Assembly and maintenance of the sarcomere night and day

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The assembly of sarcomeric proteins into the highly organized structure of the sarcomere is an ordered and complex process involving an array of structural and associated proteins. The sarcomere has shown itself to be considerably more complex than ever envisaged and may be considered one of the most complex macromolecular assemblies in biology. Studies over the last decade have helped to put a new face on the sarcomere, and, as such, the sarcomere is being redefined as a dynamic network of proteins capable of generating force and signalling with other cellular compartments and metabolic enzymes capable of controlling many facets of striated myocyte biology.

1. Introduction

The assembly of contractile proteins into the remarkably regular structure of the sarcomere has been the central theme of the structure and organization of striated muscle, from the earliest observations of muscle structure through the first generation of microscopes. Composed of the contractile proteins myosin and actin that are associated with the generation of force and the thin filament proteins that fine-tune the force generation, the sarcomere is spatially organized by the sarcomere cytoskeleton. This structure plays a major role in sarcomere stabilization and provides the connection of the force generating units to the cell membrane. The notion that extracellular signals are transmitted to the sarcomere via activated cytoplasmic kinases/phosphatases is well established. A vast body of literature has coalesced to identify the sarcomeric proteins as targets for post-translation modification, which alter their function in response to changes in the extracellular environment. However, the concept that the sarcomere is not just a recipient of signalling input but actively takes part has grown over the last few years. Consequently, the sarcomere is being redefined as a dynamic network of proteins capable of generating force, signalling with other cellular compartments, and with metabolic enzymes capable of controlling many facets of striated myocyte biology.

The aim of this review is to present an overview of the literature highlighting some of the recent advances in sarcomere assembly and the evolving function of the sarcomere. Consequently, due to the limitations in space and scope we can focus on only a small fraction of the literature. Throughout, we will refer to many other scholarly review articles that have focused on the various topics covered, as a recommended source of reading material.

2. Assembly of the sarcomere

The sarcomere is defined as the region between two Z-lines, which in turn form the boundaries of each sarcomere. The actin filaments anchored into the Z-discs via their barbed ends are decorated with the regulatory proteins troponin and tropomyosin composing the thin filaments. Extending in both directions from the Z-line denote the I-band. Interdigitated with the thin filaments are the thick filaments, composed of myosin molecules arranged as bipolar filaments in the middle of the sarcomere creating the A-band. The thick filaments are held in place by a structure known as the M-band, which forms the central point in the entire structure. While the Z-line and the M-band orders the sarcomere in the transverse plane, a third structural protein titin connects these two components in the longitudinal plane. With the N-terminus of titin anchored into the Z-discs and C-terminus into the M-band proteins, it is thought that titin provides the blueprint for the length of the sarcomere. Force is generated by the attachment of the head region of the myosin molecules to the actin molecules and in an ATP-dependent manner; the filaments slide past one another producing shortening of the sarcomere. Thus, exemplified as a structure within a structure, the sarcomere represents force producing units coupled together, which all interface...
with the cytoskeletal structure to transmit force and bring about muscle contraction.

Sarcomerogenesis, the assembly of the sarcomeric proteins into the highly organized structure, is an ordered and complex process involving an array of structural and associated proteins during the formation of the myofibrils. A great deal of our understanding of how this process is orchestrated has been derived from *in vitro* studies of cardiomyocytes. Aims at defining the sequence of events involved in the remodelling and assembly of the myofibrils, which is in part an adaptation to most two-dimensional culture environments, these studies have provided insight into some of the earliest events in the assembly process.

