20 January 2022 Joe Jean Dipak Jushuo

Inhibitors of the Ubiquitin Proteasome System block myofibril assembly in cardiomyocytes derived from chick embryos and human pluripotent stem cells

Jushuo Wang 1, Yingli Fan 1, Chenyan Wang 3, Syamalima Dube 2, Bernard J. Poiesz 2, Dipak K. Dube 2, Zhen Ma 3, Jean M. Sanger 1, and Joseph W. Sanger 1

Department of Cell and Developmental Biology 1, Department of Medicine 2, SUNY Upstate Medical University, Syracuse, NY; Department of Biomedical & Chemical Engineering, The BioInspired Institute for Materials and Living Systems 3, Syracuse University, Syracuse, NY

Correspondence: Dr. Joseph W. Sanger, Department of Cell and Developmental Biology, SUNY Upstate Medical University, Syracuse, NY 13210. Email: sangerjo@upstate.edu

Emails of authors

wangj@upstate.edu
fany@upstate.edu
cwang40@syr.edu
DubeS@upstate.edu
PoieszB@upstate.edu
DubeD@upstate.edu
Zma112@syr.edu
sangerjm@upstate.edu
sangerjo@upstate.edu

Abstract (250 words allowed; Current # = 244)

Details of sarcomeric protein assembly during *de novo* myofibril formation closely resemble myofibrillogenesis in skeletal and cardiac myocytes in birds, rodents and zebrafish. The arrangement of proteins during myofibrillogenesis follows a three-step process: beginning with premyofibrils, followed by nascent myofibrils, and concluding with mature myofibrils (reviewed in Sanger et al., 2017). Our aim is to determine if the same pathway is followed in human cardiomyocytes derived from human inducible pluripotent stem cells. We found that the human cardiomyocytes developed patterns of protein organization identical to the three-step series seen in the model organisms cited above. Further experiments showed that myofibril assembly can be blocked at the nascent myofibril by five different inhibitors of the Ubiquitin Proteasome System (UPS) stage in both avian and human cardiomyocytes. With the exception of Carfilzomib, removal of the UPS inhibitors allows nascent myofibrils to proceed to mature myofibrils. Some proteasomal inhibitors, such as Bortezomib and Carfilzomib, used to treat multiple myeloma patients, have off-target effects of damage to hearts in three to six percent of these patients. These cardiovascular adverse events may result from prevention of mature myofibril formation in the cardiomyocytes. In summary, our results support a common three-step model for the formation of myofibrils ranging from avian to human cardiomyocytes. The Ubiquitin Proteasome System is required for progression from nascent myofibrils to mature myofibrils. Our experiments suggest a possible explanation for the cardiac and skeletal muscle off-target effects reported in multiple myeloma patients treated with proteasome inhibitors.

KEYWORDS

Myofibrillogenesis, premyofibril, nascent myofibril, mature myofibril, Ubiquitin Proteasome System, proteasomes, multiple myeloma

Introduction

The repeating patterns of sarcomeres in mature myofibrils provide the foundation for contractile activity in vertebrate cardiac muscles. Despite changes in the size and contractile properties of the muscle cells during growth, aging and illness, the repeating patterns of sarcomeres persists in myofibrils for a lifetime of contractions. Understanding the details of the process of myofibril assembly is a major challenge in the muscle field (Piccirillo et al, 2014; Sanger et al., 2017). In previous studies, Wide Field Fluorescence, Confocal and Deconvolution microscopies were used to document the organization of key sarcomeric proteins in contractile units during myofibrillogenesis in cardiac and skeletal muscle cells from different vertebrate model organisms. (Rhee et al., 1994; Du et al., 2003, 2008, Sanger et al., 2017, White et al., 2014, 2018). Observations of the assembly process in fixed and living cells led to the proposal that assembly proceeded through a three-step progression in structural organization, beginning with premyofibrils, the earliest precursor structures, followed by nascent myofibrils, and terminating in mature myofibrils (Figure 1) (reviewed in Sanger et al., 2017; White et al., 2018). Assembly begins at the spreading edges of cardiomyocytes with stepwise formation of premyofibrils composed of small bands of nonmuscle myosin II interdigitating with actin filaments tethered to α-actinin containing Z-Bodies. Transition to nascent myofibril formation, occurs with the recruitment of titin, and overlapping filaments of muscle myosin II to interactions with the premyofibril core. Mature myofibrils form with aligned Z-bodies coalesced to form Z-Bands, the alignment of overlapping muscle myosin II filaments forming the parallel arrangement of muscle myosin filaments in A-Bands, and the addition of myosin-binding proteins (Myosin Binding Protein C, and myomesin), and the concurrent loss of nonmuscle myosin II (reviewed in Sanger et al., 2017; White et al., 2018).

The separation between the aligned Z-Bodies in premyofibrils and nascent myofibrils ranged from 0.3 to 1.4 μm (Sanger et al., 1984; Rhee et al., 1994; Dabiri et al., 1997). These minisarcomeres grow to the sarcomere lengths typical of mature myofibrils, i.e., 1.8 to 2.5 μm sarcomeric spacings between the Z-Bands (Sanger et al,

1986 a, b, 1989; Rhee et al., 1994; Dabiri et al.,1997). Fluorescence Recovery After Photobleaching (FRAP) analysis showed that exchange of sarcomeric proteins between proteins inthe cytoplasmic pool and the sarcomere subunits in each myofibril stage, is more dynamic in the early stages myofibril assembly (premyofibril and nascent myofibril stages) than in the mature myofibrils (Wang et al., 2005 a, 2011,2020), an indication that additional proteins inserted in mature myofibrils result in a more stable protein complex. Sensitized Emission Floruescence Resonance Energy Transfer (SE-FRET) microscopy also demonstrated changes in some Z-band proteins as Z-Bodies of premyofibrils fused to form the Z-Bands of mature myofibrils, during the stepwise model for the assembly of myofibrils (Stout et al., 2008).

This manuscript describes cardiomyoctes derived from human induced Pluripotent Stem Cells (hiPSCs) forming myofibrils in a three-step process, as reviewed above: premyofibrils to nascent myofibrils, ending with mature myofibrils. An unexpected discovery of our study is the role inhibitors of the Ubiquitin Proteasome System (UPS) play in blocking myofibrillogenesis at the nascent myofibril stages in both embryonic chick cardiomyocytes, and hiPSC-derived cardiomyocytes. Removal of four of five different UPS inhibitors tested, allowed myofibrillogenesis to continue to the mature myofibril stage. These inhibitor effects during *de novo* myofibrillogensis may explain the off-target effects on hearts reported in 3% to 6% of human patients treated with proteasome inhibitors for multiple myelomas (Bringhen et al., 2018; Guglielmi et al., 2017; Waxman et al., 2018).

RESULTS

Similarity of myofibrils in human and embryonic chick cardiomyocytes.

Cardiomyocytes derived from human induced pluripotent stem cells (hiPSC) (Lian et al, 2012; Sun et al., 2020), and cardiomyocytes, isolated from embryonic chick hearts, were grown on gelatin-coated dishes (hiPSC) or collagen-coated dishes (chick) respectively, where they attached and spread for several days in culture before fixation and staining. Muscle-specific α-actinin antibodies and phalloidin were used to provide a view of F-actin fibers, banded with α-actinin, that appear when myofibrils first assemble in the two types of cardiomyocytes (Figure 2, human, a, b, and embryonic chick c, d). α-actinin was organized in Z-bands of the mature myofibrils that were concentrated in the middle of both human and chick cardiomyocytes (long arrows, Figure 2 a, c), and in small concentrations in Z-Bodies (arrowheads) localized around the outer rim of the cells (arrowheads, Figure 2, a, c). Phalloidin staining revealed actin bands along the mature myofibrils in both kinds of cardiomyocytes (long arrows, Figure 2, b, d), as well as thin actin fibrils that surrounded the perimeters of both cardiomyocytes (arrowheads, Figure 2, b, d) demonstrating the close similarities of actin and α-actinin distributions in human and embryonic chick cardiomyocytes.

Disposition of two myosins II in human cardiomyocytes during myofibrillogenesis.

Myofibril formation, observed in cultures of a variety of embryonic skeletal and cardiac muscle cells, proceeds in a sequence of three steps that begin with an initial premyofibril stage, followed by nascent myofibrils, and ending with mature myofibrils (Figure 1). Premyofibrils have only one type of myosin II: nonmuscle myosin IIB (Rhee et al., 1994; White et al., 2018). Nascent myofibrils in the second step, have two different types of myosin II: nonmuscle myosin and muscle myosin. In the third step that concludes with mature myofibrils, just one type of myosin II, muscle myosin II, is present (Rhee et al., 1994; Dabiri et al. 1997, Du et al., 2003, 2008; Sanger et al., 2017, Wang et al., 2018**;** White et al., 2018).

To determine if the patterns of myosin II in hiPSC-CM are similar to those in embryonic chick cardiomyocytes (Rhee et al., 1994; Wang et al., 2018), we first transfected the human cardiomyocytes with mNeonGreen plasmids encoding myosin light chain 2, specific for muscle myosin II (muscle light chain 2 (MLC-2); Figure 3 a). The human cardiomyocytes expressing mNG-MLC2 were then fixed, permeabilized, and stained with antibodies directed against nonmuscle myosin IIB (Figure 3 b), and exposed to phalloidin to reveal the positions of F-actin in the cardiomyocytes (Figure 3 c). The positions of the premyofibrils (arrowheads), nascent myofibrils (short arrows), and mature myofibrils (long arrows) are marked in the same positions and focal planes in each part of Figure 3 (a to d). These transfected and doubly stained cardiomyocytes also showed three different patterns of localization of the two myosins II (MLC-2 and NMIIB) during the myofibril assembly process in the cells (Figure 3 a, b, d). The premyofibrils contained only one type of myosin II, nonmuscle myosin IIB, localized around the periphery of the human cells (arrowheads in Figure 3 b) separated from the identical positions of muscle myosin II (arrowheads in Figure 3 a). The nonmuscle myosin II colocalized with thin actin fibers (Figure 3 b, c). The mature myofibrils (positions marked by long arrows in Figure 3 a, b) contained just one type of myosin II, muscle myosin II that was uniformly aligned and organized in muscle myosin II filaments of the A-Bands. The A-Bands of the mature myofibrils (long arrows in Figure 3 a) were localized midway between the Z-Bands of the mature myofibrils marked by long arrows in Figure 3 a, c). The second stage of myofibrillogenesis, i.e., nascent myofibrils were defined by Rhee et al. (1994) in embryonic chick cardiomyocytes as containing two types of myosin II, i.e., nonmuscle myosin II and muscle myosin II. The transfected human cardiomyocytes expressing mNeon Green muscle myosin light chain-2 were fixed and stained with nonmuscle myosin IIB antibodies to detect fibers that exhibited colocalized muscle myosin II in positions of nascent myofibrils marked by short arrows in Figure 3 a, green in d, and nonmuscle myosin II proteins **in** Figure 3 b, red in d. The staining of the human cardiomyocytes with Alexa Fluor 633 labeled phalloidin reveals the distribution of F-actin in the three different stages of the fibrils during myofibrillogenesis (Figure 3 c). The F-actin stain reveals the striated patterns of

actin in the sarcomeres of the mature myofibrils whose positions are indicated by long arrows in Figure 3 c. The locations of the nascent myofibrils, indicated by short arrows in Figure 3 a to d are characterized by the presence of two different types of myosins II in some of the nonstriated F-actin fibers (Figure 3 c, d). The positions of the nascent myofibrils indicated by short arrows in Figure 3 a to d are located between the peripheral premyofibrils (arrowheads in Figure 3 a to d) and the central mature myofibrils (long arrows in Figure 3 a to d). These results support the three-step model for myofibrillogenesis illustrated in Figure 1 for human cardiomyocytes.

