compliant N2BA isoforms (>3.2 MDa). In left ventricles of healthy human heart, the isoform composition is approximately 35 % N2BA and 65 % N2B (Neagoe et al. 2002; Makarenko et al. 2004). The relative expression of titin isoforms changes during perinatal development of the cardiac muscle. Embryonic rat hearts predominantly express a long fetal N2BA isoform, which is replaced by smaller N2BA and N2B isoforms within few days after birth. As a result of the shift toward stiffer N2B titin, myofibrillar passive stiffness increases (Opitz et al. 2004; Krüger et al. 2006).

This titin isoform switch can be partly reversed in patients with end-stage heart failure and result in a higher proportion of longer and more compliant N2BA isoforms and subsequently decreased myofibrillar passive stiffness. It is currently assumed that this isoform transition is a long-term adaptation process of the failing heart to counterbalance the global increase of ventricular stiffness caused by hypertrophy and fibrosis (for review see Linke and Hamdani 2014). However, recent data showed that this isoform transition largely depends on the patient cohort and is not necessarily a hallmark of end-stage failing HCM and DCM hearts (Kötter et al. 2013).

The molecular mechanisms that control the complex splicing events of titin mRNA are still not completely understood. Cell culture experiments using embryonic rat cardiomyocytes suggested that the composition of titin isoforms is at least partly regulated by hormones, e.g., thyroid hormones or insulin, and requires activation of the PI3K/AKT signaling pathway (Krüger et al. 2008, 2010). More recent studies identified the splicing factor RBM20 as an essential player in this setting (Guo et al. 2012; Li et al. 2013).

More dynamically than by isoform transition, titin-based myofilament stiffness can be modulated by posttranslational modification. The following section will

highlight some key mechanisms by which posttranslational modification and particularly phosphorylation are thought to influence titin function.

6.2 Dynamic Modulation of Titin by Phosphorylation

6.2.1 Z-Disk and M-Band Phosphorylation

To date, phosphorylation sites have been detected in the Z-disk, I-band, and M-band portion of the titin molecule (Fig. 6.1).

The sequence insertions Zis-1 and Zis-5 at the NH_2-terminal end of titin contain various consensus sequences for proline-directed kinases, the so-called XSPXR motif repeats. These motifs were shown to be phosphorylated in vitro by extracellular signal-regulated kinases 1 and 2 (ERK1/2) and cyclin-dependent protein kinase-2 (Cdc2) (Gautel et al. 1996; Sebestyén et al. 1995). Four KSP-phosphorylation motifs were identified in the sequence insertion Mis-4 in the COOH-terminal part of M-band titin. Similar to the XSPXR repeats in Z-disk titin, the KSP motifs are phosphorylated by proline-directed kinases (Gautel et al. 1993). Phosphorylation of these motifs has been demonstrated to occur mainly in developing rather than differentiated adult muscles and may therefore be required for a proper sarcomeric integration of titin during myofibrillogenesis (Sebestyén et al. 1995; Gautel et al. 1993).

The M-band region of titin contains an autoinhibited serine/threonine kinase domain, which shares some homology with Ca^{2+}/calmodulin-regulated myosin light chain kinases (MLCK) (Gautel 2011). Activation of TK requires the removal of the C-terminal autoinhibitory tail; however, unlike other classical MLCKs the modulation of TK activity by Ca^{2+}-Calmodulin is rather weak (Mayans et al. 1998). Whether activation of the TK instead involves autophosphorylation of sites in the activation loop of the kinase is still under debate. It has further been proposed from molecular dynamics simulations that activating changes to the conformational state of the TK could be induced by mechanical stretch forces (Gräter et al. 2005; Puchner et al. 2008). Upon activation TK has been shown to interact with the ubiquitin-associated zinc-finger protein neighbor-of-BRCA1-gene-1 (Nbr1), which forms a signaling complex with p62/SQSTM1 and the muscle-specific ubiquitin E3 ligases MuRF1, MuRF2, and MuRF3 (Lange et al. 2005). Due to this interaction, TK has been suggested to be an important biomechanical stress sensor and mediator of hypertrophic signaling (Gautel 2011).

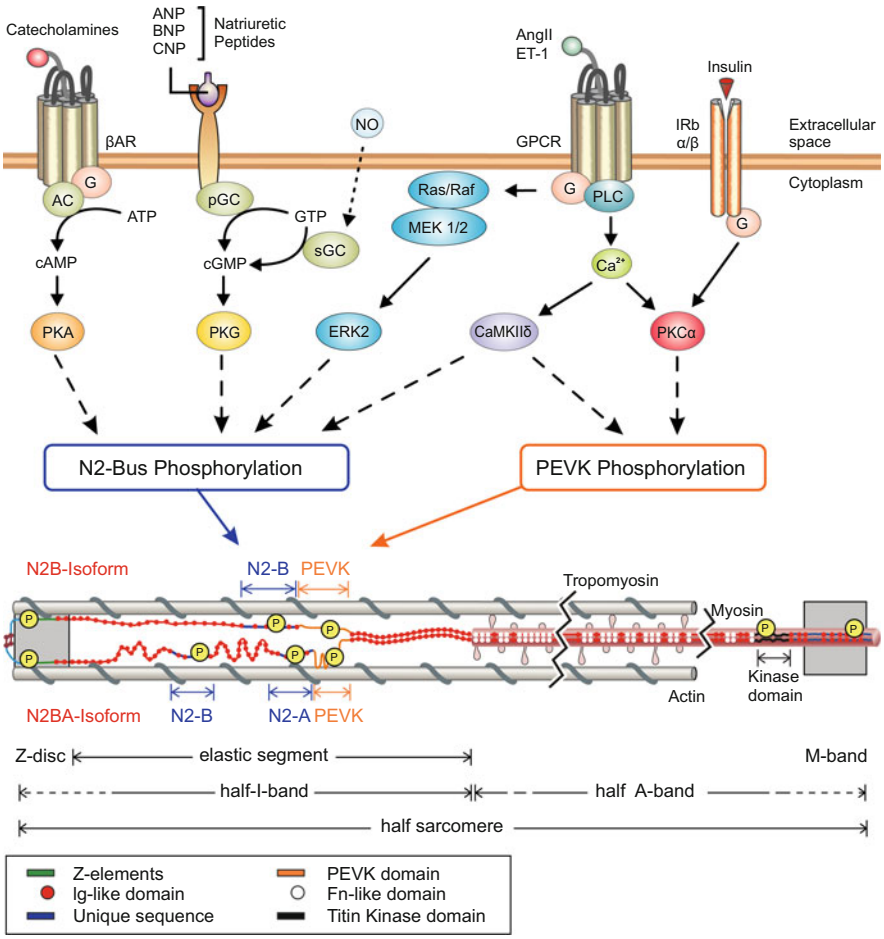

Fig. 6.1 Signal transduction pathways and kinases involved in phosphorylation of cardiac titin N2-Bus and PEVK. *AC* adenylate cyclase, *ßAR* beta-adrenergic receptor, *AngII* angiotensin II, *ANP* atrial natriuretic peptide, *ATP* adenosine triphosphate, *cAMP* cyclic adenosine monophosphate, *BNP* brain natriuretic peptide, *CaMKIIδ* Ca^{2+}/calmodulin-dependent protein kinase II delta, *CNP* natriuretic peptide, *ET-1* endothelin-1, *ERK2* extracellular signal-regulated kinase 2, *G* small G-protein, *GTP* guanosine triphosphate, *cGMP* cyclic guanosine monophosphate, *GPCR* G-protein coupled receptor, *sGC* soluble guanylyl cyclase, *pGC* peripheral guanylyl cyclase, *IRb* insulin receptor b, *NO* nitric oxide, *PKA* cAMP-dependent protein kinase, *PKC* Ca^{2+}-dependent protein kinase, *PKG* cGMP-dependent protein kinase

6.2.2 *I-Band Phosphorylation of Titin*

The main hub for titin phosphorylation is the elastic I-band region, particularly the N2-B region with its unique sequence (us) and the PEVK domain. Phosphorylation of these two titin domains dynamically modulates the passive mechanical properties of the myofilaments (Fig. 6.1).

CAMP-dependent protein kinase (PKA) has been shown to phosphorylate the cardiac-specific N2-B domain and thereby reduce passive tension in rat, bovine, and human cardiomyocytes (Yamasaki et al. 2002; Fukuda et al. 2005; Krüger and Linke 2006) (Fig. 6.2a). In human myocardium, PKA and cGMP-dependent protein kinase (PKG) both phosphorylate a serine residue in the cardiac-specific N2-Bus (Ser4185, UniProtKB accession number, Q8WZ42). Single molecule experiments using a recombinant construct of this region demonstrated that phosphorylation increases the persistence length of the N2-Bus and thereby reduces titin-based passive tension (PT) by up to 20 % (Krüger et al. 2009) (Fig. 6.2a). More recent studies applying mass-spectrometry analyses on kinase-treated recombinant titin fragments have identified further sites of PKA- and PKG-mediated phosphorylation in the N2-Bus, some of which are conserved among species (Kötter et al. 2013; Hamdani et al. 2013c) (an overview on currently identified I-band phosphorylation sites is provided in Table 6.1). The presence of multiple kinase-dependent phosphorylation motifs within this region suggests a mechanism by which titin stiffness is rapidly modulated via simultaneous hypo- or hyperphosphorylation of these sites, e.g., in response to beta-adrenergic stimulation.

In vitro kinase assays using recombinant fragments of different I-band domains of titin have shown PKG-mediated phosphorylation of several Ig domains and the N2-A domain of titin (Krüger et al. 2009). Since modification of these domains does not contribute to titin-based PT under physiological conditions (Krüger et al. 2009), the functional role of these phosphorylation motifs remains to be elucidated. The N2-A region associates with muscle ankyrin repeat proteins (MARPs) and in skeletal muscle with Calpain-3, thereby linking titin to hypertrophic signaling and the protein quality control machinery (Miller et al. 2003; Hayashi et al. 2008). Hence, phosphorylation of the N2-A region could alter protein–protein interactions and thereby modulate the respective signaling pathways.

PKA shares a conserved phosphorylation site (Ser4010) with ERK2, which has been shown to target the N2-Bus of titin at three different serine residues (see Table 6.1) (Raskin et al. 2012). Although it has not been confirmed experimentally, yet, this ERK2-mediated phosphorylation is expected to result in a reduction in titin-based PT. Interestingly, titin also associates with the four-and-a-half LIM domain proteins FHL-1 and FHL-2 in the N2-B region (Sheikh et al. 2008; Lange et al. 2002) and is thereby linked to the Ras-Raf-MEK-ERK pathway, which has an important cardioprotective role (reviewed in Wang 2007). In the absence of biomechanical stress, MEK1/2 act as scaffold proteins for ERK2 and anchor the kinase in the cytoplasm (Tanoue et al. 2000; Chuderland and Seger 2005). However, it may be speculated that the titin/FHL/MEK complex tethers a certain portion of ERK2 to the myofilaments. In response to mechanical strain, MEK then activates ERK2, which induces a conformational change that promotes its translocation to the nucleus, where ERK2 is responsible for activation of several transcription factors involved in hypertrophic growth (Mebratu and Tesfaigzi 2009). Interestingly, disruption of the FHL-Raf-MEK-ERK-signaling complex by genetic knockdown of FHL1 in mice resulted in increased myofibrillar compliance and impaired hypertrophic signaling (Sheikh et al. 2008). ERK2-mediated phosphorylation may

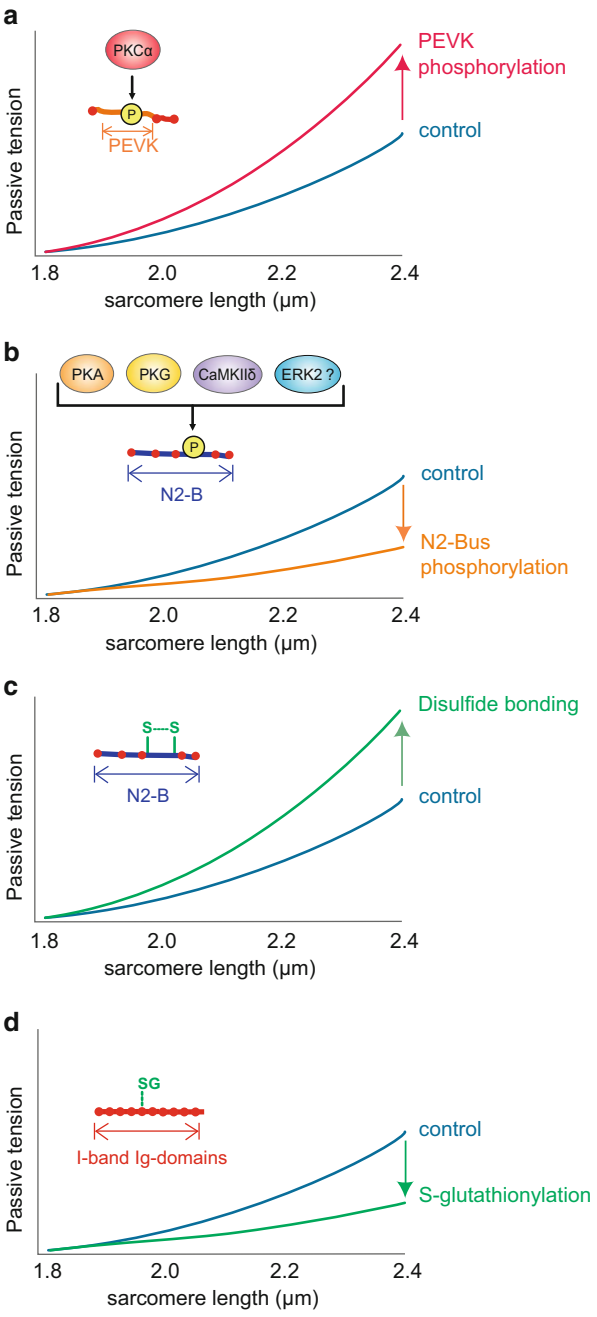

Fig. 6.2 Posttranslational modification of titin and effects on myofilament stiffness. (**a**) Phosphorylation of the PEVK region of titin by PKCα increases titin-based myofilament stiffness; (**b**) phosphorylation of the N2-B region of titin by PKA, PKG, ERK and CaMKIIδ decreased myofilament stiffness; (**c**) disulfide bonding within the N2-B region of titin increases titin-based myofilament stiffness; (**d**) S-glutathionylation (SG) of unfolded Ig domains lowers titin stiffness

Table 6.1 I-band phosphosites identified in titin N2-Bus/PEVK

Phosphosite	Titin region	Species	Kinase	Reference
Ser3744	N2-Bus	Rat	PKA/PKG	Kötter et al. (2013)
Ser3750	N2-Bus	Conserved	CaMKIIδ	Hidalgo et al. (2013)
Thr3756	N2-Bus	Human	CaMKIIδ	Hamdani et al. (2013c)
Thr3761	N2-Bus	Human	CaMKIIδ	Hamdani et al. (2013c)
Ser3787	N2-Bus	Human	CaMKIIδ	Hamdani et al. (2013c)
Ser3799	N2-Bus	Human	CaMKIIδ	Hamdani et al. (2013c)
Ser3862	N2-Bus	Human	CaMKIIδ	Hamdani et al. (2013c)
Thr3864	N2-Bus	Human	CaMKIIδ	Hamdani et al. (2013c)
Ser3918	N2-Bus	Human	ERK2	Raskin et al. (2012)
Ser3960	N2-Bus	Human	ERK2	Raskin et al. (2012)
Ser4010	N2-Bus	Conserved	PKA	Kötter et al. (2013)
			ERK2	Raskin et al. (2012)
Ser4012	N2-Bus	Conserved	PKA	Kötter et al. (2013)
Ser4062	N2-Bus	Conserved	CaMKIIδ	Hamdani et al. (2013c)
Ser4065	N2-Bus	Human/dog	PKA	Kötter et al. (2013)
Thr4065	N2-Bus	Rat/mouse	PKA	Kötter et al. (2013)
Ser4092	N2-Bus	Human/dog	PKG	Kötter et al. (2013)
Ser4099	N2-Bus	Conserved	PKG	Kötter et al. (2013)
			CaMKIIδ	Hamdani et al. (2013c)
Ser4185	N2-Bus	Human	PKA/PKG	Krüger et al. (2009), Kötter et al. (2013)
Thr4203	N2-Bus	Human	CaMKIIδ	Hamdani et al. (2013c)
Ser4209	N2-Bus	Conserved	CaMKIIδ	Hidalgo et al. (2013)
Ser11878	PEVK	Conserved	PKCα	Hidalgo et al. (2013)
			CaMKIIδ	Hidalgo et al. (2013)
Thr11922	PEVK	Human	CaMKIIδ	Hamdani et al. (2013c)
Thr11932	PEVK	Human	CaMKIIδ	Hamdani et al. 2013c
Thr11969	PEVK	Human	CaMKIIδ	Hamdani et al. (2013c)
Thr12007	PEVK	Conserved	CaMKIIδ	Hamdani et al. (2013c)
Ser12009	PEVK	Conserved	CaMKIIδ	Hamdani et al. (2013c)
Ser12022	PEVK	Conserved	PKCα	Hidalgo et al. (2009, 2013)
			CaMKIIδ	Hamdani et al. (2013c)

Residues are numbered according to the human titin sequence (UniProtKB accession number, Q8WZ42-1)

therefore be another regulatory mechanism that contributes to the biomechanical stress sensing mediated at the N2-Bus of titin.

Finally, phosphorylation of the N2-B region is induced by Ca^{2+}/calmodulin-dependent protein kinase II delta (CaMKIIδ). Using mass spectrometry ten different serine and threonine residues have been identified as putative CaMKIIδ targets in the N2-Bus (Hidalgo et al. 2013; Hamdani et al. 2013c). CaMKIIδ-mediated phosphorylation of single murine cardiomyocytes markedly reduced titin-based

passive tension by up to 50 % (Fig. 6.2a). This finding is in line with the observation that phosphorylation of the CaMKIIδ-targeted phosphosites and titin-based PT is significantly reduced in CaMKIIδ-depleted mice (Hamdani et al. 2013c).

Interestingly, mass spectrometry further identified seven CaMKIIδ-targeted phosphorylation motifs in the PEVK region (Hidalgo et al. 2013; Hamdani et al. 2013c), another elastic titin domain that is functionally modified by phosphorylation (Table 6.1). Previous studies had shown that phosphorylation of Ser11878 and Ser12022 in the PEVK region by Ca^{2+}-dependent protein kinase C α (PKCα) reduces the persistence length of the respective PEVK peptide and thereby increases titin stiffness by >20 % in skinned myocytes from mouse and pig heart (Hidalgo et al. 2009; Hudson et al. 2010) (Fig. 6.2b). Interestingly, although the identified PKCα-targeted serine residues are conserved among these species, this effect was not observed in cardiomyocytes from dog heart (Hamdani et al. 2013a).

The observation that phosphorylation of the N2-Bus by PKA, PKG, or CaMKIIδ leads to an increase of the persistence length and decreases myofilament passive tension, whereas phosphorylation of the PEVK domain by PKCα reduces the persistence length of the respective region and increases passive tension, may seem rather contradictory at first sight. A possible explanation is based on the amino acid sequences of the respective titin segments: The N2-Bus contains a high amount of acidic (negatively charged) amino acids, resulting in a low isoelectric point of this domain. In contrast, the PKCα-targeted region of the PEVK domain comprises many basic residues and therefore has a much higher isoelectric point than the N2-Bus. Introduction of a negatively charged phosphate group into an already negatively charged environment could lead to intramolecular electrostatic repulsion. This, in turn, could influence the intrinsically disordered structure of the N2-Bus and thereby increase its distensibility, reflected by an increased persistence length. In the PEVK region, the negatively charged phosphate residues are added to a basic environment, which could prevent electrostatic repulsion and potentially generate additional intramolecular ionic interactions. Such interactions likely reduce the persistence length of the PEVK domain and therefore impair its distensibility. According to this view, phosphorylation of several residues within each region should multiply the phosphorylation-induced effect on the molecular elasticity of titin and thereby allow a dynamic and presumably nonlinear modulation of titin stiffness (Kötter et al. 2013).

This hypothesis also implies that the effect of a few phosphorylated residues in one titin domain, e.g., in the PEVK region, may be counterbalanced or even offset by hypophosphorylation of the other elastic titin domain, e.g., the N2-Bus. This consideration may also provide an explanation for the observation that CaMKIIδ-mediated phosphorylation of titin results in a decrease in titin stiffness, although the kinase targets both elastic domains, N2-Bus and PEVK (Hamdani et al. 2013c).

Analysis of titin phosphorylation is an emerging field and online routines (e.g., GPS, Scansite, NetphosK) predict phosphorylation of the titin sequence at numerous additional sites and by various other kinases. Therefore, identification of more

phosphorylation motifs and characterization of their functional relevance seem just a matter of time.

6.3 Regulation of Titin Phosphorylation in Health and Disease

6.3.1 Altered Titin Phosphorylation in Heart Failure

The cellular mechanisms that regulate titin function via domain-specific phosphorylation are only beginning to be understood. However, the dynamic modulation of titin does not only provide possibilities for its adaptation to various physiological conditions, but also represents a potential target for disturbances in the setting of cardiac diseases. It is therefore not surprising that there is a growing body of evidence showing altered titin phosphorylation in different types of heart failure.

First evidence for PKG-dependent hypophosphorylation of total titin in failing hearts was provided from PKG back-phosphorylation assays performed on heart samples from patients with dilated cardiomyopathy (DCM) (Krüger et al. 2009). This finding was supported by a study that, by using the phosphospecific protein stain ProQ-Diamond (Invitrogen), demonstrated a hypophosphorylation of total titin and raised cardiomyocyte resting tension in biopsies of heart failure (HF) patients (Borbely et al. 2009). Interestingly, PKA treatment of skinned cardiac myocytes from these samples effectively lowered passive myocyte stiffness to that of control cells (Borbely et al. 2009).

However, the commonly used back-phosphorylation assay and the phosphospecific protein stain ProQ-Diamond both fail to distinguish the phosphorylation state of different titin domains and can therefore not indicate differently altered kinase activities. Moreover, it turned out that the ProQ-Diamond stain does not reliably depict changes in the phosphorylation of the PEVK region (Hudson et al. 2011). Hence, the experimental identification of kinase-specific phosphorylation motifs in the two elastic titin regions has brought about important advances, in that phosphosite-directed antibodies could be generated and now allow domain- and kinase-specific analysis of the titin phosphorylation status.

In a mouse model with transverse aortic constriction-induced HF (TAC model), Western blot analyses using phosphosite-directed antibodies showed a significant increase in the relative phosphorylation levels of Ser11878 in the PEVK spring element of titin. Interestingly, the changes in titin phosphorylation overruled the switch in titin isoform expression toward more compliant N2BA isoforms and resulted in an increase in titin-based passive myocardial stiffness (Hudson et al. 2011). In a set of samples from human DCM and HCM patients without concomitant changes in titin isoform composition, Western blot analysis of the titin phosphorylation status revealed a significantly decreased N2-Bus phosphorylation at Ser4010 (PKA- and ERK-targeted), Ser4099 (PKG-targeted), and Ser4185

(PKA- and PKG-targeted). In addition, PKCα-mediated phosphorylation of Ser11878 in the PEVK region was significantly enhanced. The changes in titin phosphorylation status resulted in largely increased passive cardiomyocyte stiffness. The study therefore demonstrated that hypophosphorylation of the N2-Bus and increased phosphorylation of the PEVK domain can act complementary to elevate passive tension in failing human hearts (Kötter et al. 2013). A similar finding was reported in a patient-mimicking hypertensive dog model of heart failure with preserved ejection fraction (HFpEF) that showed pronounced titin stiffening caused by altered isoform composition, hypophosphorylation at Ser4010/4099 within titin N2-Bus, and increased relative phosphorylation of Ser11878 in titin-PEVK (Hamdani et al. 2013a). Western blot analyses performed on failing human hearts also detected increased relative phosphorylation levels for two CaMKII-δ-dependent phosphosites, S4062 within the N2-Bus region and S12022 in the PEVK region (Hamdani et al. 2013c).

Interestingly, the reported changes in the titin phosphorylation status were more pronounced in the stiff N2B than in the more compliant N2BA isoform (Borbely et al. 2009; Kötter et al. 2013). From a physiological point of view, promoting the stiffness of the shorter and stiffer N2B-titin molecule may have a more effective impact on titin stiffness than modulation of the more compliant N2BA isoform, especially as it has higher relative expression levels in the human heart. Another possible explanation for this observation could be the limited accessibility of the phosphorylation motifs in N2BA-titin, which, unlike N2B-titin, is barely stretched in the physiological sarcomere length range.

The majority of the studies discussed above show a reduction in the relative phosphorylation levels of the PKA-targeted Ser4010 and the PKG-targeted Ser4099 in titin N2-Bus, suggesting that impaired PKG and PKA signaling account for part of the differences observed in failing hearts. In early stages of cardiac insufficiency, overactivation of the β-adrenergic system is a common compensatory mechanism of the heart. During disease progression this compensatory response may cause a neurohormonal overstimulation and lead to downregulation of the β-receptor density (Murphree and Saffitz 1989; Packer 1995) and subsequently to disturbed PKA signaling (Lohse et al. 2003). In turn, impaired PKA activation could result in chronic hypophosphorylation of titin N2-Bus, raise titin-based myofilament stiffness, and thereby contribute to passive myocardial stiffening during progression of HF. This idea is supported by a study showing that beta-blockade reversed myofilament dysfunction and enhanced myofilament responsiveness to protein kinase A in remote myocardium from pig after myocardial ischemia (Duncker et al. 2009).

Abnormal PKG signaling has been associated with ventricular hypertrophy and heart failure in animal models as well as in human patients (reviewed in Tsai and Kass 2009) and has further been associated with increased passive stiffness in myocytes from HFpEF patients (van Heerebeek et al. 2012). Therefore, restoring PKG signaling by increasing the availability of cGMP through phosphodiesterase-5 (e.g., by sildenafil) inhibition or increasing guanylyl cyclase activity may be a useful strategy to recover PKG-dependent titin phosphorylation, lower passive tension, and thereby improve cardiac dysfunction. A first successful approach has

recently been reported in a hypertensive dog model with diastolic dysfunction: treatment with sildenafil and brain natriuretic peptide increased cGMP levels and resulted in higher titin phosphorylation and lowered titin-based cardiomyocyte stiffness (Bishu et al. 2011).

Both overactivation of PKCα and increased CaMKII activity have frequently been observed during heart failure and could explain the reported alterations in titin domain phosphorylation by these kinases (reviewed in Solaro 2008; Zhang and Brown 2004). The involvement of CaMKII and PKCα in many aspects of cardiac hypertrophy and heart failure suggests that both kinases would have potential as therapeutic targets in HF patients.

6.3.2 *Hormonal Influences on Titin Phosphorylation: Relevance for Metabolic Diseases*

It has previously been demonstrated that titin isoform composition is partly regulated by hormone-induced activation of PI3K/AKT signaling (Wu et al. 2007; Krüger et al. 2008). However, emerging evidence indicates an important role for hormones such as insulin in dynamic modulation of titin phosphorylation in health and disease.

First evidence for insulin-induced titin phosphorylation was provided from cell culture experiments showing that insulin treatment of cultured embryonic rat cardiomyocytes induces a rapid increase in total titin phosphorylation (Krüger et al. 2010). Considering this observation, disturbed insulin signaling in the setting of diabetes mellitus should result in substantial hypophosphorylation of titin and increased titin-based stiffness. First titin analyses in hearts from a rat model with streptozotocin-induced type-1 diabetes mellitus did not reveal any changes in total titin phosphorylation when using the ProQ-diamond detection method (Krüger et al. 2010). Nonetheless, the importance of insulin signaling for titin phosphorylation was recently demonstrated in human cardiac biopsies from patients with coronary artery disease and type-2 diabetes mellitus, as well as in a rat metabolic risk model with HFpEF. In both studies altered insulin signaling was associated with significant hypophosphorylation of titin and increased myofilament stiffness (Falcão-Pires et al. 2011; Hamdani et al. 2013b). Future studies will certainly provide more insight into the signal transduction pathways that control insulin-induced titin phosphorylation and identify the kinases involved in this mechanism. Hopefully, the results of these studies will provide further insight into the development and treatment of cardiac dysfunction in patients with diabetic cardiomyopathy.

6.4 Posttranslational Modification of Titin in Response to Oxidative Stress

Over the past decades, clinical and experimental studies have provided substantial evidence that oxidative stress, defined as an excess production of reactive oxygen species (ROS), is enhanced in HF (reviewed in Tsutsui et al. 2011). The importance of oxidative stress is increasingly recognized as it may represent one key pathophysiological mechanism leading to maladaptive myocardial remodeling (Takimoto and Kass 2007). It has been suggested that oxidative stress also promotes structural modifications of titin that modulate titin-based myocardial stiffness. Under oxidizing conditions the human N2-B-unique sequence (N2-Bus) contains up to three disulfide bridges (Grützner et al. 2009). Stretch experiments performed on isolated myofibrils from human heart showed that the reducing agent thioredoxin increases titin-based stiffness by altered extensibility of the N2-Bus (Fig. 6.2c). It was therefore concluded that increased oxidant stress can elevate titin-based cardiomyocyte stiffness and may therefore contribute to the global myocardial stiffening frequently seen in the aging or failing heart (Grützner et al. 2009).

More recently, titin has been identified as a target of S-glutathionylation, an end product of the nitric-oxide-signaling cascade (Avner et al. 2012). In response to myofilament stretch titin immunoglobulin (Ig) domains unfold mechanically and thereby seem to expose cysteine residues, which can be S-glutathionylated (Alegre-Cebollada et al. 2014). Further investigations applying atomic force microscopy demonstrated that S-glutathionylation decreases mechanical stability of the modified Ig domains. This Ig domain instability favors a more extensible state of titin and thereby promotes the elasticity of cardiomyocytes (Alegre-Cebollada et al. 2014) (Fig. 6.2d). These observations raise the possibility that titin-based myofilament stiffness could be substantially modulated under oxidative stress conditions, such as cardiac ischemia.

6.5 Conclusions

The recent advances in the field of titin research have convincingly demonstrated that titin is an important target for dynamic posttranslational modifications under physiological as well as under pathological conditions. Most of the mechanisms that regulate kinase-specific modulation of titin stiffness are only beginning to be understood. Considering the size of the titin molecule and its complex integration into a variety of cardiac signaling processes, the identification and characterization of further phosphorylation sites and other posttranslational modifications, such as glycosylation, seem just a matter of time. The results of future studies will not only permit a better understanding of the mechanisms that lead to diastolic dysfunction

in different settings of cardiac diseases, but could also be important for the development of therapeutic strategies for the treatment of diastolic heart failure.

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Chapter 7
The M-Band: Not Just Inert Glue but Playing an Active Role in the Middle of the Sarcomere

Irina Agarkova and Elisabeth Ehler

Abstract The M-band is the region in the middle of the sarcomere that holds the thick (myosin) filaments in place by linking them to the elastic filament system made up of titin. It was recently suggested to play an important role by averaging the lateral misbalances of thick filament forces in the activated sarcomere and to actively support titin to restore the lateral order in the sarcomere. Its marker constituent in vertebrates is myomesin, but the rest of its molecular composition depends on the developmental stage and type of striated muscle and eventually results in a more rigid or a more elastic phenotype. The studies of myomesin domain interactions suggest that thick filaments might be cross-linked in their central "bare" zone by antiparallel myomesin dimers that bind myosin with their N-terminal domain and interact end to end with their C-terminal domains. In addition to its role in buffering active contractile force, the M-band is the origin of signalling processes that can go all the way to the nucleus to affect gene transcription. The mechanical deformations of the M-band filaments during muscle contraction allow the integration of smart molecular sensors to monitor the activity of the sarcomere and initiate physiological adaptations. While it is known that the composition of the M-band is altered in cardiomyopathy, only few mutations in M-band components were characterised so far that lead to hereditary cardiomyopathy, but results obtained from next-generation sequencing are bound to expand on this.

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7.1 The M-Band Is Part of the Sarcomere Cytoskeleton

In striated muscle the contractile proteins actin and myosin are arranged into paracrystalline structures, the myofibrils, which have the sarcomeres as their basic unit. A sarcomere is defined as the region between two Z-discs, where the actin (thin) filaments are anchored (Fig. 7.1). The bipolar thick filaments composed of about 300 molecules of myosin are cross-linked in the middle of the sarcomere by the M-band. Single molecules of titin span between the Z-disc, where their N-terminus is situated (Young et al. 1998), and the M-band, where their C-termini overlap (Obermann et al. 1996). Thus, the two transverse scaffolds, Z-discs and M-bands, together with the longitudinal connection of both via the elastic titin filaments constitute the basic sarcomeric skeleton essential for the structural integrity of the contraction unit (Ehler and Gautel 2008).

Over the years the Z-disc has attracted a lot of attention, and a multitude of proteins were identified that localise there. These range from mainly structural components such as alpha-actinin to signalling proteins such as MLP (muscle LIM protein) and phosphatases such as calcineurin (reviewed in Frank and Frey 2011; Luther 2009). The M-band is much less studied and only a handful of bona fide components are known at present (Lange et al. 2005a; see also Fig. 7.1). These comprise proteins with mainly a structural function such as the three members of the myomesin family but also enzymes such as MM-CK (muscle creatine kinase)

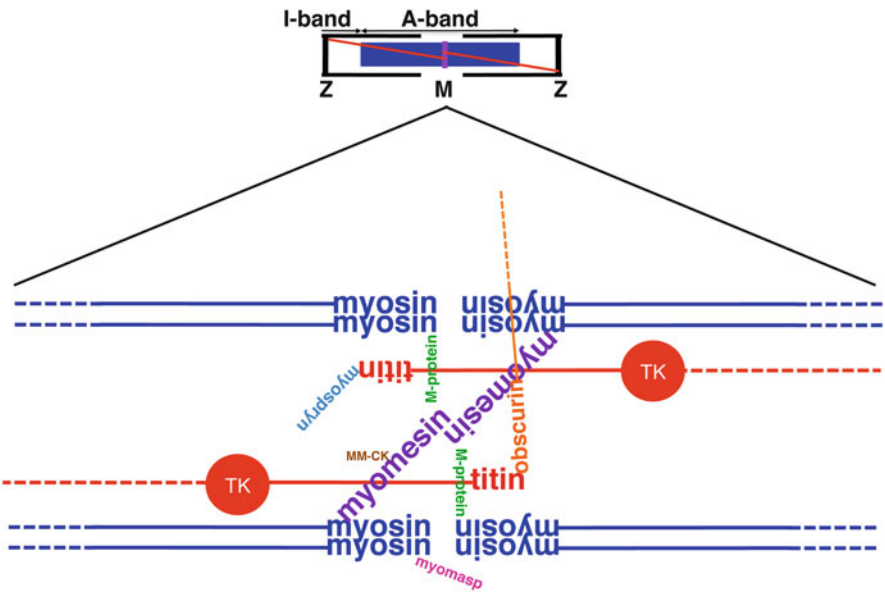


Fig. 7.1 Schematic representation of a sarcomere with a scheme showing its main molecular constituents below. Myosin is represented in *blue*, titin in *red* and the M-band in *purple*. *Dashed lines* indicate that the proteins extend beyond the font. *TK* titin kinase domain

and proteins containing signalling domains such as obscurin (Young et al. 2001; Fig. 7.1). The aim of this review is to describe the M-band in more detail and to discuss its role in the sarcomere beyond myofibrillogenesis and in particular in cardiomyopathy.

7.2 M-Band Function: Balancing the Forces in the Contracting Sarcomere

The muscle structure is rather elastic, and the original almost crystalline order of the contractile filaments is restored even after substantial deformations caused either by passive force applied from the outside or the forces that arise by active contraction of the muscle. It is achieved due to the smart integration of numerous viscoelastic elements into the sarcomere cytoskeleton. The M-band makes up an important part of this construction, and its elasticity allows for reversible deformations of the myosin filament lattice (Tskhovrebova and Trinick 2012).

However, in addition to acting as an elastic spacer and restoring the order after deformation, the M-band might have a distinct function in the contracting muscle. This role is suggested by the variable appearance and distinct protein composition of the M-band in different muscle types.

The thick filaments are attached to the Z-discs by titin filaments, the elasticity of which keeps them in the central position in the passive sarcomere. However, this central position is intrinsically unstable during sarcomere activation. Indeed, any small force imbalance between the two filament halves, caused, for example, by a different proportion of activated crossbridges or by slightly different overlap with the actin filaments on one side, would lead to the deviation of the myosin filaments from the centre position and accordingly to a higher displacing force. The dramatic changes that occur to the arrangement of the A- and M-bands in an activated muscle were demonstrated in electron micrographs by Horowits and Podolsky in the 1980s (Horowits and Podolsky 1987).

A simple calculation based on single-molecule measurements of titin elasticity shows that the restoring force produced by titin alone might not be sufficient to compensate for the intrinsic instability of individual myosin filaments. However, the cross-links at the M-band might be rather helpful in this respect, as they provide an averaging of the force imbalances between all myosin filaments present in one sarcomere (Agarkova and Perriard 2005). Assuming that force differences are completely random, the total force reduction due to the M-band cross-links is equal to the square root of the thick filament number, giving a factor of 30 (as the standard sarcomere consists of about 1,000 myosin filaments). Thus, theoretically, due to the presence of the M-band, the restoring force generated by titin filaments in the whole sarcomere is substantially amplified by 30 times.

7.3 Structure of the M-Band as Studied by Electron Microscopy

The M-band is apparent as a series of transverse dark lines in electron micrographs of longitudinal sections of striated muscle in the middle between two Z-discs. Three to five M-lines separated by 22 nm intervals are discernible in the central "bare" zone of the myosin filaments that lacks the myosin heads. The numbers of lines and their intensity vary between striated muscle types, which is probably due to distinct molecular composition (Lange et al. 2005a; Schoenauer et al. 2008). In the cardiac muscle the appearance of the M-band depends on the developmental stage and on the functional state. For example, in electron micrographs of the embryonic heart, it is not possible to discern any discrete M-band pattern (Manasek 1968), while in adult cardiac muscle, usually five lines can be distinguished in the M-band region, with slight variation in intensity between different species (Pask et al. 1994). This picture changes in disease, where the ultrastructure of the M-band becomes less distinct again (Ehler et al. 2001).

7.4 The Myomesin Protein Family

The main players in the M-band are the three members of the myomesin protein family. Historically the first discovered family member was M-protein (myomesin-2, MYOM2), originally detected by Masaki and Takaiti (1974) and subsequently characterised more closely by Eppenberger et al. (1981). The "proper" myomesin (myomesin-1, MYOM1) was identified a few years later using monoclonal antibodies against purified M-band fractions and was subsequently shown to be a 185 kDa protein (Grove et al. 1984). These two M-band components were correlated to the appearance of the M1 (M-protein) and M4, M4′ lines (myomesin) on electron micrographs of the sarcomere (Obermann et al. 1996). The additional M6, M6′ lines may be due to the presence of myomesin-3, which was identified recently by comparative sequence analysis of a mouse gene database (Schoenauer et al. 2008).

The most important member of the myomesin protein family is the protein myomesin (Fig. 7.2). It is found in all types of vertebrate striated muscles that were investigated (Agarkova et al. 2000) as well as in the first sarcomeres formed in the developing heart (Ehler et al. 1999). Accordingly, it is expressed in a nearly stoichiometrical ratio to myosin in various muscle types (Agarkova et al. 2004; see also Fig. 7.3). In contrast to myomesin, the other two protein family members, M-protein and myomesin-3, show restricted, muscle type-specific expression patterns. M-protein is found in adult heart and fast skeletal muscle (Grove et al. 1985). Myomesin-3 appears in fibres of intermediate speed in skeletal muscle and is differentially expressed in the mouse and human heart (Schoenauer et al. 2008; see also Fig. 7.3).

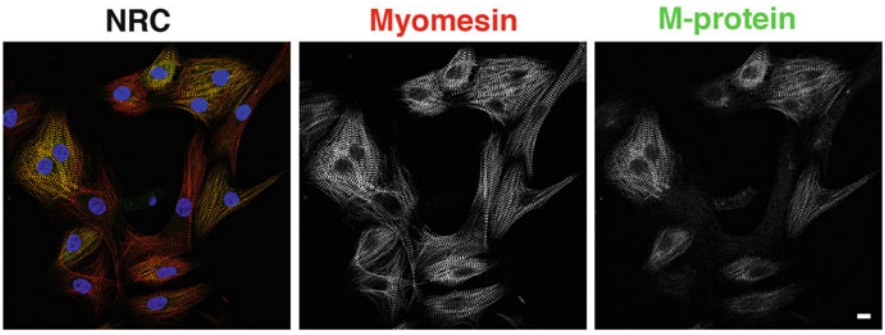


Fig. 7.2 Myomesin and M-protein are differentially expressed in neonatal rat cardiomyocytes (NRC) in culture. Confocal micrographs of NRC stained with antibodies against myomesin (*red signal* in overlay) and against M-protein (*green signal* in overlay) show that myomesin is present in every single M-band in every NRC; some of the NRC have not yet switched on M-protein expression and thus lack a *yellow signal* in the overlay due to colocalisation. Nuclei were stained with DAPI and are depicted in *blue*. Scale bar equals 10 μm

7.5 Myomesin Protein Family: Dimers That Cross-Link Myosin Filaments

The genes that encode the three members of the myomesin family are closely related, suggesting a common ancestor. The published genome of the non-vertebrate *Ciona intestinalis* contains only one myomesin gene. This indicates that the myomesin protein family may have arisen as a result of two genome-wide duplications and subsequent functional diversification, which occurred in the vertebrate lineage after separation from the common ancestor of all chordates (Putnam et al. 2008). In addition to a close relation on the gene level, the three members of the myomesin family share an identical domain arrangement. The N-terminal domain of myomesin, My1, binds to myosin and is predicted to have an intrinsically disordered/unstructured conformation (Obermann et al. 1995). Such unstructured protein fragments are often associated with molecular recognition and target-induced binding. According to amino acid comparison, the N-terminal domains of the three myomesin family members are the most diverse part of the molecule. It is suggested that they may serve to target each family member to a certain position in the bare zone of the thick filament (Schoenauer et al. 2008). The remainder of the molecule is comprised of identically arranged immunoglobulin-like (Ig) and fibronectin type III (Fn) domains (Schoenauer et al. 2008; Steiner et al. 1999; see Fig. 7.4). The C-terminal domains of myomesin, My13, are able to dimerise (Lange et al. 2005b; see Fig. 7.4). The crystal structure of this immunoglobulin-like domain revealed an antiparallel dimerisation mediated by intermolecular beta-sheets (Pinotsis et al. 2008). The same molecular mechanism is observed in assembly complexes of titin and also of filamin (Pinotsis et al. 2009). Despite the high sequence identity, the corresponding C-terminal domains of all three myomesin family members interact only in a homotypic manner, i.e. My13 only interacts with

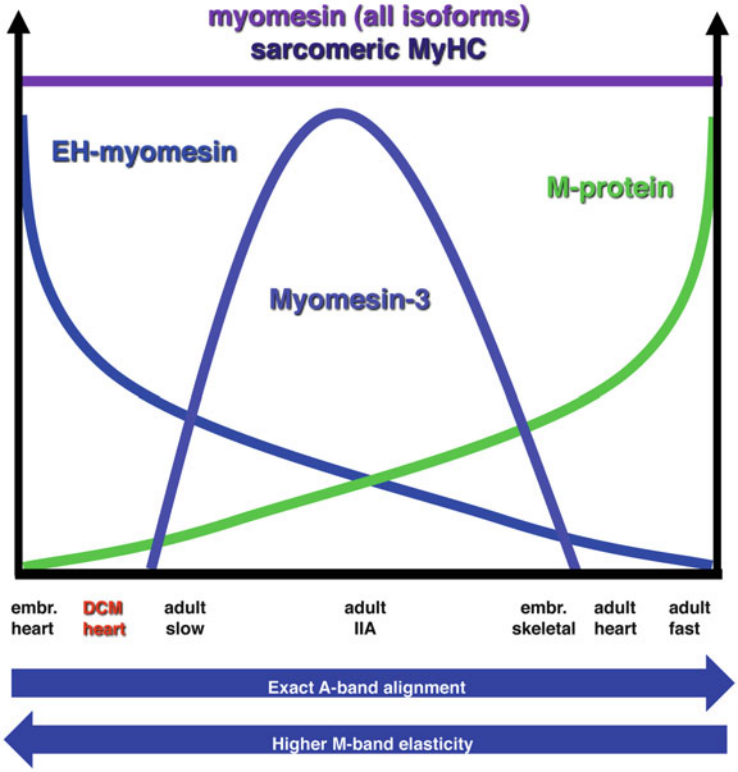
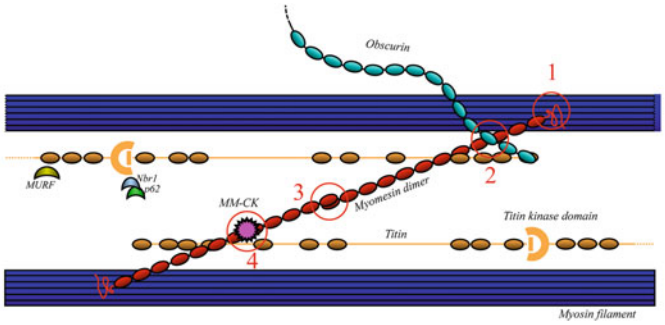


Fig. 7.3 Chart showing the expression of different myomesin family members in different muscle types. While the expression levels of myomesin and sarcomeric myosin are always tightly correlated, the adult heart expresses high levels of M-protein, while the embryonic heart expresses high levels of the splice variant EH-myomesin. Myomesin-3 is expressed in intermediate contraction speed fibres. The combination of myomesin family members expressed affects the elasticity and also the alignment of the A-bands and hence the clear visibility of M-lines in the electron microscope

itself but not with the corresponding domains of M-protein or myomesin-3 (Schoenauer et al. 2008). This suggests that they all form antiparallel dimers which act as a structural bridge between neighbouring myosin molecules, while the dimer of each myomesin family member has a specific lateral position along the central zone of the myosin filament, which is defined by the sequence of its N-terminal domain.