It was initially proposed that a series of actin stress fibre-like cables served as a template for the elements necessary to form a myofibril. These cables coupled by the focal adhesions to the extracellular environment establish a polygonal morphology with the resulting myofibrils running at oblique angles. Unlike the highly organized structure *in situ*, it was thought that the loss of organization was due to reorganization of the intermediate filament protein desmin, which serves as a connecting network between the myofibrils maintaining the overall architecture. However, a recently identified protein obscurin may play a pivotal role in aligning the myofibrils as discussed later. Nevertheless, these types of studies have defined the different stages of myofibrillar assembly. It is generally accepted that the most mature myofibrils with the regular cross-striations are found in the perinuclear region of the cell whereas in the periphery of the cell, certain components of the Z-disc are organized while components of the thick filament are more diffuse. Thus, this is the transition between these two regions that represents the major site of assembly within the cell. Consequently, two predominant hypotheses were formed to explain the earliest steps of assembly taking place in this transition zone. The first being the assembly of independent subunits, whereby components of the I-Z-I bands assemble in regions separate from where the components of the thick filaments assemble. These partially assembled units are then joined together by titin promoting their assembly into a mature myofibril. The second being the formation of premyofibrils along the plasma membrane containing actin, α-actinin, nonmuscle myosin II, closely spaced Z-discs and no titin. With the addition of titin, the spacing between the Z-discs is increased so they are aligned to form Z-lines and nonmuscle myosin is replaced by muscle myosin. Finally, myosin binding protein C and the M-band proteins clamp and align the thick filaments in the A-band during the formation of a mature myofibril.

Supporting this series of events are data derived from embryonic myocytes expressing α-actinin fused to GFP. Tracking the incorporation of the tagged α-actinin into the structures of live cells demonstrated the fluorescent protein localized in small aggregates along with actin and co-localized with nonmuscle myosin II along the periphery of the cell. Thus, the analysis of myofibrillogenesis over several days, unlike previous studies using fixed and antibody stained cultured cells, suggests the existence of premyofibrils denoted by the presence of nonmuscle myosin II B represent the earliest assembly of sarcomeric proteins.

Knockout of the nonmuscle myosin II gene in mice results in numerous cardiac defects and death by embryonic day 15.8 Surprisingly, the absence of nonmuscle myosin II B in these mice resulted in myocyte enlargement, particularly in the transverse diameter with evidence of mild myofibrillar disarray. Analysis of the underlying mechanism leading to myocyte enlargement revealed a defect in cytokinesis with a higher incident of binucleation in myocytes, but no indication of defective myofibrillogenesis during embryonic development.9 Utilizing a transgenic approach to evaluate the impact of nonmuscle myosin II B gene dosage in the heart revealed that adult mice with low levels of nonmuscle myosin II B expression develop pathology earlier compared to mice with higher levels and that nonmuscle myosin II B localizes to the Z-lines in differentiated myocytes.10,11 Together, these data implicate the importance of nonmuscle myosin II B in cytokinesis during development and suggest a role in the mechanotransduction of cardiac myocytes, but do not support the need for nonmuscle myosin II B during myofibrillogenesis.

An underlying concern regarding the examination of myofibrillogenesis in cardiomyocytes isolated from embryonic or neonatal stages is the pre-existence of myofibrils in these cells. Thus, raising the question of whether myofibrillar assembly in these models faithfully recapitulates *de novo* assembly. Examination of myofibrillogenesis in explants of pre-cardiac mesoderm and in cells isolated from the posterior lateral blastoderm of the chick embryo, support many of the earlier observations made in cells derived from foetal and neonatal stages.12,13 Nonetheless, adaptation to a two-dimensional environment, reassembling disassembled myofibrils and recycling of pre-existing myofibrillar proteins are often cited as being limitations to the interpretation of the events taking place in these culture models. Even though, disassembly, reassembly and recycling of sarcomeres may be necessary in the more fluid context of embryonic and neonatal myocytes with the ongoing capacity to undergo cell division. This apparently does occur before cytokinesis by first disassembling the proteins associated with the Z-discs and thin filaments before thick filament components.14