Embryonic chick cardiomyocytes contain nonmuscle myosin IIB, but not the IIA isoform, while their cardiac fibroblasts contain both the IIA and IIB isoforms (Rhee et al., 1994). HiPSC-cardiomyocytes are known to contain the IIA and IIB isoforms of nonmuscle myosins (Chopra et al., 2018). The human cardiomyocytes in our cultures stained with both antibodies directed against the IIA and IIB isoforms, but not with the IIC isoform of nonmuscle myosin IIs (data not shown). The localization patterns of the IIA and IIB isoforms in the human cardiomyocytes were identical. The hiPSCs stained with the IIA and IIB antibody isoforms, but not with the IIC isoform (data not shown). Interestingly, the stress fibers in the few stromal cells or fibroblasts derived from the hiPSCs in the cultures of hiPSC-human cardiomyocytes stained with all three isoforms of the nonmuscle myosin IIs (data not shown).

Detection of short muscle myosin II filaments in spreading edges of the human cardiomyocytes.

It had been a long held dogma that in vertebrate muscles myosin II filaments appeared at their final length of some 1.5 microns (reviewed in Sanger et al., 2017). However, Du et al. (2008) reported in avian in situ hearts, and in cultured isolated avian cardiomyocytes that short muscle myosin II filaments were detected in early hearts that were about half the lengths of the 1.6 micron long thick myosin II filaments in the mature myofibrils. Sanger et al. (2009) reported a similar finding in the nascent myofibrils in the skeletal muscle cells intact tails of embryonic zebrafish. In our studies of the human cardiomyocytes, the muscle myosin II antibody F59 stains only the region of the myosin

filaments that bear cross bridges (Miller and Stockdale. 1985). Muscle myosin filaments are able to be identified by the staining of the ends of the muscle myosin II filaments, and the nonstaining of the middle of their elongated stained filaments (Miller and Stockdale, 1985; Du et al., 2008; Sanger et al., 2009). Figure 4 (a to d) illustrates an example of a human cardiomyocyte fixed and stained with a muscle specific myosin II antibody (F59) and stained with the F-actin probe phalloidin after spreading in culture for several days. The outer edge of the cell in Figure 4 (c, d) is marked by the phalloidin where F-actin is present (arrow in d), and where muscle myosin is absent (arrow in c). Thus, these actin fibers in (b) with the absence of muscle myosin II (a) are premyofibrils as illustrated by Figure 1, and defined by Rhee et al. (1994). Short muscle myosin filaments are first detected away from the edge of the cell and away for the mature myofibrils near the middle of the cell (Figure 4 c). This would be the neighborhood of the nascent myofibrils (Figures 1, 3 a, b). Several of the short myosin II filaments follow the peripheral course of the actin fibers in Figure 4 c, d; numbers 2 to 4; 6 to 8. Two short myosin filaments, (Figure 4 c, d; # 1 and 5) are almost at right angles to the two nearest actin fibers. Muscle myosin II filaments were measured in the regions of the nascent myofibrils and in the mature myofibril regions of the spreading cardiomyocytes (Figure 5). The average lengths of muscle myosin II filaments in the nascent myofibrils regions are 0.80 microns +/- 0.16 (n=144),while the average lengths of A-Bands in mature myofibrils are 1.45 μm +/- 0.19 (n=116).

Titin organization in the assembly of mature myofibrils in hiPSC-cardiomyocytes in tissue culture.

Titin is first detected in nascent myofibrils (Figure 1; short arrows in Figure 6 b, d). It is not observed in premyofibrils in the human cardiomyocytes (Figure 1; arrowheads in Figure 6 b, d), and reaches its most organized state in the mature myofibrils (Figure 1; Figure 6 b, d). The titin antibody used in this study stains titin epitopes that are localized on either side of the Z-Bands (Wang et al., 1988), yielding a doublet in immunostained confocal images (long arrows in Figure 6 b, d). Phalloidin staining reveals the total cellular distribution of F-actin in the stained human cardiomyocytes derived from hiPSCs (Figure 6 c). Premyofibrils exhibit nonstriated actin

fibers (arrowhead in Figure 6 c), but there is an absence of both muscle myosin II (arrowheads in Figure 6 a, c, d), and titin (arrowheads in Figure 6 b, c, d). Nascent myofibrils display nonstriated F-actin (small arrow in Figure 6 c), but there is the appearance of overlapping muscle myosin II filaments (small arrow in Figure 6 a), and unorganized titin filaments (small arrow in Figure 6 b). F-actin, titin and muscle myosin II reach their greatest organization in mature myofibrils (long arrows in Figure 6 a to c). Nonmuscle myosin IIB present in the first two steps in the assembly of the mature myofibrils, i.e., premyofibrils and nascent myofibrils, are not detected in the final step of myofibrillogenesis, i.e., the mature myofibrils (Figure 1; Figures 3 to 5). The staining results obtained in this study of human cardiomyocytes derived from hiPSCs are identical to results reported for embryonic chick cardiomyocytes (Rhee et al.,1994), and for mouse neonatal cardiomyocytes (White et al., 2018) leading to a proposed common three step model for myofibrillogenesis in cardiomyocytes (Figure 1).

Myomesin is a late assembling protein in the formation of mature myofibrils.

Myomesin is a protein that is present in the middle of the A-Bands in mature myofibrils in both vertebrate cardiac and skeletal muscles (Lange et al., 2020; Sanger et al., 2017). White et al. (2014, 2018) reported that the M-Band protein, myomesin, is a late assembling protein, detected for the first time in mature myofibrils in cultured primary mouse skeletal myotubes (White et al., 2014), and in cultured mouse neonatal cardiomyocytes (White et al., 2018). Its position in the middle of the A-Bands suggested that myomesin was involved in aligning the thick filaments in mature myofibrils in cross-striated muscle cells (Sanger et al., 2017). To determine if myomesin first appeared in the mature myofibrils of embryonic chick (Figure 7) and in hiPSC-human (Figure 8) cardiomyocytes, we transfected the cardiomyocytes with plasmids encoding mNeonGreen α-actinin, and then fixed, permeabilized, and stained with myomesin antibodies, and phalloidin. Myomesin was not detected in the spreading edges of the cardiomyocytes where both premyofibrils and nascent myofibrils are located (arrowheads in Figures 7 and 8: a, b, c). Myomesin was only detected in the middle of sarcomeres in mature myofibrils (long arrows **in** Figures 7 and 8: a to d). These myomesin patterns are identical to those reported for both mouse skeletal muscle cells

(White et al., 2014) and mouse neonatal cardiomyocytes (White et al.,2018), and are illustrated in the three-step model for mature myofibril assembly (Figure 1).

The effects of Ubiquitin Proteasome System Inhibitors on Myofibril Assembly in cardiomyocytes.

The effects of several different inhibitors of the Ubiquitin Proteasome System (UPS) on the assembly of mature myofibrils have recently been shown to prevent the transition of nascent myofibrils to mature myofibrils in cultured embryonic skeletal muscle cells (Wang et al. 2020). Three of the five proteasome inhibitors used, MG132, MG262 and Bortezomib, were reversible, i.e., mature myofibrils formed upon washout of the inhibitors. Two of the proteasome inhibitors, Carfilzomib and epoxomicin, could not be reversed. These results support a role for the UPS system in myofibrillogenesis in skeletal muscle cells as nascent myofibrils progress to mature myofibrils (Wang et al., 2020).To determine if the UPS played a similar in cardiomyocytes, human iPSC-derived cardiomyocytes (Figure 9 a) and embryonic chick cardiomyocytes (Figure 9 b) were treated with proteasomal inhibitors (**1** μM MG132, Figure **9** c, d**,** or 2 μM Bortezomib, Figure **9** e, f) for 24 hours while undergoing myofibrillogenesis. Cells were stained with sarcomeric anti-α-actinin antibody to compare the effect of the inhibitors on myofibril assembly. The control cardiomyocytes (Figure 9 a, b) were filled with mature myofibrils with mature Z-band spacings (long arrows) and peripheral staining of small α-actinin Z-Bodies found in premyofibrils and nascent myofibrils (arrowheads). Human and embryonic chick cardiomyocytes cells that had been treated with proteasomal inhibitors showed reduced populations of mature myofibrils (long arrows in Figure 9 c to f), but increased numbers of small α-actinin bands typically detected in premyofibrils and nascent myofibrils (arrowheads in Figure 9 c to f). Both proteasomal inhibitors prevented the maturation of mature myofibrils in human (Figure 9 c, e) and embryonic chick (Figure 9 d, f) cardiomyocytes. Embryonic chick cardiomyocytes (Figures 10, 11) and hiPSC-cardiomyocytes (Figures 12, 13) were also exposed to another proteasomal inhibitors (Calfilzomib), an inhibitor of p97 (i.e., DBeQ), and MLN4924, an inhibitor of the neddylation of E1 that is involved the complex series of reactions necessary for the

ubiquitination of sarcomeric proteins for the eventual delivery to the proteasomes for proteolysis (Wang et al., 2020). The control and UPS-treated cells were fixed and stained with muscle specific myosin II antibodies and phalloidin to readily identify nascent myofibrils (over lapping patterns of myosin II), and mature myofibrils (A-Bands). All of these inhibitors inhibited the formation of mature myofibrils at the nascent myofibril stage in both avian (Figures 10, 11) and human (Figures 12, 13) cardiomyocytes.

Control, i.e., untreated, and UPS treated embryonic chick cardiomyocytes (Figure 14), and human cardiomyocytes (Figures 15) were rinsed with normal medium, trypsinized, spun down, rinsed with control medium, and then replated on to collagen or gelatin coated surfaces in control medium (Dabiri et al., 1999). The control and different previously UPS inhibitor treated cardiomyocytes spread in culture over two days. The cardiomyocytes were fixed and stained with anti-muscle-α-actinin to reveal the assembly of mature myofibrils in embryonic chick (Figure 14), and in human cardiomyocytes (Figure 15). With the exception of the proteasomal inhibitor Carfilzomib treated cardiomyocytes (Figure 14 d; 15 d), all of the other UPS inhibitors could be reversed with washout of these drugs on the embryonic chick (Figure 14 b, c, e, f) and human cardiomyocytes (Figure 11 b, c, e, f).

Cultures of embryonic chick cardiomyocytes exposed to 1 µM MG132 for different times, were collected for gel electrophoresis experiments, stained with a ubiquitin antibody to reveal the presence of ubiquitinated bands (Figure 16). There was a time dependent increase in ubiquitin staining over the four to 24 hours in the 200 KD range as the cardiomyocytes were exposed to MG132 (Figure 16). The gel was then stripped, and stained with a muscle myosin II specific antibody (F-59; Miller et al., 1985). The major muscle protein in this 200 KD ubiquitinated region was muscle myosin II (Figure 16).