1. Interaction of N-terminal domain of myomesin with myosin
2. Myomesin interaction with obscurin
3. Antiparallel homodimerisation of C-terminal domains of myomesin
4. MM-CK binding to myomesin

Fig. 7.4 Schematic representation of the molecular domain interactions of the three M-band components from which structural data is available, myomesin (*red*), titin (*brown*) and obscurin (*turquoise*). The antiparallel myosin tails are depicted as a single *blue bar*

7.6 Alternative Splicing of Myomesin

In contrast to M-protein and myomesin-3, myomesin has splice variants. In the embryonic heart, a splice variant of myomesin is predominant that contains an insert of about 100-amino acid length between domains My6 and My7 (Agarkova et al. 2000). This myomesin isoform is found also in slow and extraocular muscles (Agarkova et al. 2004; Wiesen et al. 2007). Single-molecule experiments showed that the alternatively spliced EH (embryonic heart) insert has a disordered conformation and functions like an entropic spring, similar to the PEVK domain in titin (Schoenauer et al. 2005). EH-myomesin was proposed to confer more elasticity to the M-bands in myofibrils working under eccentric contraction conditions (Agarkova and Perriard 2005). In a healthy adult heart, expression of this splice variant is restricted to the conduction tissue (Meysen et al. 2007). However, in dilated cardiomyopathy, which among other subcellular defects is characterised by less well-aligned myofibrils (Ehler et al. 2001), an upregulation of the EH-myomesin isoform is correlated to the functional status of the failing heart (Schoenauer et al. 2011).

7.7 Correlation of M-Band Appearance and Molecular Composition

How are all these different components arranged in three dimensions, and is there any correlation between molecular composition and M-band appearance in the electron microscope? The problem is that the electron dense appearance of the M-lines on electron micrographs is rather due to the decoration with the muscle isoform of creatine kinase (MM-CK) which sits on the myomesin molecule than the myomesin molecules themselves (Hornemann et al. 2003). The chains of immunoglobulin- and fibronectin-like domains alone are not visible on electron micrographs. However, some parallels are established. The most prominent M4/M4′ lines may represent the MM-CK molecules attached to myomesin which is found in all muscles. Expression of M-protein correlates to the presence of the central M1 line (Obermann et al. 1996), while the M6/M6′ lines are linked to the expression of myomesin-3 in intermediate speed fibres (Schoenauer et al. 2008). In addition, the M-lines are not discernible in electron micrographs of embryonic heart muscle, which is currently explained by a more extended and flexible M-band structure of this muscle leading to tiny misalignments and thus a more diffuse distribution of the MM-CK dots that decorate the M-band proteins (Agarkova et al. 2000). In transverse sections, the M-band shows a hexagonal arrangement that can be very well correlated with models that include myomesin dimers (Lange et al. 2005a). Hopefully, a three-dimensional single-particle analysis of electron micrographs of negatively stained muscle, as beautifully shown by Al-Khayat for the myosin head-containing zone of the A-band, will be available soon to shed more light on the M-band structure as well (Al-Khayat et al. 2013).

7.8 Other M-Band Components

Apart from these structural components of the M-band, two obvious additional components must be considered, the C-termini of the titin molecules, which stretch all the way from the Z-disc, and the myosin tails that constitute the bare zone of the A-band. As far as titin is concerned, it is assumed that C-termini from opposite Z-discs overlap in an antiparallel fashion (Obermann et al. 1996). At the edge of the M-band region, the titin molecule contains a kinase domain, which can be activated by mechanical stretch (Gräter et al. 2005). While there is some information on the organisation of myosin in the rest of the A-band (Al-Khayat et al. 2013), how the very tails of this molecule are arranged in the M-band is still a mystery.

Until the beginning of this century the above-mentioned proteins were the only known structural components of the M-band. However, in the last decade a number of novel players were identified that move the M-band from a merely structural connection site to a potential node for signalling (Fig. 7.1). The first of these was the finding that obscurin, another huge modular protein, mainly composed of

immunoglobulin-like domains, is found predominantly at the M-band in the adult heart (Young et al. 2001). Obscurin has a molecular weight of 800 kDa and possesses a calmodulin-binding IQ motif and a Rho guanine nucleotide exchange factor domain at its C-terminus. Splice variants exist that replace this stretch with one or two kinase domains (Small et al. 2004). So far a signalling role of obscurin has remained elusive, and obscurin knockout mice do not show any M-band phenotype, but have a defect in their sarcoplasmic reticulum organisation (Lange et al. 2009). Recently a molecular link of obscurin to the utmost C-terminal domain of titin, M10 and domains My4 and My5 in myomesin was demonstrated (Fukuzawa et al. 2008). Reducing myomesin expression levels in cardiomyocytes leads to a loss of obscurin at the M-band (Fukuzawa et al. 2008). A smaller relative of obscurin, ObsL-1 shows the same molecular interactions, and it is assumed that while obscurin is the main interaction partner at the periphery of the myofibrils, ObsL-1 is the main link within the myofibrils between titin M10 and myomesin. However, the titin M10 domain appears to be a busy site for interactions, since it was also shown recently to bind to myospryn (Sarparanta et al. 2010). This is especially interesting, since myospryn can also bind a regulatory subunit of protein kinase A (PKA), which in turn is known to phosphorylate the linker between My4 and My5 in myomesin that binds to obscurin (Obermann et al. 1997). Currently the significance of this web of interactions and how it is correlated to different states of M-band activity and function is unclear.

Apart from these rather large proteins, a number of smaller molecules were found to target to the M-band region, often due to their association with the stretch of titin that spans from its kinase domain to its C-terminus. Among these is DRAL/FHL2, which binds to the is2 region of M-band titin and seems to act as a hub for the association of metabolic enzymes such as creatine kinase, adenylate kinase and phosphofructokinase (Lange et al. 2002). The E3 ubiquitin ligase MURF1 associates with a domain in titin close to the titin kinase domain, suggesting a possible link to titin protein turnover (Centner et al. 2001). More recently, Myomasp, which is a leucine-rich protein, was found to locate to the M-band, and a direct interaction with the C-terminus of the myosin heavy chain was shown (Will et al. 2010).

7.9 What Happens if Myomesin Is Stretched

The force-buffering function of the M-band relies on the robustness of the cross-links between myosin filaments. In the relaxed sarcomere, the myomesin dimers are in a compact zero-force conformation and keep the myosin filaments in register. During sarcomere activation the myomesin dimers may work as force-transmitting bridges to balance the shifting forces between the neighbouring myosin filaments. A series of brilliant single-molecule studies revealed the smart molecular mechanisms, which allow the myomesin dimers to establish that tough but yet extensible link. It became clear that the stability of the M-band bridges in all possible working regiments of the muscles is achieved by a remarkable mechanical hierarchy of

unfolding—refolding processes of the various structural modules that make up the myomesin molecule (Xiao and Grater 2014). The first study from 2005 used AFM to characterise the "mechanical fingerprints" of the typical modules that constitute the myomesin molecule, including one Fn-like domain (My6), one Ig-like domain (My10) and the alternatively spliced EH-fragment (Schoenauer et al. 2005). The EH-insert was found to be largely unfolded and was considered to function as an entropic spring, analogous to the PEVK domain of titin. According to the single-molecule measurements, inclusion of two EH-inserts in the myomesin bridge allows for about 70 nm elastic extension, which nearly doubles the predicted length of the whole dimer. This considerably increases the working range of the M-band bridges without the need to unfold the Fn-/Ig-like domains. However, in muscles lacking the EH-myomesin isoform, the elongation of the myomesin molecule is achieved by other mechanisms. After the initial finding that myomesin forms antiparallel dimers via its My13 domain (Lange et al. 2005b), a structural analysis was carried out, and it was found that the dimerised domains make up a beta-sheet complex similar to filamin dimerisation and obscurin/titin interaction domains (Pernigo et al. 2010; Pinotsis et al. 2008).

When the structural analysis was extended towards the N-terminus spanning My9–My13, it was revealed that unlike many other linkers between modular domains, the linkers between domains My9 and My13 of the myomesin molecule attain an alpha-helical conformation (Pinotsis et al. 2012). Using a unique approach, this study has identified the superhelical coil arrangement of this dimer, composed of the C-terminal portion of myomesin, My9–My13. The unfolding of the alpha-helical linkers allows for an extension of up to 2.5-fold of the original length (Pinotsis et al. 2012).

Myomesin is a molecular spring: in the relaxed state it is in a coiled conformation. Its behaviour during extension might be modelled as a series of elastic springs with different stiffness together with the viscous elements, corresponding to the unfolding of individual domains. According to the mechanical hierarchy, the stretching would first unravel the whole Fn/Ig domains chain, followed by the extension of the EH-segment. In those muscles, which do not have EH-myomesin, a stretching force of about 30 pN (lacking the EH-segment) will lead to the sequential unfolding of the alpha-helical linkers in the C-terminal part of the molecule. In contrast to the extension of the elastic regions, this process will constantly absorb the energy. The Fn and Ig domains demonstrate substantially higher rupture forces and might be used as "shock absorbers" of the last bastion, because the refolding of these domains might need a longer relaxation time. The mechanical stability of the Ig domains increases towards the C-terminal part of the myomesin, while the dimerising My13 domain is the most robust one (Berkemeier et al. 2011). Molecular dynamics simulations have shown that the stability of the antiparallel dimer outperforms all other Ig domains of myomesin, ensuring the intactness of the myomesin cross-link even when all intermediate domains are unfolded (Xiao and Grater 2014). Thus, myomesin acts as a serial link of the various structural modules, whose complex viscoelastic properties allow for the reversible extension, and thus safeguards the integrity of the M-band bridges during muscle contraction.

7.10 Signalling from the M-Band

In addition to its function as mechanical link between the myosin and the titin filaments in the middle of the sarcomere, recent studies have also pointed out the potential role of the M-band in sensing stress and in signalling to the nucleus. The titin kinase (TK) domain at its edge is known to be mechanically activated (Gräter et al. 2005), and while at present it is under discussion whether TK is actually an active kinase or a pseudokinase (Bogomolovas et al. 2014), this activation definitely opens up interaction sites for potential binding partners of TK. Among those are MURF1 (Centner et al. 2001) but also the signalling cascade that originates from a TK–nbr1 interaction and goes via a stepwise cascade involving p62, MURF2 and SRF (serum response factor) all the way to the nucleus (Lange et al. 2005c). SRF is a well-known transcription factor that regulates the expression of actin and associated proteins (Olson and Nordheim 2010). SRF-dependent gene transcription was also affected in Myomasp knockdown cardiomyocytes (Will et al. 2010), suggesting signalling from the M-band via SRF may not only be restricted to the TK pathway. Transcription of M-band proteins such as myomesin and M-protein was suggested to be under the control of Mef2, since Mef2c knockout animals display a defect in myofibril maintenance due to the lack of myomesin expression (Potthoff et al. 2007). Interestingly, translation of Mef2 appears to be directly controlled by muscle activity via the mTor signalling pathway (Yogev et al. 2013), suggesting that muscle activity and translation of transcription factors that are required to replenish sarcomeric proteins such as myomesin are directly linked.

7.11 The M-Band and Disease

Up to now neither conventional nor conditional knockout mice exist for the members of the myomesin family that would substantiate their proposed indispensable role for myofibrillogenesis. When the expression of myomesin-3, which is mainly found in intermediate speed fibres such as IIA (Schoenauer et al. 2008), was abolished by gene trap experiments in slow muscle of zebrafish embryos, no defects in myofibrillogenesis were seen (Xu et al. 2012). However, there are at least five different myomesin genes in zebrafish; therefore, a lack of phenotype is most likely due to redundancy (Xu et al. 2012). Nevertheless there are several indirect strands of evidence available that support the importance of myomesin family members for myofibril maintenance and most likely also for myofibrillogenesis. For example, the lack of the entire C-terminus of titin and hence the absence of binding sites for myomesin leads to a total absence of organised myosin or myomesin complexes in differentiating embryonic stem (ES) cells (Musa et al. 2006). In case of the deletion of a smaller part of the titin C-terminal domain, where the myomesin binding site M4 is missing but the three most C-terminal Ig domains in titin (M8–10) are retained, myofibrils are initially assembled, as indicated by alternating striations

of sarcomeric alpha-actinin and myomesin, but subsequently fall apart during use (Weinert et al. 2006). Interestingly the signal for myomesin is much broader in these mice than in wild type (Weinert et al. 2006), which may indicate that the targeting of myomesin to the middle of the sarcomere may be mediated in this scenario via its obscurin/ObsL-1–titin M10 link rather than the conventional interaction with titin M4. This interaction may be sufficient for subcellular targeting, but in the absence of the link between titin and myomesin, the proposed integration of thick filaments at the M-band does not work properly and the sarcomeres come apart during contraction.

There are several indications that the composition of the M-band may be altered in different types of cardiomyopathy. Upregulation of EH-myomesin expression can be consistently correlated with a DCM phenotype in mice and humans (Schoenauer et al. 2011), and the switch to this isoform may help to buffer active contractile force in myofibrils that are not perfectly registered in parallel. A downregulation of M-protein expression was reported for hypertrophic cardiomyopathy (Rozanski et al. 2013), and myomesin-3 is re-expressed in a subset of DCM cases (Schoenauer et al. 2008). The signalling pathways that govern this isoform switch and changes in gene expression in cardiomyopathy are unclear at present. However, recently discovered links of Mef2 translation and thus the expression of myomesin and M-protein to muscle activity via the mTor signalling pathway are intriguing (Yogev et al. 2013). The same pathway is known to regulate the expression of E3 ubiquitin ligases (MURF, atrogin-1) that may serve to degrade sarcomeric proteins such as myosin but potentially also myomesin (Sandri et al. 2004). Thus compromised activity could result in increased protein turnover of M-band components, which may in the long run lead to a defect in myofibril maintenance and heart failure.

For a long time the only identified hereditary mutations in a component of the M-band were in the is7 and the M10 domain of titin, which result in tibial muscular dystrophy or limb–girdle muscular dystrophy type 2j, but did not show any involvement of the heart and also did not present with any major defects in sarcomere ultrastructure (Hackman et al. 2002). Only recently were truncating mutations in titin published that also lead to cardiomyopathy (Carmignac et al. 2007), but it may require mutated versions of titin from both parents (e.g. one point and one truncating) to result in a heart condition (Chauveau et al. 2014). It is somewhat surprising that truncated titin can be tolerated in a heterozygous state and throws an interesting light on the regulation of its expression and turnover in the sarcomere. While heterozygous mutations that truncate titin in the I-band region were postulated to be a major cause of DCM (Herman et al. 2012), analysis of DCM patient material displayed no difference in the intensity of signals of M-band titin epitopes also in cases where a titin mutation had been identified (Pluess et al. 2015; and data not shown). This suggests a preferential recruitment/expression of the allele encoding a full-length version of titin and might hint at a certain degree of removal of truncated titin mRNA via nonsense mediate decay, as suggested in the case of mutations that lead to truncated MyBP-C (Vignier et al. 2009).

The only other mutation in an M-band protein that was published so far is a V1490I missense mutation in My12 of myomesin in a family with hereditary HCM. This mutation seemed to affect the thermal stability of myomesin dimers in vitro, although My12 on its own was not affected (Siegert et al. 2011). The observation that a relative subtle missense mutation in myomesin can potentially lead to familial HCM suggests that we are just looking at the tip of the iceberg and that next-generation sequencing is bound to yield many more links of cardiomyopathy with mutations in M-band components.

7.12 Conclusion

Recent research has shown that the M-band is not a rigid structure but rather an elastic protein network that involves the C-terminal part of titin, the giant protein obscurin and three closely related members of the myomesin family in addition to the myosin tails. Its composition is adapted according to the mechanical demands in a particular muscle, making it more or less elastic. In addition to its role in buffering active contractile force, the M-band has also been shown to be a focus point for signalling processes that are regulated by mechanical activity and can go all the way to the nucleus to affect gene transcription. As more sequencing data becomes available, mutations in M-band components will be shown more and more to be at the basis of hereditary cardiomyopathies. The M-band is by no means just boring glue, but is fine-tuned to function under certain operational conditions.

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Chapter 8
Sarcomeric Signaling

Robert Frauen, Derk Frank, and Norbert Frey

Abstract Sarcomeres consist of a highly elaborate protein assembly and are the backbone of the contractile apparatus of cardiac and skeletal muscle. The sliding filament mechanism is the basis of contractility. Furthermore, sarcomeres contain a multitude of regulatory proteins, which are not directly involved in force generation. Many of them interact with key parts of intracellular signaling cascades. Thus, sarcomeres are increasingly noted as central hubs for the regulation of signaling pathways. The exact protein composition of the sarcomere is tightly controlled and tuned by a comprehensive system that regulates protein degradation and turnover. Sarcomeric components are involved in the perception of mechanical stress, which is an essential prerequisite for cellular adaption to different conditions. Oxidative stress, which occurs in context of numerous pathologic states, induces extensive modulations of many sarcomeric proteins. Connected to appropriate signaling pathways, sarcomeric proteins can modulate gene expression. Mutations in sarcomeric proteins are causally involved in the pathogenesis of inherited cardiomyopathies. In summary, the sarcomere is centrally positioned in a network of incoming and outgoing signaling pathways. In the first part of this chapter, structure and contractile function of the sarcomere are described. Then, an overview of important signaling pathways associated with sarcomeric proteins is given.

8.1 Introduction

Sarcomeres provide the structural basis for contraction of skeletal and cardiac muscle. They are structural units of specifically assembled proteins (Clark et al. 2002). In addition to components that are mainly responsible for force generation, many other proteins are located at the sarcomere that have either regulatory functions or are integrated in intracellular signaling pathways.

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The contractile apparatus constitutes of thin filaments, the main component of which is fibrillary actin, and thick filaments, which are chiefly formed by myosin filaments. These filaments are aligned side by side. Each thick filament is hexagonally surrounded by six thin filaments. Furthermore, titin makes up a third filament system. Like a spring, it primarily adjusts the elasticity of a sarcomere. In cardiac muscle, nebulette forms a fourth filament system and localizes to the sarcomeric Z-disc (Frank et al. 2006).

The uniform structure of each sarcomere is divided into different sections (Fig. 8.1). The Z-disc represents the boundary of a sarcomere. The width of the Z-disc in cardiac muscle is about 100–140 nm. Neighboring sarcomeres are connected to each other at the Z-disc. The backbone of the Z-disc consists of α-actinin. All actin filaments are anchored to the Z-disc with their barbed ends (Luther 2009). In addition, multiple regulatory proteins are located at this structure, which makes it a nodal point in sarcomeric signaling (Frank et al. 2006). The M-line or M-band forms the very middle of each sarcomeric unit. Myosin filaments are tethered to the M-line and reach in both directions toward the extremities of the sarcomere. Myosin-containing parts of the sarcomere are called A-bands, while the remaining zones are termed I-bands. Directly adjacent to the M-line lies the H-zone, which comprises only myosin but no actin filaments.

Sarcomeres are aligned in series to allow force transmission from one to the next and finally to the sarcolemma. Z-discs of adjoining sarcomeres are connected via desmin, an intermediate filament (Frank and Frey 2011). At the lateral boundaries of the cell, Z-disc-associated proteins interact with a sarcolemmal protein complex

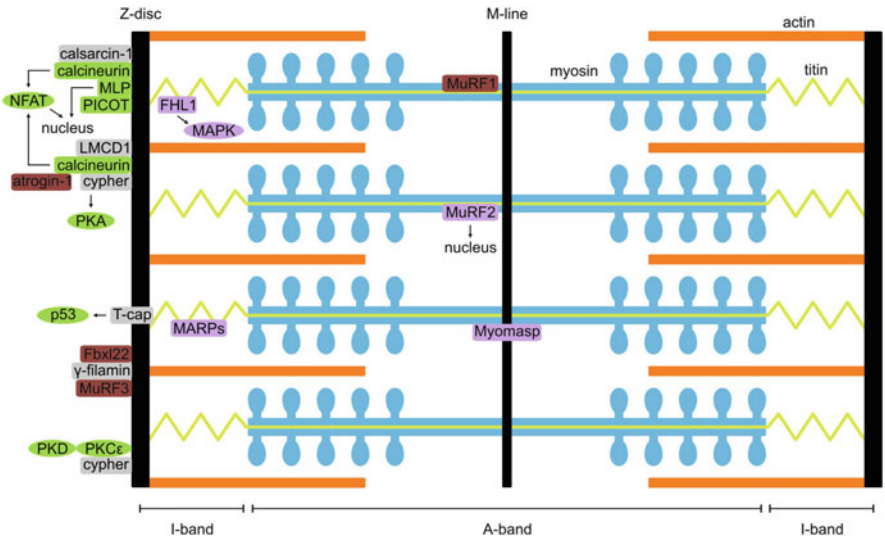

Fig. 8.1 Schematic structure of a sarcomere. Illustrated proteins are described throughout this chapter. *Gray*: Z-disc-associated proteins; *lilac*: proteins involved in mechanotransduction; *brown*: proteins involved in protein turnover and degradation; *green*: proteins associated with additional signaling pathways

termed costamere to ensure lateral anchorage and force transmission as well as signal transduction (Ervasti 2003).

Muscular contraction is generated by a sliding filament mechanism within the sarcomeres. Actin and myosin filaments slide along each other in an energy-consuming process, translating chemical energy into mechanic force. The combined action of all serially aligned sarcomeres leads to contraction of the muscle cell. The head part of myosin directly interacts with an actin filament. The neck part of myosin has the ability to perform a conformational change, which is the basis for a step-by-step movement of myosin along the actin filament. Each step requires the hydrolysis of ATP, is therefore energy consuming, and involves a detachment and reattachment of the myosin head to actin (Schmidt et al. 2004).

Tropomyosin and troponin are important regulatory proteins of the sliding filament mechanism (Kobayashi et al. 2008). Tropomyosin is a filamentary protein that is wound around actin filaments and inhibits the interaction between myosin and actin. In the event of a muscular contraction, a local increase of the calcium concentration leads to a conformational change of troponin. Since troponin is connected to tropomyosin, this leads to a displacement of tropomyosin and facilitates the interaction between myosin and actin.

The structural and force generating components of the sarcomere are closely connected to diverse intracellular signaling pathways. These interactions regulate the process of contraction, facilitate cellular adaption to different physiological and pathological stimuli, and link sarcomeric components to sarcolemmal structures as well as to the gene expression machinery within the nucleus. Of note, mutations in a multitude of sarcomeric proteins are known to cause familial cardiomyopathies (Frank et al. 2007; Kimura 2010; van Eldik and Passier 2013). The most important forms are hypertrophic cardiomyopathy (HCM) and dilated cardiomyopathy (DCM). Especially hypertrophic cardiomyopathy is in most cases based on a mutation in a sarcomeric protein. Most frequently, mutations are found in the genes coding for cardiac β myosin heavy chain, cardiac myosin-binding protein C, and cardiac muscle troponin T, but other sarcomeric proteins, including Z-disc proteins, may be involved as well (Frey et al. 2012).

In this chapter we will focus on the emerging role of the sarcomere as a hub for signal transduction in cardiomyocytes. First, we will describe how mechanotransduction, a process involving cellular sensing of mechanical stress, takes place at the level of the sarcomere. Next, we provide an outline on the regulation of degradation and the turnover of sarcomeric components. We will then discuss the modulation of sarcomeric proteins and their functional properties under conditions of oxidative stress. Finally, we will describe how eminent signaling pathways involving phosphatases and kinases are linked to the sarcomere.

8.2 Mechanotransduction

Mechanotransduction is the sensing of a mechanical stimulus and its conversion into a cellular response. A multitude of proteins have been shown to be involved in this process in cardiomyocytes. Sensing of mechanical stress is essential for cardiac adaption to hemodynamic load as well as for the induction of hypertrophic response in response to increased load due to different pathological and physiological conditions. The sarcomere at the interface between the contractile apparatus and the sarcolemma/costamere is ideally situated as a "hot spot" for proteins with a regulative function in mechanotransduction (Knoll et al. 2003; Frank et al. 2006, 2007; Buyandelger et al. 2014). Other mechanosensitive proteins are localized at the sarcolemma or in the cytosol (Sadoshima and Izumo 1997). Sarcomeric proteins are closely related to the dynamic structures of the contractile apparatus. They may sense mechanical stress and activate intracellular signaling pathways, which coordinate a cellular response. The highly structured protein complexes of the Z-disc and the M-band are centrally involved in the process of mechanotransduction (Gautel 2011). Mechanical stress induces profound alterations in gene cardiomyocyte expression (Frank et al. 2008). Some well-characterized proteins with mechanosensitive functions are discussed below.

8.2.1 Titin and Associated Signaling Pathways

The giant protein titin is one of the main structural components of the sarcomere. Titin acts as a scaffolding protein for myofibrillar assembly, serves as molecular ruler for the tick filaments, fixes the position of the myosin filaments to the middle of the sarcomere, and is a molecular spring, which regulates sarcomeric elasticity. Different titin isoforms vary in their stiffness. The elastic elements of titin are located in the I-band region. The properties of the molecular spring can be fine-tuned by phosphorylation at different sites (Kruger and Linke 2011), and as recently demonstrated, also by S-glutathionylation of cryptic cysteine residues (Alegre-Cebollada et al. 2014). Of note, the latter mechanism appears to be a general mechanism to regulate tissue elasticity.

Proteins from the four-and-a-half LIM domain family are highly expressed in cardiac and skeletal muscle. Four-and-a-half LIM protein 1 (FHL1) is upregulated in the context of pathological cardiac remodeling (Chu et al. 2000). FHL1 binds to titin at the N2B domain in the I-band region. FHL-1 knockout mice exhibit a reduced hypertrophic remodeling after transverse aortic constriction, which is a strong mechanical stress stimulus. The connection between FHL1 and an elastic part of titin indicates a distinct function in mechanotransduction. In addition, FHL1 acts as a scaffold protein for components of the MAP-kinase signaling pathway. Thus, FHL1 provides a link between this important pathway involved in cardiac hypertrophic remodeling and the sarcomeric protein titin (Sheikh et al. 2008).

Finally, FHL1 mutations have been shown to cause hypertrophic cardiomyopathy in humans (Friedrich et al. 2012). Interestingly, this also involves impaired proteasome function, implying a pathophysiological link between altered mechanotransduction and protein degradation. Moreover, several members of the muscle ankyrin repeat protein (MARP) family interact with titin at its elastic regions. Upon mechanical stretch, MARPs show an intracellular redistribution. Thus, MARPs are part of a mechanosensitive element (Miller et al. 2003). Proteins of the MARP family are thought to be another key part of the stress response system in muscles, since they are induced under different pathological conditions like starvation, stretch, injury, or denervation.

Finally, titin also contains a kinase domain, which is located near the M-line. Titin kinase is activated by mechanical stretch, also linking this enzymatic domain to mechanotransduction (Puchner et al. 2008). Titin kinase interacts with a protein complex containing nbr1, p62, and MURF2. Upon mechanical inactivity, MURF2 translocates to the nucleus, where it serves as an interaction partner of serum response factor (SRF). This pathway provides a direct link between mechanosensing and altered gene expression and thereby contributes to the development of atrophy (Lange et al. 2005).

8.2.2 Telethonin/T-Cap

Telethonin, also called T-cap, is a sarcomeric Z-disc protein that binds to the N-terminal part of titin (Zou et al. 2006). In addition to that, telethonin has several other binding partners like the calsarcins, myostatin, or ion channels (Frank et al. 2006). Telethonin provides a link between mechanical stress sensing and apoptosis. Telethonin influences the function of p53, a key regulator of the cell cycle and tumor suppressor gene, and thus connects the sarcomeric Z-disc to the regulation of apoptosis. Telethonin knockout mice do not exhibit a cardiac pathology spontaneously. However, under conditions of mechanical stress, caused by transverse aortic constriction, an increase in apoptosis is evident, which might contribute to the development of heart failure (Knoll et al. 2011).

8.2.3 Muscle Lim Protein

Muscle Lim protein (MLP) is highly expressed in striated muscle tissues. Besides other binding partners at different cellular structures, MLP interacts with sarcomeric proteins like α-actinin and telethonin/t-cap (Knoll et al. 2002; Heineke et al. 2005; Frank and Frey 2011). MLP-deficient mice develop a severe cardiomyopathic phenotype (Arber et al. 1997). MLP has been proposed to be a component of a mechanical stretch sensor. Mechanical stretch is accompanied by increased expression of BNP, a marker protein of hypertrophic remodeling. In

cardiomyocytes from MLP knockout mice, the induction of BNP expression after mechanical stretch is markedly blunted while the response to pharmacological stimuli remains unaltered (Knoll et al. 2002). Mechanical strain and pharmacological stimulation of cardiomyocytes lead to nuclear translocation of MLP. This observation also applies to rat hearts that were subjected to aortic banding or myocardial infarction (Boateng et al. 2007, 2009). In addition to its function as a nucleoplasmatic shuttling protein in the context of hypertrophic remodeling, MLP is also associated with the calcineurin pathway (see below). However, the exact cardiac function of MLP is not completely understood and controversially discussed. As described above, compelling data support a role for MLP in the regulation of cardiac remodeling. Yet, MLP transgenic mice do not exhibit a pathological cardiac phenotype. In addition to that, the extent of cardiac remodeling as a reaction to different pathological stimuli like aortic constriction or infusion of angiotensin II is equal in MLP transgenic and wild-type mice (Kuhn et al. 2012).

8.2.4 LRRC39/Myomasp

LRRC39/Myomasp is a novel M-band protein with a potential function in mechanical stress sensing (Will et al. 2010). It exhibits a strong enrichment in cardiac and skeletal muscle. Myomasp localizes to the sarcomeric M-band in cardiomyocytes and directly binds to the thick filament protein myosin heavy chain. Mechanical stretch of cardiomyocytes leads to a reduced expression of myomasp, indicating a connection to mechanosensation. This is further supported by a marked cardiac downregulation of myomasp in mice that underwent transverse aortic constriction, which causes mechanical strain in vivo. Furthermore, a knockdown of myomasp is associated with reduced expression of other structurally important M-band-bound proteins like myosin heavy chain or myomesins 1 and 2, as well as an impaired contractile function. These alterations can partly be explained by a reduction of SRF-dependent gene expression. On the other hand, a knockdown of myomasp induces an enhanced expression of typical stretch responsive marker proteins like BNP and GDF15, which generally denotes an activation of stress-associated pathways. A knockdown of the myomasp orthologue in zebrafish leads to contractile dysfunction, underscoring the importance of an intact M-band structure for proper sarcomeric function.

8.3 Protein Degradation and Turnover

An exact equilibrium between protein synthesis and protein degradation is essential for the maintenance of cardiac function. This principle applies in particular to sarcomeric proteins, which have to be assembled in a precise stoichiometry to build the backbone of the cardiomyocyte's force generation. The degradation of

sarcomeric proteins is thus tightly regulated and specific mechanisms for the degradation of certain components have been elucidated (Lyon et al. 2013).

Protein degradation is mainly performed by the ubiquitin-proteasome system, by autophagy, and by the calpain system. These systems provide the basis to recycle or degrade misfolded, unused, or damaged proteins. They have complementary functions, as for example the autophagy system is able to handle larger components like protein aggregates and even entire organelles (Portbury et al. 2011).

The ubiquitin-proteasome system consists of three classes of enzymes, which are necessary to select a protein for degradation and mark it with ubiquitin. In the first step an ubiquitin monomer is activated by an E1 enzyme in an ATP-dependent process. The second step is the transfer of ubiquitin to an E2 enzyme. Finally, an E3 enzyme transfers the ubiquitin moiety from the E2 enzyme to a selected protein and hereby marks it for degradation. E3 enzymes, which are also called ubiquitin ligases, ensure substrate specificity of the ubiquitin-proteasome system (Pickart 2001). Additional ubiquitin monomers bind to the ubiquitinated protein and form an ubiquitin chain. After that, the ubiquitinated protein is disassembled by a proteasome unit. The amino acids set free in the degradation process are substrates for the synthesis of new proteins. Several ubiquitin ligases are expressed in cardiomyocytes and some are expressed in a heart- and/or skeletal-muscle-specific fashion, implying an important role of this machinery in contractile cells. Not surprisingly, different cardiac pathologies have been linked to an altered function of components of the ubiquitin-proteasome system (Willis et al. 2014). Impairment of the ubiquitin-proteasome system is involved in the pathogenesis of inherited cardiomyopathies like HCM and DCM (Schlossarek et al. 2014). A connection between ubiquitin-proteasome impairment and HCM is, for example, well characterized for mutations in the MYBPC3 gene, coding for myosin-binding protein C. Mutations in MYBPC3 frequently lead to the formation of a truncated protein (Schlossarek et al. 2011). These mutated proteins are predominantly degraded by the ubiquitin-proteasome system. The excessive utilization of this system may cause its inability to ensure an adequate protein turnover, which potentially contributes to the pathogenesis of HCM (Sarikas et al. 2005). Mice with a knock-in of a truncated version of MYBPC3 exhibit a phenotype of cardiac hypertrophy and show an impairment of the ubiquitin-proteasome system with increasing age (Schlossarek et al. 2012).

Autophagy is another mechanism for the degradation of different cellular components, including sarcomeric proteins. Selected substrates are coated by a membrane. These vesicles then fuse with a lysosome, which contains acid hydrolase enzymes (Portbury et al. 2011; Lyon et al. 2013). Autophagy is required for a regular cardiac structure and function. Knockout of Atg5, a central component of the autophagy system, in mice leads to cardiomyopathy. This phenotype is associated with disarrangements of sarcomeric structures and a morphological disorganization of mitochondria (Nakai et al. 2007). In humans, defective autophagy due to mutations in the autophagy regulator EPG5 has been shown to cause Vici syndrome, a multisystem disorder with severe cardiomyopathy (Cullup et al. 2013). Autophagy and the ubiquitin-proteasome system are connected, so that

ubiquitinated proteins may also be degraded by transferring them to the lysosome (Kirkin et al. 2009).

In the following we will focus on cardiac-enriched proteins and their potential role in cardiomyocyte protein turnover as well as cardiac (patho)physiology.

8.3.1 Muscle-Specific RING Finger Proteins

Muscle-specific RING finger (MuRF) proteins are a group of muscle-specific ubiquitin ligases. Their function in protein degradation and turnover has been studied in cardiac and skeletal muscle. Besides other cellular compartments, MuRF proteins localize to the sarcomeres and bind to several sarcomeric proteins (McElhinny et al. 2004; Witt et al. 2005). MuRF1 binds to the sarcomeric protein titin in the M-band region (Centner et al. 2001). It regulates the degradation of the thin filament-associated protein troponin I in cardiomyocytes. Overexpression of MuRF1 in cardiomyocytes in vitro is associated with changes in the contractile properties of the cells (Kedar et al. 2004). MuRF3 regulates the turnover of the sarcomere-associated proteins γ-Filamin and of four-and-a-half LIM domain protein 2 (FHL2) (Fielitz et al. 2007). Several in vivo studies in mouse models point to a function of MuRF proteins in the regulation of cardiac and skeletal muscle mass. MuRF1 and MuRF2 seem to have some functional redundancy in this regard, since MuRF1 and MuRF2 knockout mice develop normally, while double knockout mice exhibit severe cardiac and skeletal muscle hypertrophy (Witt et al. 2008). Conversely, MuRF1 transgenic mice have thin left ventricular walls and a reduced contractile performance (Willis et al. 2009b). MuRF1-mediated protein degradation thus seems to be an important mechanism in the process of cardiac atrophy (Willis et al. 2009a). Certain mutations in the TRIM63 gene, coding for MuRF1, lead to impaired protein degradation and have been identified in patients with HCM. Underscoring the pathophysiological relevance, significant cardiac hypertrophy is observed in mice expressing a mutated variant of MuRF1 (Chen et al. 2012).

8.3.2 F-Box and Leucine-Rich Repeat Protein 22

F-Box and Leucine-Rich Repeat Protein 22 (Fbxl22) is a recently described ubiquitin ligase with important functions regarding the turnover of cardiac sarcomeric proteins (Spaich et al. 2012). Fbxl22 exhibits a highly cardiac-enriched expression pattern. On the subcellular level, Fbxl22 localizes to the sarcomeric Z-disc. Components of a known ubiquitin ligase complex such as cullin and SKP1 are direct binding partners of Fbxl22. Protein degradation of two major sarcomeric proteins, α-actinin and γ-filamin (Filamin C), by the proteasome is mediated via the specific ubiquitin ligase function of Fbxl22. Conditions of cardiac stress, which typically cause cardiac hypertrophy, lead to a reduced expression of Fbxl22.

Targeted knockdown of Fbxl22 in zebrafish embryos induces a severe cardiomyopathic phenotype, again underscoring the fundamental importance of a tightly regulated turnover of sarcomeric proteins.

8.3.3 Atrogin-1

Atrogin-1 is another muscle-specific protein from the group of F-Box proteins. Together with the cullin-RING protein cullin 1 and other proteins, atrogin-1 forms an E3 ubiquitin ligase complex. Atrogin-1 has the function of a substrate-specific adaptor protein (Lyon et al. 2013). It localizes to the sarcomeric Z-disc and directly binds to α-actinin-2 (Li et al. 2004). Until now, there is no evidence for a role of atrogin-1 in the mediation of protein turnover of sarcomeric proteins under physiological conditions. However, atrogin-1 provides a link to the phosphatase calcineurin, a prominent part of a key pathway of cardiac hypertrophy, which is associated with the sarcomeric Z-disc. Atrogin-1 directly binds to calcineurin at the Z-disc and mediates its degradation via the ubiquitin-proteasome system. When overexpressed, atrogin-1 hereby represses the development of cardiac hypertrophy as a reaction to different pathological stimuli. In addition to that, atrogin-1 regulates the ubiquitination of a truncated mutant of the thick filament-associated protein myosin-binding protein C, which is known to cause familial hypertrophic cardiomyopathy (Mearini et al. 2010).

8.3.4 Calpains

The calpains form a group of Ca^{2+}-dependent proteases. Their proteolytic functions are involved in the degradation of sarcomeric proteins in cardiac and skeletal muscle (Portbury et al. 2011). Doxorubicin induces a calpain-mediated degradation of the sarcomeric protein titin (Lim et al. 2004). Cardiac troponins are substrates for proteolysis by calpains as well (Ke et al. 2008). Calpains and the ubiquitin-proteasome system act in concert in the degradation of sarcomeric proteins. Overexpression of calpain-1 in cardiomyocytes leads to a degradation of specific proteins and is accompanied by an increase in protein ubiquitination. Calpain-1 transgenic mice exhibit an enhanced cardiac protein ubiquitination and proteasome activity. Depending on the level of overexpression in these mice, increased lethality is observed, which is associated with the occurrence of characteristics of heart failure (Galvez et al. 2007).

Taken together, the turnover of sarcomeric proteins is cooperatively coordinated by several protein degradation systems. Malfunctions in components of these systems result in disturbed protein homeostasis, which contributes to the pathogenesis of cardiomyopathies.

8.4 Oxidative Stress

Oxidative stress is characterized by an increased formation of reactive oxygen species (ROS) like hydrogen peroxide. It occurs in different cardiac pathologies. ROS interfere with normal cellular functions either by directly damaging proteins and other components or by activating ROS-sensitive enzymes. ROS modify sarcomeric proteins and hereby influence their proper function. In addition to that, several ROS-sensitive enzymes with sarcomeric targets are activated during oxidative stress (Sumandea and Steinberg 2011).

In the presence of NO during oxidative stress formation of peroxynitrite occurs. Peroxynitrite is a highly reactive compound that can induce nitration of tyrosine residues. ROS usually react with thiol moieties of cysteines. Especially cysteines flanked by aromatic or basic amino acids are susceptible to interactions with ROS. Modified residues within the amino acid sequence can lead to conformational changes of a protein or form bonds with other residues (Steinberg 2013). Overall, ROS modifications due to oxidative stress induce extensive changes in cellular functions. ROS usually modify diverse targets and sometimes at different sites. The type of oxidant species and the level of oxidative stress influence the cellular responses.

8.4.1 Direct Modification of Sarcomeric Proteins

ROS lead to changes in contractile function partially by direct modification of sarcomeric proteins. ROS modifications of myosin induce changes in protein structure and inhibit the activity of its ATPase in the head domain (Tiago et al. 2006; Passarelli et al. 2008). In context of ischemia/reperfusion injury, oxidative modifications of actin and tropomyosin occur (Canton et al. 2004). Glutathionylation of actin leads to a reduced myosin head ATPase activity and influences the dynamics of the actin–myosin binding (Pizarro and Ogut 2009). Disulfide cross-bridge formation in tropomyosin as a result of oxidative stress is associated with contractile dysfunction (Canton et al. 2006). ROS modifications also increase the stiffness of titin (Grutzner et al. 2009). Thus, passive elasticity of the sarcomere is reduced under conditions of oxidative stress. While most ROS modifications of sarcomeric proteins lead to a depressed contractile function, nitroxyl (HNO) has an opposite effect. HNO-induced modifications of myofilaments increase their Ca^{2+} responsiveness and enhance force generation (Gao et al. 2012).

8.4.2 *Modification of Sarcomeric Proteins by ROS-Sensitive Enzymes*

Several kinases involved in this process of posttranslational modifications are ROS-sensitive enzymes. Oxidative stress generally increases protein phosphorylation by activation of kinases and simultaneous inhibition of phosphatases. Particularly titin, certain subunits of troponin and the thick filament-associated protein (cMyBP-C) are phosphorylated by ROS-activated kinases (Sumandea and Steinberg 2011; Steinberg 2013).

Ca^{2+} sensitivity and functional properties of troponin I are modulated via phosphorylation by protein kinase A, CβII, Cδ, D, and p90 ribosomal S6 kinase (Haworth et al. 2004; Itoh et al. 2005; Brennan et al. 2006; Wang et al. 2006b; Sumandea et al. 2008). Protein kinase A (Type I) contains ROS-sensitive residues within its regulatory subunits. Modification of these sites by hydrogen peroxide induces formation of a disulfide bond and a translocation to myosin heavy chain, which functions as an A kinase anchoring protein (AKAP). This enhances phosphorylation of troponin I and cMyBP-C at the sarcomere by protein kinase A independently of the usual β-adrenoceptor-associated pathway. In consequence, oxidative stress enhances contractility (Brennan et al. 2006). Protein kinase Cδ can be activated in a hydrogen peroxide-induced mechanism that includes a certain phosphorylation by Src kinases. In this context, oxidative stress leads to a lipid-independent activation of protein kinase Cδ (Rybin et al. 2004). Protein kinase D is activated by hydrogen peroxide via a protein kinase C-dependent pathway (Waldron and Rozengurt 2000). However, activation of protein kinase D under conditions of oxidative stress is associated with a translocation of this enzyme to the nucleus, while an enhanced phosphorylation of sarcomeric proteins is unclear (Waldron et al. 2004).

Troponin T is phosphorylated by Raf-1, protein kinase Cα, or apoptosis signal-regulating kinase-1 (ASK-1), which is a ROS-sensitive enzyme involved in the regulation of apoptosis (He et al. 2003; Sumandea et al. 2003; Yamaguchi et al. 2003; Pfleiderer et al. 2009). Phosphorylation of tropronin T by protein kinase Cα is associated with a decreased Ca^{2+} sensitivity of the myofilaments, a diminished myosin ATPase activity, and a reduced contractility (Sumandea et al. 2003). ROS-induced activation of ASK-1 leads to contractile dysfunction. However, besides increased phosphorylation of the sarcomeric protein troponin T, changes in calcium handling may be a different explanation for this effect (He et al. 2003). In summary, phosphorylation of sarcomeric proteins by ROS-induced kinases has a significant effect on cardiomyocyte contractility.

8.4.3 ROS-Induced Cleavage of Sarcomeric Proteins

Oxidative stress leads to an increased cleavage of sarcomeric proteins, which may contribute to contractile dysfunction. Ischemic injury induces a degradation of sarcomeric proteins, including troponin I, troponin T, and myosin light chain 1 (MLC-1) (Van Eyk et al. 1998; Zhang et al. 2006). These protein cleavages are linked to an activation of the calpain system (Steinberg 2013). Matrix metalloproteinase 2 (MMP-2) is usually excreted to the extracellular matrix and cleavage of MMP-2 by other MMPs leads to its activation. MMP-2 has important functions in extracellular tissue remodeling. However, MMP-2 is not exclusively secreted from the cardiomyocytes but to some extent remains intracellular (Ali et al. 2012). MMP-2 serves as a central mediator of ROS-induced sarcomeric protein degradation, which contributes to impaired contractility (Ali et al. 2011). Overexpression of MMP-2 leads to reduced contractility and decreased responsiveness to inotropic stimuli (Wang et al. 2006a). The proteolytic activity of MMP-2 is initially inhibited by an interaction between a cysteine residue in the propeptide domain and the catalytic domain (Steinberg 2013). MMP-2 can be activated by ROS-induced modifications at this cysteine residue (Viappiani et al. 2009). Ischemia/reperfusion injury leads to a degradation of MLC-1, MLC-2, troponin I, and titin by MMP-2 (Wang et al. 2002; Sawicki et al. 2005; Ali et al. 2010; Doroszko et al. 2010). In the context of oxidative stress, MMP-2 also cleaves the sarcomeric Z-disc protein α-actinin (Sung et al. 2007).