Many of the observations made in cultures derived from embryonic tissue have shown similarities to those steps in myofibrillogenesis *in situ*, with the main differences being the absence of stress fibre cables and the existence of premyofibrils. Myofibrillogenesis in whole mount preparations from the developing chick heart appears to occur with the organization of α-actinin, the N-terminus of titin and actin into dense bodies along the cell membrane. These then detach from the membrane as titin unfolds and the C-terminus of titin becomes integrated into the thick filaments, a process mediated by the M-band protein myomesin.15 Somewhat similar to the assembly of independent subunits, this model proposes the formation of a sarcomeric cytoskeletal framework consisting of α-actinin, titin and myomesin as being the steps necessary for myofibrillogenesis. The members of this sarcomeric cytoskeletal framework have grown to include nebullet and obscurin as members of the muscle specific giant proteins, along with titin. One of the major challenges in understanding the hierarchy of assembly of the main structural proteins has been able to determine the regions/domains involved in the assembly process. However, a number of different approaches directed at disrupting protein function have started to address the role of some of these proteins in the assembly of the sarcomeres.
Titin is a central player in the assembly process. It is a multifunctional protein containing multiple binding sites for structural and signalling proteins, as well as an inherent kinase domain, and also serves as a molecular spring. The N-terminus of titin spans the Z-disc and contains several small motifs termed z-repeats of ~45 amino acids each, sandwiched between flanking regions containing Ig domains. Linking this region to GFP demonstrated that the z-repeats play the major role in targeting titin fragments to the Z-disc whereas over-expression of these z-repeat constructs produced a dominant-negative effect associated myofibrillar disassembly. Several lines of evidence indicate that this region of titin interacts with a network of both structural and nonstructural proteins in the Z-line, creating a complex involved in stabilization of the Z-disc and signalling in response to stretch. It has been proposed that the kinase domain in the C-terminus of titin phosphorylates telethonin/T-cap, which is part of the N-terminus complex, and may be involved in the control of myofibrillogenesis. Conditional deletion of the M-band exons (Mex1 and 2), which includes the kinase domain using Cre-lox recombinase technology, resulted in embryonic lethality when activated early in development and sarcomere disassembly when activated later in the developing heart. However, using constitutive knockout approach to investigate the role of the same region of titin still yielded an embryonic lethal phenotype with evidence of abnormal sarcomere thickness but normal sarcomere assembly. In contrast, a gene-targeting approach in mouse embryonic stem cells in which the kinase domain and the entire M-band region of titin was deleted demonstrated that myosin and other M-band proteins displaying a diffuse distribution as well as disruption of the Z-discs. These results show that integration of the M-band region of titin is required for myosin filament assembly, M-band formation and maturation of the Z-disc suggesting that the entire region must be intact for assembly of sarcomeres.

Obscurin, a giant sarcomeric protein (~800 kDa) initially identified as a Z-disc protein associated with titin, also extends into the M-band. The exact role obscurin plays in the assembly of myofibrils has recently been investigated. Immunolocalization places obscurin in the M-band of the newly formed sarcomeres with weak immunostaining associated with the early α-actinin-titin dense bodies at the periphery of the cell. Increased immunoactivity within the myofibrils coincided with the process of lateral alignment of myofibrillar bundles with obscurin co-localized to the M-bands and the Z-lines, suggesting that it may serve as a linking protein between myofibrils. Knocking down obscurin expression with siRNA resulted in impaired alignment of the myofibrils, with branching and bifurcation of the myofibrils. Additionally, the organization of titin and myosin were perturbed suggesting that obscurin is necessary for the stabilization and integration of myosin into myofibrils. Interestingly, it has been shown that the C-terminus of obscurin interacts with isoforms of ankyrin1 localized on the sarcoplasmic reticulum. Thus, obscurin may provide a structural link in the organization of the sarcoplasmic reticulum with respect to the myofibrils.

Nebulette (the cardiac homologue of nebulin) serves as a cross-linking protein binding to α-actinin through the Src homology domain at the C-terminus and actin through a series 35-residue repeats (nebulin repeats). Expression of the various regions of nebulin repeats to GFP creates a dominant-negative effect and was used to probe the role of nebulin in myofibril assembly. Disruption of the C-terminus of nebulin results in the disruption of the thin filament proteins without affecting the integrity of the Z-line and the thick filaments. Disruption of the repeat domain perturbed myofibrillogenesis with the myofibrillar proteins becoming entangled in a conglomerate of recombinant proteins, indicating that nebulin is an integral component of the sarcomeric cytoskeleton and may regulate actin filament assembly. Akin to the notion that titin serves as the ruler for sarcomere length, nebulin is proposed to serve as the ruler for actin filament length.