Similar five day cultures of control and 1 µM MG132 treated embryonic chick cardiomyocytes were extracted and processed for 2D gel electrophoresis (Figure 17). There was a large increase in specific ubiquitin staining of the proteins in the

cardiomyocytes treated with MG132 for one day when compared with the control (Figure 17 c versus 17 d). Seven discrete bands in the 220 kD range were cut from the Coomassie-stained gel in Figure 17 b. The proteins were extracted from these seven bands for subsequent analyses using Mass Spectroscopy. These bands were heavily labeled with anti-ubiquitin antibodies (Figure 17 d). LC-MS (liquid chromatography-mass spectroscopy) studies revealed four out of seven selected bands (bands # 1, 2, 3, and 5 of Figure 7 c, d) were found to contain muscle myosin heavy chain (7B) (Table 1). Band # 5 also contained non-embryonic spectrin alpha chain, and fibronectin (Table 1 Spot #5). In addition, five of seven spots/bands (2, 3, 4, 5 and 6) contain identical polyubiquitin peptides "TITLEVEPSDTIENVKAK" (part of polyubiquitin- B1 chain), which presumably ran that high because it was bound to other high molecular weight proteins.

Absence of BAG3 (Bcl2-associated athanogene-3) in hiPSC-cardiomyocytes does not affect the three step process for the *de novo* assembly of mature myofibrils.

There are two major Protein Quality Control (PQC) systems in vertebrate cells, particularly in abundance in cardiomyocytes and skeletal muscle cells, i.e., the Ubiquitin Proteasome System (UPS; Patel and Majetschak, 2007) and the BAG3 responsive autophagy pathway (Kirk et al., 2021). BAG3 has been reported to play an important role in myofibril structure and stress related integrity (Homma et al., 2010; Knezevic et al., 2015), particularly via its binding to CapZ that binds to the barbed end of F-actin filaments embedded in the Z-Bands of mature myofibrils (Hishiya et al., 2010; reviewed in Marzullo et al., 2020). Wang et al. (2007) reported that CapZ first appears in the nascent myofibrils in embryonic quail skeletal muscle myotubes. Some proteasome inhibitors (e.g., MG132, Bortezomib) have been reported to induced BAG3 increased expression (Wang et al., 2008; Kirk et al., 2021). However, the proteasome inhibitor, calfilzomib, does not upregulate the level of BAG3 in treated cells (Dimopoulos et al., 2016; Kirk et al., 2021). The availability of hiPSC BAG-/- cells allowed us to determine if mature myofibrils formed in hiPSC-derived BAG3-/- cardiomyocytes (Judge et al., 2017; McDermott-Roe et al., 2019) follow a three step process that we detected in hiPSC

BAG3 containing cardiomyocytes. The cardiomyocytes derived from hiPSCs lacking BAG3 was permitted to spread for four days in culture before fixation, permeabilization, and staining with (Figure 18 a) a titin antibody (9D10), a fluorescently tagged secondary antibody, and (Figure 18 b) phalloidin. Premyofibrils lacking titin are located near the edge of the cardiomyocyte marked by the arrowheads in (Figure 18 a, b). The long arrows mark the positions of the mature myofibrils (Figure 18 a, b) in the center of the cell, where (Figure 18 a) titin is a doublet marking the positions of the Z-Bands. The short arrows in (Figure 19 a, b) indicate the position of the nascent myofibrils where titin is first detected in this cardiomyocyte lacking BAG3. These results are identical to the wild type hiPSC-cardiomyocytes (+/+BAG3) stained with titin antibodies and phalloidin (Figure 6 b to d). We conclude that a three step model of *de novo* myofibril assembly takes place initially in hiPSC derived cardiomyocytes lacking BAG3. The role of BAG3 in Protein Quality Control becomes more important in the maintenance of assembled myofibrils undergoing stresses (Liu et al., 2021).

DISCUSSION

Myofibrillogenesis in cardiomyocytes

Analyses of myofibril assembly in cultured embryonic chick cardiomyocytes fixed and stained after different times of spreading with specific anti-muscle proteins and two different nonmuscle myosin II antibodies lead to a proposal of a three step process for the assembly of myofibrils: premyofibril to nascent myofibrils to mature myofibrils (Rhee et al., 1994). This proposal has been updated, revised, and is summarized in Figure 1 (Sanger et al., 2004; 2006; 2017; Wang et al., 2018; White et al., 2018; Wang et al., 2020). Support for this three step model came from a number of subsequent experiments since the basic model proposed by Rhee et al. in 1994. LoRusso et al. (1997) studied the spreading of cardiomyocytes isolated from adult cats and rats in tissue culture, and reported that initially there were no signs of premyofibrils or nascent myofibrils, only mature myofibrils were detected after one day in culture. These recently isolated adult cardiomyocytes had no sign of nonmuscle myosin IIB. However, when these adult cardiomyocytes began to spread at one end of the cell, the synthesis of nonmuscle myosin IIB was initiated, with the detection of premyofibrils, nascent myofibrils and mature myofibrils in this new spreading area of the cell over the next several days in culture (LoRusso et al., 1997). Dabiri et al. (1997) used plasmids encoding GFP-muscle-α-actinin to transfect living cultured embryonic chick cardiomyocytes to follow the assembly of new premyofibrils in living cells. They were able to follow the same living cardiomyocytes as Z-Bodies were formed on the edges of the cells, and then follow the movements of linear arrays of Z-Bodies as they moved inward towards the central nucleus of each of these cells. Z-Bodies of adjacent premyofibrils fused with one another that led the formation of beaded Z-Bands, and then the Z-Bands of the mature myofibrils. The distances between the Z-Bodies and Z-Bands grew apart until the sarcomeres of the mature myofibrils reached the length of two microns. Some of the cultures of the transfected cardiomyocytes were fixed and stained with antibodies for nonmuscle myosin IIB. This nonmuscle myosin IIB antibody stained the fibers with Z-Bodies, but not the mature myofibrils identified by Z-Bands (Dabiri et al., 1997). Du et al. (2003) studied the formation of the first myofibrils in the

first cardiomyocytes that formed from isolated quail precardiac mesoderm explants using a battery of specific antibodies. Their results were identical to those reported on the spreading of embryonic chick cardiomyocytes in culture reported by Rhee et al. (1994), and supportive of the premyofibril model (Figure 1). Du et al. (2008) met the challenge of studying the formation of mature myofibrils in the intact hearts of the Japanese quail embryos. They applied non-enzymatic procedures and deconvolution imaging techniques to investigate early heart forming regions *in situ* at 2- to 13-somite stages, a time span of about 23 hours (beating in hearts begins at the 9-somite stage). Their results supported the premyofibril model described in Figure 1. Further support for the Premyofibril model (Figure 1) was obtained by White et al. (2018) in a study on myofibrillogenesis in isolated neonatal mouse cardiomyocytes. The results that we have reported in this manuscript on human iPS cell-derived cardiomyocytes suggest a common three step model for the assembly of mature myofibrils in a large number of hearts from different model organisms (chicks, quails, cats, mice, rats) as well as in humans. In the original model for the assembly of mature myofibrils in embryonic chick cardiomyocytes it was suggested that zeugmatin, a Z-Band protein, might be responsible for the fusion of the Z-Bodies of nascent myofibrils to form Z-Bands of the mature myofibrils (Rhee et al., 1994). The zeugmatin antibody pattern was identical to that Rhee et al. (1994) reported for titin staining. Titin and actin filaments were proposed to be responsible for the initial binding of myosin filaments to the Z-Bodies of the nascent myofibrils, and then the alignment of the thick myosin filaments to form A-Bands of the mature myofibrils (Rhee et al., 1994). Subsequent work from our laboratory demonstrated that zeugmatin was not a new Z-Band protein, but a truncated part of the N-terminal targeting site of titin embedded in Z-Bands (Turnacioglu et al., 1996, 1997a, 1997 b). Turnacioglu et al. (1996) reported that the purified truncated region of titin bound purified muscle α-actinin, the actin cross linking molecules in Z-Bodies of nascent myofibrils and the Z-Bands of mature myofibrils, supporting the original suggestion of Rhee et al. (1994) that the N-terminal region of titin (i.e., zeugmatin) could bind neighboring α-actinin regions of Z-Bodies of nascent myofibrils to form Z-Bands. Over-expression of different truncated parts of the Z-Band region of titin (zeugmatin) lead to the loss of existing mature myofibrils in both cardiac and skeletal

muscle cells (Ayoob et al., 2000). These experiments demonstrated that full length titin was required for the assembly and maintenance of mature myofibrils. Interestingly, there is a line of myofibroblasts (Baby Hamster Kidney, BHK-21/C13) cells) that can assemble myofibrils with muscle specific proteins including titin (Schaart et al., 1991; Jäckel et al, 1997; Mayer and Leinwand, 1997; van der Ven et al., 2000). A subclone of this BHK cell line arose spontaneously that lacked part of the N-terminus of titin, i.e., BHK-21-Bi (Jäckel et al., 1997). These BHK-21-Bi cells were unable to assemble myofibrils (van der Ven et al., 2000), adding support to the important roles of full length titin in the assembly and maintenance of Z-Bands and A-Bands in mature myofibrils as first reported by Turnacioglu et al. (1996, 1997 a, b) and later by Ayoob et al. (2000) for both cultured cardiac and skeletal muscle cells. Expression of truncated titins in mouse hearts (Gotthardt et al., 2003) and hiPSC-derived cardiomyocytes (Chopra et al., 2018) lead to loss of sarcomeric structures. It is striking that the same Premyofibril model for cardiomyocytes has been found to be also applicable for myofibrillogenesis in skeletal muscle cells from chicks (Sanger et al.,1986; Turnacioglu et al., 1997 a, b) , quails (Wang et al., 2005 a, b; 2018), zebrafish (Sanger et al. 2008), and mice (White et al., 2014). Our results on the localization of nonmuscle myosin IIs and sarcomeric proteins (α-actinin, muscle myosin II heavy and light chains, and titin) during myofibrillogenesis in the human cardiomyocytes are also consistent with the three step model first proposed by Rhee et al. in 1994 using cultured embryonic chick cardiomyocytes. Rhee et al. (1994) also reported that muscle myosin II and titin first appeared in nascent myofibrils. Similar results were also discovered in the human cardiomyocytes reported in this work (Figures 3, 6). The initial detection of the short muscle myosin II filaments were directed at different angles along the actin fibrils of the nascent myofibrils (Figure 4). This is consisted with the ability of the muscle α-actinin in Z-Bodies to bind both F-actin and titin, and would enable these two long filaments to capture and align the muscle myosin II filaments in the nascent myofibrils and mature myofibrils (Sanger and Sanger, 2001). Thus truncated or loss of titin would inhibit the assembly of mature myofibrils as reported by several investigators (Turnacioglu et al., 1996, 1997a, 1997 b; Jäckel et al., 1997; Ayoob et al., 2000 Chopra et al., 2018). Another new result for both the embryonic chick and human cardiomyocytes is the first detected appearance of

myomesin in the mature myofibrils. It meets the title of a late assembling protein that Rhee et al. (1994) proposed for muscle myosin binding protein C first detection in mature myofibrils in embryonic chick cardiomyocytes. White et al. (2018) also reported the late assembly status for myomesin, and telethonin in mature myofibrils in mouse neonatal cardiac muscle cells.