8.5 Phosphatases and Kinases

Phosphatases and kinases are key enzymes that regulate cellular functions via phosphorylation or dephosphorylation of target proteins. A plethora of intracellular signaling pathways are controlled by phosphatases and kinases. Of note, several phosphorylation cascades are also integrated in signaling pathways that connect sarcomeric structures to other compartments of the cardiomyocyte, in particular the nucleus. Specifically, the function of many sarcomeric proteins, including titin, myosin-binding protein C, myosin regulatory light chain, and troponin subunits, is modulated by phosphorylation (James and Robbins 2011; Kamm and Stull 2011; Solaro and Kobayashi 2011; Hidalgo and Granzier 2013).

8.5.1 Calcineurin and Modulators of the Calcineurin Pathway

Calcineurin is the central part of one of the most important signaling pathways that regulate cardiac hypertrophy and remodeling. As a serine/threonine phosphatase,

calcineurin dephosphorylates transcriptions factors from the NFAT family (Hogan et al. 2003). After dephosphorylation, these transcription factors shuttle to the nucleus and activate a prohypertrophic gene program. Calcineurin-transgenic mice develop a severe cardiac hypertrophy, which progresses to heart failure within a few weeks (Molkentin et al. 1998). The calcineurin signaling pathway and the sarcomeric Z-disc are closely connected (Frank et al. 2006; Frank and Frey 2011). Several modulators of calcineurin reside at the Z-disc and regulate its function directly adjacent to this structure (Frey et al. 2000; Zou et al. 2001; Li et al. 2004; Heineke et al. 2005; Jeong et al. 2008). Moreover, calcineurin is regulated by multiple other proteins with cytosolic and/or sarcolemmal localization.

The calsarcins form a protein family with a muscle-specific expression profile. Calsarcin-1 is the only isoform that is expressed in cardiomyocytes. The calsarcins localize to the sarcomeric Z-disc and bind to several typical Z-disc proteins like α-actinin, telethonin/T-Cap, and γ-filamin (Frey et al. 2000). Functionally, calsarcin-1 is negative regulator of calcineurin at the Z-disc. It directly binds to calcineurin and inhibits its activity. Calsarcin-1 knockout mice develop an exacerbated hypertrophic phenotype in response to certain pathological stimuli due to a loss of calcineurin inhibition by calsarcin-1 at the sarcomere. However, while mechanical stress induces an aggravated hypertrophic remodeling in calsarcin-1 knockout mice, the response to a pharmacological stimulation remains unaltered (Frey et al. 2004). In this regard, MLP, another Z-disc protein is specifically integrated in a pathway that regulates the cardiac response to mechanical stress (see above). Muscle LIM protein (MLP) anchors calcineurin to the sarcomeric Z-disc. In heterozygous MLP knockout mice calcineurin loses its connection the Z-disc, which leads to a reduced activity in the calcineurin pathway and an adverse cardiac remodeling in response to myocardial infarctions (Heineke et al. 2005). As described above, MLP has additional functions in cardiomyocytes.

Recently, a link between calcium signaling and sarcomeric integrity has been described. Inactivation of the fish orthologue of ORAI1, a calcium release-activated calcium channel, leads to heart failure and skeletal muscle weakness in zebrafish. ORAI1 deficiency induces gross abnormalities in sarcomeric structure associated with a loss of calsarcin-1 Z-disc localization as well as diminished expression (Volkers et al. 2012).

Another negative regulator of calcineurin activity at the Z-disc is PICOT. This protein directly binds to MLP. PICOT disturbs the interaction between MLP and calcineurin. In consequence, calcineurin loses its connection to the Z-disc and hypertrophic remodeling is inhibited (Jeong et al. 2008). This further underscores the importance of the sarcomeric Z-disc protein complex for the regulation of cardiac hypertrophy. Another recently described sarcomeric modulator of the calcineurin pathway is LMCD1 (LIM and cysteine-rich domains 1), also called dyxin. LIM domains mediate protein–protein interactions and numerous LIM domain-containing proteins, like ALP, ZASP/Cypher/Oracle, or Enigma, localize to the cardiac Z-disc (Frank et al. 2006). In cardiac and skeletal muscle, LMCD1 exhibits a sarcomeric Z-disc and an additional sarcolemmal enrichment. LMCD1 promotes the development of cardiac hypertrophy by activating the calcineurin

pathway. A knockdown of LMCD1 in cardiomyocytes in vitro blunts a hypertrophic response after mechanical or pharmacological stimulation. The latter is accompanied by an abrogated calcineurin activation. Thus, LMCD1 is a sarcomeric protein that is important for an adequate activity of the calcineurin pathway in the context of cardiac hypertrophic remodeling (Frank et al. 2010). The Z-disc protein Cypher/ZASP/Oracle directly interacts with the phosphatase calcineurin and, as an A-kinase anchoring protein (AKAP), also with protein kinase A (PKA), which is an important mediator of posttranslational modifications. This has functional implications for Cypher/ZASP/Oracle, since it also binds to the L-type calcium channel, whose phosphorylation state can be modified by calcineurin and PKA (Lin et al. 2013).

In summary, the phosphatase calcineurin is integrated in several signaling pathways that mediate cardiac hypertrophy, which in turn are extensively regulated at the sarcomeric Z-disc.

8.5.2 Protein Kinase C

Protein kinase C proteins, which are serine/threonine kinases, mediate a large variety of intracellular processes. Members of the protein kinase C family are activated via Gq-proteins that are associated with G-protein coupled receptors (GPCR). In cardiomyocytes, protein kinase C proteins are involved in the regulation of hypertrophic remodeling. Receptors for important prohypertrophic agonists like norepinephrine, phenylephrine, angiotensin II, or endothelin-1 activate this pathway (Dorn and Force 2005). The intracellular localization of activated protein kinase C proteins is regulated by binding to anchoring proteins called receptors for activated C kinases (RACKs) (Frank et al. 2006).

Protein kinase C epsilon (PKCε) is an isoform with an important function in the regulation of cardiac hypertrophy. Activated PKCε localizes to the sarcomeric Z-disc (Huang et al. 1997; Robia et al. 2001). One of its anchoring proteins at the Z-disc is the LIM domain protein ZASP/cypher/oracle, which in turn directly binds to α-actinin (Zhou et al. 1999). Mechanical stress leads to an increase in cardiac PKCε activity (Gu and Bishop 1994). PKCε overexpression in mice induces a mild hypertrophic phenotype, which features attributes of physiological rather than pathological hypertrophy (Takeishi et al. 2000). The formation of a protein complex containing PKCε and protein kinase D1 (PKD1) at the sarcomeric Z-disc is essential for the mediation of hypertrophy induced by adrenergic agonists (Iwata et al. 2005). Furthermore, MuRF-1 inhibits the function of PKCε in the context of hypertrophic remodeling, in this case by impeding a translocation of PKCε to focal adhesions (Arya et al. 2004).

The connection between PKCε signaling and the sarcomeric Z-disc again emphasizes the central position of this structure in the regulation of cardiac hypertrophy.

8.6 Conclusion

As we have described in this chapter, sarcomeric structures are embedded in a variety of intracellular signaling pathways. The contractile function of sarcomeric proteins and the activity of their regulatory proteins can be precisely regulated by posttranslational modifications. Sensing mechanical stress and initiating a cellular response of the cardiomyocytes is an essential attribute of sarcomeric proteins. The protein assembly of the sarcomere is accurately balanced by complex systems for protein degradation. As illustrated with examples throughout this chapter, mutations in many different sarcomeric proteins can cause hereditary cardiomyopathies. Deciphering the complex system of signaling pathways and the function of single proteins will enhance our understanding of the pathophysiology of these diseases. This is the prerequisite for the establishment of future targeted therapies (Kho et al. 2012).

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Chapter 9
The Nuclear Envelope in Cardiac Health and Disease

Daniel Brayson and Catherine M. Shanahan

Abstract The nuclear envelope (NE) is a double membrane bilayer, which serves to compartmentalise the nuclear environment from the cytoplasm and regulate the movement of molecules in and out of the nucleus. Another important role of the NE is to physically mediate communication between the nuclear and cytoplasmic domains, which is facilitated by the linker of nucleoskeleton to cytoskeleton (LINC) complex consisting of filamentous lamins on the inner surface and large rod-like nesprins on the outer surface, linked by SUN domain proteins that bridge the double membrane bilayer. On the outer membrane, nesprins link, via N-terminal binding domains, to cytoskeletal components such as actin and microtubules. These physical connections allow rapid communication of mechanical perturbations into the nucleus resulting in gene expression responses designed to adapt the cell to environmental changes. Mutations to these components result in disease phenotypes, which vary in severity and tissue specificity and are often broadly termed 'premature ageing disorders'. One of the key phenotypes frequently present is cardiomyopathy. As a result, the NE is becoming increasingly relevant in the context of cardiac cytoarchitecture. The discovery that cardiomyopathies consistently arise from mutations to lamins in particular and also emerin and nesprins has led to a body of research intended to elucidate the mechanisms leading to NE-associated cardiac decline and accurately define the role of the NE in the cardiomyocyte.

9.1 Introduction

Recently, the nuclear envelope (NE) has been implicated in cardiac cytoarchitecture and function. The stimulus for this has come from the discovery that a number of clinically distinct diseases, many manifesting with cardiac

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complications, arise as a result of mutations in genes encoding proteins that comprise and/or maintain the NE (Agarwal 2003; Bonne et al. 1999; Cao and Hegele 2000; Chen et al. 2003; Wulff et al. 1997; Eriksson et al. 2003).

The NE (Fig. 9.1) primarily consists of an outer nuclear membrane (ONM) and an inner nuclear membrane (INM) that are separated by a ~50 nm luminal gap known as the perinuclear space (PNS). These two lipid membranes serve to provide a physical barrier between the nucleoplasm and cytoplasm, thus facilitating compartmentalisation of the nuclear environment. Additionally, the NE is punctured with nuclear pore complexes (NPCs), which facilitate the trafficking of macromolecules across the NE. The NE is also characterised by sequentially tethered protein complexes, which span the depth of the NE and beyond, in order to provide structure, stability and a communications interface between the nuclear and cytoplasmic environment. This complex is known as the linker of nucleoskeleton to cytoskeleton (LINC) complex (Crisp et al. 2006). The primary components of the LINC complex are nesprins, SUN proteins and the type V intermediate filament (IF) proteins known as the nuclear lamins.

9.1.1 Nesprins and the ONM

The ONM provides the primary interface for the anchoring of the rod-like NE spectrin repeat proteins (nesprins). Nesprins are derived from the *SYNE* family of genes encoding four main proteins (nesprin-1, nesprin-2, nesprin-3 and nesprin-4), which can also undergo alternate initiation and termination to produce a number of isoforms that vary in structure and function. Full-length nesprins possess a C-terminal KASH (Klarsicht/ANC-1/SYNE-1 homology) transmembrane-spanning domain, required to provide anchorage within the nuclear bilipid layer (Zhang et al. 2001; Starr and Fischer 2005). Extending into the cytoplasmic domain is a series of spectrin repeats, the number of which varies depending on the nesprin isoform. The largest proteins are nesprin-1 and nesprin-2, encoded by *SYNE1* and *SYNE2*, which extend deep into the cytoplasmic domain with 74 and 56 spectrin repeats, respectively. Both also have N-termini containing calponin homology (CH) domains that link to cytoskeletal actin filaments (Padmakumar et al. 2004; Zhen et al. 2002). Nesprin-3 and nesprin-4 are smaller protein isoforms with fewer spectrin repeats. They differ at the N-termini compared to nesprin-1 and nesprin-2 and link to intermediate filaments such as desmin, via plectin, and microtubules, respectively (Roux et al. 2009; Wilhelmsen et al. 2005). Nesprin isoform expression is also highly cell type specific. Some nesprin-1 and nesprin-2 isoforms show highest expression in muscle tissue types (Zhang et al. 2002) and are likely to play a major role in maintaining the function and integrity of striated muscle as evidenced by their involvement in Emery-Dreifuss muscular dystrophy and cardiomyopathy (Puckelwartz et al. 2010; Zhang et al. 2007).

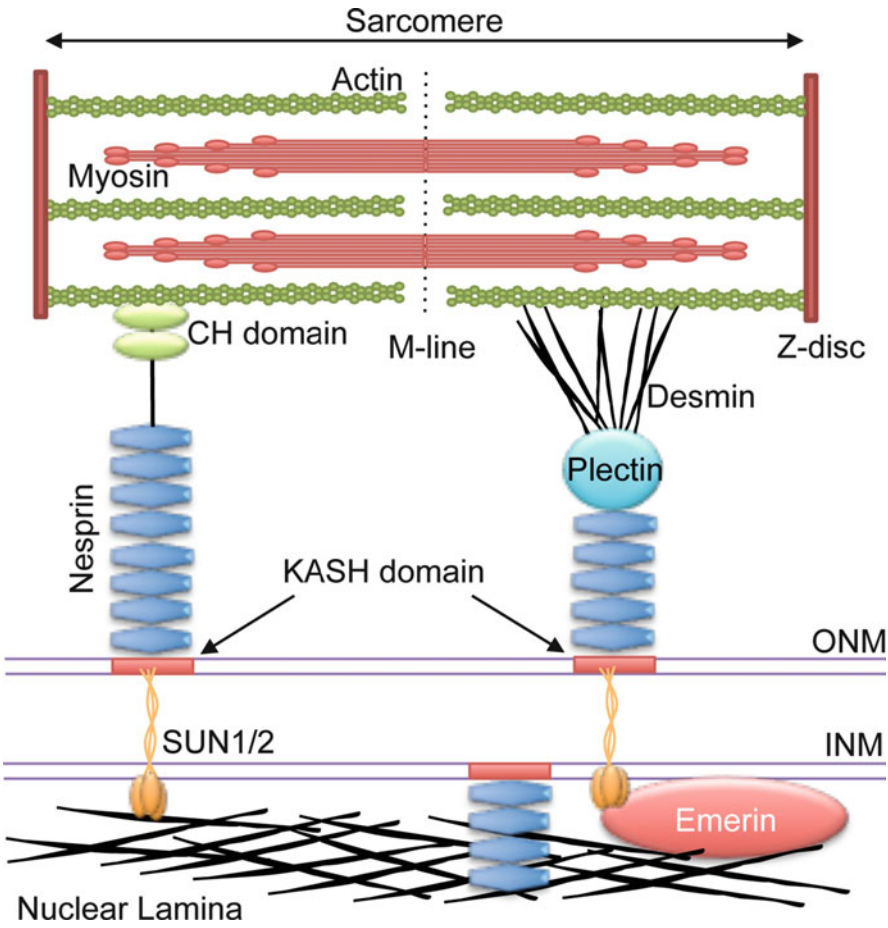

Fig. 9.1 The nuclear envelope and the nuclear lamina. The nuclear envelope provides a physical barrier for nuclear compartmentalisation and a platform for communication between the nucleus and cytoskeletal domains via a group of proteins termed the LINC complex. The outer nuclear membrane (ONM) contains the KASH domain protein nesprins, which bind to cytoplasmic filament proteins such as actin. The inner nuclear membrane (INM) contains SUN proteins and LEM domain proteins that connect the KASH domain to the nuclear lamina, consisting of lamins A, C and B. It is thought that the LINC complexes play key roles in nuclear integrity and are thought to regulate transcription, DNA damage response (DDR) and mitosis. Mutations to LINC proteins are known to cause a plethora of disease phenotypes known as laminopathies, often characterised by premature ageing or cardiomyopathy

9.1.2 SUN Domain Proteins

The perinuclear space is occupied by the Sad1 and UNC (SUN) domain proteins, of which there are two main isoforms, SUN1 and SUN2 (Hodzic et al. 2004; Malone et al. 1999). SUN proteins form coiled-coil trimers, which are bound at their

C-termini by peptides at the terminus of the nesprin KASH domain on the ONM interface of the NE. The SUN coiled-coil trimer extends across the luminal space of the NE penetrating through the INM and into the nucleoplasmic domain where the N-termini assume a globular shape and form a strong association with the nuclear lamins (Haque et al. 2006; Sosa et al. 2012).

9.1.3 Lamins and the INM

The nuclear lamina is a meshwork of proteins lining the nuclear interface of the INM, consisting of type V IF proteins known as A- and B-type lamins, which form parallel coiled-coil dimers and head-to-tail dimers to produce a large network that covers the entire INM (Ben-Harush et al. 2009). The predominant A-type lamins are lamin A and lamin C which are alternate splice products of the same gene, *LMNA* (Lin and Worman 1993). Unlike lamin A, lamin C is translated as a mature protein, whereas lamin A is translated as a precursor, prelamin A, which must undergo a number of modifications before becoming mature lamin A (Davies et al. 2009). B-type lamins are lamin B1 arising from the *LMNB2* gene and lamins B2 and B3 from the *LMNB3* gene (Harborth et al. 2001). Whilst A-type lamins are thought to be specific to differentiated cell and tissue types, B-type lamins are thought to be present ubiquitously (Broers et al. 1997). Lamins have a postulated role in the regulation of gene expression, DNA damage repair, nuclear stability and structure and heterochromatin organisation (Misteli and Scaffidi 2005). To supplement these roles, the INM also has a set of distinct membrane-associated proteins that include lamina-associated polypeptide 2 (LAP 2), emerin and MAN1 (collectively termed the LEM domain proteins) (Lin et al. 2000). Alongside lamins, these proteins are also postulated to play roles in the regulation of gene expression and particularly in the case of emerin, in maintaining the integrity of the nuclear lamina (Bengtsson and Wilson 2004).

Emerin is the 254 amino acid product of the *EMD* gene and is a ubiquitously expressed integral protein of the INM (Bione et al. 1994; Manilal et al. 1996; Nagano et al. 1996). Structurally, emerin contains a 220 amino acid N-terminal nucleoplasmic domain, a single transmembrane domain and a short C-terminal tail situated in the NE lumen (Cartegni et al. 1997). As well as the aforementioned roles in gene expression and structural nuclear integrity, emerin is also hypothesised to have a role in regulation of cell cycle and nuclear assembly during mitosis and has a known association with β-catenin which is thought to restrict accumulation of β-catenin in the nucleus (Markiewicz et al. 2006).

The sequential tethering of these NE spanning proteins is thought to be crucial for functions including structural stability and nuclear form, nuclear positioning, chromatin scaffolding and facilitating rapid physically mediated communication to the nucleus, from the cytoplasmic and extracellular environment via mechanotransduction and mechanosignalling.

Table 9.1 Known nuclear envelopathies caused by mutations to *LMNA* and *ZMPSTE24*, *SYNE1*, *SYNE2* and *EMD*

Disease	Gene	Heart involvement	Reference
Autosomal dominant Emery-Dreifuss muscular dystrophy	LMNA, SYNE1, SYNE2	Yes	Bonne et al. (1999), Zhang et al. (2007)
Cerebellar ataxia	SYNE1	No	Gros-Louis et al. (2007)
Charcot-Marie-Tooth disease	LMNA	No	De Sandre-Giovannoli et al. (2002)
Dilated cardiomyopathy	LMNA, SYNE1	Yes	Charniot et al. (2003), Puckelwartz et al. (2010)
Familial partial lipodystrophy of the Dunnigan type FPLD	LMNA	Yes	Cao and Hegele (2000)
Hutchinson-Gilford progeria syndrome	LMNA, ZMPSTE24	Yes	Eriksson et al. (2003)
Limb-girdle muscular dystrophy	LMNA	Yes	Muchir et al. (2000)
Lipoatrophy with diabetes, hepatic steatosis, hypertrophic cardiomyopathy and leukomelanodermic papules (LDHCP)	LMNA	Yes	Caux et al. (2003)
Mandibuloacral dysplasia with type A lipodystrophy (MADA)	LMNA	Yes	Novelli et al. (2002)
Mandibuloacral dysplasia with type B lipodystrophy (MADB)	LMNA, ZMPSTE24	Yes	Agarwal (2003)
Restrictive dermopathy	ZMPSTE24	Unknown	Navarro et al. (2004)
X-linked Emery-Dreifuss muscular dystrophy	EMD	Yes	Bione et al. (1994)

9.1.4 Nuclear Envelopathies

The general importance of the NE is highlighted by the unique set of pathogenic conditions that arise as a result of perturbation to components of the NE termed nuclear envelopathies. These include a subset of the laminopathies (Table 9.1), which are a set of tissue-specific disorders, often affecting striated muscle tissue and/or with cardiac involvement and displaying elements of premature ageing. Research into the characteristics and mechanisms of such diseases has helped identify a major role for the NE in cardiac cytoarchitecture and disease.

9.1.5 The LINC Complex in Cardiomyocytes

In the cardiomyocyte (CM), the LINC complex is postulated to mediate a physical interaction between the NE and the sarcomere. It is thought that within the cytoplasm this occurs via interactions between the actin-binding CH domain of nesprins, which can potentially bind actin of the sarcomere, but also via interactions between nesprins, plectin and desmin (Fig. 9.1). The importance of this link is twofold. Firstly, the nucleus is likely to be crucial to the structural integrity of the CM and therefore important in dealing with the perpetual mechanical stress imparted upon the cytoskeleton during contraction. Indeed, mutations in LINC complex components disproportionately affect tissues which are under mechanical strain suggesting that these links are crucial for the structural integrity of CMs. Secondly, the nucleus constantly senses the dynamic changes in the cytoplasmic (and potentially also the extracellular) domain due to mechanosignalling via the LINC complex and the cytoskeleton and consequently adjusts to such changes with appropriate gene expression responses (Meinke et al. 2014). Evidence suggests that these are the principal processes that become deregulated in disease settings, leading to the onset of cardiomyopathies.

9.2 The Role of the NE in Cardiac Disease

Cardiac abnormalities associated with NE mutations are clinically varied and include dilated cardiomyopathy (DCM), hypertrophic cardiomyopathy (HCM) and arrhythmogenic right ventricular cardiomyopathy (ARVC). Often these present in combination with other muscle phenotypes, such as limb girdle muscular dystrophy, LGMD, or more commonly Emery-Dreifuss muscular dystrophy, EDMD, reflecting the major influence of NE perturbations on mechanical tissues. EDMD is a condition in which patients experience early-onset contractures of the elbows, ankles and spine and endure subsequent humero-peroneal muscle wasting, and by adulthood, patients develop cardiomyopathy with conduction defects (Emery 1987, 1989, 2000). EDMD occurs by two primary means, both arising from components of the NE; one is described as X linked, in which mutations to the emerin gene *EMD* are identified as causative (Bione et al. 1994), and also autosomal dominant EDMD due to mutations in *LMNA* (Bonne et al. 1999). Of the NE components, *LMNA* mutations are currently thought to be the most common cause of NE-associated DCM. In fact, mutations in the *LMNA* gene are relatively commonly identified in patients with sporadic or inherited DCM and often lead to sudden cardiac death (van Berlo et al. 2005). As a result of this, *LMNA* is also the most rigorously studied NE protein in the context of heart disease.

HCM has also been described in conjunction with *LMNA* mutations. One instance involved a patient with familial partial lipodystrophy type 2 (FPLD2) caused by a missense mutation to *LMNA* that confers an amino acid substitution

at residue 591 (Araújo-Vilar et al. 2008). This proband was also insulin resistant indicating a potentially wider role for lamins in heart disease, as perturbations to lamins may also be involved in diabetes and metabolic syndrome (Hegele 2000, 2003). Additionally, there was a case reported whereby a patient with the R644C mutation, arising in the C-terminal domain in close proximity to the prelamin A processing site, displayed pathogenicity consistent with HCM (Mercuri et al. 2005).

LMNA mutations have also been shown to be responsible for ARVC phenotypes. ARVC is characterised by pathological remodelling primarily of the right ventricle, in which myocyte death, inflammation and fibrofatty replacement of the myocardium are the main histological features (Sen-Chowdhry et al. 2010). ARVC arises primarily due to mutations in genes whose protein products contribute to the composition of the desmosomes such as desmoplakin, desmoglein, plakoglobin and plakophilin. Desmosomes are found at the plasma membrane in the intercalated disc (ID) regions where adjacent CMs join. The role of the desmosome is to tether the IFs of one cell to the cytoplasmic membrane of another to create a lattice structure that provides mechanical strength to tissue. However, a number of patients presenting with ARVC who did not have mutations to desmosomal genes were found to have *LMNA* mutations, suggesting that NE perturbations can influence CM cytoarchitecture at peripheral subcellular locations (Larsen et al. 2012; Quarta et al. 2011).

Mutations of the *LMNA* gene in humans have most frequently been shown to result in DCM leading to conduction defects resulting in arrhythmias and even leading to sudden cardiac death (Cowan et al. 2010). However, the recurrence of common DCM-causing mutations is low; the university mutation database states that there are currently 132 unique DCM-causing mutations to *LMNA*. As such, correlations between genotype and phenotype provide little idea on the mechanisms leading to cardiomyopathy. There are a subset of laminopathies which are caused by a few 'hot spot' mutations which lead to specific phenotypes and are likely to share mechanistic pathways (Scharner et al. 2010) including classical Hutchinson-Gilford progeria syndrome (HGPS), Werner syndrome, Charcot-Marie-Tooth disorder and MAD. However, the more common nuclear envelopathies, which are primarily striated muscle disorders, A-EDMD, LGMD1B and DCM, are caused by seemingly indiscriminate and promiscuous mutations which give little indication of the mechanistic milieu. For example, mutations that cause DCM have been found in all 12 exons of the *LMNA* gene. Moreover, mutations that cause DCM can also cause EDMD, LGMD1B and FPLD in separate individuals or can lead to any of these diseases with associated DCM. The level of pleiotropy seen with some of these mutations is unusual. For example, one curious mutation to the *LMNA* gene, R644C, displays extreme phenotypic diversity in the clinic (Rankin et al. 2008). These findings suggest that epigenetic or other extrinsic factors may be important in disease pathogenesis and progression. This diversity makes mechanistic analysis challenging, although some DCM-causing *LMNA* mutations have been investigated in order to provide some insight into the workings of *LMNA*-associated DCM and are discussed in a later section (Table 9.2).

Table 9.2 Phenotypic comparison of existing models of *LMNA*-associated DCM

Model	Survival	Heart pathology	Fibrosis	Inflammation	Cell death	Nuclear morphology defects
Lmna$^{-/-}$	6–8 weeks	DCM and heart failure	+	–	+	+
Lmna$^{+/-}$	Long-lived	Late-onset DCM-CD	+	–	+	+
Lmna$^{N195K/N195K}$	12–14 weeks	DCM and heart failure	+	–		+
Lmna$^{H222P/H222P}$	Males: 4–9 months Females: 9–13 months	DCM and heart failure	+	–	–	+
Lmna$^{G609G/G609G}$	3–4 months	LQT and arrhythmia	?	–	?	?
Zmpste24$^{-/-}$	3–4 months	Nonspecific	–	+	–	?
Lmna$^{MHC\text{-}M371K}$	2–7 weeks and prenatal lethal	Acute and subacute heart failure	–	–	–	+
Lmna$^{nPLAO/nPLAO}$	Males live up to 40 weeks and females live up to 80 weeks	DCM and heart failure	+	?	?	?

Regarding nesprins, the situation is also complex. Mutations to *SYNE1* can lead to autosomal recessive cerebellar ataxia due to nonsense mutations causing truncation of the protein (Li et al. 2014). However, *SYNE1* and *SYNE2* mutations have also been identified in probands with EDMD (Zhang et al. 2007) and DCM (Puckelwartz et al. 2010), and these missense mutations occur near the C-terminus of SYNE1/2 which encode muscle-specific isoforms that bind to other LINC complex components including emerin, lamin A/C and SUN (Zhang et al. 2007), suggesting that uncoupling of the LINC complex may contribute to cardiomyocyte dysfunction.

As mentioned above, mutations to emerin lead to EDMD with associated DCM. The mutations involved lead to loss of function of emerin and correlations with phenotype suggest that a fully functional emerin is required for skeletal muscle development and maintenance, whilst also important for cardiac muscle maintenance (Bione et al. 1994; Nagano et al. 1996; Patel et al. 2014).

Mutations to SUN proteins alone have recently been putatively associated to the onset of musculoskeletal and cardiomyopathic disease (Meinke et al. 2014). They have also been identified in combination with mutations to myosin-binding protein C, lamin A/C, emerin and LAP2α, in all instances leading to EDMD-like

or cardiomyopathic phenotypes (Meinke et al. 2014; Li et al. 2014). This digenic pattern of mutation accumulation, whether sporadic or heritable, is believed to have disease-modifying effects, including early onset of disease and increased disease severity (Meinke et al. 2014). Mechanistically, it was shown, in vitro, that the identified mutations to SUN proteins led to defective myonuclear positioning via nuclear-microtubule uncoupling in human and C2C12 murine myotubes (Meinke et al. 2014).

9.3 Mechanistic Insights into NE-Associated Cardiomyopathy

There are two prominent hypotheses to explain why defects in the NE lead to cardiac cell dysfunction. The first of these suggests that mutations to NE proteins lead to structural changes that have a detrimental effect on the nucleus-cytoskeleton coupling and render cells more susceptible to mechanical stress (Worman and Courvalin 2004; Worman and Bonne 2007). The second suggests that mutations in NE proteins lead to abnormal gene expression (Worman and Courvalin 2004; Worman and Bonne 2007; Capell and Collins 2006). These hypotheses are not mutually exclusive and observations have identified the deregulation of multiple cell signalling pathways and cell maintenance systems, defects to gap junctions and fibrosis of cardiac tissue in a number of in vivo mouse models, suggesting that perturbations to the NE can have a disruptive influence on tissue organisation and integrity.

9.3.1 Mechanical Efficiency and Structural Instability

The stability and structure of a cell is undoubtedly important to its function, and striated muscle cells are uniquely designed to both exert force and cope with extreme loads. At the tissue level, perturbations of the LINC complex result in histological reports of myocyte disarray (Pendás et al. 2002), whilst at the cellular level, misexpression and mislocalisation of intermediate filament proteins such as desmin (Nikolova et al. 2004), therefore disrupting the connection of the NE to the sarcomeres of the myocyte, is apparent, suggesting uncoupling of the entire structural framework of the cell. In such scenarios, it is hypothesised that muscle is unable to exert the required force or cope with sufficient loads for efficient mechanical functionality.

In vivo, signs that mechanical instability occurs in cardiac tissue as a result of NE perturbations are compelling. The first animal model developed to investigate the role of the nuclear lamina in DCM was the lamin A/C global knockout mouse. *Lmna*$^{-/-}$ mice die between 6 and 8 weeks of age because of DCM and heart failure

(Nikolova et al. 2004). Phenotypically, hearts of *Lmna*$^{-/-}$ mice have irregular patterns of heart tissue histology and skeletal muscle dystrophy (Sullivan et al. 1999) and succumb to severe systolic dysfunction (Nikolova et al. 2004). Detailed cardiac characterisation has shown *Lmna*$^{-/-}$ CMs to be mechanically unsound. They have nuclear morphology defects and appear to undergo disruption of the LINC complex via nesprin1α leading to uncoupling of the nucleus from the cytoskeleton resulting in mechanical instability and defective force transmission (Nikolova-Krstevski et al. 2011). Additionally, the IF protein desmin, which serves to link the sarcolemma to the sarcomere and also the sarcomere to the nucleus, is mislocalised in these mice further supporting this hypothesis (Nikolova et al. 2004). However, *Lmna*$^{-/-}$ mice are not relevant in the clinical setting therefore a number of animal knockin models of human mutations have been developed.

The H222P-*LMNA* mutation causes EDMD and DCM in humans and has been investigated using *Lmna*$^{H222P/H222P}$ mice. Whilst these mice are born healthy, at 16 weeks, they display a classical DCM progression leading to heart failure and death in males at 5–9 months and in females at 7–13 months (Arimura et al. 2005). Moreover, studies of mice lacking the C-terminus and KASH domain of nesprin-1, which mimic to some extent human mutations observed in DCM, also showed a DCM-like phenotype, characterised by reduced fractional shortening, indicating reduced contractility of the myocardium (Puckelwartz et al. 2010).

Additionally, the importance of the NE for structural stability in CMs has also been highlighted by experiments performed in vitro, which showed that when DCM-causing mutations were introduced to the genes of the NE components emerin and lamin A/C, and the cells then subjected to mechanical stress, uncoupling of the nucleoskeleton from the cytoskeleton was observed (Zwerger et al. 2013). In this instance, investigators were able to show that when healthy cells are stretched at the plasma membrane, the nucleus is displaced, indicating that the nucleus, cytoskeleton and plasma membrane are mechanically linked. However, when cells with mutated lamin A/C were stretched, the nucleus was unmoved, suggesting the link between the plasma membrane and the nucleus had been broken (Zwerger et al. 2013).

9.3.2 Mechanosignalling and Gene Regulation

Mechanosignalling is the conversion of physical cues into intracellular signals (biochemical and biophysical) that mediate a cellular response to external stimuli. Indeed, research has shown that the NE plays a pivotal role in the biophysical signalling cascade, which can regulate gene transcription and elicit a physiological response (Wang et al. 2009). Furthermore, it has been suggested that the inability of the cell to convert environmental/external signals efficiently into a transcriptional response makes the cell susceptible to stress and contributes to the onset of pathogenesis, especially in the case of NE- and LINC-associated proteins (Isermann and Lammerding 2013; Jaalouk and Lammerding 2009).

There is evidence from animal models to suggest that A-type lamins play a role in mechanosignalling. In *Lmna*$^{-/-}$ mice, activation of genes appears to be attenuated, and the suggestion is that *Lmna*$^{-/-}$ mice are unable to adapt to DCM progression with compensatory hypertrophy, which accounts for the speed of disease progression and short lifespan. Moreover, it has been reported that haploinsufficiency of *Lmna* ameliorates pressure overload-induced hypertrophy in a mouse model of transverse aortic constriction (Cupesi et al. 2010). The mechanisms identified imply that impaired activation of mechanosensitive genes such as *Egr1*, which encodes the transcriptional regulator early growth response protein 1, is crucial to this phenotype. In this study, haploinsufficiency of the *LMNA* gene was investigated using *Lmna*$^{+/-}$ mice, which have a baseline phenotype of early-onset cardiac conduction system (CCS) disease and late-onset DCM (Wolf et al. 2008).

In vitro, single-cell models of mechanical stretch showed that mouse embryonic fibroblasts (MEFs) lacking emerin have impaired mechanotransduction associated with reduced *Egr1* gene expression (Lammerding et al. 2006). Similarly, MEFs lacking *Lmna* experience attenuated gene expression responses to mechanical strain and cytokine stimulation via reduced nuclear factor-kappa-B (NF-κB) transcription (Lammerding et al. 2004). Moreover, *Lmna*-null MEFs and MEFs harbouring the N195K mutation to *Lmna* are known to experience impaired nuclear translocation and downstream signalling of the mechanosensitive transcription factor megakaryoblastic leukaemia 1 (MLK1), a member of the myocardin family thought to be crucial to cardiac development and function (Meinke et al. 2014). It has also been shown that the NE may be able to detect perturbations in force and convert these perturbations into adaptive or pathological gene expression responses (Lammerding et al. 2004, 2005; Lammerding and Lee 2005).

9.3.3 Intercalated Disc Disorganisation

Intercalated discs (IDs) are polarised regions of the CM plasma membrane, which serve to mechanically couple adjacent CMs to one another and facilitate the conductance of the action potential between cells. This occurs via numerous junctional complexes, which characterise the structure and function of IDs. The importance of IDs in the heart is highlighted by the discovery that mutations to ID proteins are known to lead to cardiomyopathies and arrhythmia in humans, including plakophilin (Gerull et al. 2004), desmoplakin (Sen-Chowdhry et al. 2005) and N-cadherin (Li et al. 2005). The idea that IDs might be involved in NE-mediated cardiomyopathy stems from the observation that conduction defects and arrhythmia are also frequently observed in DCM patients with NE mutations. In vivo, several lamin models, including lamin-deficient mice and mice harbouring the N195K mutation, exhibited mislocalisation and aberrant expression of connexins (Cx) 40 and 43 (Frock et al. 2012; Mounkes et al. 2005). Connexins are gap junction (GJ) proteins whose primary role is to allow the conduction of stimulatory

potassium current from one CM to the next, thereby facilitating contractility and synchronous heart rhythm across the myocardium. Consequently, deregulation of these processes potentially leads to systolic dysfunction and arrhythmia phenotypes and is the primary cause of death in these models. Accordingly, postmortem analysis of cardiac tissue from patients who suffered sudden cardiac death showed significantly reduced Cx43 expression (Chen and Zhang 2006). Currently, it is not known how perturbations to the NE lead to disorganisation of GJs.

Another ID protein, β-catenin that forms part of the adherens junction (AJ) is regulated by emerin. In CMs, emerin also localises to the AJs, some distance away from the typical NE localisation. This interaction appears to be crucial to AJ organisation, because when CMs from emerin-null mice were analysed, β-catenin distribution, and subsequently ID architecture, was found to be aberrant. Loss of emerin also deregulates β-catenin signalling in CMs, which may provide a common mechanistic link between EDMD-associated DCM and ARVC phenotypes as ARVC pathogenesis has been attributed to altered Wnt/β-catenin signalling.

Interestingly, atypical localisation of LINC complex proteins in CMs has also been reported for nesprins. Nesprin-1 was shown to localise to the Z-disc of the sarcomere in human cardiac tissue (Zhang et al. 2002) indicating that mutations to nesprin-1 that lead to EDMD or cardiomyopathy could potentially cause pathogenesis by also disrupting sarcomere structure and function; however, this has not been experimentally tested as yet.

9.3.4 Autophagy and Related Signalling Mechanisms

Autophagy is a cellular housekeeping process involving the sequestration of cytoplasmic debris by double membrane vacuoles termed autophagosomes, which then fuse with lysosomes for the degradation of potentially toxic matter. It is activated in response to nutrient deprivation so that cells can scavenge amino acids for use as an emergency fuel source. In the context of the heart, autophagy has been found to protect the heart from haemodynamic stress. Activation of autophagy is reliant on complex signalling cascades, and aberrant modulation of these signalling cascades can prevent the activation of autophagic responses in circumstances in which they are required.

Evidence from models of NE-mediated cardiomyopathy suggests that NE perturbations are capable of inhibiting the autophagic response. In global $Lmna^{-/-}$ mice, for example, autophagy was found to be impaired (Ramos et al. 2012). Evidence suggested that this impairment was caused by hyperactivation of the Akt/mTOR signalling pathway. Subsequently, intervention with the mTOR inhibitor rapamycin significantly improved cardiac function and survival in $Lmna^{-/-}$ mice, suggesting a key role for mTOR signalling in *LMNA*-associated DCM. Additional analysis suggests a role for dual specificity phosphatase 4 (Dusp4), which is elevated in $Lmna^{H222P/H222P}$ mice. Conditional overexpression of Dusp4 leads to DCM similar to that of *LMNA*-associated DCM and mechanistically results

in activation of the Akt/mTOR signalling pathway and subsequently, negatively regulates autophagy (Choi et al. 2012a), reiterating the phenomenon that was observed in *Lmna*$^{-/-}$ mice. Treatment of the *Lmna*$^{H222P/H222P}$ mice with an inhibitor of mTOR, temsirolimus, subsequently led to activation of autophagy, coupled with amelioration of cardiac decline (Choi et al. 2012b).

The proteasome, another method of protein degradation independent of autophagy, has also been implicated in regulating *LMNA*-mediated disease. Deletion of lysine at position 32 in the lamin A amino acid sequence is known to cause severe congenital muscular dystrophy phenotypes in humans (Quijano-Roy et al. 2008). Recapitulation of this mutation in vivo, using *Lmna*$^{\Delta K32/+}$ mice, also led to pathogenesis (Cattin et al. 2013). These mice presented with a DCM manifestation that resulted in death between 35 and 70 weeks. Mechanistically, this phenotype was characterised by dysfunction of the ubiquitin proteasome system (UPS) leading to nuclear aggregation of the toxic Δ32 lamin variant.

9.3.5 Mitogen-Activated Protein Kinase Signalling

Mitogen-activated protein kinase (MAPK) signalling is activated in response to a diverse number of stimuli such as mitogens, osmotic stress, heat shock and cytokines. Moreover, it is known to be involved in the myocardial response to pathological stimuli such as haemodynamic stress (Heineke and Molkentin 2006). It coordinates the cell response to these stimuli potentially activating or suppressing responses such as cell proliferation, gene expression, mitosis, differentiation, cell survival or apoptosis.

In murine models of NE-mediated heart disease, MAPK signalling has also been implicated. Analysis of the heart tissue from *Lmna*$^{H222P/H222P}$ mice suggests that MAPK signalling is activated in these hearts, specifically the extracellular signal-regulated kinase (ERK1/2) and c-jun N-terminal kinase (JNK) branches of MAPK signalling. Pharmacological regulation of these pathways showed that a number of pathological mechanisms associated with DCM could be retrieved. Inhibition of JNK and ERK1/2 in mice significantly improved LV functional parameters, led to a reduction in fibrosis and collagen mRNA expression, normalised myosin light chain (MLC) mRNA expression and improved survival (Muchir et al. 2009; Wu et al. 2010a, b). Moreover, the clinical relevance of activated ERK1/2 signalling in DCM has been confirmed in postmortem analysis of *LMNA*-associated DCM cardiac tissue expressing other mutations, *LMNA* ΔK261 and *LMNA* IVS9 + 1g>a (Muchir et al. 2012). Additionally, analysis of the emerin-deficient *Emd*$^{-/-}$ mice, which have a mild cardiomyopathy phenotype, showed activation of the ERK1/2 branch of MAPK signalling (Muchir et al. 2007). These data provide a potential therapeutic lead as selumetinib, an ERK1/2 inhibitor, which is currently undergoing trials for treatment of cancer, reports improved survival rates and minimal adverse effects (Janne et al. 2013).

9.3.6 *Other Signalling Pathways Deregulated by Perturbations to the NE*

Cardiac fibrosis observed in *Lmna*$^{H222P/H222P}$ mice was associated with increased nuclear translocation of Smad proteins and TGFβ signalling, which are established mechanisms for fibrosis (Arimura et al. 2005). Furthermore, peroxisome proliferator-activated receptor γ (PPARγ) is a transcriptional regulator of genes associated with fatty acid oxidation and has been linked with cardiomyopathy and is a therapeutic target for a number of cardiovascular and metabolic diseases. Phenotypically, cardiac-specific knockdown of PPARγ in mice leads to cardiac hypertrophy and exacerbation of cardiac fibrosis in response to haemodynamic stress, by way of chronic angiotensin II infusion (Caglayan et al. 2008). In *Lmna*$^{-/-}$ mice, PPARγ expression is decreased. This reduction is potentially mediated by reduced nuclear import of the lamin-binding partner, sterol response element-binding protein 1 (SREBP1), and may in some way explain fibrosis observed in *Lmna*$^{-/-}$ mice (Nikolova et al. 2004).

As well as loss of connexins, the conduction defects observed in *Lmna*$^{N195K/N195K}$ mice could also be explained by an observed reduction in mRNA expression of *HF1b*/*Sp4,* a transcription factor that is important in the development of the cardiac conduction system (Nguyen-Tran et al. 2000).

Finally, mutation of the lamin-binding partner and transcriptional regulator four-and-a-half LIM domain protein 1 (FHL1) has been implicated with in development of a hypertrophy phenotype (Gossios et al. 2013). This observation reinforces the idea that the NE may have a role in hypertrophic signalling and further investigation is required.

9.4 Premature Ageing and Cardiomyopathy

The most extreme laminopathy is HGPS where patients are asymptomatic until around 2 years of age when they start to develop tissue-specific symptoms of premature ageing including subcutaneous fat loss, brittle skin, hair loss and skeletal dysplasia (DeBusk 1972; Gordon et al. 2007, 2011; Hennekam 2006; Kieran et al. 2007). Subsequently, HGPS patients die in the second decade of life primarily due to cardiovascular complications, usually in the form of an ischaemic event, a myocardial infarction or a stroke. Although data suggests the main cause of death is due to vascular defects, recent clinical studies on a relatively large number of patients have also identified cardiac defects. HGPS patients that survive to older ages, upwards of 14 years, were shown to display hypertrophy of the cardiac chambers (Gerhard-Herman et al. 2012). This is possibly because the vascular stiffening and calcification observed in HGPS patients leads to a hypertensive phenotype and subsequent cardiac overload, leading to hypertrophy. However, a small study of younger patients (8–9 years) identified the onset of cardiomegaly in

subjects in the weeks prior to death. This is suggestive of end-stage cardiac dilatation and is most likely of a primary cause (Olive et al. 2010).

At a cellular level, the HGPS phenotype is typified by the presence of a protein termed progerin, a mutated form of the lamin A/C precursor, prelamin A (Gruenbaum et al. 2005). Normally, prelamin A is processed by four post-translational steps to produce mature lamin A (Fig. 9.2), a process mediated and performed exclusively by the zinc metalloproteinase FACE1 transcribed from the *ZMPSTE24* gene (Corrigan et al. 2005). In HGPS, the final enzymatic cleavage is abolished and the peptide remains permanently farnesylated and cannot be inserted efficiently or completely into the nuclear lamina (De Sandre-Giovannoli et al. 2003a). In the most common form of HGPS, this occurs because of a single-nucleotide C > T transition mutation in the *LMNA* gene which confers no change to amino acid sequence, G608G-*LMNA*, but creates a donor splice site in exon 11 leading to a 50 amino acid deletion which removes the FACE1 cleavage site (Eriksson et al. 2003; De Sandre-Giovannoli and Levy 2006; De Sandre-Giovannoli et al. 2003b). It is also the case that loss-of-function mutations to *ZMPSTE24* lead to prelamin A accumulation and can also drive HGPS phenotypes in humans (Shackleton et al. 2005).