The M-band forms a series transverse lines in the centre of the sarcomere and ensures regular packaging of the myosin molecules. The M-band proteins (myomesin, M-protein) are related and share similar immunoglobulin-like (Ig) and fibronectin type III (Fn) domains. Myomesin plays an important role in myofibrillogenesis, organizing myosin during embryonic development whereas M-protein appears to be involved during postnatal development. Indeed the formation of the M-band is one of the last steps during sarcomerogenesis and a marker of mature sarcomeres. To date, genetic models lacking either of the M-band proteins have not been generated so the effects of the M-proteins on myofibrillogenesis and sarcomere structure are still unknown. However, the perception that this region is a static assembly point is changing due to increasing number of signalling proteins that appear to be localized to the M-band.

3. Anchoring the structure

Sarcomeres are connected in series within the myofibril at the Z-disc and bundling the myofibrils together aligns the sarcomeres in parallel longitudinally. The entire structure is anchored into the cell membrane across the lateral axis at the costameres and along the longitudinal axis at the intercalated discs. The complexity of these structures has been described elsewhere, but they represent the hubs via which mechanical forces are transduced. Proteins critical to the formation of these structures and anchoring of the myofibrils have been identified and their function examined. Below are just a few studies published over last few years that have helped define the necessity of many of these proteins in the assembly of the myofibrils.

N-RAP is an actin binding LIM protein implicated in the formation of the earliest myofibril precursors at the cell membrane. Expression of deletion mutants demonstrated that the super repeats region of N-RAP co-localized with α-actinin via its LIM domain during Z-body formation and may serve as a scaffold for actin polymerization during the formation of the I-Z-I complex. Knockdown of N-RAP expression using RNA interference supports the role and necessity of N-RAP in the assembly of the early precursors in myofibrillogenesis.

The junctional structures formed between cardiac myocytes, fascia adherens junctions, desmosomes and gap junctions all form the intercalated disks. It is proposed that the intercalated disks serve as the longitudinal stretch sensing structures. N-Cadherin belongs to a superfamily of calcium-dependent transmembrane adhesion proteins that mediates adhesion in the intercalated disks at the termini of cardiac myocytes. N-Cadherin co-localizes with α-actinin in the
peripheral Z-discs serving as an anchor for the myofibrils at cell–cell contacts. Conditional knockout of N-cadherin in the adult mouse heart results in compressed sarcomeres with wider Z-lines and a decreased sarcomere length, presumably reflecting a loss of myofibril anchorage at the plasma membrane. \(^{37}\)

The costameres are the rib-like structures that flank the Z-lines and function by linking the cytoskeleton to the cell membrane and extracellular matrix (ECM). Three macromolecular complexes, the integrin-based focal adhesions, the dystroglycan complex and spectrin–ankyrin membrane cytoskeleton, connect to the myofibrils through various linkage systems and serve as the lateral force sensing structures. Excision of the β1-integrin gene within the ventricles using a conditional knockout system resulted in an 80% reduction in expression but normal survival. Along with impaired cardiac function and a reduced ability to tolerate an increased haemodynamic load, there was evidence of impaired cardiac function and a reduced ability to tolerate an increased haemodynamic load, there was evidence of focal dissolution of the myofibrils in the mutant mice demonstrating that β1-integrin is an important mechano-transducer in cardiac myocytes. \(^{38}\)