Rhee et al. (1994) proposed a role for non-muscle myosin II for the assembly of oppositely polarized actin filaments into the initial sarcomeric units at the cell surface, i.e., the premyofibrils. They stated that "We have no evidence that the initial muscle thick filaments in the chick cardiomyocytes increase in length as the thick filaments do in the later stages of insect myofibrillogenesis (Aronson, 1961 ; Auber, 1969), nor do we suggest this takes place in our model of vertebrate myofibrillogenesis…" Since this statement in 1994, advances in imaging have led to the first discovery of shorter muscle myosin II filaments in both cardiac and skeletal muscle by our group (Du et al., 2008; Sanger et al., 2009). Du et al. (2008) used antibodies that stained the muscle myosin II near the cross bridges, leaving the middle of the thick filaments and A-Bands unstained (Wachsberger et al.,1983; Sanger et al., 1986 a). There are no cross bridges in this area of the thick filament or middle of the A-Bands (Huxley, 1963). These short myosin II filaments had an average length of 0.8 microns in the 2 somites staged embryos versus the average length of A-Bands of 1.6 microns in the 13 somites staged embryos. As mentioned first above, beating in quail hearts begins at the 9-somite stage, and the time span between these two time points(2 to 13 somites) is about 23 h (Du et al., 2008). Du et al. (2008) reported in the same paper that they isolated cardiomyocytes from ten day old quail embryonic hearts, and placed them in tissue culture to be fixed, stained, and examined using the same antibody and imaging techniques applied to the early *in situ* quail hearts previously described by Du et al. (2003). In this case there were different populations in the same cultured cardiomyocytes. The short muscle myosin II filaments were about 0.7 microns in the spreading edges of these cultured cardiomyocytes, and the A-Bands of the mature myofibrils were about 1.5 microns. A similar anti-muscle II antibody that stained only the two cross-bridge areas of the thick filaments in mature myofibrils (F59; Miller and Stockdale, 1985) plus the use of

deconvolution imaging techniques enabled Sanger et al. (2009) to detect short muscle myosin filaments **in** fixed and stained zebrafish skeletal muscle cells. Sanger et al. (2009) detected short myosin II filaments in the unbanded nascent myofibrils in the zebrafish somites. These small muscle myosin II filaments ranged in lengths from 0.4 to1.1 microns with an average length of 0.9 microns. In contrast, the A-Bands of the mature myofibrils in older somites ranged from 1.3 to 1.7 microns with an average length of 1.5 microns. In this report on hiPSC-CMs, we have used the same F59 muscle myosin antibody and deconvolution methods to identify short muscle myosin filaments in fixed cultured spread hiPSC-derived cardiomyocytes (Figure 4). A striking feature of these short muscle myosin II filaments is that they are at different angles to the actin cables. We propose a model illustrated in Figure 20 to show how the one micron long titin filaments could attach to the alpha-actinin containing Z-Bodies at their N-terminal regions, while the titin muscle myosin II binding region leading towards its C-termini could bind and then reel in these scattered short myosin II filaments to form the nascent myofibrils. Sanger et al. (2017) have speculated that these short muscle myosin II filaments may be able to take over the proposed role by Rhee et al. (1994) of nonmuscle myosin II for organizing the initial antipolar arrangement of the F-actin thin filaments in the first stage of myofibril assembly if nonmuscle myosin II could be deleted from the muscle cells. A recent paper by Chopra et al. (2018) reported that elimination of nonmuscle myosin IIA and nonmuscle myosin IIB still leads to the formation of mature myofibrils in hiPSC-cardiomyocytes. We suggest that this genetic experiment allowed the short muscle myosin II filaments to take over the establishment of antipolar actin filaments role postulated for nonmuscle myosin II in normal cardiac and skeletal muscle cells (Rhee et al., 1994; Sanger et al., 1997). We predict that future experiments testing for the distribution between the short spacings of the aligned Z-Bodies in these nonmuscle myosin II null hiPSC-CMs will have short muscle myosin II filaments localized in the minisarcomeres of their premyofibrils. Such a result would indicate the similarities of myofibrillogenesis in vertebrate and invertebrate myofibrillogenesis (Aronson,1961; Auber, 1969; Sanger et al., 2017). It is important to keep in mind that nonmuscle myosin II is found in *in situ* avian hearts (Du et al., 2008), and in *in situ* skeletal muscle cells (Sanger et al., 2008) during myofibrillogenesis.

Effects of UPS on myofibrillogenesis in cardiomyocytes

Our Ubiquitin Proteasomal System (UPS) results on embryonic chick and hiPSC cardiomyocytes suggest that some protein (s), i.e., muscle myosin II, must be removed and proteolyzed from the nascent myofibrils to form mature myofibrils. Wang et al. (2020) demonstrated that UPS inhibitors (MG132, Bortezomib, DBeQ and MLN4924) reversibly inhibited myofibrillogenesis in cultured quail embryonic myotubes at the nascent myofibril stage. In living skeletal muscle cell experiments Wang et al. (2020) discovered that the proteasomal inhibitor MG132 treated myotubes continued to elongate and to form premyofibrils and nascent myofibrils, but did not form mature myofibrils. Removal of several UPS inhibitors (MG132, Bortezomib, DBeQ and MLN4924) led to the formation of mature myofibrils in the skeletal muscle cells (Wang et al., 2020). We have discovered that these same four UPS inhibitors also reversibly inhibited the progression of nascent myofibrils to mature myofibrils in the chick and human cardiomyocytes. (Figures 14, 15). Interestingly, the proteasomal inhibitor Carfilzomib could not be reversed by its removal via wash out and replating for both embryonic chick and hiPSC-derived cardiomyocytes (Figures 14, 15). These previously Carfilzomib exposed cardiomyocytes could attach, spread and assemble fibrils with linear arrays of Z-Bodies that are typical of premyofibrils and nascent myofibrils, but mature myofibrils do not reform after two days in culture (Figure 14 d, 15 d). Carfilzomib is considered to be an irreversible proteasomal inhibitor since it forms a covalent bond with some of the proteases in the proteasomes (Downey et al., 2015). These UPS inhibitor experiments are supportive of a three step model for myofibrillogenesis in both cardiac (Figure 19) and skeletal (Wang et al., 2020) muscle cells. BAG3 is an important member of the second Protein Quality Control (PQC) system involving autophagy of misfolded proteins. Our experiments on human BAG-/- cardiomyocytes demonstrate that myofibrils form via a three-step process identical to that of wild type human cardiomyocytes, indicating that BAG3 is not involved in the process of de novo assembly myofibrillogenesis.

What could be the basis for the required participation of the UPS system to permit the formation of mature myofibrils from the nascent myofibrils? Two UPS

inhibitors (MG132, DBeQ) inhibited the dynamics of muscle myosin II heavy and light chains in nascent myofibrils but not in mature myofibrils of quail cultured skeletal muscle cells (Wang et al., 2020). In an earlier paper by Wang et a. (2018) they reported that FRET (Fluorescence Resonance Energy Transfer) experiments revealed that some nonmuscle myosin IIB and muscle myosin II were copolymerizing in the the nascent myofibrils in cultured quail skeletal muscle cells. How does a nascent myofibril lose its nonmuscle myosin II to form a mature myofibril that has just one type of myosin II, i.e. the muscle myosin II isofrom? Wang et al. (2020) demonstrated that there was a large accumulation of ubiquitinated muscle myosin II in the skeletal muscle cells when they were exposed to the MG132 proteasomal inhibitor. We have demonstrated in this report that there is a similar buildup of ubiquinated cardiac myosin II. These results indicate that the formation of the mature myofibril in cardiac and skeletal muscle cells required the destruction of the initial muscle myosin II in the nascent myofibrils, some of which had copolymerized with nonmuscle myosin II, to permit new muscle myosin II filaments to form the A-Bands that lack nonmuscle myosin II of the mature myofibrils (Wang et al., 2018, 2020). Figure 1 shows that premyofibrils contain one type of myosin II, i.e., nonmuscle myosin II, and that mature myofibrils contain one type of myosin II, i.e., muscle myosin II. The nascent myofibril in Figure 1 illustrates the presence of two different types of myosin II, i.e., nonmuscle myosin II and muscle myosin II. Figure 1 also summarizes the importance of titin in myofibrillogenesis, i.e., it makes its appearance in the nascent myofibril, and it is well organized in the mature myofibril. Turnacioglu et al. (1996, 1997 a, b) demonstrated that the N-terminal region of titin is in the Z-Bands of mature myofibrils, and that this region of titin binds α-actinin. The major part of titin extending from the Z-Bands to the middle of the sarcomeres (M-Band, site of myomesin, Figure 1) in the mature myofibrils is associated with binding to the thick muscle myosin II filament and associated proteins (Furst et al., 1989; Labeit et al., 1992; Gotthardt et al., 2003). Future experimental approaches will be needed to determine what proteins are involved preventing muscle myosin II from binding nonmuscle myosin II proteins in mature myofibrils.

Insights into use of USP inhibitors for cancer patients

Proteasome inhibitors, such as Bortezomib (also called Velcade) and Carfilzomib, have been used successfully in the treatment of patients with multiple myeloma, a cancer of plasma cells, but a cure has not yet been found (Sherman and Li, 2020). Bortezomib received FDA approval in 2003, and Carfilzomib received approval in 2012. The median age for the detection of multiple myolomas in the USA is 65 years old (Naymagon and Abdul-Hay, 2016), and it is known that proteasome activity decreases with age (Bulteau et al., 2002). Several years of treatment of different patients with the~~se~~ two inhibitors revealed that there were adverse events occurring in hearts in three (Bortezomib) to six (Carfilzomib) percent of treated patients (Nowis et al., 2010; Hasinoff et al., 2017; Waxman et al., 2018; Bringhen et al., 2018). We propose a novel mechanistic model in Figure 19 that predicts that the cardiotoxic effects of proteasomal inhibitors for multiple myeloma patients are due not to off-target effects of the inhibitors, but to the facts that the heart (and kidney) has the highest proteasomal activity in mammalian bodies (Patel and Majetschak, 2007), and thus, the hearts in older patients are particularly sensitive to the use of proteasomal and other UPS inhibitors. We suggest that the effects of these drugs in inhibiting the progression of nascent myofibrils to mature myofibrils may account for some of the cardiac muscle off-target effects in multiple myeloma patients treated with these compounds. The preclinical and clinical treatments to test UPS and nonUPS drugs in patients takes many years to determine any adverse events, e.g., effects on muscle, particular the heart (Li et al., 2018, Lin et al., 2019; Sherman and Li, 2020). We propose that different UPS and nonUPS inhibitors being used, developed, and then applied in clinical settings should first be tested in the muscle culture systems. If any of these inhibitors prevent the assembly of mature myofibrils, this would elicit an early warning sign that this drug/inhibitor may have future effects on the patient's muscle cells, particularly in the heart of cancer patients. We will be able to determine if there is an effect on the cells, i.e., cardiomyocytes derived from avian embryos, and cardiomyocytes derived from human inducible pluripotential cells (hiPSCs), and whether we can reverse the effect of the inhibitor when the inhibitor is removed from the culture dish. This approach would be an important part of the goal of rational drug design for combatting different cancers including multiple myelomas.