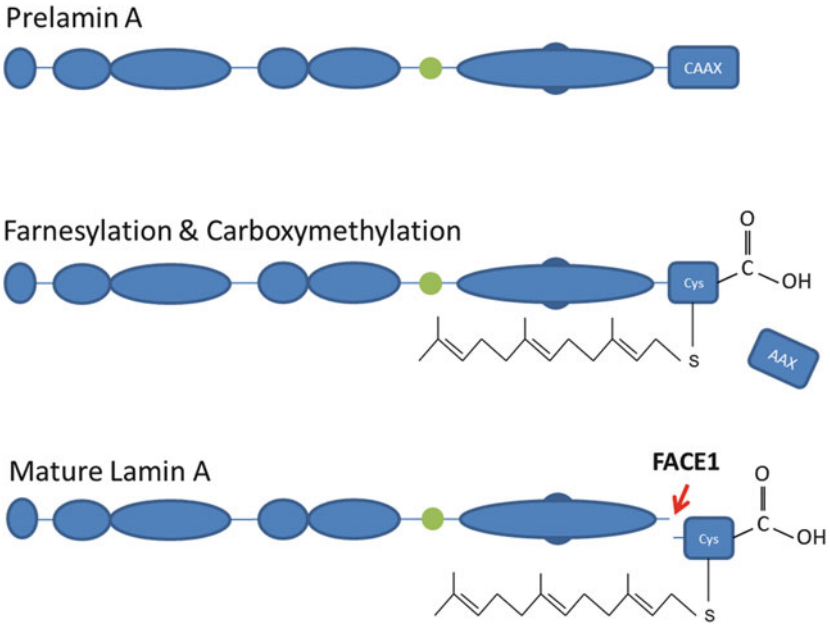

Fig. 9.2 Prelamin A is processed by four post-translational modifications to produce mature lamin A. Firstly, farnesylation of the C-terminal cysteine from the CAAX motif followed by cleavage of the remaining three amino acids. Carboxymethylation of the cysteine occurs and then finally cleavage of the entire farnesyl carboxymethylated tail. Mature lamin A is then inserted into the nuclear lamina to provide structure and mediate nuclear signalling

As previously discussed, disruption to the lamina and subsequently the LINC complex is thought to have deleterious effects on mechanical coupling of cell compartments and subsequently leads to structural instability and susceptibility to mechanical load and stress (Verstraeten et al. 2008). These processes are also thought to be of importance in HGPS; however, it is likely that additional mechanisms may also be operating. Fibroblast cells isolated from HGPS patients display nuclear morphology defects, disorganised heterochromatin distribution and, importantly, also increases in DNA damage (Goldman et al. 2004; Liu et al. 2006, 2008). It is hypothesised that increases in DNA damage are caused by defective recruitment of DNA repair factors due to unstable heterochromatin caused by lamina disruption (Misteli and Scaffidi 2005; Liu et al. 2005). Subsequently, the increases in DNA damage are thought to result in permanent cell cycle arrest in a number of cell types in HGPS patients and lead to cellular senescence, defined as the point at which cells no longer divide and/or respond to external stimuli. Current understanding suggests that these processes are crucial in regulating the pathological response in HGPS.

A number of mouse models expressing mutations designed to mimic progeria have given detailed insight into the possible pathological mechanisms that arise from *LMNA* mutations in relation to progeria and that also have implications for cardiac disease. For example, the L530P-*LMNA* variant that leads to progeria in humans was modelled in vivo by $Lmna^{L530P/L530P}$ mice (De Sandre-Giovannoli et al. 2003b). Whilst these mice showed a progeria phenotype, they also showed cardiac abnormalities. Loss of the processing enzyme ZMPSTE24 was modelled by $Zmpste24^{-/-}$ mice where prelamin A accumulates in the nuclear rim. These mice were progeric and showed fibrosis and lymphocytic infiltration of the myocardium and thinning of the ventricular wall, suggestive of cardiomyopathy (Pendás et al. 2002). Another study showed that accumulation of non-farnesylated prelamin A, studied by the use of $Lmna^{nPLOA}$ mice, led to DCM in mice expressing a homozygous 'non-farnesylated prelamin A-only' allele (Davies et al. 2010). This is cause for interest because the data imply that different stages of prelamin A processing may lead to diverse cellular responses. Another mouse model of HGPS, $Lmna^{G609G/G609G}$ mice (Osorio et al. 2011), showed compelling evidence for cardiac dysfunction as the mice developed bradycardia between 9 and 15 weeks coupled with a prolonged QRS complex, suggesting an inhibition of ventricular depolarisation and potentially conduction defects, providing strong evidence for arrhythmia. However, these mice did not appear to develop DCM. Nevertheless, the frequency in which cardiac perturbations occur in NE-associated disease models of premature ageing suggests that primary cardiac manifestations may play a role in human premature ageing disorders.

9.4.1 Therapeutic Potential

It is currently thought that accumulation of unprocessed prelamin A or progerin intermediates is toxic to cells. At the molecular level, the main difference between

prelamin A and mature lamin A is the retention of a farnesylated residue 15 amino acids from the C-terminus of the translated protein. Previous investigations have suggested that retention of the farnesyl tail was a key component in instigating pathology. This was discovered by the use of farnesyl transferase inhibitors (FTIs) which when administered to isolated HGPS fibroblasts led to a rescue of both the nuclear morphology defects and heterochromatin stabilisation in the nuclear periphery (Glynn and Glover 2005). This finding was verified in murine HGPS models of disease when FTIs led to amelioration of phenotypic traits of disease and significant increases in lifespan (Fong et al. 2006; Yang et al. 2006). Consequently, FTIs were approved for phase III clinical trials in HGPS patients, and initially, the findings were somewhat discouraging (Gordon et al. 2012). However, a recent update of this trial is suggestive of more promising clinical outcomes, as treated patients appear to have increased lifespan (1.6 years) (Gordon et al. 2014). There may also be relevance for these findings in cardiac disease. For example, DCM-causing mutations have been shown to accumulate prelamin A in a variety of systems. The M371K-*LMNA* mutation is known to cause EDMD and late-onset DCM in humans. In vivo, $Lmna^{MHC\text{-}M371K}$ mice, which modelled this mutation in a cardiac-specific manner, showed expression of unprocessed prelamin A intermediates in cardiac tissue (Wang et al. 2006). Additionally in vitro, isolated cell cultures that were subjected to viral transduction and expression of R89L-*LMNA*, a known cause of clinical DCM (Cowan et al. 2010; Taylor et al. 2003), deregulated prelamin A processing and caused accumulation of prelamin A (Brodsky et al. 2007). It is not yet known whether prelamin A accumulation occurs in the presence of other *LMNA* mutations known to cause DCM. However, if it does, then FTI treatment may be an important direction in the future treatment of *LMNA*-associated DCM. Other potential treatments for DCM associated with the NE defects may include modulation of the Akt/mTOR signalling pathway and activation of autophagy, which showed promising increases in survival in the murine $Lmna^{H222P/H222P}$ model after treatment with temsirolimus, an mTOR inhibitor (Choi et al. 2012b). Finally, as mentioned above, modulation of the MAPK signalling cascade may provide therapeutic potential in NE-associated DCM.

9.4.2 Prelamin A and 'Premature Cardiac Senescence'

The notion that a terminally differentiated cell, such as a CM, which exits the cell cycle during maturation, can undergo senescence is controversial. However, it is becoming clear that senescent phenotypes are not necessarily reliant on only the cessation of cell cycle. The ability of the cell to respond to stimuli is also considered important. This is regulated by many of the aspects discussed in this chapter, such as mechano- and cell signalling, structural stability and DNA damage. Deregulation of these pathways is linked to external factors often associated with environmental stimuli, such as UVB and IR radiation and air pollution as well as cigarette smoke, alcohol consumption, diet and exercise, and are known to be crucial in determining

the progression of age-related diseases. In such settings, cells are thought to become senescent prematurely and become susceptible to tissue-level changes, such as vascular remodelling and atherosclerosis, and eventually heart disease caused by persistent high blood pressure or ischaemia. Importantly, prelamin A has been identified as a biomarker for ageing in a number of tissues including vascular tissue (Ragnauth et al. 2010). In cardiac tissue, the picture is unclear but new insights are emerging. The pleiotropic effects of single *LMNA* mutations such as the R644C mutation, for example, which can lead to several separate disease phenotypes, many with cardiac involvement (Mercuri et al. 2005; Rankin et al. 2008), suggest that complex genetic and environmental factors can contribute to disease. Further evidence that cell-extrinsic pathways may be important comes from Osorio et al., who investigated Zmpste24 mosaic mice, null for *Zmpste24* on the autosomal *Zmpste24* locus but with an extra copy of *Zmpste24* placed on the X chromosome at the *Hprt* locus. These animals accumulated prelamin A in half of cells whilst the other half produced wild-type lamin A. The results were intriguing. Contrary to the original *Zmpste24*$^{-/-}$ model, mice were long-lived despite the expression of prelamin A, and the authors hypothesised that non-progeric cells rescued the phenotype of progeric cells and that as such cell-extrinsic mechanisms were pre-eminent in the development of progeroid syndromes (de la Rosa et al. 2013).

The finding that cell-extrinsic mechanisms are potentially required for prelamin A to mediate its toxic impact on cells and tissues suggests that prelamin A accumulation causes cells and tissues to become susceptible to external stressors accelerating cellular senescence. Given these observations, it is curious that researchers have not explored the notion of myocardial senescence in models of *LMNA*-associated DCM. The observation that mTOR signalling plays a key role in *LMNA*-associated DCM theoretically supports a myocardial senescence paradigm, as the Akt/mTOR signalling axis is commonly implicated in senescence studies (Burtner and Kennedy 2010). Moreover, there are no published observations regarding DNA damage accumulation in *LMNA* models of DCM. Most of the *LMNA*-associated DCM models show nuclear morphology defects and some show heterochromatin disorganisation (Fig. 9.3). These phenotypic features are also observed in HGPS and are associated with the accumulation of DNA damage and the processes that underlie premature senescence in these patients. Potentially, they may also be involved in NE-associated DCM and therefore require investigation.

Current evidence displays the variety of nuanced cardiac manifestations arising from NE perturbations, especially with regard to defective NE organisation and the nuclear lamina. Moreover, DCM observed in the setting of some *LMNA* mutations may be a pathologic end point of prelamin A accumulation and a ‘senescence-like phenotype’. More insights are needed to confirm the presence or importance of prelamin A accumulation in cardiac pathogenesis. Additionally, if one considers the notion that dilatation of the cardiac chambers is an end-stage process of progressive heart disease, often occurring with age, it could be postulated that *LMNA*-associated DCM is a form of ‘premature cardiac ageing’.

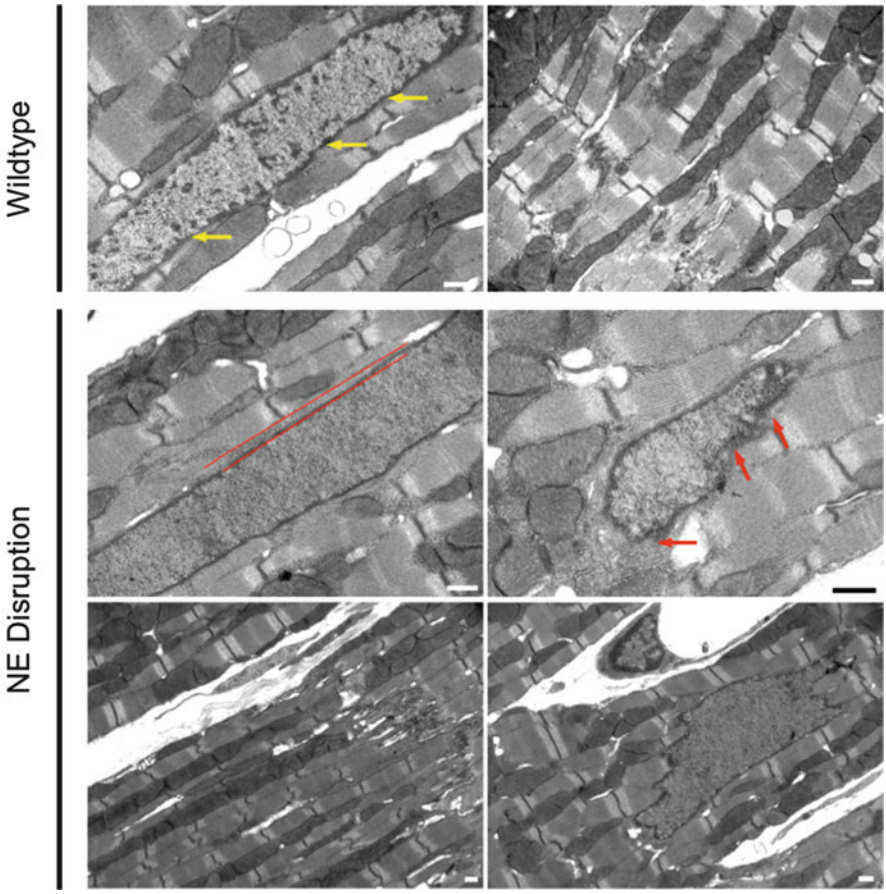

Fig. 9.3 NE perturbation leads to nuclear morphology defects, loss of heterochromatin and disruption of the nuclear membrane-sarcomere junction. Perfusion fixed hearts were processed and stained for transmission electron microscopy. The sarcomere organisation and nuclear morphology was normal in Wt hearts as was the appearance of heterochromatin (dark spots within the nucleus). Comparatively, nuclei of models with perturbed NE were often dysmorphic with a loss of heterochromatin especially at the nuclear periphery. The distance between the sarcomere and nuclear membrane appeared to be increased in hearts with perturbed NE (highlighted here with *red borderlines* and *red arrows*) compared to normal wild-type mice, which had no observable space between the nuclear membrane and sarcomere (*yellow arrows*). Scale bar = 500 nm

9.5 Summary

The role of the NE and LINC complex is to provide a physical and mechanical association between the nucleus and cytoskeleton, thereby maintaining structural integrity of the nucleus and cell as a whole. Additionally, the LINC complex is crucial for facilitating mechanosignalling and regulating gene transcription and DNA damage responses. Disruption of NE components deregulates these processes

and leads to a range of phenotypically diverse, tissue-specific nuclear envelopathies, often with features of premature ageing disorders such as HGPS. Mutations to the NE, especially A-type lamins and emerin, also lead to multiple cardiomyopathic phenotypes characterised mechanistically by susceptibility to mechanical strain, aberrant mechanosignalling, deregulated cell signalling pathways and dysfunctional cell maintenance systems. Accumulating evidence from clinical and in vivo murine studies suggests that diseases arising from perturbations to the NE in cardiac tissues are influenced by extrinsic factors and may form part of a 'premature cardiac senescence' paradigm involving the accumulation of prelamin A, although additional investigation is required. Finally, mTOR inhibitors currently provide the most realistic therapeutic potential for *LMNA*-associated DCM, whilst the use of FTIs in HGPS patients may have future relevance.

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Chapter 10
AMP-Activated Protein Kinase: A Metabolic Stress Sensor in the Heart

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Abstract AMP-activated protein kinase (AMPK) is a central cellular signaling hub that senses and responds to different kinds of stress, mainly those triggered by impaired cellular energy homeostasis. Since this is of major importance for the heart, the kinase plays important roles for cardiovascular function in human health and disease. Here, we review recent progress on the molecular structure and role of AMPK and summarize regulation and biological actions of the AMPK pathway, in particular those relevant for the heart. Activation of the kinase is involved in the myocardial response to ischemia, pressure overload, and heart failure. Pharmacological activation of AMPK may prove to be a useful therapeutic strategy in the treatment of these pathologies.

The heart is one of the most energy-requiring organs, and it needs a perfect match of energy supply with energy demand to maintain its continuous contractile performance. The most relevant protein kinase in the context of metabolic stability and energy homeostasis is the AMP-activated protein kinase (AMPK). Many studies have confirmed the importance of AMPK signaling for a correct functioning of the cardiovascular system (reviewed in Arad et al. 2007; Dyck and Lopaschuk 2006; Kim et al. 2009; Young 2008; Zaha and Young 2012).

AMPK can be characterized as gatekeeper of cellular energy homeostasis and key regulator of energy metabolism, since it plays a central role in sensing and regulating energy state at the cellular, organ, and whole-body level (Winder and Hardie 1999; Hardie and Carling 1997). It is an evolutionary conserved and ubiquitously expressed protein kinase (Hardie 2007, 2011) which is thought to have evolved as one of the first kinase signaling pathways in unicellular eukaryotes in response to starvation for a carbon source. It only later during evolution developed into a more general metabolic and energy stress sensor (Hardie 2011, 2014b; Hardie and Ashford 2014).

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Activation of AMPK is triggered by a diverse array of signals linked to limited energy availability in physiological and pathological situations, including extracellular (e.g., hormones, cytokines, nutrients) and intracellular stimuli (e.g., AMP, ADP, Ca^{2+}) (Hardie et al. 2012a). AMPK activation occurs in the context of metabolic stress that decreases ATP and increases intracellular AMP, ADP, or Ca^{2+} concentrations. These include nutrient starvation, hypoxia (Hardie et al. 1999; Marsin et al. 2002), metabolic poisons (e.g., that inhibiting mitochondrial ATP production), or muscle contraction (Winder and Hardie 1996). AMPK activation involves covalent phosphorylation and allosteric effects of AMP, ADP, and possibly other metabolites (Calabrese et al. 2014). Generally, these activation mechanisms cooperate in a very complex manner, even though new findings suggest they may also occur independently (Scott et al. 2014). They are coordinated to activate AMPK in situations of energy deficit and aim at compensating ATP loss, mostly via accelerated catabolism and inhibited anabolism. However, AMPK exerts pleiotropic control not only of metabolic pathways but also of other physiological functions more indirectly linked to cell energetics. These include growth, proliferation, shape, and motility of cells, autophagy and apoptosis, and even central, systemic control of appetite in the hypothalamus (reviewed in Steinberg and Kemp 2009). In all these cases, AMPK mediates fast (acute) effects by regulating the activity of metabolic key enzymes and others and slow (chronic) effects by tuning gene expression of these proteins. The downstream targets of AMPK have made this kinase also a prime pharmacological target for treating type II diabetes, cancer, and other pathologies (Hardie 2008b; Zhang et al. 2009; Inoki et al. 2012; Srivastava et al. 2012).

It is to note that the majority of our knowledge on AMPK comes from noncardiac cells, and part of it may not be applicable to the heart. For example, this may concern the nature of activating stimuli and/or the threshold of activation (Zaha and Young 2012). The heart is a quite unique organ in several aspects, in particular in the context of its energy metabolism. The cardiac metabolic network is characterized by an unusual stability. Energy homeostasis in the heart is maintained by multiple regulatory mechanisms controlling cellular ATP production, utilization, and transfer, including allosteric regulations and feedback loops, microcompartmentation, and metabolic channeling with concerted action of several metabolic and signaling kinases (Neumann et al. 2007; Saks et al. 2006). AMPK activation in the heart, in contrast to most other tissues, seems rather to act as a last safeguard during severe energy deprivation and in pathological situations. It plays an important role in the myocardial response to pathological stimuli like ischemia reperfusion (Kudo et al. 1995; Russell et al. 2004), pressure overload (Kim et al. 2012a; Tian et al. 2001), or heart failure (Sasaki et al. 2009). Thus, it functions also in the heart as a metabolic master regulator, orchestrating the cardiac response to various stress-related stimuli (Arad et al. 2007; Kim et al. 2009; Young 2008; Zaha and Young 2012). Importantly, pharmacological activation of AMPK also holds promise as a therapeutic strategy for treating different cardiovascular diseases (Calvert et al. 2008; Sasaki et al. 2009; Shinmura et al. 2007).

Here, we briefly review the AMPK structure, the way the kinase is activated, the regulated metabolic pathways and cellular functions relevant for the heart, as well as the available AMPK-targeted drugs. For any more extensive information on these or other issues of AMPK signaling, the reader is referred to the many excellent general reviews (Hardie 2014a, b, c; Hardie et al. 2012a; Mihaylova and Shaw 2011; Steinberg and Kemp 2009) and reviews on the role of AMPK in the heart (Kim and Dyck 2015; Kubli and Gustafsson 2014; Wang et al. 2012b; Zaha and Young 2012b).

10.1 Evolving Physiological Roles

AMP-activated protein kinase (AMPK) was first described in studies on the regulation of lipid metabolism, where an enzyme activity was identified that is responsible for phosphorylation and inhibition of acetyl-CoA carboxylase (ACC) and 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase, HMGR) (Beg et al. 1980), and which was activated by AMP (Ferrer et al. 1985). Since both phosphorylations are inhibitory, AMPK was first perceived as a downregulator of lipid synthesis.

However, it then became evident that AMPK more generally functions as a cellular "fuel gauge" and can regulate many metabolic processes related to the cellular energy state. With the discovery that both AMP and ADP are activators (Oakhill et al. 2011; Xiao et al. 2011), AMPK regulation became quite similar to what has been described already 50 years ago by Atkinson as the so-called energy charge regulation of cell metabolism (Atkinson 1968). In fact, AMPK is able to respond to low energy charge by reorganizing energy and metabolic fluxes toward a nonstressed state. It thus represents a true control point for maintaining energy and metabolic homeostasis.

The role of AMPK has been even further extended, with AMPK activity also depending on physiological stimuli independent of the cellular energy charge like hormones and nutrients (Minokoshi et al. 2002, 2004). As mentioned above, more recently identified AMPK substrates reveal that AMPK signaling acts far beyond the control of primary metabolism, as, e.g., in proliferation, autophagy, and central appetite control. Thus, AMPK can now be defined as a "metabolic sensor" or "metabolic master switch." However, also in these cases, AMPK signaling somehow acts to prevent a low energy state of cells, tissues, or the entire organism, by preventing ATP-consuming processes (growth, motility) or favoring potentially ATP-generating processes (autophagy, appetite).

10.2 Molecular Structure

The AMPK family consists of evolutionary conserved and ubiquitously expressed serine/threonine kinases that present complex structural and functional features. Structurally, AMPK occurs in vertebrates exclusively as an obligatory heterotrimeric protein complex composed of a catalytic subunit, α, and two regulatory subunits, β and γ. As a first layer of complexity, in vertebrates, each subunit occurs as different isoforms (α1, α2, β1, β2, γ1, γ2, and γ3) encoded by distinct genes (Carling 2004; Hardie et al. 2003), including some splice variants (of γ2 and γ3), generating a large variety of heterotrimeric complexes. Alternative promoters can further increase this complexity (Kahn et al. 2005). The precise physiological significance of all these isoforms is not yet entirely clear.

Much has been learned about the molecular structure of AMPK by crystallographic studies on AMPK domains and heterotrimeric core complexes (carrying larger truncations) (Townley and Shapiro 2007; Amodeo et al. 2007, 1; Chen et al. 2009, 2012, 2013; Oakhill et al. 2011; Xiao et al. 2007, 2011) and most of all by the most recently published structures of AMPK holo-complexes (Xiao et al. 2013; Calabrese et al. 2014; Li et al. 2014). The so far most complete X-ray structure covers the entire α2-subunit (with some internal truncations), the β1-subunit (lacking only the flexible N-terminal portion), and the entire γ1-subunit (Xiao et al. 2013) (Fig. 10.1). This work, stimulated by a growing interest in AMPK as a putative drug target, revealed the overall topology of the heterotrimer, the conserved global fold of large parts of the subunits, and suggested putative activation mechanisms. However, a high-resolution structure of full-length heterotrimeric complex in both active and inactive states is still lacking.

α-Subunit The α-subunit contains a typical Ser/Thr protein kinase catalytic domain in its N-terminal part, with features conserved throughout the entire protein kinase superfamily (Hanks et al. 1988). It harbors a typical activation loop carrying the critical Thr172 residue which is phosphorylated for activation by AMPK upstream kinases like liver kinase B1 (LKB1) or Ca^{2+}-calmodulin-dependent protein kinase kinase beta (CamKKβ ; see Chap. 4) (Hawley et al. 2003, 2005; Woods et al. 2003, 2005). This phosphorylation is considered as essential for AMPK activity (Hawley et al. 1996), although this has been recently challenged (Scott et al. 2014). Thr172 phosphorylation is also often used as a readout for AMPK activity, although this may not always be correct (see Chap. 4). The C-terminal part of the α-subunit carries different and important functionalities. Immediately downstream of the catalytic domain follows the autoinhibitory domain (AID) (Crute et al. 1998), which when fused to the kinase domain reduces AMPK activity as compared to kinase domain alone (Chen et al. 2009; Pang et al. 2007). In the further C-terminal sequence follows the so-called linker peptide and finally the very C-terminal region (~150 amino acids) which is required for association with the β-subunit. The latter also contains a long Ser/Thr-rich loop (not resolved in X-ray structures), as well as a nuclear export sequence [known to be functional in α2 (Kazgan et al. 2010)].

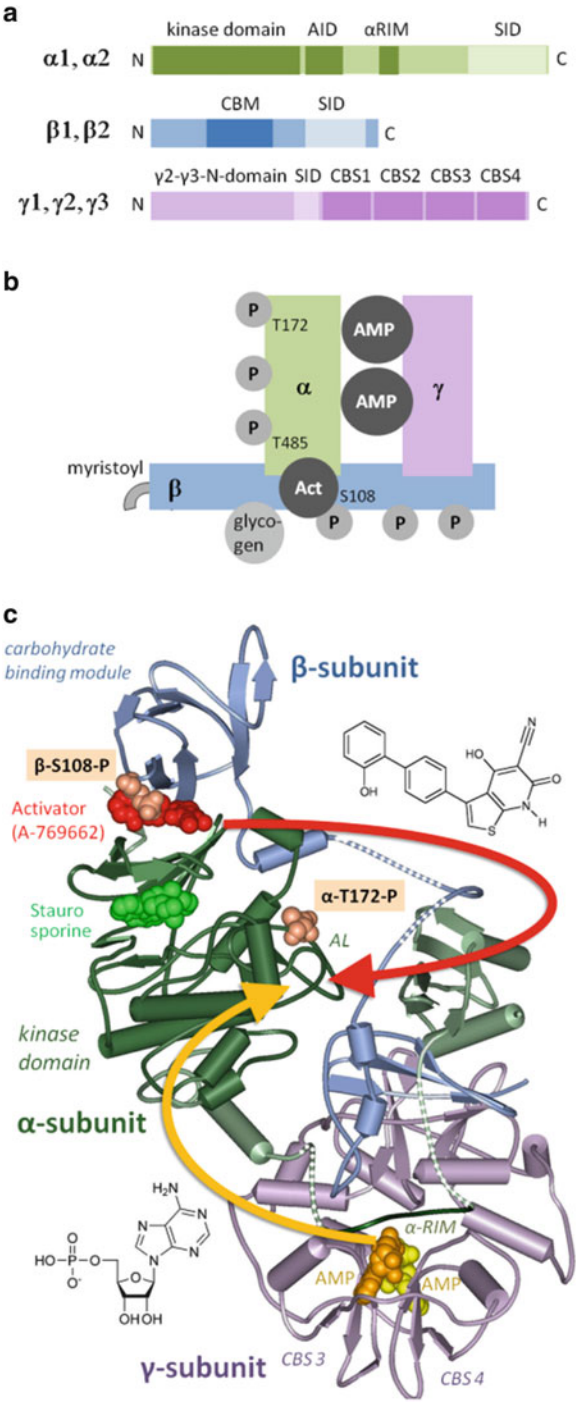

Fig. 10.1 AMPK structure. (**a**) AMPK domain structure of the three AMPK subunits (SID, subunit interaction domains; AID, autoinhibitory domain; CBM, carbohydrate-binding module;

Of particular importance is the linker peptide, since it wraps around the γ-subunit like in a close embrace. A part of this linker peptide, first identified as α-hook, closely contacts γ-subunit (Xiao et al. 2011). Chen et al. (2013) more recently corrected the amino acid register for the electron density in this region, revealing the true γ-interacting sequence termed α-regulatory subunit interacting motif (α-RIM), interacting with CBS site 3 (see below) and with a pocket formed by a newly observed β-subunit loop. More recent structures of the near full-length heterotrimers confirmed these interactions of α-linker with β- and γ-subunits (Xiao et al. 2013) and its role in moving AID away from the kinase domain in the activated state (Li et al. 2014). In addition to Thr172, the α-subunit can be phosphorylated on several other residues both in vitro and in vivo. Most of these phosphorylations occur in the Ser/Thr-rich loop and seem to inhibit the activating Thr172 phosphorylation (see Chap. 4). Further structural studies will be necessary to delineate covalent and non-covalent activation of the kinase domain in molecular detail.

β-Subunit The regulatory β-subunit represents the core of the heterotrimeric complex, since it provides a scaffold for binding of catalytic α- and regulatory γ-subunits. The N-terminal domain of the β-subunit carries an additional regulatory element, the conserved carbohydrate-binding module (CBM; also called glycogen-binding domain, GBD). Its structure complexed to beta-cyclodextrin has been solved (Polekhina et al. 2005), and it was shown that glucose α1-6-branched glycogen can behave as allosteric inhibitor, negatively regulating AMPK phosphorylation by its upstream kinases (McBride et al. 2009). In addition, CBM may serve to recruit AMPK to glycogen-bound downstream targets such as glycogen synthase (Hardie and Sakamoto 2006). The recent near full-length AMPK structures confirmed that glycogen binding moves CBM away from the α-kinase domain, while binding of pharmacological activators 991 and A769662 and autophosphorylation of β-S108, both at the α/β-interface (see Chap. 4), closely attach CBM to the kinase domain (Xiao et al. 2013; Li et al. 2014). The former conformation seems to be rather inhibitory, while the latter strongly activates AMPK. Thus, CBM is part of an allosteric regulatory site, which may also sense

Fig. 10.1 (continued) CBS, cystathionine-β synthase). (**b**) AMPK complex topology. Subunit interactions, secondary modifications (phosphorylations; myristoylation), and allosteric interactors (AMP; Act, putative activator at α/β interface). (**c**) Molecular structure and activation of the full-length AMPK heterotrimer (PDB 1CFF; Xiao et al. 2013). Binding of activating AMPK ligands AMP (γ-subunit) and A-769662 (β-subunit) has to be transduced to the α-subunit kinase domain for activation (see *arrows*), involving conformational changes. AMPK subunits α (*green*), β (*blue*), and γ (*magenta*) with α-subunit kinase domain, activation loop (AL), and regulatory interacting motif (α-RIM; Chen et al. 2013) indicated (*dark green*) and β-subunit carbohydrate-binding module (CBM) labeled; sequences missing in the structure (*dashed*) include the α-autoinhibitory domain (AID). Activation-relevant phosphosites (α-T172 in the activation loop and β-S108 in the CBM; *red brown*), activating ligands [A-769662 (*red*) and AMP (*orange/yellow*)], and kinase inhibitor staurosporine in the active site (*green*) are shown in spacefill representation. For further details see text (Figure modified from Viollet et al. 2014)

cellular energy reserves in the form of glycogen and mediate effects of certain pharmacological activators. The large very N-terminal portion of the β-subunit is not resolved in the known X-ray structures, and its function is not entirely clear. An N-terminal myristoylation affects activation (Oakhill et al. 2010) and could mediate membrane interaction. The sequence could also be involved in the interaction of AMPK with other proteins (Klaus et al. 2013).

γ-Subunit All γ-subunits contain in their C-terminal part four tandem cystathionine β-synthase (CBS) repeats, a motif named by analogy to the cystathionine β-synthase in which it was first identified (Bateman 1997). In AMPK γ-subunits, the four CBS sites (numbered CBS 1 to CBS 4 according to their occurrence in the sequence) constitute a flattened disk with one CBS repeat in each quadrant and two pairs of CBS motifs assembling into a so-called Bateman domain. Four potential binding sites for adenylates (AMP, ADP, ATP) are created in the cleft between the CBS motifs, numbered according to the CBS motif that provides the conserved Asp for adenine ribose interaction (Kemp 2004). According to several crystal structures of the mammalian γ1-subunit in the presence of various nucleotides (Chen et al. 2012, 2013; Xiao et al. 2007, 2011, 2013), it appears that only CBS sites 1, 3, and 4 are functional, while the site 2 is different and always empty. The γ2- and γ3-isoforms contain long N-terminal extensions, which can be subject to truncation by RNA splicing, and whose molecular structure and function are currently unknown. The different γ-subunit isoforms and splice variants may be involved in protein/protein interaction and confer different cellular localization and function (Pinter et al. 2012).

10.3 Localization

Tissue Specificity; Cardiac AMPK AMPK isoforms show some differences in their tissue- and developmental-specific expression patterns, although the physiological significance is still uncertain. There is no strict tissue specificity of AMPK isoforms, but increasing evidence suggests that a given tissue expresses a specific subset of AMPK heterotrimers which may be linked to particular signaling pathways in this tissue (Table 10.1). Studies with transgenic mice lacking specific α- and β-subunits have contributed to progress in this field (Viollet et al. 2009). While the α1β1γ1 complex is probably the most abundant in a vast majority of cell types, differences seem to occur in the amount of additional isoforms in a given tissue.

The heart contains high levels of the α2-isoform, which is much less expressed in the skeletal muscle and liver and almost absent in the brain. The β2-isoform is abundant in the heart and also in the muscle and brain. In addition to the γ1-isoform, the heart expresses a specific intermediate-length γ2-splice variant (γ2-3B), while γ3 seems to be quite specifically expressed only in the skeletal muscle (Stapleton et al. 1996; Thornton et al. 1998; Pinter et al. 2012). There are also pathological and developmental changes in cardiac AMPK expression. The α2-, β2-, and γ2-isoforms are all upregulated by pressure overload or heart failure in rodents,

Table 10.1 Tissue expression of AMPK subunit isoforms

Isoform	Heart	Skeletal muscle	Brain	Liver	Lung
Alpha 1	++	++	++	++	++
Alpha 2	+	+		+	
Beta 1	++	+	++	++	+
Beta 2	++	++	++		+
Gamma 1	++	+	++	+	+
Gamma 2	+[a]	+	+		
Gamma 3		+			

Data from Mahlapuu et al. (2004), Quentin et al. (2011), Stapleton et al. (1996), Thornton et al. (1998)
[a]Specific splice variant (Pinter et al. 2012)

although in patients rather the content of α1, β1, and γ2 (an intermediate form) increases with different forms of cardiomyopathy (Tian et al. 2001; Kim et al. 2012a). During embryonic development in rodents, γ1 increases, while high levels of γ3 disappear, and the embryonically predominant full-length γ2-form is replaced by γ2-3B in the heart but by short γ2b in other tissues (Pinter et al. 2012). These developmental and tissue particularities may also explain why γ2-gene mutations in the CBS domains cause hereditary hypertrophic cardiomyopathy but no other pathological symptoms (see Chap. 6). Full-length γ2 and γ2-3B share an N-terminal domain with unknown function that could localize the AMPK complex to specific compartments or signaling pathways (Pinter et al. 2012). Total cardiac AMPK activity increases after birth, contributing to the switch toward the predominant use of fatty acids (Makinde et al. 1997). AMPK levels may also be determined by ubiquitin-dependent protein degradation (Qi et al. 2008; Moreno et al. 2010).

Subcellular Distribution The subcellular distribution and recruitment of AMPK isoforms to specific cellular sites is increasingly recognized as an important factor for their signaling function. AMPK is generally observed as a soluble complex with diffuse cytosolic localization. However, at least α2-containing complexes in their activated form, e.g., after exercise in the skeletal muscle, also translocate into the nucleus to phosphorylate nuclear substrates such as transcription factors, histones, and histone deacetylases (McGee et al. 2003, 2008; Suzuki et al. 2007). Minor portions of AMPK may associate with cellular structures like specific membranes, where processes are regulated by AMPK (e.g., ion channel activity, cell polarity, or cell junction formation) (Forcet and Billaud 2007; Andersen and Rasmussen 2012; Nakano and Takashima 2012; Ramírez Ríos et al. 2014). Myristoylation of the AMPK β-subunit can localize the kinase complex to membranes and increases its activability, thus possibly favoring activation of membrane-bound complexes (Suzuki et al. 2007; Oakhill et al. 2010).

Multiprotein Complexes AMPK can also be recruited into specific complexes via interaction with its upstream kinases, downstream substrates, or more general with

scaffolding proteins. However, the AMPK interactome is only partially known so far from some targeted and non-biased interaction studies conducted by us and others (e.g., Behrends et al. 2010; Klaus et al. 2012), and more research is needed on this issue, in particular in the heart. AMPK interaction with LKB1, its major upstream kinase in the heart, could recruit AMPK to places of LKB1 localization, including the mitochondrial surface or E-cadherin in adherens junctions (Sebbagh et al. 2009). Close co-localization of both, AMPK and LKB1, can also be mediated by membrane interaction of both, farnesylated LKB1 and myristoylated AMPK (Houde et al. 2014).

Scaffolding proteins can in principle provide high specificity in cell signaling by isolating activated kinases from bulk signaling and directing the information flow into specific pathways. In the heart, for example, AMPK competes with p38 MAPK for binding to the scaffolding protein TAK-1–binding protein-1, thus blunting p38 activation during ischemia (Li et al. 2005). Mitochondrial VDAC may represent yet another anchoring protein that recruits AMPK to this organelle (Strogolova et al. 2012). Most interestingly, the scaffold protein axin together with the Ragulator complex at the lysosomal surface has been proposed as important regulators of AMPK activation (Zhang et al. 2013, 2014). These data support a model where axin bound to LKB1 recruits AMPK in the AMP-bound state, leading to AMPK phosphorylation and activation. Further, in particular under nutrient-poor conditions, the axin-LKB1-AMPK complex seems to interact with the Ragulator complex which is tethered via its LAMTOR1 component to the lysosomal surface. The Ragulator complex, apparently by its interaction with the lysosomal v-type ATPase, seems to be an independent sensor of cellular nutrient conditions. It is known to recruit the nutrient-signaling TORC1 complex (see Chap. 5) to lysosomes under nutrient-rich conditions (Bar-Peled and Sabatini 2014), thus suggesting reciprocal recruitment and activation of axin-LKB1-AMPK or mTORC1, depending on the cellular nutrient state (Hardie 2014c). It is currently unknown whether such regulation exists in the heart.

There is also some evidence that AMPK subunit isoforms determine specific protein/protein interactions. The β-subunit may in some cases confer substrate specificity, as has been shown in yeast (Vincent and Carlson 1999) and plants orthologs (Polge et al. 2008), but with mammalian AMPK [IntAct database (Kerrien et al. 2012)]. We recently found the β2-isoform interacting with Mu- and Pi-type glutathione transferases (GSTs) to favor glutathionylation of the α-subunit (Klaus et al. 2013). However, in the case of fumarate hydratase (FH), we identified a specific interaction with α2-containing AMPK complexes to facilitate FH phosphorylation (Klaus et al. 2012).

10.4 Activation

AMPK integrates various intra- and extracellular signals and maintains cross talk with other signaling pathways. This makes the kinase a central signaling hub in sensing and regulating cellular energetics and ATP-dependent functions. Indeed, the most recent research revealed that AMPK activation is much more complex than initially anticipated and that it depends on multiple covalent modifications and allosteric effectors (Fig. 10.2). Such AMPK regulation evolved from a more simple state as, e.g., in the yeast AMPK homologues that lack allosteric activation by AMP to the more complex regulation present in vertebrates.

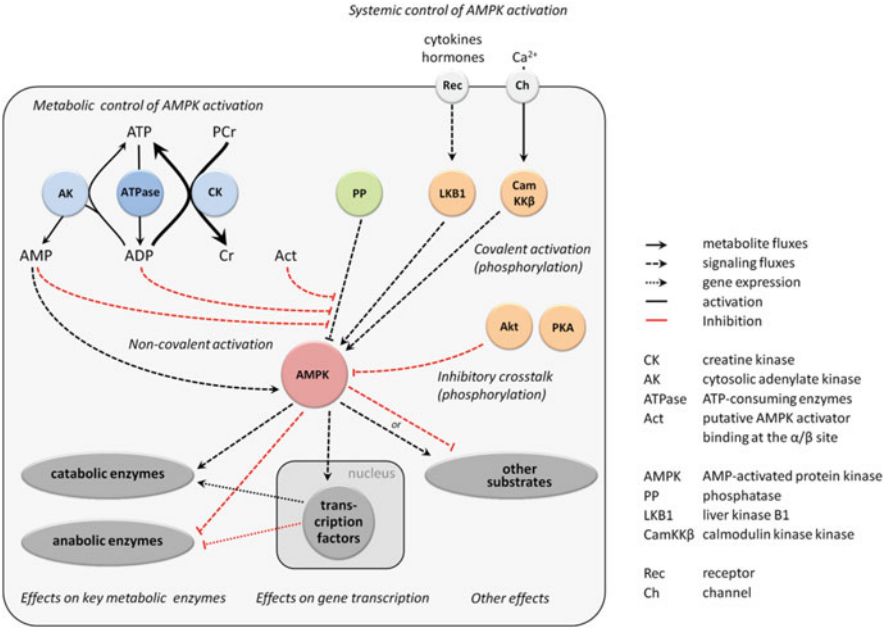

Fig. 10.2 AMPK signaling. AMPK is activated by intra- and extracellular metabolic and endocrine signals and affects various downstream processes. Activation of AMPK is triggered by upstream kinases (covalent activation by LKB1, CamKKβ, inhibition by Akt and PKA) and phosphatases. They mediate mainly extracellular signals carrying, e.g., information on the energy and nutrient state of the cellular environment and the entire organism (endocrine signals; systemic control of AMPK). Covalent activation also depends on some intracellular parameters (Ca^{2+}, possibly also ROS/RNS), as well as the allosteric ligands. The second layer of regulation is represented by AMPK activation via AMP and ADP (allosteric regulation), both acting as second messengers of cellular energy stress (metabolic control of AMPK). This signaling is linked to conversion of nucleotides via the adenylate kinase (AK) and creatine kinase (CK) reactions. Activated AMPK compensates for ATP loss by accelerating catabolism and inhibiting anabolism and exerts further effects on cell motility, growth, proliferation, and others, via regulation of key enzymes and transcription factors. For further details see text

Covalent Regulation by Phosphorylation The phosphorylation state of the conserved threonine within the kinase domain activation loop (conventionally referred as Thr172) determines the primary activation of AMPK. As compared to an inactive state, this phosphorylation can increase kinase activity by more than 100-fold (Suter et al. 2006). The AMPK phosphorylation state depends on the balance between the activity of different upstream kinases and phosphatases. The two well-established upstream kinases are tumor suppressor kinase complex LKB1-STRAD-MO25 (Woods et al. 2003; Shaw et al. 2004; Hawley et al. 2003) and Ca^{2+}-calmodulin-dependent protein kinase kinases (CamKK), in particular CamKKβ (Hawley et al. 2005; Woods et al. 2005; Hurley et al. 2005). LKB1 is the major AMPK kinase in most cells, including cardiomyocytes. However, this kinase seems to mostly exhibit constitutive activity and may thus not be the limiting step in AMPK activation. More recent studies suggest that close co-localization of LKB1 with AMPK involving the scaffold protein axin and the lysosomal surface may be necessary for efficient AMPK activation via the LKB pathway (see Chap. 3; Zhang et al. 2013, 2014). In some cell types, in particular in the brain but much less in the heart, AMPK is activated predominantly in a Ca^{2+}-dependent manner by CamKKβ. Such CamKKβ-mediated AMPK activation might anticipate an increasing energy turnover that accompanies a rise in cytosolic Ca^{2+} during muscle contraction, but its role in the heart is not well understood. Transforming growth factor-β (TGF-β)-activated kinase 1 (TAK1) has been suggested as another AMPK kinase (Herrero-Martín et al. 2009) and also as an AMPK substrate (Kim et al. 2012b). TAK-1 is present in the heart, but not activated during ischemia, and it is unclear whether it acts via direct AMPK phosphorylation (Xie et al. 2006b).

Protein phosphatases are possibly the more critical parameter governing the α-Thr172 phosphorylation state, and this may also apply to the heart. AMPK covalent activation is modulated by the protein phosphatase 1 (PP1) (Garcia-Haro et al. 2010), the protein phosphatase 2C (PP2Cα) (Sanders et al. 2007a), and the calcium-mediated protein phosphatase 2A (PP2A) (Park et al. 2013). It was also proposed that α-SNAP may exhibit phosphatase activity on AMPK Thr172 according to in vitro dephosphorylation assay (Wang and Brautigan 2013). However, in tissues including the heart and endothelial cells, especially expression levels PP2C and 2A, respectively, correlate well with AMPK activation (Wang and Unger 2005; Wu et al. 2007).