### 4. Remodelling of the sarcomeres

Remodelling and growth of the sarcomeres occurs not only during development but also in response to a number of circumstances associated with pathology. The direction the myocyte grows has major consequences for the mechanical output of the heart. In pressure overload, the myocytes develop large cross-sectional area (concentric hypertrophy), whereas in response to volume overload, the myocytes elongate (eccentric hypertrophy). These two remodelling processes occur at the level of the sarcomere. Understanding the functional mechanisms of mechanical transduction at the cellular level is critical to understanding how external forces regulate the synthesis and assembly of new sarcomeres. In culture, it has long been noted that contractile activity and cell–cell contacts regulates myofibrillar organization and assembly. \(^{39,40}\) However, cells grown on two-dimensional flattened rigid surfaces develop a stellate shape with their myofibrils growing on a single planar level. Growing myocytes on distensible silicone membranes showed that myofibrillar assembly, organization and protein turnover could be effected by passive stretch in a directional manner. Sustained uniaxial stretching of aligned myocytes along the long axis retained their linear array, but did not increase protein accumulation. Uniaxial stretch across the short axis led to branching, a loss of alignment along the long axis retained their linear array, but did not increase protein accumulation. Uniaxial stretch across the short axis led to branching, a loss of alignment along the long axis preserved sarcomeric periodicity. Conversely, stretch transverse to the longitudinal axis resulted in a loss of striations. \(^{44}\) While these data support the notion that directional strain is differentially transduced at the level of the sarcomere and parallel remodelling involves sarcomere rearrangement and increased protein accumulation, they do not provide evidence of sarcomeric remodelling in series. This may be a limitation of the model since it did not provide for attachment of the myocytes along the sides of the cells since they were seeded on the top of narrow channels. Growing cardiomyocytes on a microfabricated peg-and-groove silicone surface permitted cells to align along the bottom of the grove, as well as make contact along the bottom, sides and with neighbouring cells. This model was used to test the impact of uniaxial strain along the longitudinal axis on sarcomere structure and remodelling. In these cells, the sarcomeres of the aligned cardiomyocytes lengthened almost immediately with the onset of strain, but then recovered to their original resting length by 4 h. From these data, it was noted that approximately one sarcomere per hour could be added to the pre-existing myofibrils to reset the resting sarcomere length. Translational inhibition prevented recovery of resting sarcomere length suggesting that protein synthesis is a requirement in the recovery process of sarcomere length. \(^{45}\) In the same model, examination of where the new sarcomeres were being incorporated showed a disruption of the sarcomeric patterning along the length of the myofibrils and intense α-actinin and N-cadherin staining at the ends of the myocytes. Thus, it appears that serial remodelling occurs by insertion of new sarcomeres throughout the cell length and at the ends of the myofibrils, along with the remodelling of the intercalated discs. \(^{46}\)

In the same context, tubular scaffolds composed of ECM proteins are being used to construct three-dimensional models to examine embryonic cardiac myocyte development. In this model, embryonic ventricular myocytes undergo the transition from a hyperplastic to a hypertrophic phenotype. Interestingly, the cells display the different stages of myofibrillogenesis over time with the earliest cells having unorganized myofibrils located in the periphery, which become organized with aligned Z-discs over time, along with the formation of cell–cell connections. \(^{47}\) Further development and exploitation of these and similar models may push the balance back in favour of using these types of systems to probe the function of the critical components involved in the mechanical transduction and remodelling processes. Coupled with techniques such as RNA interference and expression of epitope tagged truncated or domains of proteins, provide an impressive array of applications, tissue culture systems with different surface topographies may provide further insight into how the three-dimensional mechanical forces a myocyte experiences are sensed and transduced in the remodelling of the sarcomeres in vivo.

Micropatterned silicone membranes fabricated with linear arrays of ECM proteins on the surface permit growth of aligned cardiomyocytes forming cell–cell contacts. These myocytes develop an in vivo-like morphology with the formation of intercalated discs and regular sarcomeric pattern. Bi-axial stretch achieved by increasing the strain relative to the aligned myocytes showed that stretch parallel to the longitudinal axis of the myofibrils resulted in preserved sarcomeric periodicity. Conversely, stretch transverse to the longitudinal axis resulted in a loss of striations. \(^{44}\) While these data support the notion that directional strain is differentially transduced at the level of the sarcomere and parallel remodelling involves sarcomere rearrangement and increased protein accumulation, they do not provide evidence of sarcomeric remodelling in series. This may be a limitation of the model since it did not provide for attachment of the myocytes along the sides of the cells since they were seeded on the top of narrow channels. Growing cardiomyocytes on a microfabricated peg-and-groove silicone surface permitted cells to align along the bottom of the grove, as well as make contact along the bottom, sides and with neighbouring cells. This model was used to test the impact of uniaxial strain along the longitudinal axis on sarcomere structure and remodelling. In these cells, the sarcomeres of the aligned cardiomyocytes lengthened almost immediately with the onset of strain, but then recovered to their original resting length by 4 h. From these data, it was noted that approximately one sarcomere per hour could be added to the pre-existing myofibrils to reset the resting sarcomere length. Translational inhibition prevented recovery of resting sarcomere length suggesting that protein synthesis is a requirement in the recovery process of sarcomere length. \(^{45}\) In the same model, examination of where the new sarcomeres were being incorporated showed a disruption of the sarcomeric patterning along the length of the myofibrils and intense α-actinin and N-cadherin staining at the ends of the myocytes. Thus, it appears that serial remodelling occurs by insertion of new sarcomeres throughout the cell length and at the ends of the myofibrils, along with the remodelling of the intercalated discs. \(^{46}\)
approaches to probe gain or loss of function in response to strain at the cellular level.