In summary, analyses of myofibril assembly in embryonic chick cardiomyocytes, neonatal mouse cardiomyocytes, adult cat and rat cardiomyocytes, and now human iPS cell-derived cardiomyocytes suggest a common three step model for the assembly of myofibrils (Figure 1). Our UPS inhibitor results support this three step model, and suggest that some protein(s) must be removed and proteolyzed from the nascent myofibrils to form mature myofibrils (Figure 13). Our experiments using different inhibitors of the UPS to inhibit myofibrillogenesis in both embryonic chick cardiomyocytes and in hiPSC-cardiomyocytes suggest a possible explanation for the off target effects of the proteasomal inhibitors Bortezomib and Carfilzomib on the hearts of multiple myeloma patients, i.e., the inhibition of progression of nascent myofibrils to the formation of mature myofibrils (Figure 19). We predict that testing of UPS and nonUPS drugs being developed for cancer treatments on cultured avian and hiPSC muscle cells would provide an early indicator of potential adverse effects on cardiac and skeletal muscle cells leading to improved risk versus outcomes for cancer patients

MATERIALS AND METHODS

Generation of hiPSC-derived cardiomyocytes

The human induced pluripotent stem cell (hiPSC) wild type line, and the hiPSC BAG3-/-line were obtained from the Bruce R. Conkin lab at the Gladstone Institute of Cardiovascular Disease. The cardiomyocyte differentiation and purification protocols have been described in detail in previous publications (Lian et al., 2012; Judge et al., 2017 Sun et al., 2020). Briefly, hiPSCs were cultured on Geltrex (Thermo Fisher Scientific, Ca#A1413302) coated 6-well plates with Essential 8 (E8) medium (Thermo Fisher Scientific, Ca#A1517001) until 90% confluency (Day 0). At day 0, the differentiation was initiated by the treatment of 6 μM GSK3 inhibitor (CHIR99021; Stem Cell Technologies, Ca#72052) in RPMI1640 medium (Thermo Fisher Scientific, Ca#A1895601) with B27 minus insulin supplement (Thermo Fisher Scientific, Ca#A1517001) (RPMI1640-B27-I). After 24 h, CHIR99021 was removed and the culture medium was switched to RPMI1640-B27-I. On the next day, cells were treated with 5 μM Wnt inhibitor (IWP4; Stem Cell Technologies, Ca#72554) in RPMI1640-B27-I for 48 h. After another 2 days of culture in RPMI1640-B27- I, cells were maintained in RPMI1640 medium with B27 complete supplement (Thermo Fisher Scientific, Ca#17504044) (RPMI-B27+C). At around day 20, differentiated cardiomyocytes were dissociated by the treatment with 1 mg/ml collagenase type II (Thermo Fisher Scientific, Ca#17018029). Harvested cells were re-plated and purified using 4 mM sodium L-lactate (Sigma Aldrich, Ca#L7022) for three consecutive cultures until there were no significant numbers of non-cardiomyocytes. Purified cardiomyocytes were cultured continuously in RPMI-B27+C medium or cryopreserved using a freezing medium composed of 90% fetal bovine serum (FBS) (Thermo Fisher Scientific, Ca#A3160502) and 10% dimethyl sulfoxide (Sigma Aldrich, Ca#D2650). We also obtained Ncyte hiPSC-derived cardiomyocytes (4 million per vial, cat# Nc-CM-CUS), and Cardiomyocyte Culture Medium (Nc-M-CMCM-250) from Ncardia (Hillsborough, NJ). The Ncardia cardiomyoctes were cultured following a protocol that came Ncardia cells. These cardiomyocytes were the equal of the other hiPSC-derived cardiomyoctes

described above, as judged by various immunohistochemical stainings of control and UPS inhibitor treated cells.

Cell Culture, Transfections and Immunostaining

Embryonic chick cardiomyocytes were isolated from of 9-day-old chick embryos and plated on MatTek dishes (MatTek Corp., Ashland, MA) at a density of 10^5 cells per dish (Dabiri et al., 1999). The MatTek dishes for the chick cardiomyocytes were coated with collagen (Corning™ Collagen I, Rat cat CB-40236, Thermo Fisher Scientific). The hiPSC-cardiomyocytes were grown on MatTek dishes coated with gelatin (Attachment Factor Protein 1X catalogue number S006100 from Thermo Fisher Scientific). The embryonic chick and hiPSC-derived cardiomyocytes were transfected after 2 days of culture with plasmids encoding mNeonGreen-fused sarcomeric proteins using FuGene HD transfection reagent (Promega, Madison, WI) according to previous methods (Wang et al., 2005 a, 2014; 2018, 2020). mNeonGreen plasmids were obtained from Dr. Nathan Shaner (Shaner et al., 2013; Allele Biotechnology, San Diego, CA). The preparation of the probes for mNeonGreen-fused sarcomeric proteins were described previously (Wang et al., 2005 a, 2014, 2018; 2020). Both types of cardiomyocytes intended for immunostaining were fixed with 3% paraformaldehyde, permeabilized and stained with anti-sarcomeric α-actinin antibody (Sigma Aldrich, St. Louis, MO), non-muscle myosin IIB for only the chick cardiomyocytes (CMII 23, Hybridoma Bank, Iowa City, IA; Conrad et al., 1991) , or anti-muscle myosin antibody F59 (Hybridoma Bank, Iowa City, IA; Miller et al., 1985), or anti-myomesin antibodies (Hybridoma Bank, Iowa City, IA; Grove et al., 1984), or anti-titin antibodies (Hybridoma Bank, Iowa City, IA; Wang and Greaser, 1985). The hiPSC-cardiomyocytes were stained with Sigma Aldrich (St. Louis, MO) purchased rabbit polyclonal antibodies directed against nonmuscle myosin IIs (IIA, M8064; IIB, M7939; IIC, SAB45003174). The anti-ubiquitin antibody was purchased from LifeSensors. The secondary goat anti mouse antibodies were labeled with Alexa Fluor 555 (Goat anti-Mouse IgG (H+L); Highly Cross-Adsorbed Secondary Antibody) were purchased from Invitrogen (Carlsbad, CA). Some cultures were stained with Alexa Fluor 633 (for three colors; if 2 then with Rhodamine Red Fluor) labeled phalloidin (Invitrogen, Carlsbad, CA)

to localize F-actin in the fixed chick cardiomyocytes and hiPSC-derived cardiomyocytes (Wang et al., 2014, 2018; 2020). All images were acquired with a Leica SP8 confocal microscope at the Leica Center of Excellence for Advanced Microscopy at SUNY Upstate Medical University.

Inhibitors Treatment and Recovery

MG132, Bortezomib/Velcade, NAE inhibitor MLN 4924, AAA-ATPase p97 inhibitor DBeQ were purchased from LifeSensors (Malvern, PA). Carfilzomib were obtained from APExBIO technology. All inhibitors were dissolved in DMSO. The inhibitors were added to the muscle cells after 4 days of culture. To reverse the drug effects, the treated cells were washed, trypsinized, spun down, rinsed with control medium, and then replated onto gelatin-coated dishes (hiPSC) or collagen-coated dishes (chick) in control medium. The control and different previously UPS inhibitor treated cardiomyocytes spread in culture over two days for recovery.

Cell Lysis, Western Blotting with antiubiquitin antibodies, and LC-MS/MS analyses

Cultured embryonic chick cardiomyocytes were washed and collected by scraping and centrifugation using PBS with protease inhibitor (cOmplete, Sigma) and deubiquitylating enzymes inhibitor PR619 (50µM). Protein was extracted with cell extraction buffer and Western blotting was performed as described previously (Wang et al. 2012). Two-dimensional (2-D) Western blotting, and subsequent mass spectroscopy analyses were performed for us by Kendrick Labs, Inc. (Madison, WI;) as described in Wang et al. (2012). The protein concentrations of the samples were determined using the BCA Assay (Pierce Chemical Co., Rockford, IL). In addition, to verify transfer and loading, the PVDF membranes after transfer are stained with Coomassie brilliant blue R 250 and an image taken. The stained membranes were compared for similar loading before destaining and western blotting. The F59 muscle myosin II antibody (Miller et al., 1985) was purchased from the Developmental Studies Hybridoma Bank (Iowa City, Iowa). The antiubiquitin antibody was purchased from LifeSensors (Malvern, PA).

Data processing and protein identification.

The raw data were processed using ProteinLynx Global Server (PLGS, version 2.4) software as previously stated [Wang et al. 2012]. The following parameters were used: background subtraction of polynomial order 5 adaptive with a threshold of 30%, two smoothings with a window of three channels in Savitzky-Golay mode and centroid calculation of top 80% of peaks based on a minimum peak width of 4 channels at half height. The resulting pkl files were submitted for database search and protein identification to the public Mascot database search (www.matrixscience.com, Matrix Science, London, UK) using the following parameters: databases from Expasy or NCBI (all organisms), parent mass error of 1.3 Da, product ion error of 0.8 Da, enzyme used: trypsin, one missed cleavage, propionamide as cysteine fixed modification and Methionine oxidized as variable modification. The best fit obtained by the for the sequencing against all organisms was the chicken.

ACKNOWLEDGEMENT

This work was supported by a NIAMS grant (R01 AR-57063 awarded to JMS and JWS. JWS was also supported by the Hendricks Fund, SUNY Upstate Medical University. Gant support from the SUNY Upstate Medical University Cancer Center Fund was essential for experiments undertaken for this report (JW, BP, DKD, JMS and JWS).This work was also supported by the following grants to ZM: NIH NICHD (R01HD101130) and NSF (CBET-1804875 and CBET-1943798). Different parts of this manuscript were reported at meetings of the American Society of Cell Biology in 2018, 2019, 2020 and 2021.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon request.

REFERENCES

Aronson J. 1961. Sarcomere size in developing muscles of a tarsonemid mite. J Biophys Biochem Cytol 11:147-156.

Auber J. 1969. La myofibrillogenese du muscle strie. I. Insectes. J Micros (Paris) 8: 197-232.

Ayoob JC, Turnacioglu KK, Mittal B, Sanger JM, Sanger JW. 2000. Targeting of cardiac titin fragments to Z-bands and dense bodies of living muscle and non-muscle cells. Cell Motil Cytoskeleton 45: 67-82.

Bringhen, S, Milan A, Ferri C, Wasch R, Gay F, & Larocca A et al., Engelhardt M. 2018. Cardiovascular adverse events in modern myeloma therapy-incidence and risks. Haematologica 103:1422-1432.

Bulteau A_L, Szeda LI, Friguet B. 2002. Age-dependent declines in proteasome actinvity. Arch Biochem Biophys 397: 298-304.

Chopra A, Kutys ML, Zhang K, Polacheck WJ,Sheng CC, Luu RJ, Eyckmans J, Hinson JT, Seidman JG, Seidman CE, Chen CS. 2018. Force generation via beta-cardiac myosin, titin, and alpha-actinin drives cardiac sarcomere assembly from cell-matrix adhesions Developmental Cell 44, 87–96.

Conrad AH, Jaffredo T, Conrad GW. 1995. Differential localization of cytoplasmic myosin II isoforms A and B in avian interphase and dividing embryonic and immortalized cardiomyocytes and other cell types in vitro. Cell Motil Cytoskel 31:93-112.

Dabiri GA, Turnacioglu KK, Sanger JM, Sanger JW. 1997. Myofibrillogenesis in living embryonic cardiomyocytes. Proc Natl Acad Sci USA 94: 9493-9498.

Dabiri GA, Ayoob JP, Turnacioglu KK, Sanger JM, Sanger JW. 1999. Use of Green Fluorescent Proteins linked to cytoskeletal proteins to analyze myofibrillogenesis in living cells. Methods in Enzymology (Optical Imaging and Green Fluorescent Proteins) (Editor: P. M. Conn) Academic Press (San Diego, CA) 302: 171-186.