There seems to be a cross talk of AMPK with many other cellular signaling pathways. Mainly the α-Thr172 phosphorylation state is negatively controlled by hierarchical phosphorylation at other sites in the AMPK heterotrimer, in particular in the α-Ser/Thr-rich loop. Protein kinase B (PKB/Akt) that is activated under glucose-rich conditions by insulin signaling is inhibiting AMPK by phosphorylation at rat α1-Ser485 (much less so at the equivalent α2-Ser491) which reduces phosphorylation at the activating α-Thr172 (Hawley et al. 2014; Horman et al. 2006). Thus, hyperactivation of PKB/Akt as occurring in many tumor cells, and also in the heart under doxorubicin treatment (see Chap. 6), can strongly downregulate AMPK activation, with negative effects on proliferation control

and cell energetics, respectively. Similar inhibitory phosphorylations of AMPK were reported for protein kinase GSK3β (Suzuki et al. 2013) and protein kinase A (PKA) (Hurley et al. 2006; Djouder et al. 2010). In the latter case, not the Ser/Thr-loop phosphorylations (including Ser485/Ser491) seem to be inhibitory but rather another one at α-Ser173 (Djouder et al. 2010). The physiological rationale underlying AMPK inhibition by GSK3β and PKA is, however, less obvious. AMPK is further negatively controlled by the Ras/Raf/MEK/ERK pathway in a more complex manner, involving negative feedback loops. While active AMPK can reduce MEK/ERK signaling via phosphorylation of upstream B-Raf (Shen et al. 2013), active ERK can reduce AMPK signaling by inhibitory phosphorylation of the AMPK upstream kinase LKB1 (Zheng et al. 2009). Further phosphorylation sites were identified in both AMPK α- and β-subunits, many of them targeted by autophosphorylation, but their functional role remains uncertain. As recently discovered, (auto)phosphorylation of β-Ser108 close to the glycogen-binding domain seems to be an important second allosteric regulatory site (see below).

Endocrine Signals Information about the cellular environment and whole-body energy and nutrient state is linked to AMPK signaling via endocrine, paracrine, and autocrine mechanisms. These include a diverse array of hormones and cytokines (Table 10.2). They regulate AMPK mainly by triggering AMPK phosphorylation via upstream kinases, and this regulation often occurs in a tissue-specific manner. Best studied are probably the orexigenic/anorexigenic hormones ghrelin and leptin. In peripheral tissues, leptin activates AMPK to regulate fatty acid oxidation and glucose uptake. In hypothalamus, leptin inhibits and ghrelin activates AMPK to decrease and increase appetite, respectively, in order to regulate food intake (Steinberg and Kemp 2009; Steinberg 2013). Other endocrine factors that affect AMPK activity are sex hormones that act via LKB1 (McInnes et al. 2012) and angiotensin 2 (Nagata et al. 2004; Steinberg 2013). Endocrine signals in the heart are discussed in Chap. 6.

Calcium Signals As described above, cellular calcium can regulate the Thr172 phosphorylation state of AMPK via calcium-homeostasis-related kinases in phosphatases, in particular CamKKβ (Hawley et al. 2005) and PP2A (Park et al. 2013), respectively. This can also be demonstrated with calcium ionophores (e.g., A23187) in LKB1-deficient cells. Ca^{2+}- and AMP-dependent AMPK activation occurs independently and can be synergistic, since AMP binding (see below) protects the Ca^{2+}-induced phosphorylation (Fogarty et al. 2010).

Non-covalent Regulation The second major mechanism of AMPK activation relies on non-covalent, allosteric regulation. It mainly occurs by AMP and ADP, competing with MgATP for binding to the γ-subunit CBS domains. At a low cellular energy state, increases of AMP and, as discovered more recently, also of ADP can be sensed by AMPK as altered AMP/ATP and ADP/ATP concentration ratios (Oakhill et al. 2011; Xiao et al. 2011). In many cell types and in particular in the heart and skeletal muscle, breakdown of ATP to ADP at the onset of high workload or cellular stress has only minor immediate effects on ATP levels. Due to

Table 10.2 Hormones and cytokines affecting AMPK activity

Compound	Effect	Mechanism	Tissue	Ref.
Leptin	+	AMP increase	Muscle	Minokoshi et al. (2002)
Leptin	–	Melanocortin receptor signaling?	Hypothalamus	Minokoshi et al. (2004)
Interleukin-6	+	Not known	Muscle	Carey et al. (2006)
Tumor necrosis factor α	–	Increased PP2C expression	Muscle	Steinberg et al. (2006)
Resistin	–	Not known	Liver, muscle, adipose	Banerjee et al. (2004)
Ghrelin	+	G protein coupled receptor signaling CamKK activation	Hypothalamus, heart	Kola et al. (2005), Nakazato et al. (2001)
Ghrelin	–		Liver	Barazzoni et al. (2005)
Adiponectin	+	Adiponectin receptor 1 signaling?	Muscle, adipose, hypothalamus	Kubota et al. (2007)
Estrogen	+	Not known	Muscle	D'Eon et al. (2005)
Testosterone; dihydrotestosterone	–	Decrease in LKB1 mRNA	Adipocytes	McInnes et al. (2012)
17β-estradiol	+	Increase in LKB1 mRNA	Adipocytes	McInnes et al. (2012)
Angiotensin 2	+	AT1R-NADPH oxidase axis	Vascular smooth muscle cells	Nagata et al. (2004)

the energy buffer and transfer function of the CK/PCr system, global and local ATP pools are rapidly replenished (Schlattner et al. 2006; Wallimann et al. 2011). Thus, ATP is not a very suitable signal for indicating developing energy deficits. However, minor decreases in ATP levels lead to more pronounced relative increases in free ADP and even more in AMP due to the adenylate kinase (AK) reaction. Under these conditions, AK uses two ADPs to regenerate ATP and AMP, thus increasing AMP concentrations from the sub-micromolar range under resting conditions to the lower micromolar range (Hardie et al. 2011). To a lesser extent, AMP levels also depend on pyrophosphates (cleaving the β-phosphate bond of ATP) and the activity of AMP degradation pathways [AMP-deaminase and 5′-nucleotidase, whose inhibition may be useful to activate AMPK (Kulkarni et al. 2011)]. As a consequence, a decrease in ATP levels by only 10 % translates into a ten- to 100-fold increase in AMP, making AMP an ideal second messenger of energy stress. Regulation of AMPK activation by the balance between ATP, ADP, and AMP concentrations resembles to what was put forward by Atkinson 50 years ago as "energy charge"

regulation (Atkinson 1968; Hardie and Hawley 2001; Xiao et al. 2011; Oakhill et al. 2011).

The molecular basis of AMPK activation by AMP and ADP is not yet fully understood but involves binding to CBS sites on the γ-subunit that trigger multiple interconnected mechanisms. Binding of AMP leads to an up to a ~tenfold allosteric activation of AMPK (Gowans et al. 2013). Earlier in vitro studies suggested that the α2-subunit has a higher sensitivity to this allosteric activation (Salt et al. 1998a). In addition, AMP and ADP binding increase the phosphorylation status of α-Thr172 through protection of the α-subunit activation loop from dephosphorylation by phosphatases (Davies et al. 1995; Xiao et al. 2011). In addition, AMP (but not ADP) promotes α-Thr172 phosphorylation by LKB1 but not by CamKKβ (Gowans et al. 2013). The γ-subunit CBS sites involved in these allosteric effects are sites 1, 3, and 4. However, there is some debate on the role of these sites, in particular which sites mediate direct allosteric activation and which ones the protection of dephosphorylation. There is a consensus that changes of AMP and ADP concentrations in the physiological range are mainly sensed at sites 1 and 3, called exchangeable binding sites. Here, free AMP and ADP probably compete mainly with free ATP, since the most abundant Mg^{2+}-complexed ATP has tenfold lower affinity for the CBS sites (Xiao et al. 2011). Sites 1 and 3 differ about 30-fold in their affinity for adenylates, and initial evidence suggested site 1 as high-affinity site, sensing low micromolar concentrations of AMP for allosteric activation, and site 3 as low affinity site, involved in protection of dephosphorylation at higher AMP and ADP concentrations (Xiao et al. 2011). However, the role of CBS sites may not be defined as clearly. A more recent study suggests that site 3 is the most important for allosteric activation (Chen et al. 2012). Indeed, mutation of site 3 residues abrogates allosteric AMPK activation (Chen et al. 2012; Scott et al. 2004), and this site is also in contact with the α-subunit (see below). In addition, site 4 may play a role in allosteric activation. This is a tight AMP-binding site, generally reported as non-exchangeable site since purified protein or protein crystals always retain AMP in this site, even when treated with ATP. However, Chen et al. (Chen et al. 2012) observed ATP at site 4 when co-crystallizing AMPK core complex in the presence of 2 mM free ATP, a very high concentration that may not be physiologically relevant. However, ATP binding to site 4 forces site 3 to remain empty, and this affects allosteric AMPK activation, consistent with the model of CBS site 3 being the major site of allosteric regulation. A complicating fact is that some nucleotide-binding CBS residues can interact with nucleotides at different sites, thus precluding a clear-cut functional assignment of CBS sites (Hardie 2014c).

All known direct AMPK activators act via allosteric effects (see Chap. 7). They either act like AMP at the CBS sites (e.g., 5-aminoimidazole-4-carboxamide riboside, AICAR; Giri et al. 2004) or they exert their effects by binding to an entirely different site, discovered only recently (e.g., A-769662; Scott et al. 2008). This site is situated in a cleft between the α-kinase domain and the β-CBM domain and stabilized by autophosphorylation of the β-Ser108. Occupation of this α/β site

confers protection of dephosphorylation. It can be speculated that there exists an endogenous activating metabolite binding at the α/β site, and/or an endogenous activating kinase, able to phosphorylate Ser108 (Hardie 2014c).

All these allosteric mechanisms, whether they involve binding events at the CBS sites or at the novel α/β site, require close communication between the sensing subunit (γ or β) and the catalytic subunit (α). We and our collaborators have proposed that subunit communication and activation occur via a conformational switch within the AMPK full-length complex (Riek et al. 2008; Chen et al. 2012). Indeed, AMP-induced conformational changes have been evidenced through structural studies by SAXS (Riek et al. 2008), electron microscopy (Zhu et al. 2011), and X-ray crystallography (Chen et al. 2012) within different parts of the AMPK heterotrimer. Recent structures of the holo-AMPK complex in its active state, as well as low-resolution structures in Thr172 phosphorylated and unphosphorylated states, suggest that conformational changes and intramolecular movements involve α-RIM, α-AID, the two lobes of the α-kinase domain, as well as the entire γ-subunit (Chen et al. 2013; Xiao et al. 2011; Calabrese et al. 2014; Li et al. 2014). High-resolution apo-AMPK structures of holo-AMPK complex will be necessary to answer the remaining questions, in particular how a different occupation of CBS sites communicates via α-RIM and α-AID with the kinase domain. Collectively, these non-covalent AMPK activation mechanisms add an important layer to the regulation of AMPK activity, since they allow a direct response to intracellular metabolites.

Exercise and Hypoxia Given the sensitivity of AMPK for adenine nucleotides, any physiological or pathological situation that changes adenylate ratios will affect AMPK signaling. AMPK is activated by a plethora of stimuli such as metabolic stresses and drugs and xenobiotics that either (1) inhibit ATP production, such as starvation for glucose (Salt et al. 1998b) and oxygen (Marsin et al. 2002), or metabolic poisons or (2) increase ATP consumption, such as muscle contraction (Lantier et al. 2014). Muscle contraction and exercise in general trigger rapid activation of AMPK (Chen et al. 2003), and this may be one of the fastest mechanisms that mediate metabolic adaptation to exercise. When AMPK is knocked out in the skeletal muscle of β1β2 transgenic mice, they lose exercise tolerance and glucose uptake during contractions, become physically inactive, and present an importantly impaired capacity for running linked to reductions in skeletal muscle mitochondrial content (O'Neill et al. 2011). During hypoxia, from the early stage on, a drastic drop in the ATP/AMP level occurs, resulting in AMPK activation.

Other Covalent and Non-covalent Regulations In addition to the above-described conventional regulation of AMPK, there is increasing evidence for additional activation and inactivation mechanisms. Here, different secondary protein modifications play an important role. Myristoylation at Gly2 in the β-subunit increases the sensitivity of AMPK for allosteric activation and promotes Thr172 phosphorylation (Oakhill et al. 2010). The β2-subunit, but not β1, is sumoylated by the E3-small ubiquitin-like modifier (SUMO) ligase protein inhibitor of activated

STAT (PIASy), which attaches SUMO2 but not SUMO1 moieties. This seems to enhance AMPK activity and competes with ubiquitination that results in inactivation of AMPK complex (Rubio et al. 2013). Ubiquitination of AMPK occurs via complexes of laforin (a dual-specificity protein phosphatase) and malin (an -E3-ubiquitin ligase), mainly at the β-subunit, and leads to K63-linked ubiquitin chains that are involved in functions different from proteasome degradation (Moreno et al. 2010). Glutathionylation at Cys299 and Cys304 in the α-subunit activates the kinase under oxidative conditions in cellular models and is favored by binding to certain GST isoforms (Klaus et al. 2013). This latter mechanism may be part of a more general redox regulation of the kinase (Han et al. 2010; Jeon et al. 2012). ROS and RNS activate AMPK, but it is unclear whether this happens via increases in ADP and AMP concentrations or whether noncanonical mechanisms at the level of AMPK (like glutathionylation) or upstream kinases play a role. Vice versa, AMPK regulates NADPH homeostasis and an entire battery of ROS-detoxifying enzymes. Another non-covalent allosteric regulator is glycogen as well as other synthetic branched oligosaccharides that inhibit AMPK activity by binding to the β-CBM domain (McBride et al. 2009) (see above).

10.5 Regulation

Metabolism AMPK regulates cellular metabolism at many levels, reducing anabolism (ATP-demanding processes) and upregulating catabolism (ATP-generating processes) to restore a healthy energy status at a cellular and whole-body level. To do so, AMPK directly acts on metabolic key enzymes and signaling proteins (acute effects) or on transcription factors (chronic effects, see Fig. 10.2) (Hardie et al. 2012b). Interestingly, drugs of the two main classes of antidiabetic drugs, biguanides (e.g., metformin) and thiazolidinediones (e.g., rosiglitazone), both act at least in part through activation of AMPK (Morrison et al. 2011; Musi et al. 2002). In the heart, AMPK is part of the signaling network that allows a predominant use of fatty acid oxidation for ATP generation and also provides the metabolic flexibility to respond to changes in substrate availability, thus continuously matching ATP generation and demand. Failing of AMPK to provide this flexibility under certain pathological conditions can contribute to the pathogenesis of heart failure (see Chap. 6, reviewed in Kim and Dyck 2015).

Lipid Metabolism Activated AMPK induces transfer of fatty acid transporter (FAT/CD36) to the plasma membrane to increase fatty acid uptake (Luiken et al. 2003). AMPK further inhibits ATP-consuming lipid synthesis, notably in the liver and in the adipose tissue, but stimulates lipid catabolism for ATP generation. Phosphorylation of acetyl-CoA carboxylase (ACC) decreases ACC-catalyzed formation of malonylCoA a precursor in the fatty acid synthesis pathway. At the same time, reducing malonylCoA levels will relieve their inhibition of carnitine palmitoyltransferase 1 (CPT-1), which triggers fatty acid import into mitochondria

and subsequent β-oxidation. AMPK also phosphorylates and inhibits other anabolic enzymes: 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR), a key enzyme in cholesterol synthesis that converts 3-hydroxy-3-methylglutaryl-CoA into mevalonic acid, and glycerol phosphate acyltransferase, involved in triglyceride and phospholipid synthesis (Liao et al. 2014). Since AMPK acts by stimulating lipolysis and inhibiting lipogenesis, its pharmacological activation seems to be useful to treat obesity, diabetes type 2, and more generally the metabolic syndrome (Hardie 2008a; O'Neill et al. 2013).

Carbohydrate Metabolism AMPK also interferes with carbohydrate metabolism at different levels, including carbohydrate uptake, glycolysis, and glycogen synthesis. Activated AMPK promotes cellular glucose uptake via glucose transporters GLUT1 (expressed in most cells except muscle, liver, and adipose tissue) and GLUT4 (expressed mainly in adipose tissue and striated muscle). AMPK activation promotes GLUT4 translocation to the plasma membrane (Kurth-Kraczek et al. 1999) and stimulates GLUT4 transcription by phosphorylation of the transcription repressor histone deacetylase 5 (HDAC5) which reduces its affinity for the GLUT4 promoter (McGee et al. 2008). GLUT1-dependent glucose uptake is activated via an unclear mechanism that involves GLUT1 already located at the plasma membrane (Barnes et al. 2002). Notably in case of energy deprivation in the heart, AMPK phosphorylates and activates the 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFKFB) to increase the steady-state concentration of fructose-2,6-bisphosphate (Marsin et al. 2000). This metabolite then acts as an allosteric activator of glycolysis by stimulating the glycolytic enzyme 6-phosphofructo-1-kinase (PFK1), a rate-limiting glycolytic enzyme. Once activated, AMPK also represses anabolic glucose storage into glycogen by directly phosphorylating and inactivating glycogen synthase (Bultot et al. 2012). Finally, AMPK affects carbohydrate metabolism indirectly by phosphorylation of the mTor–raptor complex, which was proposed to modulate insulin sensitivity by regulating protein levels of IRS-1 (Haruta et al. 2000; Kahn et al. 2005).

Transcription AMPK phosphorylates and regulates various transcription factors and coregulators, including forkhead box O (FoxO) proteins FoxO1 and FoxO3 (Kubli and Gustafsson 2014) and peroxisome proliferator-activated receptor (PPAR) γ coactivator-1a (PGC-1α) (Patten and Arany 2012), both having important roles in the regulation of cardiac energetic homeostasis and beyond. PGC-1α is a central transcriptional coactivator that orchestrates mitochondrial biogenesis and dynamics, fuel transport and/or consumption, angiogenesis, and antioxidative effects. PGC-1α phosphorylation by AMPK results in improved metabolism of fatty acids and more efficient energy utilization (Schilling and Kelly 2011). FoxO transcription factors regulate expression of genes involved in the antioxidative stress response and in the balance between apoptosis, autophagy, and energy metabolism. These functions are critical for cardiac function (Ronnebaum and Patterson 2010). FoxO-regulated genes also encode proteins that contribute to improved energy metabolism, including FAT/CD36 and GLUT4 indirect metabolic effects.

Growth and Proliferation Many effects of AMPK on cell growth, cell cycle, and autophagy are mediated by another evolutionary conserved serine/threonine protein kinase further downstream, the mammalian target of rapamycin (mTor). mTor occurs as two functional multiprotein complexes, mTORC1 and mTORC2 (Loewith et al. 2002). mTORC1 comprises mTOR, raptor, mLST8, and PRAS40 and is regulated by cellular energy and nutrient state, whereas mTORC2 is not. Raptor also plays a significant role in intracellular localization of mTORC1 in response to amino acid availability, which is an essential cellular signal for mTORC1 activation (Sancak et al. 2008). Activation of mTORC1 occurs at the lysosomal surface as a part of complex, multiprotein assemblies (Bar-Peled and Sabatini 2014). Active mTORC1 stimulates several ATP-demanding cellular processes such as translation, transcription (protein synthesis), ribosome biogenesis, mitochondrial metabolism, proliferation, and autophagy. Unlike mTORC2, mTORC1 is sensitive to rapamycin, a molecule used as immunorepressor due to its capacity to downregulate protein synthesis, notably of antibodies.

Two important substrates of mTORC1 in its response to nutrients and cellular energy status are S6 kinase (S6K) and eIF4E binding proteins (4EBPs). Raptor, a component of mTORC1, functions as a scaffolding protein to recruit such substrates for phosphorylation (Nojima et al. 2003). S6K is a ribosomal kinase regulating translation initiation, mRNA processing, and cell growth and notably enhances protein synthesis once phosphorylated. 4EBPs are translational repressors that are inactivated upon phosphorylation by mTORC1. To precisely regulate these mTORC1-dependent, energy-demanding processes, AMPK inhibits mTORC1 signaling through two distinct mechanisms (Inoki et al. 2012). First, it directly phosphorylates raptor at the conserved Ser722 and Ser792, leading to recruitment of 14-3-3 protein and an inactive mTORC1 complex (Gwinn et al. 2008). Second, it phosphorylates tuberous sclerosis protein 2 (TSC2), a GTPase-activating protein (GAP), thus stimulating the downstream GTPase Ras homologue enriched in brain protein (Rheb). This transforms Rheb from its GTP-bound form that activates mTORC1 into its inactive, GDP-bound form (Inoki et al. 2003). This latter pathway of mTORC1 regulation by AMPK may depend on cell type and tissue (Wolff et al. 2011). Collectively, the AMPK and mTORC1 pathways serve as a signaling nexus to regulate cellular metabolism, energy homeostasis, and cell growth. Disorder of each pathway may strongly contribute to the development of pathologies such as type II diabetes or cancer.

Autophagy and Apoptosis While AMPK activation by upstream kinases is well studied, much less is known about regulation of AMPK stability and activity by components of the ubiquitin–proteasome system, responsible for cellular recognition and degradation of proteins. Growing evidence suggests that AMPK regulates overall proteasome activity and individual components of the ubiquitin–proteasome system (Ronnebaum et al. 2014). Autophagy is important for maintaining homeostasis when nutrient supply becomes limiting. It is important for the cellular turnover of proteins and organelles and is rapidly upregulated during stress. In metabolic disorders including obesity and diabetes, autophagy is reduced, leading

to accumulation of protein aggregates and dysfunctional organelles which can contribute to pathogenesis.

10.6 Cardiac Signaling in Health and Disease

Cardiac AMPK activity is increased by many stimuli, acting either via upstream kinases or modulation of adenylate levels under both pathological and physiological stress and involving various hormones and cytokines (Fig. 10.2; Zaha and Young 2012). However, within the physiological range, the role of cardiomyocyte AMPK is possibly different from other cell types, mainly because of the remarkable metabolic stability of this organ maintained by multiple other mechanisms. Two classical physiological stimuli of AMPK, exercise and hypoxia, also act on cardiac AMPK (Coven et al. 2003; Frederich et al. 2005; Musi et al. 2005) and promote the metabolism of glucose and fatty acids via its different downstream targets. However, it is unclear whether this activation is due to altered energy state as in the skeletal muscle or rather relies on alternative upstream signaling. At least one other physiological AMPK stimulus, nutrient deprivation, seems not to operate in a canonical manner in the heart (Clark et al. 2004). Apart of these key roles, cardiac AMPK mediates the cardiomyocyte response to a variety of other physiological or pathological situations, including some forms of pressure overload, heart failure, intracellular calcium overload, or reactive oxygen and nitrogen species (Dolinsky et al. 2009; Zaha and Young 2012).

Ischemia As a pathological stimulus, ischemia is the best studied both in form of no-flow and partial ischemia in isolated perfused animal hearts, as well as regional ischemia due to coronary ligation in vivo (Kim et al. 2011; Kudo et al. 1996; Paiva et al. 2011; Russell et al. 2004; Wang et al. 2009), for a review see Young (2008). They lead to rapid and lasting AMPK activation. As already mentioned, oxidative stress may be a determinant of such activation, acting through different forms of ROS (Sartoretto et al. 2011; Zou et al. 2002). In endothelial cells, it is rather peroxynitrite formation that affects AMPK via the protein kinase Cζ-LKB1 axis (Xie et al. 2006a; Zou et al. 2004), while in other non-excitable cells, it may be rather an ROS-induced Ca^{2+} release that triggers the CamKKβ axis (Mungai et al. 2011). ROS-facilitated glutathionylation of AMPK (see Chap. 4) as observed in cellular systems represents yet another direct activation mechanism but still has to be verified in cardiomyocytes (Klaus et al. 2013; Zmijewski et al. 2010). However, the signaling function of ROS may be lost at more intense oxidative stress that inactivates AMPK (Gratia et al. 2012). Stress resulting from many but not all forms of pressure overload also results in AMPK activation, mainly increasing glucose uptake and glycolysis (Allard et al. 2007; Li et al. 2007; Tian et al. 2001; Zhang et al. 2008), as well as changing the gene expression profile (Hu et al. 2011).

Endocrine Regulation Cardiac AMPK is also regulated by extracellular signals as adiponectin (Shibata et al. 2004), leptin (Minokoshi et al. 2002), resistin (Kang

et al. 2011), ghrelin (Kola et al. 2005), IL6 (Kelly et al. 2004), and CNTF (Watt et al. 2006). Best studied are probably the hormones adiponectin and leptin (the latter at least during ischemia; McGaffin et al. 2009) which activate cardiac AMPK or the proinflammatory cytokine IL-6 which appears to reduce AMPK content and activity (Zaha and Young 2012). Cardiac AMPK seems to be involved in the positive effects of adiponectin for cardioprotection during ischemia and for reduced cardiac hypertrophy (Shibata et al. 2004, 2005). Also, leptin may modulate AMPK in the heart, since impaired leptin signaling correlates with reduced AMPK activation and metabolic defects or reduced postconditioning after ischemia (Bouhidel et al. 2008; McGaffin et al. 2009). Proinflammatory cytokines like IL-6 rather reduce AMPK protein and activation (Ko et al. 2009), although there may be opposite effects in specific tissues like skeletal muscle due to a specific autocrine–paracrine effect (Kelly et al. 2004). Another cytokine with functions in the heart is macrophage migration inhibitory factor (MIF), which is involved in AMPK activation during ischemia and hypoxia, and its decrease with age in mice seems to reduce AMPK activation during ischemia (Ma et al. 2010; Miller et al. 2008).

Protein Turnover AMPK has been suggested to play an important role in regulating cardiac turnover of proteins and organelles (Baskin and Taegtmeyer 2011; Zaha and Young 2012), also during ischemia. This process is critical for the survival and self-renewal of terminally differentiated cells as cardiomyocytes and requires tightly regulated degradation of misfolded and damaged proteins or damaged/dysfunctional organelles (as, e.g., mitochondria) and their replacement by new and functional entities. Recent evidence suggests that AMPK regulates degradation at two levels. Individual proteins are eliminated by the ubiquitin–proteasome system, with AMPK activating the cardiac ubiquitin ligases atrogin-1 and MuRF. Whole organelles are digested by stimulation of autophagy via activation of ULK1 and inhibition of mTOR (Baskin and Taegtmeyer 2011; Hardie et al. 2012a; Zaha and Young 2012). Thus, under conditions of metabolic stress, AMPK activation inhibits protein synthesis (via mTOR) and activates degradation of proteins and organelles. The recycling of nutrients from breakdown of cellular components (macromolecules and organelles) contributes to the maintenance of the cellular ATP-regenerating capacity, to the control of protein and organellar quality, as well as to the maintenance of cardiomyocyte size and their survival. It is to note that slowing down protein synthesis also prevents accumulation of unfolded proteins under stress situation such as hypoxic or ischemic injury and the related endoplasmic reticulum stress (Terai et al. 2005).

Inflammation As mentioned above, cytokines can directly regulate cardiac AMPK activity. On the other hand, AMPK has the capacity to repress inflammatory responses and exert anti-inflammatory and immunosuppressive effects in a variety of cell types by interfering with cytokine signaling (Salminen et al. 2011; Salt and Palmer 2012). There is evidence that in several tissues, including the cardiovascular system, activation of AMPK impairs leukocyte infiltration (an early key step in development of inflammation) by reducing expression of chemokines and adhesion molecules (Salt and Palmer 2012). This is important for the heart, since

inflammation is a critical component in the pathogenesis of many common cardiovascular diseases (Pankuweit et al. 2004), including the diabetic heart (Ko et al. 2009).

Cardioprotection Most mechanisms triggered by active cardiac AMPK, though possibly not all, are recognized to promote cardioprotective effects. For example, AMPK-dependent stimulation of glucose metabolism (glucose uptake by GLUT4 and stimulated glycolysis by PFK2) is of particular importance for the anaerobic ATP synthesis during ischemia and thus for protection of the ischemic heart (Young 2008). It is to note, however, that AMPK activation persisting after ischemia during early reperfusion is considered rather detrimental, because excessive stimulation of fatty acid oxidation impairs glucose oxidation via Randle cycle/uncoupling of enhanced glycolysis from glucose oxidation (Dyck and Lopaschuk 2006). The net result of AMPK activation during an ischemia/reperfusion episode can still be considered as beneficial (Zaha and Young 2012). Finally, AMPK was suggested to mediate the cardiac response to different known cardiac protectants, mostly in pathological setting, as, e.g., during ischemic episodes or pathological hypertrophy (review in Kim et al. 2009). For example, AMPK contributes to the cardioprotective effects of adiponectin and metformin during coronary occlusion in mice (Calvert et al. 2008; Shibata et al. 2005), as well as to cardiac preconditioning by regulating the activity and recruitment of sarcolemmal K (ATP) channels (Sukhodub et al. 2007).

As AMPK activation has predominantly pro-survival character, it is considered as promising potential therapeutic target in the treatment of different cardiovascular diseases (Inoki et al. 2012; Kim et al. 2011; Zaha and Young 2012).

Cardiomyopathies Mutation of specific CBS residues is associated with pathological disorders (Kemp 2004; Ignoul and Eggermont 2005). Mutations in the CBS domains of the AMPK γ2-subunit, expressed at particularly high levels in the heart, cause the Wolff-Parkinson-White (WPW) syndrome, a hereditary cardiomyopathy of varying severity, involving cardiac hypertrophy, contractile dysfunction, and arrhythmias. Mutations impair adenylate binding and thus AMPK activation (Scott et al. 2004; Burwinkel et al. 2005), but the major cause for the cardiomyopathy is the increased AMP-independent basal AMPK activity. This leads to higher glucose uptake, accumulation of glycogen in cardiac myocytes, and finally impairment of heart muscle development (Burwinkel et al. 2005; Davies et al. 2006).

Cardiac Contractility Cardiac troponin I was identified in a yeast 2-hybrid screen to interact with the AMPK γ2-subunit N-terminal domain and to be phosphorylated by AMPK at Ser150 in vitro and during ischemia in the heart (Oliveira et al. 2012). This results in increased myocyte contraction and prolonged relaxation by an increase in myofilament Ca^{2+} sensitivity. These effects were also triggered by the AMPK activator AICAR, suggesting that pharmacological AMPK activation could improve heart function.

Doxorubicin-Induced Cardiotoxicity The anthracycline antibiotic doxorubicin (Adriamycin; DXR) remains one of the most largely prescribed chemotherapeutic drugs for the treatment of a variety of human cancers (Eschenhagen et al. 2011; Ewer and Ewer 2010; Gianni et al. 2008; Minotti et al. 2004, 2010). It is still a cornerstone of combination therapies together with more targeted, new generation drugs. Unfortunately, the potent antitumor effect of DXR is accompanied by a number of unwanted side effects, in particular a serious cardiac toxicity. This complication represents a major obstacle when using the drug for prolonged time and/or at a higher cumulative dose (Curigliano et al. 2012; Menna et al. 2008). Detrimental effects of DXR are thought to be mediated by different kinds of stress induced by the drug: energetic stress, oxidative stress, and genotoxic stress (Gratia et al. 2012). Given the stress-sensing function of AMPK, an activation of the kinase is expected in the heart as a result of drug action. Paradoxically, it seems that in the heart DXR does not increase but rather decrease the basal phosphorylation of AMPK, thus inactivating the kinase. Such AMPK inhibition has been observed in different model systems of acute and chronic cardiotoxicity, including cultured cardiomyocytes (Konishi et al. 2011; Wang et al. 2012a), perfused heart (Gratia et al. 2012; Tokarska-Schlattner et al. 2005), and in vivo models of rat (Cai et al. 2010; Gratia et al. 2012; Russell 2003) and mice (Kawaguchi et al. 2012; Kim et al. 2010; Konishi et al. 2011). AMPK appears to be an early and sensitive DXR target: in rat hearts perfused with rather low clinically relevant DXR concentrations, AMPK is inhibited already after 1–2 h, well before changes in myocardial function can be observed. In rats, AMPK inactivation persists several weeks after the end of treatment (Gratia et al. 2012; Konishi et al. 2011). Thus, it seems that DXR generates conditions which normally should activate AMPK but instead inhibits the stress response of AMPK. This may create a vicious cycle for the heart, important for drug toxicity.

DXR-induced inhibition of cardiac AMPK signaling is, at least partially, due to the negative cross talk with other signaling pathways, in particular Akt and ERK. These two pro-survival kinases respond in the heart to a variety of stimuli (Kehat and Molkentin 2010; Sussman et al. 2011) and are activated by DXR (Gabrielson et al. 2007; Gratia et al. 2012; Horie et al. 2010; Khalil et al. 2012; Kobayashi et al. 2010; Lee et al. 2006; Lou et al. 2005; Tokarska-Schlattner et al. 2010). Both kinases are known to inhibit AMPK (Du et al. 2008; Esteve-Puig et al. 2009; Hahn-Windgassen et al. 2005; Horman et al. 2006; Kovacic et al. 2003; Soltys et al. 2006).

This interplay of AMPK with Akt seems especially pronounced in heart, and AMPK inhibition by the Akt pathway has been also reported for other cardiac pathologies (Dyck and Lopaschuk 2006). We could substantiate the role of the Akt–AMPK cross talk for drug-induced AMPK inhibition by using the specific Akt inhibitor MK2206 (Gratia et al. 2012). Akt is mainly activated by DNA-damage signaling in response to strong DNA damage that is induced in cardiomyocytes by the drug. This involves DNA-dependent protein kinase (DNA-PK), activated by DNA double-strand breaks which are a typical consequence of DXR action.

Interestingly, several most recent studies indicate a more general relationship between a reduced LKB1–AMPK signaling and cardiac disease (Dolinsky et al. 2009; Ikeda et al. 2009; Shaw 2009). Decreased activation state of the LKB1–AMPK axis occurs in several cardiac pathologies, and in some of them a similar AMPK inhibition by cross talk with Akt has been suggested as underlying mechanism (Hahn-Windgassen et al. 2005; Horman et al. 2006; Kovacic et al. 2003; Soltys et al. 2006). In spontaneously hypertensive rats, another mechanism has been put forward, namely, oxidative damage of LKB1 due to formation of adducts between 4-hydroxy-2-nonenal (HNE, product of lipid peroxidation) and LKB1 which inhibits LKB1 and thus also AMPK activity (Dolinsky et al. 2009). Taken together, these data suggest that AMPK activation as a potential preventive or therapeutic strategy during DXR treatments.

10.7 Pharmacological Activation

AMPK controls metabolic pathways and cellular processes that are critical to the etiology of various, otherwise unrelated pathologies. For many of them, including cardiovascular disease, activation of AMPK has been recognized as a potential treatment, mimicking, for example, the positive effects of exercise on many of these pathologies. However, one has to keep in mind that systemic, constitutive AMPK activation by drugs also carries risks. These include, as already described above, a risk of cardiac pathologies as seen in the WPW syndrome and are due to the highly pleiotropic target spectrum of AMPK and in particular its central effects as, e.g., in hypothalamic appetite control.

A large panel of natural or synthetic agents were reported to activate AMPK (Yun and Ha 2011). However, the caveat with most of these molecules is that, where analyzed, they do not directly activate AMPK and have numerous cellular effects (Hardie 2014a). They often include mild inhibition of mitochondrial ATP generation by interfering with either the respiratory chain or mitochondrial ATPase (El-Mir et al. 2000; Gledhill et al. 2007), thus leading to a small but chronic increase in cellular AMP/ATP and ADP/ATP ratios. This group of compounds includes antidiabetic drugs like metformin and many plant polyphenols like resveratrol contained in grapes, green tea, peppers, garlic, or traditional products of Chinese medicine (Gu et al. 2010; Huang and Lin 2012; Kim et al. 2012c; Wang et al. 2009; Yang et al. 2012).

Also, some direct activators act via the allosteric mechanisms known for AMP. The long-known 5-aminoimidazole-4-carboxamide riboside (AICAR; Giri et al. 2004) is metabolized within the cell to ZMP, an AMP analogue that binds to CBS domains and acts like AMP. However, such AMP analogues may affect any AMP-sensitive processes, a condition potentially avoided by the novel AMP analogue, 5-(5-hydroxyl-isoxazol-3-yl)-furan-2-phosphonic acid or C2. This compound was reported to be 1,000-fold more potent than ZMP in vitro (Gómez-Galeno et al. 2010). All these allosteric mechanisms involve the γ-subunit CBS sites, and

activation is lost in cells expressing mutant AMPK insensitive to AMP (Hawley et al. 2010).

A third group of AMPK activators exerts its effects by binding to an entirely different site, situated at the interface of α-kinase domain and β-CBM domain. Its occupation confers protection of dephosphorylation and allosteric activation. Activators binding at this site include A-769662 (Scott et al. 2008), the 991 compound (Xiao et al. 2013) and its derivatives, as well as salicylate (Hawley et al. 2012).

As a result of almost 15 years of research, a total of 26 patents have been disclosed, describing 10 classes of direct AMPK activators (Giordanetto and Karis 2012). However, no direct AMPK activator has succeeded so far in clinical studies, although promising trials are ongoing (Ballantyne et al. 2013). Abbott Laboratories were the first in 2005 to identify thienopyridones as direct AMPK activators (Abbott Laboratories 2005). High-throughput screening first identified A-592107 that was then optimized to yield the more potent A-769662 (EC_{50}: 0.8 μM) which did not show immediate signs of cytotoxicity or activity at secondary biological targets (Cool et al. 2006). Discovery of A-769662 represented a shift in AMPK-targeted pharmaceutical research, since it demonstrated that the kinase can be directly activated by non-nucleotide ligands. Since then, A-769662 has been used as a standard AMPK activator in basic research, and much has been learned about its action mechanism and pharmacology. A-769662 activates AMPK allosterically and by inhibition of Thr172 dephosphorylation mediated by PP2C, similar to AMP (Goransson et al. 2007; Sanders et al. 2007b). However, it does not bind to the γ-subunit CBS sites but to a novel allosteric regulatory site identified at the interface between the α-kinase and the β-CBM domain (Calabrese et al. 2014; Xiao et al. 2013). Importantly, A-769662 has a much stronger inhibitory effect on β1-containing complexes as compared to those containing β2. This showed for the first time that isoform-specific small molecule activators can be developed that would allow a more tissue-specific pharmacological modulation of AMPK activity.

Most recently, it was shown that AMP and A-769662 have a synergistic effect on AMPK activation (Scott et al. 2014; Ducommun et al. 2014) that even allows to bypass phosphorylation of Thr172, a step thought to be essential for AMPK activity (Scott et al. 2014; Viollet et al. 2014). This observation has put a note of caution onto the widespread use of Thr172 phosphorylation as a reliable readout for AMPK activity (Scott et al. 2014; Viollet et al. 2014). Although in the meantime also some off-target effects of A-769662 have been observed (Benziane et al. 2009; Treebak et al. 2009), the compound remains a very useful tool to explore AMPK-mediated cellular processes.

Most other direct AMPK activators that have been described bear structural similarities to A-769662, such as the 991 compound (Xiao et al. 2013). Only more recently, compounds that do not resemble to such thienopyridones have been described. For most patented direct AMPK activators, there is limited documentation available concerning their selectivity profile across the various AMPK isoform combinations (Giordanetto and Karis 2012; Yun and Ha 2011) apart from A-769662 (Scott et al. 2008; Goransson et al. 2007; Sanders et al. 2007b) and the 991 compound (Xiao et al. 2013). Given the plethora of processes controlled by AMPK, heterotrimer-specific AMPK activation would allow to preferentially target

a specific tissue and thus has the strongest potential for pharmacological applications.

Taken together, very few structurally different AMPK activators are known so far (Giordanetto and Karis 2012; Yun and Ha 2011). Although this could represent a true limitation of potentially AMPK-activating compounds, it may also be related to the applied screening procedures. A critical point in screening chemical libraries may be the readout system used to identify AMPK activation. AMPK activators bind at allosteric sites, but the readout generally relies on the activity of the AMPK kinase domain. This implies the use of MgATP and accumulation of ADP and even traces of AMP during the assay. The presence of these nucleotides may obscure effects of activating compounds, at least of those acting at the CBS domains. Tools that report AMPK activation without the need of kinase assays would therefore have a clear advantage.

10.8 Concluding Remarks

Defects in cardiac energy metabolism contribute to the pathogenesis of cardiovascular disease and heart failure. AMPK is now recognized as the central sensor and regulator of cellular energetics, and AMPK activation has been proposed as a suitable strategy for the treatment of insulin resistance/type II diabetes, cancer, and some other pathologies (Steinberg and Kemp 2009). However, the potential for pharmacological activation of AMPK in the heart has not yet been analyzed in much detail. For its application in cardiac pathologies, more work is still necessary to evaluate efficacy and safety in short-term and long-term activation protocols. There is continued interest of the pharmaceutical industry in developing AMPK agonists. Indeed, the search for clinically applicable, specific AMPK activators remains an urgent need to evaluate and fully exploit the pharmacological potential of AMPK.

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Chapter 11
How Cardiac Cytoarchitecture Can Go Wrong: Hypertrophic Cardiomyopathy as a Paradigm for Genetic Disease of the Heart

Thomas J. Cahill and Katja Gehmlich

Abstract The genetic cardiomyopathies are a group of inherited heart disorders with variable pathophysiology and clinical phenotype. As an example, hypertrophic cardiomyopathy will be discussed in this chapter. Clinical features and therapeutic options will be outlined, followed by a review of the underlying genetics of disease. The pathomechanisms of causative mutations will be discussed, with reference to both sarcomeric and non-sarcomeric genes. Finally, the potential and pitfalls of next-generation sequencing as applied to genetic cardiomyopathies will be analysed.

11.1 Introduction

Genetic cardiomyopathies are a group of heritable disorders affecting the heart muscle, with variable pathophysiology and clinical phenotype (Arbustini et al. 2014). These diseases are caused by mutations in genes coding for cardiac proteins with structural and/or signalling functions, leading to perturbations in cardiac cytoarchitecture and function. Broadly, the genetic cardiomyopathies can be classified by phenotype into the following categories: hypertrophic cardiomyopathy (HCM), dilated cardiomyopathy (DCM), arrhythmogenic cardiomyopathy (AC), left ventricular non-compaction cardiomyopathy (LVNC) and restrictive cardiomyopathy (RCM).

Hypertrophic cardiomyopathy was the first familial cardiomyopathy to be described (Teare 1958) and remains the paradigm for studying genetic disease in the heart. In 1990 linkage analysis identified the first causative mutation in a large

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family affected by HCM in the beta-myosin heavy chain gene (*MYH7* p. R403Q) (Geisterfer-Lowrance et al. 1990). Soon mutations in other sarcomere genes, e.g. *TPM1, TNNT2*, *MYBPC3* (Thierfelder et al. 1994; Watkins et al. 1995), were found to cause the same disease. HCM was postulated to be a 'disease of the sarcomere', suggesting a common disease pathway for all these mutations. The initial findings of monogenetic disease with high penetrance also paved the way for genetic screening and predictive testing in cardiomyopathy patients and their family members.

More than two decades after the first description of an HCM mutation, clinical, genetic and in vitro research findings have provided insights into the pathomechanisms of disease. In parallel, unforeseen complexity has emerged in both the cardiomyopathy phenotype and genetics. Some of these complexities will be discussed here, before giving an overview about the genetics and cellular pathophysiology of HCM as an exemplar of the other forms of cardiomyopathy. In the final section, the advances and challenges of applying high-throughput sequencing into the clinical practice of cardiomyopathies will be discussed.

11.2 Complexity Beyond One Mutation, One Gene, One Phenotype

The clinical classification of cardiomyopathies is based on the disease phenotype, relying on morphological and functional assessment by noninvasive imaging. Improvement of diagnostic tools, especially high-resolution imaging techniques, e.g. cardiac MRI (Kumar et al. 2013), and sensitive assessment of cardiac function, e.g. echo tissue Doppler (Afonso et al. 2008), have led to progressive refinement of the phenotype.

Despite this refinement, the substantial variability and overlap between cardiomyopathy phenotypes means that clinical diagnosis remains challenging. For example, although HCM is typically associated with left ventricular hypertrophy and normal systolic function, it can resemble DCM in patients who develop 'burnt-out' disease, associated with impaired systolic function. Likewise, AC with predominant left ventricular involvement may be misdiagnosed as DCM (Sen-Chowdhry et al. 2008). Such diagnosis might be revised once the underlying genetic mutation has been identified ('molecular/genetic classification of cardiomyopathy').

For each cardiomyopathy, numerous disease genes and mutations within these genes have been found to cause the same phenotype. On the other hand, the very same mutation can cause phenotypes of variable severity in different individuals, often even within the same family. Incomplete penetrance, i.e. the absence of disease expression in the presence of a pathogenic mutation, is observed for certain HCM mutations (Moolman et al. 2000) and quantitative differences of phenotypic disease expression (expressivity) are common. These observations suggest that

modifier genes, ethnicity and lifestyle may contribute to the phenotype of an individual. Furthermore, the level of epigenetic modulation is poorly understood (Haas et al. 2013; Mahmoud and Poizat 2013).

Moreover, mutations in one disease gene may cause different types of cardiomyopathy in different patients. For example, mutations in *TNNI3* can cause HCM, DCM or RCM (Carballo et al. 2009; Gomes et al. 2005; Kimura et al. 1997) and *TTN* mutations have been described for DCM, HCM and AC cases (Chauveau et al. 2014). For some disease genes, such as *FHL-1* or *LMNA*, mutations can either cause an isolated cardiomyopathy or syndromic forms with multi-organ involvement (Azibani et al. 2014; Friedrich et al. 2012; Schessl et al. 2011).

Even though the majority of cardiomyopathy cases are inherited as autosomal-dominant traits, cases of recessive, X-linked and maternal transmissions are also known. Further complexity is caused by compound or digenic heterozygosity. In compound heterozygosity, two or more mutations in the same gene contribute to disease, while in digenic heterozygosity mutations in two genes are involved. Often at least one of the variants is of uncertain pathogenicity, complicating genetic evaluation further.