5. Integration and exchange

Once the sarcomere is established, integration and exchange of new proteins into the structure occurs continually. The half-life of the contractile proteins is quite long, the half-life of myosin in the heart is \( \sim 15 \) days. The turnover rate of the myofibrillar proteins varies; the subunits of troponin have a turnover rate of 3–5 days under steady-state conditions, suggesting there is an unassembled pool of myofibrillar proteins available for exchange. Incorporation of newly synthesized epitope-tagged thin filament proteins into the myofilaments indicates that the site of incorporation and rate of incorporation differs between proteins. Studies conducted on targeting of sarcomeric proteins indicate that the information required for targeting resides within the protein domains. However, little is known about the fate of newly synthesized sarcomeric proteins and the mechanism of incorporation into the sarcomere. It has been shown that folding of a chimeric myosin-GFP construct can be improved in the presence of muscle cell extract, suggesting the involvement of muscle factors. Immunolocalization of the myosin-GFP folding intermediates with conformational sensitive antibodies showed that early myosin intermediates co-localize with the protein chaperones Hsc70 and Hsp90 in C2C12 cells. Incubation with an antibiotic that specifically binds the Hsp90 ATPase pocket and traps substrates in the chaperone complex, led to an accumulation of myosin intermediates and prevented assembly into the myofibrils. Both Hsc70 and Hsp90 are ubiquitous but it is thought that muscle proteins maybe efficiently targeted by muscle specific adaptors. In Caenorhabditis elegans, UNC-45 is a member of the UCS-domain containing family, a region that interacts with myosin in fungi, and is essential for normal muscle development. UNC-45 binds directly to myosin preventing aggregation and also binds to Hsp90 through its tetratricopeptide repeat domain in vitro. Two UNC-45 homologs exist in mammals, the general cell homolog expressed in all tissues (unc-45A) and the striated muscle homolog (unc-45B). Knocking down the unc-45b gene in zebrafish by antisense morpholino oligonucleotide injection results in both skeletal and cardiac abnormalities. Injected fish lack myosin in the sarcomeres of their trunk muscle and also fail to undergo proper cardiac morphogenesis, supporting the role of UNC-45 in sarcomere maintenance downstream of muscle differentiation in vertebrate systems. A recently identified point mutation in the steif/unc-45b gene leads to truncation of the USC-domain and causes a loss of organized myofibrils in both skeletal and cardiac muscle. Gene rescue approaches restore myofibrillar organization confirming that the point mutation in the steif/unc-45b gene was responsible for the phenotype. Hsp90 is an interacting partner and knocking down Hsp90 expression phenocopies the steif/unc-45b mutant phenotype in skeletal muscle but surprisingly not in the heart. These data support the role of a chaperone complex involved in the assembly of the contractile apparatus in vertebrate skeletal muscle but the mechanism and its role in cardiac muscle are yet to be elucidated.

6. Signalling

It is now appreciated that the sarcomere is not just a force-producing unit, but is also organized into subcompartments with specific signalling functions. These signalling functions within the sarcomeres appear to play a role in the assembly, remodelling and mechanotransduction either from the external environment or within the sarcomeres. A basic model of how these processes are regulated places the Z-discs and associated proteins as being a nodal point within the sarcomere for the integration of many signalling functions. With the explosion of data regarding the integration of the mechanotransduction signals occurring at the Z-disc and more recently at the M-band, it is not too surprising that the myofibrils appear to house proteins involved in regulating the daily activity and metabolism of the cardiac myocyte. Described below is some of the recent literature that implicates the sarcomere as central in the regulation of the daily metabolic activity.