Dimopoulos MA, Moreau P, Palumbo A, Joshua D, Pour L, Hájek R, Facon T, Ludwig H, Oriol A, Goldschmidt H, Rosiñol L, Straub J, Suvorov A, Araujo C, Rimashevskaya E, Pika T, Gaidano G, Weisel K, Goranova-Marinova V, Schwarer A, Minuk L, Masszi T, Karamanesht I, Offidani M, Hungria V, Spencer A, Orlowski RZ, Gillenwater HH, Mohamed N, Feng S, Chng WJ; ENDEAVOR Investigators. 2016. Carfilzomib and dexamethasone versus bortezomib and dexamethasone for patients with relapsed or refractory multiple myeloma (ENDEAVOR): a randomised, phase 3, open-label, multicentre study. Lancet Oncol 17:27-38.

Downey S L, Florea BI, Overkleeft HS, and Kisselev AF. 2015. Use of proteasome inhibitors. Current Protocols in Immunology, 109, 9.10.1–9.10.9.

Du, A, Sanger, JM, Linask, KK, Sanger, JW. 2003. Myofibrillogenesis in the first cardiomyocytes formed from isolated quail precardiac mesoderm. Dev. Biol. 257: 382-394.

Du, A, Sanger, JM, Sanger, JW. 2008. Cardiac myofibrillogenesis inside intact embryonic hearts. Developmental Biology 318: 236-246.

Furst, DO, Nave R, Osborn, M., and Weber, K. 1989.
Repetitive titin epitopes with a 42 nm spacing coincide in relative position with A band striations also identified with myosin-associated proteins. J Cell Sci 94:119-125.

Gotthardt M, Hammer RE, Hubner N, Monti J, Witt CC, McNabb M, Richardson JA, Granzier H, Labeit S, Herz J. 2003. Conditional Expression of Mutant M-line Titins Results in Cardiomyopathy with Altered Sarcomere Structure. J. Biochem Chem 278: 6059-6065.

Grove BK, Kurer V, Lehner C, Doetschman TC, Perriard J-C, Eppenberger HM. 1984. A new 185,000-dalton skeletal muscle protein detected by monoclonal antibodies. J Cell Biol 98:518-524.

Guglielmi V, Nowis D, Tinelli M, Malatesta M, Paoli L, Marini M, Manganotti P, Sadowski R, Wilczynski GM, Meneghini V, Tomelleri G, Vattemi G. 2017. Bortezomib-induced muscle toxicity in multiple myeloma. J Neuropathol Exp Neurol 76:620-630.

Hishiya A, Kitazawa T, Takayama S. 2010. BAG3 and Hsc70 interact with actin capping protein CapZ to maintain myofibrillar integrity under mechanical stress. Circ Res. 107:1220-1231.

Hasinoff BB, Patel D, Wu X. 2017. Molecular mechanisms of the cardiotoxicity of the proteasomal-targeted drugs bortezomib and carfilzomib. Cardiovasc Toxicol 17: 237-250.

Homma S, Iwasaki M, Shelton GD, Engvall E, Reed JC, Takayama S. 2006. BAG3 deficiency results in fulminant myopathy and early lethality. Am J Pathol 169: 761-73.

Huxley HE. 1963. Electron microscope studies on the structure of natural and synthetic protein filaments from striated muscle. J Mol Biol 7: 281-308.

Jäckel M, Witt, C, Antonova O, Curdt I, Labeit S, Jockusch H.
1997. Deletion in the Z-line region of the titin gene in a baby hamster
kidney cell line, BHK-21-Bi. FEBS Lett. 408, 21-24.

Judge LM, Perez-Bermejo JA, Truong A, Ribeiro AJ, Yoo JC, Jensen CL, Mandegar MA, Huebsch N, Kaake RM, So PL, Srivastava D, Pruitt BL, Krogan NJ, Conklin BR. 2017. A BAG3 chaperone complex maintains cardiomyocyte function during proteotoxic stress. JCI Insight 2:e94623. doi: 10.1172/jci.insight.94623.

Kirk JA, Cheung JY, Feldman AM. 2021. Therapeutic targeting of BAG3: considering its complexity in cancer and heart disease. J Clin Invest 16;131(16):e149415.

Knezevic T, Myers VD, Gordon J, Tilley DG, Sharp TE 3rd, Wang J, Khalili K, Cheung JY, Feldman AM. 2015. BAG3: a new player in the heart failure paradigm. Heart Fail Rev. 20:423-34.

Labeit S, Kolmerer B. 1995. Titins: giant proteins in charge of muscle ultrastructure and elasticity. Science 270, 293-296.

Lange S, Pinotsis N, Agarkova I, Ehler E. The M-band: The underestimated part of the sarcomere. Biochim Biophys Acta Mol Cell Res. 2020 Mar;1867(3):118440.

Li X, Elmira E, Rohondia S, Wang J, Liu J, Dou QP. 2018. A patent review of the ubiquitin ligase system: 2015-2018. Expert Opin Ther Pat.

Lian X, Hsiao C, Wilson G, Zhu K, Hazeltine LB, Azarin SM, Raval KK, Zhang J, Kamp TJ, Palecek SP. 2012. Robust cardiomyocyte differentiation from human pluripotent stem cells via temporal modulation of canonical Wnt signaling. 2012. Proc Natl Acad Sci 109: E1848-E1857.

Liu L, Sun K, Zhang X, Tang Y, Xu D. 2021. Advances in the role and mechanism of BAG3 in dilated cardiomyopathy. Heart Failure reviews 26:183-194.

LoRusso, SM, Rhee D, Sanger JM, Sanger JW: Premyofibrils in spreading adult cardiomyocytes in tissue culture: evidence for reexpression of the embryonic program for myofibrillogenesis in adult cells. Cell Motil Cytoskel 37: 183-198, 1997.

Marzullo L, Turco MC, De Marco M. 2020. The multiple activities of BAG3 protein: Mechanisms. Biochim Biophys Acta Gen Subj1864:129628.

Mayer D C, and Leinwand L A. 1997. Sarcomeric gene expression and contractility in myofibroblasts. J. Cell Biol 139, 1477-1484.

McDermott-Roe C, Lv W, Maximova T, Wada S, Bukowy J, Marquez M, Lai S, Shehu A, Benjamin I, Geurts A, Musunuru K. 2019. Investigation of a dilated cardiomyopathy-associated variant in BAG3 using genome-edited iPSC-derived cardiomyocytes. JCI Insight. 4(22):e128799.

Miller JB, Crow MT, Stockdale FE. 1985. Slow and fast myosin heavy chain content defines types of myotubes in early muscle cell cultures. J Cell Biol 101: 1943-1650.

Naymagon L, Abdul-Hay M. 2016. Novel agents in the treatment of multiple myeloma: a review about the future. J Hematol Oncol 30;9(1):52. doi: 10.1186/s13045-016-0282-1.

Nowis D, Maczewski M, Mackiewicz U, Kujawa M, Ratajska A, Wieckowski MR, Wilczyński GM, Malinowska M, Bil J, Salwa P, Bugajski M, Wójcik C, Siński M, Abramczyk P, Winiarska M, Dabrowska-Iwanicka A, Duszyński J, Jakóbisiak M, Golab J. 2010. Cardiotoxicity of the anticancer therapeutic agent bortezomib. Am J Pathol. 176: 2658-2668.

Patel MB, Majetschak M. Distribution and interrelationship of ubiquitin proteasome pathway component activities and ubiquitin pools in various porcine tissues. 2007. Physiol Res 56:341-350.

Piccirillo, R, Demontis, F, Perrimon, N, Goldberg, AL. 2014. Mechanisms of muscle growth and atrophy in mammals and Drosophila. Dev Dyn 243:201-15.

Rhee D, Sanger, JM, Sanger, JW. 1994. The premyofibrils: evidence for its role in myofibrillogenesis. Cell Motil. Cytoskeleton 28: 1-24.

Sanger, JW, Mittal B, Sanger JM. 1984. Formation of myofibrils in spreading chick cardiomyocytes. Cell Motility 4: 405-416, 1984.

Sanger, JM, Mittal, B, Pochapin, M, Sanger, JW. 1986 a. Myofibrillogenesis in living cells microinjected with fluorescently labeled α-actinin. J Cell Biol 102: 2053-2066.

Sanger JM, Mittal B, Pochapin MB, Sanger JW. 1986 b Observations of microfilament bundles in living cells microinjected with fluorescently labeled contractile proteins. J. Cell Sci Suppl. 5,17-44.

Sanger JW, Sanger JM, Franzini-Armstrong C. 2004. Assembly of the skeletal muscle cell. In: Myology, 3rd Edition, (Editors: A. G. Engel and C. Franzini-Armstrong), McGraw-Hill, New York 45-65.

Sanger JW, Kang K, Siebrands C, Freeman N, Du A, Wang J, Stout AL, Sanger JM. 2006. How to build a myofibril. J Muscle Res Cell Motility 26:343-354.

Sanger, JW, Wang, J, Holloway, B, Du, A, Sanger, JM. 2009. Myofibrillogenesis in skeletal muscle cells in zebrafish. Cell Motil Cytoskeleton 66: 556-566.

Sanger, JW, Wang J, Fan Y, White J, Sanger JM. 2010a. Assembly and dynamics of myofibrils. Journal of Biomedicine and Biotechnology, Volume 2010. Article ID 858606, 8 pages. (www.hindawi.com/journals/jbb/2010/858606.html)

Sanger JW, Wang J, Fan Y, White J, Mi-Mi Lei, Dube DK, Sanger JM, Pruyne D. 2017. Assembly and maintenance of myofibrils in living muscle cells. Handbook of Experimental Pharmacology 235: 39–75.

Sanger JM, Sanger JW. 1980. Banding and polarity of actin filaments in interphase and cleaving cells. J. Cell Biol. 86:568-575, 1980.

Sanger JW, Sanger JM. 2001. Fishing out proteins that bind to titin. J Cell Biol 154: 21-24.

Schaart G, Pieper FR, Kuijpers HJ, Bloemendal H, and Ramaekers FCS. 1991. Baby hamster kidney (BHK-21/C13) cells can express striated muscle type proteins. Differentiation 46, 105-115.

Shaner NC, Lambert GG, Chammas A, Ni Y, Cranfill PJ, Baird MA, Sell BR, Allen JR, Day RN, Israelsson M, Davidson MW, Wang J. 2013. A bright monomeric green fluorescent protein derived from *Branchiostoma lanceolatum.* Nat Methods10:407-9.

Sherman DJ and Li Jing, 2020. Proteasome Inhibitors: Harnessing Proteostasis to Combat Disease. Review: Molecules 25(3):671.

Stout AL, Wang J, Sanger JM, Sanger JW. 2008. Tracking changes in Z-Band organization during myofibrillogenesis with FRET imaging. Cell Motil. Cytoskeleton 65, 353-367.

Sun S, Shi H, Moore S, Wang C, Ash-Shakoor A, Mather PT, Henderson JH, Ma Z. (2020). Progressive myofibril reorganization of human cardiomyocytes on a dynamic nanotopographic substrate. ACS Appl Mater Interfaces 12: 21450–21462.

Turnacioglu K, Mittal B, Sanger JM, Sanger JW. 1996. Partial characterization and DNA sequence of zeugmatin. Cell Motil Cytoskel 34: 108-121.

Turnacioglu KK, Mittal B, Dabiri G, Sanger JM, Sanger JW. 1997a. Zeugmatin Is part of the Z-Band targeting region of titin. Cell Struct. Funct 22: 73-82.

Turnacioglu KK, Mittal B, Dabiri G, Sanger JM, Sanger JW. 1997b. An N-terminal fragment of titin coupled to green fluorescent protein localizes to the Z-bands in living muscle cells: overexpression leads to myofibril disassembly. Mol. Biol. Cell 8: 705-717.

van der Ven PFM, Bartsch JW, Gautel M, Jockusch H, Fürst DO. 2000. A functional knock-out of titin results in defective myofibril assembly. J Cell Sci 113: 1405-1414.