11.3 Hypertrophic Cardiomyopathy

11.3.1 Clinical Features and Therapeutic Options

Hypertrophic cardiomyopathy is the most common genetic heart disease, with a prevalence of 1:500 (Maron 2002). It is autosomal dominant with variable penetrance and expressivity. The characteristic feature is inappropriate myocardial hypertrophy, classically asymmetric hypertrophy of the interventricular septum, in the absence of stimuli which drive physiological or reactive hypertrophy such as athletic training or hypertension. Hypertrophy, myocyte disarray and interstitial fibrosis disrupt cardiac cytoarchitecture and provide the substrate for arrhythmia and diastolic heart failure (Varnava et al. 2001).

The clinical features and phenotype of HCM are highly variable. The disease may be subclinical for prolonged periods and can present at any age. HCM is infamous for causing sudden cardiac death (SCD) in young adulthood, which is frequently the first manifestation of the disease (Maron et al. 2014). It remains the most common cause of death in US athletes (Maron et al. 2009). Asymmetric septal hypertrophy and systolic anterior motion of the mitral valve can cause obstruction of the left ventricular outflow tract, leading to symptoms of chest pain, breathlessness and presyncope. HCM can also cause a heart failure syndrome through a variety of mechanisms: systolic dysfunction due to 'burnt-out' HCM, pressure overload from LV outflow tract obstruction, or diastolic dysfunction, in turn predisposing to atrial fibrillation (Cahill et al. 2013).

Assessment of an individual may be triggered by symptoms, ECG screening or diagnosis of a family member with HCM. Diagnosis of HCM relies primarily on the demonstration of unprovoked cardiac hypertrophy in a septal, concentric, apical or segmental pattern by echocardiography or MRI imaging. In current clinical practice, genetic testing is performed on the index HCM patient to assist with diagnosis of family members, who may be carriers of a pathogenic mutation but without overt disease features (Maron et al. 2014). Family members who do not carry the pathogenic mutation can be excluded from clinical surveillance. Despite early hopes, it has become clear that identification of a specific HCM pathogenic mutation provides limited insight into prognosis or SCD risk. Genetic testing does have the potential to assist in diagnosis, for example, identification of phenocopies or differentiating athlete's heart from HCM, but the yield of a definite pathogenic mutation in this setting currently remains low (Pelliccia et al. 2012).

The clinical management of HCM revolves around risk stratification and prevention of SCD, control of symptoms, prevention of complications such as stroke due to atrial fibrillation and genetic counselling. No pharmacological treatment has been shown to alter the prognosis of HCM, but trials to date have been small and heterogeneous (Spoladore et al. 2012). Symptoms related to left ventricular outflow tract obstruction can be improved by beta-blockade, verapamil and disopyramide. Interventional options to reduce the size of the interventricular septum include alcohol septal ablation and surgical myectomy. HCM management has been revolutionised by the implantable cardioverter defibrillator (ICD), demonstrated to prevent SCD by terminating episodes of ventricular tachycardia or ventricular fibrillation (Maron et al. 2000). For end-stage, burnt-out HCM, heart transplantation is an option (Garcia-Pavia et al. 2011; Maron et al. 2010).

11.3.2 Genetics of HCM

11.3.2.1 Sarcomeric HCM Mutations

In approximately 60 % of HCM patients, a genetic mutation can be found in the eight most common known disease genes. Among them, *MYH7* and *MYBPC3* mutations are the most frequent, each accounting for approximately 25 % of cases (Richard et al. 2003).

MYH7 codes for beta-myosin heavy chain, the predominant form of myosin in the cardiac thick filament. *MYH7* p. R403Q was the first mutation identified by linkage analysis in a large HCM family and subsequent Sanger sequencing of candidate genes (Geisterfer-Lowrance et al. 1990).

The majority of the more than 200 *MYH7* mutations are missense mutations generating 'poisonous peptides'. There do not appear to be specific mutational hotspots, but founder mutations, i.e. mutations carried by individuals who founded a distinct population, have been identified. *MYH7* p.R1053Q is a founder mutation in the Finnish population (Jaaskelainen et al. 2014), accounting for 5.6 % of the

HCM patients studied (a cohort of 306). The founder mutation *MYH7* p. A797T has been described in South Africans (Moolman-Smook et al. 2000). For some of *MYH7* mutations, genotype-phenotype correlations have been studied (Lopes et al. 2013a; Moolman-Smook et al. 2000). *MYH7* mutations are associated with marked hypertrophy at relatively young age, the phenotype varies significantly. In patients with *MYH7* missense mutations, substantial deviations from the expected 50 % mutant/wild-type beta-myosin heavy chain protein levels (for heterozygous mutations) have been observed (Becker et al. 2007). This variable level of mutant protein may be one of the many factors contributing to a more mild or severe phenotype in an HCM patient.

Mutations in *MYBPC3* are an equally common cause of HCM. *MYBPC3* codes for cardiac myosin-binding protein C, a thick filament protein, which stabilises the thick filament and regulates myosin-mediated contractility (Ackermann and Kontrogianni-Konstantopoulos 2011). In addition, myosin-binding protein C binds to actin and modulates thin filament activity (Craig et al. 2014). Of note, many HCM-causing *MYBPC3* mutations are frameshift or nonsense mutations, i.e. the majority of mutations code for truncated proteins, which were found to be prone to nonsense-mediated decay and hence are not expressed (Carrier et al. 2010; van Dijk et al. 2009). Reduced protein levels of cardiac myosin-binding protein C are also observed in the presence of *MYBPC3* missense mutations (Marston et al. 2012). Thus, the lack of functional protein (haploinsufficiency) seems the dominant mode of action of HCM-causing *MYBPC3* mutations (Marston et al. 2009).

MYBPC3 p.Q1061X is a founder mutation in the Finnish population, found in 11 % of the 306 HCM patients studied (Jaaskelainen et al. 2013). There are three founder mutations in the Dutch population: *MYBPC3* c.2373_2374insG (coding for p.W792fsX17), *MYBPC3* c.2864_2865delCT (coding for P955fsX95) and *MYBPC3* p.R943X mutation (Christiaans et al. 2010). *MYBPC3* c.2373_2374insG accounts for almost 25 % of all Dutch HCM cases (Alders et al. 2003); the other two mutations are found in at least 5 % of the Dutch HCM population (Christiaans et al. 2010).

Other thick filament proteins known to harbour HCM mutations are myosin light chains (Flavigny et al. 1998; Poetter et al. 1996). However, mutations in *MYL2* and *MYL3* are rare causes of HCM.

In the thin filament, HCM mutations have been identified in *TNNT2*, *TNNI3*, *TPM1* and *ACTC1*. *TNNT2* and *TNNI3* code for cardiac troponin T and troponin I, respectively. Together with troponin C they constitute the troponin complex, which regulates the calcium-mediated contractile cycle. HCM-causing mutations in *TNNT2* are associated with a high risk of SCD, but mild ventricular hypertrophy (Moolman et al. 1997; Pasquale et al. 2012). Mutations in *TPM1* (coding for alpha-tropomyosin) and *ACTC1* (coding for cardiac actin) are generally rare; however, *TPM1* p. D175N is a founder mutation in the Finnish population, found in 6.5 % of HCM patients (Jaaskelainen et al. 2013). Mutations in *ACTC1* cause primarily apical hypertrophy and are associated with a high risk of SCD (Arad et al. 2005;

Song et al. 2011). Phenotypic overlap with LVNC is observed for the recurrent mutation *ACTC1* p. E101K (Monserrat et al. 2007b).

11.3.2.2 Mutations in Non-contractile Proteins Causing HCM

In 40 % of HCM patients, no causative mutation in a sarcomeric (contractile) gene can be identified, which has led to the assumption that unidentified HCM genes must exist. Indeed, mutations in a few genes coding for non-contractile proteins have been identified in patients with HCM phenotypes, including *CSRP3* (Geier et al. 2003), *ANKRD1* (Arimura et al. 2009), *TCAP* (Hayashi et al. 2004) and *MYOZ2* (Osio et al. 2007). All of these proteins have signalling functions in cardiomyocytes (Fig. 11.1).

CSRP3 codes for muscle LIM protein (MLP), a protein specific for cardiac and slow skeletal muscle. It was initially described as a Z-disc component (Knoll et al. 2002), but later found to be primarily cytoplasmic (Geier et al. 2008), with potential functions also in the nucleus (Boateng et al. 2009). Four different missense mutations in *CSRP3* were found to be causative for HCM, and the second zinc finger of the first LIM domain appears to be a mutational hotspot. The best studied mutation is *CSRP3* p. C58G. Linkage analysis and co-segregation in a large German family underline the pathogenicity of the mutation, and functional studies suggest that the mutation renders the protein less stable; hence, a lack on functional MLP protein could be the driver of HCM in this case (Gehmlich et al. 2004; Geier

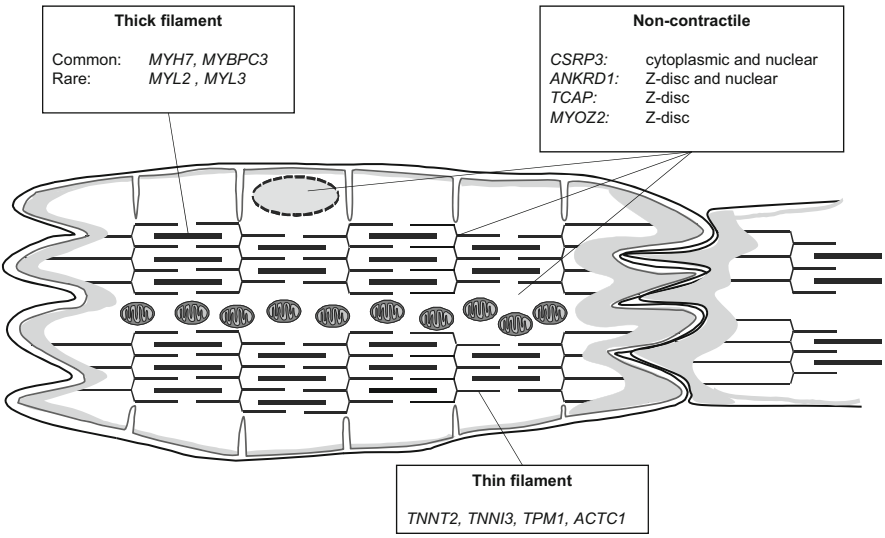

Fig. 11.1 Schematic drawing of a cardiomyocyte with the localisation of the eight most common sarcomeric HCM disease genes in the thick and thin filament. Additionally, the localisation non-contractile proteins implicated in HCM is indicated

et al. 2008). Mutations in *CSRP3* are rare and account for less than 1 % of the HCM cases.

CSRP3 p. W4R was initially described as a DCM-causing mutation (Knoll et al. 2002) and later also found in HCM patients (Bos et al. 2006). However, it is now known as a common variant (with an allelic frequency of approx. 0.9 % in the German population (Geier et al. 2008), 0.37 % at Exome Variant Server, EVS, http://evs.gs.washington.edu/EVS/) and discussed as a modifier gene for HCM (Knoll et al. 2010).

For the other non-contractile HCM genes, the majority of mutations were found by candidate gene screening approaches, and an attribution of the pathogenic role was based on the presence of a mutation in single individuals with HCM and its absence in small control cohorts (a few hundred individuals). Most reports fail to show cosegregation in large families and/or genome-wide linkage, which is the gold standard to describe novel disease genes. With more sequencing data being available now due to next-generation sequencing (NGS) technology, some reported HCM-causing mutations have now been found at low frequencies in the normal population (Andreasen et al. 2013). Future studies will reveal how these variants contribute to HCM, e.g. as modifier genes. So far MURFs and *FHL1* have been identified as modifier genes in HCM (Christodoulou et al. 2014; Su et al. 2014).

TTN mutations have been found in HCM patients, with a high proportion being missense variants (Lopes et al. 2013b). However, variants in *TTN* seem to occur also in the normal population and future research is needed to classify and evaluate them (see below).

Overall, mutations in genes coding for non-contractile proteins are rare and certainly do not account for the 'missing 40 %' of HCM patients, where no mutation in the common HCM genes has been identified. Indeed, the majority of those 40 % of HCM patients are unlikely to have Mendelian disease. Also they behave clinically as a distinct disease entity, e.g. they have milder microvascular dysfunction and less fibrosis (Olivotto et al. 2011).

11.3.2.3 Phenocopies of HCM and Mitochondrial Disease

The main phenocopies of HCM are Wolff-Parkinson-White syndrome caused by mutations in *PRKAG2* [the gamma2 subunit of AMP kinase (Murphy et al. 2005)], Fabry disease caused by mutations in *GLA* [alpha-galactosidase (Monserrat et al. 2007a)] and Danon disease caused by mutations in *LAMP2* [lysosome-associated membrane protein 2 (Yang et al. 2005)]. Of note, Fabry disease and Danon disease are X-linked. Likewise, mutations in *FHL1* (coding for four-and-a-half-LIM-domains 1) cause X-linked myopathy with HCM, but isolated HCM cases have been reported (Friedrich et al. 2012; Hartmannova et al. 2013).

Moreover, gene mutations affecting the mitochondria can cause HCM-like phenotypes (Govindaraj et al. 2014). If they are caused by mutations in the mitochondrial genome, maternal inheritance patterns are observed (Taylor et al. 2003).

11.3.3 Pathomechanisms of HCM

Following closely behind the genetics of HCM, in vitro and animal model experiments have yielded significant insights into disease mechanisms. On a cellular level, HCM is associated with abnormal sarcomeric calcium handing, energy homeostasis, biomechanical stress signalling, fibrosis and microvascular changes, which are all proposed to underlie or contribute to the pathogenesis of disease.

The vast majority of HCM mutations affect proteins of the cardiac contractile apparatus. HCM mutations typically increase calcium sensitivity and actin-dependent ATPase activity (Robinson et al. 2002), leading to increased myofilament contractility (Robinson et al. 2007; Sequeira et al. 2013; Witt et al. 2001). Increased cross-bridge cycling and ATPase activity (Belus et al. 2008) has a higher energy requirement to produce a given force—'tension cost' (Frey et al. 2006). This points at inefficient energy usage and relative energy depletion, which itself is proposed to drive disease pathogenesis (Ashrafian et al. 2003).

The energy depletion hypothesis is supported by the finding that HCM patients have a lower ratio of cardiac phosphocreatine (PCr) to adenosine triphosphate (ATP), independent of the underlying HCM mutation and occurring prior to the development of hypertrophy (Crilley et al. 2003). Cellular energy depletion has also been observed in mouse models and in vitro experiments (Ferrantini et al. 2009; Song et al. 2013; Witjas-Paalberends et al. 2014). Additionally, an HCM-like phenotype develops in a range of metabolic and mitochondrial disorders which compromise cellular energetics. Treatment of HCM patients with perhexiline, which optimises cardiac substrate metabolism with a shift towards carbohydrate use, improves the PCr/ATP ratio and improves functional capacity (Abozguia et al. 2010).

Downstream of the sarcomere, HCM mutations are associated with altered calcium signalling and calcium handling proteins such as SERCA2a and phospholamban (PLN). Changes in calcium kinetics have been proposed to underlie arrhythmogenesis, which can occur in the absence of significant hypertrophy (Knollmann et al. 2003). Cardiac troponin T HCM mouse models show alterations in SERCA2a:PLN ratio, PLN phosphorylation pattern and sarcoplasmic reticulum calcium uptake (Guinto et al. 2009). In support of a disease-causing role for aberrant calcium signalling, neonatal adenoviral delivery of SERCA2a to a tropomyosin mutant HCM mouse model was found to delay the development of hypertrophy (Pena et al. 2010).

Patients with HCM develop significant cardiac fibrosis, although the molecular triggers remain incompletely defined (Ho et al. 2010). Fibrosis occurs early, often prior to overt hypertrophy, and is a cause of arrhythmia and diastolic dysfunction and an important predictor of adverse survival (O'Hanlon et al. 2010). In addition, intimal hyperplasia and medial hypertrophy of the intramyocardial microvasculature lead to reduced flow and ischaemia, further promoting fibrosis and leading to impaired contractile function (Olivotto et al. 2011).

The pathomechanisms of HCM caused by mutations in non-contractile proteins remain enigmatic and will be the subject of future research. A role for downstream biomechanical stress signalling is suggested by identification of mutations in signalling molecules such as *CSRP3* and *ANKRD1*. Dissecting the cellular pathomechanisms in HCM holds great promise for developing clinical treatments, for patients with not only cardiomyopathy but also other diseases characterised by inappropriate hypertrophy or heart failure.

11.4 Next-Generation Sequencing: 'Opening Pandora's Box'?

Next-generation sequencing (NGS) technology has dramatically enhanced understanding of the genetic architecture of disease. For genetic cardiomyopathies, NGS holds great promise for elucidating the more complex genetic background to these diseases, for example, in HCM patients where a mutation is not identified by traditional candidate gene screening. However, the increased sensitivity of genetic sequencing also raises the substantial challenge of how to interpret variants of unknown clinical significance (VUS) and whether they are causal, contributory or bystanders to a disease phenotype.

Rare coding variation in the genome has historically been significantly underestimated (Tennessen et al. 2012). Overall, in a study of over 5,000 exomes, over half the sequence variants identified were unique to one patient (Pan et al. 2012), although this was substantially lower for known cardiomyopathy genes. Previously, pathogenicity has been ascribed to 'mutations' identified in candidate gene sequencing studies on the basis of absence from a small sample of controls. It is now clear that many of these variants are present in the normal population at low frequency, casting significant doubt on their pathogenicity in disease (Andreasen et al. 2013; Christensen et al. 2010).

NGS thus poses a significant challenge to researchers—how to pick out genuine disease-causing mutations from rare, benign variants existing at low frequency in the genome. Furthermore, genetic variants may not be acting in isolation, but as compound mutations, which are disease-causing when in the presence of another mutation, or as modifiers of the phenotype. Early use of targeted high-throughput sequencing in patients with HCM hints at the level of genetic complexity to be unravelled: 11 % had multiple candidate variants within the eight sarcomeric genes alone. Over 40 % of this HCM clinical cohort also had VUS in ion channel and AC candidate genes (Lopes et al. 2013b).

NGS has also been employed to overcome the technical challenges inherent in sequencing the enormous *TTN* gene, containing 363 exons (Herman et al. 2012). Using massively parallel sequencing, truncating variants were identified in 22 % patients with idiopathic DCM, significantly higher than in HCM or controls. Confirming pathogenicity in a given patient remains difficult, however, given that

TTN truncating variants were also found to exist in the control population at a frequency of 3 %. Presumably pathogenicity is influenced by as yet unknown modifying genetic or epigenetic loci, alongside environmental factors such as hypertension.

The strongest evidence for pathogenicity for a given mutation comes from linkage analysis or co-segregation with a phenotype—as established for the sarcomeric HCM genes—but frequently this is not possible, for example, in small families, for sporadic disease or where penetrance is low. In general, disease-causing variants would be expected to have a low frequency in libraries of control populations (e.g. 1,000 Genomes Project, the Exome Variant Server http://evs.gs.washington.edu/EVS/ and Exome Aggregation Consortium Browser http://exac.broadinstitute.org/) and to be enriched in disease cases (e.g. ClinVar http://www.ncbi.nlm.nih.gov/clinvar/). Pathogenic mutations are more likely to be those which disrupt the amino acid reading frame. *In silico* or isolated in vitro effects on protein function or structure are supportive but are a 'low bar' to meet (Watkins 2013) and have low sensitivity and specificity. Recapitulation of the phenotype in animal models, with subsequent rescue by silencing the variant, is strong evidence but costly and time-consuming. Hence, new cost-effective and efficient test systems, e.g. cardiac-specific cellular assays, are required to evaluate the pathogenicity of variants.

Overall, the resources needed for NGS interpretation are substantial and for the moment remain prohibitive outside a research environment. In addition to VUS assessment and interpretation, there are challenges inherent in bioinformatic processing, management of incidental findings and updating results over time as VUS are identified as benign or pathogenic (Dewey et al. 2014). As large databanks of genomes are acquired and automation and filtering of sequence results are improved, NGS will become an extremely powerful tool in probing beyond the 'one mutation, one gene' description of genetic disease of the heart.

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Chapter 12
Cardiac Cytoarchitecture: How to Maintain a Working Heart—Waste Disposal and Recycling in Cardiomyocytes

Jordan Blondelle and Stephan Lange

Abstract Normal development and maintenance of the heart is determined by the balance between protein synthesis and degradation. The regulation of this balance is critical, as increased protein synthesis is linked to hypertrophy of the heart, whereas increased degradation is usually associated with atrophy. Hypertrophy and atrophy of the heart are just two examples of cardiomyopathies, where the cellular equilibrium between synthesis and degradation is out of balance. It has become increasingly clear that impaired degradation of cardiac proteins is associated with the development of many cardiomyopathies. Here we discuss the functions of cardiac waste disposal and recycling systems, examine their regulation, and summarize recent developments that outline how cardiac-specific as well as ubiquitously expressed components of the cellular degradation systems contribute to the development and maintenance of a healthy heart.

12.1 Cellular Degradation Systems

The well-regulated degradation and recycling of cardiac proteins and macromolecular complexes plays significant roles not only during development of the heart (e.g., change in expression profile) but also for cardiac maintenance (e.g., removal of damaged and misfolded proteins) and for adaptive responses to stress (e.g., during cardiac remodeling). The removal of proteins in the heart depends on three cellular degradation systems: the ubiquitin-proteasome system (UPS), the autophagy/lysosome system, and proteolytic actions of proteases, such as caspases and calpains.

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12.1.1 The Ubiquitin-Proteasome System

The ubiquitin-proteasome system is responsible for the degradation of the majority of cytoplasmic proteins (Goldberg 2003). A cascade of enzymes that consists of the E1-ubiquitin activating enzyme Uba1, around 40 mammalian E2-conjugating enzymes, and a plethora of E3-ligases ensures the regulated ubiquitylation of cellular substrate proteins (Grabbe et al. 2011). This process is initiated by the proteolytic activation of the small protein ubiquitin. Ubiquitin is an evolutionary highly conserved 76 amino acid protein that belongs to the family of ubiquitin-like proteins (Fig. 12.1). The proteolytic cleavage of the pro-ubiquitin, which is usually expressed as poly-ubiquitin or ubiquitin with a C-terminal peptide extension (Ozkaynak et al. 1987), is achieved by a family of ubiquitin C-terminal hydrolases (UCHs) that consist in mammals of UCH-L1, UCH-L3, UCH37, and BAP1. These hydrolases create mono-ubiquitin that has at its C-terminus the di-glycine motif. Ubiquitin monomers are then coupled to the E1-enzyme Uba1 in an ATP-driven step, to form an energetically rich Ub~E1 thioester bond between the ubiquitin C-terminus and a cysteine residue on the E1-activating enzyme. Newer research uncovered that this reaction coincides with remarkable conformational changes of the E1-enzyme (Lee and Schindelin 2008). Additionally, once the cysteine residue in Uba1 links to the ubiquitin C-terminus, the E1-enzyme is adenylating the next ubiquitin, thereby loading the enzyme with two ubiquitin molecules. This double-loaded E1-enzyme is best primed to recruit an E2-conjugating enzyme (sometimes also referred to as ubiquitin-carrier protein) (Haas et al. 1988; Pickart et al. 1994). After docking, the bound ubiquitin is transferred to a catalytically active cysteine on the E2-enzyme in a thioester transfer reaction (Olsen and Lima 2013). Ubiquitin-loaded E2-enzymes are then recruited by ubiquitin E3-ligases that link to protein substrates for ubiquitylation. Substrate specificity is achieved by the variety and high number of these E3-ligases, which fall into three categories: RING-type, HECT-type, and U-box-type E3-ligases. How E3-ligases transfer ubiquitin onto protein substrates is dependent on the type of E3-enzyme. RING-type and U-box-type E3-ligases coordinate the direct transfer of the ubiquitin from the E2-enzyme onto an epsilon-amine of a lysine residue in the substrate protein. In contrast, HECT-type ligases catalyze a thioester transfer reaction that links ubiquitin first to a cysteine residue in the E3-ligase, before transferring it onto an epsilon-amine of a lysine in the substrate protein.

While substrate proteins may be released from the E3-ligase once they have undergone mono-ubiquitylation, marking them for degradation requires the formation of poly-ubiquitin chains. This poly-ubiquitylation is possible by the occurrence of seven lysine residues within the ubiquitin protein itself, such as lysine 48 (K48) or lysine 63 (K63), which can be utilized for attachment of another ubiquitin, and results in the formation of serially linked ubiquitins (also called poly-ubiquitin chains). Several models exist that explain how E3-enzymes promote ubiquitin chain formation (reviewed in Hochstrasser 2006). The most prominent of these models features the sequential addition of a single ubiquitin to a growing ubiquitin

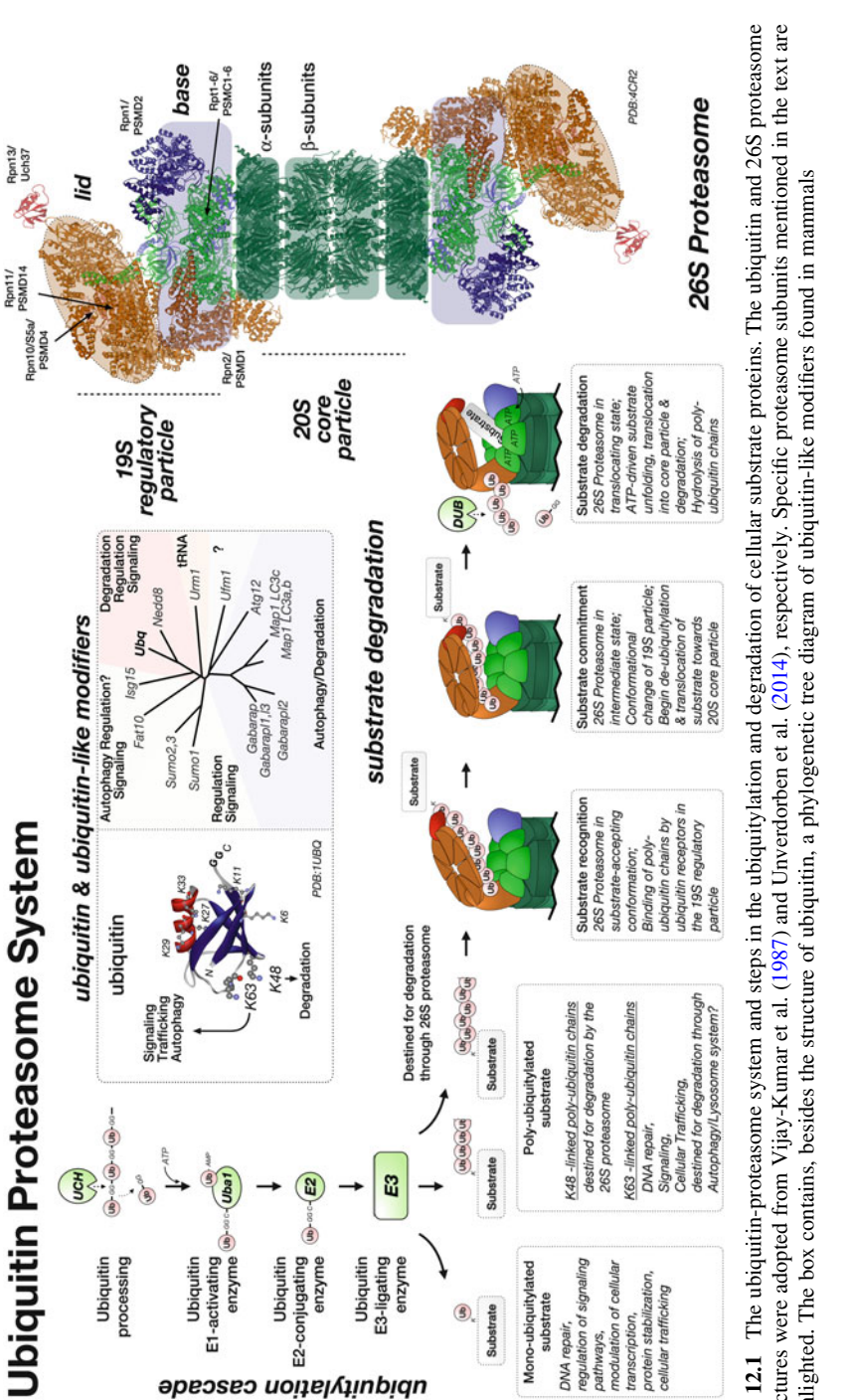

Fig. 12.1 The ubiquitin-proteasome system and steps in the ubiquitylation and degradation of cellular substrate proteins. The ubiquitin and 26S proteasome structures were adopted from Vijay-Kumar et al. (1987) and Unverdorben et al. (2014), respectively. Specific proteasome subunits mentioned in the text are highlighted. The box contains, besides the structure of ubiquitin, a phylogenetic tree diagram of ubiquitin-like modifiers found in mammals

chain on the substrate protein. HECT-type E3-ligases, however, may first assemble the poly-ubiquitin chain onto a ubiquitin that is linked through a thioester bond to a cysteine within the enzyme and then transfer it onto the substrate protein (Verdecia et al. 2003). In addition, some E2-enzymes can assemble poly-ubiquitin chains without the aid of E3-enzymes (Chen et al. 1991), or poly-ubiquitin chain generation may be facilitated by formation of E2-enzyme homo- or heterodimers (Chen et al. 1993; Varelas et al. 2003).

Several factors have been reported that influence the ubiquitin chain type (e.g., K48-linked vs. K63-linked). These include pseudo E2-enzymes (also called ubiquitin E2-variant proteins) that resemble E2-enzymes but lack the catalytically active cysteine for ubiquitin thioester bond formation (Moraes et al. 2001; VanDemark et al. 2001). Other factors comprise conformational side-chain arrangements in E2-enzymes (Petroski and Deshaies 2005) or simply E3-ligase specificity that favors one type of poly-ubiquitin linkage over another (reviewed in Dye and Schulman 2007).

Mono-ubiquitylation of substrates, or modification of substrate proteins with poly-ubiquitin chains other than K48-linked, is typically associated with signaling events (e.g., DNA damage, transcriptional activation) or cellular trafficking (reviewed in Chen and Sun 2009; Shih et al. 2000). Typically, only substrates that have been modified by K48-linked poly-ubiquitin chains are routed for degradation through the 26S proteasome (Chau et al. 1989; van Nocker and Vierstra 1993). However, newer research indicates that some substrates modified by K63-linked ubiquitin chains may be also routed to the 26S proteasome (Saeki et al. 2009) or via p62/SQSTM1 to another cellular degradation system, the autophagy/lysosome system (Pankiv et al. 2007; Seibenhener et al. 2004). This specificity of the 26S proteasome for poly-ubiquitylated substrates modified by K48-linked (and K29-linked) ubiquitin chains is achieved through the essential Rpn10/S5a/PSMD4 and Rpn13/ADRM1 subunits, located in the 19S regulatory particle of the proteasome (Deveraux et al. 1994; Hamazaki et al. 2007; Husnjak et al. 2008; Peth et al. 2010; Zhang et al. 2009). Although biochemical studies indicated that Rpn10 is able to interact with K6-, K11-, K29-, and K48-linked poly-ubiquitin chains (Baboshina and Haas 1996; van Nocker et al. 1996), only poly-ubiquitin chains linked via K11, K29, K48, and K63 have been observed in vivo (Arnason and Ellison 1994; Chau et al. 1989; Spence et al. 1995; van Nocker and Vierstra 1993; Xu et al. 2009). After substrate recognition by the (poly-)ubiquitin receptors Rpn10 and Rpn13, the 19S particle undergoes extensive conformational changes that lead to substrate de-ubiquitylation and translocation of the substrate protein towards the 20S core particle. De-ubiquitylation (DUB) is achieved by one of the three proteasome subunits that possess DUB-function: Rpn11/PSMD14, Rpn13/Uch37/ADRM1, and Ubp6/Usp14 (reviewed in Finley 2009; Voges et al. 1999). While all these enzymes have de-ubiquitylation activity, their processivity differs: Rpn11 activity is ATP dependent, Rpn13 releases mono-ubiquitin, while Ubp6 was shown to release di- and tri-ubiquitin from poly-ubiquitin chains (Hanna et al. 2006; Lam et al. 1997). Following binding of poly-ubiquitylated substrates to ubiquitin receptors Rpn10 and Rpn13, and the

processing of the poly-ubiquitin chain by proteasome subunits, the proteasome becomes committed to substrate degradation in an ATP-dependent step. This commitment step involves the recognition of unstructured regions in the substrate (Prakash et al. 2009) and activity of the six AAA-ATPase subunits (Rpt1–6/PSMC1–6) of the 19S proteasome base (Peth et al. 2010). The ATPases unfold globular substrate proteins and translocate the polypeptide chain towards the 20S core particle of the proteasome (Smith et al. 2007). The commitment of the proteasome towards a poly-ubiquitylated substrate was also shown to allosterically open a gate in the 20S core particle, which allows for entry of the unfolded polypeptide (Bech-Otschir et al. 2009; Li and Demartino 2009; Schreiner et al. 2008). The core particle consists of four ring-like multi-protein complexes (two heptameric alpha- and two heptameric beta-subunits) that form a central channel in which the unfolded polypeptide chain of the substrate translocates into (reviewed in Kunjappu and Hochstrasser 2013). Alpha subunits of the 20S core interact with proteins in the 19S regulatory particle to control gate opening, while the beta-subunits, specifically beta1, beta2, and beta5, harbor the caspase-like, trypsin-like, and chymotrypsin-like enzymatic activity to proteolytically cleave the substrate polypeptide into smaller fragments (reviewed in Tanaka 2013).

12.1.2 Autophagy/Lysosome

Eukaryotic cells developed another pathway for the removal of less degradable proteins, large molecular complexes, and even cellular organelles: the autophagy/lysosome system. Autophagy is commonly divided between directed or "selective" autophagy of specific substrates and so-called macro- or nonspecific autophagy of larger complexes, macromolecules, or cellular organelles, such as mitochondria (autophagy of mitochondria is termed "mitophagy"). The substrate is in either case encapsulated in a double-membraned vesicle called the autophagosome. Many of the studies that identified functions of autophagy-related genes/proteins (also abbreviated as ATG or APG) have been characterized in yeast (Klionsky et al. 2003); however, they are readily translatable to higher organisms, such as mammals, due to their high degree of evolutionary conservedness (Hale et al. 2013; Mizushima et al. 2011).

Degradation of substrates through the autophagy/lysosome system starts with the small protein LC3 (Map1LC3/ATG8) in an activation cascade that involves E1-like activating, E2-like conjugating, and E3-like lipidating enzymatic reactions. These reactions are similar to the canonical ubiquitylation cascade (Figs. 12.1 and 12.2). Moreover, a second enzymatic cascade involving the related ATG12 protein (Fig. 12.2 and box in Fig. 12.1) is required to assemble the ATG12-ATG5 E3-like lipidating enzyme for LC3/ATG8 (Ohsumi 2001).

Both LC3/ATG8 and ATG12 are proteolytically processed to enable the thioester bond formation between the C-terminal glycine residue in LC3/ATG8 or ATG12 and a cysteine residue on the E1-like activating enzyme ATG7. The

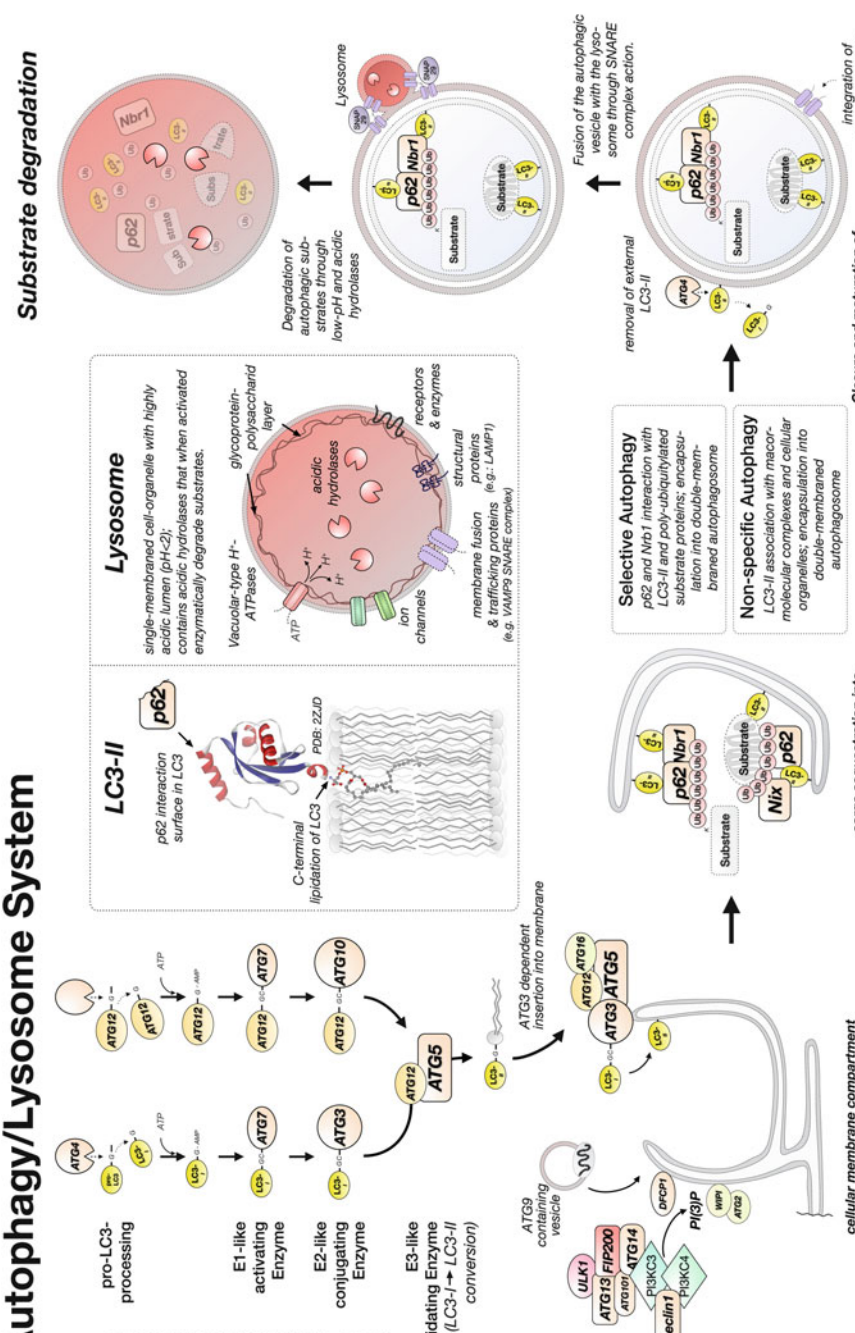

Fig. 12.2 The autophagy/lysosome system and steps in the degradation of cellular substrates. The structure of LC3/ATG8 was adopted from Ichimura et al. (2008)

proteolytic cleavage of the pro-LC3/ATG8 C-terminus is done by one of four ATG4 cysteine proteases (Kirisako et al. 2000), with ATG4B displaying the broadest processivity that extends to the closely related Gabarap and Gabarap-like family proteins (Gabarapl1, Gabarapl2/GATE16, and Gabarapl3; box in Fig. 12.1) (Kumanomidou et al. 2006; Li et al. 2010; Tanida et al. 2006). Once proteolytically processed, the LC3/ATG8 or ATG12 C-terminal glycine residue is adenylated in an ATP-dependent step and then coupled onto a cysteine residue in ATG7 through thioester bond formation. ATG7 works somewhat differently from the "canonical" ubiquitin E1-activating enzyme Uba1. LC3/ATG8- or ATG12-bound ATG7 forms homodimers (Komatsu et al. 2001). Once coupled to the ATG7 homodimer, LC3/ATG8 is transferred onto a cysteine residue in the E2-like conjugating enzyme ATG3, in a trans-thiolation reaction (Noda et al. 2011). A similar mode of action has been proposed for the thioester transfer reaction of ATG7-bound ATG12 onto ATG10 (Kaiser et al. 2013). ATG12 is finally irreversibly transferred onto ATG5 (Mizushima et al. 1998) through isopeptide bond formation and forms with the help of ATG16 a multimeric ~350 kDa ATG12-ATG5 complex (Kuma et al. 2002; Mizushima et al. 1999, 2003; Parkhouse et al. 2013). This multimeric ATG12-ATG5 complex now serves as E3-like enzyme in the transfer of LC3/ATG8 from ATG3 onto a phospholipid, phosphatidylethanolamine (PE) (Hanada et al. 2007; Ichimura et al. 2000). Newer research unveiled that the lipidation reaction proceeds in an ATG3-dependent fashion most efficiently at strongly deformed membrane structures present on the forming autophagosome rim (Nath et al. 2014) (Fig. 12.2). The un-lipidated and lipidated versions of LC3/ATG8 are sometimes referred to as LC3-I and LC3-II, respectively, and can be detected on immunoblot gels due to a slight shift in their molecular weights (Kabeya et al. 2004). Similarly, the closely related Gabarap and Gabarap-like proteins were also shown to exist in two versions: form I and form II, representing the un-lipidated and lipidated ubiquitin-like proteins, respectively.

Formation of the nascent autophagosome (sometimes also referred to as phagophore or omegasome) is thought to involve the endoplasmic reticulum membrane, although other cellular membranes, such as the plasma membrane, the membrane of the Golgi apparatus, or the outer mitochondrial membrane, have been suggested as autophagosome origin (Axe et al. 2008; Hailey et al. 2010; Moreau and Rubinsztein 2012; Yen et al. 2010). The autophagosome formation is thought to be initiated by a starvation signal originating from the mTORC1 pathway that acts on a protein complex consisting of the uncoordinated 51-like kinase 1 (ULK1/ATG1), ATG13, ATG14, ATG101, and FIP200/ATG17 (Hara et al. 2008; Itakura and Mizushima 2010; Karanasios et al. 2013; Kim et al. 2011; Mercer et al. 2009). The activated complex localizes to the nascent autophagosome (Hosokawa et al. 2009; Jung et al. 2009). Another signal for autophagosome initiation is connected to phosphatidylinositol 3-phosphate PI(3)P formation by class III phosphoinositide 3-kinase complexes, consisting of PIK3C3/Vps34, PIK3R4/p150/Vps15, Beclin1/ATG6, and ATG14 (Kihara et al. 2001; Suzuki et al. 2007; Tassa et al. 2003). Newly generated PI(3)P attracts another protein complex consisting of WIPI (WD40-repeat domain phosphoinositide-interacting

protein; ATG18 analogs) family proteins and ATG2, as well as DFCP1 (Axe et al. 2008; Itakura and Mizushima 2010; Polson et al. 2010; Proikas-Cezanne et al. 2004) (Fig. 12.2). Finally ATG9, a transmembrane protein localized in endosomes, is thought to provide the nascent autophagosome with membrane vesicles (Mari et al. 2010; Orsi et al. 2012; Yamamoto et al. 2012).

Lipidated LC3-II/ATG8 in the nascent autophagosome membrane is then able to attract substrates for degradation. This feat is achieved by interacting with adaptor proteins that anchor the substrates to the lipidated LC3-II/ATG8. Examples of LC3-II/ATG8 interacting adaptor proteins are p62/SQSTM1, Nbr1, or NIX. p62/SQSTM1 and Nbr1 serve as adaptor proteins for poly-ubiquitylated substrates (Bjorkoy et al. 2005; Kirkin et al. 2009; Lamark et al. 2009; Pankiv et al. 2007). NIX resides in the mitochondrial membrane and plays an essential role for mitophagy (Ding et al. 2010; Novak et al. 2009). Encapsulation of substrates and closure of the autophagic vesicle may require actions of ATG4 proteases, presumably to remove LC3-II/ATG8 from the external membrane, and allow for autophagosome maturation (Betin et al. 2013; Yu et al. 2012). After encapsulation and closure, the mature autophagosome merges with the lysosome through SNARE complex-mediated membrane fusion (Itakura et al. 2012). Syntaxin-17, a SNARE complex protein, is specifically incorporated into membranes of mature autophagosomes and was recently shown to interact with SNAP29 and VAMP9, localized in lysosomal membranes. After lysosome fusion, the inner membrane will dissolve and the acidic environment as well as acidic hydrolases and proteases (e.g., cathepsins) from the lysosome will degrade macromolecular complexes, proteins, and membranes (Koike et al. 2005; Tatti et al. 2012).

12.1.3 Proteases

While protease functions are associated with the ubiquitin-proteasome system (e.g., de-ubiquitylating enzymes, beta-subunits of the proteasome) and the autophagy/lysosome system (ATG4 cysteine proteases, cathepsin proteases of the lysosome), “free” cellular proteases have also been shown to play important functions for signaling and clearance of substrates. Proteases are enzymes that hydrolyze peptide bonds within proteins. While comparative genomic and proteomic approaches identified more than 500 proteases in mammals, their actions, substrate specificity, and tissue-specific expression vary widely (Puente et al. 2003) (Table 12.1, Fig. 12.3). Proteases can be classified into five groups according to their mechanism of action: aspartic, metallo-, serine , threonine, and cysteine proteases. Aspartic and metalloproteinases require an activated water molecule in their active site for a nucleophile attack on the peptide bond in the substrate protein. In the other proteases, specific serine, threonine, or cysteine residues in the active site are utilized for the nucleophilic attack on the peptide bond, which gave rise to their respective names (serine, threonine, or cysteine proteases).