7. The myofilaments and potential circadian regulation

The diurnal alternation between light and dark influences the behaviour and activities of all forms of life. This daily fluctuation in physical exertion influences the activity and function of many internal organs, including the heart. In the human myocardium, heart rate and blood pressure vary depending on the time of day. This variability in heart function is thought to be regulated by an internal and external ‘clock’ that enables the organ to meet changes in demand. Other work suggests that the heart can also alter its efficiency, contractile performance, carbohydrate oxidation, oxygen consumption and the expression of metabolic genes accordingly. These changes are thought to be under the influence of circadian proteins by a mechanism that is still poorly understood. The heart expresses a number of circadian genes including Clock, BMAL1 (brain and muscle ARNT-like protein1), cryptochrome (CRY) and the period genes (PER1, PER2 and PER3). These genes are thought to regulate numerous cellular processes, including metabolism. Clock protein forms a heterodimer with BMAL1 which translocates to the nucleus, where it increases the transcription of target genes including PER and CRY genes. PER and CRY proteins in turn dimerize and enter the nucleus, repressing the activities of the Clock/BMAL1 complex. As a result, these proteins form a cyclic feedback loop that regulates gene expression.

Recent work has shown that Clock protein is found within the myofilament Z-disc, co-localizing with \( \alpha \)-actinin as shown in Figure 1. Clock protein adds to the growing number of proteins being discovered within this complex region of the myofilaments. Nuclear translocation of Clock increases in response to phenylephrine treatment but decreases with either the calcium channel blocker verapamil or butanedione monoxime, which inhibits myofilament cross-bridge cycling. These data show that myocyte contractility can directly alter the subcellular distribution of Clock protein. It remains to be determined if contractile activity influences other circadian proteins. The subcellular distribution of other circadian proteins and their function in relation to Clock also needs to be determined since these proteins function in a coordinated manner.
The Clock/Bmal1 complex forms part of the positive component of the circadian cycle in cells and its translocation to the nucleus in response to increased cross-bridge cycling would be expected to result in the activation of numerous genes.

The complex can directly influence gene expression through the remodelling of chromatin as a result of its histone acetyl transferase activity. \(^{66,67}\) Work is ongoing to determine the genes that are under the direct regulation of the Clock/Bmal complex, however initial reports suggest that there may be many hundreds. Many of these target genes regulate the expression of proteins associated with metabolism including glucose transporter proteins. With Clock protein localized within the myofilaments, this raises a fascinating possibility that the circadian complex may provide a direct link between the sensing of energy expenditure from within the myofilaments and the regulation of energy supply. A proposed model of this activity is shown in Figure 2.

There is cross talk between the circadian protein network and other signalling pathways. These clock proteins are regulated by phosphorylation, through a growing number of enzymes including the phosphatase PP1, protein kinase A, \(\text{Ca}^{2+}/\text{calmodulin-dependent kinase II}, \text{Ca}^{2+}\)-dependent protein kinase C and the extracellular signal-regulated kinase. \(^{68–70}\) Many questions still remain though. Is an alteration in myocardial circadian protein regulation or function implicated in cardiac disease and how does the environment alter their activity? Certainly cardiac hypertrophy induced by aortic constriction has been shown to blunt the diurnal variations in heart efficiency and function. \(^{60}\) This may prevent the heart from adequately adjusting to the diurnal variation in energy demand leading to increased myocardial stress. Disruption of the normal circadian rhythm can also adversely affect cardiac function. Sleep deprivation for example increases C-reactive protein, a predictor of future cardiovascular morbidity. \(^{71}\) Sleep-related breathing disorder, which interrupts normal sleep, blunts maximum exercise capacity and heart rate reactivity in subjects. \(^{72}\) A more recent study showed that in aortic-banded animals, circadian gene expression and hypertrophic genes were abnormally expressed when the animals were forced in to a disruptive 20 h rhythm. These aberrant changes could be restored when the normal 24 h cycle was resumed. \(^{73}\)

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**Figure 1** Clock and \(\alpha\)-actinin staining in cultured rat neonatal cardiac myocytes. (A) Immunostained image of the Z-disc protein \(\alpha\)-actinin (in green). (B) Immunostained image of the circadian protein Clock (in red) in the same cells. (C) The combined images of Clock and \(\alpha\)-actinin along with nuclei stained with Dapi (in blue). The two proteins co-localize at the Z-disc.