Wachsberger P, Lampson L, and Pepe FA. 1983. Non-uniform staining of myofibril A-Bands by a monoclonal antibody to skeletal muscle S1 heavy chain. Tissue Cell 15: 341-349.

Wang HQ, Liu HM, Zhang HY, Guan Y, Du ZX. 2008. Transcriptional upregulation of BAG3 upon proteasome inhibition. Biochem Biophys Res Commun 365:381-385.

Wang J, Shaner N, Mittal B, Zhou Q, Chen J, Sanger JM, Sanger JW. 2005 a. Dynamics of Z-band based proteins in developing skeletal muscle cells. Cell Motil Cytoskeleton 61: 34-48.

Wang J, Sanger JM, Sanger JW. 2005 b. Differential effects of latrunculin-A on myofibrils

In cultures of skeletal muscle cells: Insights into mechanisms of myofibrillogenesis. Cell Motil Cytoskeleton. 62: 35-47.

Wang J., Sanger JM, Kang S, Thurston H, Abbott LZ, Dube DK, Sanger JW. 2007. Ectopic expression and dynamics of TPM1α and TPM1κ in myofibrils of avian myotubes. Cell Motil Cytoskeleton 64: 767-776.

Wang J, Dube DK, Mittal B, Sanger JM, Sanger JW. .2011. Myotilin dynamics in cardiac and skeletal muscle cells. Cytoskeleton 68: 661-670.

Wang J, Dube DK, White J., Fan Y, Sanger JM, and Sanger JW. 2012. Clock is not a component of Z-bands. Cytoskeleton 69: 1021-1031.

Wang J, Fan Y, Dube DK, Sanger JM, Sanger JW. 2014. Jasplakinolide reduces actin and tropomyosin dynamics during myofibrillogenesis. Cytoskeleton 71: 513-529.

Wang J, Fan, Y, Sanger JM, Sanger JW. 2018. Nonmuscle myosin II in cardiac and skeletal muscle cells. Cytoskeleton 75: 339-351.

Wang J, Fan Y, Dube S, Wanko Agassy NW, Dube DK, Sanger JM, Sanger JW. 2020. Myofibril Assembly and the Roles of the Ubiquitin Proteasome System. Cytoskeleton 77:456-479.

Wang SM, Greaser ML. 1985. Immunocytochemical studies using a monoclonal antibody to bovine cardiac titin on intact and extracted myofibrils. J Muscle Res Cell Motil 6:293-312.

Wang SM, Greaser ML,Schultz E, Bulinski JC, Lin JJ-C, Lessard JL. 1988. Studies on cardiac myofibrillogenesis with antibodies to titin, actin, tropomyosin and myosin. J Cell Biol 107: 1075-1083.

Waxman AJ, Clasen S, Hwang WT, Garfall A, Vogl DT, Carver J, O'Quinn R, Cohen AD, Stadtmauer EA, Ky B, Weiss BM. 2018. Carfilzomib-Associated Cardiovascular Adverse Events: A Systematic Review and Meta-analysis. JAMA Oncol. Mar 8;4(3):e174519. doi: 10.1001/jamaoncol.2017.4519. Epub 2018 Mar 8.

White J, Barro MV, Makarenkova HP, Sanger JW, Sanger JM. 2014. Localization of sarcomeric proteins during myofibril assembly in cultured mouse primary skeletal myotubes. Anat Record 297: 1571-1584.

White J, Wang J, Fan, Y, Dube, DK, Sanger JW**,** Sanger JM. 2018. Myofibril assembly in cultured mouse neonatal cardiomyocytes. Anat Rec 301: 2067-2079.

FIGURE LEGENDS 20 January, 202 Corrected Figures 1 to 20 with input from Dipak, Jushuo, and Jean

Figure 1. Three-Step Model of Myofibrillogenesis.
The first of three steps leading to the formation of mature, contractile myofibrils in cardiomyocytes occurs with the assembly of mini-sarcomeres organized in premyofibrils forming at the cells' spreading edges. These mini-sarcomeres form when the barbed ends of actin filaments bind to the muscle-specific α-actinin in Z-Bodies attached to the cell membranes, and the unattached pointed ends of actin filaments interdigitate with filaments of non-muscle myosin IIB. This arrangement of premyofibril sets up the anti-polar alignment of actin filaments that wnecessary the basis for contractions (Huxley, 1963). As the cardiomyocyte cell surface extends along the substrate, premyofibrils transform to nascent myofibrils and then to mature myofibrils (Dabiri et al., 1997). The arrangement of parallel actin filaments on opposite sides of the Z-Bodies, will allow Z-Bodies to fuse laterally, and form Z-Bands without steric problems (Sanger and Sanger, 1980). In a second step, nascent myofibrils form when (a) titin and muscle myosin II filaments wrap around a core of banded fibers of nonmuscle myosin IIB, and (b) beaded Z-Bands form (Rhee et al., 1994). The third step, the transition to mature myofibrils occurs when solid Z-Bands form and myomesin binds to the middle of aligned muscle myosin II filaments in the A-Bands (White et al., 2014; 2018). Nonmuscle myosin IIB, is present in both premyofibrils and nascent myofibrils, and is absent from mature myofibrils (Rhee et al., 1994; Wang et al., 2018). Muscle myosin II is present in both nascent and mature myofibrils, but absent in premyofibrils (Sanger et al., 2017; Wang et al., 2018).

Figure 2. Cardiomyocytes derived from human induced pluripotent stem cells (hiPSC-CM) (a, b), and from embryonic chick chearts (c, d) were grown in culture and stained with muscle-specific anti-α-actinin antibody (a, c) and phalloidin (b, d) to compare the assembly of α-actinin and actin in myofibrils in human and chick cardiomyocytes. In both types of cardiomyocytes, α-actinin is organized in Z-bands in mature myofibrils (a, c, long arrows) in the middle of both human and chick cardiomyocytes, and localized in

small concentrations in Z-Bodies around the outer edges of the premyofibrils (a, c, arrowheads). After exposure to phalloidin, banded actin fibers were prominent in the mature myofibrils (b, d, long arrows). Thin actin fibrils were visible around the cell perimeter (b, d arrowheads) in both human and chick cardiomyocytes. The two labels (F, F), adjacent to the chick cardiomyocyte, in (d) mark the positions of fibroblasts displaying stress fibers of phalloidin-stained fibrous actin. These same two fibroblasts labeled F, F in (d) do not react with anti-muscle α-actinin antibodies (F, F in c), and therefore are not visible in (c).

Figure 3. A single hiPSC-CM was transfected with mNeonGreen muscle myosin light chain-2 (MLC-2) (a), and counter-stained with non-muscle myosin IIB antibody (NMIIB) (b), and phalloidin (c). The cardiomyocyte is shown in color, and with arrowheads and arrows displaying the summation of the single images in a and b. Arrowheads and arrows mark identical positions in each cell image (a to d). (a) Long arrows point to mature myofibrils with muscle myosin II (MLC-2) in A-bands (green in d). (b) Arrowheads and short arrows point to small bands of nonmuscle myosin IIB (NMIIB) in fibers along the perimeter of the cardiomyocyte (red in d). (c) Phalloidin staining reveals the distribution of all actin filaments in the cardiomyocyte. Arrowheads in (b, red in d) mark the positions of premyofibrils that have nonmuscle myosin IIB, and F-actin (c), but lack muscle myosin II (a, green in d). Nascent myofibrils, indicated by short arrows in a to d, have two different types of myosins II (MLC-2 and NMIIB) in some actin fibers (c).

Figure 4. Detection of short muscle myosin II filaments in spreading edges of a human cardiomyocyte. A spreading hiPSC-CM was fixed on day four to five days in culture, then stained with (a) a muscle-specific myosin II antibody (F-59), and (b) counterstained with phalloidin. The same areas within the boxes (a, b) were enlarged in (c, d). The upper edge of the same cell is indicated by arrows in (c, d). (c) In a higher magnification short muscle myosin II filaments are marked by short yellow lines indicating the lengths of the short muscle myosin II filaments (# 1 to 8), and longer yellow lines marking longer muscle myosin filaments in A-Bands (# 9 to 11). (d) This same enlarged region of the cardiomyocyte in (b) demonstrates the locations of the F-

actin staining by phalloidin. The single arrow in (d) marks the edge of this F-actin stained cell. The single arrow in (c) marks the edge of the cell as determined by F-actin staining in (d). The positions of the short and longer myosin filaments from (c) are indicated here by the same short lines (# 1 to 8) in (d) to indicate their distributions in the F-actin network. It is striking that these myosin II rodlets (c) are at different angles with respect to the circumferential F-actin fibers (d). The longer muscle myosin filaments in (c) are shown in the actin staining image (d, # 9 to 11) to be between the Z-Bands of early mature myofibrils. Note the increasing number of short myosin II filaments as one moves from the edge of the cell (arrowhead in c) towards the early mature myofibrils that are present in this cell (c, d). Note the absence of muscle myosin II filaments near the arrowhead in (c). The same position in (d) shows stained actin fibers marking the positions of premyofibrils that lack muscle myosin II.

Figure 5. Bar graph of the distribution of short muscle myosin II filaments in nascent myofibrils, and longer ones in mature myofibrils in hiPSC-cardiomyocytes. The number of muscle myosin II filaments are plotted versus lengths that were measured in nascent myofibrils (blue bars) versus mature myofibrils (green bars).

Figure 6. hiPSC-CM transfected with (**a**) mNeonGreen muscle myosin II light chain-2 (MLC-2), and (b) stained with titin antibody (9D10), and with (**c**) Alexa 633 phalloidin. The long arrow in (a) points to the position of an unstained Z-Band localized in the space between A-Bands containing muscle myosin II in a mature myofibril. The short arrow (a) marks overlapping muscle myosin II fibers in a nascent myofibril before A-Bands have formed. The arrowhead (a) is positioned at the edge of the cardiomyocyte where muscle myosin II filaments have not yet formed. In the image showing titin antibody staining (**b**), the long arrow points to doublets of titin localized on either side of a Z-Band in a mature myofibril**.** The identical position is marked by the long arrow between two A-Bands in (a). Titin is absent at the edge of the cell (b, arrowhead), and is in single bands in nascent myofibrils (b, small arrow) bordering the mature myofibrils. (c) Phalloidin-staining of F-actin identifies fibers of F-actin concentrated in the cardiomyocyte. Actin is concentrated in Z-Bands (long arrow in c) aligned along mature

myofibrils; and is unbanded in nascent myofibrils (short arrow in c) and in newly assembling actin fibrils at the cell edge (arrowhead in c). (d) A merged color view of the images shows phalloidin-stained F-actin fibers in red, and titin in green. Titin is absent along F-actin fibers at the edge of the cell (short arrow in d), and present only in small concentrations in nascent fibers (b, d) situated between the fibers at the cell perimeter **(**arrowhead in c, d) and the mature myofibrils (long arrow in a to d). Titin is shown in green arrayed in a doublet pattern spaced along mature myofibrils (d, long arrow), and in small particles on nascent myofibrils (d, short arrow).