Table 12.1 Select proteases and known cardiac substrates

Protease	Select potential and confirmed substrate	Disease link
Matrix metalloproteinases (MMP2)	ECM Alpha-actinin (Sung et al. 2007) Troponin-I (Wang et al. 2002) Titin (Ali et al. 2010) Myosin light chain (Sawicki et al. 2005) Myosin (Rouet-Benzineb et al. 1999)	Cardiac remodeling in MMP2 overexpressing mice (Bergman et al. 2007) Cardiac remodeling, fibrosis, inflammation (Passino et al. 2014) Angiogenesis, heart development, ischemia (Luttun et al. 2000) Heart failure (Muller and Dhalla 2011)
Cathepsins (cathepsin-L)	Autophagosomes (Koike et al. 2005; Tatti et al. 2012) Calsarcin (Petermann et al. 2006) Desmin (Petermann et al. 2006) Tropomyosin (Petermann et al. 2006) Alpha-actinin (Sun et al. 2013) Myosin (Sun et al. 2013) Connexin-43 (Sun et al. 2013) H-cadherin (Sun et al. 2013)	Cardiac hypertrophy, contractile dysfunction (Hua et al. 2012) Heart failure (Muller and Dhalla 2011) Cardiac inflammation, autophagosome accumulation (Pan et al. 2012) Cardiomyocyte apoptosis (Roberg and Ollinger 1998)
Calpains	Titin and MARPs (Hayashi et al. 2008; Laure et al. 2010; Suzuki et al. 1996) Troponin-I (Maekawa et al. 2003; van der Laarse 2002) Troponin-T (Ho et al. 1994) Myosin (Pemrick and Grebenau 1984) Nebulin (Taylor et al. 1995) Calponin (Croall et al. 1996; Tsunekawa et al. 1989) Desmin (Galvez et al. 2007; O'Shea et al. 1979) Dystrophin (Yoshida et al. 1992) Filamin (Davies et al. 1978) Ankyrin-B, -R (Harada et al. 1997) Cadherin (Bush et al. 2000; Sato et al. 1995) Beta-catenin (Li and Iyengar 2002) spectrin (Stabach et al. 1997; Yoshida et al. 1995) Talin (Muguruma et al. 1995) Vinculin (Taylor et al. 1995) Calcineurin (CnA) (Lakshmikuttyamma et al. 2004)	Limb-girdle muscular dystrophy LGMD2A (Kramerova et al. 2008; Ojima et al. 2010; Richard et al. 1995) Dilated cardiomyopathy in calpastatin transgenic mice (Galvez et al. 2007) Increased fibrosis, sarcolemmal defects in calpain 4 knockout hearts (Taneike et al. 2011) Ischemia-reperfusion (Neuhof and Neuhof 2014; Singh et al. 2004) Heart failure (Muller and Dhalla 2011) Cardiovascular disease (Sorimachi and Ono 2012)

(continued)

Table 12.1 (continued)

Protease	Select potential and confirmed substrate	Disease link
	SERCA2 (Singh et al. 2004) Phospholamban (PLN) (Singh et al. 2004) RyR (Singh et al. 2004) p53 (Kubbutat and Vousden 1997) PKCalpha (Kang et al. 2010) PKCdelta (Yamakawa et al. 2001) PKCepsilon (Saido et al. 1992) CaMK II, IV (Hajimohammadreza et al. 1997; McGinnis et al. 1998) MyLCK (Ito et al. 1987)	
Caspases	Actin (Maravei et al. 1997; Mashima et al. 1999) Myosin light chain (MyLC) (Moretti et al. 2002) Myosin light-chain kinase (Petrache et al. 2003) Gelsolin (Martin et al. 2010) Troponin-T (Communal et al. 2002; Lancel et al. 2005) Filamin (Browne et al. 2000; Umeda et al. 2001) Alpha-actinin (Communal et al. 2002) PKC-δ (Narula et al. 1999) mammalian sterile 20-like kinase 1 (Mst1) (Yamamoto et al. 2003) Serum response factor (SRF) (Chang et al. 2003) ATG4D (Betin and Lane 2009) Beclin1 (Wirawan et al. 2010)	Roles of caspases in: Cardiomyopathies (Birks et al. 2008; Ghosh et al. 2005; Lancel et al. 2005; Sharma et al. 2007) Heart failure (Muller and Dhalla 2011) Myocardial infarction (Olivetti et al. 1996; Piro et al. 2000; Zidar et al. 2006) Ischemia-reperfusion (Fliss and Gattinger 1996; Gottlieb et al. 1994; Kang et al. 2000)
ATG4	Pro-LC3 and delipidation of LC3-II Pro-Gabarap(l1-3) and delipidation of Gabarap(l1-3)-II	
DUBs (de-ubiquitinating cysteine proteases)	Poly-ubiquitin chains Pro-ubiquitin	
Mitochondrial proteases (Lon protease)	SOD2 (Bayot et al. 2010) RIP1 (Bayot et al. 2010) mtHSP60 (Bayot et al. 2010) F_1F_0-ATPAse subunit 1 and 2 (Bayot et al. 2010) StAR (Bayot et al. 2010) Aconitase (Bota and Davies 2002)	Aging (Delaval et al. 2004; Ngo and Davies 2007) Cardiac fibroblast survival (Anuka et al. 2013; Ondrovicova et al. 2005) Pressure overload-induced cardiomyopathy (Hoshino et al. 2014) Friedreich's ataxia (Guillon et al. 2009)

12.1.3.1 Matrix Metalloproteinases

The matrix metalloproteinase (MMP) system plays important functions for degradation and remodeling of the extracellular matrix in hearts (Liu et al. 2006). These calcium- or zinc-dependent enzymes are able to degrade most extracellular proteins, like collagen (MMP1, 8, 13, and 18), elastin, laminin, or fibronectin (e.g., MMP3, 7, 10), and have been shown to play critical roles for cardiac remodeling after myocardial infarction or in fibrosis (for a review see Hua and Nair 2015; Luttun et al. 2000; Passino et al. 2014). MMPs are expressed as inactive proenzymes (zymogens), which become active after proteolytic cleavage (Massova et al. 1998). While most substrates for MMPs are located in the extracellular space, intracellular functions for MMP2 have been reported on sarcomeric proteins, such as alpha-actinin, troponin-I, titin, or myosin light and heavy chains (Ali et al. 2010; Rouet-Benzineb et al. 1999; Sawicki et al. 2005; Sung et al. 2007; Wang et al. 2002) (Table 12.1). Indeed, transgenic overexpression of MMP2 leads to cardiac remodeling, diastolic dysfunction, and development of dilated cardiomyopathy (Bergman et al. 2007). The action of MMPs can be regulated by the four members of the tissue inhibitor family of matrix metalloproteinases (TIMPs) (Woessner 1991). While all cardiac cell types, including cardiomyocytes, have been demonstrated to express MMPs, cardiac fibroblasts and endothelial cells constitute the main sources for MMPs and TIMPs in the heart under basal conditions (Coker et al. 1999; Tyagi et al. 1995). It was also demonstrated that during cardiac remodeling infiltrating inflammatory cells (neutrophiles, macrophages) may contribute additional MMPs (Romanic et al. 2002). Newer research indicated that besides TIMPs, MMP2 action in cardiomyocytes, cardiac fibroblasts, and endothelial cells can be inhibited by the scaffolding domain of caveolins 1 and 3 (Chow et al. 2007; Kandasamy et al. 2009) as well as by inhibitors of other proteases, such as calpastatin (Kandasamy et al. 2009).

12.1.3.2 Cathepsins

Cathepsins are proteases that can be found in the lysosome and contribute to autophagic degradation of cellular substrates. Cathepsins can be classified either as serine, aspartic, or cysteine proteases (Hua and Nair 2015). Recent studies also described cathepsin action in neutral conditions, such as the cytosol of cells (Sever et al. 2007). Specifically, cathepsin-L has been shown to play important roles for the heart, as mice lacking this protease develop dilated cardiomyopathy with accumulation of lysosomes, mitochondrial degeneration, and aggregation of sarcomeric proteins alpha-actinin and myosin, as well as higher levels of connexin-43 and H-cadherin (Petermann et al. 2006; Spira et al. 2007; Stypmann et al. 2002; Sun et al. 2013) (Table 12.1). Other cathepsins, such as cathepsin-K, -S, or -D, have also been demonstrated to play roles during hypertrophy, contractile dysfunction,

cardiac inflammation, or oxidative stress (Hua et al. 2012; Pan et al. 2012; Roberg and Ollinger 1998).

12.1.3.3 Calpains

Calpains are a large family of calcium-dependent papain-like cysteine proteases. Calpain proteases can be classified according to their calcium requirements into *m-calpains*, which require millimolar amounts of calcium for their activity, and μ-*calpains* that work in the presence of micromolar amounts of calcium (Cong et al. 1989). Further classification of the 15 mammalian calpains can be done as specified by their domain layout into *typical-calpains*, which possess a C-terminal calmodulin-like domain and *atypical-calpains* that lack this domain (Sorimachi and Ono 2012; Suzuki et al. 1995). Lately, calpain nomenclature has been unified according to the gene products defined by the Human Genome Organization Gene Nomenclature Committee (Sorimachi et al. 2011).

Despite the ubiquitous expression of many calpains, some of them show distinctive tissue-specific expression patterns, like calpain-3, which is prominently expressed in skeletal muscle cells and, when mutated, can be the molecular cause for a form of muscular dystrophy (Kramerova et al. 2008; Ojima et al. 2010, 2011; Richard et al. 1995). Although mainly expressed in skeletal muscles, calpain-3 may also play a role in the heart (Fougerousse et al. 2000; Sarparanta et al. 2010; Taveau et al. 2002). Calpains 1 and 3 have been shown to localize to the myofibrils at the M-band and I-band of the sarcomere, indicating specific roles for these proteases at these sites (Hayashi et al. 2008; Raynaud et al. 2005). Targets for calpains in muscle include titin and titin-binding proteins of the muscle ankyrin repeat domain-containing protein (MARP) family (Hayashi et al. 2008; Laure et al. 2010; Suzuki et al. 1996), troponin-I (Maekawa et al. 2003; van der Laarse 2002), desmin (Galvez et al. 2007), calcineurin (CnA) (Lakshmikuttyamma et al. 2004), SERCA2-ATPase, phospholamban (PLN), ryanodine receptor (RyR) (Singh et al. 2004), calmodulin-dependent protein kinase (CaMK) (McGinnis et al. 1998), p53 (Kubbutat and Vousden 1997), or PKCalpha (Kang et al. 2010) (Table 12.1). Although the variety of substrates for calpains render it difficult to find conserved proteolytic motifs, targeted sequences are usually intrinsically unstructured and show increased abundance of proline, glutamic acid, serine, and threonine residues, similar to PEST sequences that were found to be susceptible for degradation by the ubiquitin-proteasome machinery (Shumway et al. 1999).

Several factors have been shown to regulate calpain activity. These are in addition to calcium, calpastatin (Murachi 1989), and phosphorylation by protein kinases, such as the extracellular regulated kinase (ERK) (Inserte et al. 2009).

Calpain function is required for cardiac development and cardiac function. Mice that overexpress calpain inhibitor calpastatin, causing a 50 % reduction of calpain activity, develop dilated cardiomyopathy (Galvez et al. 2007). Moreover, conditional knockout mice for calpain 4, a regulatory subunit for calpains 1 and 2, displayed increased fibrosis and sarcolemmal defects under hemodynamic stress,

indicating important functions for these proteases in cardiac adaptation and remodeling (Taneike et al. 2011).

12.1.3.4 Caspases

Caspases are cysteine proteases that are best known for their role during signaling and cell apoptosis (Stephanou et al. 2001; Yuan et al. 1993, reviewed in Chowdhury et al. 2008). Similar to matrix metalloproteinases, caspases exist first as inactive form (precursor zymogen), before becoming active through a proteolytic cleavage event that separates the tripartite proenzyme. Further classification of caspases can be done depending on their function into group I caspases that play a role for inflammatory responses (caspases 1, 4, 5, 12–14), group II caspases that initiate apoptosis (caspases 2, 8–11), and group III caspases that are also called effector or executioner caspases (Caspases 3, 6, 7) (Deveraux et al. 1998). Activation of caspases is achieved through a proteolytic cleavage event after conserved aspartic acid residues either by autoactivation or through the action of other caspases (Yang et al. 1998, reviewed in Chowdhury et al. 2008). After processing, caspases assemble into a hetero-tetramer consisting of two large subunits that each contains a catalytic protease site and two smaller subunits that structurally link the complex together (Yang et al. 1998).

As caspase activity is linked to apoptosis and cell death, most investigations into the role of caspases for the heart have been done in various forms of cardiomyopathy (Birks et al. 2008; Ghosh et al. 2005; Sharma et al. 2007), myocardial infarction (Olivetti et al. 1996; Piro et al. 2000; Zidar et al. 2006), and ischemia-reperfusion (Fliss and Gattinger 1996; Gottlieb et al. 1994; Kang et al. 2000). Besides activating the cellular apoptotic signaling cascade, caspases may also directly cleave myofibrillar substrates, as injection of active caspase 3 into healthy cardiomyocytes rapidly degraded sarcomeric structures (Laugwitz et al. 2001). Indeed, potential substrates include actin (Maravei et al. 1997; Mashima et al. 1999), myosin light chain (MyLC) (Moretti et al. 2002), myosin light-chain kinase (Petrache et al. 2003), gelsolin (Martin et al. 2010), troponin-T (Communal et al. 2002; Lancel et al. 2005), filamin (Browne et al. 2000; Umeda et al. 2001), or alpha-actinin (Communal et al. 2002) (Table 12.1).

12.1.3.5 Mitochondrial Proteases

While degradation of whole mitochondria within cells occurs preferentially through the autophagy/lysosome system (mitophagy), mitochondria developed a protein quality control and degradation system to manage misfolded or oxidatively damaged and denatured proteins (Wang et al. 1993). The best characterized system involves actions of the Pim1/Lon and Clp proteins, mitochondrial proteases that localize to the mitochondrial matrix (Kang et al. 2002; Suzuki et al. 1994). Both proteases have been identified in most organisms and show a remarkable degree of

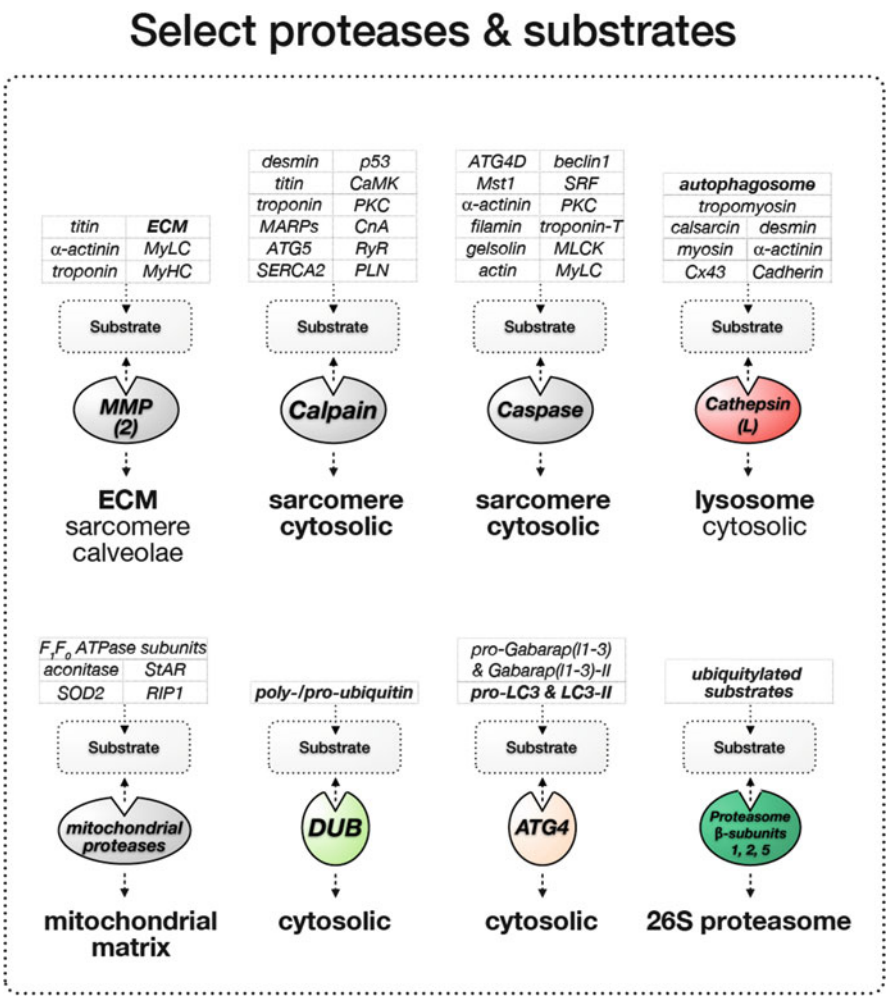

Fig. 12.3 Select proteases and cardiac substrates

evolutionary conservedness. Lon protease contains in addition to its protease domain an AAA-ATPase domain, which is important for target selection, unfolding, and translocation of the substrate protein towards the protease domain (similar to AAA-ATPase subunits of the 26S proteasome) (Ondrovicova et al. 2005). The structure of ClpP proteases was found to be remarkably similar to the beta-subunits of the 26S proteasome, forming a doughnut-shaped heptameric ring structure (de Sagarra et al. 1999; Kessel et al. 1995).

While degradation signals, such as poly-ubiquitylation, have not been identified for substrate proteins of mitochondrial proteases, Lon protease was shown to specifically degrade the oxidized (damaged) version of aconitase over the functionally active native version of the enzyme (Bota and Davies 2002). Aconitase is an

essential mitochondrial enzyme that is particularly susceptible to oxidative damage and has been demonstrated to be of importance for cardiac function and in disease (Bota and Davies 2002; Huang et al. 2001; Lin et al. 2009; Rotig et al. 1997; Yan et al. 1997). It has been shown for yeast that the activities of mitochondrial proteases, such as Lon, in combination with chaperones, protect this cell organelle from protein aggregation (Bender et al. 2011). Other substrates for mitochondrial proteases, such as Lon protease, may include SOD2, RIP1, mtHSP60, F_1F_0-ATP synthase subunits 1 and 2 (Bayot et al. 2010), or the steroidogenic acute regulatory protein (StAR), which is expressed in cardiac fibroblasts and may play a role for their survival (Anuka et al. 2013; Ondrovicova et al. 2005) (Table 12.1). The decrease in activity of this system during aging and in degenerative disease could be particularly harmful, as mitochondrial dysfunction may lead to an increase in cellular reactive oxygen species, triggering caspase activation, and result in cellular apoptosis (Bota et al. 2005; Delaval et al. 2004; Ngo and Davies 2007). Results from mice undergoing pressure overload-induced hypertrophy indicated inactivation of Lon-protease activity through its oxidation (Hoshino et al. 2014), further hinting at important roles of this class of proteases for cardiac maintenance. On the other hand, a mouse model for Friedreich's ataxia shows upregulation of mitochondrial proteases Lon and ClpP that correlate with a loss of mitochondrial Fe-S proteins and progression of the disease (Guillon et al. 2009). Taken together, the data indicate that mitochondrial proteases are important regulators of mitochondrial homeostasis. More recent data suggest further that their function may extend beyond degradation of damaged proteins, as they bind to mitochondrial DNA and RNA (Liu et al. 2004), and may be involved in the replication of the mitochondrial genome.

12.1.4 Chaperones and Heat-Shock Proteins

Chaperones and heat-shock proteins serve multiple functions within cardiac cells. They form an important part of the ERAD system by detecting misfolded proteins, assist in the folding of newly generated polypeptides, aid in the integration and removal of proteins into larger macromolecular complexes (e.g., integration of myosin into the sarcomere), and protect proteins from aggregating. Each of these diverse functions requires a specialized subset of chaperones.

Well-characterized chaperones in cardiac muscles and fibroblasts are Hsp90, Hsp70, Unc45B, the family of HspB proteins, or Hsp47 (for a review see Nagata 1998; Willis et al. 2009b). Proteins of the HspB family (small heat-shock protein family, also called sHSP) with important cardiovascular functions are the muscle-specific HspB7/cvHSP (cardiovascular heat-shock protein), alphaB-crystallin/HspB5, and Hsp27/HspB2 or Hsp20/HspB6. Among these, alphaB-crystallin/HspB5 and Hsp27/HspB2 were shown to localize to the sarcomere (Lutsch et al. 1997; Yoshida et al. 1999). The heat-shock protein alphaB-crystallin/HspB5 reportedly binds to the titin N2B-region and protects this spring-like element from unfolding (Bullard et al. 2004). Knockouts for alphaB-crystallin/HspB5 and/or

HspB2 demonstrated separate functions for the two chaperones, indicating that each of the HspB family members may exert special roles for muscles (Brady et al. 2001; Morrison et al. 2004; Pinz et al. 2008). Mutations in alphaB-crystallin/HspB5 have been shown to cause desmin-related myopathies and cardiomyopathies, such as dilated cardiomyopathy (Dalakas et al. 2000; Wang et al. 2001). Assembly of myosin into sarcomeres is aided by the functions of the striated muscle Unc45B, Hsp70, and Hsp90 (Barral et al. 2002; Srikakulam and Winkelmann 2004), which also display a sarcomeric localization pattern (Etard et al. 2008). While these chaperones were shown to have important functions for specific cardiac proteins (e.g., myosin or desmin), another heat-shock protein, Hsp27, was identified to have a more developmental role, suggested by its importance for heart tube formation (Brown et al. 2007). In addition, many cardiac chaperones and heat-shock proteins have been shown to be differentially modified and regulated in a variety of cardiomyopathies and during heart failure (Fan and Kranias 2010; Scheler et al. 1999; Stark et al. 2010).

Besides cardiomyocytes, heat-shock proteins play also important roles in cardiac fibroblasts and endothelial cells. The heat-shock protein Hsp47 forms part of the endoplasmic reticulum-associated degradation (ERAD) system in cardiac fibroblasts, where it transiently associates with procollagen to support the protein quality control. Therefore, Hsp47 functions play a crucial role during cardiac remodeling and fibrosis (Hagiwara et al. 2011; Nagata 1996, 1998).

12.1.5 Cooperativity Between Degradation Systems

It is important to point out that none of the degradation systems work in isolation but that there is in fact a high degree of cooperativity and cross talk between the UPS, autophagosome/lysosome system, cellular proteases, as well as chaperones. Examples for the regulation of cellular autophagy include the degradation of autophagy-related proteins ATG13 and ATG5 by the UPS (Hammerling and Gustafsson 2014; Mercer et al. 2009), the clearance of poly-ubiquitylated proteins via autophagy (Fan et al. 2010; Lamark et al. 2009; Novak et al. 2009; Seibenhener et al. 2004), or the regulation of autophagy by calpain1- and caspase-dependent cleavage of ATG5 (Xia et al. 2009; Yousefi et al. 2006) or beclin1 (Wirawan et al. 2010).

The activity of cellular proteases was shown to be dependent on the action of E3-ubiquitin ligases, as mono-ubiquitylation of caspases 3 and 7 has been demonstrated to regulate their activity (Huang et al. 2000). Cytoplasmic proteases and their inhibitors have also been shown to regulate matrix metalloproteinases (Kandasamy et al. 2009) and are important for the proteasomal degradation of a subset of myofibrillar proteins (Galvez et al. 2007).

12.2 Roles of Degradation Systems in the Heart

While the functions and mechanisms of the various cellular degradation systems are similar in all cell types, cardiomyocytes and cardiac fibroblasts show cell-type-specific adaptations to suit their specific needs and tissue-specific requirements.

12.2.1 Balance Synthesis/Degradation

Under physiological conditions, the heart mass is determined by the balance between muscle hypertrophy and muscle atrophy, which is directly linked to the regulation of protein synthesis and degradation. Deleterious diseases such as cardiomyopathies can result from defects in this balance, and comprehension of the underlying signaling pathways that influence protein synthesis and degradation is a field of intensive research (Heineke and Molkentin 2006; Razeghi and Taegtmeyer 2006). The increase in protein synthesis during hypertrophy largely contributes to the increase in cardiomyocyte size and the cardiac mass. The main signaling pathways underlying cardiac hypertrophy comprise, but are not restricted to, the activation of the calcineurin-NFAT pathway (Wilkins and Molkentin 2004), the calcium and calmodulin-dependent kinase II (CaMKII)-HDAC pathway (Swaminathan et al. 2012; Zhang et al. 2003; Zhu et al. 2000) that leads to activation of MEF2 (Wu et al. 2006), or the pathway encompassing insulin and insulin growth factor 1 (IGF1), PI3K, AKT, and GSK3-beta (Heineke and Molkentin 2006; Yu et al. 2010). Another way of promoting hypertrophy through the PI3K-AKT pathway includes activation of the mTORC1 protein complex. This complex is then able to promote protein synthesis through the activation of the p70/85 S6 kinase-1 (S6K1) and p54/56 S6K2, resulting in an increase of ribosomal biosynthesis (Heineke and Molkentin 2006).

During cardiac atrophy, the rate of protein degradation is predominant over the rate of protein synthesis. As a consequence, cell size and cardiac mass are decreased. The ubiquitin-proteasome system (UPS) plays a prominent role in atrophy, as UPS components like ubiquitin-B and UbcH2, as well as poly-ubiquitylated proteins, are generally found to be increased (Razeghi et al. 2006; Razeghi and Taegtmeyer 2006). The discovery of muscle-specific UPS components, such as E3-ligases of the MuRF family, or substrate adaptors for cullin-based E3-ligases, like atrogin1/MAFbx, triggered a multitude of investigations into molecular mechanisms, substrates, and roles during heart development and in disease.

12.2.2 Protein Quality Control and the ERAD System

Protein degradation is an important mechanism to maintain cardiac cell function. Indeed, misfolded proteins have been identified as molecular causes underlying the development of cardiac diseases. For example, production of misfolded alphaB-crystallin/HspB5 leads to crystallinopathies which include restrictive, hypertrophic, and dilated cardiomyopathies, as well as heart failure (Sanbe 2011). Another example are desminopathies (McLendon and Robbins 2011), where the accumulation of misfolded desmin is causative of the cardiomyopathy. Indeed, aberrant proteins need to be identified early during their synthesis or maturation and either refolded through the actions of chaperones or degraded.

The best characterized protein quality control mechanism is the endoplasmic reticulum-associated degradation (ERAD) system. Detection of abnormal proteins in the endoplasmic reticulum (ER) triggers the unfolded protein response (UPR). Misfolded proteins that are recognized by the ER trigger activation of chaperones, including the immunoglobulin heavy chain-binding protein (BiP), a lumenal Hsp70 protein (Brodsky 2007; Haas 1994), and the protein disulfide isomerase (PDI) (Molinari et al. 2002). Once identified, aberrant peptides are extracted from the ER through the retranslocon complex. This complex is constituted of membrane proteins assembled around E3-ubiquitin ligases, forming a pore in the membrane through which targeted proteins exit the ER (Carvalho et al. 2010; Zhang and Ye 2014). The translocation of proteins from the ER is believed to be highly energy dependent. While crossing the membrane, abnormal proteins undergo poly-ubiquitylation. A protein complex formed by p97/VCP [an AAA-ATPase protein (ATPases Associated with diverse cellular Activities)], Npl4, and Ufd1 was found to be required for the extraction of aberrant peptides from the ER (Zhang and Ye 2014). Once in the cytosol, the poly-ubiquitylated substrates are degraded by the 26S proteasome.

12.2.3 The Role of Substrate-Ubiquitylation Beyond Degradation

The ubiquitylation of proteins is a highly conserved posttranslational modification that serves versatile functions within cells. While the role for ubiquitin in protein degradation is now well characterized, its involvement in other biological pathways is emerging. There are numerous examples, where ubiquitylation of substrate proteins in cardiac cells modulates their functionality significantly.

Gap junctions are specialized plasma membrane domains enriched in connexin proteins that form channels (connexons) between adjacent cells. These channels are located at the intercalated discs (ID) and were found to be essential for cardiac action potential propagation, thus playing an important role in the intercellular communication (Kurtenbach and Zoidl 2014). As observed during injury, gap

junctions are often and rapidly remodeled or disassembled. Indeed, the protein half-life of connexin-43 (Cx43), a major constituent of cardiac gap junctions, is thought to be between 1 and 3 h (Beardslee et al. 1998). While Cx43 has been shown to be degraded by the UPS and the lysosome, its mono-ubiquitylation may serve as an initial trigger that leads to clathrin-dependent internalization of the connexon into the cytoplasm, its disassembly, and ultimately poly-ubiquitylation (Leithe and Rivedal 2004a) (Fig. 12.4). This tightly regulated process is thought to be under control of several cellular signaling pathways and protein kinases (Lampe and Lau 2000; Leithe and Rivedal 2004a, b) and allows gap junctions to be quickly remodeled in response to cellular stress.

In a similar fashion to clathrin-mediated endocytosis of Cx43, caveolae are sites where cells internalize membranes, molecules, transmembrane, and membrane-bound proteins. Caveolin-1 (Cav1), -2, and -3 proteins are structural constituents of caveolae expressed in cardiomyocytes and were shown to play important roles in cardiac signaling and physiology (Panneerselvam et al. 2012; Yang et al. 2014). Interestingly, it was shown that ubiquitylation of the Cav1 N-terminus constitutes a signal for its internalization and sorting into lysosomes (Kirchner et al. 2013). Endo-lysosomal sorting of ubiquitylated Cav1 proteins was shown to be p97/VCP dependent, pointing out a new role for p97/VCP unrelated to ERAD (Ritz et al. 2011).

Endocytotic mechanisms for substrate internalization play also a role for cardiac ion channels. Indeed, major cardiac voltage-dependent channels are tightly regulated in a quantitative manner via endocytosis (Ishii et al. 2012). Ubiquitylation is a very common posttranslational modification observed on cardiac ion channels and is thought to control their removal from the sarcolemma (Rougier et al. 2010). Interestingly, members of NEDD4 E3-ligases that are enriched in cardiac tissue were shown to ubiquitylate various ion channels, such as the voltage-gated cardiac potassium channel KCNQ1 or hERG1 (Cui and Zhang 2013; Rougier et al. 2010).

Ubiquitylation of sarcolemmal ion channels constitutes therefore a rapid way to modulate the density of ion channels at the surface of the membrane of cardiomyocytes and may represent an important mechanism for controlling the duration of the cardiac action potential.

Another example for the role of protein-ubiquitylation beyond its function as a degradation tag are caspases, where mono-ubiquitylation of caspase 3 and 7 by E3-ligases of the IAP family has been shown to act as an intracellular targeting signal, which in addition modulates caspase activity (Huang et al. 2000).

Similarly, posttranslational modification of calmodulin (CaM) by ubiquitin is thought to modulate the functions of this intracellular calcium receptor and signaling protein (Berchtold and Villalobo 2013; Swulius and Waxham 2008). It was shown that CaM can be reversibly ubiquitylated by ubiquitin-calmodulin ligase in the presence of calcium in cardiac cells (Jennissen and Laub 1988; Ziegenhagen and Jennissen 1988). Interestingly, mono-ubiquitylated CaM was able to strongly decrease its signaling function and the activity of binding partners such as phosphorylase kinase (Laub et al. 1998). These studies illustrate that (mono-) ubiquitylation of CaM is not priming the protein for degradation by the UPS but is important for the modulation of its activity.

Mono-ubiquitylation of substrate proteins was also shown to be important for the regulation of transcription factors. Transcription factors of the FoxO-family (Forkhead box O) were demonstrated to be posttranslationally modified by the cullin1-atrogin1 E3-ligase via K63-linked poly-ubiquitin chains that led to their activation (Li et al. 2007a). Intriguingly, activation of FoxO transcriptional activity through this mechanism resulted in the expression of atrogin1, suggesting a feed-forward loop that may counteract the induction of cardiac hypertrophy.

12.2.4 Cardiac-Specific Adaptations of the Degradation Machineries

Sarcomeres are the basic contractile units of striated muscles. Therefore, their maintenance is crucial for the correct function of the muscles. Indeed, the discovery of UPS-related proteins localizing at the sarcomere is not surprising. These include de-ubiquitylating (DUB) enzyme complexes such as Abro1 (Cilenti et al. 2011), members of the ubiquitin-specific protease family USP17 (Shin et al. 2006), USP22 (Lee et al. 2006), USP2 (Gousseva and Baker 2003), or USP28 (Valero et al. 2001), as well as E3-ligases and their substrate adaptor proteins, like the muscle RING-finger proteins (MuRFs) or atrogin1.

Intriguingly, many of the muscle-specific, but also some of the more ubiquitously expressed degradation system components were found to be associated with the sarcomeres. Their subcellular localization may result from interaction partners and/or ubiquitylation substrates like titin, troponin, or myosin, which are integral parts of the sarcomeric apparatus. In addition, interaction of degradation machinery components with the sarcomere may regulate their function. Examples may include MuRF1 and other UPS components that interact with the titin M-band region. Degradation system components may also be sequestered to the sarcomere in order to "inhibit" their action under baseline conditions (e.g., calpain 3). Once activated, such as during cellular stress, their sarcomeric localization is lost.

This principle may also work in reverse, where cellular stress results in the sarcomeric association of proteins that are normally found in the cytoplasm (e.g., chaperones). Here stress on the myofilaments may result in the unfolding of sarcomeric components and trigger association of chaperones and other degradation pathway components.

12.2.5 The Cardiac Degradation Process

It is generally thought that chaperones and proteases release proteins and protein complexes from the myofilaments, other macromolecular complexes, or cellular organelles (Goll et al. 2003). These actions may be supported by E3-ligases, which

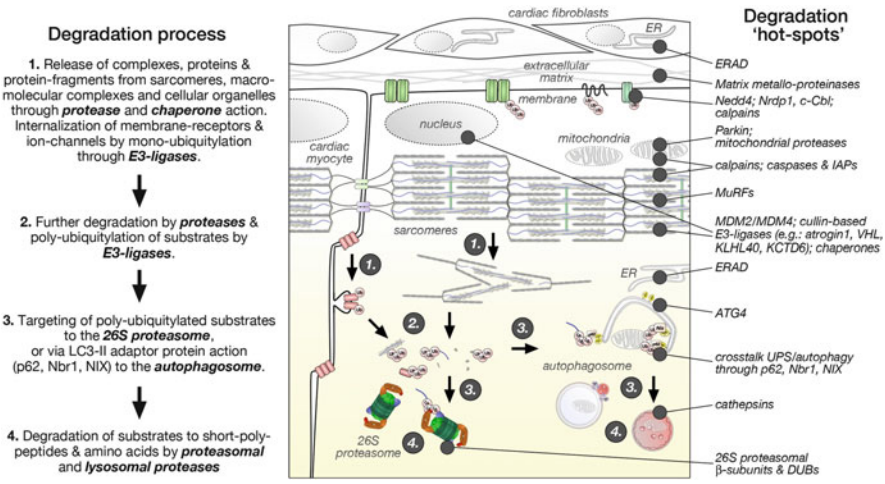

Fig. 12.4 The degradation process of cardiac proteins (adopted from Goll et al. 2003) and cellular "hot spots" for degradation pathway components

were shown to regulate membrane-receptor and ion-channel internalization through mono-ubiquitylation. Protein substrates are then poly-ubiquitylated by substrate-specific E3-ligases for subsequent degradation by the UPS and/or sequestered into nascent autophagosomes for lysosomal degradation (Fig. 12.4).

The following paragraph highlights specific E3-ligases with known cardiac roles and discusses their involvement in cardiac development and for cardiomyopathies.

12.3 E3-Ligases with Cardiac Roles

12.3.1 MuRF Protein Family

The E3-ubiquitin ligase family of muscle RING-finger (MuRF) proteins consists of three highly related members: MuRF1/TRIM63/RNF28, MuRF2/TRIM55/RNF29, and MuRF3/TRIM54/RNF30. These muscle-specific E3-ligases are among the first ubiquitin-proteasome components that were shown to localize to the sarcomere in cardiac and skeletal muscle cells (Centner et al. 2001; McElhinny et al. 2004; Spencer et al. 2000). All members of the MuRF family display a similar domain layout, with an N-terminal RING domain, a centrally located BBox domain followed by coiled-coil domains, and a MuRF-specific unstructured region towards the C-terminus that may contain another motif, the COS-box (Short and Cox 2006). While MuRF2 was shown to express four different splice isoforms that are developmentally regulated in the heart (Perera et al. 2010), MuRF1 and MuRF3 are both expressed in adult striated muscles (including the heart) (Centner et al. 2001).

The N-terminally located RING domain in MuRF proteins, in combination with E2-conjugating enzymes of the UBCH5 family (Fielitz et al. 2007a; Kedar

et al. 2004), is required for the ubiquitylation of substrates (Fig. 12.5). In addition it was shown that the RING domain may also interact with Sumo3, a small ubiquitin-like modifier (Dai and Liew 2001) (box in Fig. 12.1). This association of MuRF with the sumoylation machinery is further validated by interactions with the ubiquitin-conjugating enzyme 9 (Ubc9) as well as isopeptidase T-3 (ISOT-3); both enzymes are involved in the sumoylation cascade (McElhinny et al. 2002).

The centrally located Bbox domain was shown to attract various interaction partners and potential substrates, including FHL2/DRAL, calsarcin-2, or CARP1/Ankrd1, a member of the muscle ankyrin repeat domain-containing protein (MARP) family (Witt et al. 2008) (Table 12.2 and Fig. 12.5 for more potential

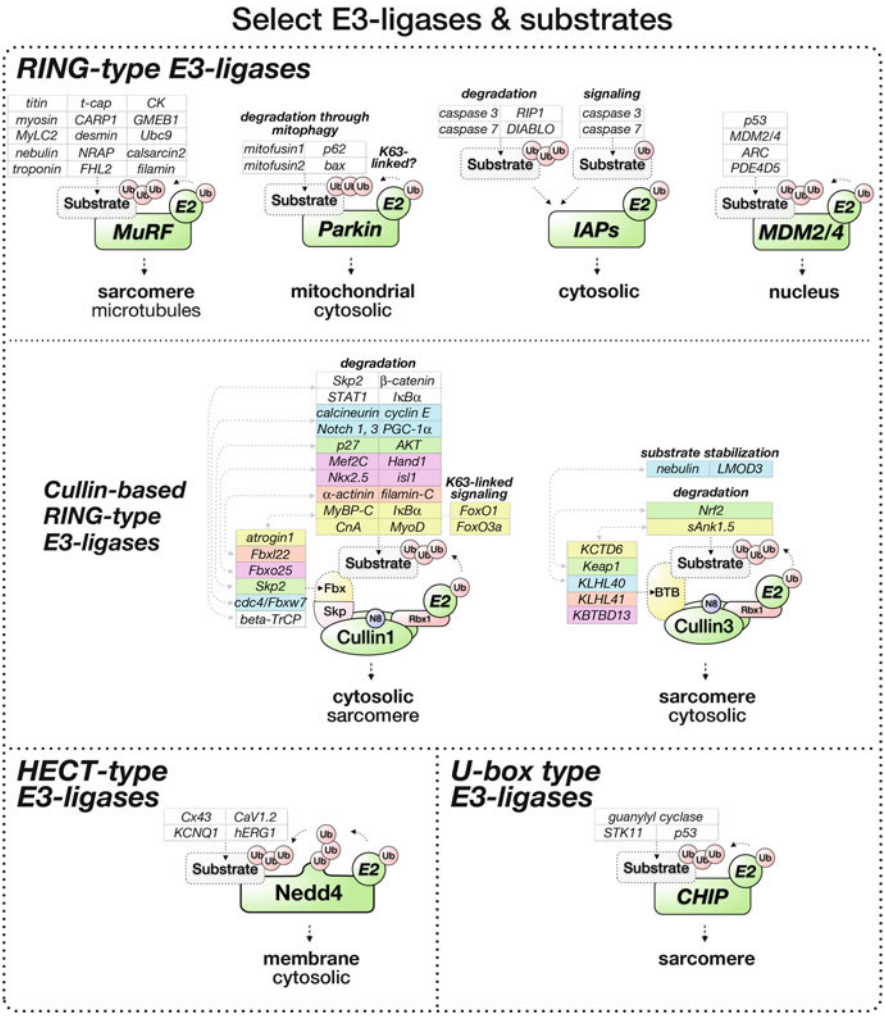

Fig. 12.5 Select E3-ligases and their cardiac substrates for ubiquitylation

Table 12.2 Potential and confirmed cardiac substrates for muscle RING finger (MuRF) E3-ligases

E3-ligase	Select potential and confirmed substrates	Disease link
MuRF	FHL2 (Fielitz et al. 2007b; Witt et al. 2008) Calsarcin-2 (Witt et al. 2008) Calcineurin-A (CnA) (Maejima et al. 2014) CARP1/Ankrd1 (Witt et al. 2008) Telethonin/T-cap (Witt et al. 2005, 2008) Desmin (Witt et al. 2008) Troponin-I (Kedar et al. 2004; Witt et al. 2005, 2008) Troponin-T (Witt et al. 2005) Troponin-C (Kedar et al. 2004) Myotilin (Witt et al. 2005, 2008) Titin (Witt et al. 2005, 2008) Nebulin (Witt et al. 2005, 2008) Nebulette (Witt et al. 2008) NRAP (Witt et al. 2005) Filamin (Fielitz et al. 2007b; Witt et al. 2008) Vimentin (Rubel et al. 2013) p62 (Witt et al. 2008) mtATP synthase (Rubel et al. 2013; Witt et al. 2005) mtHSP60/HSPD1 (Rubel et al. 2013) Creatin kinase (CK) (Koyama et al. 2008; Witt et al. 2008) Adenylate kinase (Witt et al. 2005) Aldolase A (Witt et al. 2005) 3-Hydroxyisobutyrate dehydrogenase (Koyama et al. 2008; Witt et al. 2005) GMEB1 (Koyama et al. 2008; McElhinny et al. 2002; Witt et al. 2008) PIAS (Witt et al. 2008) Sumo-3 (Dai and Liew 2001) Ubc9 (McElhinny et al. 2002) ISOT-3 (McElhinny et al. 2002) Myosin light chain (Kedar et al. 2004; Rubel et al. 2013; Witt et al. 2005) Myosin (Clarke et al. 2007; Fielitz et al. 2007a) Myosin binding protein-C (Kedar et al. 2004; Witt et al. 2008) Microtubules (Koyama et al. 2008; Rubel et al. 2013; Short and Cox 2006; Spencer et al. 2000) MuRF1 (Rubel et al. 2013)	Hypertrophy (Maejima et al. 2014; Wadosky et al. 2014; Willis et al. 2007, 2009a; Witt et al. 2008) Muscle wasting/atrophy (Bodine et al. 2001; Kamalov et al. 2013; Moresi et al. 2010; Polge et al. 2012) Heart failure (Willis et al. 2009a) Metabolic syndrome (Willis et al. 2009a; Witt et al. 2008) Aging (Witt et al. 2008) Myosin storage myopathy (Fielitz et al. 2007a)

and verified MuRF substrates). The coiled-coil domains in MuRF proteins indicate the potential to form dimers. Indeed, the formation of homo- and heterodimers between all members of this family has been shown experimentally (Centner et al. 2001; Witt et al. 2005). While MuRF proteins display sequence variability in their C-terminal regions, this part of the protein contains a sequence motif called COS-box (also named acid-rich/AR region). This region was demonstrated to be responsible for the reported interaction and regulation of microtubules (Koyama et al. 2008; Short and Cox 2006), alongside the coiled-coil domains (Spencer et al. 2000).

The best characterized sarcomeric interaction partners for MuRF1 are titin domains A168–A170, which are located adjacent to the stretch-sensitive protein kinase domain in titin (TK) (Lange et al. 2005; Mrosek et al. 2007; Muller et al. 2007). Indeed, transgenic mice for MuRF1 were found to display changes to the sarcomeric M-band, where this region of titin is located (Willis et al. 2009a). Moreover, the M-band region of titin may serve as a hot spot for the regulation of muscle gene expression and turnover, as MuRF2, p62/SQSTM1, and Nbr1 were also found to associate with titin-kinase in a stretch-dependent manner (Lange et al. 2005), and titin M-band knockout mice develop a severe form of atrophy (Peng et al. 2005). Both p62/SQSTM1 and Nbr1 link the ubiquitin-proteasome system with the autophagy/lysosome system (Bjorkoy et al. 2005; Kirkin et al. 2009; Lamark et al. 2009; Pankiv et al. 2007), indicating myofilament-dependent cross talk between these two degradation systems in muscles, including the heart (Perera et al. 2010).

MuRF functions for skeletal and cardiac muscles were widely assessed in knockout and transgenic mouse models. Single and double knockouts for MuRF1 (Bodine et al. 2001; Fielitz et al. 2007a; Koyama et al. 2008; Maejima et al. 2014; Wadosky et al. 2014; Willis et al. 2007, 2013; Witt et al. 2008), MuRF2 (Willis et al. 2007, 2013; Witt et al. 2008), and/or MuRF3 (Fielitz et al. 2007a, b), as well as transgenic mouse models for MuRF1 (Wadosky et al. 2014; Willis et al. 2009a), highlighted the importance of these muscle-specific E3-ligases for muscle development and maintenance, as well as for muscle atrophy and hypertrophy.

Sequencing of MuRF1 genes in patients affected with hypertrophic cardiomyopathy led to the discovery of three abnormal variants (Chen et al. 2012). These mutated MuRF1 proteins mis-localized to sarcomeric Z-discs and displayed reduced substrate ubiquitylation, resulting in the pathological accumulation of myosin heavy chain 6, cardiac myosin-binding protein-C, calcineurin, and mTOR in cardiomyocytes.