**Figure 2** Diagram showing a possible role of Clock protein in cardiac myocytes. (Part 1) Contractile activity and energy usage within the myofilaments leads to nuclear translocation of Clock protein. (Part 2) Clock protein in the nucleus activates the transcription of genes that regulate metabolism including the glucose transporters. (Part 3) Increased glucose transporters lead to increased glucose uptake by myocytes, thereby increasing the energy supply.
The myofilaments require maintenance, and circadian regulation may enable 'repairs' to occur during periods of reduced activity, when both heart rate and blood pressure are at their lowest. Unlike skeletal muscle, which stops contracting during sleep, the heart needs to maintain its activity continually. A plausible hypothesis is that myofilament turnover, maintenance and energy replenishment all occur during periods of reduced activity, as occurs during sleep. A number of components of the Ubiquitin complex responsible for protein turnover are also circadianly regulated. Anything that interferes with this circadian activity may prevent the myocardium from adapting most efficiently to its environment. A growing body of evidence suggests that there is a direct link between circadian activity and cardiac function with the possibility that this may be mediated at least in part through the myofilaments.

8. The myofilaments and regulation of metabolism

As mentioned previously, the myofilaments may regulate myocyte metabolism indirectly through circadian proteins like Clock. However, the myofilaments may also regulate myocyte metabolism more directly through their interaction with mitochondria. These organelles are usually more numerous in regions with the greatest energy demand and the myofilaments are among the highest source of myocyte energy expenditure. Muscle LIM protein (MLP) is a mechanosensor in the myofilament Z-disc and has been shown to interact directly with metabolic enzymes including d-lactate dehydrogenase. In MLP knockout mice, the distribution of mitochondria around the myofilaments is much reduced so that they no longer accumulate in the regions of high-energy demand. This suggests that cytoskeletal MLP may be part of an energy sensing mechanism allowing mitochondria to concentrate in the regions of ATP-dependent cross-bridge cycling. In myocytes, the intermediate filament protein desmin is associated with the myofilaments and connects the Z-disc with the sarcolemma. In desmin null mice, there is an abnormal proliferation of mitochondria and an activation of the apoptotic pathway in cardiac muscle following increased workload. However, over-expression of the anti-apoptotic protein BCL-2 was able to rescue this abnormality. The assembly and function of desmin appears to be regulated through phosphorylation by protein kinase C. These studies all strongly suggest a functional signalling link between the myofilaments and metabolism in the myocardium.

9. Understanding the sarcomere in the 21st century

The sarcomere has shown itself to be considerably more complex than was ever envisaged and may well be considered one of the most complex macromolecular assemblies in biology. Understanding this structure will remain a formidable challenge to future researchers and it seems there still is a tremendous amount of work to be done to decipher the interactions between the proteins of this massive complex. Combinations of new approaches and models have provided insights into the role and necessity of the major proteins, particularly from the structural perspective. In the context of the present intense research effort directed at cell-based approaches to repair the damaged myocardium, sarcomere assembly appears to be at the very centre of the issue of making a new myocyte. Understanding the dynamics of maintaining and remodelling the structure once built in both physiologic and pathologic contexts is offering new challenges. Other model organisms, novel in vitro models, coupled with new microscopic techniques for the analysis of real-time cellular processes will undoubtedly help unlock some of the dynamic aspects of sarcomerogenesis. Fluorescent speckle microscopy and spatiotemporal image correlation spectroscopy have been used to measure the kinetics (velocity and direction) of epitope-tagged cytoskeletal proteins in the assembly and disassembly of the cytoskeleton, plus its interactions with the focal adhesion complexes in live cells. Utilizing these technologies to examine sarcomeric protein dynamics is probably under development and will undoubtedly add another new facet to sarcomere assembly. This structure is constantly being built and remodelled and is in constant need of maintenance. It cannot be taken off-line for repair and now it appears that it senses when to 'wake up and feed'.

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