Figure 7. An embryonic chick cardiomyocyte transfected with mNeonGreen-α-actinin (a) expressed α-actinin in Z-Bands in mature myofibrils (long arrow) and in Z-bodies near the perimeter of the cardiomyocyte (arrowhead). Myomesin antibody (b), stained in the space between Z-Bands (long arrow), but was absent at the cell perimeter (arrowhead). Phalloidin (c) stained actin in Z bands (long arrow) and actin near the perimeter of the cell (arrowhead). A merged view in color (d) marks alternating staining of α-actinin (green), and myomesin (red) in the mature myofibril (long arrow) The cardiomyocyte in (a) expressing α-actinin is surrounded by four fibroblasts (Fs) that stain only with phalloidin (c). Long arrows (a to d) point to the same Z-Band position in the identical mature myofibril with banded patterns of α-actinin (a), myomesin (b) and actin (c). (b, d) Myomesin is localized only in the middle of sarcomeres (red bands in d) between Z-Bands (green bands in d). Arrowhead in (a) points to area of premyofibrils with small bands of muscle α-actinin near the cell periphery. (c) Arrowhead points to premyofibrils with F-actin fibers around the cell perimeter. (b) Note the absence of myomesin in the premyofibril region at the cell edge (arrowheads in b, d). Myomesin is localized solely in mature myofibrils (b, d), thus it is a late assembling sarcomeric protein

Figure 8. An hiPSC-CM transfected with mNeon Green α-actinin (a), stained with myomesin antibody (b), and phalloidin (c). (d) A merged view shows α-actinin (green), and myomesin (red). Long arrows (a to c) point to Z-Band positions in mature myofibrils with banded patterns of α-actinin (a), myomesin (b) and actin (c). (b, d) Myomesin is

localized in the middle of sarcomeres (red bands in d) between Z-Bands (green bands in d). Arrowhead in (a) points to premyofibrils with small bands of muscle α-actinin around the cell periphery. (c) Arrowhead in (c) point to premyofibrils with unbanded F-actin fibers around the cell perimeter. (b) Note the absence of myomesin in the regions away from the mature myofibrils whose positions are indicated by long arrows in a to d.

Figure 9. Human iPSC-derived cardiomyocytes (a, c, e) and embryonic chick cardiomyocytes (b, d, f) were treated with proteasomal inhibitors (MG132, c, d, or Bortezomib, e, f) for 24 hours while undergoing myofibrillogenesis: (a, b) control, (c, d) 1 μM MG-132 treatment, and (e, f) 2 μM Bortezomib exposure. Cells were fixed, permeabilized, and stained with sarcomeric anti-α-actinin antibody to compare the effects of the inhibitors on myofibril assembly. Control cells (a, b) were filled with mature myofibrils with mature Z-band spacings (long arrows) and peripheral staining of small α-actinin Z-Bodies found in premyofibrils (arrowheads). Compared to control cells (a, b) inhibitor treated cells had reduced populations of mature myofibrils (long arrows in c to f), but increases in the number of small α-actinin bands typically detected in premyofibrils and nascent myofibrils (arrowheads in c to f).

Figure 10. Isolated embryonic chick cardiomyocytes were placed in three different dishes, each containing control medium for two days. The control cells (a-b) were placed in fresh control medium for another day. The cells in the second dish (c-d) were treated with 1 μM MG132 for one day, and the cells in the third dish (e-f) were exposed to 1 μM Bortezomib for one day. The control (a-b) and UPS inhibitor treated cells (c-d, e-f) were fixed, permeabilized and stained with a specific muscle myosin II antibody (a, c, e), and counter-stained with red phalloidin (b, d, f). Abundant mature myofibrils (a, b) are present in the control cells. In contrast to the control cells, the cardiomyocytes exposed to UPS inhibitors exhibit a large number of nascent myofibrils (c-d; e-f), and few or no mature myofibrils

Figure 11. Embryonic chick cardiomyocytes were placed in three dishes containing control medium for two days, and then exposed to medium containing one of three

different UPS inhibitors for one day (a-b 1 µM Carfilzomib; c-d DBeQ 10 µM; e-f 0.1 µM MLN4924). The UPS inhibitor treated cells were fixed, permeabilized and stained with a specific muscle myosin II antibody (a, c, e), and counter-stained with red phalloidin (b, d, f). The cells exposed to these different UPS inhibitors all exhibit a large number of nascent myofibrils (a-b, c-d, e-f), and few or no mature myofibrils.

Figure 12. hiPSC-derived cardiomyocytes were placed into three groups, and placed in dishes containing control medium for two days. The medium in a control dish of cells (a-b) was replaced with fresh control medium for another day. The cells in the second dish (c-d) were treated with 1 µM MG132 for one day, and cardiomyocytes in the third dish (e-f) were exposed to 1 µM Bortezomib for one day. The control cells and UPS-inhibitor treated cells were fixed, permeabilized and stained with a specific muscle myosin II antibody (a, c, e), and counter-stained with phalloidin (b, d, f). (a-b) Abundant mature myofibrils formed in the control cells, whereas the cells exposed to UPS inhibitors exhibited numerous nascent myofibrils (c-d; e-f), and few or no mature myofibrils.

Figure 13. hiPSC-derived cardiomyocytes were divided into three groups and placed into three different dishes, each containing control medium for two days, and then treated with one of three different UPS inhibitors for an additional day (a-b 1 µM Carfilzomib; c-d DBeQ 10 µM; e-f 0.1 µM MLN4924). The UPS inhibitor-treated cells were fixed, permeabilized and stained with a specific muscle myosin II antibody (a, c, e), and counter-stained with red phalloidin (b, d, f). Each of the three dishes of cells exposed to one of the UPS inhibitors were filled with nascent myofibrils, and did not form mature myofibrils (a-b; c-d; e-f).

Figure 14. Reversal of chick embryonic cardiomyocytes after exposure to UPS inhibitors. Control and one day UPS inhibitor-treated embryonic chick cardiomyocytes were trypsinized, spun down, rinsed with control medium several times, and plated on culture dishes to restart myofibrillogenesis in the absence of inhibitors. After spreading in control media for two days, the cells were fixed, permeabilized and stained with muscle-specific α-actinin antibodies. (a) The control cardiomyocyte reformed mature

myofibrils, and fibers with short spacings of muscle α-actinin-stained Z-Bodies. UPS inhibitors (b, MG132, c, Bortezomib, e, DBeQ and f, MLN-4924) were removed from the medium by replating in control media led to the reformation of mature myofibrils in the embryonic chick cardiomyocytes. (d) However, removal of the UPS inhibitor Carfilzomib did not restore the formation of mature myofibrils. The fibers in this cell (d) containing short spacings of muscle α-actinin stained Z-Bodies are typical of premyofibrils and nascent myofibrils. Bar = 10 microns.

Figure 15. Control cardiomyocytes and one day UPS inhibitor-treated ihPSC-cardiomyocytes were trypsinized, spun down, rinsed with control medium several times, and plated on culture dishes to restart myofibrillogenesis in the absence of inhibitors. After spreading in control media for two days, the cells were fixed, permeabilized and stained with muscle specific α-actinin antibodies. (a) A control cardiomyocyte reformed mature myofibrils, and fibers with short spacings of α-actinin stained Z-Bodies. Removal of the UPS inhibitors (b, MG132, c, Bortezomib, e, DBeQ and f, MLN-4924) from the medium led to the formation of mature myofibrils in the human cardiomyocytes. (d) However, removal of the UPS inhibitor Carfilzomib, specifically a proteasome inhibitor, did not lead to the reformation of mature myofibrils. The fibers in this cell contain short spacings of α-actinin stained Z-Bodies, typical of premyofibrils and nascent myofibrils. Bar = 10 microns.

Figure 16. Gel electrophoresis experiment demonstrates the accumulation of ubiquitinated proteins at the level of muscle myosin II in embryonic chick cardiomyocytes. Embryonic chick cardiomyocytes were exposed to 1 μM of MG132 for 0 (control, lane 1), 4 hours (lane 2), 8 hours (lane 3), and 24 hours (lane 4). The same concentration of four different samples were run on acrylamide gels, and then stained with Ponceau Red (upper panel). The proteins from the top gel were transferred to a nitrocellulose membrane **(**Invitrogen), and then stained with an anti-ubiquitin antibody (middle panel). The amount of ubiquitin labeling increases from the control cells over 4, 8, and 24 hours treatment with 1 μM MG132. The gel was stripped, and stained with a muscle specific F59 antibody (bottom panel). The major muscle protein in this 250 KD

(marker gel) region that labels with the ubiquitin antibody is the muscle myosin II heavy chain.

Figure 17. 2D Western blot analysis of protein extracts from four day-old cultures of embryonic chick cardiomyocytes. The Coomassie stained (a) control and (b) sample of one day MG132-treated chick cardiomyocyte proteins are shown in their respective gels. The MG132-treated cells were in control medium for three days, and then in M132 medium for one day before protein extraction. (c) The PVDF filter of control protein gel (a) was stained with anti-ubiquitin monoclonal antibody, followed by treatment with secondary antibody, and subsequently treated with ECL followed by exposure to x-ray film. The most prominent ubiquitin signal in in the extracted control cardiomyocytes (c) is at the 220 KD marker level (muscle myosin II heavy chain gel marker). (d) The PVDF filter of the MG132-treated cardiomyocyte protein gel was treated similarly as the control protein extract gel in (a, c). There was a very large increase in the ubiquitin signal at the 220 KD level (muscle myosin II heavy chain gel marker) in the MG132-treated embryonic cardiomyocytes (d) compared to the control chick cardiomyoctyes in (c). Seven spots outlined in the upper right corner of the gel in (b) 1, 2, 3, 4, 5, 6, and 7 were excised and used for extraction of protein for subsequent mass spectrometric analyses (see the results in Table 1).

Figure 18. Effect of myofibril assembly in a cardiomyocyte lacking BAG3. A cardiomyocyte derived from hiPSCs lacking BAG3 was allowed to spread for four days in culture prior to fixation, permeabilization, and staining with (a) a titin antibody (9D10), and fluorescently tagged secondary antibody, and (b) counter-stained with phalloidin. Arrowhead in (a) marks the absence of titin at the edge of the cardiomyocyte where premyofibrils are located as seen with phalloidin staining (arrowhead in b). The long arrows mark the positions of the mature myofibrils (a, b) in the center of the cell, where (a) titin binds in a doublet marking the positions of the Z-Bands, a characteristic of mature myofibrils. The short arrows in (a, b) indicate the position of the nascent myofibrils where titin is first detected in this cardiomyocyte lacking BAG3. These images

support a three step model of myofibril assembly in hiPSC derived cardiomyocytes lacking BAG3.

Figure 19. Three-step model of myofibrillogenesis, and suggested roles for the UPS system to advance myofibril assembly from nascent myofibrils to mature myofibrils. **Top sequence:** Three-step model of myofibrillogenesis: premyofibrils to nascent myofibrils to mature myofibrils. Three different types of UPS inhibitors arrest the formation of mature myofibrils at the nascent myofibril stage. **Lower sequence:** UPS steps from neddylation to ubiquitination to extraction of ubiquitinated proteins to proteolysis are all required for the progression of nascent myofibrils to mature myofibrils. Thus, the UPS system is essential to advance myofibril assembly from nascent myofibrils to mature myofibrils.

Figure 20. Model of how the appearance of titin molecules (one micron in length) attaching to the Z-bodies in the forming nascent myofibrils could capture the newly formed short muscle myosin II filaments that are at different angles to the component actin bundles. The N-terminal regions of titin bind to α-actinin molecules in Z-Bodies, while the muscle myosin binding repeats along the titin filaments, leading towards its C-termini, can bind the short muscle myosin filaments.