12.3.2 Cullin-Type E3-Ligases

Cullin-based E3-ligases belong to one of the largest families of E3-ubiquitin ligases. Although the cullin family consists of only eight members (cullin1, cullin2, cullin3, cullin4A, cullin4B, cullin5, cullin7, and cullin9/PARC), their modular

assembly poises them for a large number of possible targets (Fig. 12.5). Cullins do not bind their substrate proteins directly, but rely on an array of substrate adaptor protein families. Each of these adaptor protein families contains a specific domain that allows them to interact with a specified set of cullin proteins. Cullin1- and cullin7-based E3-ligases rely on the small protein Skp1 to interact with F-box-domain-containing proteins, while cullin3-based E3-ligases require BTB/POZ-domain-containing substrate adaptors to link to their targets for poly-ubiquitylation (reviewed in Sarikas et al. 2011).

Most cullin proteins (with the exception of cullin7 and cullin9/PARC) contain in their N-terminus three cullin repeats, which moderate the interaction with their substrate adaptor protein families (Table 12.3). A cullin-homology domain, followed by a conserved lysine that is used for neddylation of the protein, is found towards the C-terminus. The C-terminus of most cullins binds to Rbx1/ROC1 (regulator of cullin), which in turn attracts the activated E2-conjugating enzyme (Fig. 12.5). Rbx1 is the protein that carries the RING domain and fulfills thereby the E3-ligase functionality. Therefore, cullin proteins resemble in their role more that of a scaffolding protein, to attract all binding partners into a functioning E3-ligase complex that subsequently (poly-)ubiquitylates a specific cellular substrate protein.

Studies of knockout mice revealed that cullins are important for cell cycle, apoptosis, tumorigenesis, spermatogenesis, as well as vasculogenesis (Zhou et al. 2013). Cullin proteins are widely expressed in the organism, whereas their substrate adaptor proteins usually display more restricted expression patterns.

12.3.2.1 Regulation

Activation of cullin-based E3-ligases is extremely complex and involves a multitude of proteins and signalosome complexes, among them the COP9 signalosome complex, the small ubiquitin-like modifier nedd8 (box in Fig. 12.1), and CAND1/Tip120. Un-neddylated cullins bind to CAND1/Tip120a (Liu et al. 2002). Tip120b, a muscle-specific isoform of TIP120a, was shown to be regulated by a HECT-type E3-ligase (Aoki et al. 1999; You et al. 2003).

The assembly of the active E3-ligase requires the posttranslational modification of cullins by nedd8 (neddylation) and their homodimerization. Neddylation of cullin releases CAND1 and allows further assembly of the E3-ligase. Besides CAND1, the COP9 signalosome complex was found to be important for the regulation of cullin activity, as it proteolytically removes the nedd8 modification, resulting in an inactivation of the E3-ligase (reviewed in Kato and Yoneda-Kato 2009; Schwechheimer 2004). The importance of this signalosome complex for the heart function can be seen in cardiac-specific knockouts for some of its components. Most notably, ablation of COP9 subunit CSN8 results in dilated cardiomyopathy (Su et al. 2010, 2011, 2013), while silencing of CSN5 was shown to influence L-type calcium channels (Kameda et al. 2006).

Table 12.3 Select substrate adaptors and substrates for cullin-based E3-ligases

E3-ligase	Select potential and confirmed substrates	Disease link
Cullin1-atrogin	Myosin binding protein-C (Mearini et al. 2009) Calcineurin-A (CnA) (Li et al. 2004) MyoD (Tintignac et al. 2005) IkappaBalpha (Usui et al. 2011) FoxO transcription factors (K63-linkage) (Li et al. 2007a)	Atrophy (Bodine et al. 2001; Gomes et al. 2001) Hypertrophy (Li et al. 2007a) Pressure overload (Li et al. 2004) Chronic heart failure (Li et al. 2007b) Phenylephrine and pressure overload-induced hypertrophy, heart failure (Spaich et al. 2012)
Cullin1-Fbxl22	Alpha-actinin (Spaich et al. 2012) Filamin-C (Spaich et al. 2012)	
Cullin1-Fbxo25	Nkx2.5 (Jang et al. 2011) Isl1 (Jang et al. 2011) Hand1 (Jang et al. 2011) Mef2C (Jang et al. 2011)	
Cullin1-Skp2	Cyclin-dependent kinase inhibitor p27 (Chen et al. 2008; Pramod and Shivakumar 2014; Tamamori-Adachi et al. 2004) Akt (Chan et al. 2012)	Ischemia-reperfusion after myocardial infarction (Tamamori-Adachi et al. 2004) Herceptin-induced cardiotoxicity (Chan et al. 2012)
Cullin1-cdc4/Fbxw7	Notch 1 and 4 (Tetzlaff et al. 2004) Cyclin E (Tetzlaff et al. 2004) Calcineurin (Kishi et al. 2007) PGC-1alpha (Olson et al. 2008)	Mitochondrial function (Haemmerle et al. 2011) Myocardial infarction (Liepinsh et al. 2013) Diabetic cardiomyopathy (Palomer et al. 2013)
Cullin1-beta-TrCP	STAT1 (Soond et al. 2008) IkappaBalpha (Li et al. 2013; Winston et al. 1999) Beta-catenin (Wei et al. 2007; Winston et al. 1999) Skp2 (Wei et al. 2007) cdc4/Fbxw7 (Wei et al. 2007)	
Cullin2-VHL	HIF1alpha, HIF2alpha (Maxwell et al. 1999; Pozzebon et al. 2013)	Metabolic syndrome, obesity, or diabetes (Lei et al. 2008)
Cullin3-KCTD6	sAnk1.5 (Lange et al. 2012)	
Cullin3-KLHL40	LMOD3 (Garg et al. 2014) Nebulin (Garg et al. 2014)	Nemaline myopathy (Gupta et al. 2013; Ravenscroft et al. 2013; Sambuughin et al. 2010)
Cullin3-KLHL41	NRAP (Lu et al. 2003)	
Cullin3-KBTBD13		Nemaline myopathy (Sambuughin et al. 2010)
Cullin3-Keap1	Nrf2 (Fourquet et al. 2010)	ROS-induced cardiotoxicity (Muthusamy et al. 2011; Nordgren and Wallace 2013)
Cullin4-ctd2/pcna	p21 (Jackson and Xiong 2009; Nishitani et al. 2008)	

(continued)

Table 12.3 (continued)

E3-ligase	Select potential and confirmed substrates	Disease link
Cullin4-DDB2	Histones H2A, H3, H4 (Kapetanaki et al. 2006; Wang et al. 2006)	Cardiac development (Sakamaki et al. 2012) Ischemia-reperfusion injury and heart failure (Fan et al. 2013)
Cullin4-SV5	STAT (Jackson and Xiong 2009)	
Cullin5-SOCS	IRS1 (Hu et al. 2013; Rui et al. 2002) IRS2 (Rui et al. 2002)	Viral myocarditis (Knowlton 2008) Diabetic cardiomyopathy (Palomer et al. 2013)
Cullin7-Fbxw8	IRS1 (Xu et al. 2008)	

12.3.2.2 Cullin1-Atrogin1

Atrogin1/MAFbx/Fbxo32 is a muscle-specific cullin-1 substrate adaptor protein that localizes to the sarcomeric Z-disc (Li et al. 2004). The protein contains an F-box domain that binds to Skp1, a nuclear localization domain, and a PDZ-binding motif in its C-terminus (Gomes et al. 2001). Although atrogin1 localizes to the sarcomere due to its interaction with alpha-actinin, its overexpression did not alter alpha-actinin expression levels, indicating that alpha-actinin may not be a substrate for the atrogin-containing cullin-1 E3-ligase (Li et al. 2004). Confirmed substrates for the E3-ligase activity of cullin1-atrogin1 are a mutated version of myosin-binding protein-C that causes familial hypertrophic cardiomyopathy (Mearini et al. 2009), calcineurin-A (Li et al. 2004), as well as several transcription factors, including members of the Forkhead family of transcription factors (FoxO1, FoxO3a) (Li et al. 2007a), MyoD (Tintignac et al. 2005), and IkappaBalpha (Usui et al. 2011) (Table 12.3). While most of the investigated cullin1-atrogin1 substrates are channeled to the proteasome complex for degradation, the K63-linked poly-ubiquitylation of FoxO transcription factors acts as co-activation signal for their transcriptional activity (Li et al. 2007a) (Fig. 12.5). How the cullin1-atrogin1 E3-ligase differentiates between K48- and K63-linked poly-ubiquitylation of its substrates remains to be further investigated.

12.3.2.3 Other Cullin-1 Substrate Adaptors

Atrogin1 is not the only cullin-1-specific substrate adaptor protein with important roles for the heart. Recently, the cardiac-enriched F-box and leucine-rich repeat protein 22 (Fbxl22) was reported to mediate cullin-1-dependent degradation of alpha-actinin and filamin C (Spaich et al. 2012). Fbxl22 contains an N-terminal F-box domain, followed by a largely unstructured C-terminus. Intriguingly, the

protein localizes, like atrogin1, to the Z-disc of the sarcomere, where its two characterized cardiac substrates are located.

Fbxo25, a close homologue of atrogin1 (Gomes et al. 2001), was recently identified as substrate adaptor for several important cardiac transcription factors (Jang et al. 2011). Among other tissues, Fbxo25 is enriched in the fetal heart, and its expression is downregulated during heart development (Jang et al. 2011; Tacchi et al. 2010). The protein displays a nuclear localization (Hagens et al. 2006; Jang et al. 2011) and aggregates in a sub-compartment of the nucleus (Manfiolli et al. 2008). Here, Fbxo25 is thought to specifically ubiquitylate the cardiac transcription factors Nkx2.5, Isl1, Hand1, and Mef2C, which are then routed to the proteasome for degradation (Jang et al. 2011). Hence, developmental downregulation of this cullin1 substrate adaptor may drive cardiac differentiation and development.

Skp2, another F-box domain-containing protein, was also shown to play important functions for cardiomyocyte development and in disease. The cullin1-Skp2 E3-ligase was recently demonstrated to regulate the degradation of cyclin-dependent kinase inhibitor p27, possibly in an ERK2-dependent fashion (Chen et al. 2008; Pramod and Shivakumar 2014; Tamamori-Adachi et al. 2004). Terminally differentiated cardiomyocytes display low levels of Skp2 protein, which is actively ubiquitylated, leading to accumulation of the cullin1-Skp2 substrate p27 and cell-cycle arrest. Overexpression of Skp2 led to p27 degradation and stimulation of CDK2, which stimulated proliferation in D1NLS-/CDK4-transfected cardiomyocytes (Tamamori-Adachi et al. 2004). The same group of authors used the expression of Skp2 in D1NLS/CDK4 virally infected cardiomyocytes to investigate improvement of ischemia-reperfusion injury during myocardial infarction (Tamamori-Adachi et al. 2008).

More recently, a link between Skp2 ubiquitylation of Akt and Herceptin sensitivity was uncovered (Chan et al. 2012), indicating that Skp2 may play a role in Herceptin cardiotoxicity (Fuller et al. 2008).

Knockout mice for cdc4/Fbxw7, another F-box domain-containing protein, display malformation of the heart chamber and die at embryonic day 10.5, suggesting significant roles for cullin1-cdc4/Fbxw7-dependent protein degradation mechanisms during cardiac development (Tetzlaff et al. 2004). Tissues from these mice show accumulation of cyclin-E and Notch proteins 1 and 4, presumably through failed ubiquitylation of the putative substrates by this E3-ligase. The PPARgamma (peroxisome proliferator-activated receptor gamma) co-activator PGC-1alpha was also shown to be ubiquitylated through cullin1-cdc4/Fbxw7 E3-ligase (Olson et al. 2008). Regulation of PPAR through PGC-1alpha was shown to be of importance for the metabolic regulation of mitochondrial function (Haemmerle et al. 2011), during myocardial infarction (Liepinsh et al. 2013) and in diabetic cardiomyopathy (Palomer et al. 2013). Although only demonstrated in yeast, the F-box domain-containing protein cdc4/Fbxw7 was recently shown to promote degradation of calcineurin (Kishi et al. 2007). While the action of the cullin1-cdc4/Fbxw7 E3-ligase on calcineurin awaits confirmation in skeletal or

cardiac muscle cells, they open another intriguing way to manipulate calcineurin activity and putatively NFAT signaling (Crabtree and Olson 2002).

The beta-transducin repeat-containing protein 1 (beta-TrCP; also called F-box/WD repeat-containing protein 1A) contains, besides its F-box domain, several WD40 repeats towards the C-terminus of the protein. Several lines of evidence exist that indicate a role for the cullin1-beta-TrCP E3-ligase in the ubiquitylation of beta-catenin, IkappaBalpha (Li et al. 2013; Wei et al. 2007; Winston et al. 1999), and STAT1 (Soond et al. 2008), thereby influencing cardiac signaling pathways. Recent evidence also hints at an involvement of the cullin1-beta-TrCP E3-ligase in the degradation of other cullin1 substrate adaptors, namely, Skp2 and cdc4/Fbxw7 (Wei et al. 2007). Intriguingly, the NAD-dependent deacetylase SIRT1 was shown to regulate beta-TrCP degradation (Woo et al. 2013), indicating regulation of this protein presumably via acetylation and subsequent degradation via E3-ligase mediated poly-ubiquitylation.

12.3.2.4 Cullin-2

Cullin2-based E3-ligases bind to their respective substrate adaptor proteins with the help of the elonginBC heterodimer (Kamura et al. 1998). Substrate adaptor proteins that interact with elonginBC contain a characteristic BC-box motif.

One of the best characterized substrate receptors for cullin2-based E3-ligases is the BC-box domain-containing VHL protein (von Hippel-Lindau tumor suppressor). The cullin2-VHL E3-ligase is known to play important roles for HIF (hypoxia inducible factor) ubiquitylation and degradation (Maxwell et al. 1999; Pozzebon et al. 2013). Degradation of the heterodimeric HIF complex is regulated by oxygen, whereby normal cellular oxygen levels lead to the hydroxylation of the O2-sensitive HIFalpha complex subunit by prolyl-hydroxylases (Semenza 1999). The hydroxylated HIF complex is then recognized by cullin2-VHL as substrate and poly-ubiquitylated for subsequent degradation by the 26S proteasome. Decreased cellular oxygen levels, such as during ischemia, lead to inactivation of prolyl-hydroxylases and stabilization of the HIF complex, which then acts as transcription factor. Analysis of HIF pathway functions for the heart implicated its role in a wide range of diseases, including metabolic syndrome, obesity, or diabetes (Girgis et al. 2012).

A recent publication that investigated VHL functions using adipocyte-specific knockouts found that these mice develop a lethal form of postnatal cardiomegaly within the first week after birth, with dramatically hypertrophied ventricular and septal walls, as well as increased angiogenesis (Girgis et al. 2012). Intriguingly, adipocyte-specific deletion of HIF2alpha but not HIF1alpha rescued the cardiomelagy phenotype completely.

In contrast, mice with cardiac-specific deletion of VHL developed heart failure, malignant transformation of the heart, and premature death by 5 months of age (Lei et al. 2008).

12.3.2.5 Cullin-3

In contrast to cullin1, cullin3-based E3-ligases with importance for cardiac development and function are just starting to be investigated. Cullin3 itself was found to associate with the Z-disc in cardiomyocytes (Lange et al. 2012), like many of its substrate adaptor proteins (Bowlin et al. 2013; du Puy et al. 2012; Gupta et al. 2013). Substrate adaptors for this E3-ligase typically contain a BTB/POZ domain, which interacts with a binding site in the cullin3 protein (Canning et al. 2013) (Fig. 12.5).

Recently, KCTD6 was shown to act as cullin3-based substrate adaptor for the muscle-specific isoform of Ank1, called small ankyrin 1.5 (sAnk1.5) (Lange et al. 2012). KCTD6 localized to the M-band and Z-disc region of the sarcomere. The interaction of KCTD6 with sAnk1.5 was mediated by one of the obscurin-binding domains in the regulatory C-terminus of ankyrin, which is also partially present in non-muscle isoforms of Ank1, indicating regulatory functions for the ubiquitously expressed KCTD6 that extend beyond its role in muscle.

Functions of the cullin3-based substrate adaptor KLHL40/KBTBD5 were lately uncovered to be important for thin filament regulation. KLHL40 mutations cause an autosomal recessive form of nemaline myopathy (Ravenscroft et al. 2013). Although mainly characterized as a skeletal muscle disease, nemaline myopathy has been shown to be associated with cardiomyopathy, depending on disease mechanism and origins (Gatayama et al. 2013; Mir et al. 2011; Nagata et al. 2011; Taglia et al. 2013).

It emerged that cullin3-KLHL40 E3-ligases promote stability of nebulin and leiomidin 3 (LMOD3), thereby increasing thin filament durability in cross-striated muscles (Garg et al. 2014). Although further investigations are needed to uncover the exact mechanism, it is thought that cullin3-KLHL40 decreases the K48-linked poly-ubiquitylation of substrates, thereby promoting their folding and stability. KLHL40 is expressed specifically in heart and skeletal muscle (Bowlin et al. 2013), where it localizes to the sarcomeric I-band and A-band. Knockout mice for KLHL40 display neonatal lethality and show severe disruption of sarcomeres in skeletal muscles only (Garg et al. 2014). Intriguingly, two closely related BTB-domain proteins, KLHL41 and KBTBD13, were also implicated in nemaline myopathy development (Gupta et al. 2013; Sambuughin et al. 2010).

Mutations in KLHL41 (also known as KBTBD10, Krp1, sarcosin) were correlated with nemaline myopathy in several patients, who occasionally also displayed cardiac abnormalities, like ventricular septum defects (VSDs) (Gupta et al. 2013). Functional loss in a zebrafish model showed presence of nemaline bodies and led to diminished motor function and sarcomeric disorganization. KLHL41 is expressed in heart and skeletal muscles and displayed strong association with the I-band and Z-disc of the sarcomere as well as occasionally the M-band (du Puy et al. 2012; Gupta et al. 2013). Here it may associate with the nebulin-related anchoring protein (NRAP), which was recently identified as an interaction partner for KLHL41 (Lu et al. 2003). Knockdown experiments indicated that KLHL41 may also be

involved in the lateral fusion of adjacent myofibrils (Greenberg et al. 2008). Mature myofilaments were found to be severely decreased in knockdown cells, which may suggest a similar mechanism to KLHL40-mediated stabilization of myofilaments. Further investigations are needed to better understand the molecular pathway by which this substrate adaptor protein regulates myofibril assembly in cardiomyocytes.

Similar to KLHL40 and KLHL41, mutations of KBTBD13 in patients were found to cause an autosomal-dominant form of nemaline myopathy (Sambuughin et al. 2010). KBTBD13 is another substrate adaptor for cullin3-based E3-ubiquitin ligases (Sambuughin et al. 2012). The protein contains an N-terminal BTB/POZ domain that permits interaction with cullin3 and promotes its homodimerization. KLHL40 harbors in its C-terminus a KELCH domain, which is known to mediate protein–protein interactions, presumably linking up to various substrate proteins. In contrast to KLHL40 and KLHL41, KBTBD13 was found to be largely cytoplasmic and did not colocalize with the myofilaments (Sambuughin et al. 2010).

Another cullin3-based substrate adaptor protein with important cardiac functions is Keap1 (Chauhan et al. 2013). Keap1 is best known for its role as cellular mechanism against oxidative stress through its interaction with the transcription factor Nrf2 (Nfe2l2, nuclear factor (erythroid-derived 2)-like 2) (Nordgren and Wallace 2013). Under normal conditions, Keap1 targets Nrf2 for degradation by the ubiquitin-proteasome system. Oxidation of Keap1 by reactive oxygen species leads to disulfide-bridge formation between two Keap1 molecules, thereby altering its conformation, resulting in the stabilization of Nrf2, which is now able to translocate into the nucleus (Fourquet et al. 2010). This pathway is also important in the acute cellular response to doxorubicin-induced ROS production (Nordgren and Wallace 2013) or ROS production after acute exercise stress (Muthusamy et al. 2011).

Keap1 may also be involved in the clearing of poly-ubiquitylated substrates through the autophagy/lysosome pathway. Intriguingly, Keap1 is able to interact with p62/SQSTM1 and LC3 in a stress-dependent manner (Fan et al. 2010). Indeed, genetic ablation of Keap1 led to accumulation of poly-ubiquitylated aggregates and compromised autophagosome formation through decreased LC3 lipidation. The interaction of Keap1 with both p62 and LC3 may also be the reason for the finding that Keap1 itself, as well as three other BTB-domain-containing proteins, GAN1, ENC1, and KLHL41, get degraded in a cullin3-dependent way, however, independent of the ubiquitin-proteasome machinery (Zhang et al. 2005b).

12.3.2.6 Cullin-4

Similar to cullin3, the functions of cullin4-based E3-ubiquitin ligases for the heart are just emerging. Experiments with zebrafish indicated that cullin4a is responsible for positively modulating cardiac development by upregulating the transcription factor tbx5 (Zhao et al. 2015). Cullin4a morphants were found to display greatly reduced cardiac proliferation and heart looping defects. This effect may be due to nuclear functions of cullin4 family members, cullin4a and cullin4b. Indeed,

mammalian cullin4b was shown to associate with the polycomb-repressive complex (PRC2) to mono-ubiquitylate histone-2A (H2A) at K119 (Hu et al. 2012). Further experiments in Xenopus identified FADD (Fas-associated death domain-containing protein), an adaptor protein that transmits apoptotic signals and regulates NfkappaB signaling as interaction partner. Correct regulation of FADD proteins plays important roles during heart development. Embryonic manipulation of FADD protein levels led to induction of apoptosis or changes in cardiac chamber sizes (Sakamaki et al. 2012). Inhibition of FADD has been demonstrated to protect against ischemia-reperfusion injury and in heart failure (Fan et al. 2013).

Substrate adaptors for cullin4-based E3-ligases contain a DWD domain (DDB1-binding WD40) that links them via the DDB1 protein to the cullin scaffold (Jackson and Xiong 2009). Identified substrates for this E3-ligase include p21 [via cullin4-CTD2/PCNA (Jackson and Xiong 2009; Nishitani et al. 2008)]; histones H2A, H3, and H4 [via cullin4-DDB2 (Kapetanaki et al. 2006; Wang et al. 2006)]; or STAT1 through 3 [via cullin4-SV5 (Jackson and Xiong 2009)] (Table 12.3).

12.3.2.7 Cullin-5

Little is known about the biological functions of cullin5-based E3-ligases for cardiac development and function. However, a cullin5 substrate adaptor of the SOCS (suppressor of cytokine signaling) protein family has been linked to the stress-induced degradation of insulin receptor substrate-1 and -2 (IRS1, IRS2) (Hu et al. 2013; Rui et al. 2002). All SOCS proteins have been shown to act as cullin5-based substrate adaptors for the degradation of cytokine-induced signaling intermediates, thereby inhibiting the signaling cascade (Babon et al. 2009). Regulation of cytokine signaling in the heart was shown to be of importance during viral myocarditis (Knowlton 2008) or during diabetic cardiomyopathy (Palomer et al. 2013). Interaction of all SOCS proteins with cullin5 requires the elonginBC heterodimer as a cofactor (Babon et al. 2009).

12.3.2.8 Cullin-7

Similarly to cullin1, E3-ligases that are based on cullin7 require Skp1 as intermediary to bind to their respective substrate adaptors (Sarikas et al. 2011). One identified substrate for cullin7-Fbxw8 E3-ligase is insulin receptor substrate-1 (IRS1) (Scheufele et al. 2013; Xu et al. 2008). Loss of cullin7 in muscles was shown to hyperactivate IRS1, leading to phosphorylation of PI3K/AKT and ERK signaling pathways. This role of cullin7 on IRS1 regulation and in insulin signaling may be of importance during ischemia-reperfusion injury (Nagoshi et al. 2005), or in diabetic cardiomyopathy (Liu et al. 2014), although a clear link needs to be established.

A functional role for cullin7 in myocardial infarction was also shown in transgenic mice that express a mutant version of cullin7 (p193/cul7) (Hassink et al. 2009). The reasoning behind these experiments was that cardiomyocytes expressing this cullin7 mutant permit the reentry or preservation of cell-cycle activity (Nakajima et al. 2004). Indeed, experiments with transgenic p193/cul7 mutant mice reported a modest reduction in scar formation and enhancement of cardiac physiology compared to controls.

12.3.3 Other RING-Type E3-Ligases

12.3.3.1 Parkin

Parkin is a HECT-RING hybrid E3-ubiquitin ligase that has been shown to play important roles for mitochondrial homeostasis. A systematic approach to identify dynamically regulated parkin E3-ligase functions identified hundreds of mitochondrial substrate proteins, including bax, mitofusins 1 and 2, and p62/SQSTM1 (Sarraf et al. 2013) (Table 12.4). Although parkin is best characterized for its role

Table 12.4 Select cardiac targets for other RING-type E3-ligases

E3-ligase	Select potential and confirmed substrates	Disease link
Parkin	p62 (Sarraf et al. 2013) bax (Sarraf et al. 2013)	Myocardial infarction (Kubli et al. 2012) Aging (Kubli et al. 2013)
cIAP1, cIAP2, xIAP	Caspase 3 (Huang et al. 2000; Suzuki et al. 2001) Caspase 7 (Huang et al. 2000) RIP1 (Bertrand et al. 2008) Smac/DIABLO (Hu and Yang 2003)	Protection of cardiac fibroblasts from oxidative damage (Philip and Shivakumar 2013) Upregulation in pressure overload-induced hypertrophy (Balasubramanian et al. 2006; Johnston et al. 2009)
Nrdp1/ RNF41	Neuregulin receptors (ErbB1–4) (Diamonti et al. 2002; Yen et al. 2006)	Ischemia-reperfusion injury (Zhang et al. 2011b) Doxorubicin-induced cardiotoxicity (Zhang et al. 2011a)
MDM2, MDM4	p53 (Haupt et al. 1997; Pei et al. 2012; Schuster et al. 2007; Toth et al. 2006) MDM2 (Fang et al. 2000) PDE4D5 (Li et al. 2009) ARC (Foo et al. 2007)	Cardiac and vascular development (Grier et al. 2006; Zhang et al. 2012) Cardiac fibrosis (Zhang et al. 2014) Dilated cardiomyopathy (Xiong et al. 2007) Pressure overload-induced hypertrophy (Balasubramanian et al. 2006)
c-Cbl	ErbB1 (Waterman and Yarden 2001) EGFR (Levkowitz et al. 1999) FAK (Rafiq et al. 2011)	Ischemia-reperfusion injury (Rafiq et al. 2014) Tissue injury (Rafiq et al. 2011) Pressure overload-induced hypertrophy (Balasubramanian et al. 2006) Diabetes (Gupte and Mora 2006)

in Parkinson's disease, recent work established a function for this E3-ligase during myocardial infarction (Kubli et al. 2012) and in aging (Kubli et al. 2013). Parkin knockout mice are viable (Goldberg et al. 2003) and display no baseline cardiac phenotype (Kubli et al. 2012). However, parkin deficiency during stress, such as ischemia-reperfusion, led to defects in mitophagy and the accumulation of damaged mitochondria. Consequently, overexpression of parkin protected cardiomyocytes against hypoxia-mediated cell death (Kubli et al. 2012).

The mechanism of parkin-induced mitophagy involves the mitochondrial protein kinase PINK1 (PTEN-induced putative kinase 1) and the outer mitochondrial membrane proteins mitofusin 1 and 2 (Chen and Dorn 2013; Sarraf et al. 2013; Tanaka et al. 2010). Parkin binds and promotes K63-linked poly-ubiquitylation of mitofusins in a PINK1-dependent manner (Chen and Dorn 2013; Olzmann and Chin 2008). Poly-ubiquitylation of mitofusin and other outer mitochondrial membrane proteins attracts NIX and p62/SQSTM1, which actively bind to lipidated LC3 to encapsulate mitochondria in the nascent autophagosome (Ding et al. 2010; Narendra et al. 2010) and promote the initiation of mitophagy (Figs. 12.2 and 12.5).

12.3.3.2 IAPs

Inhibitor of apoptosis proteins (cIAP1, cIAP2, xIAP) are RING-domain-containing E3-ligases that ubiquitylate proteins in the apoptosis pathway, including RIP1, Smac/DIABLO (second mitochondria-derived activator of caspases), and several caspases (Bertrand et al. 2008; Hu and Yang 2003; Huang et al. 2000) (Table 12.4). E3-ligases cIAP1 and cIAP2 were shown to constitutively poly-ubiquitylate RIP1 (Bertrand et al. 2008), while xIAP (x-linked IAP) was found to promote poly-ubiquitylation of active caspase 3 (Suzuki et al. 2001) (Fig. 12.5). Intriguingly, cIAP2 may also modulate its activity and promote the mono-ubiquitylation of caspases 3 and 7 (Huang et al. 2000). Although the mechanism remains unclear, the authors of the study suggest that mono-ubiquitylation of caspases may act as an intracellular targeting signal modulating the activity of these proteases (Shih et al. 2000).

E3-ligases of the IAP family have been demonstrated to be important for the heart, mainly due to their function as inhibitors of apoptosis and cell death. In cardiac fibroblasts, cIAP2 was shown to protect the cells from oxidative damage (Philip and Shivakumar 2013). IAP E3-ligases were also found to be upregulated in pressure overload-induced hypertrophy and heart failure (Balasubramanian et al. 2006) and may protect cardiomyocytes in a beta3-integrin-dependent way (Johnston et al. 2009).

12.3.3.3 Nrdp1/RNF41

Nrdp1/RNF41 is a member of the TRIM (tripartite interaction motif) protein family and shows thereby similarities to the domain organization of the MuRF proteins.

Most of Nrdp1's functions have been associated with the ubiquitylation of membrane receptors ErbB2, ErbB3, or ErbB4 of the neuregulin receptor family (Diamonti et al. 2002; Yen et al. 2006) (Table 12.4), indicating that this E3-ligase associates with the cellular membrane. Mechanistically, monomeric Nrdp1 is sufficient to ubiquitylate neuregulin receptors through the actions of its RING domain and the unique protein C-terminus. However, newer research indicated that this E3-ligase may also oligomerize through its N-terminal BBox domain, which leads to auto-ubiquitylation and lability of the protein, indicating a putative mechanism for its regulation (Printsev et al. 2014). Cardiac expression of the neuregulin receptors (like ErbB2) has been shown to be important for cardiovascular development, and their targeted deletion or inhibition through the anti-cancer drug Herceptin (monoclonal antibody against ErbB2) results in either embryonic lethality or cardiotoxic effects (Fuller et al. 2008). Therefore, it is not surprising that cardiac overexpression of the E3-ligase, which in turn regulates ubiquitylation of neuregulin receptors, exacerbates ischemia-reperfusion-induced cardiac injury (Zhang et al. 2011b) through downregulation of ErbB3 or doxorubicin-induced cardiac dysfunction (Zhang et al. 2011a).

12.3.3.4 MDM2, MDM4/MDMX

The MDM2 (murine-double minute) E3-ligase is best known for its role in the degradation of the proapoptotic transcription factor p53 (Honda et al. 1997; Toth et al. 2006) (Table 12.4). Its function to poly-ubiquitylate p53 is enhanced by the closely related MDM4/MDMX (Pei et al. 2012). Both E3-ligases harbor the substrate-binding site (e.g., for p53) in the N-terminal part of the protein, while the C-terminus contains the RING domain (Cheok et al. 2010). Depending on protein levels, MDM2 is able to mono- or poly-ubiquitylate p53 (Li et al. 2003). Poly-ubiquitylation of p53 leads to its nuclear export, where it is degraded by the 26S proteasome (Haupt et al. 1997; Pei et al. 2012; Schuster et al. 2007). In addition to p53 poly-ubiquitylation, MDM2 was also demonstrated to be able to undergo auto-ubiquitylation and to ubiquitylate MDM4/MDMX (Chao 2015). Substitution of the RING domain in MDM2 with that of an unrelated E3-ligase of the RING-type family did not affect the ability of MDM2 to promote auto-ubiquitylation. However, the chimeric MDM2 protein was unable to ubiquitylate p53, suggesting some level of specificity of the RING domain towards p53 (Fang et al. 2000).

Global and cardiac-specific knockout mice for MDM2 exhibit early embryonic lethality due to abnormally increased p53 levels, which can be rescued by genetic deletion of p53 (Grier et al. 2006; Jones et al. 1995; Montes de Oca Luna et al. 1995). Inducible global p53 knockout mice became moribund within 1–2 days and displayed drastic cardiac fibrosis after interrupted tamoxifen treatment (Zhang et al. 2014). While embryonic deletion of MDM2 in a cardiomyocyte-specific MDM2 knockout mouse model displayed no obvious cardiac differences at embryonic stage E9, all knockout mice died at embryonic stage E13.5 and displayed severe morphological defects of the heart (Grier et al. 2006).

Endothelial/endocardial-specific knockout of MDM2 also resulted in early embryonic lethality, displaying severe vascular and morphogenic defects (Zhang et al. 2012). Overexpression of MDM2 on the other hand promoted cardiomyocyte survival, blocked cardiac hypertrophy, and protected against ischemia-reperfusion injury in a cell-culture model (Toth et al. 2006).

Similarly to MDM2, loss of MDM4/MDMX had an impact on the heart. While deletion of MDM4 in the embryonic heart does not cause any developmental defects (Grier et al. 2006), cardiac-specific knockouts for this E3-ligase showed severe edema and heart failure by 3 months of age. In the same study, mutant MDM4 mice developed dilated cardiomyopathy in a p53-dependent fashion and died of heart failure by 8 months of age (Xiong et al. 2007).

More targets for the E3-ligase function of MDM2 include PDE4D5 (cAMP phosphodiesterase-4D5) (Li et al. 2009) and ARC (apoptosis repressor with caspase recruitment domain) (Foo et al. 2007). For PDE4D5, beta-arrestins were required as a scaffold to first mediate the mono-ubiquitylation of the enzyme at Lys-140, which primes the protein for subsequent poly-ubiquitylation at three other lysine residues in the C-terminus of the protein. PDE4D5 in combination with beta-arrestin is known to influence cardiac signaling through the beta2-adrenergic receptors and adenylyl cyclase. ARC is expressed in terminally differentiated tissues, such as cardiomyocytes, where its degradation is dependent on MDM2 action and subsequent poly-ubiquitylation (Foo et al. 2007). Pressure overload-induced cardiac hypertrophy was found to be associated with increased MDM2 (Balasubramanian et al. 2006) and decreased ARC levels (Foo et al. 2007).

12.3.3.5 Cbl

Members of the Cbl (Casitas b-lineage lymphoma) E3-ubiquitin ligase family were shown to regulate receptor or non-receptor tyrosine kinases and other proteins known to be associated with membranes (Rao et al. 2002) (Table 12.4). Three mammalian Cbls are known: Cbl, the related b-Cbl, and the more distant c-Cbl. All family members contain the central RING domain and share in addition an N-terminal tyrosin kinase-binding domain (TKB). The C-terminus of Cbl and b-Cbl harbors in addition a leucine-zipper motif, which is absent in c-Cbl. While both Cbl and b-Cbl display ubiquitous expression, c-Cbl has a more tissue-specific expression pattern mostly in epithelial cells and the epidermis; however, the protein displays negligible expression levels in unstressed hearts (Griffiths et al. 2003; Keane et al. 1999; Kim et al. 1999). Global knockouts for c-Cbl are viable and show no obvious abnormalities (Griffiths et al. 2003). Nevertheless, newer research unveiled that c-Cbl exhibits important functions during ischemia-reperfusion injury (Rafiq et al. 2014), in a model for acute tissue injury (Rafiq et al. 2011), during pressure overload-induced hypertrophy (Balasubramanian et al. 2006), and in obesity and diabetes (Gupte and Mora 2006). In most cases, receptor tyrosine kinases, such as the epidermal growth factor receptor (EGFR) (Levkowitz et al. 1999), focal adhesion kinase (FAK) (Rafiq et al. 2011), or members of the

neuregulin receptor kinase family (ErbB) (Waterman and Yarden 2001), have been identified as targets for c-Cbl's E3-ubiquitin ligase activity. Intriguingly, ubiquitylation of receptor kinases by c-Cbl may be dependent on the activation of these tyrosine kinases. Phosphorylation of specific tyrosine residues within c-Cbl was found to be absolutely necessary for its ubiquitin ligase activity (Levkowitz et al. 1999) and may therefore represent a negative feedback to suppress receptor function. Ubiquitylation of receptor kinases results in their degradation either through the 26S proteasome (FAK, EGFR) and/or the autophagy/lysosome system (EGFR, ErbB).

The role of several other RING-type E3-ligases or cullin-based substrate adaptors for cardiac development and maintenance is just emerging and needs to be further investigated. These include the APC (anaphase-promoting complex protein/cyclosome) (Chang et al. 2004; Yamada et al. 2011), mitsugumin/TRIM72 (MG53) (He et al. 2012; Kohr et al. 2014; Levy et al. 2013; Masumiya et al. 2009), RNF19 (Larsen et al. 2012), malin (DePaoli-Roach et al. 2010; Garyali et al. 2014), RNF156/Mgrn1 (Mahogunin ring finger 1) (Cota et al. 2006; Perez-Oliva et al. 2009), Trim32/HT2A (Cohen et al. 2014; Kudryashova et al. 2005; LaBeau-DiMenna et al. 2012) , Trim37 (Kallijarvi et al. 2005; Lipsanen-Nyman et al. 2003), Neurl2 (Ozz) (Nastasi et al. 2004), or myospryn (cardiomyopathy associated 5) (Durham et al. 2006; Nakagami et al. 2007).

12.3.4 HECT-Type E3-Ligases

In contrast to RING-type ligases that do not form a physical bond with ubiquitin during poly-ubiquitylation of their substrate proteins, HECT-type E3-ubiquitin ligases first transfer the ubiquitin from the loaded E2-conjugating enzyme onto their C-terminus via thioester bond formation/trans-thiolation (Verdecia et al. 2003). A model for the mechanism of these E3-ligases indicates how they are able promote the formation of poly-ubiquitin chains. In these models, a newly loaded E2-conjugating enzyme may enter its binding site, permitting the transfer of another ubiquitin onto the existing ubiquitin already attached to the E3-ligase C-terminus. Once HECT-type enzymes are loaded with mono- or poly-ubiquitin chains, they transfer these onto a lysine residue in the substrate protein (Fig. 12.5).

12.3.4.1 Nedd4 E3-Ligases

The E3-ligases family of Nedd4-proteins contains nine members: Nedd4-1, Nedd4-2, WWP1, WWP2, Smurf1, Smurf2, ITCH, NEDL1, and NEDL2 (An et al. 2014). All NEDD4 E3-ligases harbor an N-terminal C2 domain that enables the protein to interact with phospholipids in a calcium-dependent manner and targets this class of ligases to the membrane. The centrally located WW domains serve important functions for the binding of substrates, by interacting with the proline-rich motifs

PPXY or LPXY in target proteins. The C-terminal HECT domain fulfills the E3-ligase functionality and contains the catalytic cysteine residue required for attachment of ubiquitin. There is growing evidence that the activity of Nedd4 E3-ligases may be modulated by kinases (An et al. 2014) and Nedd4 family-interacting proteins (NDFIP) (Mund and Pelham 2009).

Cardiac substrates of Nedd4-2 include the voltage-gated potassium channels KCNQ1 or hERG1/KCNH2 (Cui and Zhang 2013; Rougier et al. 2010), the voltage-gated sodium channel Nav1.5 (Rougier et al. 2005), the voltage-gated L-type calcium channel CaV1.2 (Kawaguchi et al. 2006), or Cx43 (Leykauf et al. 2006; Mollerup et al. 2011) (Table 12.5). The activity of Nedd4 E3-ligases and their modulation is important for hearts, as their ion-channel substrates play critical roles for the action potential and repolarization of the cardiomyocyte during contraction. Hence it is not surprising that Nedd4-ligases family members were implicated with cardiac hypertrophy (Rajagopalan et al. 2013) or dilated cardiomyopathy (Molina-Navarro et al. 2014). Indeed, knockouts for Nedd4-2 showed an imbalance in their salt regulation manifested by higher blood pressure and developed cardiac hypertrophy with depressed cardiac functions on a high salt diet (Shi et al. 2008). In addition, Nedd4 family members Smurf1 and Smurf2 may play critical roles for TGFbeta signaling in cardiac fibroblasts and were consequently found to be modulated in fibrosis (Cunnington et al. 2009; Wang et al. 2012).

Table 12.5 Select HECT- and U-box-type E3-ligases and their cardiac targets

E3-ligase	Select potential and confirmed substrates	Disease link
Nedd4 family (Nedd4-2)	KCNQ1 (Rougier et al. 2010) hERG1 (Cui and Zhang 2013) NaV1.5 (Rougier et al. 2005) CaV1.2 (Kawaguchi et al. 2006) Cx43 (Leykauf et al. 2006; Mollerup et al. 2011)	High blood pressure, cardiac hypertrophy (Rajagopalan et al. 2013; Shi et al. 2008) Dilated cardiomyopathy (Molina-Navarro et al. 2014) Fibrosis (Cunnington et al. 2009; Wang et al. 2012)
Ube3a/E6AP		Deregulated in cardiomyopathy (Balasubramanian et al. 2006; Zhang et al. 2013)
PRP19	p53 (Kleinridders et al. 2009)	Dilated cardiomyopathy (Kleinridders et al. 2009)
CHIP	p53 (Esser et al. 2005) Guanylyl cyclase (Xia et al. 2007) STK11 (Schisler et al. 2013)	Ischemia-reperfusion (Xu et al. 2013) Pressure overload-induced hypertrophy (Schisler et al. 2013)

12.3.4.2 Ube3a/E6AP

Little is known about the functions of this HECT-type E3-ligase for the heart. Ube3a was found to be deregulated in various forms of cardiomyopathies (Balasubramanian et al. 2006; Zhang et al. 2013) (Table 12.5). Similar to Nedd4-ligases, Ube3a activity may be regulated by protein kinases (Chan et al. 2013). Recent evidence suggests that Ube3a may be part of the ERAD and protein quality control system, as it is recruited to aggresomes and poly-ubiquitylates HSP70-bound misfolded proteins (Mishra et al. 2009).

12.3.5 U-Box-Type E3-Ligases

Six mammalian U-box-type E3-ligases have been reported in the literature. These are UBE4A/Ufd2b and Ube4B/Ufd2a, CHIP (carboxy terminus of Hsp70-interacting protein), Ubox5/Rnf37/KIAA0860, CYC4 [cyclophilin-like protein Cyp-60; also known as peptidyl-prolyl cis-trans isomerase-like 2 (PPIL2)], and PRP19/Pso4 homologue. While the domain layout of U-box-type E3-ligases is quite different among the members of this group, they all contain a conserved U-box domain (Hatakeyama et al. 2001). This type of domain is predicted to have a similar three-dimensional structure compared with RING domains, but lacks the metal-chelating histidine or cysteine residues required for zinc-ion coordination. Lack of these residues is, however, compensated by the appropriation of other residues in U-box-containing proteins, which stabilizes the domain and permits the interaction with the E2-conjugating enzyme (Aravind and Koonin 2000). Ube4a, PRP19, and CHIP are three U-box E3-ligases that have been shown to be important for the heart (Table 12.5).

12.3.5.1 Ube4A

Ube4a displays high expression levels in heart and skeletal muscles, where it localizes to the nucleus (Contino et al. 2004). Recently, several tissue-specific splice variants of Ube4a were identified, including one that is upregulated during cross-striated muscle development. While Ube4a was shown to interact with p97/VCP, a component of the ERAD system, the muscle-specific insertion specifically disrupts this interaction (Mammen et al. 2011).

12.3.5.2 PRP19

This U-box E3-ligase was shown to be essential for embryonic development, as knockout mice die at day 1.5 postfertilization. The conditional inactivation of

PRP19 in heart and skeletal muscles resulted in a severe case of dilated cardiomyopathy caused by increased cardiomyocyte apoptosis. Tissue-specific knockout mice displayed a 5 % survival rate after 28 days postnatal development. On a molecular level, PRP19 deletion was linked to increased p53 (Kleinridders et al. 2009), indicating important roles for this E3-ligase in the control of cellular p53 protein levels.

12.3.5.3 CHIP (C-Terminus of HSP70-Interacting Protein)

The chaperone-associated E3-ubiquitin ligase CHIP is best known to target p53 for poly-ubiquitylation and degradation through the 26S proteasome (Chao 2015; Esser et al. 2005) (Fig. 12.5). This feature is achieved with the help of HSP90 and HSP70, two cellular chaperones important for cardiac function. Similar to HSP70 (Paulsen et al. 2009), CHIP was found to have a myofibrillar localization in cardiomyocytes (Schisler et al. 2013). Deletion of CHIP in the heart does not result in a baseline phenotype. However, when hearts are challenged by ischemia-reperfusion, CHIP knockouts developed ventricular arrhythmias and displayed decreased survival rates (Zhang et al. 2005a). Analysis of heat-shock protein expression in hearts of these mice indicated that CHIP is required for maximal induction of HSP70 expression.

Transgenic mice for CHIP displayed increased cardiac performance and decreased angiotensin-II-induced fibrotic remodeling of the heart, indicating that this E3-ligase has beneficial effects to counteract cellular stresses (Yang et al. 2012). The same group of authors reported that positive effects of CHIP overexpression were also noted upon ischemia-reperfusion injury following myocardial infarction, including higher survival rates (Xu et al. 2013).

Other putative substrates for CHIP include soluble guanylyl cyclase (sGC) (Xia et al. 2007) and the serine/threonine protein kinase STK11/LKB1 (Schisler et al. 2013).

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